Improvement in the quality of heterotopic allotransplanted mouse ovarian tissues with basic fibroblast growth factor and fibrin hydrogel

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STUDY QUESTION: Does basic fibroblast growth factor (bFGF) in combination with fibrin hydrogel improve follicle development and revascularization of heterotopically transplanted mouse ovarian tissues?

SUMMARY ANSWER: Treatment of transplanted ovarian tissues with higher concentrations (75, 100 and 150 μg/ml), but not lower concentrations (25 and 50 μg/ml), of bFGF significantly improved primordial follicle survival and angiogenesis, while apoptosis of follicles and stromal cells was significantly decreased.

WHAT IS KNOWN ALREADY: Use of transplanted ovarian tissues in female fertility preservation is limited by the massive loss of follicles and ischemia-reperfusion injury due to the expected delay in revascularization.

STUDY DESIGN, SIZE AND DURATION: Ovarian tissues from 18-day-old ICR mice were encapsulated in fibrin hydrogel mixed with different concentrations of bFGF, then transplanted under the skin of adult female mice for 1 week. The ovarian tissues treated without fibrin hydrogels and bFGF were designated as Control group I, and the ovarian tissues treated with fibrin hydrogels but without bFGF were designated as Control group II. The ovarian tissues treated with 25 and 50 μg/ml bFGF were designated as the lower concentration group, and the ovarian tissues treated with 75, 100 and 150 μg/ml bFGF were designated as the higher concentration group.

MATERIALS, SETTING AND METHODS: Assessment of follicular quantity and follicle classification was carried out by histologic analysis. Follicle proliferation was evidenced by immunostaining with proliferating cell nuclear antigen and apoptosis was verified by anti-active caspase-3 staining. Epithelial cells of new blood vessels were stained using CD31 antibody to evaluate neoangiogenesis, and the blood vessel density was analyzed by immunohistochemistry.

MAIN RESULTS AND THE ROLE OF CHANCE: The ovarian tissues were recovered 1 week post-transplantation. Compared with the control group, the survival and proliferation of the follicles was significantly increased, the apoptosis of follicles and stromal cells was significantly decreased, and angiogenesis was significantly enhanced when the transplanted ovarian tissues were treated with a higher concentration of bFGF. Treatment with a lower concentration of bFGF did not improve follicle survival and blood revascularization.

LIMITATIONS, REASONS FOR CAUTION: The results obtained may not be fully extrapolated to humans because of the physiologic differences between mice and humans.

WIDER IMPLICATIONS OF THE FINDINGS: For the first time, the present study investigated the role of bFGF in transplanted ovarian tissues and demonstrated that bFGF might significantly improve the quality of transplanted ovarian tissues by increasing follicle quantity and
Introduction

As a result of earlier diagnoses and advanced treatments, the survival rate of young cancer patients has greatly increased; however, the cytotoxicity of ionizing radiation and chemotherapeutic drugs can injure the ovaries, frequently leading to premature ovarian failure and resulting in loss of both endocrine and reproductive functions, a condition with serious long-term hormone-related consequences and infertility (Abir et al., 2011; Shikanov et al., 2011; Soleimani et al., 2011; Friedman et al., 2012). To overcome this predicament, the concept of female fertility preservation was developed, including oocyte, fertilized embryo and ovarian tissue preservation (Donnez and Dolmans, 2011). Of these three methods, ovarian tissue preservation has displayed superiority because it decreases the potential drug side effects associated with controlled ovarian hyperstimulation for oocyte recovery and fertilization. In addition, there are other advantages of ovarian tissue preservation. Specifically, ovarian tissue preservation preserves gametogenic function not only for women of childbearing age, but for prepubertal girls (Chen et al., 2006). Primordial follicles can be stored to conserve fertility, and transplantation back to patients can produce healthy offspring (Grynberg et al., 2011). Of these three methods, ovarian tissue preservation has displayed superiority because it decreases the potential drug side effects associated with controlled ovarian hyperstimulation for oocyte recovery and fertilization. In addition, there are other advantages of ovarian tissue preservation. Specifically, ovarian tissue preservation preserves gametogenic function not only for women of childbearing age, but for prepubertal girls (Chen et al., 2006). Primordial follicles can be stored to conserve fertility, and transplantation back to patients can produce healthy offspring (Grynberg et al., 2011). Another advantage of ovarian tissue preservation is that steroidogenic function is maintained, and this will avoid the potential side effects of long-term medications for endogenous gonadotrophin deficiency (Weissman et al., 1999; Oktay et al., 2001; Kim et al., 2009).

