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ORIGINAL ARTICLE *Reproductive biology*

Basic fibroblast growth factor promotes the development of human ovarian early follicles during growth *in vitro*

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STUDY QUESTION: What is the effect of basic fibroblast growth factor (bFGF) on the growth of individual early human follicles in a three-dimensional (3D) culture system *in vitro*?

SUMMARY ANSWER: The addition of 200 ng bFGF/ml improves human early follicle growth, survival and viability during growth *in vitro*.

WHAT IS KNOWN ALREADY: It has been demonstrated that bFGF enhances primordial follicle development in human ovarian tissue culture. However, the growth and survival of individual early follicles in encapsulated 3D culture have not been reported.

STUDY DESIGN, SIZE, DURATION: The maturation *in vitro* of human ovarian follicles was investigated. Ovarian tissue ($n = 11$) was obtained from 11 women during laparoscopic surgery for gynecological disease, after obtaining written informed consent. One hundred and fifty-four early follicles were isolated by enzymic digestion and mechanical disruption. They were individually encapsulated into alginate (1% w/v) and randomly assigned to be cultured with 0, 100, 200 or 300 ng bFGF/ml for 8 days.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Individual follicles were cultured in minimum essential medium α (αMEM) supplemented with bFGF. Follicle survival and growth were assessed by microscopy. Follicle viability was evaluated under confocal laser scanning microscope following Calcein-AM and Ethidium homodimer-1 (Ca-AM/EthD-1) staining.

MAIN RESULTS AND THE ROLE OF CHANCE: After 8 days in culture, all 154 follicles had increased in size. The diameter and survival rate of the follicles and the percentage with good viability were significantly higher in the group cultured with 200 ng bFGF/ml than in the group without bFGF ($P < 0.05$). The percentage of follicles in the pre-antral stage was significantly higher in the 200 ng bFGF/ml group than in the group without bFGF ($P < 0.05$), while the percentages of primordial and primary follicles were significantly lower ($P < 0.05$).

LIMITATIONS, REASONS FOR CAUTION: The study focuses on the effect of bFGF on the development of individual human early follicles in 3D culture *in vitro* and has limited ability to reveal the specific effect of bFGF at each different stage. The findings highlight the need to improve the acquisition and isolation of human ovarian follicles.

WIDER IMPLICATIONS OF THE FINDINGS: The *in vitro* 3D culture of human follicles with appropriate dosage of bFGF offers an effective method to investigate their development. Moreover, it allows early follicles to be cultured to an advanced stage and therefore has the potential to become an important source of mature oocytes for assisted reproductive technology; particularly as an option for fertility preservation in women, including patients with cancer.

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Key words: human follicle / *in vitro* 3D culture / basic fibroblast growth factor / *in vitro* growth

Introduction

The long-term survival rate of young cancer patients has been significantly improved with great development in the management of childhood malignancies. Unfortunately, the ovaries are particularly sensitive to chemotherapy and radiotherapy, which often result in the loss of both reproductive and endocrine functions (Dolmans *et al.*, 2006). The option of cryopreserving ovarian tissue prior to the initiation of cancer treatment and re-implanting the frozen/thawed ovarian tissue in patients, after they have recovered, has already achieved successful pregnancy and delivery (Donnez *et al.*, 2004). However, the transplantation of frozen/thawed ovarian tissue is not suitable for all cancer patients. For example, a risk of re-introducing malignant cells in the cryopreserved ovarian tissue has been reported in patients with leukemia (Donnez *et al.*, 2006; Dolmans *et al.*, 2010; Rosendahl *et al.*, 2010). To avoid transferring malignant cells, the isolation and *in vitro* culture (IVC) of ovarian follicles is a better option.

Fresh (Hovatta *et al.*, 1997; Telfer *et al.*, 2008) and cryopreserved (Picton and Gosden, 2000) human cortical tissues have been used to research human follicle growth *in vitro*. Follicle culture systems have been developed for mouse (Eppig and Schroeder, 1989; Spears *et al.*, 1994; Eppig and O'Brien, 1996; O'Brien *et al.*, 2003; Xu *et al.*, 2006a,b; Jin *et al.*, 2009; Hornick *et al.*, 2012), large animals (Gutierrez *et al.*, 2000; Wu *et al.*, 2001; Picton *et al.*, 2003; Thomas *et al.*, 2007; Xu *et al.*, 2009b, 2013) and human (Roy and Treacy, 1993; Abir *et al.*, 1997, 2001; Hovatta *et al.*, 1997; Wright *et al.*, 1999; Telfer *et al.*, 2008; Amorim *et al.*, 2009; Xu *et al.*, 2009a; Vanacker *et al.*, 2011). Nevertheless, it is still challenging to coordinate the growth factors and hormones required in the processes of follicle development and oocyte maturation *in vitro*. These systems have successfully supported the growth of mouse secondary follicles that finally produced healthy offspring, but have not yet produced competent oocytes in the human. The effect of growth factors and hormone signals on human follicle growth *in vitro* needs to be investigated further.

