

Fibrin Encapsulation and Vascular Endothelial Growth Factor Delivery Promotes Ovarian Graft Survival in Mice

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Ovarian cryopreservation before chemotherapy and autotransplantation post-treatment can restore fertility to women with premature ovarian failure. Although the majority of primordial follicles survive the cryopreservation cycle, the follicular pool is reduced after transplantation due to ischemic death. Therefore, we engineered a biomaterial-based system to promote angiogenesis in a mouse model of ovarian transplantation. To mimic the clinical situation of sterility, a bilateral ovariectomy was performed 2 weeks before transplantation, during which time serum levels of follicular stimulating hormone rose to menopausal levels. Before transplantation, vitrified/thawed ovarian tissue from 12-day-old C57Bl/6J pups was encapsulated in fibrin modified with heparin-binding peptide (HBP), heparin, and loaded with 0.5 μ g vascular endothelial growth factor (VEGF). The group transplanted with fibrin-HBP-VEGF had twice as many surviving primordial follicles and an increased number of blood vessels relative to the no biomaterial control. Transplanted tissue was viable and supported natural conception that led to live and healthy offspring. The timeline of live births with VEGF delivery suggested that primary follicles survived transplantation, and provided the gametes for the first litter. Thus, VEGF delivery from fibrin supported integration of the transplant with the host, promoted angiogenesis, and enhanced engraftment and function of the tissue.

Introduction

THE CRYOPRESERVATION AND autotransplantation of ovarian tissue is emerging as a powerful approach for preserving fertility for patients that are losing ovarian function, which may be genetically based, an autoimmune disorder, or results from chemotherapy treatments. Hormonal stimulation, and subsequent egg or embryo banking, is the traditional approach for fertility preservation; however, this option has constraints on its applicability, such as the nature of the disease, pubertal status of the patient, and availability of a sperm donor.¹ The ovary at any age contains mainly primordial follicles (>70%), which is the most immature stage of follicle development, along with follicles at multiple stages of development (primary, preantral, and antral). Primordial follicles have the greatest potential to survive cryopreservation and transplantation because of their small size (30 μ m) relative to follicles that have been activated into the growing pool (80 to 500 μ m in mice). Transplantation of tis-

sue with a large number of follicles could restore both endocrine function and could produce multiple cycles for conception. Thus far, 13 live births in humans have been reported from the transplantation of cryopreserved ovarian tissue.^{2–11} Despite the potential of this technology, the success of ovarian tissue transplantation has multiple challenges, such as a low number of follicles in the graft that may impact its longevity, and survival of the tissue during *ex vivo* processing and subsequent transplantation.

Hypoxia in the transplant from delayed revascularization is a primary cause of premature follicle depletion. Ovarian cortical pieces are grafted without vascular anastomosis, so graft survival depends on revascularization. The onset of revascularization happens within 48 h at the earliest in rodents,¹² whereas human ovarian tissue stays hypoxic for up to 5 days post-transplantation.¹³ After transplantation, vascular endothelial growth factor (VEGF) transcription in the graft is upregulated due to hypoxia. Within the ovary, VEGF infusion¹⁴ or exogenous gonadotropin administration¹⁵ after

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ovarian grafting in primates failed to improve graft function and follicle survival. However, the controlled release of VEGF from biomaterials has been effective in other systems to promote angiogenesis.¹⁶ Additionally, lack of the physical connectivity between the host and graft may also limit revascularization by restricting cell infiltration from the host and paracrine communication. For ovarian transplants, graft-host communication is additionally required to restore endocrine function and cyclicity. A biodegradable and adhesive biomaterial can spatially connect the host and graft tissue to promote their crosstalk.

We hypothesized that a controlled biomolecular environment that physically bridges the transplant and host tissue and releases angiogenic factors would promote revascularization and follicle survival. Fibrin hydrogels were employed to encapsulate the tissue to act as both a vehicle for the controlled release of growth factors as well as to form a continuous path for cellular infiltration and bidirectional crosstalk between the host and graft. Controlled release is achieved through encapsulation of heparin into a fibrin hydrogel modified with heparin-binding peptide (HBP) and heparin, which provides a reservoir of noncovalently bound VEGF. The fibrin-HBP-VEGF hydrogel serves as a cell infiltration matrix and allows active release due to cellular degradation of the matrix rather than passive diffusion.^{17–20} The controlled release will maintain growth factor concentrations and gradients during the time required for initiating functional revascularization. This system is applied for ovarian tissue transplantation, with investigation of early stage follicle survival, vascularization, graft function, and live births. The described approach may enhance the utility of ovarian tissue transplantation as a mechanism for fertility preservation.

Materials and Methods

Ovarian tissue vitrification

Ovaries were isolated from 12-day-old C57Bl/6J mice, bisected, and transferred to an equilibration solution at room temperature containing 7.5% dimethyl sulfoxide (DMSO) and 7.5% ethylene glycol for 20 min, followed by a second equilibration in 20% DMSO, 20% ethylene glycol with 17% sucrose for 5 min at room temperature. The ovarian tissue was placed on a thin copper strip and submerged directly into liquid nitrogen. The strips and vitrified tissue were transferred to cryovials and stored in liquid nitrogen for at least 24 h or longer.

Ovarian tissue thawing

Ovarian tissue was thawed before transplantation by removing the cryovial from the liquid nitrogen and submerging the copper strip with the attached ovarian tissue in prewarmed to 37°C thawing solution containing 1 M sucrose for 3 min, followed by 5 min in 0.5 M sucrose solution at room temperature. Then, tissue was equilibrated for additional 10 min in L15 media at room temperature before encapsulation.

