Mechanical stress accompanied with nuclear rotation is involved in the dormant state of mouse oocytes

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The most immature oocytes remain dormant in primordial follicles in the ovary, ensuring the longevity of female reproductive life. Despite its biological and clinical importance, knowledge of mechanisms regulating the dormant state remains limited. Here, we show that mechanical stress plays a key role in maintaining the dormant state of the oocytes in primordial follicles in mice. Transcriptional and histological analyses revealed that oocytes were compressed by surrounding granulosa cells with extracellular matrix. This environmental state is functionally crucial, as oocytes became activated upon loosening the structure and the dormancy was restored by additional compression with exogenous pressure. The nuclei of oocytes in primordial follicles rotated in response to the mechanical stress. Pausing the rotation triggered activation of oocytes through nuclear export of forkhead box O3 (FOXO3). These results provide insights into the mechanisms by which oocytes are kept dormant to sustain female reproductive life.

INTRODUCTION

Oogenesis is a unique sequence of differentiation processes that produces functional eggs. The process starts from primordial germ cells (PGCs), which are specified in the pluripotent epiblast at embryonic day 6.5 (E6.5) (1). Then, PGCs migrate into the embryonic gonads at around E10.5. Soon after the migration, the PGCs enter meiosis, thereby becoming primary oocytes, in response to the female gonadal environment (2, 3). In the fetal ovary, oocytes form clusters that are connected to each other through intercellular bridges. Then, at the perinatal stage, the intercellular bridges are broken down, and a large proportion of primary oocytes are eliminated by apoptosis. The remaining oocytes form a follicle structure in which single oocytes are enclosed by granulosa cells (4). In the neonatal ovary in mice, there are two kinds of follicles: One immediately undergoes the first wave of oogenesis to produce mature oocytes, and the other remains immature (5). The latter are called primordial follicles and function as a storehouse of the oocytes (6). In the primordial follicles, oocytes are arrested at the dictyate stage of meiotic prophase I, and the cell size, a critical criterion of oocyte growth, remains small (<20 μm in diameter) (7). This “dormant” state of oocytes in the primordial follicles is a basis of sustainability of the oocyte pool in the ovary, ensuring the longevity of the reproductive life of females. Therefore, the mechanism maintaining the dormant state is a fundamental issue in reproductive biology and medicine.

In many mammals, dormant oocytes are surrounded by squamous granulosa cells and are located in the cortex region of the ovary. Upon follicular activation, the morphology of granulosa cells becomes cuboidal, forming a primary follicle, and the follicle moves toward the medullar region of the ovary. Despite the fact that the follicular stages have been well characterized by histological criteria, the mechanisms underlying the balance between dormancy and activation of follicles are still enigmatic. Genetic analysis and in vitro culture experiments revealed that the Akt/P13K (phosphatidylinositol3-kinase) pathway intrinsically triggers oocyte growth (8, 9). The signal from this pathway mediates phosphorylation of FOXO3, a key transcription factor for maintenance of the dormant state, resulting in export of FOXO3 from the nucleus to the cytoplasm, thereby triggering oocyte growth. FOXO3 plays a central role in maintenance of the dormant state: Overexpression of Foxo3 makes oocytes refractory to growth (10), and disruption of the Foxo3 gene accelerates oocyte growth, causing premature loss of oocytes in the ovary (11). In these studies, it is noteworthy that mice with overexpression or disruption of Foxo3 still exhibited periodical cycles of oocyte growth despite the altered balance between dormancy and activation. Moreover, histological analysis of these mice showed that small oocytes somehow remained in the cortical region. These observations suggest the involvement of not only an oocyte-intrinsic factor but also an environmental factor(s) that is localized in the cortical region. However, no intensive attempts have been made to identify such an environmental factor(s).

A clue to address this issue came from the transplantation of fetal ovaries into adult mice. When fetal ovaries were transplanted into the kidney capsule or the ovarian bursa, there was very little primordial follicle formation in the transplant at 4 to 5 weeks after transplantation (12, 13). This was also the case in in vitro culture of fetal ovaries (14): Most of the oocytes in the ovary grew simultaneously, and therefore, few oocytes remained dormant. These studies indicate that the environmental conditions necessary for the maintenance of dormancy are not yet established in the fetal ovaries. There are some indications of dynamic organization in ovarian development at the perinatal stage. Cell lineage–tracing analysis demonstrated the distinct origin of cortical and medullar granulosa cells: Cortical granulosa cells were derived from the surface epithelium of the ovary at around birth. Consistently, ovarian epithelial cells that express leucine-rich repeat containing G-protein coupled receptor 5 (LGR5), a stem cell marker in multiple cell lineages (15), invaginate into the fetal ovary and then differentiate into cells expressing p27 and FoxI2 but not Lgr5. Although these findings explain the mechanism by which distinct regions are generated in the ovary, it is still unclear how the functional differences in each region are regulated. Here, we identify the environmental factor responsible for maintaining the dormancy and possibly directing the localization of dormant oocytes in the ovary.