Basic fibroblast growth factor (bFGF), also called FGF-2, is part of an 18 (or more)-member family of mammalian fibroblast growth factors. They play roles in multiple developmental, physiological and pathological functions (Nishimura *et al.*, 1999). Their biological functions are carried out by binding to and activating high-affinity transmembrane FGF receptors (FGFR1-4) and low-affinity plasma membrane receptors (Beenken and Mohammadi, 2009). bFGF interacts predominantly with FGFR1 and FGFR2, thereby triggering phosphorylation of the receptors and tyrosine residues in the cytosolic substrates (Ibrahimi *et al.*, 2004). The low-affinity receptors mediate ligand concentration and the presentation of the FGF dimers to the high-affinity receptors in a heparan sulfate glycosaminoglycan-dependent manner (Baird, 1994).

bFGF is involved in follicle development. Studies in mammals have shown the expression of bFGF and its receptors in early ovarian follicles in rat (Nilsson *et al.*, 2001), goat (Wandji *et al.*, 1992), cow (van Wezel *et al.*, 1995) and human (Yeh and Osathanondh, 1993; Quennell *et al.*, 2004; Ben-Haroush *et al.*, 2005). Moreover, bFGF was also found to initiate the activation of primordial follicles in rat (Nilsson, *et al.*, 2001; Kezele *et al.*, 2002) and promote both the growth and survival of primordial follicles in goat (Matos *et al.*, 2007b). In human ovarian tissue culture, bFGF has been reported to play a role in estrogen production by the ovarian cortex and high doses of bFGF also enhanced primordial follicle development (Garor *et al.*, 2009). Recently, our group demonstrated that bFGF might significantly improve the quality of transplanted ovarian tissues by increasing follicle quantity and promoting neoangiogenesis (Gao *et al.*, 2013). However, the effect of bFGF on individual human early follicles in IVC remains unknown.

In this study, the effect of bFGF on the growth of individual human early follicles *in vitro* was investigated.

Materials and Methods

Ovarian tissue collection

The use of human tissue for this study was approved by the Ethics Committee of Peking University (the registration number: 2009005). After obtaining written informed consent, ovarian tissues were taken from 11 women, who underwent laparoscopic surgery for gynecological disease, such as endometrial carcinoma, cervical cancer or ovarian tumor.

Tissues were removed and immersed in Leibovitz's L-15 Medium (L-15, Invitrogen, Carlsbad, CA, USA) supplemented with 1% human serum albumin (HSA, LifeGlobal, Guilford, CT, USA), 100 IU/ml penicillin/100 μg/ml streptomycin (Invitrogen) and placed on ice. They were transported from the operating room to the research laboratory within 2 h. The medulla was removed from the biopsies using surgical scissors and scalpels. The size of the cortical pieces varied between 5 × 5 and 10 × 8 mm.

Follicle isolation

All follicles included in this study were isolated from the fresh tissue as described previously (Vanacker *et al.*, 2011). Briefly, the cortical pieces were placed in a tissue sectioner (McIlwain Tissue Chopper, The Mickle Laboratory, Guildford, UK), adjusted to 0.5 mm. The cutting procedure was swift (<5 min), and uniform-size pieces of 0.5 × 0.5 × 1 mm were obtained. The tissue was then enzymatically digested by transferring to a digestion medium, consisting of αMEM (Invitrogen) containing 0.04 mg/ml Liberase DH (Dispase High; Roche Diagnostics GmbH, Mannheim, Germany), 10 IU/ml DNase I (Fermentas, Beijing, China), 100 IU/ml penicillin and 100 μg/ml streptomycin (both Invitrogen) for 75 min at 37°C on a shaker in the incubator (Thermo Fisher, Marietta, OH, USA). The enzymatic digestion was terminated by adding an equal volume of Dulbecco's phosphate buffered saline (DPBS, Invitrogen) supplemented with 10% HSA (LifeGlobal) at 4°C. The digested cortex was washed twice with DPBS supplemented with 10% HSA. Follicles were isolated mechanically using 29-gauge needles and transferred to culture medium [αMEM (Invitrogen) supplemented with

100 IU/ml penicillin, 100 mg/ml streptomycin, 1% HSA (LifeGlobal), 1% insulin-transferrin-selenium (ITS, Invitrogen), 50 mg/ml ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA) and 50 mIU/ml FSH (Sigma-Aldrich)], and incubated at 37°C under 5% CO_2 in humidified air for 2–4 h recovery before encapsulation.

