Integration of mouse ovary morphogenesis with developmental dynamics of the oviduct, ovarian ligaments, and rete ovarii.

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Abstract

The morphogenetic events that occur during fetal development of the mammalian ovary are crucial to the establishment of adult female fertility. While our knowledge of the cellular and molecular aspects of ovary development is increasing, the structural rearrangements that give rise to the cortex and medulla and the relation of other female reproductive tissues to the ovary have not been thoroughly investigated. In this study, we used tissue clearing and lightsheet microscopy to investigate the stepwise morphogenesis of the ovary. We found that the ovary transitioned from an elongated to a crescent-shaped structure, suggestive of a folding mechanism. As this occurred, the ovarian surface epithelium wrapped from the ventral to the dorsal side of the ovary, forming the ovarian cortex and engulfing the dorsal face of the ovary to form the medulla. To identify the tissue mechanics that drive ovary folding, we investigated proximate tissues closely associated with the ovary including the Müllerian duct, the cranial suspensory ligament, and the rete ovarii. We found that relocation of the Müllerian duct to the ventral aspect of the ovary was associated with expansion of mesonephric tissue that left the ovary fully encapsulated by birth. The cranial suspensory ligament, which tethers the ovary to the body wall and to the Müllerian duct may exert mechanical tension that triggers ovarian folding. Finally, we found that the rete ovarii, a previously dismissed epithelial appendage of the ovary, significantly expanded during late gestation and may act to anchor the ovary to the mesonephros, leading to integration of extrinsic components into the ovarian medulla. This detailed atlas of ovary morphogenesis reveals novel relationships among the ovary and its surrounding tissues and paves the way towards the functional investigation of the relationship between architecture and differentiation of the mammalian ovary.

Introduction

During mouse fetal development, the gonad first arises around embryonic day 9 (E9) as an elongated population of somatic cells that accumulate from proliferation of the coelomic epithelium covering the ventral surface of the mesonephros (Brambell and Hill, 1927; Harikae et al., 2013; Karl and Capel, 1998; Windley and Wilhelm, 2015). The gonad is composed of germ cells and bipotential somatic cells, which progressively differentiate into supporting and interstitial populations after E11.5 (Stévant and Nef, 2019; Swain and Lovell-Badge, 1999; Ungewitter and Yao, 2013). In the XX embryo, the gonads develop into ovaries, where supporting cells become granulosa cells, interstitial cells become theca cells and other stromal cell types and germ cells become oocytes (Nef et al., 2019; Rastetter et al., 2014; Stévant et al., 2019; Wilhelm et al., 2013). As these cells undergo differentiation between E11.5 and birth, they progressively assemble into individual ovarian follicles, characterized by the presence of one oocyte surrounded by two or three pre-granulosa cells (Lei and Spradling, 2016, 2013; Liu et al., 2010, p. 201; Mork et al., 2012; Pepling and Lei, 2018; Pepling, 2012; Zheng et al., 2014). The advent of single cell transcriptomics has provided a wealth of information on the cellular profiles and gene expression trajectories within the developing ovary, presenting an unprecedented view of the molecular and cellular pathways at play during mammalian ovary differentiation and follicle formation (Li et al., 2017; Liu et al., 2010; Niu and Spradling, 2020; Stévant et al., 2019). However, transcriptomics alone do not address the spatial and structural components that underlie ovary regionalization and patterning. In fact, while granulosa cells and oocytes are locally assembling into follicles, the ovary as a whole undergoes dramatic changes in shape and cell composition, including the integration of extrinsic components such as lymphatics, mesenchymal theca cells, and innervation (Liu et al., 2015; McKey et al., 2019; Svingen et al., 2012). The events that occur during ovary morphogenesis are poorly defined and lack context, owing in part to the scarcity of molecular and imaging tools to study the ovary in detail. In addition, tissues closely associated with the ovary, such as the Müllerian duct, ovarian ligaments and the rete ovarii also undergo dramatic transitions during development (Adham et al., 2002; Ford et al., 2020; Lee et al., 2011; Wenzel and Odend'hal, 1985). This could in turn affect ovary morphogenesis and patterning. These external influences have been overlooked in the context of ovary development, highlighting the need for a detailed, integrated atlas of ovary morphogenesis. With recent advances in tissue clearing and 3D imaging technology, it is now possible to visualize structural changes at the level of the whole gonad or even the whole embryo (Bunce et al., 2021; Fiorentino et al., 2021). We recently developed a tissue-clearing method that combines CUBIC and iDISCO (McKey et al., 2020; Renier et al., 2014; Susaki et al., 2015), and optimized 3D imaging methods using lightsheet microscopy to visualize the fetal and perinatal ovary *in situ*, in the context of its surrounding tissues (McKey et al., 2019).

Using our 3D-imaging methods and high-resolution confocal microscopy, we provide a detailed and contextual description of mouse ovary morphogenesis from E14.5 to birth. We define three discrete hallmarks of ovary morphogenesis: (1) ovary folding, (2) specification of medullary and cortical compartments, and (3) ovary encapsulation. We integrated this morphometric approach with a detailed study of three tissues closely

associated with the ovary: the ovarian ligaments, the Müllerian duct and the rete ovarii, and found compelling correlations between the developmental dynamics of these tissues and ovary morphogenesis, suggesting that these structures influence the establishment of ovary architecture.

Results

3D models of developing ovaries provide a new perspective on ovary morphology.

