Table 3 continued

	I ask co-medical staff if a patient has an interest in fertility				I provide my patients with educational material about fertility preservation				I use LHRH analogue to preserve fertility			
	p	OR	95% CI		*p*	OR	95% CI		*p*	OR	95% CI	
			Min	Max			Min	Max			Min	Max
Knowledge												
Fair	0.242				0.125				0.653			
Not fair												
Attitude												
Conservative	0.895				0.100				0.248			
Aggressive												
Gender												
Female	0.133				0.047	1.183	0.973	1.440	0.399			
Male												
Age												
<50	0.262				0.416				0.914			
>50												
Specialty												
Surgery	0.105				0.066				0.057			
Others												
Affiliation												
University hospital/cancer center	0.795				0.046	1.000			0.656			
General hospital/private hospital						1.671	0.959	2.911				
Female physician colleague												
Present	0.793				0.026	1.919	1.014	3.632	0.259			
Absent						1.000						
Medical oncologist colleague												
Present	0.443				0.407				0.381			
Absent												
Breast cancer-specialized nurse												
Present	0.316				0.871				0.516			
Absent												
Board-certified cancer pharmacist												
Present	0.900				0.325				0.663			
Absent												
Number of breast surgeries per week												
1–5	1.000				0.273				0.402			
6–												
Number of young patients per week												
0–1	0.583				0.721				1.000			
2–												
Partner/spouse												
Present	0.192				1.000				0.828			
Absent												
Children												
Present	0.614				1.000				0.156			
Absent												

Table 3 continued

	I consult a reproductive specialist with questions about fertility issues in my patients				I refer patients who have questions about fertility to reproductive specialists			
	p	OR	95% CI		*p*	OR	95% CI	
			Min	Max			Min	Max
Knowledge								
Fair	0.442				0.162			
Not fair								
Attitude								
Conservative	0.032	1.000			0.003	1.656	1.183	2.319
Aggressive		1.599	1.014	2.798		1.000		
Gender								
Female	0.039	1.121	0.995	1.277	0.001	1.176	1.062	1.302
Male		1.000				1.000		
Age								
<50	0.264				0.004	1.424	1.110	1.828
>50						1.000		
Specialty								
Surgery	1.000				0.795			
Others								
Affiliation								
University hospital/cancer center	0.007	1.349	1.067	1.706	0.012	1.243	1.047	1.474
General hospital/private hospital						1.000		
Female physician colleague								
Present	0.051	1.467	0.995	2.164	0.123			
Absent		1.000						
Medical oncologist colleague								
Present	0.103				0.042	1.212	1.011	1.453
Absent						1.000		
Breast cancer-specialized nurse								
Present	0.710				1.000			
Absent								
Board-certified cancer pharmacist								
Present	0.803				0.138			
Absent								
Number of breast surgeries per week								
1–5	0.785				1.000			
6–								
Number of young patients per week								
0–1	0.270				0.813			
2–								
Partner/spouse								
Present	0.807				0.670			
Absent								
Children								
Present	0.197				0.209			
Absent								

of reproductive specialists or infertility clinic for referral (38%) were the major causes for them not to discuss fertility with patients.

Discussion

This study describes the attitude of the main providers of breast cancer treatment in Japan towards fertility issues in young breast cancer patients. The high response rate to our survey in a relatively short time indicates the interest of breast oncologists in fertility issues. More than 80% of the participants responded that they had a positive attitude when discussing fertility issues in the clinic, but this result may be biased by the respondents' interest in fertility issues. The recent awareness of fertility issues among Japanese breast oncologists may be related to the publication of the ASCO guideline in 2006 and the inclusion of fertility-related contents in JBCS patient guideline 2009 [2, 9]. Indeed, the JBCS treatment guideline, the standard textbook for board certification of Breast Oncologists, updated its contents to cover fertility-related issues in July 2010 [10].

The physicians with a positive attitude and working in institutions with medical oncologists and/or female colleagues had a higher likelihood of consultation or referral to reproductive specialists. The likelihood of referring to reproductive specialists was slightly higher in female physicians, which was consistent with the results of the survey in the USA [4]. These results indicate that participation of female healthcare providers in the team and a multidisciplinary working environment might enhance physicians' awareness of and behavior toward fertility-related issues. Because knowledge and attitude seem to be influenced by gender, personal experience, and the working environment of the physicians, we think that outreach with educational materials and systematic learning opportunities for healthcare providers would be helpful in expanding knowledge and performance regarding fertility issues in young breast cancer patients.

High risk of disease recurrence was considered the greatest barrier for physicians, similar to the results of other studies [5, 6]. In our previous study, patients' with higher risk of disease recurrence did not voluntarily express their concerns regarding fertility when compared to patients of lower risk of disease recurrence [3]. Both patients and physicians may refrain from discussing future fertility when the estimation of prognosis of the cancer is poor. Although early referral to reproductive specialists might increase the patients' likelihood of receiving reproductive intervention and improve the fertility outcome [11, 12], fertility preservation techniques such as embryo preservation and oocyte preservation connote ethical issues especially in patients with poor prognosis [13]. Ethical and psychosocial support is necessary in the shared decision-making process among patients, families, and physicians.

A lack of reproductive specialists or infertility clinic for referral is a real problem. A survey in the USA showed that many breast cancer clinicians reported that they do not have knowledge of or resources for fertility preservation [8, 14]. Interdisciplinary communication between reproductive specialists and oncologists is necessary.

Early case–control studies suggest that pregnancy after primary treatment of breast cancer does not have a negative impact on cancer prognosis, although "healthy mother" bias might exist [15]. Because prognostication of breast cancer has become individualized using genetic biomarkers [16, 17], further investigations to clarify the impact of pregnancy after primary treatment on an individual basis is needed so that patients can personalize their decision-making regarding both cancer treatment and fertility.

In conclusion, Japanese breast oncologists were in general positive in discussing fertility issues with young breast cancer patients. Female and younger physicans as well as physicians working in a multidisciplinary environment had more positive attitudes and behavior towards fertility preservation. The development of multidisciplinary and interdisciplinary programs is necessary to meet the fertility needs of breast cancer patients.

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Hippo signaling disruption and Akt stimulation of ovarian follicles for infertility treatment

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Primary ovarian insufficiency (POI) and polycystic ovarian syndrome are ovarian diseases causing infertility. Although there is no effective treatment for POI, therapies for polycystic ovarian syndrome include ovarian wedge resection or laser drilling to induce follicle growth. Underlying mechanisms for these disruptive procedures are unclear. Here, we explored the role of the conserved Hippo signaling pathway that serves to maintain optimal size across organs and species. We found that fragmentation of murine ovaries promoted actin polymerization and disrupted ovarian Hippo signaling, leading to increased expression of downstream growth factors, promotion of follicle growth, and the generation of mature oocytes. In addition to elucidating mechanisms underlying follicle growth elicited by ovarian damage, we further demonstrated additive follicle growth when ovarian fragmentation was combined with Akt stimulator treatments. We then extended results to treatment of infertility in POI patients via disruption of Hippo signaling by fragmenting ovaries followed by Akt stimulator treatment and autografting. We successfully promoted follicle growth, retrieved mature oocytes, and performed in vitro fertilization. Following embryo transfer, a healthy baby was delivered. The ovarian fragmentation–in vitro activation approach is not only valuable for treating infertility of POI patients but could also be useful for middle-aged infertile women, cancer patients undergoing sterilizing treatments, and other conditions of diminished ovarian reserve.

ovary | aging | YAP | CCN2 | PTEN

Between 5% and 10% of reproductive-age women are infertile due to polycystic ovarian syndrome (PCOS) (1), whereas 1% of them suffer from infertility due to primary ovarian insufficiency (POI) (2, 3). They are infertile due to aberrant follicle growth. As early as the 1930s, ovarian wedge resection (4) was used for PCOS treatment to induce follicle growth, followed by recent success based on ovarian "drilling" by diathermy or laser (5). In addition, ovarian cortices are routinely fragmented to allow better freezing and grafting for fertility preservation in cancer patients who underwent sterilizing treatment (6). Subsequent autotransplantation of ovarian fragments is associated with spontaneous follicle growth. Underlying mechanisms for these disruptive procedures to promote follicle growth are, however, unclear.

The Hippo signaling pathway is essential to maintain optimal organ size and is conserved in all metazoan animals (7–9). Hippo signaling consists of several negative growth regulators acting in a kinase cascade that ultimately phosphorylates and inactivates key Hippo signaling effectors, Yes-associated protein(YAP)/transcriptional coactivator with PDZ-binding motif(TAZ). When Hippo signaling is disrupted, decreases in YAP phosphorylation increase nuclear levels of YAP. YAP acts in concert with TEAD transcriptional factors to increase downstream CCN growth factors and baculoviral inhibitors of apoptosis repeat containing (BIRC) apoptosis inhibitors (7). CCN proteins, in turn, stimulate cell growth, survival, and proliferation (10).

Using a murine model, we now demonstrated the promotion of follicle growth following ovarian fragmentation and allo-transplantation. Ovarian fragmentation increased actin polymerization, decreased phospho-YAP (pYAP) levels, increased nuclear localization of YAP, as well as enhanced expression of CCN growth factors and BIRC apoptosis inhibitors. Fragmentation-induced follicle growth was partially blocked by CCN2 antibodies and verteporfin, a small molecule that inhibits interactions of YAP with TEAD transcriptional factors (11).

