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.

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.
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. 1A, major increases in graft weights 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. 1B). Histological analyses (Fig. SL4) and follicle counting of grafts (Fig. 1C and Fig. S1B) 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. 1C). Compared with day 10 ovaries, the grafting procedure led to decreases in absolute number of primordial, primary, and early secondary follicles (Fig. S1B). Furthermore, cutting/grafting of ovaries from older mice, including those containing early antral follicles from day 23 animals, also increased graft weights (Fig. 1D).

After grafting for 5 d, hosts received an ovulating dose of human chorionic gonadotropin (hCG). As shown in Fig. S1C, 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. S1D). Similar to mouse studies, fragmentation/auto-transplantation 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. S2.4 and B) indicated the expression of transcripts and proteins for key Hippo signaling genes in ovaries of juvenile mice. Also, immunohistochemical staining of oocytes from adult mice (Fig. S2C) 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. 2A, 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. 2B), 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. S2D). 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. 2C, 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. S3A). Immunoblotting of highly expressed CCN2 demonstrated increased CCN2 proteins in fragmented ovaries (Fig. 2D). Real-time RT-PCR analyses showed fragmentation-induced increases in CCN2 transcripts in somatic cells, but not oocytes (Fig. S3B). 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. 2E). 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. S3C), 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 ovariecтомized 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/grafting. Mean ± SEM; *P < 0.05; 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; Pm, primary; Sec, secondary; P0, 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.
ovaries for fragmentation. As shown in Fig. S4A, 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. S4B). Follicle counting of grafts indicated no loss of total follicles with verteporfin pretreatment (Fig. S4C). 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. S4D). 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. 3D). 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. 3B). 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. S6A), 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. S6B). We then thawed cryopreserved human ovarian cortical strips (1–2 mm thickness and 1 × 1 cm) and cut them into small cubes (1–2 mm³) before incubation. Real-time RT-PCR analyses indicated time-dependent increases in transcript levels for CCN2, 3, 5, and 6 (Fig. S6C). 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. 3D) and incubated them with Akt stimulators before xenografting into immune-deficient mice. Within 4 wk, antral follicles were detected, demonstrating rapid follicle growth (Fig. 3E 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. 4A). 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...
Thirty-six hours later, egg retrieval was performed (Fig. 4A) on elevated serum hCG levels. Two embryos was transferred and pregnancy was diagnosed based on elevated serum hCG levels for 4 wk. (D) Cortical strips before grafting. Arrow, a secondary follicle; arrowheads, primordial/primary follicles. (Scale bar, 1 mm.) Mean ± SEM; *P < 0.05.

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 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...
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 LAT51 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 polymerization. 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 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 acinar 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 maximize follicle growth with ovarian damage.

Figure 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 2 mm 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 cryopreservation of four-cell stage embryos. Patients then received hormonal treatments to prepare the endometrium for implantation following 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.
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 pre-ovulatory 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).

ACKNOWLEDGMENTS. We thank M. Hoshina, K. Tarumi, N. Takahashi, and S. Tsukamoto for technical assistance. No federal funds were used for the human IVF work. This work was supported by funds from the National Institutes of Health National Institute of Child Health and Human Development (US4 HD068158 as part of the Specialized Cooperative Centers Program in Reproduction and Infertility Research, to A.J.H.), and California Institute of Regenerative Medicine Grant RB2-01553 (to A.J.H.). K.K. is supported by Grant-In-Aid for Scientific Research (24390376, 23013004, and 24659722) and funds from Uehara Memorial Foundation, Naito Foundation, Terumo Life Science Foundation, Astellas Foundation, and Mochida Memorial Foundation.

SI Methods

Animals. CD-1 and B6D2F1 mice were purchased from Charles River Laboratories and housed in the animal facility of Stanford University. Immune-deficient SCID mice (CLEAR) were housed in the Akita University facility.

Ovarian Fragmentation and Grafting. Paired ovaries from CD-1 mice at different ages were excised in t-15 medium. One ovary from each animal was cut into 2-4 pieces, whereas the contralateral one remained intact. Ovaries were incubated and transplanted for 5 d with daily FSH [1 international unit (IU)/mice] injections as described (1). At the end of transplantation, grafts were collected for fixation before weighing and histological analyses. Some fragmented ovaries from day 10 mice were incubated with 1 µg/mL of CCN2 antibodies or nonimmune IgG for 18 h before grafting for 5 d and weight determination. To test the effect of Akt stimulators, ovaries from day 10 mice were treated with bisperoxovanadium (bpV) (hopic) (30 µM) and 740YP (Tocris) (150 µg/mL) for 1 d, followed by 740YP treatment for another day, before grafting.