There have been 28 live births following fresh and frozen cortical ovarian tissue transplantation reported by various groups worldwide (Grynberg et al., 2012), but some obstacles must be overcome before this method of fertility preservation can become a standard procedure. One of the major obstacles to ovarian tissue preservation is that a large number of primordial follicles are lost when the ovarian tissue is transplanted, which limits transplantation success and graft longevity. Existing evidence has shown that after transplantation, the size of the graft is significantly reduced to 30–70% of the original size, and significant fibrotic changes occur in most grafts (Kim et al., 2002). In ovarian cortex autografted in a sheep model, only a few primordial follicles survive (between 5 and 30%), despite the resumption of endocrine and fertility function (Aubard et al., 1999; Baird et al., 1999). In humans, the cyclicity of ovarian function after transplantation is of shorter duration than expected in many studies (Callego et al., 2001; Kim et al., 2004a,b). In addition, as occurs in other organs or tissues which are grafted, ischemia–reperfusion injury is a threat to survival of tissue, resulting in necrosis-apoptosis and fibrotic changes during the initial stage after transplantation (Kim et al., 2002; Liu et al., 2002). Therefore, how to improve the quality of transplanted ovarian tissue is a critical issue to be addressed.

Recently, basic fibroblast growth factor (bFGF) has been shown to improve follicle development in animal and human studies. In an animal model, the rate of follicle development in rat ovaries was accelerated, and the primordial follicles showed a corresponding decrease after 2 weeks of bFGF treatment in vivo. bFGF treatment promotes growth of bovine ovarian theca cells and stromal cells (Nilsson et al., 2001). Garor et al. (2009) reported that the number of developing follicles in human ovarian tissue samples when cultured with bFGF was significantly higher than in uncultured specimens, and that the estradiol (E2) production increased significantly while bFGF inhibitors reduced E2 secretion. Furthermore, bFGF mRNA and protein are expressed throughout follicular development, which indicates the potential roles of bFGF during follicle growth (Almeida et al., 2012). Moreover, bFGF has also been suggested to play a key role in angiogenesis and tissue healing by stimulating migration and proliferation of a variety of cell types (Akita et al., 2005; Murakami and Simons, 2008; De Laporte et al., 2011; Tassi et al., 2011). bFGF gene transfer enhances vascularization and viability of ischemic skin flaps, and localized and sustained release of bFGF promotes angiogenesis and nerve growth within spinal cord injuries (Fujihara et al., 2005; De Laporte et al., 2011). In clinical trials, administration of bFGF has been used to induce therapeutic angiogenesis for critical limb ischemia, accelerate wound healing of diabetic ulcers, and stimulate regeneration of periodontal tissues in patients with periodontitis (Marui et al., 2007; Kitamura et al., 2008; Uchi et al., 2009).

However, there has been no studies addressing the effects of bFGF priming in vivo on ovarian grafts and the resulting development of follicles because free bFGF is readily degradable in vivo, resulting in loss of biological activity and function (Yun et al., 2010). Fibrin hydrogel appears to be a promising material to encapsulate the tissue to serve as a vehicle for the controlled release of growth factors and to form a continuous pathway for cellular infiltration and bidirectional crosstalk between the host and graft because of an ability to support cellular processes and natural bioactivity (Shikanov et al., 2011). Fibrin encapsulation of the ovary may serve as a bridge between the graft and the host for endothelial cell (EC) migration and proliferation in blood vessel development. Fibrin clot degradation occurs in days to weeks (Kidd et al., 2012); therefore, fibrin may be used as an effective delivery matrix to control the release of growth factors.

