Supporting Information

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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 L-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 grating 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-Müllerian hormone (AMH) transcripts. Other 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 RNeasy Micro Kit, and cDNAs were synthesized using a Sensicript 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 mm^2 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 is 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 cm^2, 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 mm^2). 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), a PTEN enzyme inhibitor, and 150 μg/mL of 740YP, a PI3K stimulator, for 24 h followed by 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_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.

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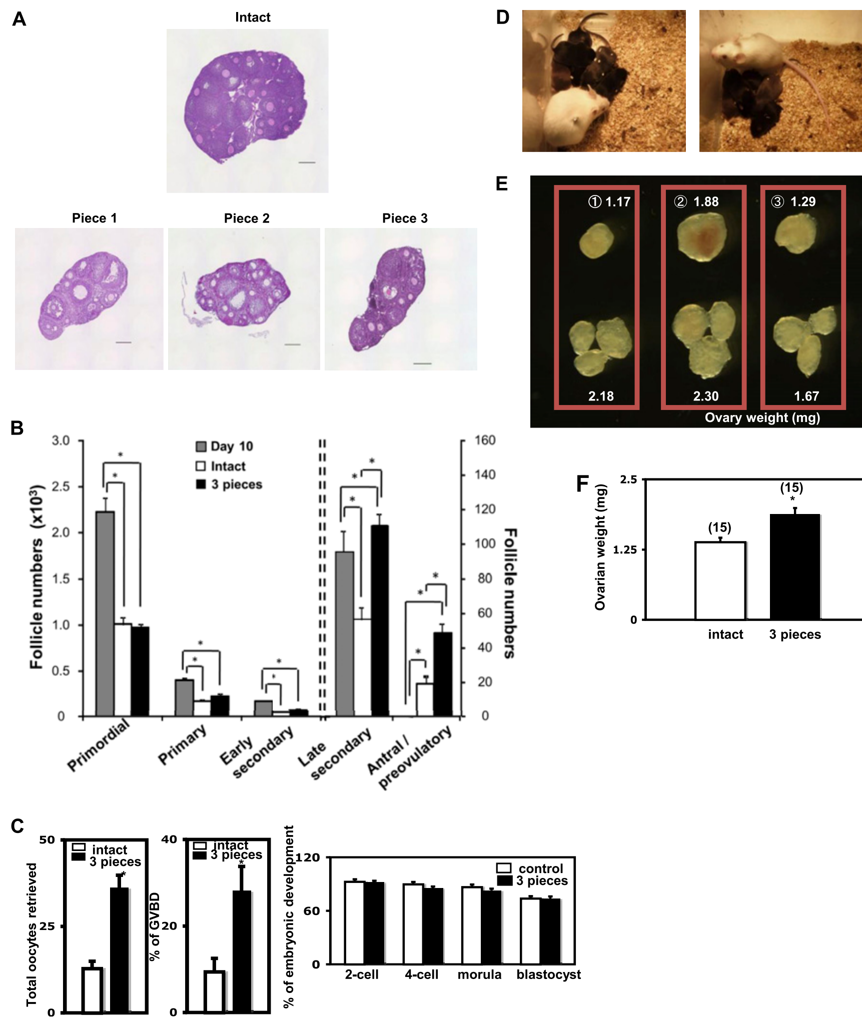

Fig. S1. Ovarian fragmentation/grafting led to viable pups. (*A*) Representative histology of ovarian grafts from day 10 mice with or without cutting into three pieces, followed by grafting for 5 d. (Scale bar, 200 μm.) (*B*) Absolute follicle numbers before and after grafting of intact and fragmented (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; $P < 0.05$.

1. Sato Y, Cheng Y, Kawamura K, Takae S, Hsueh AJ (2012) C-type natriuretic peptide stimulates ovarian follicle development. *Mol Endocrinol* 26(7):1158–1166.

