GYNECOLOGY

First pregnancies, live birth, and in vitro fertilization outcomes after transplantation of frozen-banked ovarian tissue with a human extracellular matrix scaffold using robot-assisted minimally invasive surgery

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BACKGROUND: Ovarian tissue cryopreservation is an experimental fertility preservation method and the transplantation techniques are still evolving.

OBJECTIVE: We attempted to improve the technique with the utility of a human decellularized extracellular tissue matrix (ECTM) scaffold, robot-assisted minimally invasive surgery, and perioperative pharmacological support.

STUDY DESIGN: We prospectively studied 2 subjects with hemophagocytic lymphohistiocytosis (patient A) and non-Hodgkin lymphoma (patient B) who underwent ovarian tissue cryopreservation at the age of 23 years, before receiving preconditioning chemotherapy for hematopoietic stem cell transplantation. Both experienced ovarian failure postchemotherapy and we transplanted ovarian cortical tissues to the contralateral menopausal ovary 7 and 12 years later, using a human ECTM scaffold and robotic assistance. The ECTM scaffold tissue compatibility was shown in preclinical studies. Patients also received estrogen supplementation and baby aspirin preoperatively to aid in the revascularization process.

RESULTS: Ovarian follicle development was observed approximately 10 (patient A) and 8 (patient B) weeks after ovarian tissue transplantation. Following 8 and 7 cycles of in vitro fertilization, 9 and 10 day-3 embryos were cryopreserved (patients A and B, respectively). While the baseline follicle-stimulating hormone (range 3.6-15.4 mIU/mL) levels near normalized by 7 months and remained steady postovarian transplantation in patient A, patient B showed improved but elevated follicle-stimulating hormone levels throughout (range 21-31 mIU/mL). Highest follicle yield was achieved 14 (8 follicles; patient A) and 11 (6 follicles; patient B) months postintervention. Patient A experienced a chemical pregnancy after the third frozen embryo transfer attempt. She then conceived following her first fresh in vitro fertilization embryo transfer and the pregnancy is currently ongoing. Patient B conceived after the first frozen embryo transfer attempt and delivered a healthy girl at term.

CONCLUSION: We report the first pregnancies after the minimally invasive transplantation of previously cryopreserved ovarian tissue with an ECTM scaffold. This approach seems to be associated with steady ovarian function after a follow-up of up to 2 years.

Key words: AlloDerm, fertility preservation, in vitro fertilization, ovarian cryopreservation and transplantation, robotic surgery, translational research

Introduction

Ovarian cryopreservation is one of the key strategies in fertility preservation. Utilizing the previously accumulated knowledge from animal[1] and human[2] ovarian xenografting studies, we performed the first autologous ovarian tissue transplantation (OTT) case with frozen-thawed tissue in 1999.[3,4] Although the patient did not desire pregnancy, she demonstrated ovarian

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follicle development 8 weeks after the transplant with documented function for up to 9 months.[5] The live births were to follow several years later as reported by numerous investigators.[6-9] In addition, we reported heterotopic OTT techniques where the tissues were grafted subcutaneously under the forearm or abdominal skin.[10,11] The latter resulted in oocyte retrievals and embryo development.

With the current techniques, akin to skin grafting, one has to rely on the natural process of revascularization from the recipient site. Because it takes up to 10 days for new human ovarian microvessels to reach full maturity, the graft suffers an initial ischemic injury.[12] This initial ischemic injury has been shown to result in the loss of nearly two thirds of all primordial follicles in ovarian xenograft models.[13] This inefficiency largely

explains the unpredictable longevity of ovarian transplants. The length of ovarian function has been reported to range from 1 to >7 years[14,15] with an average of 4-5 years in successful orthotopic and heterotopic ovarian transplants with frozen-banked ovarian tissue.[15]

In addition, issues with oocyte quality have been cited by some after in vitro fertilization (IVF); one possible explanation being the restricted blood flow.[16] The baseline follicle-stimulating hormone (FSH) levels remain high and antimüllerian hormone (AMH) levels tend to be low in the majority of ovarian transplants with cryopreserved tissue, possibly reflecting the restriction in blood flow.[17] Therefore, there is convincing evidence to support the view that vascularization issues curb OTT success both acutely in the form of follicle loss and chronically with limited microvascular flow.

AlloDerm (LifeCell Corp, Branchburg, NJ) is a decellularized human extracellular tissue matrix (ECTM) generated from cadaver skin. It has been used in cosmetic surgery, breast reconstruction, and dentistry and other surgical reconstructive fields to augment tissue grafts and aid in revascularization.[18] In this study we hypothesized that the cryopreservation of ovarian tissue may preserve fertility. We also hypothesized that the use of the ECTM with robotic surgical assistance may improve outcomes, presumably by aiding the revascularization process.

Materials and Methods

This translational work represents the outcomes ovarian cryopreservation and transplantation research protocol after up to 14-year follow-up of 2 subjects who consecutively underwent grafting. The study was approved by the institutional review board and the preclinical animal studies were also approved by the Institutional Animal Care and Use Committee at New York Medical College.