In this study, we determined the effects of bFGF mixed with fibrin hydrogel on transplanted ovarian tissues in vivo using a murine model. The survival of grafts, follicle growth and apoptosis, and revascularization of transplanted ovarian tissues were evaluated.

Materials and Methods

All of the chemicals used in this study were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) unless otherwise stated.
Mice
All animal experiments were approved by the Institutional Animal Care and Use Committee of Peking University Third Hospital. ICR outbred mice (CD-1; Animal Center of Medical College of Peking University) at 8 weeks of age were maintained under controlled temperature and lighting conditions in groups of five per cage in the Animal Center of Medical College of Peking University according to national legislation for animal care, and given food and water ad libitum.

Ovarian tissue collection and treatment
For this study, 150 ovaries were isolated from 75 18-day-old ICR mice. The ovaries were collected and immersed in Leibovitz-15 medium supplemented with 100 mg/ml of streptomycin and 100 IU/ml of penicillin at room temperature before encapsulation. The experiments were performed with fresh tissue to eliminate possible effects of cryopreservation. We divided the ovary into two parts using sterile blades and assigned the parts at random.

Ovarian size calculation
Ultrasoundography is commonly used to determine the average size of the ovary, but only for the 8-week-old mice. Ultrasoundography was not adopted in the current study because the size of the ovarian tissues in 18-day-old mice is too small to be detected. Therefore, we measured the length, width and height of the ovaries using a ruler, and calculated the approximate size (1.5–3 mm³). Hemi-ovaries were randomly allografted into each incision.

Ovarian tissue encapsulation
The fibrinogen solution (80 mg/ml) was made with lyophilized fibrinogen from human plasma (87% clot table protein) in 0.9% saline at 37°C. Thrombin was prepared at a concentration of 200 UN/ml in sterile 0.1% (w/v) BSA solution. Solutions were kept at pH 6.5 and stored at −20°C in plastic tubes. The thrombin solution was further diluted at 100 UN/ml for use. The fibrinogen solution was mixed with different concentrations of bFGF (25, 50, 75, 100 and 150 µg/ml; Gibco) and 5 µl droplets were placed on a hydrophobic surface. Hemi-ovaries of 18-day-old mice were transferred into the fibrinogen droplets and 2.5 µl of thrombin solution was added to cross-link for 5 min. Then, the hydrogels were transplanted subcutaneously to the posterior aspect of the necks of recipient mice.

Ovarian tissue transplantation
ICR outbred adult female mice were anesthetized with an intraperitoneal injection of 2,2,2-tro bromoethyl alcohol and tertamyl alcohol. Two subcutaneous skin incisions were made on the posterior aspects of the neck after removing hair and sterilizing the area. Ovarian tissue grafts, encapsulated in fibrin hydrogel, were randomly placed in the incisions. The skin incisions were closed with absorbable 5/0 Prolene. Two control groups were performed under aseptic conditions. One group consisted of ovaries grafted with fibrin hydrogel, were randomly placed in the incisions. The skin incisions were closed with absorbable 5/0 Prolene. Two control groups were performed under aseptic conditions. Two control groups were performed under aseptic conditions. Two control groups were performed under aseptic conditions. Two control groups were performed under aseptic conditions. Two control groups were performed under aseptic conditions. Two control groups were performed under aseptic conditions. Two control groups were performed under aseptic conditions. Two control groups were performed under aseptic conditions. Two control groups were performed under aseptic conditions. Two control groups were performed under aseptic conditions.

