CLARITY reveals dynamics of ovarian follicular architecture and vasculature in three-dimensions

Yi Feng1,2, Peng Cui1,2, Xiaowei Lu1, Brian Hsueh3, Fredrik Möller Billig1, Livia Zarnescu Yanez4, Raju Tomer3, Derek Boerboom5, Peter Carmeliet6, Karl Deisseroth3 & Aaron J. W. Hsueh1

Optimal distribution of heterogeneous organelles and cell types within an organ is essential for physiological processes. Unique for the ovary, hormonally regulated folliculogenesis, ovulation, luteal formation/regression and associated vasculature changes lead to tissue remodeling during each reproductive cycle. Using the CLARITY approach and marker immunostaining, we identified individual follicles and corpora lutea in intact ovaries. Monitoring lifetime changes in follicle populations showed age-dependent decreases in total follicles and percentages of advanced follicles. Follicle development from primordial to preovulatory stage was characterized by $3 \times 10^5$-fold increases in volume, decreases in roundness, and decreased clustering of same stage follicles. Construction of follicle-vasculature relationship maps indicated age- and gonadotropin-dependent increases in vasculature and branching surrounding follicles. Heterozygous mutant mice with deletion of hypoxia-response element in the vascular endothelial growth factor A (VEGFA) promoter showed defective ovarian vasculature and decreased ovulatory responses. Unilateral intrabursal injection of axitinib, an inhibitor of VEGF receptors, retarded neo-angiogenesis that was associated with defective ovulation in treated ovaries. Our approach uncovers unique features of ovarian architecture and essential roles of vasculature in organizing follicles to allow future studies on normal and diseased human ovaries. Similar approaches could also reveal roles of neo-angiogenesis during embryonic development and tumorigenesis.

Follicles are basic functional units of the ovary. Although most follicles remain at the primordial stage, activation of these dormant follicles leads to their growth into primary, secondary, and antral stages1. Among ~20 early antral follicles present during early follicular phase of the menstrual cycle in women, most become atretic and only one reaches antral/preovulatory stages. This dominant follicle secerts a majority of circulating sex steroids necessary for maintaining reproductive cyclicity and eventually ovulates a mature oocyte capable of developing into a new life2,3. After rupture, ovulated follicles become corpora lutea that secret progesterone important for pregnancy maintenance. Follicle growth, massive atresia, and rupture as well as luteal formation/regression are hormonally regulated and associated with neo-angiogenesis that is rarely found in adult mammals4. Thus, cyclic remodeling of ovarian structures takes place during each reproductive cycle accompanied by a lifelong decline of follicles until reproductive senescence5.

Many organs in vertebrates consist of compartmentalized branching units such as lobules in lung, liver, and mammary gland or tubules in testis and kidney. However, ovaries contain primordial follicles situated in the cortical region together with growing follicles arranged in a centripetal fashion without a clear pattern of organization6. Most traditional histological analyses deal with limited information regarding interrelationships among follicles and corpora lutea due to uncharacterized three-dimensional (3D) architecture. Based on nuclear volume

1Departments of Obstetrics and Gynecology, Stanford University School of Medicine, Stanford University, Stanford, CA 94305, USA. 2Department of Integrative Medicine and Neurobiology, State Key Lab of Medical Neurobiology, School of Basic Medical Sciences, Shanghai Medical College, and Institute of Brain Science, Brain Science Collaborative Innovation Center, Fudan University, Shanghai 200032, China. 3Department of Bioengineering, Howard Hughes Medical Institute, CNC Program, Department of Psychiatry and Behavioral Sciences, Stanford University, Stanford, CA 94305, USA. 4Department of Bioengineering, Stanford University, Stanford, CA 94305, USA. 5Centre de Recherche en Reproduction Animale, Université de Montréal, St-Hyacinthe, Québec, Canada. 6Laboratory of Angioenesis and Vascular Metabolism, Vesalius Research Center, VIB and Department of Oncology, University of Leuven, Leuven, Belgium. Correspondence and requests for materials should be addressed to Y.F. (email: fengyi17@fudan.edu.cn) or A.J.H. (email: aaron.hsueh@stanford.edu)
of the oocyte as an indicator of follicles, a recent whole mount immunofluorescence 3D imaging study begun to provide follicle dynamics from neonate to adult mice\(^2\). However, there is no analysis on follicular somatic cells together with oocytes in 3D architecture. The vascular system is generally quiescent in adult mammals and the ovary and endometrium are the only normal adult tissues capable of undergoing neo-angiogenesis\(^8\). Furthermore, the relationship between folliculogenesis and ovarian vasculature is unclear.