Patient A was diagnosed with familial hemophagocytic lymphohistiocytosis in April 2006, at the age of 23 years. Shortly before the preconditioning chemotherapy (thiotepa and fludarabine) and total body irradiation (1375 cGY) for hematopoietic stem cell transplant, we performed a laparoscopic oophorectomy. Ovarian cortical strips were cryopreserved with a slow freeze protocol.[19] In April 2013, her blood work was consistent with menopausal state (FSH 108.7 mIU/mL; luteinizing hormone [LH] 61.4 mIU/mL; estradiol [E_2] <6 pg/mL; AMH <0.16 ng/mL) and a transvaginal ultrasound showed that the remaining ovary and the endometrium to be atrophic.

Patient B was diagnosed with non-Hodgkin lymphoma at the age of 17 years and completed a chemotherapy regimen including cyclophosphamide (600 mg/m^2 × 8 courses every 3 weeks), prednisone, procarbazine, doxorubicin, bleomycin, and vinblastine in October 1996. She experienced mediastinal recurrence at the age of 23 years. We performed ovarian cryopreservation with

a protocol identical to patient A, shortly before receiving the highly gonadotoxic regimen of ifosfamide, carboplatin, etoposide, and dexamethasone prior to hematopoietic stem cell transplant. In July 2013, her FSH and LH levels were found to be 54 and 70 mIU/mL, and 47 and 33 mIU/mL 1 month apart, respectively. Her AMH was <0.16 ng/mL with ultrasound examination showing no obvious healthy follicles, confirming the diagnosis of ovarian failure.

Follicle density assessment

In patient A, because the diagnosis was noncancer and an ovarian sample to rule out metastasis was not needed at the time of ovarian tissue cryopreservation we could not assess precryopreservation follicle density. However, 1 vial of tissue was thawed and the follicle density was assessed before the OTT. This revealed a mean follicle density of 1.66 ± 0.37 follicles/mm^2. Based on these results, and after discussion with the couple, we empirically decided to thaw 5 of 10 vials, containing 10 pieces of ovarian tissue for transplantation.

Likewise, in patient B, 1 vial of tissue was thawed prior to OTT, and revealed a mean follicle density of 0.62 ± 0.32 follicles/mm^2. Although patient B was of similar age to patient A at the time of ovarian tissue cryopreservation, this density was significantly lower than that of with patient A ($P < .05$). Furthermore, when compared to patient's precryopreservation follicle density of 1.4 follicles/mm^2, >50% of follicles appeared to have been lost during freezing and thawing. Based on these results and following a discussion with the couple, we empirically decided to thaw and transplant 6 of 12 vials, containing 12 pieces of ovarian tissue.

Preoperative preparation

Before the procedure, both patients underwent a hysterosonogram and the partners were evaluated with a semen analysis. In addition, patient A also underwent a pelvic magnetic resonance imaging to rule out any obvious total body irradiation–induced uterine damage. Ten weeks prior to the procedures, both patients received transdermal

0.1 mg E_2 (Climara; Bayer Healthcare Pharmaceuticals Inc, Whippany, NJ) weekly and vaginal progesterone 100 mg (Prometrium; Schering-Plough, Kenilworth, NJ) nightly with a 2-week-on/2-week-off regimen. This regimen was continued after the transplant until a sign of ovarian function was seen. Hormone replacement was given as there is some evidence from animal studies that this may improve ovarian vascularization.[20] Again, with the aim of enhancing revascularization, both patients were also given daily baby aspirin 81 mg (Bayer Healthcare Pharmaceuticals Inc) for 7 days, which was discontinued 2 days before the surgery.
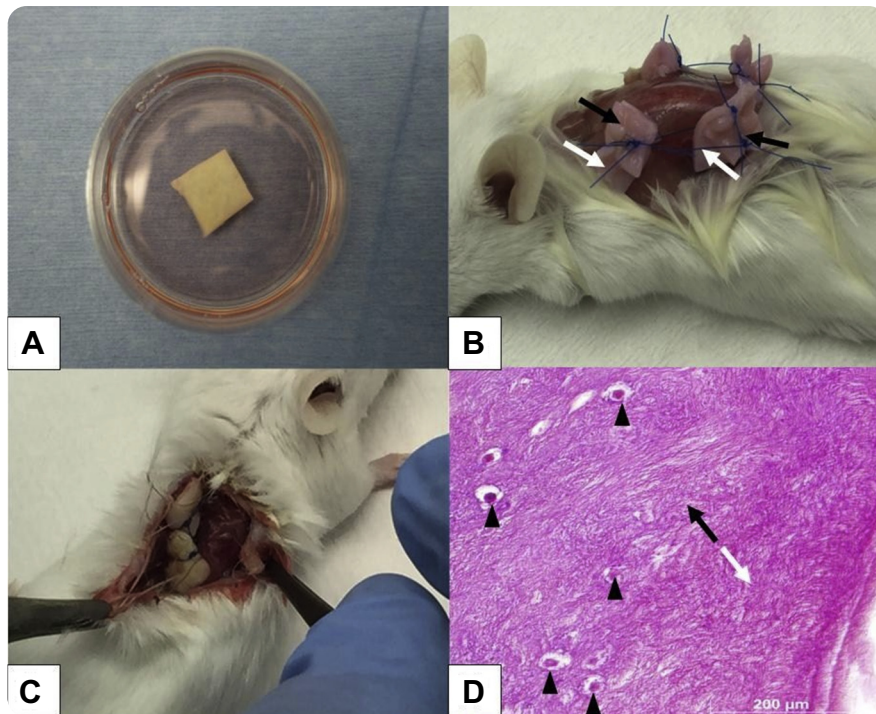
Preclinical evaluation of ECTM