RESULTS

Screening of factors required for the dormant state of oocytes

To determine the timing at which the dormant state of oocytes is established during ovarian development, we transplanted fetal and...
neonatal ovaries at various stages [E12.5, E17.5, postnatal day 0 (P0), P3, P7, and P14] into the ovarian bursae of adult immunocompromised mice. The donor ovaries were from Stella–green fluorescent protein (GFP) or Stella–cyan fluorescent protein (CFP) transgenic females, enabling us to distinguish the fluorescent donor oocytes from nonfluorescent oocytes derived from the recipient ovary. At 3 to 4 weeks after the transplantation, we evaluated the percentages of primordial follicles formed in the donor ovaries. At 3 weeks after transplantation, as expected, there were few primordial follicles left, if any, in the transplanted ovaries, but at the same time, there were a number of follicles growing to antral stage (Fig. 1, A to C). The percentage of formed primordial follicles was gradually increased in the donor ovaries of E17.5, P0, and P3 (Fig. 1, A to C). Thereafter, the percentage seemed to reach a plateau since it was unchanged in the P7 and P14 donor ovaries. These findings indicate that the pool of dormant oocytes was almost established in the ovary by P3. This is consistent with the previously documented dynamic change of histological structure in the perinatal ovaries, during which many of the oocytes are eliminated by apoptosis and then single oocytes are enclosed by granulosa cells (16). In addition, we frequently observed multioocyte follicles in the E12.5 donor ovaries (Fig. 1B), suggesting that these oocytes started to grow before the elimination of oocytes, followed by follicle formation.

To gain insight into the mechanisms involved in establishing the dormant state, we identified genes preferentially expressed in P3 oocytes by comparing them with E12.5 PGCs. As expected, transcription factors essential for early oocyte differentiation, such as Figla, Sohlh1, No box, and Lhx8, were enriched in the gene list (Fig. 1D), confirming the reliability of the analysis. Gene ontology (GO) analysis identified functional terms such as extracellular matrix (ECM), extracellular region, and collagen trimer (Fig. 1E). This suggested that oocytes respond to environmental cues that might be markedly changed during the perinatal period. A previous report showed that collagen is enriched in the cortex of ovaries, where primordial follicles reside (17). However, the function of the ECM in the ovary has remained elusive. Therefore, we examined the functionality in the following experiments.

**ECM is an environmental factor for maintenance of the dormant oocytes**

We first confirmed the results of the GO analysis by immunostaining using E12.5 and P3 ovaries. The results consistently demonstrated that components of the ECM, such as fibronectin and collagen type IV, permeated around follicle structures in the cortical region of P3 ovaries, whereas the components were faint around the germ cells in E12.5 ovaries (Fig. 2A and fig. S1). We then evaluated whether the ECM plays a role in maintenance of the dormant oocytes in a culture experiment. We incubated P7 ovaries with CTK solution [containing collagenase type IV, trypsin, and knockout serum replacement (KSR)] for 1 hour to digest the ECM in the ovary. CTK treatment induced export of FOXO3 to the cytoplasm of small oocytes (<20 μm in diameter) located at the cortical region of the ovaries (Fig. 2B). The percentage of oocytes with cytoplasmic FOXO3 was significantly higher in CTK-treated ovaries than in phosphate-buffered saline (PBS)–treated control ovaries (Fig. 2B). It is noteworthy that the small oocytes with cytoplasmic FOXO3 in the CTK-treated ovaries were located at the edge of the cortex (Fig. 2B). This is presumably because CTK infiltrated and digested ECM from the outside of the ovary during incubation. This strongly supports the notion that the ECM is involved in nuclear localization of FOXO3 in the small oocytes.

To confirm that the oocytes exit from the dormant state, we examined subsequent oocyte growth in the CTK-treated ovaries. P3 ovaries treated with CTK were subjected to an organ culture system that allows oocyte growth of up to secondary follicle formation (14). At 14 days of culture, the number of large oocytes (>50 μm in diameter) was significantly increased in the CTK–treated ovaries compared to the control ovaries (Fig. 2C). Consistently, the number of small oocytes (<20 μm in diameter) was significantly decreased in CTK–treated ovaries (Fig. 2C). Together, these results suggest that ECM is one of the components providing environmental cues essential for the maintenance of dormant oocytes.