Recently developed CLARITY approach makes intact tissues transparent and enables immunostaining of markers to elucidate detailed 3D structure of organs\(^9\). Using specific markers and advanced computer algorithms, we imaged ovarian follicles and corpora lutea in intact ovaries and generated 3D digital maps of ovarian organelles in relation to vasculatures to reveal changes in follicle sizes, location, roundness, clustering, interrelationships, and vasculature throughout reproductive life. In mutant mice with deletion of the hypoxia-response element in the vascular endothelial growth factor A (VEGFA) promoter, defective ovarian vasculature was found to be associated with lower ovulatory responses following gonadotropin stimulation. Following treatment with an inhibitor for VEGF receptor kinases, we also suppressed gonadotropin-induced ovarian neo-angiogenesis, leading to defective vasculature and ovulation. The purpose of the current study was to use the CLARITY approach to elucidate folliculogenesis and the relationship between vasculature and follicles in mice after birth to adult life.

**Results**

**Identification of follicles and corpora lutea at different developmental stages.** We used the CLARITY approach to render ovaries transparent (Figure S1A), followed by staining with different markers before digital imaging (Figure S1B). Our preliminary data (Figure S2) indicated that staining with antibodies against tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine biosynthesis, led to prominent staining in theca cells, ganglia, and corpora lutea as confirmed using antibodies against neuron-specific class III beta-tubulin (Tuj1)\(^10\) and brain-derived neurotropic factor (BDNF), consistent with earlier findings\(^11,12\). In addition, nonspecific background TH staining was found around all follicles, allowing for identification of follicular structures. As shown in Figure S1B, primordial and primary follicles both showed TH staining but could be separated based on shapes of granulosa cells (squamous for primordial; cuboidal for primary). In secondary follicles, anti-Müllerian hormone (AMH) staining was evident in granulosa cells as reported\(^13\). Also, BID (a pro-apoptosis protein) and CYP19 (the enzyme for estrogen biosynthesis) marked granulosa cells in atretic\(^14\) and antral/preovulatory follicles\(^15\), respectively (Figure S1B). Preovulatory and antral follicles were detected semi-manually according to their sizes and the antral shape and location. In addition to their unique homogenous texture, corpora lutea showed staining for VEGF important for angiogenesis\(^8\). Also, digital slicing of adjacent images showed an ovulating follicle (Figure S1B).

**Three-dimensional determination of follicle numbers throughout life.** We analyzed follicle dynamics of intact ovaries from mice at different ages following CLARITY processing and immunostaining for TH together with AMH or CYP19 staining (Figure 1). Scanned images were analyzed following 3D rendering, digital slicing, and Spot transformation to identify primordial, primary, secondary, antral, and preovulatory follicles (Fig. 1A and Figure S3). In addition, corpora lutea were distinguished based on their unique homogenous texture in ovaries of adult and aging (day 360) mice. Three-D images of a day-10 mouse ovary were generated for Spot transformation, followed by manual improvement, to identify all follicles (Movie S1). Using specific markers, follicles at all developmental stages were traced together with corpora lutea in the ovary of an adult proestrous animal to construct follicle relationship maps (Movie S2).

Based on specific markers, we estimated number of follicles at different stages of development (Fig. 1) and determined their diameters, surface areas, and volumes (Table S1). Follicle volume increased ~45 fold during transition from primordial to primary stage whereas secondary follicle development encompassed 125-fold increases in volume, indicating major growth (Tables S1 and S2). From early antral to the largest preovulatory follicles, ~15-fold increases in volume were found. Throughout folliculogenesis from the smallest primordial to largest preovulatory follicles, ~3 x 10\(^{4}\)-fold increases in follicle volume were found, accompanied by 88,000-fold increases in oocyte volume. Analyzing lifetime changes in follicle numbers indicated an age-dependent decline in total follicles, together with major decreases in primordial follicles (Fig. 1B) as previously reported\(^16\). Of interest, percentages of both primordial and primary follicles increased with age (Fig. 1C) accompanied by a drop of secondary follicles, suggesting age-dependent follicle arrest before the secondary stage.