Ovarian tissue encapsulation

Cryopreserved and thawed ovarian tissue was encapsulated in sodium alginate (55%–65% guluronic acid; FMC

BioPolymers), fibrin (prepared from human fibrinogen; Calbiochem), or in fibrin-HBP-VEGF hydrogels. Alginate was modified with adhesion peptides to support cell adhesion and infiltration.^{21–24} The HBP is a bifunctional peptide that is covalently cross-linked to fibrin by Factor XIIIa on one domain and noncovalently binds heparin on its other domain. The heparin binds heparin-binding growth factors, such as VEGF, and retains them in the scaffold. The fibrinogen solution (7.3 mg/mL final concentration) was mixed with the peptide (NQEVSPPK(βA)FAKLAARLYRKA, 0.37 mg/mL), heparin (18 kDa, 0.67 mg/mL), and VEGF₁₆₈ (Invitrogen; 0.074 mg/mL), and 5 μL droplets were placed on hydrophobic surface. Thawed and equilibrated ovarian tissue was transferred to the fibrinogen droplets and 2.5 μL of thrombin solution (Aldrich-Sigma; 125 IU/mL) was added to crosslink the hydrogels for 5 min. Tissue was similarly encapsulated in unmodified fibrin. The encapsulated tissue was transferred to the equilibration solution and transplanted within 1 hour. Ovarian tissue encapsulation in alginate was performed by adding the tissue to 0.5% w/v alginate solution in PBS, followed by extrusion of 7.5 μL of the solution with the tissue into the cross-linking solution composed of 50 mM CaCl₂ and 140 mM NaCl. The alginate beads were cross-linked for 3 min, transferred to L15 media, and transplanted to the bursal cavity.

Ovariectomy and tissue transplantation

Eighteen C57Bl/6J female mice were anesthetized and a bilateral ovariectomy was performed as previously described.²⁵ A 1% w/v alginate bead (7.5 μL) cross-linked in CaCl₂ solution (50 mM CaCl₂ and 140 mM NaCl) was inserted into the bursa to preserve the site for future transplantation (Supplementary Fig. S1a–d; Supplementary Data are available online at www.liebertonline.com/tea). Vaginal cytology was performed to verify the absence of ovarian function after the ovariectomy. Two weeks after ovariectomy, the alginate bead was removed and replaced with ovarian tissue encapsulated in unmodified fibrin, fibrin-HBP-VEGF, or left unencapsulated as a control in the bursa, with six mice used in each group. Daily vaginal cytology was performed to determine the onset of hormonal cyclicity (graft function) and weekly tail blood was collected to determine serum follicular stimulating hormone (FSH) levels.

Histological tissue analysis

Histological evaluation is the primary approach to assess ovarian tissue quality. Optimal fixation was achieved with Bouin's and 4% paraformaldehyde fixative. Parameters of histological evaluation included follicular density, tissue and oocyte general morphology, and blood vessel density. The transplanted tissue was retrieved after two full estrous cycles, fixed, and sectioned (5 μm). Follicles were counted in every two sections, and blood vessels in every five sections. Blood vessels were confirmed by immunohistochemistry staining with a CD31 antibody.

Live birth

Two additional groups of mice (6 mice in each) were ovariectomized and transplanted with control (no biomaterial) tissue and tissue encapsulated in fibrin-HBP. Two weeks after grafting, mice were paired with C57Bl/6J male breeders.

All offspring remained with the mother until weaning. The fertility of the offspring was confirmed by breeding.

Statistical analysis

The statistical analysis was performed using software program STATA (StataCorp LP). Serum FSH levels were compared at different time points to the starting levels within each group using rank-sum test. Follicle numbers at each developmental stage were compared between different experimental groups also using rank-sum test. The resumption of cyclicity was plotted using Kaplan-Meier method and the difference between the different groups was tested using log rank test. Same test was applied to determine the difference in the length of the period between transplantation and conception. In all statistical analysis $p < 0.05$ was considered statistical significant.

Results

Vitrification preserves ovarian morphology

We initially investigated ovarian tissue cryopreservation to ensure that transplanted tissue was viable. Ovarian folli-

cles, the functional unit of the ovary, are sensitive to freezing and thawing cycles because of the large volume of the oocyte, as well as the need to preserve cell-cell connections between the somatic (granulosa) cells and the oocyte in the follicle. Ovarian tissue cryopreservation is also challenged by the range of follicle sizes within the ovary. Vitrification is an ultra-rapid freezing method that was initially developed for freezing eggs and immature oocytes,^{26,27} and it avoids the formation of ice crystals that could damage cells, causing necrosis. Recently, successful vitrification of human and bovine ovarian tissue was reported,^{11,28} but the protocol could be improved by preserving a wider range of follicle sizes within the ovary. Primordial (30 μm diameter) and primary follicles (<100 μm) typically have the greatest survival relative to more mature and larger follicles. The vitrification procedures were adapted for bisected mouse ovaries. Histological evaluation verified that follicles in the vitrified tissue had a normal appearance, with circular oocytes surrounded by one or more tightly packed layers of granulosa cells (Fig. 1a–d). The density of the ovarian tissue was indistinguishable from fresh tissue, suggesting that connections between stromal cells and within the follicles were not disrupted. The average number of primordial and primary