The gonad first arises on the coelomic surface of the mesonephros. Early gonads are generally visualized and studied in complex with the mesonephros, which hosts the developing reproductive ducts. The mesonephric duct, which gives rise to the Wolffian duct (WD), is present from the onset of gonadogenesis (Brambell, 1927; Kobayashi and Behringer, 2003; Zhao and Yao, 2019). However, the Müllerian duct (MD) develops later by invagination of the mesonephric epithelium between E11.5 to E13.5 in the mouse (Huang et al., 2014; Mullen

and Behringer, 2014; Orvis and Behringer, 2007; Roly et al., 2018). While both Wolffian and Müllerian ducts initially develop in all embryos, only one is maintained depending on the sex of the embryo. In female embryos, the Wolffian duct fully regresses by E15.5 (Zhao et al., 2021), while the Müllerian duct is maintained and gives rise to the oviduct (most proximate to the ovary), the uterus, the cervix and the anterior aspect of the vagina (Kobayashi and Behringer, 2003).

To determine the optimal angles to assess ovary morphology, we first performed immunostaining of whole XX embryos at E14.5 with antibodies against FOXL2, which is expressed in pre-granulosa cells and serves as a marker of the gonadal domain, and antibodies against PAX8, which is expressed in the epithelium of the reproductive ducts and cells of the developing kidney. These samples were cleared using iDISCO and imaged with lightsheet microscopy (Bunce et al., 2021; Renier et al., 2014). We generated 3D models of the labelled structures using isosurface segmentation to provide a morphological view of the urogenital complex *in situ* (Fig. 1A, Fig. S1A, Movie S1). We found that at E14.5 the bilateral gonad/mesonephros complexes resided in the posterior third of the embryo, in a plane orthogonal to the midline axis of the embryo (Fig. 1A) located on the ventro-lateral aspect of the developing kidneys (Fig. 1B). A recent study combining contextual 3D images

of the developing gonads in the whole embryo with quantitative morphometric analysis showed that the entire genital ridge rotates inward from E10.5 to E12.5 (Bunce et al., 2021), thus by E12.5 the gonads appear on the medial aspect of the mesonephros. Consistent with these observations, at E14.5 the reproductive ducts were located lateral to the gonads, which were oriented towards each other in the medial axis (Fig. 1B). We used the 3D objects representing the ovaries and ducts in this image to define the six views that are presented throughout this study (Fig. 1C). The caudal and cranial views best illustrated the position and shifts of the ducts relative to the ovary (Fig. 1B), while the dorsal, medial, lateral and ventral views provided information on the shape and regionalization of the ovary itself (Fig. 1C). Dorsal and ventral views also provided context on the position and development of the individual ducts and mesonephric tubules inside the mesonephros (Fig. 1C, *top panels*). Lateral and medial views were optimal for observing the dorsal/ventral regionalization of the ovary and the interface between the gonad and the mesonephros (Fig. 1C, *bottom panels*).

The ovary transitions from elongated to crescent-shaped during late gestation.

Having defined the contextual components of ovary development at E14.5 and the optimal ways of visualizing morphogenetic changes, we next studied the shape of the ovary from E14.5 to birth. For this, we used immunostaining with antibodies against FOXL2 and first imaged whole-mount gonad/mesonephros complexes using confocal microscopy (Fig. 2A). At E14.5, the ovary was still shaped as an elongated tissue developing on the medial face of the mesonephros. By E17.5, the ovary adopted a rounder conformation. The dorsal side eventually resembled a crescent shape in E18.5 embryos (Fig. 2A). This change in conformation was accompanied by

a hollowing of the FOXL2 domain in the center of the dorsal face of the ovary (Fig. 2A).

We next investigated the dynamics of the FOXL2+ domain in whole ovaries cleared with iDISCO+CUBIC and imaged with lightsheet microscopy (Fig. S1A, B; Movie S2). The advantage of this method

is that the sample was imaged in its native conformation, in a cylinder of agarose, and thus did not undergo artificial deformation resulting from mounting on a slide (Fig. S1A, B). With these 3D models, we better visualized the conformation of the ovary in space, and observed a similar shift from an elongated to a crescent shape between E14.5 and birth (postnatal day 0, P0) (Fig. 2B). This was most visible on the dorsal and ventral sides of the ovary, where anterior and posterior regions appeared to rise toward the medial axis and come closer to each other. This was associated with an increase in the inflexion towards the center of the ovary, and with an ingression of the medial face of the ovary, leading to an increase in the concavity of the lateral face (Fig. 2B). Our data thus suggest that the change in conformation of the ovary is representative of a folding process resulting in the center inflexion of the ovary and relocation of anterior and posterior poles.

Upon closer observation, we found that the anterior portion of the ovary appeared to thicken and shift up above the middle of the ovary, suggesting that the anterior region is subject to more deformation than the posterior region (Fig. 2B). Caudal and cranial views of the process showed that while the ventral surface of the ovary remained smooth during ovary folding, a lateral protrusion extended from the mid-region of the dorsal face between E14.5 and E17.5 (Fig. 2C, *white arrows*).

Ovary folding results in specification of the medullary and cortical compartments of the ovary.

Previous studies have shown that the location of individual follicles inside the ovary carries functional significance (Cordeiro et al., 2015; Mork et al., 2012; Suzuki et al., 2015). Although follicles assembled during late gestation in the medullary compartment of the ovary initiate growth shortly after birth, they become atretic during the juvenile period and are all depleted by the onset of puberty. In parallel, a new wave of *de novo* follicle formation occurs around birth in the cortical compartment (Mork et al., 2012; Niu and Spradling, 2020; Zheng et al., 2014). These constitute the finite pool of ovarian follicles that remain quiescent throughout the juvenile period and begin to sequentially activate and initiate growth cyclically at the onset of puberty and until menopause, thus supporting the entire female reproductive lifespan (Duncan and Gerton, 2018; Mork et al., 2012; Zheng et al., 2014). While this functional regionalization points to the influence of ovarian

architecture on the organization and outcome of follicles within the ovary, both the origin of the cortical and medullary compartments and the nature of the signals and the mechanisms that drive this differential patterning remain unclear.