**Clustering and migration of follicles based on 3D mapping.** We identified locations of follicles in ovaries from mice at different ages (Fig. 2A). Clustering of follicles at the same developmental stage was monitored by measuring spatial homogeneity using the Ripley’s K function that quantifies the degree of object clustering/dispersion as compared to a random distribution\(^17\). As shown in Fig. 2B, clustering indexes decreased as primordial follicles developed into primary, secondary, and later stages in ovaries from animals at all ages. Of interest, there is also an age-dependent increase in follicle clustering from ovaries of day 10 to day 60 animals. We also analyzed distances of developing follicles from geometric center of the ovary using Imaris Spot and Surface algorithms. As shown in Fig. 2C, Spot plotting of distances of individual follicles from the center in adult ovaries suggested inward migration of primary, secondary, and antral follicles, consistent with earlier identification of primordial follicles near the periphery of ovaries in prepubertal and adult mice\(^6\).

**Analyses of follicles neighboring preovulatory follicles and corpora lutea.** Three-D imaging analyses indicated that individual follicles were in close contact with other follicles or corpora lutea with minimal interstitial tissues. We digitally isolated individual preovulatory follicles and corpora lutea to estimate neighboring follicles in ovaries from proestrous mice (Fig. 3A). Corpora lutea were in direct contact with follicles of all stages whereas preovulatory follicles had fewer neighboring follicles. Quantitative analyses using ovaries from...
mice at different stages (Fig. 3B) indicated that preovulatory follicles had fewer neighboring primordial, primary, and secondary follicles as compared with those adjacent to the corpora lutea. In contrast, comparable numbers of antral and preovulatory follicles were found near preovulatory follicles and corpora lutea. By monitoring corpora lutea throughout all estrous stages by daily vaginal smears, we also found decreases in volumes of corpora lutea from proestrus to diestrus (Fig. 3C and D), suggesting rapid luteolysis within one reproductive cycle.

Sphericity index analysis in follicles during development. We calculated sphericity of individual follicles. In ovaries of adult proestrous animals, roundness of follicles decreased as follicles developed into more advanced stages (Fig. 4A; left panel: whole ovary image; right panel: representative follicles at different stages). Unlike adult ovaries, no changes in sphericity were found during follicle development in ovaries of immature mice treated with equine chorionic gonadotropin (eCG) for 24 h (Fig. 4B and C), suggesting differences in ovarian rigidity. In adult, sphericity indices decreased from 0.91 in nearly round primordial follicles to 0.73 in irregularly shaped preovulatory follicles (Fig. 4C).