Graft recovery and histologic evaluation
The animals were anesthetized (vide supra) and the grafts were recovered and fixed in 4% formaldehyde 7 days after transplantation. Then, the mice were euthanized by cervical dislocation. The entire graft was embedded in paraffin and cut into serial sections, 5 µm in thickness. Ten serial sections of the graft were stained with haematoxylin and eosin (H&E) and observed with light microscopy to perform histomorphologic examinations. Follicles were counted in a high-power field (×400) at three randomly selected positions in 10 different sections when the dark staining nucleus was visualized within the nucleus of the follicles. The mean value of follicles in each section was calculated. The follicles were classified as follows: (i) primordial follicles with one layer of flattened granulosa cells surrounding the oocyte; (ii) primary follicles with one layer of cuboid granulosa cells and (iii) secondary follicles with two or three layers of granulosa cells (Chen et al., 2006).

Immunohistochemistry
Sections were deparaffinized and rehydrated in xylene and in an ethanol series of decreasing concentrations. After deproteinizing in citrate buffer (pH 6.0) for 20 min at 98°C and cooling to room temperature, 0.3% H2O2 was used to block endogenous peroxidase activity by incubation for 10 min. The sections were then incubated overnight at 4°C with primary antibodies. The sections were thricewashed with PBS for 2 min each, then the slides were subsequently incubated for 30 min at room temperature with secondary antibodies. The primary antibody was omitted in each different immunohistochemistry (IHC) staining for negative control. DAB was added to change the color by incubating for 2–5 min and counterstaining was done with haematoxylin for 5 min, after which the slides were dehydrated with an increasing ethanol concentration and xylene, then sealed.

Sections from the middle of grafts were analyzed for IHC. IHC staining against proliferating cell nuclear antigen (PCNA; Invitrogen) was performed (1:300 dilution) to confirm cell proliferation and anti-active caspase-3 (AC-3) staining (Abcam) was performed to evaluate (1:200 dilution) apoptosis in the grafted tissues in various experimental groups. Red-brown coloring of the cytoplasm/nucleus of the granulosa cells, stromal cells or oocytes was specified as positive staining (otherwise as negative staining). The positive staining cells and the total number of cells were randomly counted in a high-power field (×400) in the same area. The ratio of PCNA- or AC-3-positive cells to the total number of cells was calculated. To quantify angiogenesis, epithelial cells of new blood vessels were stained using anti-CD31 antibody (1:100 dilution; Abcam). The primary antibody was also omitted in each different IHC staining for negative control. To calculate vascular density, CD31-positively stained vessels were counted in a high-power field (×400) at three randomly selected positions in different sections. The mean value was used as the final vascular density for each graft. Referring to the manufacturer’s instructions for the antibodies, breast cancer tissues were used as a positive control for PCNA immunostaining, mouse ovaries were used as a positive control for AC-3 immunostaining after freeze-thawing, and fetal villi tissues were used as a positive control for CD-31 immunostaining.

Statistical analysis
Statistical analysis was performed with IBM SPSS Statistics 20.0 software. Statistical significance between groups was analyzed by one-way ANOVA followed by a LSD post hoc test for multiple comparisons. A P < 0.05 was considered to be statistically significant.
Results