This ECTM has been used in the surgical field but there is no description of its use in reproductive surgery. To ensure its compatibility with ovarian tissues, we performed a series of preclinical evaluations. First we evaluated various thicknesses (thin, medium, full) of the ECTM in thawing media (Figure 1, A) as well as simulating its use with ovarian cortical pieces from organ donor cadavers to determine the best thickness for handling. We found the medium-thickness ECTM to be sturdy yet malleable and hence next tested it in a xenograft model. We subcutaneously xenografted 4- × 4-mm ovarian cortical pieces together with ECTM to immunodeficient mice (Figure 1, B), as we previously described.[21] After 10 days of xenografting, the tissues were evaluated. We found that ovarian stroma had integrated into ECTM, without any pathological changes (Figure 1, C and D).

Next we cultured mouse oocytes (N = 30/group) with or without ECTM for 16 hours and assessed survival. We found that there was no difference in oocyte survival when ECTM was used, compared to controls (88.1% vs 81.6%, $P = .39$). These findings provided assurance that ECTM was compatible with ovarian tissues.

Ovarian transplant technique

Robotically assisted OTT procedures were performed in July 2013 on patient A at the age of 30 years (7 years after

FIGURE 1
Preoperative evaluation of extracellular tissue matrix (ECTM) scaffold

Preclinical evaluation of ECTM for ovarian transplantation. **A**, Medium-thickness ECTM during rehydration. **B**, Xenografting of ECTM (white arrows) with ovarian tissue (black arrows) to severe combined immunodeficiency mouse. **C**, Gross and **D**, histological evaluation showing proper integration of ECTM (white arrow) into ovarian tissue (black arrow). Transition zone begins where tails of 2 arrows oppose. Note presence of normal-appearing primordial follicles (arrowheads) near transition zone.

Oktay et al. Improving ovarian transplantation success. Am J Obstet Gynecol 2016.

ovarian cryopreservation), and on patient B in October 2013 at the age of 35 years (12 years after cryopreservation). The OTT technique is described in Supplement 1 and illustrated in Video S1.

Statistical methods
Statistical analyses were performed with software (SPSS 15 for Windows; IBM Corp, Armonk, NY) using Student t test (follicle density comparisons) or Fisher exact test (survival rates of mouse oocytes). A P value of $\leq.05$ was considered statistically significant.

Results
Patient A
The first sign of ovarian activity was detected approximately 10 weeks after OTT in October 2013, by the demonstration of a 14.5-mm follicle on a transvaginal ultrasound exam. The hormone replacement was then discontinued and the patient resumed cyclical menstruation (Figure 2, A).

Subsequently, she underwent 8 IVF cycles with peak E_2 levels as high as 833 pg/mL (Table 1) (see Supplement 2 for monitoring and IVF protocol). The highest follicle yield (8 follicles) was obtained on her last stimulation cycle, 14 months post-OTT (Figure 3, A), when 6 oocytes were retrieved. In all, 31 follicles developed in total during these 8 cycles, of which 10 were ≥17 mm on the trigger day. Of the 12 metaphase II oocytes collected during these 8 cycles (Table 1), 10 fertilized after intracytoplasmic sperm injection with a fertilization rate of 83.3%; 9 day-3 embryos were cryopreserved (Figure 3, B). We proceeded with embryo cryopreservation rather than fresh IVF-embryo transfer because the ovarian

graft longevity could not be predicted and the patients wished to preserve embryos for future pregnancy attempts.

We initially attempted pregnancy with frozen embryo transfers (FETs); the third FET resulted in a biochemical pregnancy. We then proceeded with a fresh cycle of ovarian stimulation. This resulted in the retrieval of 2 mature oocytes both of which were fertilized and developed into grade-A embryos on day 3 (Figure 3, C, left panel). Considering these together with the frozen embryo cycles, the overall fertilization rate reached 85.7%. The transfer of these 2 embryos resulted in a pregnancy. Fetal heart activity was shown on the seventh week of gestation with appropriate crown-rump length measurements (Figure 3, C, right panel). A fetal free-DNA test (MaterniT21[1]PLUS; Sequenom, San Diego, CA) performed at the 11th week of gestation showed normal complement for chromosomes 13, 18, and 21 and indicated female gender. The pregnancy is currently ongoing at the 23rd week of gestation.