To test the involvement of Hippo signaling, day 10 mice were pretreated i.p. with verteporfin (12.5 mg/kg body weight) for 3 h before obtaining ovaries for fragmentation into three pieces. At 4 h after incubation, some ovaries were used for determination of CCN2 and anti-Mullerian hormone (AMH) transcripts. Some ovaries were grafted into FSH-injected hosts for 5 d before determination of graft weights and follicle dynamics. Some ovaries with or without fragmentation were used for real-time RT-PCR to measure levels of CCN growth factors and BIRC apoptosis inhibitors, for immunoblotting to determine protein levels for Yes-associated protein (YAP)/phospho-YAP (pYAP) and CCN2, and for immunostaining to analyze changes in cellular localization of YAP after fragmentation. Quantification of transcript levels for CCN growth factors and BIRC apoptosis inhibitors was also performed using incubated ovarian samples without grafting. For some samples, somatic cells and oocytes were isolated as previously described (2). For rat studies, ovaries from day 10 rats were fragmented and incubated for 1 h before autotransplantation into kidneys of the same animals for 5 d.

Follicle Counting. Ovarian grafts were fixed in 10% (vol/vol) buffered formalin overnight, embedded in paraffin, serially sectioned, and stained with hematoxylin and eosin. Only follicles with a clearly stained oocyte nucleus were counted as described (1).

IVF and Embryo Transfer. At 5 d after transplantation, B6D2F1 host mice were treated with 10 IU equine chorionic gonadotropin (eCG) for 48 h, followed by an injection of human chorionic gonadotropin (hCG) (10 IU) to induce oocyte maturation. Twelve hours later, grafted ovarian fragments were collected for oocyte retrieval. In vitro fertilization (IVF) and embryo transfer were performed as described (1).

Ovarian Explant and Follicle Cultures. Ovaries from day 10 mice were treated with recombinant CCN growth factors for 4 d, with medium changes after 2 d of culture as described (3). At the end of culture, ovarian weights and follicle dynamics were determined. Secondary follicles (115 µm) were isolated and cultured for 2 d as described (3). Some follicles were treated with bpV (hopic) (3 µM) and 740YP (15 µg/mL) for 1 d and 740YP alone for another day. Follicle diameters were monitored.

Measurement of Actin Levels. Ratios of filamentous actin (F-actin) to globular actin (G-actin) in ovaries were determined by F-actin/G-actin in vivo assay kit (Cytoskeleton). Intact or fragmented ovaries were homogenized in the F-actin stabilization buffer. After incubation at 37 °C for 10 min., the lysate was centrifuged at 350 × g for 5 min at 37 °C to remove tissue debris. After further centrifugation at 100,000 × g for 1 h at 37 °C, the supernatant was collected. Pellets were resuspended in ice-cold water containing 8 M urea and incubated on ice for 1 h with gentle mixing every 15 min. To measure F-actin/G-actin ratios, equal amounts of supernatant (G-actin) and resuspended pellets (F-actin) were subjected to immunoblotting analysis using the panactin antibody (Cytoskeleton).

Real-Time RT-PCR Analyses. Total RNAs were extracted using an RNasea Micro Kit, and cDNAs were synthesized using a Senscript RT Kit (QIAGEN). Real-time PCR was performed using iTaq SYBR Green SuperMix (Bio-Rad) on a Smart Cycler TD system (Cepheid) as follows: 15 min at 95 °C, 45 cycles of 15 s at 95 °C, and 60 s at 60 °C. Relative abundance of specific transcripts was normalized based on GAPDH levels. Human ovarian strips (1 × 1 cm) from Caesarean-section patients were obtained. Frozen strips were thawed and some stripes were further fragmented into cubes of 1-2 mm³ before evaluating the expression of CCN growth factors using real-time RT-PCR. To normalize basal levels among patients, data were expressed as fold changes relative to the 0 h data expressed as 1.0.