Studies using phosphatase and tensin homolog deleted from chromosome 10 (*PTEN*) deletion mice indicated the stimulatory roles of Akt signaling in the development of primordial (12) and secondary follicles (13). Our earlier report demonstrated the ability of Akt stimulators to activate dormant primordial follicles (14). We now demonstrated additive increases in follicle growth when ovarian fragments containing secondary and smaller follicles were treated with Akt stimulators. Using this in vitro activation (IVA) method for infertility treatment of POI patients, we successfully promoted the growth of residual follicles in autografts and report a viable birth following oocyte retrieval and in vitro fertilization (IVF)–embryo transfer.

Significance

Human ovaries hold follicles containing oocytes. When follicles mature, they release eggs for fertilization. Patients with primary ovarian insufficiency develop menopausal symptoms at less than 40 y of age. They have few remaining follicles and their only chance for bearing a baby is through egg donation. Kawamura et al. demonstrated that Hippo and Akt signaling pathways regulate follicle growth. Using an in vitro activation approach, they first removed ovaries from infertile patients, followed by fragmentation to disrupt Hippo signaling and drug treatment to stimulate Akt signaling. After grafting ovarian tissues back to patients, they found rapid follicle growth in some patients and successfully retrieved mature eggs. After in vitro fertilization and embryo transfer, a live birth is now reported.

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Results

Ovarian Fragmentation Promoted Follicle Growth. We fragmented ovaries from juvenile (day 10) mice containing secondary and smaller follicles, followed by allo-transplantation under kidney capsules of adult hosts. As shown in Fig. 1*A*, major increases in graft sizes were evident after cutting ovaries into three pieces and grafting for 5 d compared with paired intact ovaries. Graft weights increased after cutting ovaries into 2–4 pieces or incubating fragments for up to 24 h before grafting (Fig. 1*B*). Histological analyses (Fig. S1*A*) and follicle counting of grafts (Fig. 1*C* and Fig. S1*B*) indicated a loss of total follicles following fragmentation/grafting. However, major increases in the percentage of late secondary and antral/preovulatory follicles were evident, accompanied by decreases in primordial follicles (Fig. 1*C*). Compared with day 10 ovaries, the grafting procedure led to decreases in absolute number of primordial, primary, and early secondary follicles (Fig. S1*B*). Furthermore, cutting/grafting of ovaries from older mice, including those containing early antral follicles from day 23 animals, also increased graft weights (Fig. 1*D*).

After grafting for 5 d, hosts received an ovulating dose of human chorionic gonadotropin (hCG). As shown in Fig. S1*C*, numbers of oocytes retrieved from fragmented grafts per ovary were 3.1-fold of those from intact grafts, accompanied by increased percentages of mature oocytes. Mature oocytes retrieved from fragmented grafts were fertilized and their development to early embryos was comparable to controls. After embryo transfer, healthy pups were delivered (Fig. S1*D*). Similar to mouse studies, fragmentation/autotransplantation of ovaries from rats also increased graft weights (Fig. S1 *E* and *F*).

Ovarian Fragmentation Increased Actin Polymerization and Disrupted Hippo Signaling. Real-time RT-PCR and immunoblotting analyses (Fig. S2 *A* and *B*) indicated the expression of transcripts and proteins for key Hippo signaling genes in ovaries of juvenile mice. Also, immunohistochemical staining of ovaries from adult mice (Fig. S2*C*) indicated the expression of MST1/2, salvador (SAV)1, large tumor suppressor 1/2 (LATS1/2), and TAZ mainly in the cytoplasm of granulosa cells, theca cells, and oocytes of follicles at all sizes but at lower levels in the corpus luteum.

Polymerization of globular actin (G-actin) to the filamentous form (F-actin) is important for cell shape maintenance and locomotion. Recent genome-wide RNAi screening demonstrated that induction of extra F-actin formation disrupted Hippo signaling and induced overgrowth in *Drosophila* imaginal discs and human HeLa cells (15, 16). As shown in Fig. 2*A*, a transient increase in ratios of F-actin to G-actin was detected at 1 h after ovarian fragmentation. The Hippo signaling kinase cascade phosphorylates YAP to promote its cytoplasmic localization and degradation, thus decreasing its transcriptional actions. When Hippo signaling is disrupted, decreases in pYAP increase nuclear YAP levels (17). After ovarian fragmentation and incubation for 1 h, decreases in pYAP levels and pYAP to total YAP ratios were evident (Fig. 2*B*), suggesting Hippo signaling disruption. In intact ovaries from day 10 mice, immunohistochemical staining indicated that YAP was localized in the cytoplasm of granulosa cells in most follicles at primary and secondary stages (Fig. S2*D*). At 4 h after fragmentation, nuclear staining of YAP was found in granulosa cells of primary and secondary follicles.

Disruption of Hippo signaling leads to increased expression of downstream CCN growth factors and BIRC apoptosis inhibitors (7, 8). As shown in Fig. 2*C*, ovarian fragmentation and subsequent grafting increased transcript levels for several CCN growth factors (CCN2, 3, 5, and 6) and apoptosis inhibitors (BIRC1 and 7) in fragmented ovaries. Similar changes were found following continuous culture without grafting (Fig. S3*A*). Immunoblotting of highly expressed CCN2 demonstrated increased CCN2 proteins in fragmented ovaries (Fig. 2*D*). Real-time RT-PCR analyses showed fragmentation-induced increases in CCN2 transcripts in somatic cells, but not oocytes (Fig. S3*B*). The ability of CCN proteins to promote ovarian growth was further demonstrated by dose-dependent increases in ovarian explant weights after culturing with CCN2, 3, 5, and 6 (Fig. 2*E*). Analyses of follicle dynamics indicated the ability of CCN factors to promote the development of primary follicles to the late secondary stage in ovarian explants (Fig. S3*C*), underscoring the role of CCN proteins as ovarian growth factors.

Roles of Hippo Signaling and CCN2 in Fragmentation-Induced Follicle Growth. YAP has no transcriptional activity and its actions are dependent on downstream transcriptional factors. Recent drug library screening identified a small molecule verteporfin, capable of inhibiting YAP association with TEAD transcriptional factors and suppressing YAP-induced liver overgrowth (11). Because fragmentation-induced CCN and BIRC changes were transient, we injected day 10 mice for 3 h with verteporfin before obtaining

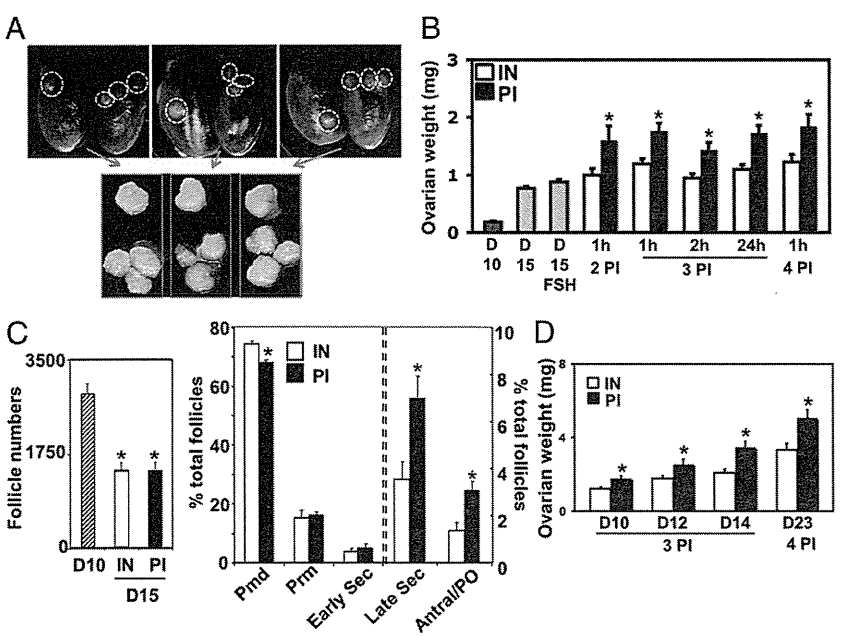

Fig. 1. Ovarian fragmentation and grafting promoted follicle growth in mice. Paired ovaries from juvenile mice were grafted into kidneys of adult ovariectomized mice (intact, IN; pieces, PI). Hosts were injected with FSH daily for 5 d before graft retrieval. (*A*) Morphology of paired ovarian grafts with or without fragmentation into three pieces. (*A*, *Upper*) Grafts inside kidney capsules. (*A*, *Lower*) Isolated paired grafts. (*B*) Weights of paired ovaries following fragmentation into 2–4 pieces from day 10 (D10) mice and incubated for 1–24 h before grafting. Ovarian weights before grafting (D10) and at 5 d after grafting with (D15 FSH) or without FSH treatment (D15) served as controls; n = 8–22. (*C*) Follicle dynamics before and after grafting of intact and fragmented (three pieces) ovaries from day 10 mice. (*C*, *Left*) Total follicle numbers. (*C*, *Right*) Follicle dynamics; n = 5. Pmd, primordial; Prm, primary; Sec, secondary; PO, preovulatory. (*D*) Weights of paired ovaries from mice at different ages following fragmentation into 3–4 pieces and grafting. Mean ± SEM; $^*P < 0.05$; n = 8–22.