Follicle encapsulation and culture

The protocol for encapsulating the selected follicles in a 1% alginate (Sigma-Aldrich) bead was a slight modification of the method previously described (Xu *et al.*, 2006a,b). Briefly, a single follicle was transferred into 20 μl of alginate solution and then sucked up together with 2.5 μl alginate and dropped into a solution of 50 mM $CaCl_2$ in 140 mM NaCl (Sigma-Aldrich) for 2 min to cross-link. Beads were washed twice in αMEM (Invitrogen). Each alginate bead was placed into a separate drop of 50 μl culture medium (see above) covered by mineral oil in a culture dish. Follicles were randomly divided into four study groups and accordingly transferred to basic culture media supplemented with bFGF 0, 100, 200 or 300 ng/ml (Invitrogen). All follicles were cultured at 37°C under 5% CO_2 for up to 8 days. Every 2 days, half of the culture media was replaced, the morphology of the follicles was analyzed and their diameter was measured. Fresh culture media was prepared weekly.

Follicle measurement and classification

Follicles were observed and photographed using a Nikon TS100 light microscope (Nikon Instruments, Inc.) equipped with an ELWD 0.3 T1-SNCP camera. Follicle diameters were calculated by averaging two perpendicular measurements taken from follicle peripheries using Nis-Element software (Nikon Instruments, Inc.). Growth and survival data were reported on Day 8 of culture. Follicles were considered to be survival unless if they had dark oocytes and/or granulosa cells (GCs) or had no integrity between the oocyte and the GCs (Xu et al., 2009b).

Early follicular stages were classified into four types based on descriptions by Gougeon (1996) and Fortune (2003). These were: primordial follicle (an oocyte surrounded by a single layer of flattened GCs); primary follicle (an oocyte surrounded by a single layer of cuboidal GCs); secondary follicle (a growing oocyte surrounded by two to five layers of GCs) and pre-antral follicle (six or more layers of GCs or diameter >120 mm).

Follicle viability assessment

For follicular viability assessment, follicles were transferred to 100 μl of DPBS containing 2 μM calcein AM and 5 μM ethidium homodimer-1 (EthD-1; Live/Dead Viability/Cytotoxicity kit; Life Technologies, Grand Island, NY USA). Follicles were incubated with the fluorescent dyes for 45 min at 37°C in the dark (Cortvrindt and Smitz, 2001). In the last 10 min, 10 μg/ml Hoechst 33342 (Sigma; which emits blue light in the cell nucleus (ex/em ~346 nm/~460 nm)) was added. Live cells have ubiquitous intracellular esterases which cleave nonfluorescent cell-permeant calcein AM to the intensely fluorescent calcein producing an intense uniform green fluorescence in live cells (ex/em ~495 nm/~515 nm). EthD-1 enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, thereby producing a bright red fluorescence in dead cells (ex/em ~495 nm/~635 nm). After exposure to fluorescent dyes, the follicles were washed in DPBS and imaged on a Zeiss LSM 510 confocal microscope using a Neoflaur 20× objective.

The follicles were classified into four categories depending on the percentage of dead GCs: Viability1(V1), live follicles: follicles with the oocyte and all the GCs viable; Viability2(V2), minimally damaged follicles: follicles with <10% of dead GCs; Viability3(V3), moderately damaged follicles: follicles with 10–50% of dead GCs; Viability4(V4), dead follicles: follicles with both the oocyte and/or >50% GCs dead (Dolmans *et al.*, 2006).

Statistical analysis

Analyses were carried out using the SPSS 18.0 program (SPSS, Chicago, IL, USA). Data were statistically analyzed by the Kruskal–Wallis and Mann–Whitney tests with *post hoc* tests, as required. To compare percentages between groups, ordinal variables were transformed to continuous variables based on a scoring system and a Kruskal–Wallis test was performed. $P < 0.05$ was considered statistically significant.

