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Patterning the gonad in 4-dimensions

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Abstract

The urogenital ridge (which includes the gonad and attached mesonephros) has proved to be a unique system to analyze the role of vascularization in morphogenesis of an internal organ. During the period of vascularization, development of the testis and ovary diverge morphologically. Time-lapse imaging of the gonad provides novel insights into the mechanisms that incorporate vasculature into an organ at the stage when structural organization of the tissue is initiated. Moreover, the divergent development of the testis and ovary provides a basis of comparison to correlate vascular development with the sex specific morphological development of the tissue. We are currently incorporating different cellular markers in this analysis to simultaneously image the endothelium and other cells in the organ. As far as we know, this is the first system for imaging vascular development in an organ undergoing extensive epithelial morphogenesis (Coveney *et al*., 2008). We are optimistic that this work will lead to further insights into the interwoven processes of cell differentiation and paracrine signaling that transform a common bipotential primordium to the highly specialized testis or ovary.

Keywords: gonad, live-imaging, ovary, testis, vascular development.

The early gonad is a bipotential primordium, capable of differentiating as a testis or an ovary. Recent experiments in the lab suggest that gonadal cells are initially balanced between two fates by antagonistic signals between *Fgf9* and *Wnt4* (Kim and Capel, 2006). The Y chromosome-linked male sex-determining gene, *Sry*, creates an imbalance in these signals by up-regulating its target gene, *Sox9*. *Sox9* and *Fgf9* work in a feed-forward loop to repress *Wnt4* and establish the commitment of the supporting cell lineage to the Sertoli cell fate, and the gonad to the testis pathway. In an XX gonad, where *Sry* is not present, the pathway is governed by *Wnt4* and *Rspo1*, two genes that activate ß-catenin signaling, repress *Sox9* and *Fgf9*, and commit the supporting cell lineage to the follicle cell fate, and the gonad to the ovary pathway (Kim *et al*., 2006).

Many lines of evidence suggest that, in addition to feed-forward loops that occur within cells, there are many signaling loops between cells that reinforce the commitment of the gonad to the testis or ovary pathway. For example, the differentiation of Sertoli cells, the male supporting cell lineage, influences the differentiation of the steroidogenic precursors to form Leydig cells (Adams and McLaren, 2002; Bowles *et al*., 2006; Brennan *et al*., 2003; Clark *et al*., 2000; Yao *et al.,* 2002). Leydig cells in turn influence the structure of the testis and the viability of germ cells (Tang *et al*., in press). Germ cells normally enter mitotic arrest in the testis according to the spermatogonial differentiation program, and secrete signals such as prostaglandin D2 that reinforce testis development (Adams and McLaren, 2002; Bowles *et al*., 2006). In contrast, the entry of germ cells into meiosis is an early characteristic of the ovarian pathway (McLaren, 2003; Baltus *et al*., 2006). Signals from meiotic germ cells, which produce *Figα*, play a critical role in the structural development of the ovary, in particular in the formation of ovarian follicles (Soyal *et al*., 2000). Interestingly, meiotic germ cells can disrupt testis cord development, suggesting that there is normally a tightly integrated network of signals between cells that canalize the gonad toward the testis or ovarian fate (Yao *et al*., 2003).

Early gonadal development is characterized by a striking difference in morphological development between the two sexes (Fig. 1). The epithelialization of Sertoli cells and the organization of testis cords, easily recognizable at the level of the light microscope, have served as a landmark of testis development for generations of reproductive biologists (Pelliniemi *et al*., 1993). Defects in these morphological processes were easily detected. In contrast, ovarian development was very difficult to analyze because very few changes in morphology were characterized during the parallel stages of ovary development. For many years, recognition that the ovarian pathway had been initiated depended solely on the identification of meiotic germ cells (McLaren, 2003). This situation is changing as expression profile experiments indicate an active ovarian expression program (Nef *et al*., 2005; Beverdam and Koopman, 2006; Bouma *et al*., 2007). A number of molecular markers are now known to characterize the commitment of the gonad to the ovarian fate. Among these, an elevation of *Wnt4* expression (Kim *et al*., 2006; Vanio *et al*., 1999), activation of *Rspo1* (Chassot *et al*., 2008), *Bmp2*, *Fst* (Yao *et al*., 2004), and *Foxl2* (Schmidt *et al*., 2004; Yao, 2005) have all been reported. Evidence that repression of the male pathway, and activation of the ovarian pathway depends, at least in part, on ß-catenin signaling is a significant step forward (Chassot *et al*., 2008; Maatouk *et al*., 2008). Further work will be required to define the stepwise morphological changes that occur, and to identify genes that act downstream of primary signals to firmly establish ovarian fate.