The process of ovary folding as well as the differences in the conformation of the ventral and dorsal surfaces of the ovary led us to investigate whether ovary folding is responsible for the regionalization of the ovary into cortical and medullary compartments. The ovarian cortex is covered with ovarian surface epithelium (OSE), which is the female-specific derivative of the gonadal coelomic epithelium. We used the dynamics of the OSE to serve as a proxy for the establishment of the ovarian cortex during morphogenesis using the *Lgr5-GFP-IRES-CreER* (*Lgr5-Gfp*) transgenic reporter, in which cells of the OSE are labelled with GFP (red in these images) (Fig. 3) (Ng et al., 2014). At E14.5, the OSE covered the ventral portion of the ovarian surface, as demonstrated by the overlap of FOXL2 staining and GFP expression, but was only visible in the most medial aspect of the ovary from the dorsal side (Fig. 3A). The OSE continued to cover the ventral and lateral faces of the ovary throughout the time-course (Fig. 3C-F). However, as the anterior and posterior domains began to fold toward the center at E15.5, the OSE became visible from the dorsal side, in the anterior and posterior regions (Fig. 3C, *top panel*). From E15.5 to E17.5, the FOXL2+/LGR5- domain was gradually restricted to the middle region of the ovary from the dorsal face (Fig. 3C, D). By P0, the ovary was fully folded, and the LGR5+ OSE domain covered about half of the dorsal side of the ovary, enclosing the FOXL2+/LGR5- domain within the center of the ovary (Fig. 3E, F). 3D models of the ovary generated using lightsheet images of *Lgr5-Gfp* samples clearly illustrated the uniform coverage of the ventral side with surface epithelium and the association between folding of the ovarian domain and the extension of the cortical domain of the ovary from the ventral to the dorsal side (Fig. 3B, D, F). The appearance of the LGR5+ domain on the dorsal side coincided with the inflexion of the middle region of the ovary, which presumably resulted in an internalization of the cells originally present on the dorsal surface of the ovary to form the ovarian medulla, and to establish the hilum, which serves as a conduit for ovary vasculature and innervation (Fig. 3D, E, F).

The expansion and relocation of the Müllerian duct leaves the ovary fully encapsulated.

Having characterized the structural changes of the ovarian domain, we next investigated the developmental dynamics of tissues closely associated with the ovary, starting with the Müllerian duct. The invagination of the Müllerian duct occurs from E12.5 to E13.5 in the mouse and is followed by a significant expansion of the duct and functional regionalization into oviduct, uterus, and vagina (Kobayashi and Behringer, 2003; Roly et al., 2018). The presumptive oviduct develops closest to the ovary and undergoes coiling during late gestation (Ford et al., 2020; Harwalkar et al., 2021). We investigated the correlations between the dramatic changes in oviduct conformation and ovary morphogenesis. For this, we first used 3D reconstructions of the ovary and Müllerian duct based on isosurface segmentation of lightsheet images of FOXL2 and PAX8 immunostaining respectively. The shift in position of the Müllerian duct with respect to the ovary was best visualized from caudal and cranial views (Fig. 4A). At E14.5, the Müllerian duct was fully invaginated and lay parallel to the entire longitudinal axis of the ovary, on its dorso-lateral aspect (Fig. 4A). The infundibulum is the

cranial opening of the presumptive oviduct, which in adults is responsible for capturing ovulated follicles. The infundibulum was initially located near the anterior pole of the ovary (Fig. 4A - *yellow asterisk*). At E17.5, while the infundibulum had only slightly shifted to the ventro-medial region of the ovary and the presumptive uterus remained parallel to the longitudinal axis of the ovary, the presumptive oviduct had expanded and shifted ventrally to the ovary (Fig. 4A). This shift was more pronounced in the anterior region of the oviduct (Fig. 4A). By P0, the presumptive oviduct had significantly expanded and coiled into its characteristic structure, and this was associated with a complete shift to the ventral side of the ovary (Fig. 4A).

We next investigated how the relocation of the Müllerian duct correlated with ovary encapsulation. We immunostained whole-mount gonads with antibodies against PAX8 to label the epithelium of the Müllerian duct and ENDOMUCIN to label the vasculature, as a way to visualize the highly vascularized ovarian capsule. These samples were imaged with confocal microscopy (Fig. 4B). In parallel, we used 3D reconstructions of the ovary, Müllerian duct, and ovarian capsule based on isosurface segmentation of lightsheet images of FOXL2, PAX8 and Hoechst labelling, respectively. Consistent with results shown in figure 4A, at E14.5, the Müllerian duct was located immediately below the ovary, with the infundibulum on the lateral side, near the anterior pole (Fig. 4B, left; C, left). At this stage, the ovary was in direct contact with the abdominal cavity and was not encapsulated. Both the mesonephros and ovary were already highly vascularized (Fig. 4Ba). We used isosurface segmentation of the Hoechst labelling in our lightsheet images to generate surfaces representing the entire ovary/mesonephros complex (Fig. 4C - grayscale) and transparency to make the FOXL2-positive ovarian domain visible (Fig. 4C - cyan). Note that the FOXL2 domain is surrounded by the OSE (not labelled in these images), which accounts for the space between the Hoechst-based isosurface and the FOXL2 domain at all stages (Figure 4C - white arrows) and should not be confused with the ovarian capsule, which is absent at