Ovarian vasculature networks surrounding follicles and corpora lutea. We investigated ovarian vasculature changes following immunostaining for platelet-endothelial cell adhesion molecule 1 (PECAM1), a marker for endothelial cells. PECAM1 staining in ovaries from mice at different ages was traced using the Imaris Filament algorithm and showed age-dependent increases in vasculature (Fig. 5A, upper panel: 3D rendering; lower panel: blood vessel tracing). In day 3 ovaries containing mainly primordial follicles, no PECAM1 staining outlining individual follicles were found despite the existence of major branches. In day 10 ovaries, growing secondary follicles showed strong staining in the theca layer whereas primordial/primary follicles in
Figure 2. Construction of 3D follicle maps indicates follicle clustering and migration. (A) Spot representation shows location of follicles in ovaries from mice at 10, 21, and 60 days of age. Follicles at primordial (green), primary (pink), secondary (purple), antral (orange), and preovulatory (red) stages are shown. Note absence of antral and preovulatory follicles in day 10 ovary whereas day 21 ovary lacks preovulatory follicles. This graph has been drawn by Imaris Vantage, plot type: XYZ 3D color and scale; different sizes in each follicular type denote different depths of follicles (smaller follicles are located the deeper from the surface). (B) Clustering analyses. Ovaries from animals at different ages were processed using Spot identification before analyzing clustering of follicles at the same developmental stage using the Ripley’s K function. The Y-axis depicts deviations of the follicle distribution (K(d)) from randomness (E(K(d))) over a given distance (X-axis) between center of follicles of the same size. Higher values indicate clustering whereas negative values indicate dispersion. (C) Follicle development from primary to antral stages was characterized by movement toward the center of the ovary. Distances from geometric center of the ovary to the center of individual follicles were determined in ovaries from mice at different ages (n = 3 ovaries). All values were normalized using location of the most peripheral primordial follicles (set at 1.0). Means + SE are shown.
Furthermore, gonadotropin treatment of immature mice led to time-dependent increases in ovarian vasculature (Fig. 5B). Diameters of the largest vessel showed a 3.7-fold increase at 48 h after gonadotropin treatment. After Spot transformation of individual follicles, a clear relationship between ovarian vasculature and follicles was observed. Figure 3. Analyses of follicles neighboring preovulatory follicles and corpora lutea. (A) Ovaries from proestrus mice were processed to reveal follicles in direct contact with preovulatory follicles (red color) and corpora lutea (CL, yellow color). Upper panel: 3D cropping of representative graphs showing selected preovulatory follicle and corpus luteum together with neighboring follicles. Lower panel: follicles shown as Spot graphs (red color: preovulatory; orange color: antral; blue color: secondary). Because preovulatory follicles and corpus lutea have irregular shapes, the Surface algorithm in Imaris was used to define their exact borders before neighboring analysis. Smaller and generally round follicles were reconstructed by using the Spot algorithm in Imaris. (B) Number of follicles neighboring individual preovulatory follicles or corpora lutea was plotted based on stages of follicle development (n = 10 from 5 ovaries). Stars indicate significant differences between numbers of follicles neighboring preovulatory follicles and CL (P < 0.05). Means ± SE are shown. (C) Changes in volumes of corpora lutea during the estrous cycle. Upper panel: representative Spot graphs of corpora lutea (yellow color) and antral/preovulatory follicles (red color) at different stages of the estrous cycle. Lower panel: Decreases in volume of individual corpus luteum (CL) from proestrus to diestrus. Means ± SE are shown.
found (Fig. 5B, lower panel). Following human chorionic gonadotropin (hCG) treatment of eCG-primed animals to induce ovulation, follicle luteinization was accompanied by rapid increases in vasculature inside preovulatory follicles and increases in volume of individual corpus luteum (Fig. 5C). In a representative adult ovary (Fig. 5D), PECAM1 staining in primordial/primary follicles was negligible whereas theca layers surrounding secondary, antral, and preovulatory follicles showed strong staining with no signal in inner granulosa cells. Serial sections of a representative corpus luteum (Fig. 5E) showed continuous PECAM1 staining throughout the solid structure. Supplementary Movie S3 showed relationship of blood vessels and antral/preovulatory follicles after eCG stimulation whereas Movie S4 showed invasion of PECAM1-stained endothelial cells into ruptured follicles during corpora lutea formation in hCG-treated animals. Movie S5 traced major vessels showing surrounding follicles in an ovary at 24 h after eCG treatment.