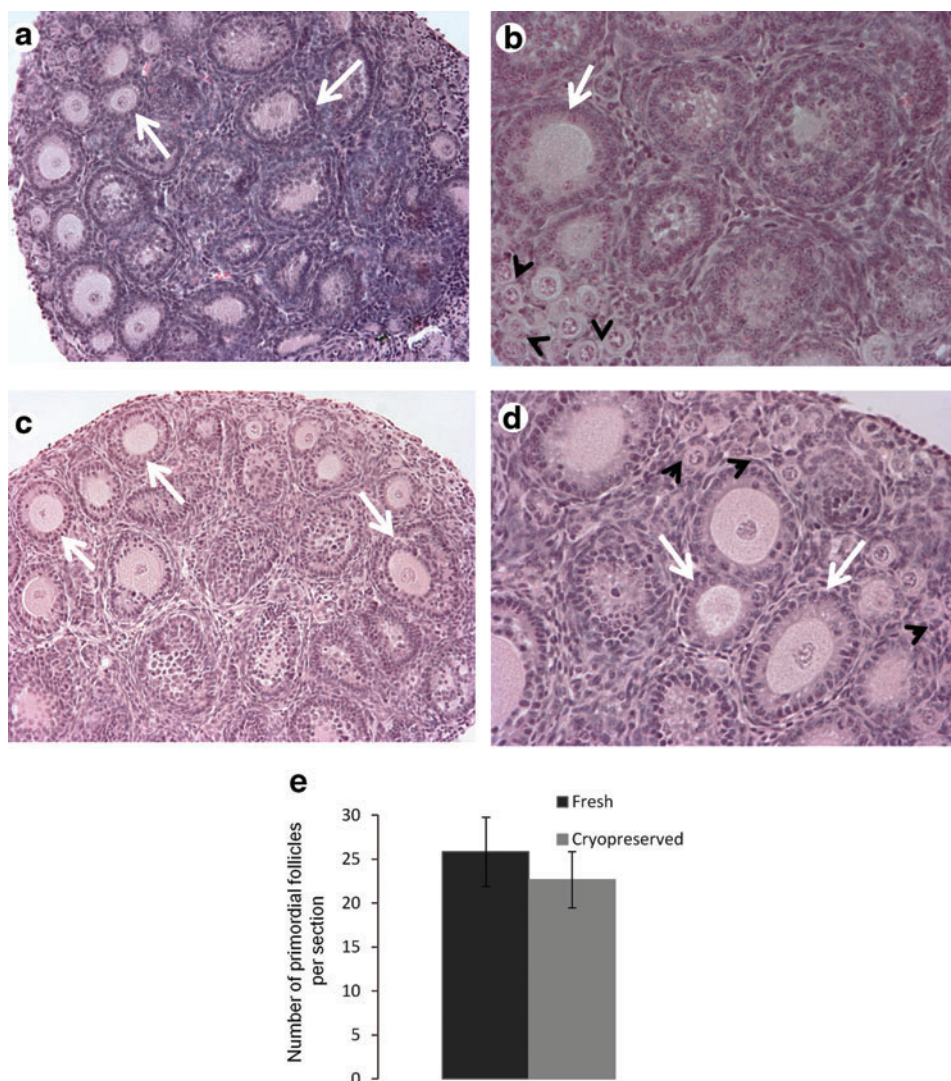


FIG. 1. Ovarian tissue morphology after cryopreservation, encapsulation, and transplantation. Representative micrograph of H&E-stained ovarian sections showing the preserved healthy secondary (white arrows) and primordial (black arrowheads) follicle morphology (during cryopreservation; (a, b) fresh ovary from a 12-day-old mouse, magnification 100 $\times$, 200 $\times$, (c, d) vitrified ovarian tissue, magnification 100 $\times$, 200 $\times$). (e) The number of primordial follicles per section was compared between fresh and cryopreserved ovarian tissue from 12-day-old pups. H&E, hematoxylin and eosin. Color images available online at www.liebertonline.com/tea

follicles per section was the same as in the fresh tissue, each with a density of 20–25 primordial follicles per section (Fig. 1e).

Fibrin encapsulated and grafted ovarian tissue is fully integrated with the host

We investigated the orthotopic transplantation of ovarian tissue to ovariectomized mice using two natural biomaterials, fibrin and alginate, to encapsulate the tissue. Alginate was selected as alginate hydrogels have been previously employed for the culture of immature ovarian follicles. The material was modified with adhesion peptides to support cell adhesion and infiltration.^{21–24} Additionally, we investigated fibrin hydrogels, which are a fast degrading, bioactive, and blood-derived biomaterial, and have also been used with *in vitro* follicle culture.²⁹ Ovarian tissue was encapsulated in fibrin or alginate and grafted in the bursa of mice 14 days postovariectomy. Grafts were removed 2 weeks post-transplantation. Alginate hydrogels had minimal cell infiltration from the surrounding tissue, which resulted in the surrounding bursa remaining largely detached from the encapsulated tissue. This limited cell infiltration was expected to severely limit revascularization of the tissue and physically block ovulation, and was not investigated further (Fig. 2a). Fibrin encapsulation (Fig. 2d–f) preserved ovarian tissue morphology, and after *in vivo* grafting the biomaterial completely degraded, leading to integration of the transplanted tissue with the host (Fig. 2b, c). Cells had infiltrated into the biomaterial, and the encapsulated tissue was apposed and integrated with the bursa.