Results

After obtaining written informed consent, ovarian tissues were taken from 11 women aged (mean ± .SD) 31.8 ± 8.36 years (Table I). The patients all underwent laparoscopic surgery for gynecological disease, such as endometrial carcinoma, cervical cancer or ovarian tumor.

bFGF improves early follicle growth and survival *in vitro*

A total of 154 follicles were cultured and all were found to have increased in size after 8 days (Fig. 1). The initial mean diameters in the groups cultured with 0 ng/ml bFGF ($n = 38$), 100 ng/ml bFGF ($n = 42$), 200 ng/ml bFGF ($n = 39$) and 300 ng/ml bFGF ($n = 35$) were 75.9 ± 20.4, 76.4 ± 22.9, 77.9 ± 20.1 and 74.6 ± 23.1 μm, respectively. There was no significant difference observed in the initial size of follicles within these different groups. By the end of the IVC at Day 8, follicular size had increased to 90.2 ± 29.8, 117.3 ± 42.5, 133.3 ± 35.1 and 110.0 ± 39.2 μm, respectively. Diameter in the group of 200 ng/ml bFGF on Day 6 and 8 (128.4 ± 35.2 and 133.3 ± 35.1 μm) was statistically significantly higher than those in the group of 0 ng/ml bFGF (88.0 ± 22.8 and 90.2 ± 29.8 μm) ($P < 0.05$) (Fig. 2A).

In addition to improving the follicle growth, culturing with bFGF also promotes follicle survival. We observed that the survival rate of follicles in the group of 0 ng/ml bFGF was only 36.8% at Day 8. While the survival

Table I Characteristics of the patients who donated human ovarian tissue and the number of follicles recovered.

Patient	Age	Diagnosis	Biopsy volume (mm^3)	Follicles (*n*)
No. 1	31	Ovarian cancer	115	11
No. 2	21	Ovarian mass	160	34
No. 3	27	Ovarian mass	85	10
No. 4	41	Cervical cancer	88	9
No. 5	30	Ovarian mass	94	7
No. 6	25	Ovarian mass	108	8
No. 7[a]	36	Endometrial carcinoma	200	13
No. 8	23	Cervical cancer	96	10
No. 9	49	Endometrial carcinoma	240	37
No. 10	34	Cervical cancer	85	7
No. 11	26	Ovarian mass	90	8

[a]Patient received chemo/radiotherapy before tissue donation.

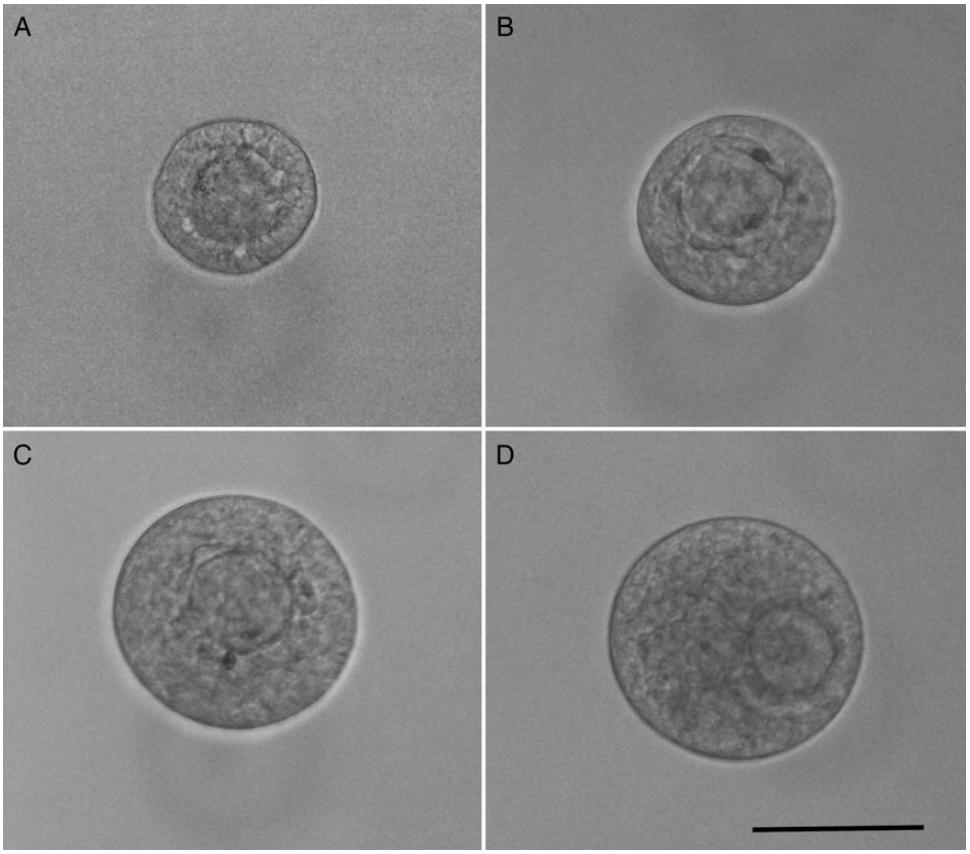

Figure 1 Characteristics of ovarian follicles growing *in vitro* with 200 ng/ml basic fibroblast growth factor. (**A**) Primary follicles (89.5 μm) were isolated from the ovarian cortex and grown in a 3D system for 8 days. (**B**) Follicles developed into secondary follicles at Day 2 (112.5 μm). (**C**) Granulosa cells continuously proliferated at Day 4 (139.5 μm), and (**D**) maintained 3D structural integrity at Day 8 (151 μm). Bar = 100 μm.