The patient's baseline E_2 and FSH levels normalized by 7 months post-OTT and remained at or near normal early follicular levels up until the time of FETs. However, on intermittent evaluations, the AMH measurements remained <0.16 ng/mL. After a pause for FETs, she underwent 2 additional baseline evaluations at approximately 22 and 23 months post-OTT. These showed baseline E_2 and FSH levels of 10 pg/mL and 3.6 mIU/mL and of 53.5 pg/mL and 14.2 mIU/mL, respectively, with 2 antral follicles on each transvaginal ultrasound. The patient conceived following the last baseline hormonal evaluation. This indicated the persistence of ovarian function for up to 2 years after the transplant (Figure 3, D).

Patient B
Four weeks after the OTT, small follicles were suspected on a transvaginal ultrasound, which prompted the discontinuation of hormone replacement therapy. Four weeks later her laboratory evaluation showed an E_2 of 264 pg/mL, FSH of 6 mIU/mL, LH of 6.3 mIU/mL, and a progesterone value of 0.5 ng/mL (Figure 2, B).

FIGURE 2
Timeline from freezing to postovarian transplant
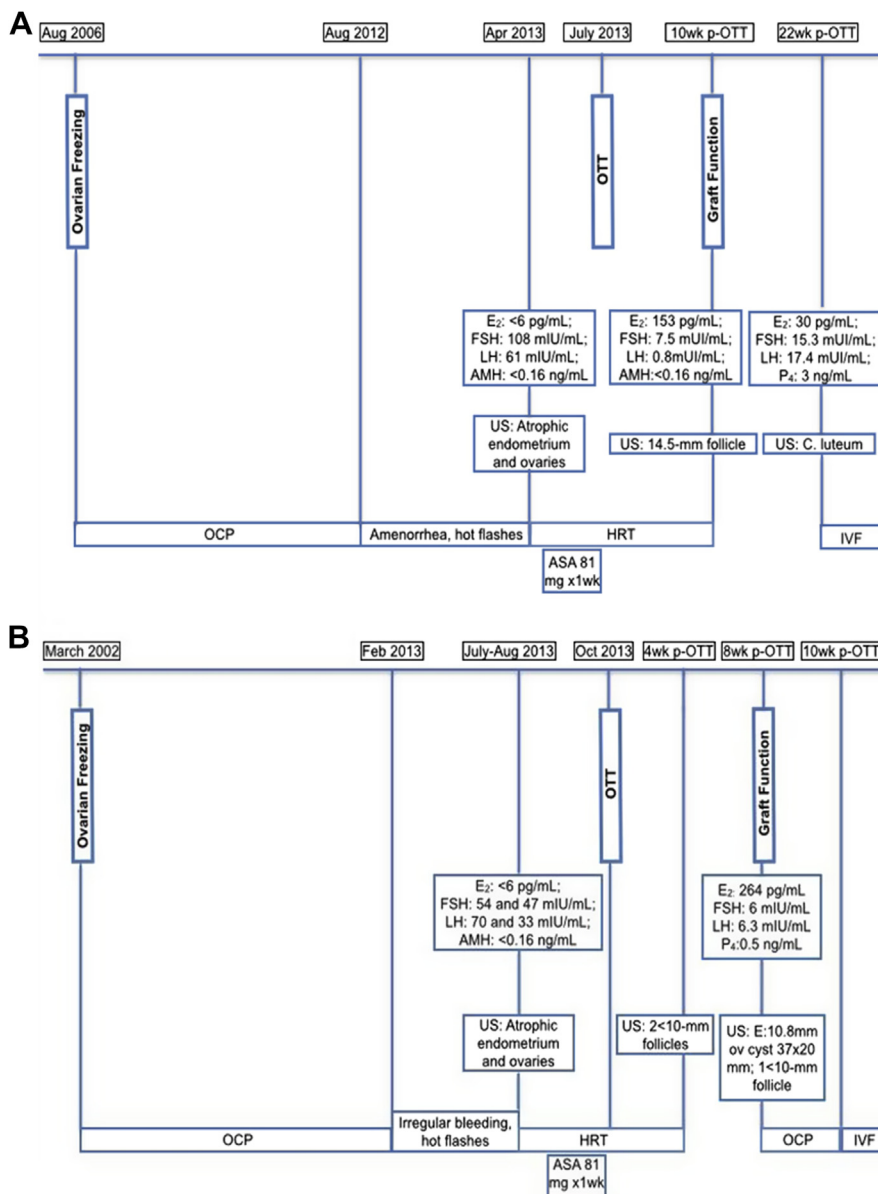
Timeline of events from ovarian cryopreservation to immediately post-transplantation. **A**, Patient A and **B**, patient B.

AMH, anti-müllerian hormone; *ASA*, acetylsalicylic acid; *C*, corpus; *E*, endometrial thickness; *E₂*, estradiol; *FSH*, follicle-stimulating hormone; *HRT*, hormone replacement therapy; *IVF*, in vitro fertilization; *LH*, luteinizing hormone; *OCP*, oral contraceptive pill; *OTT*, ovarian tissue transplant; *P₄*, progesterone; *p-OTT*, postovarian tissue transplant; *US*, ultrasound.

Oktay et al. Improving ovarian transplantation success. Am J Obstet Gynecol 2016.