Figure 4. Decreases in sphericity of follicles during development. (A) A representative ovary from a proestrous mouse showing follicles at different developmental stages (left panel), together with gallery pictures of individual follicles arranged based on stages (right panel). (B) Same presentation of an ovary from an immature eCG-treated animal. (C) Sphericity indexes decreased with advancing stages of follicle development in ovaries of adult, but not eCG-treated immature mice. Stars indicate significant differences between different types of follicle and primordial follicle in each group (**P < 0.01, ***P < 0.01). Means ± SE are shown.
Defective ovarian vasculature and ovulatory responses in VEGF promoter mutant mice. To evaluate the importance of ovarian vasculature in ovulation and luteinization, we obtained Vegfαδ/δ mutant mice with deletion of the hypoxia-response element in the VEGFA gene promoter. These animals showed reduction of VEGF expression under hypoxia and defects in gonadotropin stimulation of VEGF expression. After genotyping of wild type and heterozygous animals, we performed vaginal smearing of adult (~12 weeks of age) females and found heterozygous mutant animals were acyclic, showing prolonged diestrus. We then injected wild type and mutant animals at diestrus with eCG for 48 h, followed by hCG. At 16 h later, ovarian weights and number of ovulated oocytes were determined. As shown in Fig. 6B, mutant mice showed decreases in ovarian weights and lower number of oocytes released, together with a small decrease in body weight. CLARITY processing and immunostaining using PECAM1 antibodies indicated defective ovarian vasculature as shown by decreased total vasculature (Fig. 6C), presumably underlying the defective ovulatory responses. Furthermore, we uni-laterally injected axitinib, an inhibitor for all three VEGF receptor tyrosine kinases, into ovarian bursa of immature wild type mice, before intraperitoneally treating the mice with eCG to induce follicle development. Forty-eight h later, axitinib pre-treatment decreased ovarian weights by ~30% (Fig. 7A and B) when compared with untreated contralateral ovaries in the same animal. Image analyses showed that axitinib treatment led to decreased total vasculature and lower antral/preovulatory follicle numbers (Fig. 7C). Following subsequent...
treatment with hCG to induce ovulation, axitinib-treated ovaries released fewer numbers of oocytes into oviducts (Fig. 7D).

Discussion

Using the CLARITY approach, we performed marker staining, digital imaging, and spatial analysis to obtain 3D ovarian architecture throughout life. This method transforms intact tissues into a nanoporous hydrogel-hybridized form that is optically transparent and macromolecule-permeable. Unlike classical 2D sectional histological analyses, CLARITY provides 3D mapping of interrelationships among follicles and corpora lutea at different developmental stages. Marker staining of ovarian structures revealed changes in follicle numbers, volume, clustering, migration, and sphericity, as well as previously unappreciated association among follicles and relationship between local vasculature and follicles/corpora lutea.

We found ~3 × 10^5-fold increases in follicle volume from primordial to the preovulatory stage. This was accompanied by ~88,000-fold increases in oocyte volume, forming the largest cell in the body with a volume of
Figure 7. Unilateral intrabursal injection of a VEGF receptor inhibitor axitinib suppressed ovarian neo-
angiogenesis, ovarian weight, and ovulation. Immature mice at 21 days of age were intrabursally injected with
axitinib into the right ovary with the left side serving as controls. Control animals were injected unilaterally
with the vehicle (DMSO). Animals were then treated with eCG to stimulate follicle growth. At 48 h after eCG
injection, weights (A), morphology (B), and vasculature (C) of control and axitinib-treated ovaries were
recorded. Stars indicate significant differences between untreated and treated sides (\*P < 0.05 and \**P < 0.05).
Means \pm SE are shown. In (C), \( n \) represents number of total antral/preovulatory follicles in individual ovaries
whereas \( \Phi \) denotes the diameter of vascular trunks originating from the helium. (D) Some animals were further
treated with hCG to induce ovulation before recording number of ovulated eggs retrieved. Stars indicate
significant differences between untreated and treated sides (\( P < 0.001 \)). Means \pm SE are shown.
angiogenesis takes place following follicle rupture during luteinization and invasion of vasculature into corpora of small sizes. The present approach using large adult ovaries provides a standardized way to determine follicle approach, a recent study minimized sectional errors and quantified oocyte dynamics in murine prenatal ovaries. Extensive of local neo-angiogenesis in regulating follicle dormancy. In growing secondary and larger follicles could secret follicle activation inhibitors as predicted by 2D spatial analysis whereas decreased clustering of secondary follicles is consistent with culture studies in direct contact invariably led to the dominance of one and growth suppression of its neighbor. Consistent with rat studies, small, non-growing primordial follicles were found under the surface epithelium in prepubertal and adult mice. Once primordial follicles are activated by local factors, they migrate away from the cortical region. In contrast, advanced follicles maintained their roundness in ovaries of gonadotropin-treated immature mice. These data suggested that repeated tissue remodeling during recurring reproductive cycles could decrease tensile strength/elasticity in adult ovaries, rendering larger follicles into irregular shapes.