Follicle characteristics in the transplanted ovarian tissues

The follicle characteristics in transplanted ovarian tissues were analyzed to evaluate the effects of ovarian tissue transplantation and the effects of fibrin and bFGF treatment. Ten transplanted ovarian tissues in each group were collected on Day 7. Follicle morphology in transplanted ovarian tissues is shown in Fig. 1 in which three types of follicles were observed, including primordial follicles 30 μm in diameter and one layer of flattened granulosa cells surrounding the oocyte, primary follicles (<100 μm) with one layer of cuboid granulosa cells surrounding the oocyte and secondary follicles (>100 μm) with two or three layers of granulosa cells. The follicles in 10 ovarian tissue samples were counted and compared between the control and bFGF treatment groups. The total follicle and primordial follicle counts were significantly decreased in the grafts compared with ovaries before transplantation (Fig. 2A). Compared with both control groups, the numbers of follicles were not increased when ovarian tissues were treated with lower concentrations of bFGF (25 and 50 μg/ml; \( P < 0.05 \)), but were significantly increased with higher concentrations of bFGF (75, 100 and 150 μg/ml; \( P < 0.05 \); Fig. 2A). Furthermore, the distribution of follicles in each developmental stage was identified, including primordial, primary and secondary follicles. The number and the proportion of primordial follicles in the higher concentration groups were significantly higher than the control and lower bFGF concentration groups; however, there were no significant differences between the control and lower concentration groups for the number and proportion of primordial follicles. Moreover, there were no differences among the control, lower concentration and higher concentration groups for the number and proportion of primary and secondary follicles (\( P > 0.05 \); Fig 2B and C).

Effects of ovarian transplantation on proliferation and apoptosis of follicles

Follicles and stromal cells were immunostained with PCNA to evaluate proliferation, and with anti-AC-3 staining to evaluate apoptosis of the follicles and stromal cells in ovarian tissues. Ten transplanted ovarian tissues in each group were evaluated on Day 7. Similar to effects on the number of follicles, lower concentrations of bFGF did not improve graft survival, and a low rate of proliferation was noted, as demonstrated by PCNA staining, in the lower bFGF concentrations and control groups (\( P > 0.05 \)). More positive signals were detected in the higher concentration bFGF groups, indicating significant proliferation of follicles (\( P < 0.05 \); Fig. 3A and C, and Supplementary data Fig. S1). The numbers of PCNA-positive primordial follicles was significantly increased compared with the lower concentration and control groups, but no significant differences existed in the number of PCNA-positive primary and secondary follicles in each group (Fig. 3D). In addition, the higher concentrations of bFGF...
inhibited apoptosis of follicles compared with the lower concentrations of bFGF and control groups (P < 0.05) (Fig. 3B and C, and Supplementary data Fig. S2). The numbers of AC-3-positive primordial follicles were significantly decreased compared with the lower concentration and control groups, but no significant differences existed in the number of positive primary and secondary follicles in each group (Fig. 3E). The positive controls for PCNA and AC-3 are shown in Supplementary data Fig S3A and B.

Identification of angiogenesis in transplanted ovarian tissues

To evaluate the effect of fibrin and bFGF on neoangiogenesis, the specific cell surface marker for new blood vessels, CD31 (also known as PECAM-1), was identified in the transplanted ovarian tissues. Ten transplanted ovarian tissues were collected and stained on Day 7. The revascularization of ovarian fragments is shown by anti-CD31 antibody IHC of grafts in Fig. 4A (black arrows). A significant higher positive signal of CD31 was found in peripheral sites of the grafted ovarian. Figure 4B represents the vascular density as a histogram. Angiogenesis in both control groups was not significantly different (P = 0.051) and was significantly less than in the experimental groups (P > 0.05). The vascular density was more prominent in the ovarian tissues treated with higher concentrations of bFGF in comparison with the lower concentrations of bFGF (P < 0.05). The positive control for CD-31 is shown in Supplementary data Fig. S3C. The total surface area analyzed for CD-31-positive cells is shown in Supplementary data Fig. 4. In the ovarian tissues, CD-31-positive signals were mainly observed in antral follicles before transplantation, and in endothelial progenitor cells after transplantation.

To verify the relationship between the enhanced revascularization and the changed number of follicles, we correlated the vascular density with the number of primordial, primary and secondary follicles. A significant positive correlation was observed between the number of primordial follicles with microvascular density in the grafts (Spearman's rank correlation coefficient = 0.84, P < 0.001; Fig. 4C), indicating that the bFGF-induced neoangiogenesis contributed to postovarian transplant follicle survival; however, there was no correlation between the number of primary or secondary follicles with microvascular density in the grafts (Supplementary data Fig. S5A and B).