Immunoblotting and Immunostaining Analyses. Proteins were extracted using M-PER Mammalian Protein Extraction Reagent (Thermo) containing a protease inhibitor mixture (Thermo). Specific first antibodies (Cell Signaling Technology and Santa Cruz Biotechnology) were used for immunoblotting analyses. Immunohistochemical staining was performed using anti-SAV1 (Proteintech), anti-LATS1/2 (Abnova), anti-YAP (Cell Signaling Technology), and anti-TAZ (Cell Signaling Technology) at 1:100, 1:50, 1:200, and 1:200 dilutions, respectively. For mouse MST1/2, primary antibodies were from AbFRONTIER (1:100 dilution). For negative controls, nonimmune IgG (Dako) was used.

POI Patients, Ovarian Fragmentation, Akt Stimulation, and Autotransplantation. Primary ovarian insufficiency (POI) patients were selected based on a history of amenorrhea for >1 y before 40 y of age and serum FSH levels of >40 mIU/mL. A total of 27 patients (37.3 ± 5.8 y of age) were enrolled. Their duration of amenorrhea was 6.8 ± 2.1 y without spontaneous pregnancy. Under laparoscopic surgery, bilateral ovariectomy was performed without using electrocautery hemostasis to avoid damage to residual follicles. Ovarian cortices were immediately dissected by removing medulla, followed by cutting into small strips (1 × 1 cm², 1-2 mm thickness) for cryopreservation, and randomly selected pieces were used for histological examination. After vitrification (4), ovarian fragments were stored in liquid nitrogen. After patients recovered from surgery, cryopreserved ovarian strips were thawed and further fragmented into smaller cubes (1-2 mm³). Six to nine ovarian cubes were put on cell culture inserts (Millicell Cell Culture Insert, 12 mm; polycarbonate, 3.0 µm; Merck Millipore) and treated with 30 µM of bpV (hopic), PTEN enzyme inhibitor, and 150 µg/mL of 740YP, a PI3K stimulator, for 24 h after incubation with 740YP alone for another 24 h in DMEM/F12 medium containing 10% (vol/vol) human serum albumin (Mitsubishi Tanabe Pharma), 0.05 mg/mL ascorbic acid, 1% antibiotic/
antimycotic solution (Invitrogen), and 0.3 IU/mL FSH at 37 °C under a 5% CO\textsubscript{2} atmosphere (1). For autotransplantation, patients underwent a second laparoscopic surgery. Transplantation sites beneath the serosa of Fallopian tubes were swollen by preinjection with saline, followed by incisions of serosa to insert ovarian cubes that were washed immediately before transplantation in warmed (37 °C) culture media. Approximately 20–80 cubes were inserted beneath the serosa of each Fallopian tube before suture. The underside of serosa in Fallopian tubes was selected as the grafting site due to high vascularization, convenience for transvaginal ultrasound monitoring, and ease for oocyte retrieval.

**Follicle Growth Monitoring, IVF, Preimplantation Embryo Cryopreservation, Embryo Transfer, and Pregnancy Detection.** Follicle growth was monitored weekly or biweekly by transvaginal ultrasound together with serum estrogen levels to detect growing antral follicles. When antral follicles (>5 mm in diameter) were detected, monitoring frequency increased to every 2–3 d and follicle growth was promoted by injecting 150–300 IU recombinant FSH (GONAL-f; Merck Serono) daily until the follicle reached >16 mm in diameter or serum estradiol levels elevated to ~200 pg/mL. Once a preovulatory follicle was detected, the patient received a single injection of 10,000 IU hCG (Asuka Pharma) to induce oocyte maturation. At 36 h later, oocytes were aspirated from follicles using color Doppler ultrasound-guided transvaginal retrieval via a 19G needle. After oocyte retrieval, IVF was performed by intracytoplasmic sperm injection (ICSI) before culturing injected oocytes in the fertilization medium (Quinn’s Advantage Fertilization HTF Universal Medium; Sage) for 16 h. Because the majority of patients lived a long distance from the hospital, we routinely cryopreserved the husband’s sperm. Although cryopreservation of sperm is an established method, the fertilization ability of thawed sperm decreased severely in some patients. Thus, ICSI was routinely used to minimize fertilization failure.

Fertilized oocytes were transferred to the cleavage medium (LifeGlobal) and cultured for one more day. Preimplantation embryos at the four-cell-stage were cryopreserved by vitrification using Cryotop (Kitazato BioParma) (5) and stored in liquid nitrogen. To improve embryo implantation of POI patients, embryos were cryopreserved and then transferred after patients were treated with estrogens and then progesterone. The dosage and duration of sex hormones were determined based on hormonal and uterine endometrial status of individual patients. After embryo transfer, patients were treated with progesterone supplements for 6 wk to support early pregnancy. Establishment of pregnancy was determined by ultrasound and by measuring serum hCG levels. After the patient became pregnant, development of the fetus was monitored by routine prenatal checkups.