Grafted ovarian tissue restored ovarian function in mice

Integration between the host and transplanted tissue by fibrin encapsulation motivated subsequent studies that investigated localized delivery of angiogenic factors as a means to enhance engraftment. The fibrin hydrogels were modified with fibrin-HBP, which can be loaded with heparin and VEGF. Heparin binds to the peptide-modified fibrin, and reversible binding between the immobilized heparin and VEGF provides an affinity-based vehicle for localized delivery. Control conditions included unmodified fibrin and un-

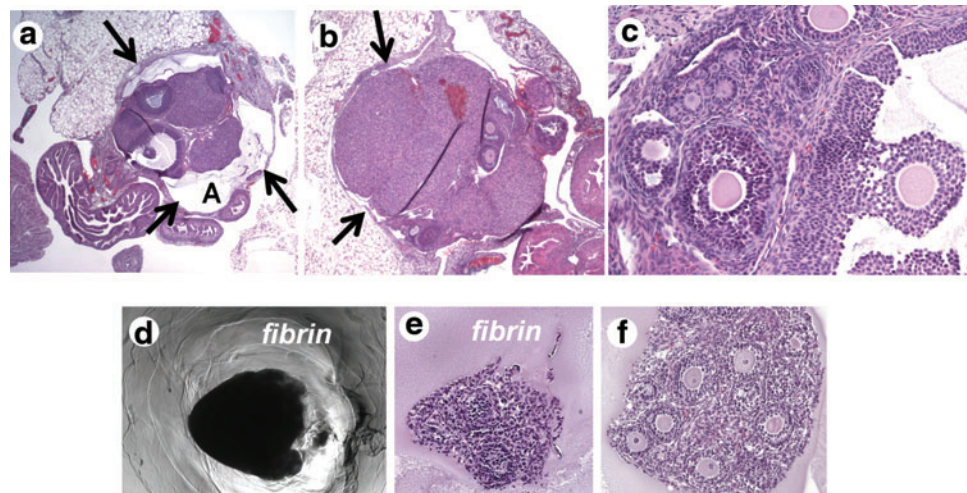
encapsulated ovarian tissue. Mice stopped cycling after bilateral ovariectomy, which was confirmed by vaginal cytology (Fig. 3a) and increased serum levels of FSH (Fig. 3b). Serum FSH levels in a fertile mouse depend on a hormone feedback from the ovary and range from 2 to 10 ng/mL. In the absence of the gonads, mice had FSH levels of 50 ng/mL at 7 days postovariectomy, and remained elevated up to 7 days after transplantation. Serum FSH levels decreased 7 days after transplantation until reaching an undetectable level (<10 ng/mL) at 3 weeks post-transplantation for all experimental groups, indicating the restoration of ovarian function and control of pituitary gonadotropins. Daily vaginal cytology supported the FSH data, because mice in all experimental groups resumed cyclicity at 2 weeks post-transplantation. Based on the cytology results, mice transplanted with fibrin-HBP-VEGF resumed cyclicity earlier than the unmodified fibrin or the un-encapsulated tissue control group. Histological evaluation of the grafts removed at 3 weeks post-transplantation revealed ovarian tissue with all classes of follicles and corpus luteum suggesting multiple ovulations. The encapsulating fibrin was fully degraded, with no barrier between grafted tissue and the host bursa (Fig. 4a–c) and the transplanted ovaries had similar morphology to the healthy fertile adult female (Fig. 4d).

We also investigated the contribution of hormone cyclicity on engraftment and function of the transplanted tissue. Mice were bilaterally ovariectomized and immediately transplanted with fibrin encapsulated ovarian tissue (designated fibrin-cycle), which ensure that the mice have normal FSH levels at the time of transplantation. Previous studies have suggested that elevated FSH and reduced AMH levels cause premature primordial pool depletion by massive recruitment of primordial follicles in the graft.³⁰ The FSH levels for fibrin-cycling increased in the first 7 after transplantation, yet reached only 28 ng/mL or approximately half the serum FSH levels in ovariectomized mice. By day 14, serum FSH levels for the fibrin-cycle group was equivalent to the other experimental conditions (Fig. 3b).

VEGF delivery increased primordial follicle survival

The survival of the follicular pool after transplantation was subsequently investigated, and indicated an increased

FIG. 2. Importance of using degradable biomaterial to allow reconnection of the recipient bursa (black arrows) and the transplanted tissue, (a) tissue transplanted in alginate (A), magnification 100×, (b, c) in fibrin, 100×, 200×; (d–f) half ovary encapsulated in fibrin, live, H&E 100×, 200×. Color images available online at www.liebertonline.com/tea



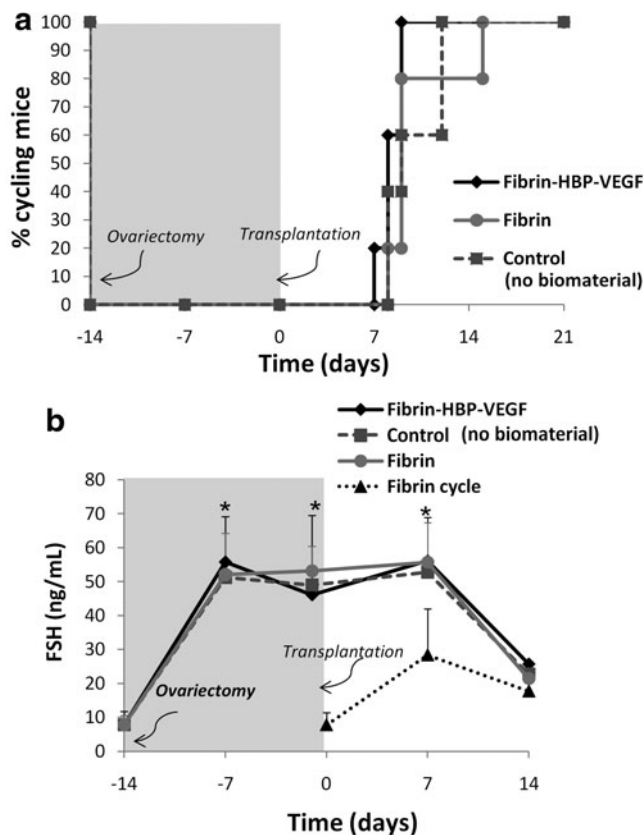


FIG. 3. Restoration of ovarian function after transplantation: **(a)** Restoration of estrous cycle after transplantation presented as Kaplan-Meier plot. At time point 0 the animals were transplanted with the ovarian tissue and the percentage of cycling animals is shown on axis y corresponding to different time points. **(b)** FSH levels were determined in tail blood drawn once a week. Day -14 corresponds to ovariectomy and day 0 to a transplantation time points. FSH serum levels were significantly higher at days -7, 0, and 7 comparing to the FSH levels before ovariectomy (day -14), rank-sum test, $p < 0.05$. FSH, follicular stimulating hormone.