Angiogenesis is very active 48 h post-transplantation in a murine model. Thus, 10 ovarian tissues in each group were collected and stained on Day 2, and CD-31 expression was also identified using an IHC method. Grafts treated with a higher concentration of bFGF (75, 100 and 150 μg/ml) demonstrated a significant increase in the density of CD31-positive blood vessels than those treated with the lower concentration bFGF and the control groups (data not shown). The density of CD-31-positive blood vessels in control I group, in which ovarian tissue was grafted without fibrin and bFGF, was significantly lower than in Control group II, in which ovarian tissue was grafted with fibrin but without bFGF (data not shown).

Discussion

In the present study, we systematically investigated the effects of bFGF and fibrin hydrogels on the quality of mouse ovarian tissues following heterotopic allotransplantation. The results indicated that treatment with high concentrations of bFGF and fibrin hydrogels significantly improved the quality of transplanted ovarian tissues by preventing follicle loss and decreasing apoptosis. Moreover, the revascularization in the transplanted ovarian tissues, which is regarded as one of the important means by which to improve the quality of transplanted ovarian tissues, was enhanced with high concentrations of bFGF.
The first clinical live birth obtained using ovarian tissue cryopreservation and re-implantation was described in 2004 (Donnez et al., 2004). Since then, this technique became a promising option to preserve the fertility of young cancer patients. However, massive loss of follicles during the initial days after ovarian transplantation, mainly due to ischemia caused by slow post-transplantation graft revascularization, has limited the success and acceptance of the procedure (Baird et al., 1999; Nisolle et al., 2000). However, regarding the trends of younger cancer patients and the development of advanced medical technology, more and more female patients under childbearing age consider the option of reproduction and hormone restoration. Therefore, it is necessary to study the methods of ovarian tissue preservation to improve the quality of post-transplantation ovarian tissues.

Currently there are two main approaches for ovarian tissues transplantation (orthotopic and heterotopic) for autotransplantation of human ovarian tissue. Although the advantage of orthotopic transplantation is natural pregnancy, the procedure requires abdominal surgery and general anesthesia, and more importantly, orthotopic location is not preferred when the risk of ovarian metastasis is high because tissue monitoring may be more difficult. Therefore, heterotopic transplantation is preferable due to the rapid restoration of endocrine function, decreasing the risk of surgery as well as the possibility of monitoring and retrieving oocytes.
repeatedly from the transplanted ovarian tissues (Oktay et al., 2001; Kim et al., 2009). However, it is still unknown where the ovarian tissues should be transplanted. Dath et al. (2010) demonstrated that four grafting sites, including intraperitoneal, ovarian bursa, subcutaneous and i.m. sites, had equal performance with respect to the follicular pool and ovarian tissue integrity after short-term transplantation of freeze-thawed human ovarian tissues. Endocrine function has been reported to persist for 7 years with heterotopic transplantation of cryobanked human ovarian tissue (Kim, 2012). Von Schönfeldt et al. (2011) generated preantral stage follicles from subcutaneously transplanted marmoset monkey ovarian tissues. The subcutaneous location was selected as the grafting site because it is easier to observe revascularization around ovarian tissue in experiments using murine models. Furthermore, the subcutaneous site is an appropriate heterotopic transplantation site for women with desired fertility, but requires restoration of ovarian hormone function.