E14.5. At E17.5, the infundibulum had progressed from the lateral to the medial portion of the ovary, and had begun travelling away from the anterior pole to the center of the ovary (Fig. 4B, *middle*; C, *middle*). This was associated with a lateral to medial expansion of cranial mesonephric tissue, the presumptive ovarian capsule, that began covering the cranial region of the ovary (Fig. 4C, *middle* - *green arrows*). At this stage, the middle portion of the Müllerian duct wrapped around the latero-ventral side of the ovary and was no longer visible from the dorsal perspective (Fig. 4B, *middle*). Thus, the process was best visualized from the lateral view, where the cranial region of the ovary was no longer directly accessible from the abdominal cavity but was instead covered by highly vascularized mesonephric tissue, lined on the medial edge by the expanding Müllerian duct (Fig. 4B, *middle*; C, *middle*). By P0, the infundibulum had continued its cranial to caudal progression and was now located at the middle of the medial side of the ovary, near the opening of the hilum (Fig. 4B, *right*; C, *right*). This change was associated with a continued expansion of cranial mesonephric tissue, which now covered the cranial half of the ovary. At this stage, we also observed a caudal expansion of tissue, and the uterine segment of the Müllerian duct was now aligned with the infundibulum on the medial side of the ovary (Fig. 4B, *right*). From the ventral side, the P0 ovary appeared fully encapsulated, with the coils of the oviduct forming the ventral rim of the ovarian capsule (Fig. 4C, *right*). By the end of this process, the infundibulum of the oviduct was embedded in the capsule, where it later will be responsible for capturing ovulated follicles.

These data demonstrate that the encapsulation of the ovary occurs during late gestation and appears intimately associated with the expansion and regionalization of the Müllerian duct to form the oviduct. This observation prompted us to investigate potential mechanical components that could be affecting structural change in both the ovary and the oviduct simultaneously.

The broad ligament and cranial suspensory ligament tether the ovary to the MD and body wall and may act as mechanical drivers of ovary folding and encapsulation.

The position of the ovary within the body cavity is maintained by two ligaments: the broad ligament (BL) and the cranial suspensory ligament (CSL). The BL and CSL are peritoneal folds that support the uterus, oviduct, and ovary, providing a conduit for vasculature, innervation, and lymphatics, and securing the position of the reproductive organs inside the body (Martin et al., 1996). While the BL is a single continuous structure, different segments are defined by the organs they support: the mesometrium supports the uterus, the mesovarium supports the ovary, and the mesosalpinx supports the oviduct. The caudal end of the BL is tethered to the dorsal pelvic body wall, while its cranial region transitions into the CSL, which tethers the ovary to the dorsal abdominal body wall near the diaphragm (Hutson et al., 1997; van der Schoot and Emmen, 1996). Because these structures are fibromuscular tethers of the ovary, we postulated that developmental dynamics of the BL and CSL could provide insight into the mechanisms of ovary morphogenesis. We first used immunostaining against the neural marker TUJ1 as a proxy for visualizing the BL in whole urogenital complexes from E15.5 to E18.5 (Fig. 5A). We found that the mesometrium, mesovarium, and mesosalpinx formed a continuous structure on the medial/dorsal aspect of the reproductive tract and ovary at E15.5 (Fig. 5A, *left*; C, *left*) labeled also with alpha smooth muscle actin (aSMA) (Fig. S2A, *left*). At E16.5, the mesovarium

was visible as a thin sheet of tissue on the medial/dorsal surface of the ovary that hosted neural projections (Fig. 5A, *middle*) and vasculature (Fig. S2A, *middle*) growing towards the ovary. At E18.5, this sheet of tissue was no longer visible on the medial/dorsal surface of the ovary, and innervation and vasculature had penetrated the dorsal ovary in the region of the hilum (Fig. 5A, *right*; Fig. S2A, *right*). These observations suggested that the mesovarium, and associated extrinsic components, became internalized into the ovarian medulla as the ovary folded. We next focussed on the development of the CSL, which we labelled with antibodies against TNC and aSMA in urogenital complexes from Tg(*Nr5a1-GFP*) mice (Stallings et al., 2002), referred to here as *Sf1-eGFP*, in which both the gonad and adrenal are labelled with GFP (Fig. 5B). We found that at E14.5, the CSL was tightly associated with the initial site of invagination of the MD and extended cranially to the ovary, along the kidney and up to the adrenal (Fig. 5B, *left*). The adrenal derives from a subpopulation of cells that segregate from gonadal cells early in gonad formation (Saito et al., 2017; Upadhyay and Zamboni, 1982), suggesting that the TNC+ CSL links these two organs prior to their separation, and