survival of primordial follicles. Grafts were removed after two full estrous cycles (17–22 days post-transplantation depending on the onset of ovarian function that differed between groups) and sectioned, and follicles were counted in every 2nd section. The fibrin-HBP-VEGF group had the greatest number of primordial follicles at 3.7 follicles per section, which is comparable to healthy ovarian tissue from an 8-week-old female. Fibrin and fibrin-cycle conditions had similar numbers of primordial follicles, (2.5 per section) and the unencapsulated tissue had the lowest number of remaining follicles (1.5 per section) (Fig. 4e). The number of primary and preantral secondary follicles was similar in all experimental conditions, and the fibrin-cycle condition had a slightly higher number of antral follicles. These results suggest that follicles were recruited to the growing pool after transplantation independently of the treatment.

Importantly, experimental studies in sheep, monkeys, and human have demonstrated a significant increase in the proportion of activated follicles, from $<20\%$ in ovaries before grafting to $>70\%$ after transplantation.³¹ This increase in growing follicles correlated with premature depletion of the

ovarian reserve. In the fibrin-HBP-VEGF condition, primordial follicles represented 62% of the total ovarian pool ($<30\%$ activated), which was greater than the primordial pool in fibrin (54%), un-encapsulated (46%), and fibrin-C (37%) groups (Table 1). Therefore, VEGF delivery may provide a protective effect to the follicular pool after transplantation.

Transplanted ovarian grafts were vascularized at 3 weeks

We subsequently characterized the extent of vascularization in the transplanted ovarian tissue and observed that VEGF delivery increased the number of blood vessels. In the ovary, the vascular supply is normally formed on a cyclic basis. Smaller primordial follicles do not possess an independent capillary network and are dependent on their proximity to the stromal vessels. As a follicle develops to the antral stage, a vascular wreath continuously develops; however, the capillaries do not penetrate the basement membrane until shortly before ovulation.³² Thus, efficient revascularization of the graft is critical for primordial follicle survival and the development of vasculature surrounding maturing follicles. The fibrin-HBP-VEGF condition had the greatest number of blood vessels (17 blood vessels/section) 3 weeks after transplantation (Fig. 5d). Fibrin had fewer blood vessels (10 blood vessels/section), demonstrating that VEGF delivery promoted the blood vessel development. The number of blood vessels in the un-encapsulated tissue was similar to the fibrin groups (<10 blood vessels/section).

Healthy, naturally conceived pups born from vitrified grafted ovarian tissue

The fertility of the mice with transplanted ovarian tissue was investigated, with each female receiving half of a 12-day-old ovary in each bursa, totaling 1 ovary per mouse. Transplant groups were fibrin-HBP-VEGF and an un-encapsulated control. The tissue size was a limiting factor for both cryopreservation and revascularization; thus, vitrification and transplantation of bisected ovaries, with approximate dimensions of 1 mm^3 , were chosen. The average litter size and average number of litters per dam was 5 mice and 2.5 litters, respectively, for both the fibrin-HBP-VEGF and control group. For comparison, a healthy C57BL/6J female has an average of 6.6 mice per litter and an average of 3.8 litters. (Table 2). The reduction in developing follicles per ovulation cycle and the duration of fertility may be due to the size of the transplants (one ovary is equivalent to the half of normal ovarian reserve), and not the process of cryopreservation or engraftment.

While litter size and the number of litters were similar between groups, the average time until birth for the first litter was significantly reduced in the fibrin-HBP-VEGF group. Mice receiving fibrin-HBP-VEGF encapsulated tissue gave birth 22 ± 3 days after breeding, whereas the un-encapsulated group required 43 ± 7 days (Table 2). The gestational period for mice is 20 days, suggesting that conception occurred on the first or second estrous cycle after breeding in the fibrin-HBP-VEGF group. The follicular pool before transplantation is composed of 80% primordial, 15% primary, and 5% secondary preantral follicles. Secondary follicles are FSH-responsive, and secrete estradiol to provide negative feedback to the pituitary gonadotropes that secrete FSH, resulting in decreasing serum FSH levels over the 2-