Our 3D imaging analyses indicated that follicles are in direct contact with each other. Although follicles at all stages were found adjacent to corpora lutea, preovulatory follicles were in close contact with antral, but fewer primordial, primary, and secondary follicles. These findings suggest that high levels of progesterone produced by the corpora lutea do not affect folliculogenesis whereas high levels of estrogen, inhibin, or other factors secreted by preovulatory follicles could confer unfavorable environments for preantral follicles. Also, we observed decreases in average volumes of corpora lutea from proestrus to the diestrous stage, suggesting rapid tissue remodeling and dissolution of the regressing structure within one estrous cycle.

In adult tissues, angiogenesis mainly occurs during wound healing and fracture whereas abnormal capillary growth is associated with pathological tumor growth, retinopathies, fibroses, and rheumatoid arthritis. Cyclic changes in ovarian and endometrial vasculature represent exceptions. Consistent with the essential role of PECAM1 in vasculogenesis and angiogenesis, we found major increase in PECAM1 staining during folliculogenesis and luteinization. Instead of randomly distributed inside the ovary, follicles are located along vascular branches. Growing follicles near vascular branches likely acquire more nutrients and secret more angiogenesis factors to promote local vasculature, resulting in a positive feedback loop. The adult ovary can be pictured as a vascular tree with 3 to 4 main branches each bearing fruits (follicles) of different sizes. Although the ovary does not have branching organelles similar to many other organs, intraovarian distribution of follicles is not a random event but is “constructed” around local vasculature.

We confirmed the avascular nature of cortical primordial follicles. Because mTOR signaling regulated by nutrient availability is pivotal for the activation of dormant primordial follicles, our data underscore the important role of local neo-angiogenesis in regulating follicle dormancy. In growing secondary and larger follicles, surrounding thecal cells have rich blood vessels that do not penetrate the basal membrane. Extensive angiogenesis takes place following follicle rupture during luteinization and invasion of vasculature into corpora lutea is regulated by local pro- and anti-angiogenic factors.

FSH stimulation of follicle growth is likely associated with hypoxia in the avascular granulosa cell compartment, leading to increased secretion of angiogenesis factors to promote neo-angiogenesis. The roles of ovarian VEGFA and neo-angiogenesis during gonadotropin induction of ovulation are demonstrated by our studies on ovariolytic responses and vasculature changes in mutant mice with deletion of the hypoxia-response element in the VEGFA promoter. Because all three VEGF receptors are expressed in the ovary, we unilaterally injected immature mice with aitinib, a pan-specific inhibitor for VEGF receptors, into ovarian bursa. Findings of defective ovarian vasculature and ovariolytic responses after inhibitor treatment further underscore importance of endogenous VEGFs during gonadotropin-induced follicle maturation and ovulation.

Most ovarian research deals with molecular, cellular, and follicular scales, revealing roles of gonadotropins, paracrine factors, and local Hippo signaling. The present approach facilitates studies at the whole ovary scale, highlighting the roles of vasculature, follicles interactions, and follicle locations in intact ovaries. The CLARITY approach provides opportunities to investigate hormonally regulated folliculogenesis, interrelationships among follicles and corpora lutea as well as remodeling of ovarian architecture during each reproductive cycle. Three-D imaging could allow elucidation of local tissue remodeling, including roles of invading macrophages during follicle rupture as well as fibribral stem cells during surface epithelium repair. CLARITY studies on cyclic ovarian remodeling confirm the theory that blood vessels direct the configuration of organs, first proposed by Aristotle.
Due to wide interests in using the CLARITY approach, recent modifications of this method has shortened the duration needed for tissue processing from weeks used here to days\(^45\). In addition, processing of larger samples is now possible\(^46\), thus allowing future evaluation of 3D architecture of the human ovary. In addition to providing models to investigate roles of neo-angiogenesis during organogenesis and tumorigenesis, future studies using the CLARITY approach could reveal ovarian architecture and vasculature underlying ovarian diseases, including polycystic ovarian syndrome, primary ovarian insufficiency, and ovarian tumorigenesis.