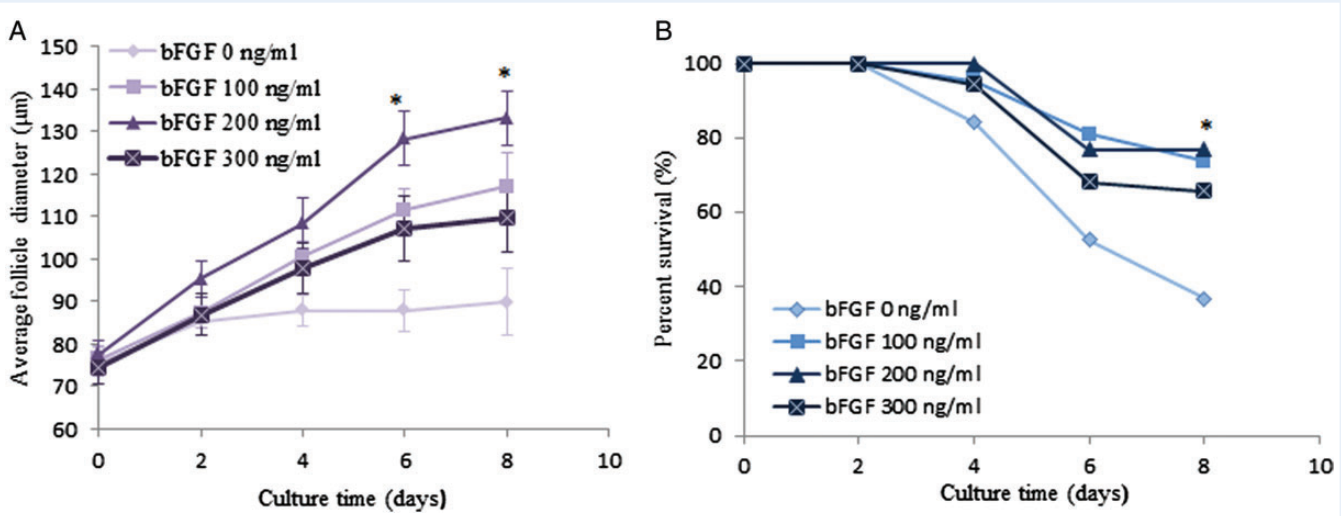

Figure 2 Basic fibroblast growth factor (bFGF) is necessary for early follicles to survive and grow *in vitro*. (**A**) Follicles grown in the group with 200 ng/ml bFGF reach statistically significantly larger diameters on Day 6 and 8 than those cultured without bFGF. (**B**) Follicles grown in the group with 200 ng/ml bFGF have increased survival during culture compared with those cultured without bFGF. $^{*}P < 0.05$.

rate of follicles cultured with bFGF increased significantly (Fig. 2B), the survival rate of follicles in the group of 100, 200 and 300 ng/ml bFGF increased to 73.8, 76.9 and 65.7%, respectively.

bFGF promotes follicle growth to the advanced stages

Data in Table II show the distribution of follicle stages of four groups before and after IVC. On Day 0, most follicles were at primordial and primary stages and there was no significant difference between any of the four groups. After 8 days in the IVC, most of the follicles had activated and differentiated into advanced follicle stages and there was a statistically significant difference between the groups of 0 and 200 ng/ml bFGF ($P < 0.05$).

bFGF improves early follicle viability *in vitro*

A total of 98 isolated human follicles were examined for viability using the fluorescent probes calcein-AM and ethidium homodimer-1 (Figs 3 and 4) on Day 8. V1 and V2 represented follicles with high viability (maximum 10% of dead GCs) and V3 and V4 follicles with poor viability (10–100% of dead GCs or a dead oocyte). Follicles in the group of 200 ng/ml bFGF showed significantly higher viability with 77.2% V1 and V2 (13.6% + 63.6%, respectively) than those in the group of 0 ng/ml bFGF with 16.7% V1 and V2 (0% + 16.7%, respectively) ($P < 0.05$). The remaining poor viability follicles (V3 + V4) accounted for 83.3% in the group of 0 ng/ml bFGF with 41.7% of dead follicles (V4), whereas in the group of 200 ng/ml bFGF, V3 + V4 accounted for 22.7% with only 9.1% of dead follicles (V4) ($P < 0.05$).