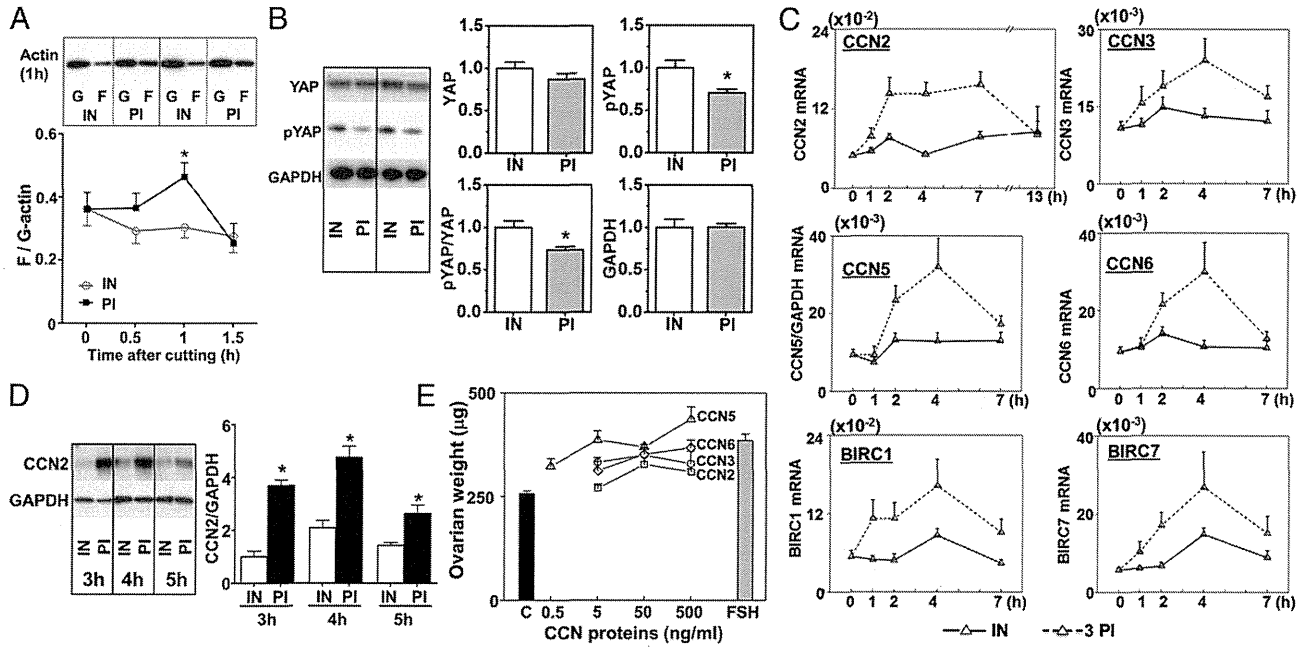

Fig. 2. Fragmentation of murine ovaries increased actin polymerization, disrupted Hippo signaling, and increased CCN growth factors and apoptosis inhibitors. (*A*) Ovarian fragmentation increased F-actin levels. Paired ovaries from day 10 mice were cut into three pieces or kept intact before immunoblotting analyses of F- and G-actin levels (*Upper*). (*A, Lower*) F- to G-actin ratios; $n = 6$–11. (*B*) Ovarian fragmentation decreased pYAP levels and pYAP to total YAP ratios. Paired ovaries with or without cutting were incubated for 1 h, followed by immunoblotting. (*B, Left*) Representative immunoblots. (*B, Right*) Ratios of different antigens; $n = 8$ pairs. (*C*) Ovarian fragmentation increased expression of CCN growth factors and BIRC apoptosis inhibitors. Paired ovaries with or without cutting were incubated for 1 h with subsequent grafting before analyses of transcript levels normalized by GAPDH. Intact ovaries, solid lines; pooled three pieces, dashed lines; $n = 10$–15. (*D*) Ovarian fragmentation increased CCN2 proteins. Paired ovaries with or without cutting were incubated for 3–5 h before immunoblotting. (*D, Upper*) Representative blots. (*D, Lower*) Quantitative analyses; $n = 3$–5. (*E*) Treatment with CCN2, 3, 5, and 6 increased ovarian explant weights. Explants from day 10 mice were cultured with different CCN growth factors for 4 d before weighing; $n = 5$–6. Mean ± SEM; $^*P < 0.05$. IN, intact; PI, pieces.

ovaries for fragmentation. As shown in Fig. S4*A*, pretreatment with verteporfin blocked fragmentation-induced increases in CCN2 transcripts without affecting those for anti-Müllerian hormone, a secondary follicle marker. In contrast to graft weight increases found between intact and fragmented ovarian pairs from vehicle-pretreated animals, no significant changes in graft weights were found between intact and fragmented pairs after pretreatment with verteporfin (Fig. S4*B*). Follicle counting of grafts indicated no loss of total follicles with verteporfin pretreatment (Fig. S4*C*). In contrast, verteporfin pretreatment prevented fragmentation-induced increases in late secondary follicles, with smaller suppression of antral/preovulatory follicles. We further incubated ovarian fragments with CCN2 antibodies for 18 h before grafting. Neutralization of endogenous CCN2 suppressed fragmentation-induced graft weight gain by 75% (Fig. S4*D*). These findings underscore the role of Hippo signaling in fragmentation-induced follicle growth.

Additive Effects of Hippo Signaling Disruption and Akt Stimulation on Secondary Follicle Growth. In addition to the stimulatory role of Akt signaling in primordial follicle development (12, 14), conditional deletion of the *PTEN* gene in granulosa cells of secondary follicles also promoted follicle growth (13). We isolated secondary follicles from juvenile mice and demonstrated the ability of Akt stimulating drugs (PTEN inhibitor and PI3K activator) to promote secondary follicle growth (Fig. 3*A*). We further tested combined effects of Akt stimulating drugs and Hippo signaling disruption on ovarian graft growth. Using ovaries obtained from day 10 mice containing secondary and smaller follicles, we found additive increases in ovarian graft weights when fragmented ovaries were incubated with Akt stimulating drugs followed by grafting (Fig. 3*B*). Counting of follicles indicated increases in late secondary and antral/preovulatory follicles induced by fragmentation and Akt stimulation (Fig. 3C and Fig. S5).

We obtained human ovarian cortical cubes containing secondary and smaller follicles. RT-PCR analyses demonstrated the expression of key Hippo signaling genes (Fig. S6*A*), whereas immunohistochemical analyses showed the expression of SAV1, LATS1/2, YAP, and TAZ in granulosa cells, theca cells, and oocytes of primordial to secondary follicles (Fig. S6*B*). We then thawed cryopreserved human ovarian cortical strips (1–2 mm thickness and 1 × 1 cm) and cut them into small cubes (1–2 mm^2) before incubation. Real-time RT-PCR analyses indicated time-dependent increases in transcript levels for CCN2, 3, 5, and 6 (Fig. S6*C*). Higher CCN growth factor expression was found in ovarian cubes after further fragmentation from strips, suggesting fragmentation-induced disruption of Hippo signaling. We then cut human cortical strips containing secondary and smaller follicles (Fig. 3*D*) and incubated them with Akt stimulators before xenografting into immune-deficient mice. Within 4 wk, antral follicles were detected, demonstrating rapid follicle growth (Fig. 3 *E* and *F*).

Hippo Signaling Disruption and Akt Stimulation as Infertility Treatment. In patients with POI, also known as premature ovarian failure, early exhaustion of ovarian function is evident due to genetic, immunological, iatrogenic, or other causes (2). POI is characterized by amenorrhea and elevated serum FSH before 40 y of age. Patients are infertile due to a lack of follicle growth and ovulation; oocyte donation is the only treatment option.

We obtained ovaries from POI patients for IVA based on Hippo signaling disruption and Akt stimulation, followed by autotransplantation and IVF–embryo transfer (Fig. 4*A*). Using laparoscopic surgery, ovaries were removed from 27 POI patients (37.3 ± 5.8 y of age; duration of amenorrhea, 6.8 ± 2.1 y), cut into strips (1–2 mm thickness and 1 × 1 cm), and vitrified