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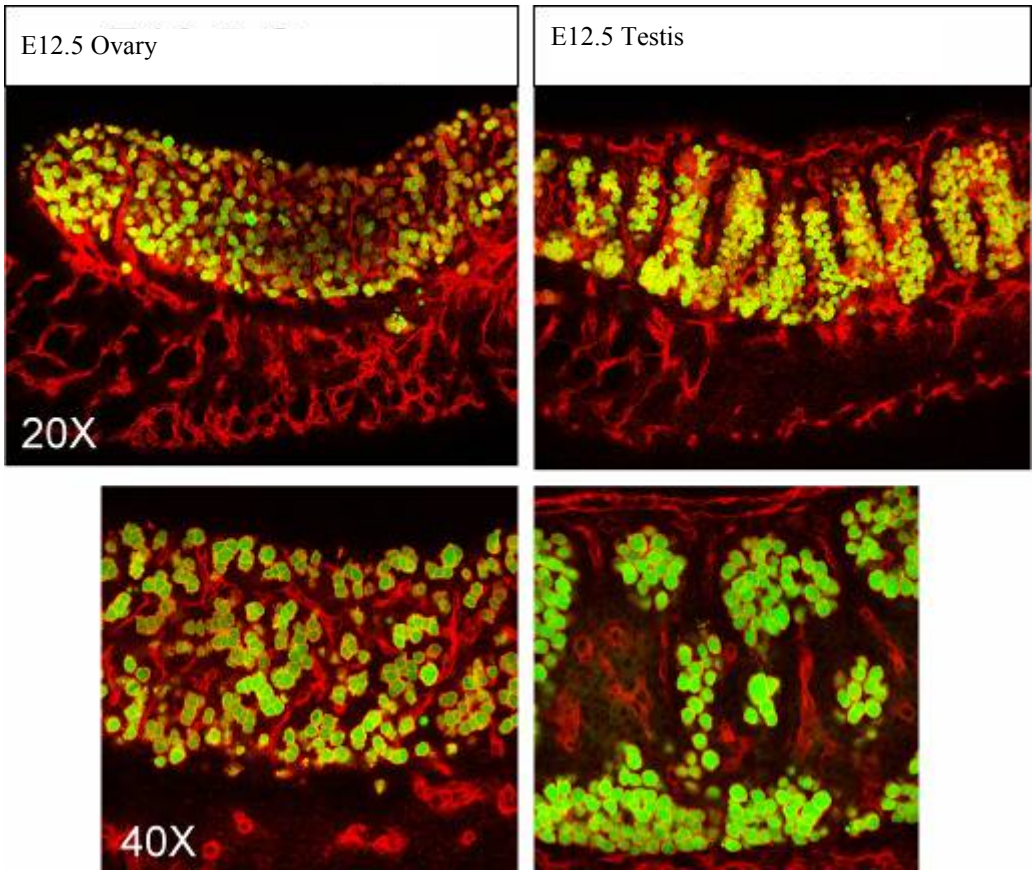

Figure 1. Confocal images of ovary (left) and testis (right) at E12.5 illustrate the dramatic morphological differences between the two organs, one day after *Sry* expression reaches its peak. In the ovary, germ cells (green) are scattered throughout the tissue and the vasculature (red) shows no specific organizational pattern. In the testis, germ cells are organized in clusters enclosed inside testis cords, while a specific coelomic vessel is apparent at the surface of the organ, and vascular branches extend between each testis cord. Top images at 20X, bottom images at 40X, courtesy of Danielle Maatouk.

For many years, my lab has investigated the early divergence in morphological development based on the hypothesis that it is a critical part of the process that reinforces testis or ovary fate. During early testis development, Sertoli cells activate *Sox9* expression, epithelialize, and undergo de novo organization around germ cells to form testis cords. This process partitions the tissue into cell populations inside testis cords (Sertoli and germ cells), and cells outside testis cords, in the interstitial space. The interstitial cell population includes the steroidogenic cells of the testis (Leydig cells), the vasculature, and other unidentified cell types (Fig. 1; for review, see Brennan and Capel, 2004).