Discussion

This study investigated, for the first time, the effect of bFGF on the development of individual human early follicles *in vitro* using a modified 3D culture system. It showed that bFGF improves the growth, survival and viability of follicles. These results indicate that with modification and optimization of culture conditions, human follicle growth efficiency can be improved and it is even possible to develop human oocytes *in vitro*.

Previous studies have shown the effect of bFGF on follicle growth *in vitro*. In rat ovaries in floating culture in the presence of bFGF, primordial follicles progressed to become developing follicles after 14 days' culture (Nilsson *et al.*, 2001). In another study, 50 ng/ml bFGF maintained the morphological integrity and stimulated the activation of goat primordial follicles and the growth of activated follicles with ovarian cortex in IVC for 5 days (Matos *et al.*, 2007b). More recently, with human ovarian cortex cultured in serum-free media for 4 weeks, bFGF was found to play a role in the E_2 production of early follicles and a high concentration of bFGF enhanced follicular development in serum-free media (Garor *et al.*, 2009). However, follicles did not grow well with cortical tissue culture, and their ability to survive decreased, resulting in a loss of follicle integrity and oocyte survival (Hovatta *et al.*, 1999; Telfer *et al.*, 2008). In this study, we use a 3D culture system to culture individually isolated follicles from ovarian cortex to limit oxidative damage and facilitate nutrient diffusion into the tissue *in vitro*. In a previous study, Sharma *et al.* (2009) used a 3D culture system for buffalo pre-antral follicles. Their culture medium contained IGF-I combined with bFGF and they showed that survival rate and mean follicle diameter decreased compared with the IGF-I alone group. They suggested that IGF-I

Table II Classification of the stages of human ovarian follicles cultured *in vitro* with basic fibroblast growth factor (bFGF).

bFGF (ng/ml)	Day 0				Day 8			
	Primordial % (*n*/*n*)	Primary % (*n*/*n*)	Secondary % (*n*/*n*)	Pre-antral % (*n*/*n*)	Primordial % (*n*/*n*)	Primary % (*n*/*n*)	Secondary % (*n*/*n*)	Pre-antral % (*n*/*n*)
0	23.7 (9/38)	47.4 (18/38)	26.3 (10/38)	2.6 (1/38)	0 (0/14)	42.9 (6/14)	50 (7/14)	7.1 (1/14)
100	33.3 (14/42)	47.6 (20/42)	9.5 (4/42)	9.5 (4/42)	0 (0/31)	32.3 (10/31)	29 (9/31)	38.7 (12/31)
200	30.8 (12/39)	38.5 (15/39)	30.8 (12/39)	0 (0/39)	0[a] (0/30)	10[b] (3/30)	30 (9/30)	60[c] (18/30)
300	25.7 (9/35)	51.4 (18/35)	11.4 (4/35)	11.4 (4/35)	0 (0/23)	47.8 (11/23)	13 (3/23)	39.1 (9/23)

[a,b]Significantly lower than the initial percentage ($P < 0.05$).
[c]Significantly higher than the initial percentage ($P < 0.05$).

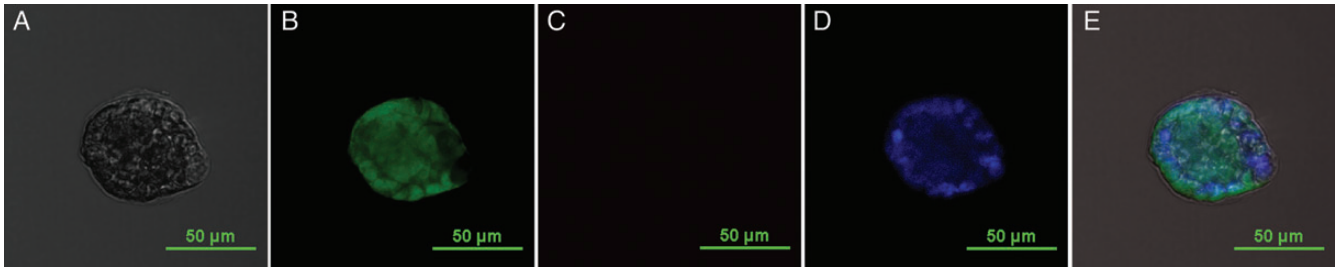

Figure 3 Human ovarian follicles cultured with basic fibroblast growth factor (bFGF) 200 ng/ml for 8 days and evaluated as highly viable. (**A**) A follicle observed without filters. (**B** and **C**) Live/dead staining: viable cells are stained by calcein-AM and emit green light (B); dead cells are stained by ethidium homodimer-1 and emit red light (C). (**D**) Cell nuclei are stained with Hoechst 33 342 and emit blue light. (**E**) All channels merged. No dead cells were detected in this follicle.