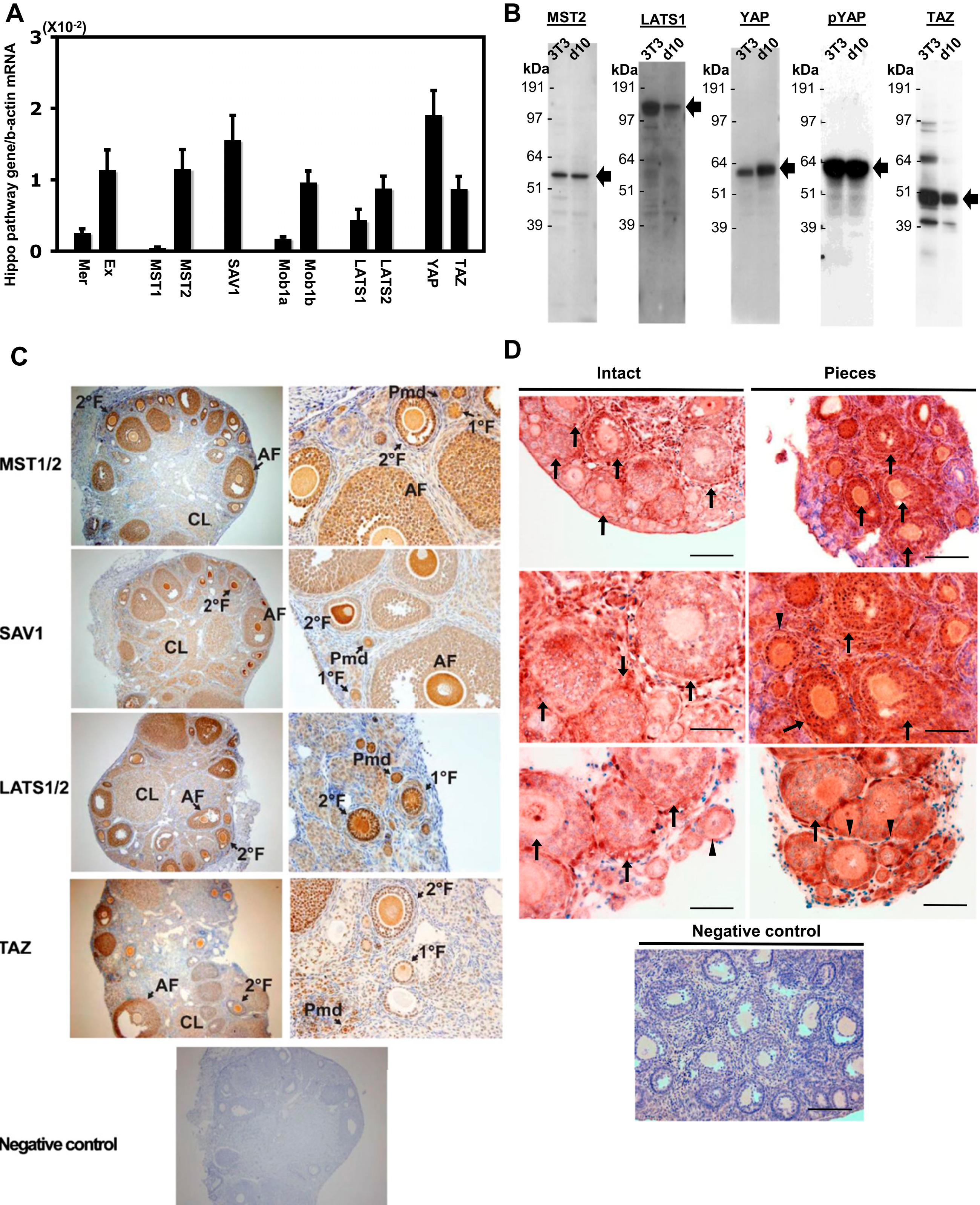

Fig. S2. Ovarian expression of Hippo pathway genes and increases in nuclear YAP localization following ovarian fragmentation. (*A*) Real-time RT-PCR analyses of transcripts for Hippo pathway genes in ovaries of day 10 mice; *n* = 6. (*B*) Immunoblotting of Hippo pathway proteins in ovaries of mice at 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

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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.