It is critical to maintain the quality and function in the process of ovarian tissue transplantation, and follicle quantity is thought to be one of the best indices to evaluate ovarian function. In the current study, we found that higher concentrations of bFGF could significantly rescue the loss of follicles, especially primordial follicles. In previous studies, bFGF added to the culture medium in vitro has been shown to significantly increase follicle survival (Nilsson et al., 2001; Garor et al., 2009; Peng et al., 2010). All of the results indicate that bFGF is beneficial for follicle development. However, the usual concentration of bFGF for culturing follicles in vitro is <500 ng/

**Figure 4** Effects of different bFGF concentrations on blood revascularization in the transplanted ovarian tissues. (A) Immunohistochemical staining for the angiogenesis-specific marker, CD31, in the transplanted ovarian tissues. Red-brown coloring was specified as positive staining (black arrow). A0: Grafted ovarian tissues in Control group I. A1: Grafted ovarian tissues in Control group II. A2–A6: Grafted ovarian tissues treated with 25, 50, 75, 100 and 150 µg/ml of bFGF, respectively. Original magnification ×200. Scale bar = 50 µm. (B) The mean number of blood vessels in different bFGF concentration groups.* indicates the P-value was <0.05 when the mean number of blood vessels in the lower bFGF concentration groups was compared with the control groups. # indicates the P-value was <0.05 when the mean number of blood vessels in the higher bFGF concentration groups was compared with that in the control groups and the low bFGF concentration groups. (C) Positive correlation of the microvascular density with the number of primordial follicles in the grafts.
ml. The results in the current study showed that there was no obvious improvement when bFGF (25 and 50 μg/ml) was supplemented for tissue transplantation in vivo, although this concentration in vivo is 50–100 times compared with in vitro bFGF. Furthermore, the higher concentration of bFGF could effectively improve follicle development, which suggests that there may be different mechanisms of bFGF access or function for in vitro and in vivo follicle development.

Our results also showed that follicles and stromal cells were rescued with bFGF supplementation. Ovarian stromal cells and extracellular matrix have been reported to have an important role in normal ovarian function, follicle growth and survival (Oktyay et al., 2000; Soleimani et al., 2011). Regulation of ovarian follicle development depends on endocrine- and paracrine-acting hormones of surrounding stromal cells (Oktem and Oktay, 2007; Woodruff and Shea, 2007). Thus, stromal cell proliferation promoted by bFGF treatment, as shown in the current study, is likely beneficial for follicle survival.

We also showed that revascularization in ovarian tissue transplants treated with fibrin and bFGF was significantly improved. Revascularization has always been a barrier for the development of ovarian transplantation. Some studies indicate that the expected delay results in the loss of massive primordial follicles due to ischemia and hypoxia in the early stage of transplantation (Soleimani et al., 2011; Friedman et al., 2012). Researchers have suggested that improvements and acceleration in neoangiogenesis is beneficial for ovarian grafting, yet there is no consensus regarding potential methods. bFGF has been used to facilitate wound healing, vascularization and regeneration in many tissues, including skin, muscle, bone and nerve, due to the ability of bFGF to induce angiogenesis (Kawai et al., 2000; Yoon et al., 2006; Hong et al., 2010; Friedman et al., 2012). bFGF is expressed throughout follicular development, playing important roles in follicle development in vitro (Nilsson et al., 2001; Almeida et al., 2012). This function is probably mediated through signal crossstalk mechanism and is closely related with improvement of vascular regeneration (David et al., 2012; Lin et al., 2012). In agreement with the results regarding improvement in follicle development, lower concentrations of bFGF did not increase angiogenesis. In previous studies, 100 μg/ml of bFGF applied to grafts improved vascularization (Yasuda et al., 2008; Katayama et al., 2010; Katsuno et al., 2011; Tengood et al., 2011). In the current study, treatment with higher concentrations of bFGF improved revascularization, and follicle development was subsequently enhanced.