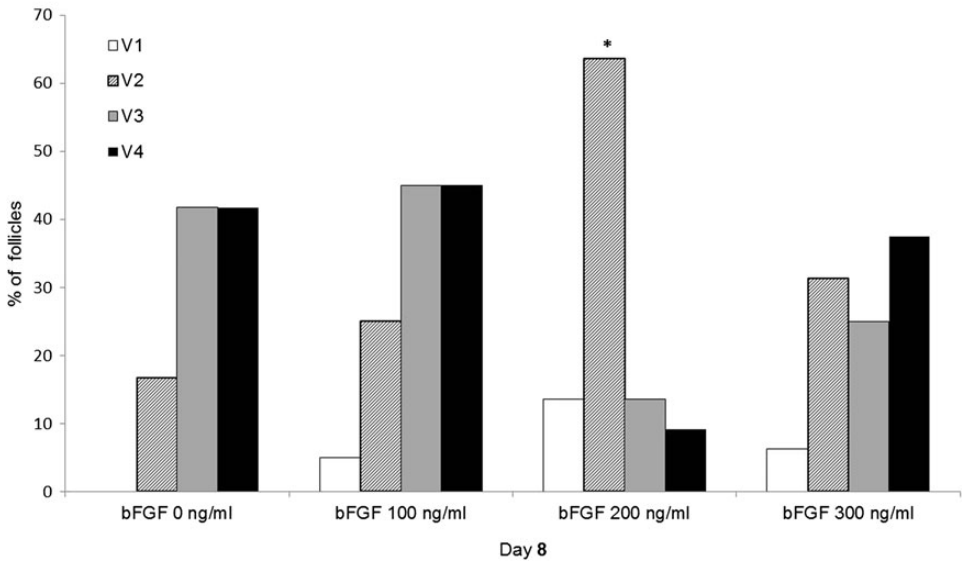

Figure 4 The viability of human ovarian follicles after *in vitro* culture for 8 days. Viability1 (V1), live follicles: follicles with the oocyte and all the granulosa cells (GCs) viable; Viability2 (V2), minimally damaged follicles: follicles with < 10% of dead GCs; Viability3 (V3), moderately damaged follicles: follicles with 10–50% of dead GCs; Viability4 (V4), dead follicles: follicles with both the oocyte and/or > 50% GCs dead. *The percentages of V1 + V2 follicles in the 0 and 200 ng/ml bFGF groups was statistically significantly different, $P < 0.05$.

suppresses the stimulatory effect of bFGF and has an adverse effect on the *in vitro* growth of buffalo pre-antral follicles but did not investigate the effort of bFGF alone compared with the control group. In a study of 3D human follicle culture, the survival rate was only 37% with the diameter increased <25 mm after 1 week of all 254 human ovarian follicles individually cultured (Vanacker *et al.*, 2011). In our study, the survival rate was improved by up to 76.9% and the diameter increased >60 mm in the group exposed to 200 ng/ml bFGF, while the control group had a similar survival rate (36.8%) and increase in diameter (around 15 mm) as the study mentioned above. This result shows that bFGF has a positive effect on the growth of follicles individually cultured *in vitro*; therefore the addition of bFGF to the culture medium should be considered.

In our study, the optimal dosage of bFGF in *in vitro* 3D culture was investigated, 200 ng/ml bFGF promoted the optimal follicle growth, survival and viability, although 100 and 300 ng/ml bFGF also promoted follicle growth. The optimal concentration of bFGF varies in different animal models and culture methods. In rats, whole ovaries were cultured *in vitro* with 40 ng/ml bFGF that induced primordial follicle activation and initiated folliculogenesis (Nilsson and Skinner, 2004). In goats, during long tissue cultures a concentration of only 50 ng/ml bFGF was sufficient to stimulate the growth of follicles by increasing the oocyte diameter and/or the number or size of GCs (Matos *et al.*, 2007b). In human cortical samples cultured with serum substitutes, high doses of bFGF (100 and 300 ng/ml) enhanced human follicular development but bFGF stimulated E_2 production at lower doses (Garor *et al.*, 2009). However,