**Compression of oocytes is required for the dormant oocytes**

We next examined the mechanisms underlying the maintenance of the dormant state by ECM. Since ECM triggers various cellular responses, such as changes in gene expression, intracellular signaling, and morphology, there are several possibilities with regard to the functional requirements of ECM. This study postulated that mechanical stress is a key factor generated by the ECM surrounding the follicle structure, since one of the major responses to ECM is the formation of contractile stress fibers that increase cellular tension, causing a flattened cell morphology (18), and this morphology is likely relevant to squamous granulosa cells surrounding the oocyte. We then examined whether stress fibers were formed in the primordial follicles. Immunofluorescence analysis showed that stress fibers were observed in squamous granulosa cells, as well as oocytes, of the primordial follicles in the P7 ovaries (Fig. 3, A and B). The stress fibers represented by the phalloidin intensity in primordial follicles were attenuated by CTK treatment (Fig. 3, A and B), especially at the edge of the ovarian cortex (fig. S2). These observations confirmed that the stress fibers were generated through ECM. Given that the surrounding squamous granulosa cells have contractile stress fibers, the oocytes surrounded by these cells would be compressed in the primordial follicles (fig. S3A). Therefore, we hypothesized that constant compression is a key requirement for maintenance of the dormant state of oocytes. If this is true, then the volume of the oocytes would be increased in CTK treatment since the contractile stress fibers would loosen. To test this possibility, we measured the volume of oocytes located at the edge of the ovary using a confocal microscope. As expected, we found that the volume of oocytes was significantly increased in comparison to those of PBS-treated ovaries (Fig. 3C). To further validate the hypothesis, we increased the compression using a pressure culture chamber (STREX AGP-3001S) (fig. S3B). Under an exogenous pressure at 33.3 kPa, the volume of the oocytes in the CTK-treated ovaries returned to that of the oocytes without CTK (Fig. 3C). Unexpectedly, immunofluorescence analysis revealed that nuclear localization of FOXO3 was restored in the oocytes cultured with exogenous pressure (Fig. 3D). In agreement with this finding, the percentage of oocytes with cytoplasmic FOXO3 was significantly reduced under culture with exogenous pressure (Fig. 3D). With respect to the subcellular localization of FOXO3, exogenous pressure almost completely canceled the effect of CTK treatment (Fig. 3D). This suggested that the cytoplasmic localization of FOXO3 induced by CTK treatment was attributable to the reduced compression in the oocytes. Together, these results demonstrate that compression, which keeps oocytes small, is a critical factor for maintenance of the dormant state of oocytes.
Fig. 1. Identification of factors potentially involved in the establishment of the dormant state of oocytes. (A) Primordial follicle formation in the transplanted ovaries. Images are Stella-GFP transgenic ovaries after transplantation into the adult ovarian bursa. The developmental stages of the donor ovaries are indicated on the left side of the images. Note that small oocytes (arrowheads), presumably in primordial follicles, were formed in the E17.5 and later ovaries, whereas large oocytes (arrows) were dominant in the E12.5 ovary. dpc, days post coitum; dpp, days post partum; BF, bright field. Scale bars, 200 µm. (B) Histology of transplanted ovaries. Images show the results of immunohistochemistry with anti-GFP antibody (brown) and hematoxylin and eosin (HE) staining of the transplanted E12.5 and P3 Stella-CFP ovaries. Arrows indicate primordial follicles. Scale bars, 200 µm. (C) Summary of the percentage of follicular stage. The bars indicate the percentages of each follicle indicated above in the transplanted ovaries. (D) DEGs from the comparison between E12.5 PGCs and P3 oocytes. The numbers of differentially expressed genes (DEGs) are shown in the plot. The X axis shows log2 average of counts per million (CPM) mapped reads. (E) Gene ontology (GO) analysis of genes enriched in P3 oocytes. Note that GO terms related to the extracellular matrix (ECM) are listed in the upper ranks. MHC, major histocompatibility complex.
Nuclear rotation in response to compression

To further investigate the effect of compression on the dormancy, we tried to identify a cellular response to the compression. A clue was revealed unexpectedly while we were measuring the volume of oocytes by the Z-scan analysis (Fig. 3C). To our surprise, we found that the nuclei of the oocytes were rotating (movie S1). More intensive live-imaging analysis confirmed that nuclear rotation was observed in the oocytes at the edge of the P7 ovary, whereas those inside the ovaries were stationary (movie S2). Although the reason for these different behaviors of the differently located nuclei remains elusive, it is possible that the edge region generates even higher mechanical stress represented by the strong signal of phalloidin staining at the edge region (fig. S2 and see below). Tracking of a Hoechst 33342–dense heterochromatic region suggested that the kinetics of nuclear rotation was likely a circular motion (Fig. 4A and fig. S4, A and B). Some of the oocytes in P7 ovaries did not show a circular motion, possibly due to the rotation being relatively more along the Z axis (fig. S4B).

There are several reports showing nuclear rotation in myotubes (19), fibroblasts (20), spermatocytes (21), and cell lines (22–24). Some of these reports have shown a different kinetics of nuclear rotation (20, 21, 23). In particular, spermatocytes, which are a male counterpart of oocytes, exhibited oscillation rather than a circular motion (21). Therefore, to evaluate the specificity of the rotation, we visualized the movement of nuclei of the oocytes at various developmental stages by live-imaging analyses. These analyses revealed that nuclei of the oocytes in E14.5 and E18.5 ovaries wiggled constantly (movies S3 and S4 and fig. S4, A to C), whereas those in P7 ovaries exhibited longer strokes (fig. S4C). These wiggling nuclei at E14.5 and E18.5 may be attributed to chromosomal pairing between the homologous chromosomes in meiotic prophase I (25, 26). The movement itself might be described as oscillation (21), since spermatocytes are also consistently the cells entering meiosis. In 4-week-old ovaries, nuclei of the oocytes at the edge of the ovary were rotating in a manner similar to P7 oocytes (movies S5 and S6 and fig. S4, A to C). Further, we still observed the rotation in

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**Fig. 2. Involvement of ECM in the dormant state of oocytes.** (A) Immunofluorescence analysis of E12.5 and P3 ovaries. Images show the results of immunostaining of fibronectin (red) and mouse vasa homolog (MVH) (cyan) antibodies. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; blue). Scale bars, 10 µm. (B) Immunofluorescence analysis of P7 ovaries treated with PBS or CTK, followed by culture for 6 hours. Images show the results of immunostaining of MVH (red) and FOXO3 (green). Nuclei were stained with DAPI (blue). Scale bars, 50 µm. The percentages of cytoplasmic FOXO3 in small (<20 µm) MVH-positive oocytes are shown in the graph. Data representing the means ± SD of four biological replicates are shown. Statistical significance was determined using Student's t test. **P < 0.01. (C) P3 ovaries were treated with PBS or CTK, followed by culture for 14 days. BF, bright field; SC, Stella-CFP. Scale bars, 500 µm. The numbers of oocytes with different diameters are shown in the graph. Data representing the means ± SD of five biological replicates are shown. Statistical significance was determined using Student's t test. **P < 0.01, *P < 0.05. ns, not significant.
20-week-old ovaries (movies S7 and S8). These results confirmed that nuclear rotation is constantly detectable in a subset of oocytes, especially those located at the edge of the ovary after birth. The kinetics of nuclear rotation in oocytes was similar to those observed in other cell types (20, 23). These results indicate that the mechanism for the rotation of nuclei is conserved among many cell types, including oocytes. The nuclei of the oocytes in P7 ovaries treated with CTK ceased their rotation (Fig. 4B and movie S9), indicating that compression drives nuclear rotation. Consistent with our observation, it has been reported that mechanical stress (stretching) induced nuclear rotation in C2C12 myofibroblasts (23). Given that compression is one of the cues for nuclear rotation, exogenous pressure would drive the rotation even in CTK-treated ovaries. We tested this possibility by culturing CTK-treated P7 ovaries with exogenous pressure at 33.3 kPa, followed by live-imaging analysis. Under exogenous pressure, the nuclei of the oocytes in CTK-treated primordial follicles were rotating (movie S10). Together, these results led us to conclude that oocytes in the primordial follicle at the edge of the ovary are subjected to sufficient mechanical stress (compression) to rotate the nuclei.