Baseline FSH levels showed continual improvement (Figure 3, E) after the OTT but remained above normal early follicular phase levels. Intermittent serum measurements showed that the AMH levels remained <0.16 ng/mL although the peak E_2 levels reached as high as 650 pg/mL. The highest number of follicles (6 follicles) developed 11 months post-OTT (Figure 3, F).

Eight months post-OTT, we performed a laparoscopic salpingectomy to remove a contralateral hydrosalpinx. Laparoscopic evaluation showed that the ECTM graft had completely integrated with the patient's native ovarian tissue and could not be discerned (Video S1 and Figure 3, G), while confirming unilateral tubal patency.

A total of 22 follicles developed in response to 7 cycles of ovarian stimulation, of which 7 were ≥17 mm. These yielded 10 metaphase II oocytes (Table 1); 100% were fertilized and cryopreserved on day 3 (Figure 3, H). We then performed a FET with 2 day-3 embryos graded as 8-cell-A and 10-cell-B, which resulted in a positive pregnancy test. A fetal free-DNA test (MaterniT21[1]PLUS) performed at 11th week of gestation showed normal complement for chromosomes 13, 18, and 21 and indicated female gender. A fetal anatomical scan on the 20th week of gestation also revealed a healthy fetus (Figure 3, I). The pregnancy has progressed to term without any complications. The patient had a normal spontaneous vaginal delivery of a healthy female child weighing 3617 g with Apgar scores of 9/9 at 39 weeks 5 days of gestation. The child is currently 4 months old and developing normally.

Comment
Ovarian cryopreservation is a highly important fertility preservation strategy, which has several advantages over gamete and embryo freezing. It can be performed in prepubertal girls and children,[22,23] does not require ovarian stimulation and delay in chemotherapy, restores ovarian endocrine function, and provides the possibility of natural conception. However, despite the mounting number of live births, the technique still remains in the experimental realm. Those who defend the experimental status for ovarian freezing and transplantation generally cite the relatively brief experience, limited number of pregnancies, and brevity of ovarian function.

In this translational study we presented 2 successful cases of OTT with a new approach, which resulted in robust ovarian function and pregnancies, with one progressing to term. Based on a literature review in all available languages including English in PubMed, as well as

TABLE 1
Ovarian stimulation outcomes in ovarian transplantation patients

Cycles	Peak E$_2$,[a] pg/mL	Follicles,[b] n	MII, n	Embryos, n	Embryo grade (and outcome)
Patient A-1	208	2 (1)	1	1	6c-B (RF)
Patient A-2	352	3 (1)	1	1	6c-B (RF)
Patient A-3	808	6 (2)	2[c]	2	8c-A, 6c-B (both TT, NP)
Patient A-4	430	4 (1)	1	1	6c-B (RF)
Patient A-5	602	3 (1)	1	0	3c (degenerated)
Patient A-6	833	3 (1)	1	1	8c-A (TT, chemical pregnancy)
Patient A-7	353	2 (2)	0	0	N/A
Patient A-8	627	8 (1)	5[d]	3	3 × 8c-A (thawed, 2 transferred as blastocysts, NP)
Patient A-9[e]	482	2	2	2	Compacting-A, 8-A (fresh transfer, ongoing pregnancy)
Patient B-1	236	2 (1)	1	1	6c-A (RF)
Patient B-2	543	4 (1)	3	3	8c-A, 10c-B, 5c-B (RF)
Patient B-3	445	2 (1)	0	0	N/A
Patient B-4	575	3 (1)	1[e]	1	6c-A (RF)
Patient B-5	168	3 (2)	2[f]	2	6c-B, 5c-B (RF)
Patient B-6	172	2 (1)	1	1	8c-A (RF)
Patient B-7	574	6 (0)	2	2	8c-A, 8c-B (both TT, live birth)

A or B on the embryo grade column, correspond to embryo grade on day 3, A being highest; c, cell; E$_2$, estradiol; MII, metaphase II oocyte; N/A, not available; NP, no pregnancy; RF, remains frozen; TT, thawed and transferred.

[a] Peak E$_2$ denotes highest measurement during stimulation, including posttrigger day; [b] Numbers in parenthesis denote those follicles ≥17 mm; [c] Only 2 oocytes could be retrieved due to premature luteinizing hormone surge and ovulation; [d] Includes 2 metaphase I oocytes in vitro matured to MII; [e] Did not mature in vitro; [f] Includes in vitro matured to MII from germinal vesicle stage.

Oktay et al. Improving ovarian transplantation success. Am J Obstet Gynecol 2016.

unpublished reports identified from review articles and abstract presentations, this is the first report of live birth and pregnancies after the use of this multipronged technique. In addition, our study has several other unique aspects. First, we for the first time reported outcomes of consecutive ovarian stimulation cycles and longitudinal follow-up for the response to such stimulation. Follow-up of baseline hormone measurements and response to ovarian stimulation for up to 2 years gave us a unique perspective as to how the function of OT may improve over time.