Testis cord formation is dependent on the migration of a population of cells from the neighboring mesonephros. Cell migration from the mesonephros is male-specific and does not occur into the XX gonad. When cell migration is blocked, testis cords do not form (Buehr *et al*., 1993; Tilmann and Capel, 1999). The specific migrating cell type required for the induction of cord formation was believed to be peritubular myoid cells, a cell type that surrounds the entire outside surface of testis cords. However, our recent experiments show that peritubular myoid cells are not among the migrating cells. Most, if not all, migrating cells are endothelial cells (Cool *et al*., 2008; Coveney *et al*., 2008). These results strongly imply that it is the endothelium that is required to induce cord formation.

In an effort to better understand the differences in vascular development between the ovary and the testis, we performed live-imaging of endothelial migration during the initial divergence of morphological pathways in the early gonad (Coveney *et al*., 2008). These time-lapse movies confirm fundamental differences in the development of the vasculature between XX and XY gonads. Endothelial cells migrate into the XY gonad from multiple positions along the mesonephric border, partitioning the gonad into approximately 10 domains where testis cords will form. Cells move directly to the coelomic domain where they organize into the characteristic coelomic vessel. Subsequently, vessels extend in the domains between cords. Although the vasculature also expands in the developing ovary during this early stage of morphological

divergence, the mechanism(s) governing the process are not yet clear.

Several lines of experiments have reinforced the idea that the endothelium is required for testis cord formation. First, if migration is blocked, using a membrane barrier, testis cords do not form (Buehr *et al*., 1993; Tilmann and Capel, 1999). Furthermore, in mutants where ovotestes form, endothelial migration occurs specifically into regions where cords are forming, but is excluded from the ovarian domains of the gonad (Albrecht *et al.*, 2000). Other laboratories have used chemical inhibitors to block migration (Cupp *et al*., 2002, 2003). However, as these inhibitors also affect Fgf and Pdgf signaling, which both have multiple roles in testis development (Colvin *et al*., 2001; Brennan *et al*., 2003; Schmahl *et al*., 2004; Kim *et al*., 2006, 2007), we were concerned that these inhibitors could affect many processes in addition to vascular migration. Typically, all of these inhibitors disrupt SOX9 expression in Sertoli cell progenitors when gonads are treated at early stages. Thus it is impossible to determine in these cases whether the disruption of cord formation is primarily a result of loss of SOX9 and Sertoli cell differentiation, or loss of the endothelium. In ongoing work in the lab, we have attempted to address this concern, by using a specific inhibitor of VEGFA (Aflibercept; Regeneron). We anticipate that results using the specific VEGFA inhibitor, combined with a genetic approach to reinforce these results, will be more convincing than results using more general inhibitors.

It remains unclear whether endothelial cells influence cord formation directly or through their effects on the interstitium. The interstitium is a poorly understood cell population that appears to arise from proliferation at the coelomic epithelium and possibly also near the gonad/mesonephric border. Interstitial progenitors segregate from Sertoli progenitors expressing SRY and SOX9 from at least E11.5 onward. Whether the interstitium plays an active role in partitioning testis cords during their formation has not been investigated. How interstitial progenitors segregate from the Sertoli population, and whether there is heterogeneity among cells in the interstitum prior to E12.5 is unknown, as no markers have been discovered that distinguish early cell types. All markers previously investigated (*Pdgfra*, *Sma*, and *Lhx9*) label the entire interstitial population between E11.5 and E12.5. Sertoli cells have been the focus of most of the research in testis development as they are the cell type where *Sry* is expressed and they are the first cells recognized to differentiate during testis development. In future work, we plan to investigate the relationship between the interstitium, the vasculature, and testis cord formation.

As if this were not complex enough, there is an additional wrinkle in the story. Leydig cells, the neuroendocrine cells of the testis, differentiate in close proximity to vascular plexes in the testis interstitium. In several mutants, there is a positive correlation between the presence or absence of vasculature and the differentiation of Leydig cells. For example, in XY Pdgfr-α. mutants, where vasculature is compromised, Leydig cells do not form, and testis cords are severely disrupted (Brennan *et al*., 2003; Tang *et al*., in press). In recent experiments, we have found that both cord morphology and germ cell viability depend on an appropriate population of Leydig cells during fetal life. Experiments in which Leydig cell numbers were significantly increased or decreased led to disruptions in cord morphology and germ cell numbers (Tang *et al*., in press). These experiments suggest that the processes of vascularization, Leydig cell differentiation, cord formation, and germ cell viability are all tightly correlated during testis development.

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