**Statistical Analyses.** Results are presented as the mean ± SEM of three or more independent assays. Statistical significance was determined by using one-way ANOVA, followed by Fisher’s protected significant difference test, with \( P < 0.05 \) being statistically significant.

**Animal and Human Subject Approval.** Mice and rats were treated in accordance with guidelines and following approval of the administrative panel on laboratory animal in Stanford and the Animal Research Committee, Akita University School of Medicine. For clinical studies, we obtained informed consent from patients and approval from the Human Subject Committee of St. Marianna University and Japan Society of Obstetrics and Gynecology. Human ovarian strips from C-section patients were obtained following informed consent from patients and approval from the Human Subject Committee of Akita University.

Fig. S1. Ovarian fragmentation/grafting led to viable pups. (A) Representative histology of ovarian grafts from day 10 mice with or without cutting into three pieces, followed by grafting for 5 d. (Scale bar, 200 μm.) (B) Absolute follicle numbers before and after grafting of intact and fragmented (three pieces) ovaries from mice at day 10 of age. (B, Left) Total follicle numbers. (B, Right) Follicle dynamics; n = 5 ovaries. Same asterisk symbols indicate significant differences (P < 0.05). (C) Retrieval of oocytes (Left) after cutting/grafting of ovaries from day 10 mice for fertilization and embryonic development. Percentage of mature oocytes (Center) and fractions of fertilized oocytes developed to different embryonic stages (Right) are shown. Controls in Right represent oocytes obtained after treatment of day 23 mice with eCG, followed by hCG to induce ovulation (1); n = 8 ovaries. GVBD, germinal vesicle breakdown. (D) Delivery of healthy pups following transfer of embryos derived from mature oocytes obtained after ovarian fragmentation/grafting. Ovaries from day 10 B6D2F1 mice were fragmented into three pieces before grafting for 5 d with daily FSH treatment. Animals were then treated sequentially with eCG (48 h) and hCG (12 h) before retrieval of mature oocytes for fertilization and embryo culture. Two-cell embryos were transferred to surrogate mothers of the CD1 strain followed by pregnancy and delivery. Pups derived from ovarian fragmentation were healthy and fertile. (E and F) Ovarian fragmentation and grafting promoted ovarian follicle development in rats. Paired ovaries from day 10 rats were autografted into kidneys of the same animals intact or in three pieces. Animals were treated with FSH for 5 d before determination of ovarian weights. (E) Morphology of isolated ovaries. (F) Ovarian weights. Numbers in parentheses are number of ovaries used. Mean ± SEM; P < 0.05.

Fig. S2. Ovarian expression of Hippo pathway genes and increases in nuclear YAP localization following ovarian fragmentation. (A) Real-time RT-PCR analyses of transcripts for Hippo pathway genes in ovaries of day 10 mice; n = 6. (B) Immunoblotting of Hippo pathway proteins in ovaries of mice at day 10 of age (d10). Extracts from 3T3 cells served as controls (3T3). Specific signals are marked with arrows. pYAP, phospho-YAP(Ser127). (C) Immunohistochemical staining of Hippo signaling genes in ovaries of adult mice. Signals were detected using specific antibodies. All antigens were found mainly in the cytoplasm of granulosa cells, theca cells, and oocytes of primordial (Pmd), primary (1°F), secondary (2°F), and antral (AF) follicles but at lower levels in the corpus luteum (CL). [Scale bar, 200 and 100 μm for low (Left) and high (Right) magnification, respectively.] (D) Fragmentation-induced increases in nuclear YAP in granulosa cells of primary
and secondary follicles. (D, Left) YAP staining in intact ovaries from mice at day 10 of age, showing predominantly cytoplasmic localization of YAP in granulosa cells of most primary and secondary follicles. (D, Right) YAP staining in ovaries at 4 h after fragmentation showing nuclear localization of YAP in granulosa cells of most primary and secondary follicles. Due to limited cytoplasm of granulosa cells in primordial follicles, it is difficult to determine cellular distribution of YAP in primordial follicles. [Scale bar, 100 and 50 μm for low (Top) and high (Middle and Bottom) magnification, respectively.] Arrows, secondary follicles; arrowheads, primary follicles.
Fig. S3. Increased expression of CCN growth factors and BIRC inhibitors after ovarian fragmentation and the ability of CCN growth factors to promote follicle development. (A) Ovarian fragmentation without subsequent grafting increased the expression of key CCN growth factors (CCN2, 3, 5, and 6) and apoptosis inhibitors (BIRC1 and 7). Paired ovaries from day 10 mice with or without cutting into three pieces were incubated for up to 7 h before analyses of transcript levels for different genes. Intact ovaries, solid lines; three pieces, dashed lines; n = 10–15. (B) Increases in CCN2 expression in somatic cells of fragmented ovaries. Paired ovaries from day 10 mice with or without cutting into three pieces were cultured for 1 h and then transplanted under kidney capsules. At 3 h after transplantation, ovaries were dissected followed by isolation of oocytes and somatic cells as previously described (1). CCN2 and GAPDH mRNA levels were measured by real-time RT-PCR; n = 4. *P < 0.05 vs. intact. (C) Treatment with CCN growth factors promoted preantral follicle development in ovarian explants. Following explant cultures with different CCN growth factors, follicle dynamics were determined by counting the percentages of follicles at different developmental stages. Mean ± SEM; *P < 0.05; n = 5.