throughout development. At P0, the aSMA-labelled CSL remained closely associated with the ovary, kidney, and adrenal (Fig. 5B, *right*). By this stage, the kidney had significantly grown in volume, resulting in a longer distance between the ovary and adrenal, and we propose that this growth exerts mechanical tension in the CSL. To investigate the link between the CSL, ovary, and MD in more detail, we labelled isolated gonad/mesonephros complexes at E14.5 and found that the TNC+ domain lined the medial ridge of the ovary and the medial edge of the infundibulum before extending towards the dorsal abdominal wall (Fig. 5C, *left*). At E18.5, the ovary had folded and the TNC+ CSL continued to line the medial edge of the infundibulum, which had relocated to the opening of the ovarian hilum (Fig. 5C, *right* - *yellow asterisk*; Fig. S2B). However, at this stage, the CSL was no longer parallel to the longitudinal axis of the ovary but instead ran through the middle of the ovary before extending towards the dorsal abdominal wall (Fig. 5C, *right*). These observations were confirmed in semi-transparent 3D models of the ovary and CSL based on lightsheet images, where the ovary was seen folding around the CSL between E14.5 and P0 (Fig. S2B). Thus, rearrangements of the CSL correlated with folding of the ovarian domain and relocation of the infundibulum. Using IF labeling for RUNX1, which is known to label granulosa cells of the ovary and epithelial cells of the MD (Laronda et al., 2013; Nicol et al., 2019), we discovered that RUNX1 also labels a discrete population of cells present at the medial edge of the aSMA+ mesovarium, which appeared to form a continuous structure from the mesovarium to the infundibulum to the body wall from E14.5 to E17.5 (Fig. 5D). These data suggest that the CSL acts as a physical tether that links the ovary to the infundibulum and to the body wall throughout development. As a consequence, any mechanical tension on the CSL, caused for example by the growth of the kidney, could direct the relocation of the infundibulum and the folding of the ovarian domain simultaneously. The false-colored 3D models in Figure 5E represent all four views of ovary/mesonephros complexes at E14.5 and E17.5, and clearly illustrate the expansion of the mesosalpinx that correlates with translocation of the MD, and results in ovary encapsulation (Fig. 5E, *yellow*) and internalization of the mesovarium during ovary folding (Fig. 5E, *green*).

Developmental dynamics of the rete ovarii are integrated with events of ovary morphogenesis.

The rete ovarii (RO) is an epithelial appendage of the ovary thought to arise from the mesonephric tubules (Sato, 1985). The RO was first described in 1870 by W. Waldeyer (as cited by Byskov and Lintern-Moore, 1973), and was believed to be a functionless female homologue of the male rete testis (Kulibin and Malolina, 2020; Wenzel and Odend'hal, 1985). It was once thought to degenerate in most individuals, however research shows that this structure persists into adulthood in large mammals (Archbald et al., 1971; Hadek, 1958; Mossman and Duke, 1975). The RO consists of three distinct regions: (1) the intraovarian rete (IOR), (2) the connecting rete (CR), and (3) the extraovarian rete (EOR) (Byskov, 1978). The IOR resides inside of the ovary and consists of thin strings of squamous cells. The EOR is a tubular epithelial structure that resides outside of the ovary, and the CR is a transitional zone between the two regions (Byskov and Lintern-Moore, 1973; Lee et al., 2011). Previous literature has not explored the development of the RO or a possible role during morphogenesis of the ovary.

As the RO is directly linked to the ovary, we hypothesized that morphogenesis of the RO might be involved in morphogenesis of the ovary. To investigate the morphological changes of the RO during development, we used antibodies against PAX8, which labels all three RO regions, to image the ovary and RO using confocal and lightsheet microscopy. Our findings demonstrated that the regions of the RO changed in proportion to one another during the embryonic and early postnatal period. At E14.5, the RO was exclusively located on the dorsal side of the ovary and all three regions were distinguishable (Fig. 6A, E14.5; B, left). The IOR was the largest of the three regions at this stage and lined most of the interface between the ovary and mesonephros. PAX8+ cells were squamous and intermingled with FOXL2+ cells, suggesting the IOR was embedded inside the ovarian domain. The rest of the structure was connected to the IOR and located within the mesonephric tissue. The region proximate to the ovary was characterized by a short region of transition from squamous epithelial cells in the CR to a columnar epithelium in the EOR, which extended in tubules outward from the ovary (Fig. 6A, E14.5; B, left). As the ovary folded between E16.5 and E17.5, the IOR adopted a U-shape (Fig. 6A, E16.5-E17.5; B, middle). At E16.5, the tubules of the EOR began to elongate and by E17.5 had wrapped around to the ventral face of the ovary (Fig. 6A, E16.5-E17.5; B, E17.5). The CR was

located alongside the dorsal protrusion of the ovary of this stage (Fig. 6A, *E16.5*; B, *middle*). At *E17.5*, the embedded IOR was restricted to the edge of the FOXL2 domain on the dorsal side (Fig. 6A, *E17.5*), and the CR was more predominant with growth of the dorsal protrusion. The EOR underwent rapid growth and convolution of the tubules, especially apparent in the 3D model, where both the dorsal and medial views demonstrated the intricacy of the tubular coiling (Fig. 6B, *right*). By *E18.5*, the IOR had slightly regressed compared to *E17.5*, and no longer extended all the way to the poles of the ovarian domain (Fig. 6A, *E18.5*). At this stage, the EOR was the largest of the three regions, and had developed into an elaborate and convoluted tubular structure that curved alongside the ovary within the ovarian capsule (Fig. 6A, *E18.5*). Between P0 and P5, the IOR mostly regressed to the lateral edge of the medulla, but a thin string of cells continued to line the dorsal boundary between the ovarian cortex and medulla, and the EOR continued to grow and wrap around the ovary (Fig. 6A, *P0-P5*; B, *right*). The location of the IOR at the boundary of the ovarian cortex coincides with the dorsal opening of the hilum. To further characterize this domain, we stained late-gestation ovaries for ENDOMUCIN and TUJ1, which label vasculature and innervation respectively. These components entered the ovary through this dorsal opening, lined with cells of the IOR (Fig. S3A, B). This suggests that the IOR plays a role in the invasion of extrinsic components into the ovarian medulla, either by mechanically restricting folding on the dorsal side, or by producing chemoattractant signals, or both.