**Nuclear rotation in the oocytes in response to compression**

Despite the previous reports showing nuclear rotation in various cell types, the biological significance of nuclear rotation has not been fully elucidated. We tried to identify the functional requirement
of nuclear rotation in the dormant state of oocytes. We then tried to inhibit the nuclear rotation. Several cytological studies indicate that nuclear rotation is mediated by dynein, a motor protein that locomotes along microtubules in the cytoplasm (20, 27). To test whether nuclear rotation in the oocytes also depends on dynein, we treated P7 ovaries with ciliobrevin D (CD), an inhibitor of dynein adenosine triphosphatase activity. Upon addition of the inhibitor, nuclear rotation immediately ceases (Fig. 4C and movies S11 and S12), demonstrating that the nuclear rotation in oocytes was dynein dependent. It was observed that inhibition of nuclear rotation in oocytes induced export of FOXO3 to the cytoplasm: The percentage of small oocytes (<20 µm in diameter) with cytoplasmic FOXO3 was significantly increased in the ovaries cultured with CD (Fig. 4D). Consistent with these findings, the number of growing follicles was also increased in the ovaries treated with CD, followed by organ culture for 14 days (Fig. 4E). It is noteworthy that the small oocytes with CD-induced export of FOXO3 to the cytoplasm were still accompanied by squamous granulosa cells (movie S11), suggesting that FOXO3 was exported to the cytoplasm upon cessation of the nuclear rotation even under the compressive condition. These results indicate that nuclear rotation in oocytes is involved in the maintenance of the dormant state.

Reconstitution of the dormant state in culture

We recently reported a culture system that produced functional mature oocytes from fetal ovaries (14). The culture system largely reproduced oogenesis in vivo with respect to gene expression, meiosis, and morphology, and thus, it was expected to be applicable as an in vitro model to improve our understanding of oogenesis. On the
other hand, few primordial follicles were formed in this culture system. This means that the environmental conditions necessary to produce a robust number of sustainably dormant oocytes have still not been reconstituted in culture. Therefore, we next used our culture system to test whether exogenous pressure is sufficient to induce the dormant state in oocytes. As expected, when E12.5 ovaries were cultured without exogenous pressure, only 8.1% of the oocytes were smaller than 20 \( \mu \)m in diameter at 21 days of culture (Fig. 5A). In contrast, the number of small oocytes was increased up to 35.6% in the culture with exogenous pressure, in which FOXO3 was localized in the nuclei (Fig. 5A). These results demonstrated that a robust number of dormant oocytes were induced in cultured E12.5 ovaries by exogenous pressure.

Using this culture system, we gained insights into the functional hierarchy for establishment of the dormant state. It is known that FOXO3 is regulated through its phosphorylation by the AKT/PI3K pathway (8, 9). Phosphorylated FOXO3 is transported into the cytoplasm, which triggers oocyte growth. When LY294002, a PI3K inhibitor, was added into the culture of E12.5 ovaries from 6 to 16 days of culture, during which intercellular bridges between the oocytes were broken down and follicle structures were formed (14), 28.6% of the oocytes were arrested at a smaller stage than 20 \( \mu \)m in diameter (Fig. 5B). In 55.0% of the small oocytes, FOXO3 was localized in the nucleus at 16 days of culture (Fig. 5C and fig. S5). When E12.5 ovaries were cultured with LY294002 and exogenous air pressure at 33.3 kPa, the percentage of small oocytes with nuclear FOXO3 was

![Figure 5. Reconstitution of the dormant state using fetal ovaries.](image)