There is scant information on IVF cycles after OTT and the few reports indicate relatively limited response.[24] We observed a relatively high follicle yield (2-8 follicles/cycle, an average of 3.87 follicles/cycle in patient A and 2-6 follicles/cycle, an average of 3.14 in patient B). Interestingly, the highest number of follicles developed during the last

stimulation cycles for both patients, 14 and 11 months after OTT, indicating a continuous improvement in ovarian function and no obvious sign of graft exhaustion.

We have also observed high fertilization rates (85.7-100%); the reported fertilization rates from previous studies with oocyte originating from orthotopic transplants range from 0-100% with a mean of 43%.[24] The embryos from both patients were in general of high quality and patient B conceived after the first transfer. Interestingly, the same patient had received a chemotherapy regimen containing an alkylating agent prior to ovarian cryopreservation. The relatively higher baseline FSH levels in patient B compared to patient A may be explained by the reduction of primordial follicle density due to exposure to alkylating agents prior to cryopreservation. While patient A failed had a chemical pregnancy with the third FET, she conceived

when 2 fresh embryos were transferred. Despite the initial setbacks with patient A, high fertilization rates and embryo grades, as well as ongoing and delivered pregnancies point to high oocyte quality following the OTT with ECTM.

However, even in cycles with a relatively large number of follicles and multiple oocyte recovery, and high-quality embryos, E$_2$ levels were not proportionately high. Furthermore, intermittent AMH measurements remained <0.1 ng/mL, lower than expected given the number of follicles developed in response to ovarian stimulation. This may indicate that especially the venous vasculature in frozen-thawed OTTs may be at variance from that in a normal ovary. This could be in the form of a weakly formed venous microcirculation,[25] which is unable to reflect AMH production from preantral follicles.

There is also very limited information on pregnancies with frozen embryos

FIGURE 3
Ovarian transplantation outcomes
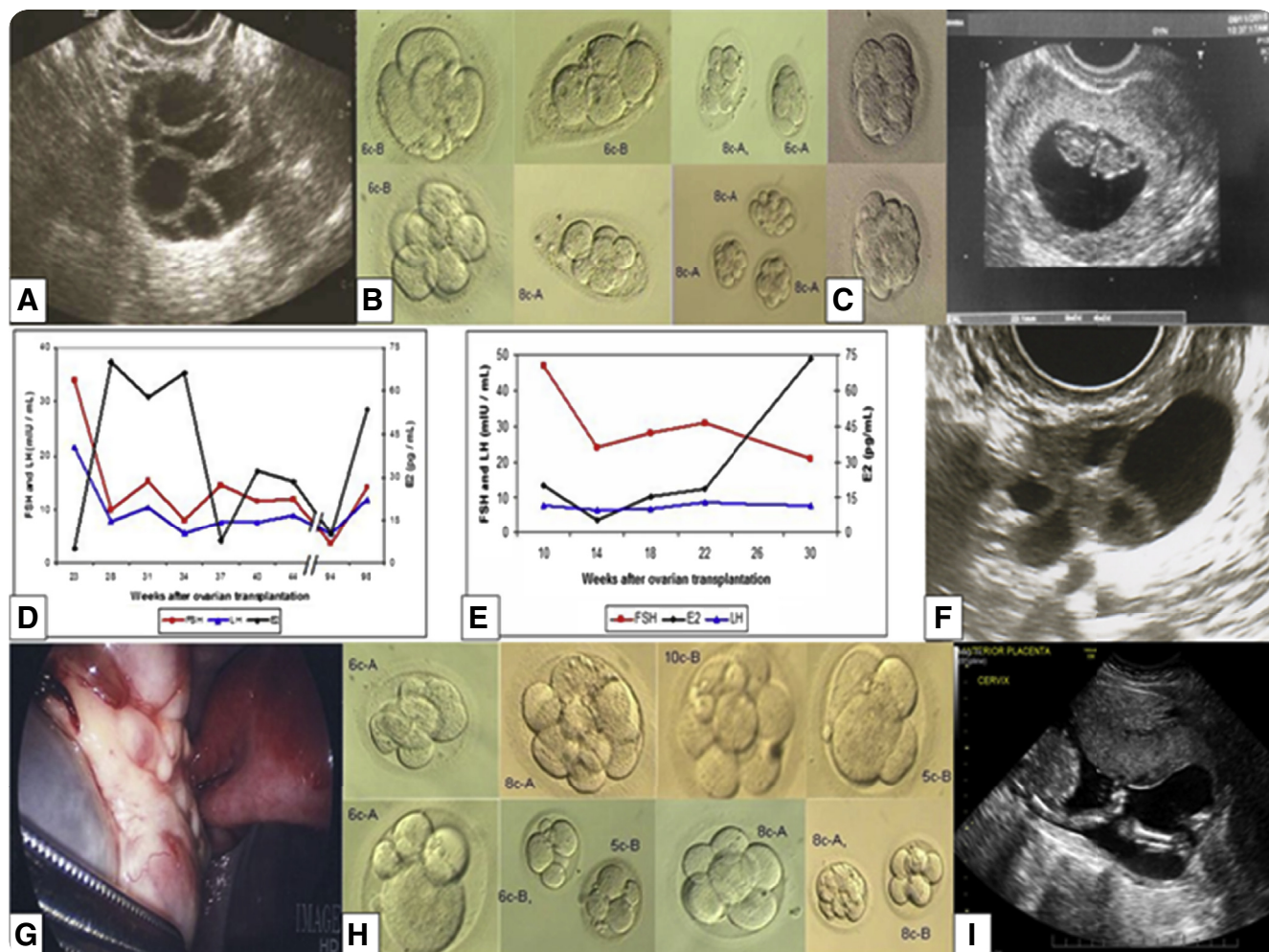
Robust ovarian function and IVF pregnancies after robot-assisted ovarian transplantation with ECTM. **A**, Robust response to ovarian stimulation in patient A, 14 months after ovarian tissue transplant (OTT). **B**, Nine embryos were frozen from patient A after 8 in vitro fertilization (IVF) cycles; embryo grades are indicated adjacent to each embryo. **C**, In addition, 2 embryos were transferred fresh in last IVF cycle, which resulted in ongoing pregnancy. **D** and **E**, Respective early follicular phase follicle-stimulating hormone (FSH), luteinizing hormone (LH), and estradiol (E2) levels in patient A and patient B, indicating improvement in ovarian function after OTT. **F**, Multiple follicle development 11 months post-OTT in patient B. **G**, Transplanted ovary at second-look laparoscopy. **H**, Eight embryos were frozen from patient B after 7 IVF cycles; embryo grades are indicated adjacent to each embryo. **I**, Twenty-week fetal anatomical scan shows normally developed fetus in patient B.