Periods of 48 h and 7 days after transplantation have been suggested as important durations of time in some studies. Normally, angiogenesis occurs within 48 h of tissue transplantation. Angiogenesis of freeze-thawed rat ovarian tissue begins within 48 h of transplantation, and takes more than 7 days to complete the process (Kim et al., 2004a,b). Although severe damage had occurred on the vascular system of the graft tissue on Day 2, single or crowds of vascular ECs were detected and significantly increased, suggesting the beginning of angiogenesis (Wu et al., 2010). Other studies have demonstrated that exogenous gonadotrophin and 5’-(N-ethylcarboxamido) adenosine increase the expression of VEGF in the grafted ovaries on Day 2, and sphingosine-1-phosphate accelerates neoangiogenesis 2 days after ovarian xenotransplantation (Yang et al., 2008; Hornzoi et al., 2011; Soleimani et al., 2011). More importantly, the formation of new blood vessels will generally be completed 7 days after tissue transplantation. Bedaiwy et al. reported that angiogenesis takes later than 48 h, although ovarian tissue is well endowed with angiogenic factors at that time. Most follicles in the grafts were apoptotic on Day 2, but recovered by Day 7. The duration of full angiogenesis into the grafted ovary may take 1 week (Bedaiwy et al., 2012). Moreover, a massive increase in the proportion of Ki-67-positive follicles, which indicates the proliferation index of granulose cells and follicles, was observed for 7 days after grafting (Dath et al., 2010). Furthermore the pO2 levels were significantly increased at ~1 week (Van Eyck et al., 2009). Human dermal microvascular ECs transplanted on biodegradable polymer matrices were shown to be dispersed throughout the sponge 1 day after transplantation, organized into empty tubular structures by Day 5, and differentiated into functional microvessels within 7–10 days. Dath et al. (2011) also observed that a well-organized and well-vascularized stromal structure was clearly observed on the seventh day after transplantation.

Free bFGF is readily degradable in vivo, resulting in loss of biological activity and function (Yun et al., 2010). In the current study, we showed that fibrin hydrogels encapsulate ovarian tissue and control the release of growth factor to provide immediate gradients of growth factors for angiogenesis. Further, fibrin encapsulation of the ovary serves as a bridge between the graft and the host for EC migration and proliferation for blood vessel development. A fibrin scaffold mimics physiologic angiogenesis, where extravascular fibrin gel is a substrate for EC growth, and bFGF delivery promotes EC adhesion, migration, proliferation, differentiation and growth factor secretion (Zampetaki et al., 2008). Thus, we have postulated that fibrin encapsulation and bFGF delivery enhances neoangiogenesis and improves follicle survival during mouse ovarian tissue allotransplantation. We observed a high rate of graft recovery when grafts were treated with fibrin, probably because the natural biological characteristics of fibrin increased adhesiveness between the ovarian tissue and the host. Our results also indicated that fibrin encapsulation improved neoangiogenesis.

This study is the first to demonstrate that bFGF and fibrin hydrogels improve follicle survival following ovarian transplantation. Accordingly, our findings indicated that bFGF treatment improves primordial follicle survival of ovarian transplantation and enhances angiogenesis and stromal cell proliferation. Further studies should be conducted to further elucidate the mechanism of this improvement and promote the application of this technology in clinical settings.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/

Authors’ roles

G.J.M. performed the experiments, including ovarian tissue treatment with bFGF and fibrin hydrogels and ovarian tissue transplantation experiments, and also took part in manuscript drafting, critical discussion and data analysis. Y.J. performed PCNA, AC-3 and CD-31 immunostaining by the IHC method, and was involved in critical discussion and data analysis. L.R. took part in the study design, manuscript drafting, critical discussion and data analysis. L.M. performed follicle separations and counting, and was involved in critical discussion and data analysis. Y.L.Y. collected the ovarian tissues. W.T.R., Z.H.C. and Z.Y. were involved in data analysis and manuscript editing. Y.Y. mainly contributed to experiment design, data analysis, manuscript drafting and critical discussion. Q.J. contributed to the study conception and design, coordinated the research and edited and submitted the manuscript.

Supplementary data

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Conflict of interest
None of the authors have any conflicts of interests to be declared.

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