**A** Culture of E12.5 gonads under exogenous pressure. Time courses of the culture experiments are shown (top). Images show the results of immunofluorescence analysis of E12.5 ovaries cultured for 21 days with or without exogenous pressure (33.3 kPa). Images show the results of immunostaining of MVH (green) and FOXO3 (red). Nuclei were stained with DAPI (blue). Dashed lines indicate the region of small oocytes characteristically observed under exogenous pressure. The percentage of oocytes with the size indicated under each culture condition. Data representing the means ± SD of three independent experiments are shown. Statistical significance was determined using Tukey’s multiple comparison test. *P < 0.05, **P < 0.01. (B) Culture of E12.5 gonads with LY294002. Time courses of the culture experiments are shown (top). Images show the results of immunofluorescence analysis of E12.5 ovaries at 16 days of culture without (Control) or with LY294002 (LY). BF, bright field; SC, stella-CFP. The percentage of oocytes with the size indicated under each culture condition. Data representing the means ± SD of three independent experiments are shown. Statistical significance was determined using Tukey’s multiple comparison test. *P < 0.05, **P < 0.01. (C) Effect of each inhibitor on the dormant state of oocytes. Time courses of the culture experiments are shown (top). The percentages of small (<20 \( \mu \)m) MVH-positive oocytes with nuclear FOXO3 are shown. Data representing the means ± SD of three independent experiments are shown. Statistical significance was determined using Tukey’s multiple comparison test. Comparisons were performed with LY294002. **P < 0.01. P, pressure. (D) Schematic illustration of pathways regulating the localization of FOXO3 in oocytes.
These observations led us to the idea that the oocytes may be abundant in the granulosa cells surrounding the oocytes (Fig. 2A). Stress fibers therefore generate sur-
therby forming stress fibers (30), which are responsible for the export of FO XO3 from the stationary nuclei in the oocytes. Together, these results demonstrate that nuclear rotation is a functional outcome of compression that regulates the nuclear/ cytoplasmic balance of FO XO3 (Fig. 5D). Although the detailed molecular mechanisms remain elusive, our findings provide the insight that mechanical stress generated by environmental cues in the ovary is required for the dormant state of the oocytes.

DISCUSSION

In adult ovaries, primordial follicles are encased in collagen fibers in the cortical region of the ovary (17), which suggests that the ECM plays a role in the maintenance of primordial follicles. However, the function of the ECM in the ovary has not been fully examined. In this study, we identified the ECM as a functional factor for maintenance of the primordial follicles by comparison between E12.5 and P3 ovaries; upon transplantation into adult mice, the former lost primordial follicles, but the latter did not (Fig. 1, A to C). In the P3 ovary, it was already observed that the ECM, as represented by fibronectin and collagen type IV, was enriched in the cortical region (Fig. 2A and fig. S1). Therefore, the properties involved in maintaining the primordial follicles are already established by the perinatal stage. ECM surrounding the primordial follicles seems functional, since the digestion of the matrix by CTK treatment induced oocyte growth accompanied with nuclear export of FO XO3 (Fig. 2, B and C). This provides a clue that may solve a long-standing question: What is the initial signal that triggers oocyte growth? Since matrix metalloproteinase, which digests ECM, is brought via blood, the initial signal to grow the follicles is triggered by digestion of the ECM around the primordial follicles near blood vessels. In the ovaries, angiogenesis is highly active to provide nutrition to growing follicles and corpora lutea, and therefore, the vascular network is constantly remodeled. It is plausible that primordial follicles, which are closed off from the blood vessels, would begin to grow in response to ECM digestion. This idea is supported by both a classical observation that the ovarian cortex is relatively avascular and a recent report that growing follicles are located around blood vessels (28). The balance between location and vascular remodeling is one of the critical factors triggering oocyte growth.

It is well known that the ECM regulates gene expression, intracellular signaling, and structural remodeling, thereby altering cell proliferation and differentiation. The initial response to ECM is concentrated in adhesion molecules, such as integrins (29), where the local adhesion is formed in a subcellular region by polymerizing actin filaments and gathering cytoskeletal molecules. Nonmuscle myosin II, which forms a bipolar structure, gives traction to the actin filaments, thereby forming stress fibers (30). Stress fibers therefore generate surface tension of the cells. In primordial follicles, actin filaments are abundant in the granulosa cells surrounding the oocytes (Fig. 2A). These observations led us to the idea that the oocytes may be continuously compressed by the granulosa cells and this mechanical stress could be important for the dormant states in oocytes. It seems that there is a feedback between oocyte size and external force, since FO XO3 is restored in the nuclei in response to the exogenous pressure. One of major pathways responsible for mechanical stress is the Hippo signaling pathway. In the ovaries, genes involved in Hippo signaling are expressed in granulosa cells, theca cells, and oocytes (31). These genes seem functional, as fragmentation of the ovaries disrupted Hippo signaling and, in turn, promoted follicular growth. It seems that the disruption of the Hippo pathway triggered follicle growth via proliferation of somatic cells, since a target growth factor protein, cellular communication network factor 2 (CCN2), was elevated only in somatic cells (31). However, key regulators for Hippo signaling, such as macrophage stimulating 1/2 (MST1/2), salvador family WW domain containing 1 (SAV1), large tumor suppressor kinase 1/2 (LATS1/2), tafazzin (TAZ), are highly expressed in oocytes in the primordial follicles (31). Furthermore, there is a synergistic effect between MST1 and FO XO proteins in other cell types (32). These findings still suggest a possibility that the Hippo pathway is a mechanismsensor of oocytes per se. From a cytological point of view, other aspects of the transduction of mechanical stress are possible. Recently, it has been increasingly revealed that mechanical stress is transduced to the nuclear membrane via the linker of the nucleoskeleton and cytoskeleton (LINC) complex (33). Cytosplasmic actin fibers connect to the LINC complex, which spans the nuclear envelope and associates with nuclear lamina. Mechanical stress induces deformation of the nuclear envelope and consequently triggers lamina remodeling, alteration of membrane tension, and modification of the nuclear pore size. These conformational changes of the nucleus are closely related to chromatin regulation and gene expression (34). Therefore, it is possible that mechanical stress provides a particular nuclear environment to establish the dormant state in oocytes, the molecular mechanisms of which should be solved in the future. It is, however, still unclear whether the mechanical stress critical for the dormant state is sensed by oocytes per se or by surrounding granulosa cells. Since our culture system provided exogenous pressure to the ovarian tissue, we cannot exclude the possibility that the mechanical stress directly targets the granulosa cells, which, in turn, provide signal(s) for maintenance of the dormant state of the oocytes. To rigorously evaluate the involvement of somatic cells, it will be necessary to develop a culture system that can provide artificial compression only to oocytes.