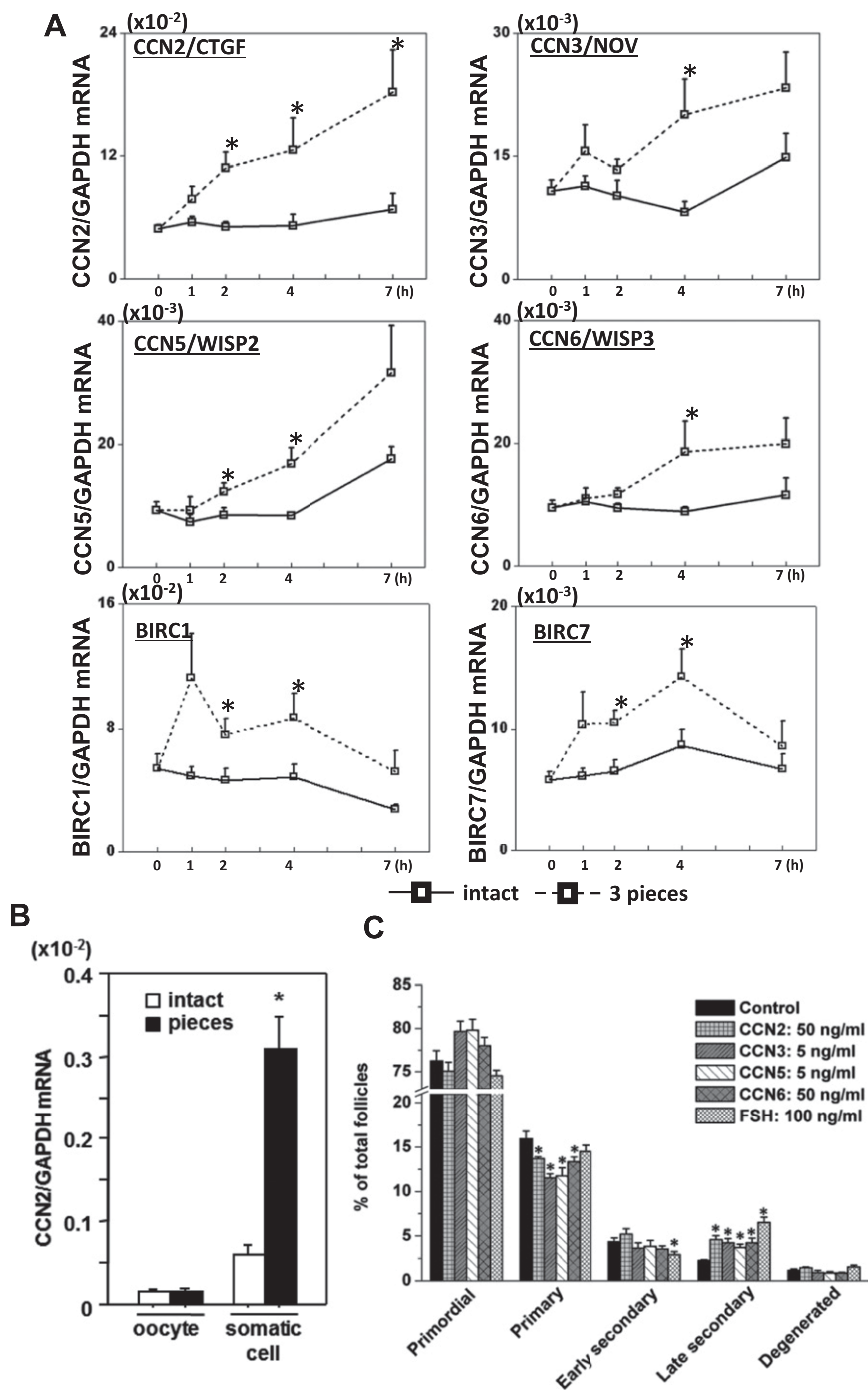

Fig. S3. Increased expression of CCN growth factors and BIRC inhibitors after ovarian fragmentation and the ability of CCN growth factors to promote follicle development. (*A*) Ovarian fragmentation without subsequent grafting increased the expression of key CCN growth factors (CCN2, 3, 5, and 6) and 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; $*P < 0.05$; $n = 5$.