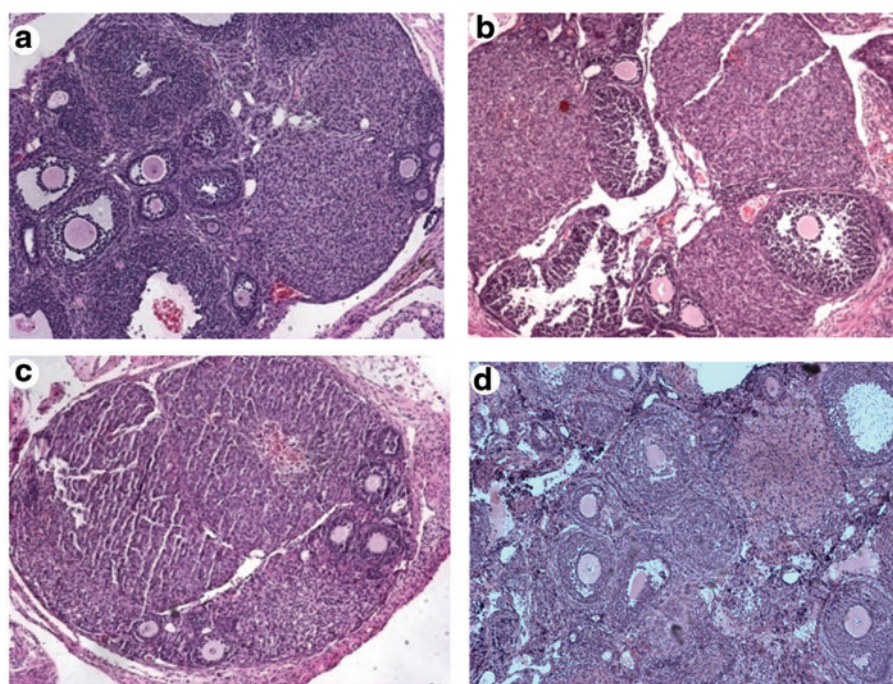


FIG. 4. H&E representative micrographs of ovarian tissue transplanted in control, fibrin, fibrin-HBP-VEGF, and normal adult ovary, magnification 100 $\times$ (**a–d**); primordial, primary, and preantral follicle counts in the transplanted ovarian tissue (**e**). The follicle numbers were compared between different groups using rank-sum test, $p < 0.05$; different letters correspond to a significantly different groups. HBP, heparin-binding peptide; VEGF, vascular endothelial growth factor. Color images available online at www.liebertonline.com/tea

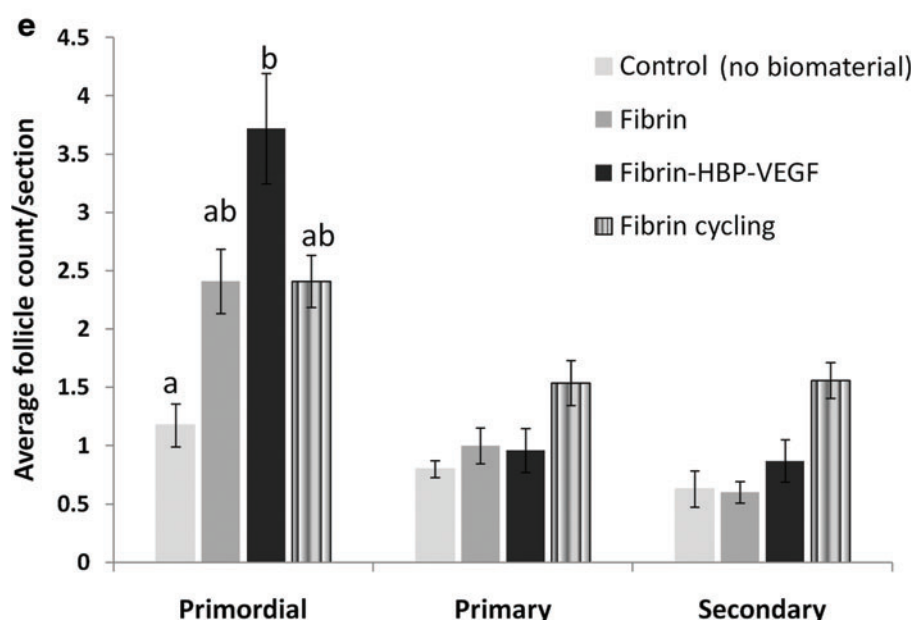


TABLE 1. PROPORTION OF THE ACTIVATED (PRIMARY AND SECONDARY) VERSUS NONACTIVATED (PRIMORDIAL) FOLLICLES IN THE TRANSPLANTED OVARIAN TISSUE

	Primordial (%)	Primary (%)	Secondary (%)
Control (no biomaterial)	42	29	23
Fibrin	54	22	13
Fibrin-HBP-VEGF	62	16	14

HBP, heparin-binding peptide; VEGF, vascular endothelial growth factor.

week period after transplantation (Fig. 3b). A secondary follicle develops to a preovulatory follicle in 12 days, whereas primary follicles and activated primordial follicles require 15–17 days and up to 19–21 days, respectively.³³ Thus, upon pairing transplant recipients with breeders 14 days after transplantation, the surviving secondary follicles were already past possible ovulation, and only the surviving primary follicles would have had sufficient time to develop for ovulation. The fibrin-HBP-VEGF group became pregnant 17 ± 3 days after transplantation, which is substantially less than the 39 ± 6 days in control conditions. The longer time required for conception in the control suggests that primordial follicles were the only follicle class that preserved fertility and that primary and secondary follicles did not