Although our culture system demonstrated that exogenous pressure induced the dormant state in the oocytes, hydrostatic pressure in culture may not be identical to the compression generated by surrounding granulosa cells. For example, the osmotic condition in the cells might be different, since the water in the oocytes could be diffused under local compression by surrounding granulosa cells, whereas it could stay in the oocytes under compression of the entire tissue by hydrostatic pressure. Since it is becoming clear that changes in osmolarity alter the tension of the nuclear membrane, chromatin configuration, and gene expressions (35), it is possible that the dormant state in culture under exogenous pressure is not identical to that in vivo. Although we found that nuclear FO XO3 was restored even by hydrostatic pressure, indicating that its localization was independent of the osmotic condition, a genome-wide gene expression profile should be tested to critically evaluate the culture system. In addition, it would be a valuable experiment to change the osmotic pressure in the culture conditions. These alternative culture conditions need to be verified in the future.

Unexpectedly, we found that the nuclei of oocytes in primordial follicles were rotating. It has been known since 1953 that nuclei are in motion, not static (36). Since then, nuclear movement has been
observed in various cell types, including fibroblasts, myogenic cells, spermatogenic cells, and cell lines such as NIH3T3 and HeLa. Although the detailed mechanisms driving nuclear movement are still elusive, it is clear that the movement is governed by cytoskeletal proteins and their associates: Depletion of either kinesin-1 or dynein abolished nuclear rotation (19, 20), while disruption of vimentin promoted nuclear rotation (22). The biological significance of nuclear rotation is largely unknown. In myogenesis, disruption of the LINC complex abrogates nuclear rotation and alters key transcription factors such as MyoD and myogenin, resulting in perturbation of the muscular differentiation in C2C12 cells (23). In wound healing by fibroblasts, knockdown of dynein abrogated nuclear rotation and disrupted the location of the nuclei in the cells, resulting in a delay of movement toward the wound region (20). In this study, inhibition of dynein function by CD treatment abrogated nuclear rotation and induced translocation of FOXO3 into the cytoplasm, which then triggered oocyte growth (Fig. 4E). Even under the compressed condition, CD treatment abolished nuclear localization of FOXO3 (Figs. 4D and 5C). These results suggested that nuclear rotation in oocytes is involved in the maintenance of the dormant state. Several possible mechanisms of the involvement of nuclear rotation in the dormant state are conceivable. As discussed above, one is that nuclear rotation changes the nuclear environment—e.g., the lamina composition, membrane tension, and nuclear pore size—which regulates chromatin configuration and gene expression. Another possibility is that nuclear rotation promotes remodeling of the endoplasmic reticulum (ER), which contributes to protein synthesis, folding, and modification as well as calcium storage and lipid synthesis. It was reported that the rotation kinetics differed between ER and the nucleus (20), suggesting a deformation of the ER membrane that is continuous with the nuclear membrane. It is known that the ER is an organelle that responds to cellular stress in a highly dynamic manner. Therefore, this conformational change of the nucleus may trigger changes in multiple functions of the ER. This study cannot exclude other possibilities, such as that the cytoplasmic localization of FOXO3 upon CD treatment is caused by other dynein function(s) and/or disruption of somatic cell function. In the future, it will be necessary to disrupt the machinery specifically involved in nuclear rotation to solve these issues.

A live-imaging system revealed that the population of oocytes in the primordial follicles was heterogeneous with respect to nuclear rotation: The rotation was more frequent in the follicles at the edge of the ovary than in those further inside. On the basis of this observation, nuclear rotation may be involved in modulating the threshold of the dormancy. It is possible that nuclear rotation is an extreme outcome arising from the satisfaction of particular conditions. One of these conditions would be the transmission of sufficient mechanical force to the nucleus. Another would be an adequate distance between the nucleus and the centrosome based on a previous report proposing that the distance is one of the requirements for rotation (27). Therefore, it is plausible that dynein constitutively plays a role in the maintenance of dormancy in oocytes, the nuclei of which appear to be static. Recently, it has been uncovered that the dilation of the nucleus may induce alterations of nuclear import and/or export (37). Our data indicate that compression may modulate the nuclear/cytoplasmic ratio of FOXO3 in the oocytes and XPO1 may be involved in this process.

Here, we have demonstrated that mechanical stress accompanied with nuclear rotation is one of the critical factors required for the maintenance of dormant oocytes. This study provides a platform for the further investigation of fundamental issues related to species perpetuation through the female germ line. In particular, our study established a culture system that produces a robust number of dormant oocytes in response to exogenous pressure. Since the mechanisms underlying the dormant state are of biological and clinical importance, this culture system should also provide a useful tool for elucidating the dormant state. Currently, it is not known how the local environment is generated in the ovary. Our study revealed that the local mechanical stress inducing nuclear rotation is a crucial clue to the local environment. This may provide an explanation for the clinical observation that fibrosis in the ovaries increases with age (38, 39), which generally makes it difficult to produce mature oocytes.