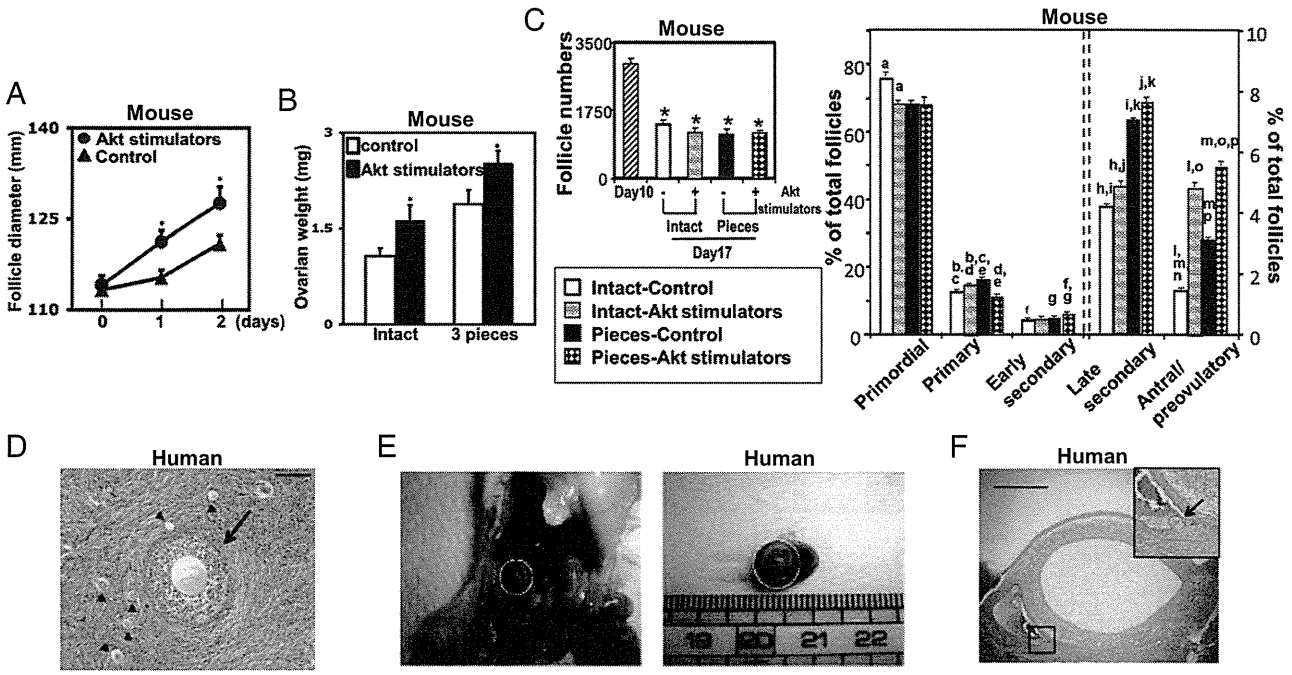

Fig. 3. Additive effects of Hippo signaling disruption and Akt stimulation promoted secondary follicle growth. (*A*) Secondary follicles were isolated from juvenile mice and cultured with Akt stimulators; $n = 30$. (*B*) Additive increases in graft weights following ovarian fragmentation and/or Akt stimulation. Paired ovaries from juvenile mice were fragmented and incubated with or without Akt stimulators for 2 d followed by allo-transplantation for 5 d before graft weight determination; $n = 8$–10. (*C*) Follicle dynamics before and after grafting of intact and fragmented murine ovaries with or without treatment with Akt stimulators. (*C, Left*) Total follicle numbers. (*C, Right*) Follicle dynamics; $n = 4$. Same letter symbols indicate significant differences ($P < 0.05$). (*D–F*) Vitrified human cortical strips were thawed and fragmented into cubes before treatment with Akt stimulators for 2 d followed by grafting into immune-deficient mice for 4 wk. (*D*) Cortical strips before grafting. Arrow, a secondary follicle; arrowheads, primordial/primary follicles. (Scale bar, 100 μm.) (*E*) A kidney graft in situ (*Left*) and after isolation (*Right*), showing an antral follicle. (*F*) Histology of two large antral follicles with the side view of one showing an oocyte at the germinal vesicle stage (arrow). (Scale bar, 1 mm.) Mean ± SEM; $^*P < 0.05$.

(18). Randomly selected pieces were used for histological analyses, and ovaries from 13 of 27 patients contained residual follicles. Before autografting, frozen ovarian strips were thawed and fragmented into ~100 cubes of 1–2 mm^2, followed by treatment with Akt stimulating drugs for 2 d. Forty to 80 ovarian cubes each were then autotransplanted beneath the serosa of Fallopian tubes (Fig. 4 *B–D*; Movie S1).

Following weekly or biweekly transvaginal ultrasound monitoring, together with serum estrogen measurement, follicle growth was found in eight patients (Fig. 4*E*), all of them belonging to those with histological signs of residual follicles. After follicles reached the antral stage (>5 mm in diameter; Fig. 4*E*, right upward arrows), patients were treated daily with FSH, followed by an injection of hCG when follicles reached >16 mm in diameter. Thirty-six hours later, egg retrieval was performed (Fig. 4*E*, upward arrows) under transvaginal ultrasound. Similar to normal menstrual cycles, usually only one follicle emerged from the growing pool to reach the preovulatory stage. Although earlier data indicated that 6 mo are needed for the development of human primordial follicles to the preovulatory stage (14, 19), all eight patients developed preovulatory follicles (~2 cm) in less than 6 mo of grafting, with some of them (patients 1, 3, 5, and 7) developing preovulatory follicles within 3 wk, indicating rapid growth of secondary follicles. In contrast, preovulatory follicles developed after 6 mo of grafting were likely derived from primordial follicles.

Mature oocytes were successfully retrieved from five patients for intracytoplasmic sperm injection (ICSI) using the husband's sperm. When embryos reached the four-cell stage, they were cryopreserved. For patient 1, one of two embryos was transferred. However, no pregnancy occurred. For patient 5, one of two embryos was transferred and pregnancy was diagnosed based on elevated serum hCG levels.

Patient 3 reached menarche at 11 y of age with regular menses. At 23 y of age, she experienced irregular cycles and became amenorrhea at 25 y of age with elevated FSH levels (>40 m international unit (mIU)/mL). Despite diverse testing including chromosome analysis, her pathogenesis was unknown. At 29 y of age, her ovaries were removed for fragmentation and Akt drug treatment. After monitoring of follicle growth and obtaining four four-cell embryos developed from six oocytes, two embryos were transferred and a successful singleton pregnancy was established. Consistent with reported safety of short-term treatment with Akt stimulators to activate primordial follicles in mice (20), a healthy baby (male; birth weight, 3,254 g; and Apgar score, 9 at 1 min/10 at 5 min) was delivered at 37 wk and 2 d of pregnancy. Physical features of the baby are normal, together with normal placenta and umbilical cord. No abnormal growth was detected in the transplanted site of the Fallopian tubes.

Discussion

Findings across multiple organ systems and model organisms have implicated Hippo signaling in the maintenance of organ sizes (7–9). However, our results uniquely document a role for Hippo signaling in mammalian ovaries. Our data indicate that ovarian fragmentation increased actin polymerization and disrupted Hippo signaling by decreasing pYAP levels together with increased nuclear localization of YAP, leading to increased expression of CCN growth factors and BIRC apoptosis inhibitors. Secreted CCN2 and related factors promoted follicle growth after transplantation (Fig. S7).

It is becoming clear that most ovarian follicles are constrained to growth under physiological conditions due to local Hippo signaling. Consistent with the role of Hippo signaling genes in restraining ovarian follicle growth, specific deletion of *SAV1* or

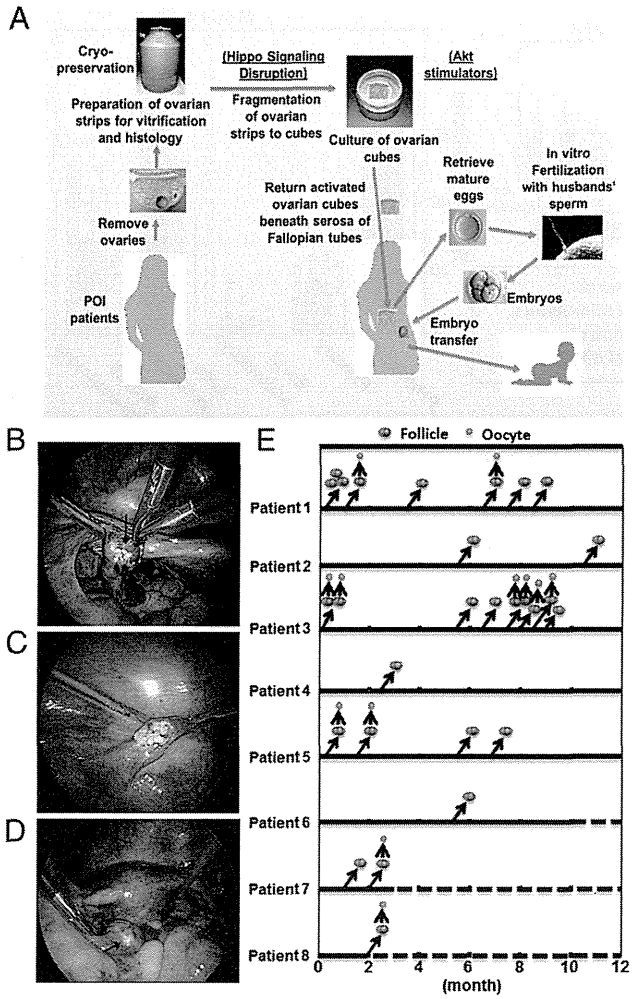

Fig. 4. Ovarian fragmentation/Akt stimulation followed by autografting promoted follicle growth in POI patients to generate mature oocytes for IVF–embryo transfer, pregnancy, and delivery. (*A*) Under laparoscopic surgery, ovaries were removed and cut into strips. Ovarian strips from POI patients were vitrified. After thawing, strips were fragmented into 1–2 mm^2 cubes, before treatment with Akt stimulators. Two days later, cubes were autografted under laparoscopic surgery beneath serosa of Fallopian tubes. Follicle growth was monitored via transvaginal ultrasound and serum estrogen levels. After detection of antral follicles, patients were treated with FSH followed by hCG when preovulatory follicles were found. Mature oocytes were then retrieved and fertilized with the husband's sperm in vitro before cyropreservation of four-cell stage embryos. Patients then received hormonal treatments to prepare the endometrium for implantation followed by transferring of thawed embryos. (*B*) Transplantation of ovarian cubes beneath the serosa of Fallopian tubes. Arrow, fallopian tube; arrowheads, cubes. (*C*) Multiple cubes were put beneath serosa. (*D*) Serosa after grafting. Ovarian cubes are visible beneath serosa (arrow). (*E*) Detection of preovulatory follicles in grafts for oocyte retrieval. Following ultrasound monitoring, follicle growth was found in eight patients. After follicles reached the antral stage (>5 mm in diameter, right upward arrows), patients were treated with FSH followed by hCG for egg retrieval (upward arrows). Double circles represent preovulatory follicles, whereas single circles represent retrieved oocytes. Dashed lines depict ongoing observation.