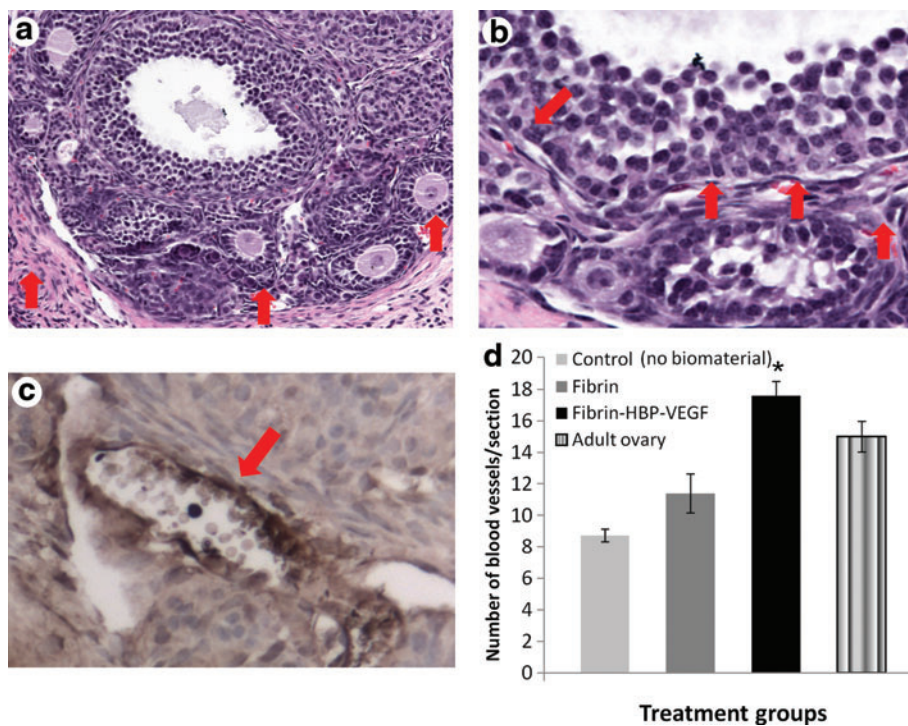


FIG. 5. Vascularization of the transplanted ovarian tissue. (a–c) Blood vessels (arrows) in ovarian tissue after transplantation in fibrin-HBP-VEGF, H&E 100 $\times$, 200 $\times$, immunohistochemistry 400 $\times$; (d) average number of blood vessels in different groups. Color images available online at www.liebertonline.com/tea

contribute to the pregnancies. Primordial follicles reside in the ovarian cortex, and may be able to survive by diffusion of nutrients from the surrounding tissue more effectively than the primary and secondary follicles that are positioned in the interior of the ovary. Taken together, the more rapid conception with fibrin-HBP-VEGF relative to the control points to the survival and function of primary follicles, as suggested by the timeline of live births, which may result from a more rapid revascularization.

Discussion

We hypothesized that encapsulating the ovarian tissue in fibrin-HBP hydrogels containing heparin-bound VEGF

TABLE 2. BREEDING RESULTS OF THE GRAFTS ENCAPSULATED IN FIBRIN-HEPARIN-BINDING PEPTIDE-VASCULAR ENDOTHELIAL GROWTH FACTOR AND THE CONTROL GROUP

	Control (no biomaterial)	Fibrin- HBP-VEGF	Wild type (C57Bl/6J)
Number (%) of fertile mice	5 (83%)	6 (100%)	N/A
Days between pairing and first litter (median)	44 $\pm$ 6 ^a (46)	22 $\pm$ 3 ^a (21)	N/A
Total litters	15	16	N/A
Mice per litter ($\pm$ STD)	5.4 $\pm$ 3	4.6 $\pm$ 2.2	6.6
Litters per dam	2.5	2.6	3.8

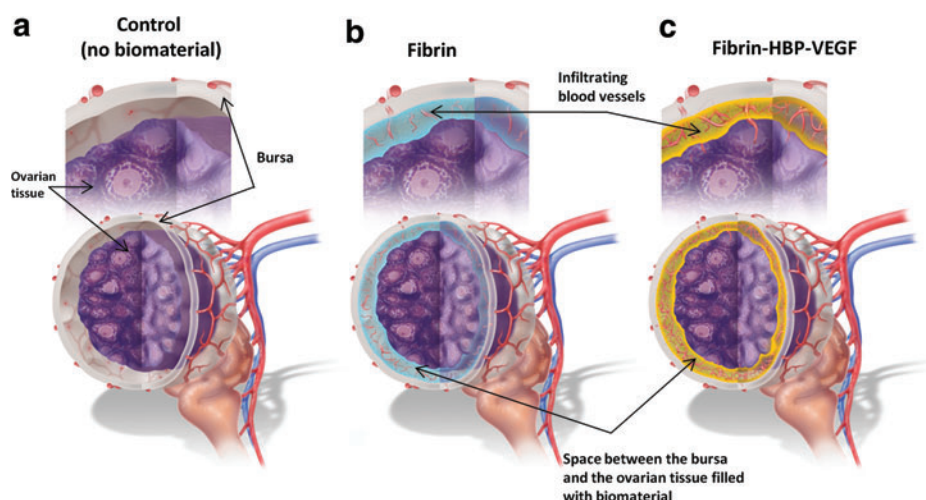
Number of days to conception after transplantation was compared between two groups by using log-rank test.

^aStatistically significant difference in the average time until the first litter was born.

would reduce post-transplantation ischemia and improve engraftment (Fig. 6). While previous reports of ovarian tissue transplants in a range of species and transplant sites are too numerous to list, the majority of these studies used the tissue as is with no attempt was made to modify its angiogenic potential with biomaterials or growth factor delivery. The modification of fibrin with HBP has been used to provide a controlled release of heparin binding growth factors, with the release rate controlled by the peptide affinity and molar ratio of peptide to heparin.^{18,19} Controlled release from the fibrin-HBP hydrogels provides immediate gradients of angiogenic factors to attract endothelial cells (ECs). Further, once ECs are activated, the fibrin encapsulating the ovary serves as a bridge between the graft and the host for EC migration and proliferation for blood vessel development. The host-graft cross talk promoted by the fibrin scaffold can additionally facilitate the restoration of the hypothalamic-pituitary-gonadal axis by exposing the more mature and gonadotropin-responsive follicles in the graft to the elevated FSH levels. In response, the follicles will secrete steroids that provide negative feedback on the HPG axis, thus restoring hormonal cyclicity. A fibrin scaffold mimics physiological angiogenesis, where VEGF delivery likely induced plasma protein leakage that can cause the formation of an extravascular fibrin gel, which is a substrate for EC growth.³⁴ The heparin and HBP immobilizes VEGF to the fibrin matrix to prolong its activity and release.^{18,19}