Oktay et al. Improving ovarian transplantation success. Am J Obstet Gynecol 2016.

originating from transplanted ovaries. While Sánchez-Serrano et al[26] cryopreserved metaphase II oocytes from 1 patient, which resulted in a pregnancy, only Suzuki et al[7] reported embryo vitrification in primary ovarian insufficiency patients undergoing OTT after follicle activation. These reports all together indicate that further cryopreservation of oocytes or embryos originating from previously frozen-thawed

and transplanted ovarian tissue may not necessarily be detrimental.

Patient B had received cyclophosphamide at doses known to cause significant ovarian damage.[27] The success with patient B also indicates that previous exposure to alkylating agents is not a contraindication for ovarian cryopreservation as some suggested.[28] Because young patients have larger ovarian reserve, they may be able to tolerate

partial losses and still be able to succeed with ovarian transplantation, even when a fraction of their tissues are thawed and transplanted as was the case in this report. In fact, 3 other studies reported pregnancies with ovarian tissues frozen after exposure to alkylating agents.[29-31]

From the current design, we cannot determine the relative contributions of peritransplant pharmacological management, the surgical site, use of ECTM,

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TABLE 2
Current outcomes and success rates with transplantation of cryopreserved ovarian tissue

Age at cryopreservation, y (range)	23.5 ± 4.7 (17–36)
Age at transplantation,[a] y (range)	30 ± 4.2 (21–38)
Maternal age at delivery, y (range)	35.6 ± 1.9 (25–39)
Gestational age at delivery,[b] wk (range)	38.1 ± 1.6 (33–41)
Clinical pregnancy/patient (%)	24/66[c] (36.3)[6,14]
Live + ongoing pregnancy/patient (%)	18/66[c] (27.2)[6,14]
Endocrine function/patient (%)	62/66[c] (93.9)[3,5,6,10,11,14,15]

Ages are mean ± SD.

[a] Seven patients had repetitive transplants from same cryopreserved tissue; [b] Four patients had consecutive deliveries after transplant; [c] Based on cited references and current report.

Oktay et al. Improving ovarian transplantation success. Am J Obstet Gynecol 2016.

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or minimally invasive surgery with robotic assistance to the success of our OTT technique. To prevent any damage to vascular bed, we refrain from cauterization during the procedure and the field of surgery may be too obscured for standard minimally invasive surgery. Robotic assistance may have advantages over standard laparoscopy because of the improved acuity of visualization with 3-dimensional view, increased dexterity with finely controlled instruments, ability to gently manipulate friable menopausal ovary as the recipient site, and more precise placement of sutures at desired locations. We also believe that it decreases the time from the removal of the tissue from the culture media to its transplantation to the vascular recipient site, thereby minimizing the time spent without sustenance. It is possible that the robust and sustained ovarian function observed with our OTT approach is the result of interaction of many factors mentioned above.