MST1/2 genes in hepatocytes resulted in enlarged livers (21, 22). Likewise, conditional deletion of *SAV1* led to enlarged hearts (23). Hippo signaling is also critical for tissue regeneration and expansion of tissue-specific progenitor cells (17). For the ovary, LATS1-null female mice exhibited a POI phenotype (24), whereas LATS1 regulates the transcriptional activity of FOXL2, a gene mutated in some POI patients (25) (Fig. S7, boxed). Genome-wide association studies also implicated YAP as a susceptibility gene for PCOS (26), whereas deletion of *CCN2/CTGF* in ovarian granulosa cells in mice led to subfertility and aberrant follicle development (27) Also, genome-wide analyses identified changes in gene copy numbers for *BIRC1* in POI patients (28).

F-actin formation in the stress fiber is required for the disruption of Hippo signaling and nuclear YAP accumulation (29). F-actin probably functions as a scaffold for Hippo signaling components because Hippo signaling genes MST1/2, merlin, and Amot all bind to actin (30). The upstream diaphanous (DIAPH) genes accelerate actin nucleation and suppress actin depolymerization. Of interest, disruption of the *DIAPH2* coding region was found in a POI family (31), whereas genome-wide association studies identified *DIAPH2* (32) and *DIAPH3* (33) as candidate genes in regulating follicle reserve and menopause (Fig. S7).

Intestinal damage using dextran sodium sulfate decreases pYAP to total YAP ratios in regenerating crypts (34). Also, CCN1/CYR61 was induced in proximal straight tubules following ischemic reperfusion injury of the kidney (35). In the obstructed bladder, expression of CCN2/CTGF and CCN1/CYR61 were also induced (36). Disruption of Hippo signaling following actin polymerization likely represents a general mechanism in regulating tissue damage and remodeling, linking mechanical alterations of structural components to intracellular signaling.

Changes in actin polymerization and downstream events induced by ovarian fragmentation were transient in nature, and increases in CCN2/3/5/6 transcript levels occurred even when frozen human ovarian strips were fragmented after thawing. CCN growth factors and apoptosis inhibitors likely induce additional downstream changes, including the PI3K–target of rapamycin (TOR) signaling pathway (37), to promote follicle growth. Although vascularization changes during grafting cannot be ruled out, treatment with CCN2 antibodies or verteporfin partially suppressed fragmentation-induced increases in graft weights, underscoring the role of Hippo signaling.

Mechanical tension associated with the rigid sclerotic capsules in some PCOS ovaries could lead to arrested follicle development. Ovarian wedge resection (4, 38) or drilling by diathermy/laser (5) in PCOS patients results in follicle growth and comparable live birth rate compared with the popular gonadotropin treatment. Our studies suggest that damage incurred by cutting or drilling PCOS ovaries could enhance actin polymerization and disrupt Hippo signaling to promote follicle growth. Local administration of actin polymerization drugs or CCN growth factors could provide new treatments for PCOS patients and minimize follicle loss associated with ovarian damage.

Conditional deletion of the *PTEN* gene in granulosa cells of secondary follicles in mice promoted follicle growth (13). We demonstrated additive increases in ovarian graft weights and follicle growth following Hippo signaling disruption (fragmentation) and Akt stimulation (treatment with PTEN inhibitors and PI3K activators). Using the present IVA protocol, rapid growth of human secondary follicles to the antral stage was found in immune-deficient mice. Although the exact stage of residual follicles in individual POI ovaries is unclear, we generated preovulatory follicles from several patients in a few weeks.

The present approach represents a possible unique infertility therapy for POI patients. This paper is a report of birth after ovarian vitrification and IVA/grafting to promote follicle growth. Our data indicated that less than half of our POI patients contained residual follicles and 62% of them responded to the therapy by showing follicle growth in grafts. Because few ovarian strips are needed for autotransplantation, we are now removing one ovary from patients and recommending continuation when residual follicles are detected based on histology. Although a healthy baby was born, more studies are needed to ensure the safety of the present IVA procedure. Because POI represents a disease with heterogeneous etiologies and >50% of our POI patients do not have residual follicles, it is important to note that patients without follicles will not respond to the present IVA treatment.

POI patients have intermittent and unpredictable ovarian functions. Although 5–10% of POI patients in reported studies have a chance to conceive, only a 1.5% pregnancy rate was found in controlled trials (39). Studies of a cohort of 358 young POI patients (26.6 ± 7.9 y of age at time of diagnosis) indicated a spontaneous pregnancy rate of 4.4% during 13 y of observation (40). In our 27 older POI patients (37.3 ± 5.8 y of age), the amenorrhea duration is 6.8 ± 2.1 y with no spontaneous pregnancy. In contrast to the rare spontaneous pregnancy found in some POI patients, the present approach represents a systematic activation of residual follicles and monitoring of follicle growth. Our detection of preovulatory follicles in eight out of 27 POI patients during <1 y of observation and successful derivation of embryos from five patients suggested that the eventual success rate could be as high as 30% (8/27) after repeated autografting and optimization of follicle monitoring and oocyte retrieval. Although five patients with histological signs of residual follicles did not respond to the present treatment, we are initiating second grafting because only fragments from selective strips were grafted.

Variable local Hippo signaling could lead to protracted preovulatory follicle development after 6 mo of grafting. In addition to POI, the present approach could be useful for fertility preservation in cancer patients undergoing sterilizing treatments and other conditions of diminished ovarian reserve. Although menopause occurs at 51 y of age, many middle-aged women between 40 and 45 y of age suffer from aging-associated infertility. Because their ovaries still contain secondary and smaller follicles (41), our approach should be effective. Without overcoming age- or environment-related increases in genetic defects in oocytes, the present approach provides more mature oocytes for embryonic development.

Methods

Animals, ovarian fragmentation/grafting, ovarian explant and follicle cultures, actin measurement, RT-PCR analyses, and immunostaining/blotting are provided in *SI Methods*. Also, included are patient treatments and human/animal subject approval. In addition, a movie of human grafting is included (Movie S1).

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—Technology Report—

Good Thermally Conducting Material Supports Follicle Morphologies of Porcine Ovaries Cryopreserved with Ultrarapid Vitrification

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Abstract. Effects of supporting materials during vitrification procedure on the morphologies of preantral follicles of pig ovaries were assessed. Ovarian cortical sections of prepubertal pigs were randomly allocated to 5 groups. The sections were vitrified ultrarapidly with 5 different vitrification devices. The sections were put on 4 fine needles (Cryosupport), on a thin copper plate, or on a carbon graphite sheet or were sandwiched between copper plates or between carbon graphite sheets before cooling. The cooling and warming rates with the graphite sheets were significantly higher than those with the copper plates ($P<0.05$). A total of 3,064 follicles were analyzed following HE staining after vitrification with 5 different devices. The morphologies follicles vitrified on the Cryosupport or on the graphite sheet were well preserved compared with those vitrified on the copper plate or between copper plates ($P<0.01$). The morphologies of follicles vitrified between copper plates were mostly damaged ($P<0.05$). Taken together, good thermally conducting material supports follicle morphologies of ovaries cryopreserved with ultrarapid vitrification.

Key words: Cryopreservation, Fertility preservation, Ovary, Ultrarapid vitrification

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Cryopreservation of preantral follicles in ovarian tissues has been expected to be an effective measure for preserving fertility of young women who need to undergo cytotoxic therapy and female animals on the endangered list.

The major damaging factors, which occur during cryopreservation, are associated with chilling injury, osmotic stress, cryoprotectant toxicity and ice crystallization [1]. In general, we are trying to reduce these damages by increasing cooling and warming rates during the vitrification protocol. In the past, vitrification was based on the combination of a high cooling rate and high concentration of cryoprotectants, which caused chemical toxicity and osmotic stress [1]. The major breakthrough in the field of vitrification came when the sample volume was reduced to a level that permitted lowering of the cryoprotectants concentration [2, 3]. For ovarian tissues, rapid cooling by direct immersion into LN_2 led to a better outcome [4–8].

Recently, an ultrarapid vitrification protocol we developed using nonhuman primates (Cryosupport vitrification) [8] supported the morphological normality of vitrified preantral follicles and oocytes compared with conventional vitrification and slow freezing [9]. Moreover, revascularization of vitrified-warmed tissues after autotransplantation was also confirmed, and normal oocytes were successfully retrieved. However, an adiabatic effect caused by nitrogen gas, which occurs on the surface of tissue, is of concerns. The Cryosupport has a size limit because it consists of 4 fine needles and the gap between needles is 2 mm.

We hypothesized that good thermally conducting material would maximize cooling and warming rates to facilitate vitrification and to prevent ice crystal injury during warming. The thermal conductivity of carbon graphite is approximately twice as high as that of copper. In this study, we assessed effects of supporting materials during cooling and warming procedures on the morphologies of preantral follicles of vitrified pig ovaries.

At the beginning, the cooling and warming rates using carbon graphite sheets and copper plates were assessed. The average values of the cooling and warming rates were 10,021 and 28,000 C/min, respectively, with the graphite sheets and 2,524 and 4,957 C/min, respectively, with the copper plates. The cooling and warming rates with the graphite sheets were significantly higher than with the copper plates ($P<0.05$).