1. Cheng Y, et al. (2013) Oocyte-derived R-spondin2 promotes ovarian follicle development. *FASEB J* 27(6):2175–2184.

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.

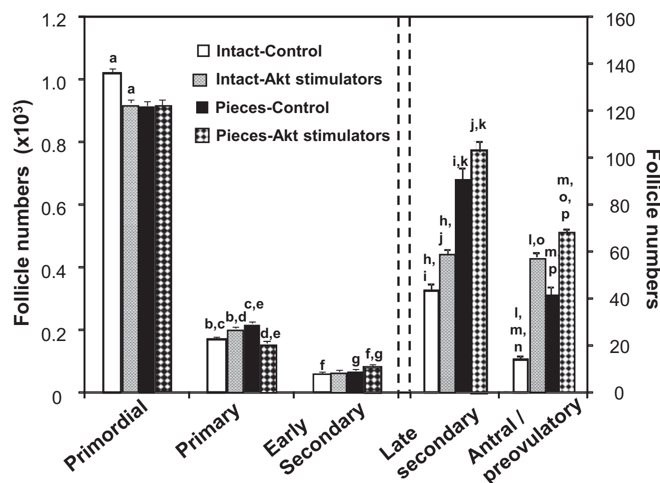

Fig. S5. Absolute follicle numbers after grafting of intact and fragmented ovaries with or without treatment with Akt stimulators. Paired ovaries from juvenile mice were fragmented and incubated with or without Akt stimulators for 2 d followed by allo-transplantation for 5 d to determine graft weights; n =4. Same letter symbols indicate significant differences (P < 0.05).