Considering the current potential live birth and unpublished reports,[6-9] the total number of children born after autologous transplantation of cryopreserved ovarian tissue for fertility preservation appears to be approximately 60.[32] However, efforts to determine success rates have been hampered by the limitation of the knowledge on the total number of attempts (denominator) as the studies tend to report only the successful attempts. When considering the few reports that included the number of attempts together with the pregnancies reported here,[6,15] the clinical pregnancy and ongoing plus live birth rates for OTT with cryopreserved tissue are 36.3% and 27.2% per patient, respectively (Table 2). As a unique feature of ovarian cryopreservation, which sets it apart from the other fertility preservation procedures, approximately 50% of the pregnancies were achieved without a need for assisted reproduction. Furthermore, 93.9% of the procedures resulted in the restoration of ovarian endocrine function, including those that were performed with heterotopic transplantation techniques (Table 2).[3,5,6,10,11,14,15]

In summation, in this translational study we reported the first live birth and an ongoing pregnancy with an OTT technique utilizing a human ECTM as a scaffold, with robot-assisted minimally invasive surgery. Ovarian tissue cryopreservation is evolving as an effective fertility preservation approach. Overall, the quest for improving ovarian transplant techniques and outcomes is continuing. This includes pharmacological agents than can accelerate the revascularization of ovarian transplants.[12] The currently reported OTT approach appears to provide robust and reproducible results and we will continue to assess its efficacy in larger trials. ∎

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Supplement 1
Ovarian transplantation technique

After the induction of anesthesia, we started with the rehydration of extracellular tissue matrix (ECTM) in warmed saline solution (up to 37°C) and the thawing process, both lasting for approximately 40 minutes. Under an operative microscope, the thawed cortical pieces were appended to ECTM with 5-0 Monocryl (11-mm 3/8C needle) (Ethicon Inc., Somerville, NJ) by positioning the cortical pieces with stromal side exposed, then passing the needle through ECTM, stroma, cortex, and then back through ECTM, and tying a knot on the opposite side (Video S1). We then trimmed the ECTM to leave a ∼5-mm tissue-free rim all around. Meanwhile, we made skin incisions and inserted 5 ports: a 12-mm for the camera, a 12-mm right upper quadrant for access, and three 8-mm ports for instrumentation. Upon gaining access to the pelvic cavity, we first exposed the remaining menopausal ovary. We then bivalved it using curved scissors, avoiding cauterization. This exposed the medulla and created a vascular bed for the graft as well as doubling the surface area available for transplantation. Next, we introduced the graft in to the abdominal cavity through the 12-mm assist port. The graft was then juxtaposed on the ovary so that the stromal sides of the cortical pieces oppose the exposed stroma of the bivalved ovary. We then anastomosed the edges of the ECTM scaffold to the edges of ovarian cortex by interrupted sutures using 4-0 Vicryl (Ethicon Inc., Somerville, NJ).

Supplement 2
Ovarian function assessment and in vitro fertilization procedures

We began to monitor the grafts by transvaginal ultrasound and endocrine assessment 4 weeks after the transplantations as ovarian function has not been reported to return sooner. Once ovarian function was confirmed, we began controlled ovarian stimulation with follicle-stimulating hormone 225-450 IU (Gonal-F; Merck & Co Inc, Whitehouse Station, NJ); a gonadotropin-releasing hormone-antagonist (Ganirelix; Merck & Co Inc) was added when the leading follicle reached 13-mm diameter. Oocyte maturation was triggered with human chorionic gonadotropin 250 μg (Ovidrel; Serono Inc, Rockland, MA) alone or using a dual trigger with human chorionic gonadotropin 125 μg (half vial) plus leuprolide acetate 60 IU (Lupron; AbbVie Inc, North Chicago, IL) when lead follicles reached ≥17 mm in diameter.

Oocyte retrievals were performed with a double lumen ovum aspiration needle (Cook; Cook Medical, Bloomington, IN). Mature oocytes (metaphase II oocyte) were subjected to intracytoplasmic sperm injection and the immature oocytes were cultured for up to 24 hours and subjected to intracytoplasmic sperm injection if matured. Because both patients wished to bank embryos for future additional pregnancies and since the longevity of ovarian transplants could not be predicted, we first intended to accumulate numerous frozen embryos before attempting pregnancy.

Embryos were cultured in Continuous Single Culture media (Irvine Scientific, Santa Ana, CA), and were vitrified in Cryotip (Irvine Scientific) on day 3.

Before vitrification, embryos were graded according to their morphological appearance. Embryos exhibiting equal blastomeres size and no fragmentation were considered grade A; embryos with blastomeres of equal/mostly equal size with minor-moderate fragmentation were classified as grade B; embryos with blastomeres of unequal size and moderate fragmentation were classified as grade C; and embryos with blastomeres of equal to unequal size with moderate to heavy fragmentation were considered grade D.

For frozen embryo transfers, the cycle was programmed first by down-regulating the pituitary during the late luteal phase with a gonadotropin-releasing hormone agonist (Lupron; AbbVie Inc), followed by estrogen 0.1 mg (Climara; Bayer Healthcare Pharmaceuticals Inc, Whippany, NJ) replacement (1-2 patches twice a week) via the transdermal route. When the endometrium reached >7-mm thickness and attained a trilaminar pattern, we added vaginal progesterone inserts 100 mg twice a day (Endometrin; Ferring, Parsippany, NJ). This was supplemented by a 1-week 50 mg intramuscular progesterone in oil treatment during the peri-implantation period. Embryo transfers were performed with Wallace Sure View Ultrasound Embryo Replacement Catheters (Smiths Medical, St Paul, MN) 6.5 days after the initiation of the progesterone supplementation.