A total of 3,064 follicles (1,552 primordial, 1,030 intermediary, 482 primary) were analyzed by light microscope to evaluate the morphologies of follicles vitrified ultrarapidly with the five different devices. Non-vitrified follicles (417 primordial, 204 intermediary, 77 primary) were also analyzed as the control. There were no differences in normality of follicles between vitrification on the Cryosupport (primordial, 92.2%; intermediary, 84.5%; primary, 77.2%; Table 1) and on the graphite sheet (primordial, 93.3%; intermediary, 81.1%; primary, 87.0%). Morphological normality of primordial follicles vitrified on the Cryosupport (92.2%) and on the graphite sheet (93.3%) was well preserved ($P<0.01$) compared with those vitrified on the copper plate (70.6%) and between the copper plates (36.4%). The morphologies of follicle vitrified between the copper

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Table 1. Proportions of morphologically normal follicles at the primordial, intermediary, and primary stages after ultrarapid vitrification

Ovarian sections were vitrified	Primordial follicles		Intermediary follicles		Primary follicles	
	Mean ± SEM	N	Mean ± SEM	N	Mean ± SEM	N
On the Cryosupport	92.2 ± 2.2^{a}	239	84.5 ± 4.4^{ab}	126	77.2 ± 7.8^{a}	89
On the graphite sheet	93.3 ± 2.0^{a}	243	81.1 ± 5.4^{ab}	153	87.0 ± 3.7^{a}	116
Between graphite sheets	84.3 ± 2.8^{ab}	647	65.8 ± 5.7^{b}	478	64.4 ± 14.2^{a}	138
On the copper plate	70.6 ± 4.3^{b}	197	69.5 ± 6.2^{ab}	65	64.1 ± 16.9^{a}	34
Between copper plates	36.4 ± 9.0^{c}	226	26.5 ± 11.6^{c}	208	23.6 ± 12.3^{b}	105
Non vitrified	90.9 ± 3.8^{a}	417	87.9 ± 3.9^{a}	204	70.4 ± 22.6^{a}	77

Four ovarian sections were used to assess the follicle morphology in each experimental condition. Data are shown as the mean ± SEM. N: number of examined follicles. Different superscript letters (a, b, c) in the same row denote significant difference at $P<0.05$ by ANOVA followed by Fisher's PLSD test.

plates were the most damaged compared with the follicles vitrified with the other devices ($P<0.05$). The architecture of the compact matrix of ovarian stromal tissue, which consists of stromal cells and bundles of collagen fibers, was damaged most in the specimens vitrified between copper sheets. A sandwich method in which an ovarian section was put between two sheets or plates was inferior to a method in which an ovarian section was put in direct contact with liquid nitrogen (putting on method), possibly due to a decrease in the cooling and warming rates.

The data of the present study clearly indicated that good thermally-conducting material supports follicle morphologies of ovaries cryopreserved with ultrarapid vitrification. In 2010, we started clinical application of ovarian vitrification using Cryosupport vitrification [8] after receiving approval from the institutional review board of St. Marianna University.

Methods

For measurement of cooling and warming rates, an ultrafine thermocouple probe (0.1 mm diameter; JT1, Chino, Tokyo, Japan) was sandwiched between copper sheets (8 mm × 15 mm × 0.1 mm, 4940372036534, Taiho Trading, Tokyo, Japan) or between carbon graphite sheets (8 mm × 15 mm × 0.1 mm, EYGS12810, Panasonic, Osaka, Japan), immersed directly into liquid nitrogen, held briefly and then warmed in a 35 C thawing solution: Hepes-buffered 199 solution (H199, 12350-039, Invitrogen, Tokyo, Japan) [8] containing 20% SSS (SSS, 99193, Irvine Scientific, Santa Ana, CA, USA) and 0.8 M sucrose (192-00012, Wako Pure Chemical Industries, Osaka, Japan). The gap between sheets was filled with vitrification solution: H199 containing 20% SSS, 35% (v/v) ethylene glycol (054-0983, Wako Pure Chemical Industries), 5% (w/v) polyvinylpyrrolidone, a synthetic polymer (PVP; PVP360, Sigma-Aldrich, St. Louis, MO, USA), and 0.5 M sucrose. Temperature changes were measured with an electronic thermometer (Model EB22005, Chino). For each method, the time required for the temperature to drop from –20 to –100 C (or the reverse) was measured during cooling and during warming with 3 replicates each, and the average cooling and warming rates were calculated.

Ovarian cortical sections (7 mm × 5 mm × 1 mm) were made by removal of the 1-mm-thick outer layer of the ovarian cortex of prepubertal pig ovaries. The ovaries were obtained from a local abattoir in Osaka Prefecture. Twenty-four sections prepared from ten ovaries were randomly allocated to six groups. The sections were vitrified ultrarapidly with the 5 different vitrification devices by direct application into liquid nitrogen following exposure to vitrification solution as described above except in the non-vitrified group. The sections were put on the Cryosupport, which consisted of 4 fine stainless steel needles [8], on the copper sheet or on the graphite sheet or were sandwiched between the graphite sheets or between the copper plates before cooling (Fig. 2). The Cryosupport was designed to minimize the surface area of the stainless steel needle to support the direct contact of ovarian tissue with liquid nitrogen [8]. After warming, the morphologies of the follicles were analyzed using light microscopy (LM). Vitrified and warmed ovarian sections were fixed in Bouin's solution. The fixed ovarian tissues were then dehydrated, embedded in paraffin wax and serially sectioned to a thickness of 5 μm. These sections were mounted and stained with hematoxylin-eosin. All sections were examined using a light microscope (Olympus, Tokyo, Japan) at a magnification of ×400. Only primordial to primary follicles that possessed oocytes surrounded by one layer of flattened or cuboidal granulosa cells were counted. Follicles with an oocyte surrounded by a single layer of flat granulosa cells were defined as primordial. Follicles with an oocyte surrounded by flat granulose cells and one or more cuboidal granulosa cells were regarded as intermediary, and those with one layer of only cuboidal granulosa cells were regarded as primary. Follicular quality was evaluated based on the morphological integrity of the oocyte, granulosa cells and basement membrane. Follicles were histologically classified as normal (1) when they contained an intact oocyte and intact granulosa cells, or degenerated (2) when they contained a pyknotic oocyte nuclei, shrunken ooplasm and/or disorganized granulose cells (e.g., enlargement in volume and/or detachment from the basement membrane, Fig. 1). Data were analyzed using the *t*-test or Fisher's PLSD test following ANOVA.

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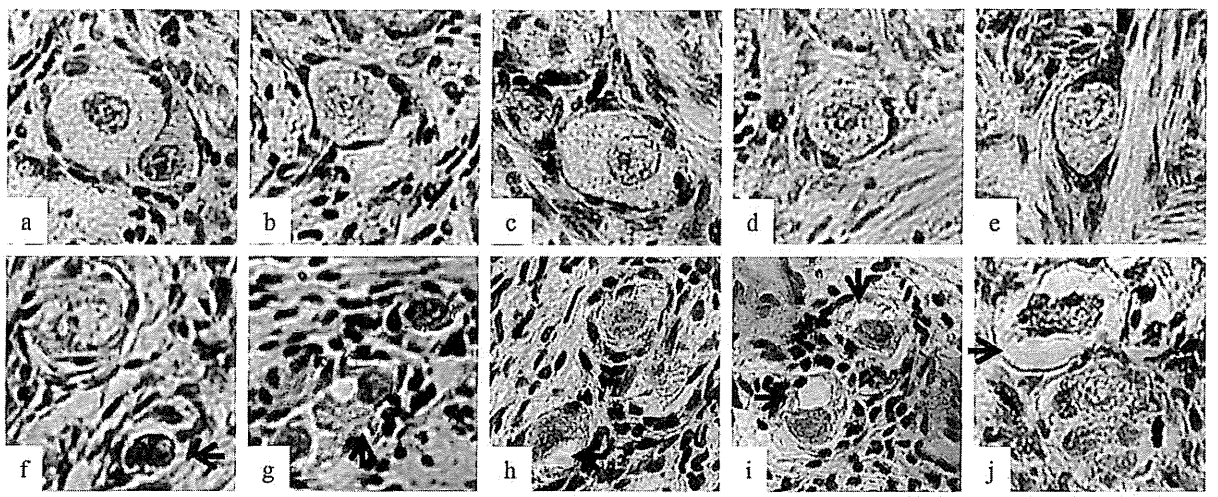

Fig. 1. Morphologies of porcine follicles after vitrification. a–e: Follicles were categorized as normal after vitrification between the graphite sheets. f–j: Follicles were categorized as damaged after vitrification on the copper sheet. Each arrow shows a pyknotic oocyte nucleus (f), shrunken ooplasm (g–j) and disorganized granulose cells (j).