### MATERIALS AND METHODS

#### Mice

Stella-CFP and Stella-GFP transgenic mice were provided by M. Saitou (Kyoto University), and ICR mice were purchased from Japan SLC (Shizuoka, Japan). Animal care was performed in accordance with the guidelines established by Kyushu University for animal use (#A30-072-0 and #A30-111-0) and recombinant DNA experiments (#1–15). Embryonic and newborn ovaries were obtained from ICR mice or by crossing Stella-CFP with ICR mice.

#### Transplantation of the reconstituted ovaries under ovarian bursa

Ovaries collected from either embryos or mice were immediately transplanted under the ovarian bursa of immunodeficient KSN females (Japan-SLC). With the animal under anesthesia, a donor ovary was inserted through a glass needle under the ovarian bursa of the recipient mouse. Transplanted ovaries were collected at 4 weeks after transplantation from the recipient females.

#### RNA sequencing analysis

To perform RNA sequencing (RNA-seq) analysis of E12.5 PGCs and P3 oocytes, a directional RNA-seq library was constructed. Polyadenylated RNAs were purified from at least 1000 P3 oocytes using a Dynabeads mRNA DIRECT Micro Kit (Invitrogen). Biologically triplicated samples were prepared. Purified RNAs were subjected to library construction using a NEBNext Ultra Directional RNA Library Prep Kit for Illumina (New England Biolabs). Complementary DNAs were enriched by a 12-cycle polymerase chain reaction. Sequencing of the libraries was performed with HiSeq 2000 (Illumina). The obtained reads were processed using the FASTX-Toolkit to remove short (<20 base pairs) and low-quality (quality score, <20) reads, followed by trimming of the adaptor sequence. Processed reads were mapped to the mouse mm10 genome using TopHat2/Bowtie2. Cuffdiff, a program in the Cufflinks software package, and QuaSR, a Bioconductor package, were used for the differentially expressed gene analyses. GO analysis using the differentially expressed genes was performed using DAVID Bioinformatics Resources 6.8 (https://david.ncifcrf.gov/).

#### Immunofluorescence analysis

For frozen sections, freshly isolated and cultured ovaries were fixed with 4% paraformaldehyde (PFA) for 3 hours at 4°C. After washing with PBS, fixed samples were sequentially soaked in 10, 15, and 20% sucrose and then treated with fresh 20% sucrose overnight. Then, the samples were embedded in O.C.T. (optimal cutting temperature)
compound (Tissue-Tek) and sectioned at 7 μm in thickness. Sections were blocked in Blocking One Histo (Nacalai) for 1 hour at room temperature and washed once in PBS. Then, the primary antibody was reacted at 4°C overnight in Blocking One Histo that had been diluted 15-fold in PBS containing 0.1% Tween 20 (PBST). The primary antibodies we used were mouse anti-fibronectin (F-3648, Sigma-Aldrich), rabbit anti–collagen type IV (ab19808), rabbit anti-DDX4/MVH (ab13840, Abcam), mouse anti-DDX4/MVH (ab27591, Abcam), rabbit anti-FOXO3a (75D8, Cell Signaling Technology), and chicken anti-GFP (ab13970, Abcam). These antibodies were used at 1:200 dilution except for anti-fibronectin (1:500) and anti–collagen type IV (1:400). The sections were washed twice in PBST and once in PBS, and then secondary antibodies were reacted at room temperature for 3 hours or at 4°C overnight in Blocking One Histo that had been diluted 15-fold in PBST. The secondary antibodies were used were anti-mouse immunoglobulin G (IgG) donkey polyclonal antibody (pAb) AlexaFluor488 (Thermo Fisher Scientific), anti-mouse IgG donkey pAb AlexaFluor568 (Thermo Fisher Scientific), anti-mouse IgG donkey pAb AlexaFluor647 (Thermo Fisher Scientific), anti-mouse IgG goat pAb AlexaFluorPlus555 (Thermo Fisher Scientific), anti-rabbit IgG goat pAb AlexaFluorPlus555 (Thermo Fisher Scientific), anti-rabbit IgG donkey pAb AlexaFluor568 (Thermo Fisher Scientific), and anti-chick IgG goat pAb AlexaFluor488 (Thermo Fisher Scientific). All of the secondary antibodies were used at 1:500 dilution. At the secondary antibody reaction, 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) was also stained with 1 μg/ml.

For the phalloidin staining, phalloidin was diluted 1:1000 in Blocking One Histo that had been diluted 15-fold in PBST and added at the secondary antibody reaction. The sections were washed twice in PBST and then once in PBS, mounted with PermaFluor (Thermo Fisher Scientific), and analyzed using an LSM700 laser microscope (ZEISS).

For whole-mount staining of cultured ovaries, the samples were detached from Transwell-COL membranes (Coaster) using micro tweezers. Then, the samples were fixed with 4% PFA for 1 hour at 4°C. After washing with PBS, the samples were soaked in 0.1% bovine serum albumin/0.3% Triton X-100 in PBS (blocking reagent) overnight at 4°C for blocking. Primary and secondary antibodies were used at the same dilutions as used for the staining of sections in blocking reagent. For the washing process, samples were washed three times in PBST for 1 hour each time. Last, the samples were mounted in Fluoro-KEEPER antifade reagent (Nacalai) and analyzed using an LSM700 laser microscope (ZEISS).