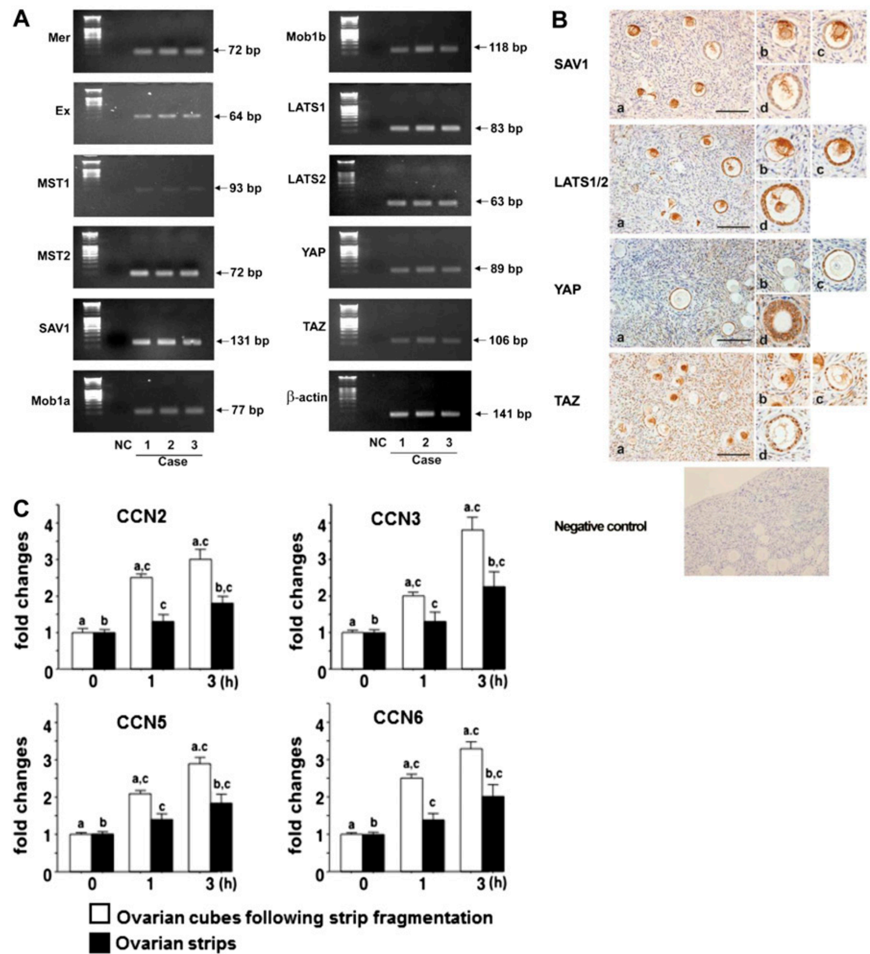

Fig. S6. Expression of Hippo signaling genes in human ovaries. (*A*) Expression of transcripts for Hippo signaling genes in human ovarian cortices. Ovarian cortical tissues were obtained from patients with a benign ovarian tumor and used for RT-PCR analyses. (*B*) Immunohistochemical staining of Hippo 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.

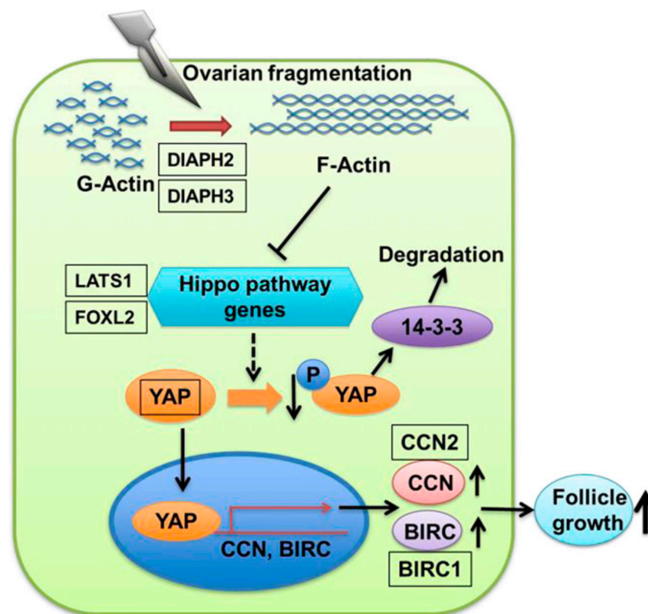

Fig. S7. Genes involved in ovarian fragmentation, Hippo signaling, and follicle growth are important for ovarian physiology and pathophysiology. Ovarian fragmentation led to changes in intercellular tension and facilitated the conversion of G-actin to F-actin. Subsequent disruption of Hippo signaling 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).

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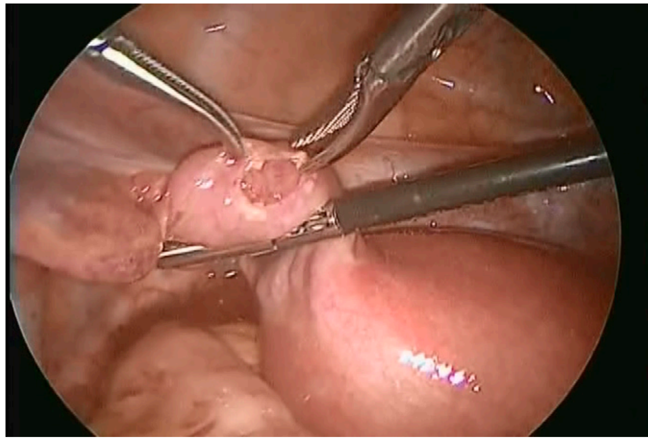

Movie S1. Grafting of ovarian cubes beneath the serosa of Fallopian tube of a POI patient.

Movie S1