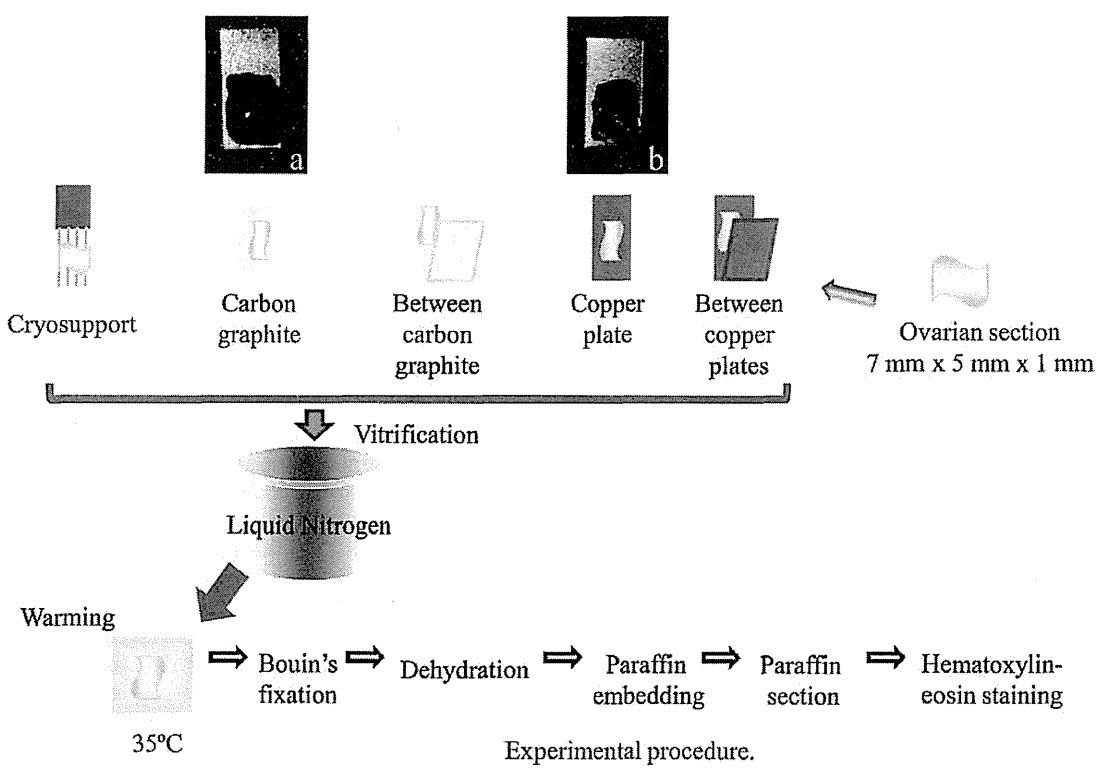

Fig. 2. Ovarian sections were put on the Cryosupport, which consisted of 4 thin needles (Cryosupport), the graphite sheet (a), or the copper plate (b) or were sandwiched between copper plates or between carbon graphite sheets before cooling. Vitrified and warmed ovarian sections were fixed in Bouin's solution. The fixed ovarian tissues were then dehydrated, embedded in paraffin wax and serially sectioned to a thickness of 5 μm. These sections were mounted and stained with hematoxylin-eosin.

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特 集
生殖系列細胞の凍結

卵巣組織の凍結保存

吉岡 伸人／鈴木 直

Summary

凍結卵巣組織を用いた自家移植による生児獲得に関する報告が2004年にこの領域のパイオニアであるDonnezによってされて以来，現在まで本技術によって20名以上の生児が誕生している。欧米では「卵巣組織凍結保存は，早期閉経発来や緊急体外受精を施行しなければならない卵巣毒性を有する治療を受けるすべての若年女性がん患者に，選択肢として提供すべき医療行為である」と認識されている。しかし，至適な凍結方法，至適な移植部位，がん細胞の再移入の可能性や安全性に関してなど，検討されるべき課題がまだ山積している。

Key words

卵巣組織凍結●卵巣組織移植
ガラス化凍結法●緩慢凍結法
がん・生殖医療

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はじめに

近年，がんに対する診断および集学的治療の進歩による治療成績向上の結果として，がんを克服する患者(cancer survivor)が増加している。それに伴ってcancer survivorのquality of life(QOL)向上に関して，特に生殖年齢患者に対する化学療法や放射線療法によって生じる妊孕性喪失について，その重要性が再認識されてきている。若年がん患者の妊孕性温存に関する診療として，古くから配偶子や受精卵の凍結保存，卵巣の位置移動術や放射線治療時の遮蔽などが施行されてきた。しかし，2004年の卵巣組織凍結・移植によるはじめての生児獲得以来，新しい妊孕性温存療法として卵巣組織凍結・移植が臨床応用されたことから，欧米ではoncofertility(がん・生殖医療)という新しい領域が確立され，若年がん患者に対する妊孕性温存の診療の考え方が見直されはじめている。本稿では，新しい妊孕性温存療法である卵巣組織凍結保存・移植に関して概説する。

緩慢凍結法による卵巣組織凍結と移植

現在の標準的な卵巣組織凍結保存は緩慢凍結法であり，これまで卵巣凍結によって得られた生児はすべて緩慢凍結法によるものである[1)2)]。しかし，緩慢凍結法によって移植後の内分泌機能がどの程度維持されるかに関してはまだ十分に検証されておらず，採卵で卵子が回収できないempty

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follicleの増加，得られた卵子の発育能の低下も明らかにされている[3]。最近Andersenらは，がん患者に対する緩慢凍結法による妊孕性温存療法に関する18症例の経験を報告している[4]。報告によると，18例の患者の年齢の中央値は28.5歳（9～38歳）で，卵巣を片側摘出した後，卵巣組織片は5×5×1mmの大きさで緩慢凍結が施行されている。移植までの期間は中央値で2年（1～5年），また移植の際，全患者とも無月経[FSH（卵胞刺激ホルモン）の中央値74IU/L（43～200IU/L）]であり，保存してある卵巣組織の20～60%（6～12片）が同所性あるいは異所性（前腹壁あるいは骨盤壁）に移植され，移植片が機能した期間の中央値は26ヵ月（0～88ヵ月）であったという。なお対象患者は，ホジキン病5例，乳がん4例，非ホジキンリンパ腫2例，自己免疫性血管炎2例，その他5例（ユーイング肉腫，子宮頸がん，再生不良性貧血，発作性夜間血色素尿症，溶血性尿毒症症候群）であった。12症例で計72周期のART治療の結果，65個の卵が獲得され，受精率は40%で最終的に5症例に妊娠が成立し[妊娠率：6.9%（5/72）]，2例の生児が獲得された[生産率：2.8%（2/72）]。対象疾患などのバイアスなどから生産率2.8%に関する評価は難しいが，Andersenらは満足のいく結果ではないと謙虚に述べている。表1にDonnezら[3]とAndersenら[5]の緩慢凍結のデータに関して記す。緩慢凍結法は，細胞外に形成される氷晶による細胞への物理的障害が予想されることから，融解後の卵胞発育過程における卵母細胞の発育と顆粒膜細胞の成熟のバランスが損なわれていると考えられている[6]。以上より，緩慢凍結法による卵巣組織凍結・移植の成功率向上には限界がある一方で，欧米では1998年以来緩慢凍結法によって数多くの卵巣組織凍結を行ってきた現状がある。しかし，Donnezらもすでに緩慢凍結法に替わる卵巣組織凍結方法としてのガラス化凍結法を基礎的に検討し模索しているが[7]，欧州の本領域におけるリーダーの1人であるMeirowによると，「すでに数百名以上の患者の卵巣組織を緩慢凍結法で凍結してあり，さらに生児獲得が得られており上手くworkしている本法を新しい方法に替える考えはない（私信）」という。

一方，Donnezらは卵巣組織内の原始卵胞への凍結によるダメージの回避――すなわち卵巣組織凍結方法の改善も重要ではあるが，移植部位の選定も重要であると述べている[8]。移植された卵巣組織の機能回復（再酸素化）には通常4～5日必要であると考えられており[9]，再酸素化に重要な因子としては卵巣組織と移植部位による血管新生と血管網の存在である。現在，残存卵巣が存在する場合には，原則として同所性移植（残存卵巣）が選択される。一方，異所性移植（腹膜など）が選択される場合には，一度移植予定の腹膜を切開しわずかな卵巣組織片を移植した後に，再度実際の卵巣組織片を移植する2段階移植法が移植卵巣組織片の血管新生をより促進し，よりよい再酸素化を作り出す可能性があると考えられている[8]。

表1　緩慢凍結法による卵巣組織凍結の評価

	採卵あたりのempty follicle率	症例あたりの未熟卵獲得率	受精率	採卵あたりの胚移植率
Donnezら[3]	29%	38%	31%	24%
Andersenら[5]	35%	33%	37%	33%

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卵巣組織凍結方法の新たな展開 —ガラス化法—

近年，ヒト卵巣組織におけるガラス化法による基礎的研究報告も散見されている。Hovattaらは，ヒト卵巣組織(帝王切開時に提供：n＝20)を緩慢凍結法(プロパンジオール，エチレングリコールの２群)あるいはガラス化法(プロパンジオール，エチレングリコールの２群で凍結保護剤への浸漬を５分，10分)で凍結した後，１～15週間後に融解した各群で卵子，顆粒膜細胞，間質細胞の形態学的変化を光学顕微鏡，電子顕微鏡を用いて評価している[10]。その結果，ガラス化法と緩慢凍結法の比較では卵胞への影響に差が認められなかった一方，ガラス化法で間質の形態が有意に良好であったと報告している。さらに近年Amorimらも，ヒト卵巣組織を用いたガラス化法[エチレングリコールとトレハロースとウシ胎仔血清(FBS)を用いた溶液]を組織学的に評価しており，長年緩慢凍結法による卵巣組織凍結を行ってきたDonnezらのグループもガラス化法の臨床への応用を模索している[7]。具体的には，22～35歳までのヒト卵巣組織を用いて(n＝９)，ジメチルスルホキシド(DMSO)＋スクロース，エチレングリコール＋DMSO，そしてエチレングリコール＋トレハロースの３群で非凍結卵巣組織の対照群と組織学的手法を用いて比較検討を行っている。その結果，エチレングリコール＋トレハロース群が最も組織学的に対象群と同等であったという。なお，凍結デバイスは閉鎖式と開放式で比較した結果，開放式でよりよい結果が得られている。さらに，Amorimらは30～41歳までのヒト卵巣組織を緩慢凍結法ならびに２種類のガラス化法[エチレングリコール＋DMSO，エチレングリコール＋DMSO＋ポリビニルピロリドン(PVP)＋スクロース]により凍結後，ヌードマウスに異種移植し１週間後にTUNEL法にてヒト前胞状卵胞のアポトーシスで凍結法を評価している[11]。その結果，卵胞の形態は高い確率(約60％)で保たれており，３群間で差が認められなかったが，TUNEL法による解析の結果，卵胞のアポトーシスはガラス化法で有意に低い傾向が認められたという(特にエチレングリコール＋DMSO＋PVP＋スクロース)。一方，間質には線維化が認められたが各群間で差はなく，さらにTUNEL解析においても各群間の差が間質では認められなかった。