Quantification of immunofluorescence analyses
For the measurement of phalloidin intensity, MVH-positive cells located at the edge of the cortex were squared and elongated to a length of 100 μm in the medulla. In the enclosed area, the intensity of phalloidin staining normalized to that of DAPI was shown as a histogram. In addition, the MVH-positive cells at the edge of the cortex were centered, and phalloidin signals of more than 0.5 μm in diameter were counted using Imaris tracking software (Bitplane). To quantify FOXO3 localization, oocytes of less than 20 μm in diameter were examined to determine whether FOXO3 was localized in their nuclei. In each sample, more than 20 randomly selected oocytes were examined for nuclear FOXO3. More than three representative samples from each experimental group are shown.

Live-imaging analysis
Ovaries were stained with Hoechst 33342 (Invitrogen) at 37°C for 30 min in a CO₂ incubator (5% CO₂). They were then moved to dishes containing a ProLong antifade solution (Invitrogen). Then, time-lapse imaging was performed using an LSM780 laser microscope (ZEISS). Pictures were taken every 20 s until 30 min (for a maximum of 90 pictures in total). Movies were generated using ZEN software (ZEISS).

Nuclear rotation assay
Nuclear rotation was analyzed by Imaris tracking software (Bitplane). Hoechst 33342 (Invitrogen)–dense heterochromatin regions were spotted and traced. Speed and velocity were calculated.

Organ culture
The in vitro culture of ovaries was performed, as previously reported (14). Briefly, gonads were isolated from E12.5 embryos and cultured on Transwell-COL membranes (Coaster) supplied with 10% KSR (Invitrogen), and 0.025% trypsin EDTA (Invitrogen). P7 ovaries were treated with PBS or CTK for 1 hour at 37°C (≏250 mmHg).

For whole-mount staining of cultured ovaries, the sections were washed twice in PBST and once in PBS, mounted with PermaFluor (Thermo Fisher Scientific), and analyzed using an LSM700 laser microscope (ZEISS).

For whole-mount staining of cultured ovaries, the samples were detached from Transwell-COL membranes (Coaster) using micro tweezers. Then, the samples were fixed with 4% PFA for 1 hour at 4°C. After washing with PBS, the samples were soaked in 0.1% bovine serum albumin/0.3% Triton X-100 in PBS (blocking reagent) overnight at 4°C for blocking. Primary and secondary antibodies were used at the same dilutions as used for the staining of sections in blocking reagent. For the washing process, samples were washed three times in PBST for 1 hour each time. Last, the samples were mounted in Fluoro-KEEPER antifade reagent (Nacalai) and analyzed using an LSM700 laser microscope (ZEISS).

Quantitative analyses of the oocyte number and volume
For the counting of oocytes, P3 ovaries were cultured for 14 days, and then images of the whole ovaries were made using a fluorescent microscope (Olympus SZX16 with DP73 Camera). Then, the numbers of Stella-CFP–positive oocytes of sizes <20 μm, 20 to 50 μm, and >50 μm were counted. To measure the oocyte volume, ovaries from P7 Stella-CFP transgenic newborns were stained with Hoechst 33342 (Invitrogen) in PBS or CTK for 1 hour at 37°C. The samples were cut in half, and the medium was replaced with S10 medium containing the ProLong antifade solution (Invitrogen). Then, Z-stack images were acquired on an LSM780 microscope (ZEISS). The images were analyzed by Imaris Surface software (Bitplane) to calculate the volume of oocytes based on Stella-CFP signals.

CTK treatment
CTK reagent was prepared as 1 μM CaCl₂, collagenase type IV (0.1 mg/ml), 20% KSR (Invitrogen), and 0.025% trypsin EDTA (Invitrogen). P7 ovaries were treated with PBS or CTK for 1 hour at 37°C in a CO₂ incubator. The ovaries were washed with PBS and then fixed to make frozen sections. In the case of nuclear transportation of FOXO3 under pressure, CTK treatment was performed under a gas-pressurized chamber (AGP-3001S, STREX). For the phalloidin

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staining and coupled FOXO3 staining, ovaries were cultured on Transwell-COL membranes (Coaster) with S10 medium for 5 hours after CTK treatment.

**CD treatment**
P7 ovaries were cultured on Transwell-COL membranes (Coaster) with S10 medium in the presence of 25 μM dynein inhibitor CD (Merck) for 6 hours. Then, the ovaries were fixed to make frozen sections. For the P3 ovarian culture, CD treatment was administered for 12 hours every 3 days for a total of 9 days (three applications).

**Statistical analysis**
For comparisons between two groups, data represent the means ± SD of independent experiments. Statistical significance was determined using Student’s t test for both sides. For comparisons among more than three groups, data represent the means ± SD of independent experiments. Statistical significance was determined using Tukey’s multiple comparison test for both sides.

**SUPPLEMENTARY MATERIALS**
Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/5/6/eaav9960/DC1

**Fig. S5.** Localization of FOXO3 in the ovaries treated with inhibitors and exogenous pressure.

**Fig. S4.** Different kinetics of nuclear rotation in ovaries at various stages.

**Fig. S2.** Distribution of actin filaments in the ovary treated with CTK.

**REFERENCES AND NOTES**
33.3 kPa. Movie S12. A 30-min live imaging of the CTK-treated P7 ovary with exogenous pressure at 33.3 kPa. Movie S11. A 30-min live imaging of the P7 ovary shown in movie S10 before CD treatment. Movie S9. A 30-min live imaging of the P7 ovary treated with CTK. Movie S8. Another 30-min live imaging of the ovary at 20 weeks old. Movie S5. A 30-min live imaging of the ovary at 4 weeks old. Movie S4. A 30-min live imaging of the E18.5 ovary. 

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