the bFGF concentrations used in previous studies are all for cortex or ovary tissue culture. No attempt to culture individual follicles with bFGF has been done in the past. Theoretically, low concentrations are expected to be enough to sustain growth of isolated follicles due to the direct access to the follicle compared with the layers of cells in cortical/ovarian culture. But we tried 0–100 ng/ml of bFGF and there was no significant improvement on follicle development in our preliminary experiments. This implied that higher concentrations bFGF may be required for the follicle growth *in vitro*. In a study by Sharma et al. (2009), they also tried low concentration of bFGF in a buffalo follicle IVC system combined with IGF. Similar to our result, they found no effect of bFGF on improving follicle growth, the concentration being too low. We speculated that the reason is, in the cortex or ovary tissue culture system, besides the exogenous bFGF added to the medium, bFGF could be secreted by stroma cells in the ovarian cortex through a paracrine pathway so that the concentration becomes sufficient to stimulate follicle growth. By contrast, in individual follicle culture, stroma cells were removed so that no endogenous source of bFGF exists, so that a higher concentration of bFGF needs to be added to the culture medium.

During 8 days of IVC, the viability of follicles from the bFGF groups remained very high and the 3D structure was a well maintained. Follicular diameter was also significantly increased in the group of bFGF 200 ng/ml compared with the control group, suggesting that bFGF has a role in early folliculogenesis. Diameter increase could be attributed to follicular growth because of the loss of inhibitory factors in ovarian stroma (Telfer *et al.*, 2008), or to an increase in the volumes of GCs after fluid absorption (Abir *et al.*, 1999). However, we found that bFGF markedly increased the growth in follicular diameter. bFGF may promote primordial follicle initiation and growth by acting on oocytes, stromal cells or pregranulosa cell (Shikone *et al.*, 1992; Wandji *et al.*, 1992; Manova *et al.*, 1993; Motro and Bernstein, 1993). Studies in rats (Nilsson *et al.*, 2001) and cattle (van Wezel *et al.*, 1995) show that bFGF is located to ovarian pre-antral follicles. Another study suggests that bFGF can increase the transition rate from primordial to primary and secondary follicles in rats (Kezele *et al.*, 2002). Matos *et al.* (2007a) indicated growth and survival rates of goat primordial follicles can be increased by adding bFGF in combination with FSH, and these effects can be blocked by a bFGF neutralizing antibody (Nilsson and Skinner, 2004). Tang *et al.* (2009) demonstrated that FSH combined with bFGF could promote the survival, activation and development of primordial follicles in the long-term culture of cattle ovarian cortex. Studies in human showed that the expression of FGF receptors-2 (FGFR-2), FGFR-3 and FGFR-4 were detected in all oocytes of different follicular stages and bFGFR was identified in GCs from female adolescents/adults but not from fetuses. However, FGFR-1 was not detected in human follicles (Ben-Haroush *et al.*, 2005). These results indicate that during primordial and early follicles growth, bFGF may play an important role to enhance ovarian granulosa, stromal and theca cell proliferation.

This study has shown that both survival and viability of early follicles—from primordial to pre-antral follicles stages—can be improved with the use of 3D culture with bFGF for up to 8 days. However, human follicle development is a complex and lengthy process and the challenge now is to optimize the culture conditions to permit long-term culture beyond 8 days. Up to now, the longest IVC time of human follicles has been 30 days. Alginate and matrigel 3D culture systems were used to make secondary human follicles develop into small antral follicles and complete GV oocyte growth (Xu *et al.*, 2009a). However, the efficiency of growing early follicles into antral follicles was still very low and the functional oocytes could not be retrieved with this procedure. These data suggest that to improve the culture medium, multiple growth factors including bFGF should be added to give full play of their individual or combined effects on follicle development. More importantly, considering the complexity and feature of the different follicle stages in human follicular development, sequential culture media with various growth factors might be needed in each follicular stage until a mature healthy competent human oocyte could be obtained in IVC.

In conclusion, we have shown that the growth of human follicles at early stage can be promoted by bFGF in 3D system. Supplementation with a proper concentration of bFGF was found to be essential for human follicle activation and development. The optimization of human follicles culture *in vitro* might provide more, precious oocytes for female fertility preservation.

Authors' roles

T.R.W. performed the experiments, including ovarian follicle isolation, encapsulation and culturing with bFGF, and took part in manuscript drafting, critical discussion and data analysis. L.Y.Y. contributed to follicle isolation and culture. J.Y. performed follicle viability assessment by calcein AM and ethidium homodimer-I straining. C.L.L. was involved in critical discussion and data analysis. X.X. and T.L.Y. performed follicle isolation and measurement. X.H.Z. contributed to data analysis and manuscript editing. J.M.G. and T.D. were involved in critical discussion and data analysis. W.H.H. and H.Y.G. collected the ovarian tissues. R.L. took part in the study design, manuscript drafting, critical discussion and data analysis. J.Q. contributed to the study conception and design, coordinated the research and edited and submitted the manuscript.

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Conflict of interest

None declared.

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