その他，ガラス化法が卵子の生存率も高く(緩慢凍結法42％ vs ガラス化法89％)[12]，ラットの実験モデルにおいて単位面積あたりの卵胞数が多いという結果であったものの(ガラス化法：DMSO)[13]，臨床における優位性はまだ証明されていない。そこで，われわれの研究グループ(IVFなんばクリニック：森本義晴，橋本　周，矢持隆之，近畿大学生物理工学部遺伝子工学科：細井美彦，イブバイオサイエンス研究所：竹之下誠，聖マリアンナ医科大学産婦人科学：石塚文平，五十嵐豪，洞下由記，高江正道，杉下陽堂，星名真理子)は2006年以降霊長類であるカニクイザルを用いた前臨床試験(ガラス化法による新しい卵巣組織凍結方法の開発)を開始し，新デバイスであるクライオサポートを用いたガラス化法を開発し，霊長類ではじめてガラス化法(エチレングリコール＋PVP＋スクロースを用いた溶液)を用いた自家移植卵巣組織から質の高い卵子の採取と，顕微授精による受精卵の獲得に成功している[14)-16)]。なお，エチレングリコールは最も一般的に使用される浸透性の耐凍剤で，細胞毒性が低く，浸透も速いとされている。また，非浸透性耐凍剤であるPVPや糖類(スクロース)などの高分子物質は，細胞を効果的に脱水させ，耐凍剤による曝露時間を減少させると考えられており，耐凍剤による曝露時間の減少は卵巣組織内の卵母細胞の生存能力を支えるために不可欠となってくる。以上より，われわ

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表2　ガラス化法溶液の組成内容比較

Hashimoto ら[14]	5.64M(35% v/v)EG+ 5 % PVP+0.5M スクロース 平衡時間：5分
Hovatta ら[10]	0.38M EG+0.35M DMSO+0.38M PrOH 0.75M EG+0.7M DMSO+0.75M PrOH 1.5M EG+1.4M DMSO+1.5M PrOH 平衡時間：5分あるいは10分
Amorim ら[11]	26% EG+10% DMSO+2.5% PVP+ 1 M スクロース 平衡時間：11分

EG：エチレングリコール，PrOH：プロパンジオール

れは表2に示すガラス化法溶液を用いて卵巣組織凍結を行っている。

現在，聖マリアンナ医科大学倫理委員会によって承認された臨床試験「若年女性がんおよび免疫疾患患者のQOL向上を志向した卵巣組織凍結ならびに自家移植」を進め，本邦ではじめて臨床応用を開始し，2013年3月現在，57症例(早発卵巣機能不全症例も含む)に対して卵巣組織凍結を施行している。将来ガラス化卵巣を用いた妊孕性温存療法によって生児が獲得された暁には，本技術の評価が卵巣組織凍結という妊孕性温存療法の再評価として重要となる。

卵巣組織凍結に関する指針

欧米では，腫瘍医と産婦人科医，そして患者間におけるがん・生殖医療(oncofertility treatment)に関する情報共有のネットワークがすでに2006年以降構築されている。一方，本技術の臨床応用への成功を機に，International Society for Fertility Preservation (ISFP：初代ISFP会長Donnez)による第1回会議(World Congress on Fertility Preservation)が2009年にベルギーで開催され，2年に1度世界中の研究者が集まり議論を展開している。そして，ISFPの2代目会長のKimらにより「若年がん患者に対する妊孕性温存療法の推奨」が2012年6月に「Journal of Assisted Reproduction and Genetics」誌に掲載され，本推奨が若年がん患者の妊孕性温存療法に関する最新の指針となっている[17]。ISFP推奨の適応疾患(がん)はリンパ腫と乳がんとなっており，一方白血病は卵巣内におけるがん細胞の存在の可能性によって適応から除外されている。しかし，リンパ腫に対する標準的化学療法であるABVD療法(アドリアマイシン，ブレオマイシン，ビンブラスチン，ダカルバジン)は卵巣機能不全に対してリスクの低い治療法であることから，リンパ腫は骨髄移植/造血幹細胞移植施行症例かあるいは緊急的な妊孕性温存療法として行う場合にはオプションとして卵巣組織凍結が適応となるとしている。一方，卵巣機能不全のリスクが高い治療法を受ける思春期前のリンパ腫患者に対しては卵巣組織凍結が唯一の妊孕性温存療法となる。

一方，2006年に米国臨床腫瘍学会(The American Society of Clinical Oncology；ASCO)は，がん患者における妊孕性温存に関する指針を示している[18]。そのなかですでに確立された治療法として胚凍結，放射線療法時の卵巣遮蔽，卵巣位置移動術などが記されており，卵凍結や卵巣組織凍結，GnRHアナログやアンタゴニストによる卵巣機能の保護は臨床試験の段階の技術として記されている[18]。ASCOのがん患者における妊孕性温存

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に関するガイドラインは近日中に見直しが計画されているが(Oktay 私信)，しかし oncofertility という概念の生みの親でもある米国の Woodruff が構築した Oncofertility コンソーシアムにおいても，卵巣組織凍結は研究的技術ではあるが早急に妊孕性温存を考慮する際の最高のオプションとなりうると推奨されている。Oncofertility コンソーシアムにおける卵巣組織凍結の適応外疾患は白血病，リンパ腫そして卵巣がんとなっている[19]。一方，2006年に世界ではじめてのがん患者の妊孕性温存ネットワークとして構築されたドイツ語圏を中心とした FertiPROTEKT[20]でも，卵巣組織凍結の適応外疾患として白血病と卵巣がんを挙げている。凍結卵巣を移植する際には卵巣組織内の微小残存がん病巣(minimal residual disease；MRD)が問題となることから適応疾患を慎重に選択すべきであり，白血病では組織所見ならびに免疫組織化学染色で MRD が認められなかった症例の75％で染色体異常が PCR 法にて検出されたとの報告もあることから[21]，これまで述べてきたように白血病は卵巣組織凍結の適応とはならない。また Andersen らによる同様の報告も存在し，免疫組織染色にて残存腫瘍細胞が陰性であった組織に対して real time qPCR を施行した結果，7例中4例が陽性であったという[22]。しかしそれらの組織をヌードマウスに移植し，5ヵ月後にがん細胞の再発について検索を行ったが，real time qPCR では再発を確認できなかったことから，ヌードマウスへの移植によって悪性腫瘍の発生の有無を確認する実験系の限界が示唆されている[22]。

乳がんに関しては，初期の乳がん患者51症例の HE ならびに WT-1 を用いた免疫組織化学染色による報告では，卵巣組織に転移は認められなかったという結果もある[23]。現在，欧米においては進行乳がん患者が妊孕性温存療法の適応とならないことから，卵巣組織凍結適応となる乳がん患者は卵巣への転移に関して安全であるとされており，乳がん患者が卵巣組織凍結保存の適応疾患の上位となっている。

おわりに
—がん・生殖医療における卵巣組織凍結・移植の実践—

Donnez による，はじめての卵巣組織凍結施行の報告から15年が経過し，はじめての生児獲得の報告から9年が経過した現在，欧米では本技術はすべての若年女性がん患者への選択肢として提供すべき医療行為となっている。そして，卵巣組織凍結が可能な施設が本邦でも増えつつある。最新の指針である世界の21の主要がんセンターの NPO 団体である National Comprehensive Cancer Network(NCCN)の Adolescent and Young Adult (AYA：思春期・若年成人，15～39歳が対象) Oncology ガイドライン(2012年版，患者向けは2013年版[24])では，卵巣組織凍結は卵子凍結と GnRH アナログによる卵巣保護と同様に，依然として本法は実験的段階の診療であると記されている。しかし，卵巣組織凍結保存はより多くの卵子を保存できるだけでなくエストロゲン分泌によるホルモン補充ができるというメリットがあり，妊孕性の温存だけでなく卵巣欠落症状の改善やエストロゲン低下による心血管系障害の予防や骨密度低下を緩和することができる可能性も有している。さらに，卵巣組織凍結による妊孕性温存療法は，目の前の恐怖と不安のなかでがん治療に臨む若年がん患者の精神的支えとなる可能性もある。今後，より至適な卵巣組織凍結法が開発され，一方でその技術が高い倫理観をもって臨床応用される考え方が，医師のみならず患者にも浸透することが望まれる。

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