The morphological difference between cells of the EOR and CR/IOR prompted us to investigate the expression of tubular epithelium markers KRT8 and E-Cadherin. We found that these markers were significantly enriched in the EOR (Fig. S4A, B). The EOR was also the only region to be wrapped in a sheath of α SMA, presumably fibromuscular tissue of mesenchymal origin (Fig. S4C). We also used antibodies against SOX9, a broader epithelial marker, and noted that all three regions of the RO were labelled (Fig. S4D). A small subset of the CR and IOR cells were positive for the pre-granulosa marker FOXL2, suggestive of a transitional region. The CR and IOR also expressed several ovarian markers, including RUNX1, SF-1 and GATA4, reinforcing the notion that cells of the IOR are closely related to ovarian cells (Fig. S4E, F, G). While expression of RUNX1 was lower in the CR, this was the only gonadal marker to be expressed in the tubular cells of the EOR (Fig. S4E). In addition, we found that some cells of the IOR and CR expressed both PAX8 and the gonadal markers Nr5a1 (SF1-eGFP) and FOXL2 (Fig S4F, H - *white arrows*). Interestingly, PAX8⁺ cells of the IOR were observed in close contact with oocytes labeled with HuC/D, suggesting that the IOR was intermingled with ovarian follicles in the medullary region (Fig. S4H). We then investigated the presence and structure of the RO in adult mouse ovaries using tissue clearing and lightsheet microscopy. The RO was labelled with PAX8 antibodies, and the ovarian domain was identified using antibodies against AMH (Anti-Müllerian Hormone - a marker of preantral and antral follicles). All three regions of the RO persisted into adulthood (Fig. 6C), suggesting that the role of the RO is not limited to the embryonic period. In adulthood, the EOR remained the largest of the regions followed by the CR and IOR (Fig. 6C).

Discussion

Using lightsheet and confocal imaging, we provide a robust image dataset that catalogs the complex events of ovary morphogenesis in the context of the extrinsic structures incorporated into the ovary and the other female reproductive structures that surround it. Our goal is to make this dataset available to the field as a resource to advance our understanding of ovary development and its role in the establishment of female fertility. While the location of individual follicles within the cortex or medulla of the ovary appears to be crucial in determining their outcome (Cordeiro et al., 2015; Mork et al., 2012; Suzuki et al., 2015), the mechanisms that control regionalization of the ovary remain poorly understood. Importantly, the morphogenetic data presented here is an important first step towards integrating mechanical and architectural aspects of ovary development with the establishment of the medullary and cortical domains of the ovary. In the present study, we propose a model of ovary morphogenesis that links the establishment of ovarian architecture and the functional regionalization of the ovary into cortical and medullary compartments with the morphogenesis of the oviduct and the CSL.

The dorsal face of the ovary is the center of action for most architectural changes during ovary morphogenesis. While the ovary initially develops on top of the mesonephros, the dorsal aspect of the ovary becomes progressively coated with the mesovarium, the ovarian portion of the broad ligament. The cranial region of the oviduct, the infundibulum, remains tethered to the original anterior pole of the ovary throughout ovary morphogenesis through their mutual attachment to the CSL, which tethers the ovary to the dorsal abdominal body wall. The CSL originally appears on the medial ridge of the ovary, parallel to its longitudinal axis, and tethered to the infundibulum near the anterior pole of the ovary. Ovary folding is associated with relocation of the infundibulum to the medio-ventral opening of the ovarian hilum and incorporation of the CSL within the fold. The relocation of the oviduct and infundibulum is driven by the dorsal expansion of the mesovarium and the cranial expansion of the mesosalpinx, the region of the broad ligament that supports the oviduct (Harwalkar et al., 2021). This process leaves the ovary fully encapsulated, with the ovarian capsule arising from the growth and fusion of the mesovarium and the mesosalpinx (Agduhr, 1927). Our hypothesis is that mechanical tension exerted on the fibromuscular CSL, likely due to growth of the kidney and the ovary away from the body wall, drives the process of ovary folding. As the ovary folds toward its dorsal face, the mesovarium and associated vasculature and innervation, which were located on the medio-dorsal aspect of the ovary, become internalized inside the folded ovary, contributing new tissue to the initial gonad primordium. As the ovary folds, the OSE, located as a canopy on the ventral face of the ovary at E14.5, becomes visible from the dorsal face. This process establishes the ovarian cortex and medulla. Oocytes and pre-granulosa cells that were originally in the dorsal region of the ovary are now located in the medulla, in close proximity to newly internalized extrinsic components. This suggests that the allocation of follicles into medullary and cortical domains is driven by structural changes of the ovary during morphogenesis. It will be an exciting endeavor to integrate the morphogenetic approach presented here with the dynamics of ovarian follicle formation to decipher the contribution of extrinsic molecular and mechanical signals to the establishment of the ovarian reserve.