**Methods**

**Study design.** The experiments presented here were designed to investigate relationships among ovarian follicles, corpora lutea, and vasculatures as well as to test the hypothesis that ovarian vasculature dynamics are playing an important role in folliculogenesis and ovarian development. Using a newly established three-dimensional imaging technique, we provided evidences on vasculature control of folliculogenesis with the help of whole tissue imaging, together with immunostained markers in mouse ovaries. Furthermore, genetic and pharmacological mouse models were employed to regulate angiogenesis induced by VEGF signaling for evaluating folliculogenesis and ovulation. All experiments and analyses were repeated at least three times and were in accordance with the "Guide for the Care and Use of Laboratory Animals" (NIH publication 85–23, revised in 1996) and the "Principles of Laboratory Animal Care" (National Society for Medical Research). These experimental protocols and animal care were approved by regional committees (Stanford University, Project Nr. 10347 and Fudan University, Project Nr. 20150119-019).

**Animals and experimental design.** Female C57BL/6 mice at different ages were obtained from Charles River Laboratories (Wilmington, MA). Aging C57BL/6 mice were from Shanghai Research Center for Model Organisms (Shanghai, China). Animals were housed in animal facilities at Stanford University or Fudan University under 12 h dark/light with free access to food and water. To investigate gonadotropin regulation of folliculogenesis, immature mice at 21 days of age were injected i.p. with 2 IU eCG (Pelemx), followed at 48 h later by an injection of 10 IU hCG (Asuka Pharma) for up to 72 h. Animals were sacrificed at different times to obtain ovaries for CLARITY processing. To analyze atretic follicles, immature mice were treated with a single injection of 5 IU eCG for 96 h\(^47\). For studies using axitinib (Tocris), a pan-specific inhibitor for all three VEGF receptor tyrosine kinases\(^22\), mice at 21 days of age were anesthetized using isoflurane (Piramal Group) and injected with axitinib (5 or 10 \( \mu \)g dissolved in 10 \( \mu \)l DMSO; Tocris Inc.) through the ovarian fat pad into the bursa of the right ovary. This was followed by an injection of 5 IU eCG to stimulate follicle development, before collection of ovaries at 48 h after eCG treatment for weighing and CLARITY processing. To estimate ovulatory potency, animals were injected with 10 IU hCG at 48 h after eCG treatment before counting numbers of oocytes in oviducts at 16 h later. Each group included 6 to 12 mice.

**Genotyping and ovulation induction of mutant mice.** Vegfa\(^{d/d}\) mice were generated by mating male and female heterozygous animals for the transgenic allele and maintained on a C57BL/6 genetic background. Wild type C57BL/6 mice were obtained from the Charles River Laboratory. Genotyping analyses were performed by PCR on DNA obtained from tail biopsies using the oligonucleotide primers 5'-GCTTATCTGAGCCCCTGTGTGAT-3' (Vegfa genotype sense probe); 5'-GATGAAAGCCTAGGGTAGTG-3' (Vegfa genotype antisense probe); 5'-GACTACAGTGACCTGTGTGAAATG-3' (mutant-sense probe); and 5'-AGACTACAGTGACCTGTGTGGT-3' (wild type-antisense probe) based on the following PCR conditions: 1 min at 94 °C for one cycle, 30 sec at 94 °C, 30 sec at 60 °C, and 30 sec at 72 °C for 29 cycles, and 10 min at 72 °C for one cycle. Primers were designed to generate PCR products of 482 bp and 208 bp for the wild type Vegfa allele, or 482, 303, and 208 bp for the heterozygous Vegfa\(^{d/d}\) allele. Adult (10–11 weeks of age) wild type and heterozygous mutant mice were injected i.p. with 7.5 IU of eCG on the diestrous day, followed by a single injection of hCG (10IU) at 48 h later. At 16 h after hCG injection, ovarian and body weights were determined together with counting the number of ovulated oocytes in oviducts to estimate ovulatory potency. Some ovaries were further processed using CLARITY and immunostaining to evaluate ovarian vasculature.