Fig. S4. Pretreatment with verteporfin and CCN2 antibodies suppressed fragmentation-induced ovarian growth. (A) Pretreatment with verteporfin suppressed fragmentation-induced increases in CCN2, but not AMH, transcript levels. Juvenile mice were injected i.p. with verteporfin for 3 h before retrieving ovaries for fragmentation. At 4 h after incubation, transcript levels for CCN2 and AMH were determined by real-time RT-PCR; n = 6–12. (B) Pretreatment with verteporfin partially suppressed fragmentation-induced graft weight increases. Ovaries from juvenile mice preinjected with verteporfin for 3 h were fragmented and incubated for 1 h before grafting into FSH-treated hosts for 5 d followed by determination of graft weights. Numbers in parentheses are number of samples used. (C) Follicle dynamics of intact and fragmented ovarian grafts from animals with or without verteporfin (VP) pretreatment; n = 3–5. (D) Incubation of fragmented ovaries with CCN2 antibodies suppressed ovarian weight increases. Paired ovaries were fragmented into three pieces followed by incubation with CCN2 antibodies or non–immune-IgG for 18 h before grafting for 5 d. Mean ± SEM; *P < 0.05.

Fig. S5. Absolute follicle numbers after grafting of intact and fragmented ovaries with or without treatment with Akt stimulators. Paired ovaries from juvenile mice were fragmented and incubated with or without Akt stimulators for 2 d followed by allo-transplantation for 5 d to determine graft weights; n = 4. Same letter symbols indicate significant differences (P < 0.05).
Fig. S6. Expression of Hippo signaling genes in human ovaries. (A) Expression of transcripts for Hippo signaling genes in human ovarian cortices. Ovarian cortical tissues were obtained from patients with a benign ovarian tumor and used for RT-PCR analyses. (B) Immunohistochemical staining of Hippo signaling proteins in human ovarian cortices. Expression of SAV1, LATS1/2, YAP, and TAZ antigens in ovarian cortices was performed. All antigens were found in cytoplasm of granulosa cells. (Scale bar, 100 μm.) (C) Increases in CCN transcripts after fragmentation of human ovarian tissues. Thawed cortical strips were cut into pieces or left intact before incubation for different intervals followed by real-time RT-PCR analyses; n = 4–8.
Fig. S7. Genes involved in ovarian fragmentation, Hippo signaling, and follicle growth are important for ovarian physiology and pathophysiology. Ovarian fragmentation led to changes in intercellular tension and facilitated the conversion of G-actin to F-actin. Subsequent disruption of Hippo signaling decreased pYAP to total YAP ratios, leading to increased expression of downstream CCN growth factors and BIRC apoptosis inhibitors. Secretion of CCN growth factors stimulated follicle growth. Genetic studies found DIPAH2 (1) and FOXL2 (2) mutations in POI families, whereas deletion of LATS1 (3) or CCN2 (4) led to infertility phenotypes in mice. Genome-wide association studies identified DIAPH2 (5) and DAIPH3 (6) as candidate genes for follicle reserve and menopausal ages, whereas copy number changes for BIRC1 (7) were found in POI patients. YAP was found not only as a candidate gene for PCOS (8) but also as an oncogene for ovarian surface epithelial cancer (9).

Movie S1. Grafting of ovarian cubes beneath the serosa of Fallopian tube of a POI patient.