This proposed model for ovary morphogenesis points to the CSL as the driver of the cascade of events that lead to the establishment of the architecture of the ovary. A recent study demonstrated that the gut mesentery, which is a fibromuscular connective tissue similar to the broad ligament and CSL, plays a crucial role in the establishment and maintenance of gut looping by exerting contractile forces on the developing intestine (Savin et al., 2011). Whether the CSL and/or broad ligament have contractile properties remains to be investigated, and further studies will be required to test the functional significance of the CSL in the process of ovary folding. While the CSL originally develops in both males and females, it is maintained only in females to secure the ovary to the abdominal wall. In males, the CSL regresses to accommodate testis descent (Adham et al., 2002; Hutson et al., 1997; van der Schoot and Emmen, 1996). Perhaps coincidentally, the testis does not fold the way the ovary does. It will be interesting in the future to determine how this dimorphism relates to the presence of the CSL. In addition, the ovary integrates extrinsic components within the medulla, which appears more permissive than the testis (McKey et al., 2019; Svingen et al., 2012). Our data suggest that the presence of vasculature and innervation within the ovary is, at least in part, a result of folding over the growing mesovarium. The integration of these components likely influences the functional regionalization of the ovary.

This study also illuminates the developmental dynamics of the RO. We found that the CR remains at the interface between the ovary and mesonephric tissue throughout development, and corresponds to the dorsal protrusion of the ovarian domain. One hypothesis is that the CR acts as a tether of the ovary to the mesonephros. The IOR may act as ribs of an umbrella restricting folding to allow integration of the mesovarium and other extrinsic components. Alternatively (or in addition) the cells of the CR and/or IOR may produce guidance molecules that promote the invasion of extrinsic tissues into the ovary. Given the location of the IOR at the boundary between the ovarian cortex and medulla, it will be relevant to investigate the mechanical and/or molecular contribution of the RO to the functional regionalization of ovarian follicles in the perinatal period. The EOR develops inside the ovarian capsule in fetal life, but later resides in the periovarian fat pad in postnatal mice, leading to the hypothesis that the fetal ovarian capsule may give rise in postnatal life to both the ovarian bursa and the periovarian fat pad. Functional models will be required to test these hypotheses and determine the role of the RO during ovary morphogenesis. Importantly, while the RO is often dismissed as a vestigial remnant of the mesonephric tubules believed to degenerate in adulthood, we show that all three regions of the RO are maintained with the post-pubertal mouse ovary, and that the EOR remains a prominent structure that resides in the periovarian fat tissue. These observations highlight the necessity of better understanding the biological function of the rete ovarii, and its potential role in female fertility.

Materials and methods

Mice. Unless otherwise stated, experiments were performed on wild-type CD-1 embryos, or mixed CD-1 and C57BL/6J embryos. All transgenic lines were maintained on the C57BL/6 background. The *Lgr5-Gfp-IRES-CreER* and *Tg(Nr5a1-eGFP) (Sf1-eGFP)* lines were previously described (Ng et al., 2014; Stallings et al., 2002). To collect embryos at specific developmental stages, males were set up in timed mating with several females. Females were checked daily for the presence of a vaginal plug. Date of the plug was

considered embryonic day 0.5. All mice were housed in accordance with National Institutes of Health guidelines, and experiments were conducted with the approval of the Duke University Medical Center Institutional Animal Care and Use Committee (IACUC protocol # A089-20-04 9N).

Whole embryo collection and genotyping. Whole embryos at E14.5 were dissected in PBS^{-/-}, fixed in 4% PFA/PBS overnight at 4°C and gradually dehydrated using methanol dilutions for 45min each at room temperature: 25% MeOH/PBS; 50% MeOH/PBS; 75% MeOH/PBS; 100% MeOH. Embryos were then stored at -20°C in 100% MeOH until required for staining. The amniotic sac of each embryo was used for sex genotyping: the tissue was incubated overnight at 55°C in tissue lysis buffer with Proteinase K. The following day, genomic DNA was precipitated with isopropanol and purified by centrifugation. The pellet was resuspended in sterile dH₂O and processed for PCR genotyping for Sry. Primers used for PCR genotyping are listed in Supplementary Table 1.

Reproductive complex collection. Ovary/mesonephros/Müllerian duct complexes at desired stages were dissected in PBS^{-/-}, with as little removal of surrounding tissue as possible to maintain the integrity of the complex. Samples were fixed for 30min at room temperature in 4%PFA/PBS. Following two 15min washes, samples were gradually dehydrated into MeOH dilutions for 15min each at room temperature: 25% MeOH/PBS; 50% MeOH/PBS; 75% MeOH/PBS; 100% MeOH. Samples were then stored at -20°C in 100% MeOH until required for staining.

iDISCO+ clearing and immunostaining for lightsheet imaging of whole embryos. When ready for analysis, E14.5 XX embryos were incubated overnight in 33% methanol, 66% dichloromethane (DCM) rocking at room temperature. Samples were then treated with 5% H₂O₂ in methanol overnight at 4°C. After washes in a reverse MeOH/PBS gradient (75% MeOH/PBS; 50% MeOH/PBS; 25% MeOH/PBS), rehydrated tissues were washed 15min in PBS 0.2% Triton X-102 (PTx.2), and permeabilized overnight at 37°C in iDISCO permeabilization solution (PTx.2, 2.3% glycine, 20% DMSO). The following day, samples were transferred to iDISCO blocking solution (PTx.2 10% DMSO, 1.5% horse serum) for 6h at 37°C. Tissues were then incubated in primary antibodies diluted in PTwH (PTx.2 with 0.001% heparin) 3% horse serum, 10% DMSO for 72 hours at 37 °C. Samples were then washed three times for 1hr in PTwH and incubated for 48hr in secondary antibodies diluted in PTwH 3% horse serum at 37°C. After three 1hr washes in PtWH, embryos were transferred to glass scintillation vials and progressively dehydrated into 100% methanol and incubated for 3h in 33% methanol, 66% DCM rocking at room temperature. Following two 30min washes in 100% DCM, samples were transferred into 100% dibenzylether (DBE) for final clearing. All primary antibodies and fluorophore-conjugated secondary antibodies used in this study are listed in Supplementary Tables 2 and 3 respectively.