Efficient revascularization after tissue grafting contributed to the resumption of ovarian function and the longevity of the graft. For a metabolically active tissue such as the ovary, diffusion is limited to a couple hundred microns³⁵ and the presence of empty follicles in the grafts is believed to be a result of ischemia as was reported by Dolmans and colleagues.³⁶ Therefore, the survival of developing follicles is dependent on revascularization. To limit the duration of

FIG. 6. Schematic presentation of the hypothesis: **(a)** ovarian tissue transplanted in the bursal cavity lack the spatial connection to the host; **(b)** ovarian tissue encapsulation in degradable fibrin allows physical connectivity that may result in improved communication between the host and the graft and serve a continuous path for cell infiltration; **(c)** additionally to the described advantages of **(b)** controlled release of VEGF promoted revascularization. Color images available online at www.liebertonline.com/tea



revascularization, slices of ovarian cortex not thicker than 500 μm are used because follicles are sensitive to hypoxia. Further, primordial follicles reside exclusively in ovarian cortical strips that can be grafted only avascularly.¹¹ An increase in follicle survival within the transplanted ovarian tissue may enhance the duration over which the ovarian tissue functions. Microsurgical transplantation of an intact ovary is an alternative that can use the vasculature of the donor ovary. This approach is technically more difficult, and is limited by the absence of procedures to effectively cryopreserve an intact whole ovary.¹¹

The results suggest that the biomaterial support synergizes with protein delivery to enhance engraftment and function. The extracellular matrix (ECM) normally provides critical support for vascular endothelium primarily through adhesive interactions with integrins on the EC surface for their organization into blood vessels. Although much emphasis has been placed on the role of angiogenic cytokines, such as VEGF in EC migration, survival, and proliferation, considerable evidence indicates that ECM is equally or more important.³⁷ Moreover, in most cases, cytokine function is entirely dependent on EC adhesion to ECM to promote migration, which is particularly important during sprouting of new blood vessels from the existing vasculature. Evidence continues to increase that the ECM also drives capillary morphogenesis through sustained signaling, resulting in persistent EC cytoskeletal reorganization and changes in cell shape.^{37,38} Sprouting ECs may migrate in response to both chemotactic gradients of angiogenic factor and haptotactic gradients in ECM. Regardless, all motility-promoting stimuli are dependent on EC adhesion to ECM.³⁷

Our results are consistent with previous experimental studies that indicate a considerable decrease in the number of primordial follicles, ~60%–70%,^{39–41} in grafted tissue that is related to delayed tissue revascularization. The transplant site can influence revascularization, with grafting to the back muscle region resulting in less primordial follicle loss and a better blood supply in comparison to other heterotopic locations such as kidney capsule or subcutaneous space.⁴² However, in heterotopic grafting, the absence of the ovarian environment may have an unknown effect on egg quality, and the need to perform *in vitro* fertilisation may be limiting. We report herein that tissue encapsulated in fibrin-HBP-

VEGF had three times more primordial follicles comparing to unencapsulated tissue. In the fibrin-HBP-VEGF and control groups, primordial follicles represented 62% and 37% of the total follicle pool, respectively, demonstrating that less primordial follicles are activated as a result of the faster restoration of normal ovarian function. The fibrin-HBP-VEGF group resumed cycling before the control group, providing additional support that the fibrin scaffold provided a beneficial environment for both the immature primordial follicles as well as primary follicles that were responsible for faster restoration of ovarian function.

Orthotopic ovarian tissue transplantation restores endocrine function and fertility and allows natural conception. The fibrin-HBP-VEGF group started cycling earlier, became pregnant in the first week of pairing, and delivered their first litter 22 ± 3 days after, which is 3 weeks earlier than the control group. Based on the timeline of follicle development, earlier ovarian function and fertility result from the function of primary follicles that reside in deeper layers of the ovarian tissue, and thus can be more sensitive to delayed revascularization. The timeline suggests that rescued primary follicles were responsible for the first litter of offspring in the fibrin-HBP-VEGF group, whereas in the control group the pregnancies developed from primordial follicles. The litter size and the total number of litters, as well as the duration of fertility (5 months) were not significantly different between the two groups, which is expected based on mouse physiology. Obstruction of the oviduct that is connected to the bursa was a concern when transplanting tissue or a biomaterial. However, fibrin-HBP-VEGF and control groups naturally conceived and delivered pups, which proves complete degradation of the fibrin and functioning oviduct and uterus despite the 2-week menopausal period after ovariectomy.

This study demonstrates the potential of engineering biomaterials to enhance angiogenesis during engraftment of ovarian tissue and, to our knowledge, represents the first study utilizing biomaterials for ovarian transplantation. Silber *et al.*⁴³ recently published a long-term follow-up of a series of fresh and cryopreserved transplants in humans, and reported promising results of ovarian function and fertility for 1 to greater than 4 years post-transplantation. The importance of orthotopic location, careful preparation of thin wafers of tissue, and close apposition of the graft to the

vascular bed is required for successful graft function. Fibrin is currently used in clinics as glue in surgical procedures in the form of Hemaseel™; thus, application of fibrin-HBP-VEGF between the graft and host could be readily translated to human applications. For applications to cancer patients, the risk of metastatic involvement (e.g., leukemia, neuroblastoma, and breast cancer) must be considered because of the potential for cancer cell transmission in the graft.

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Disclosure Statement

The authors of this article have no conflicts of interest to disclose.

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