**CLARITY processing of ovaries.** Mice were anesthetized i.p. with 2, 2, 2-Tribromoethanol (250 mg/kg body weight, Sigma-Aldrich) before transcardially perfused with 10 (postnatal day 3 or 10) or 20 ml (postnatal day 21 or older) of ice-cold 1 × PBS (Invitrogen GIBCO), followed by 10 or 20 ml of ice-cold hydrogel solution with 4% (weight/volume) paraformaldehyde (Electron Microscopy Sciences), 4% acrylamide (Bio-Rad), 0.05% bis-acrylamide (Bio-Rad), 0.25% VA-044 (Wako), 0.05% Saponin (Sigma) and 1 × PBS for 2 min as described\(^4\). Fixed ovaries were immediately extracted and immersed in 10 ml conical tubes containing 6–7 ml of the hydrogel solution for 3–4 days at 4 °C. Then, tubes were de-gassed with dry ice to remove oxygen and submerged in a 37 °C water bath for 3 h to polymerize hydrogel monomers. Embedded samples were then transferred into 10 ml of the clearing solution (200 mM boric acid (Sigma), 4% (wt/vol) sodium dodecyl sulfate (SDS) (Sigma) dissolved in distilled H\(_2\)O, with the addition of NaOH (EMD) to reach pH 8.5\(^3\). The clearing solution was replaced 2–3 times/week and ovaries gradually became transparent after 4–8 weeks depending their sizes and ages of the animal. We performed CLARITY without the electrophoretic step to allow better antibody staining and to minimize damage of small and fragile murine ovaries.

**Immunofluorescence staining.** Cleared ovaries were transferred into 10 ml glass bottles containing PBST (PBS with 0.1% Triton-X (Sigma)) before shaking slowly in PBS for 1 day to remove residual SDS. Samples were then incubated with primary antibodies for two days, washed in buffer for 1 day, followed by secondary antibodies for 2 days. Before mounting and imaging, samples were incubated in PBST for at least 1 day in the FocusClear solution (CelExplore, Taiwan) for 1 h to correct for refractive index. All procedures were conducted with shaking at 37 °C. Detailed antibody information is shown in Table S3.
Digital imaging and data analysis. Intact ovaries were mounted on glass slides using MountClear (CelExplore, Taiwan) and Willco-Dish (Ted Pella, CA). Images were obtained using Leica SP5 confocal microscope (20 × water-immersion objectives; numerical aperture 1.0; working distance 2.0 mm) and Olympus confocal microscopes (25 × water-immersion objectives; numerical aperture 0.95; working distance 2.5 mm; single-photon excitation). Ovary scanning was taken at the maximum resolution using the Leica tandem resonant scanner (image resolution 1024 × 1024 pixels) and images were stored in the TIFF format. Two-D and 3D imaging as well as data analysis were performed using the Fiji ImageJ and Imaris software (V 8.0, Bitplane, Switzerland). Spot transformation was based on the Imaris algorithm. Because imperfection of staining, some follicles were identified semi-manually using the Spot algorithm in the Imaris software (Figure S3). Spatial homogeneity was determined using Ripley’s K function for point pattern analysis by quantifying the degree of object clustering/dispersion as compared to a random distribution.7 Roundness of follicles was determined using Imaris Surface algorithm semi-manually. After defining follicle borders according to signals in the related sections, the software automatically made an object that was measurable by the statistic option of the Imaris software. Whole mount analysis of branching structures was generated semi-manually based on Filament algorithms developed for studies on kidney glomeruli.49 After setting the starting point of the blood vessel, the software detected signals to make a continuous line along the vessel. By setting the signal thresholds in the software, the cylinder shape of the vessels could be formed. To finalize the 3D construction of vessels with the Filament option, data related to the designed branching structures were measured by using the Imaris software.

Statistical analysis. All data were presented as standard errors of mean (SE). Kolmogorov-Smirnov test was performed for evaluation of the data normality. For multiple groups, statistical analysis was done with independent sample t-test or one-way ANOVA and Tukey post-hoc test (SPSS for Windows, version 20, SPSS Inc, Chicago, Illinois, USA). The P values of less than 0.05 referred to statistically significance.

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Additional Information

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Author Contributions

Y.F., P.C.1., and X.L. performed the experiments; Y.F. prepared all figures; Y.F. and A.J.W.H. designed the experiments and wrote the manuscript; D.B. and P.C.2. provided transgenic mice, K.D., B.H., F.M.B., L.Z.Y., and R.T. assisted in sample processing and data analyses; All authors reviewed the manuscript.

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