Immunostaining for confocal imaging. Samples were gradually rehydrated into PBS through 10min washes in a reverse methanol gradient (75% MeOH/PBS; 50% MeOH/PBS; 25% MeOH/PBS), and transferred to PBS 0.1% Triton X-100 for 30min. Samples were then transferred to blocking solution (PBS; 1% Triton X-100; 10% horse serum) for 1hr, and incubated overnight in primary antibodies diluted in blocking solution at 4°C. The next day, samples were washed three times for 30min in PBS 0.1% Triton X-100 and incubated overnight in secondary antibodies and Hoechst vital dye diluted 1:500 in blocking solution. On day 3, samples were washed twice for 15min in PBS 0.1% Triton X-100 and transferred to PBS at 4°C until ready to mount for confocal imaging (1hr - 48hr). Samples were mounted for imaging in polyvinyl alcohol mounting solution (stored at 4°C) or DABCO mounting solution (stored at -20°C) and stored until imaged. To image the dorsal and ventral sides of the same sample, we used 3D-printed reversible slides that can be flipped and imaged from both sides. The 3D model is available for download on the NIH 3D Print Exchange website at <https://3dprint.nih.gov/discover/3DPX-009765>.

iDISCO+CUBIC clearing and immunostaining for lightsheet imaging. Fetal reproductive complexes were cleared and immunostained as previously described (McKey et al., 2020, 2019). Briefly, isolated ovary/mesonephros samples went through the steps described above for iDISCO clearing. After incubation in DBE overnight, samples were transferred to 100% MeOH and progressively rehydrated to PBS through a reverse methanol gradient. Samples were then transferred into a 1:1 dilution of CUBIC Reagent-1 in dH₂O (1/2Cubic R-1) for overnight incubation at 37°C. The next day samples were transferred into 100% CUBIC R-1 for 24-48hr. When sufficiently cleared, samples were washed in PBS and transferred to a 1:1 dilution of CUBIC Reagent-2 in PBS for 6hr and 24-48hr at 37°C in 100% Cubic R-2 containing Hoechst nuclear dye. Once sufficiently cleared, samples were embedded in 2% agar/CUBIC R-2 in 1mL syringes and stored in the dark at RT until imaged. Samples were imaged in CUBIC reagent-2 using the Z.1 Zeiss Lightsheet microscope (Carl Zeiss, Inc.).

Image acquisition. Images of the E14.5 iDISCO+-cleared whole embryo were acquired using a LaVision BioTec Ultramicroscope II lightsheet microscope, equipped with zoom body optics, an Andor Neo sCMOS camera, an Olympus MVPLAPO 2X/0.5 objective and a 5.7 mm working distance corrected dipping cap (total magnifications ranging from 1.3X to 12.6X with variable zoom). The whole embryo was mounted in a sample holder and submerged in a 100% DBE reservoir. The sample was imaged at 1.26X (0.63X zoom), using the three lightsheet configuration from both sides with the horizontal focus centered in the middle of the field of view, an NA of 0.026 (beam waist at horizontal focus = 28 µm), and a lightsheet width of 90%. Spacing of Z slices was 10 µm. Confocal images of uncleared fetal ovary/mesonephros complexes were captured in the longitudinal plane on Zeiss LSM710 or LSM880 confocal microscopes and the affiliated Zen software (Carl Zeiss, Inc., Germany) using a 10X objective. Images of iDISCO+CUBIC-cleared isolated ovary/mesonephros complexes were acquired using a Zeiss Z.1 Lightsheet (Carl Zeiss, Inc., Germany). Lightsheet images were collected using the 5x NA 0.16 dry objective. Images were captured at 0.5 to 1X zoom, using the dual or single

side lightsheet illumination (depending on size of sample), a lightsheet thickness of 2.5µm, and a Z-interval of 1.2 to 2µm. Image acquisition was performed with Zen software (Carl Zeiss, Inc., Germany).

Image processing. Confocal Z-Stacks were imported into FIJI software for minor processing, including cropping and rotations. All images of individual ovary/mesonephros complexes were oriented with the anterior end of the gonad to the left and posterior end to the right. Unless otherwise stated, all images presented in this study are maximum intensity projections of confocal Z-Stacks generated with FIJI. Final processing, such as channel overlays, brightness and contrast adjustments and figure compositions were made in Adobe Photoshop CC (Adobe, Inc., CA). Lightsheet Z-stacks were converted into .ims files using the Imaris file converter and imported into Imaris software for processing (Version 9.6; Bitplane, Inc., United Kingdom). Upon opening the file for the first time, x and y dimensions were downsampled to 50% of the original size (1920x1920x1.2px to 960x960x1.2px) for better file management. 3D volumes were rendered using the *maximum intensity* render mode, and isosurfaces were generated using the *surface creation wizard*, using an absolute threshold specified for each image and channel. To display specific regions and tissues, the *clipping plane* tool was applied. In cases where the antibodies labelled more than the region desired for display, the *cut surface* tool was used. Each channel was individually adjusted for brightness and contrast to capture still images of native data or 3D models using the *snapshot* tool (images captured as fixed size 1000 x 1000px transparent PNGs). Videos were captured using the *animation* tool in Imaris and edited using Adobe Premiere Pro CC software (Adobe, Inc., CA). All source images and 3D models will be made available upon request.

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