

Illuminating Testis Morphogenesis in the Mouse

Liesl Nel-Themaat¹, Gabriel Gonzalez¹, Haruhiko Akiyama² and Richard R. Behringer¹

¹Department of Genetics, University of Texas, M. D. Anderson Cancer Center, Houston, Texas 77030;

²Department of Orthopedic Surgery, Graduate School of Medicine, Kyoto University, Kyoto, Japan.

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Correspondence: Richard Behringer, Department of Genetics, University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030, Tel: 713-834-6327, Fax: 713-834-6339;
Email: rrb@mdanderson.org

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Abstract

The mammalian testis is a complex organ composed of multiple cell types that are organized into seminiferous tubules and an interstitium, producing spermatozoa and hormones, respectively. During embryogenesis, the testis forms from the genital ridge associated with the embryonic kidney called the mesonephros. After germ cells migrate into the genital ridge, the Sertoli-germ cell mass forms and undergoes morphogenetic changes to generate testis cords, the precursors of the seminiferous tubules. Static images of the fetal testis at sequential stages of development provide structural information about cord formation. Transgenic mice that express fluorescent protein reporters offer new opportunities for time-lapse imaging to visualize live cells and their behaviors during testis differentiation and morphogenesis.

Keywords: Fetal gonad, seminiferous tubule, fluorescent protein, time-lapse imaging.

Introduction

The mammalian testis is composed of spermatozoa-producing seminiferous tubules surrounded by interstitial cells. The seminiferous tubules are composed of the somatic Sertoli cells that nourish internally-located spermatogenic cells that continuously produce spermatozoa. The seminiferous tubules are enveloped by peritubular myoid cells. The interstitium includes the hormone-secreting Leydig cells, a vasculature composed of endothelial cells, and undefined stromal cells.

In most mammals, testis formation occurs in XY individuals. Although the development of the mammalian testis has been studied intensely, it has been difficult to examine the cellular behaviors that lead to cord and interstitium formation. The use of novel imaging and data processing techniques, in conjunction with the generation of several new transgenic mouse models that express fluorescent proteins, have recently led to a detailed description of the dynamic interplay between different cell types and tissues during testis morphogenesis. This review focuses on recent findings and technologies that examine testis formation in the mouse.

The testis forms from the genital ridge in XY embryos

The testes form in XY embryos from a pair of gonad precursors associated with the mesonephroi. In the mouse, at tail somite stage 8 (TS8, E10.5), the gonad precursor or genital ridge is a thickening of the coelomic epithelium on the ventromedial side of the mesonephros (for review see (Swain and Lovell-Badge, 2002). Primordial germ cells (PGC) migrate into the genital ridges between E10.5 and E11.5 (Ginsburg et al, 1990; Lawson and Hage, 1994; Anderson et al, 2000). At E11.5, whole mount views (**Fig. 1**) and histology of the fetal testes show a homogeneous mixture of PGCs and somatic cells with no obvious structures. One day later at E12.5 and older stages, testis cords can be seen when viewed with a stereodissecting microscope and histology reveals the formation of testis cords (**Fig. 1**) composed of Sertoli cells and germ cells that are surrounded by an interstitium. In addition, the fetal testis has developed a heterogeneously organized vasculature (**Fig. 1**) that is distinct from the fetal ovary which at the same stage has a diffuse network of endothelial cells.

Structures and cellular components of the fetal testis

Testis cords

Germ cells

After germ cells arrive in the genital ridge, they become subjected to sex-specific influences of their surroundings, independent of the chromosomal sex of the germ cells (i.e. XX and XY germ cells behave the same in an ovarian or testicular environment). Germ cells in the fetal testis undergo mitotic arrest at E13.5-E15.5, which is maintained until 3-4 days after birth when spermatogonia become mitotic (reviewed by Peters, 1970). Germ cells of the fetal testis are in close contact with Sertoli cells, initially forming a Sertoli-germ cell mass that will subsequently be partitioned into testicular cords. Although germ cells are an important component of the developing fetal testis, they are not required for testis formation because cords can form in XY gonads lacking germ cells (McCoshen, 1983).

Sertoli cells

Sertoli cells are the primary somatic component of testis cords in the embryo and adult. Their main role is to provide a nurturing environment for germ cells (Mruk and Cheng, 2004). Sertoli cells originate from the coelomic epithelium between TS15-17 and migrate into the genital ridge (Karl and Capel, 1998). The first sign of testis differentiation is an increase in cell proliferation in Sertoli cells at TS17-18 (Schmahl et al, 2000; Schmahl and Capel, 2003). This leads to a rapid increase in gonadal size, specifically the number of cell layers that contributes to the gonad (Karl and Capel, 1995). Interestingly, there is a temporary cessation in Sertoli cell proliferation between E11.5 and E12.5 (Schmahl et al, 2000), suggesting that the gonadal thickness increase observed during this time is due to cell migration or rearrangement of Sertoli cells and possibly other cell types. Morphometric analysis of testis formation demonstrated that the gonad roughly doubles in width every 24 hours from E11.5 to E13.5 (Nel-Themaat et al, 2009). It was suggested that the increase in gonad thickness during this window of time is a result of Sertoli and other cell types migrating more towards the center of the gonad, because there was a concomitant reduction in gonadal length. Cell proliferation is reinitiated at E12.5, reaching its highest rate at E14.5 and continues until after birth (Schmahl et al, 2000).

Peritubular myoid cells

Sertoli cells of the testis cords are surrounded by a layer of squamous cells called peritubular myoid cells. PTM cells are believed to interact with Sertoli cells to generate the basement membrane of the epithelial Sertoli layer that surrounds the cords (Skinner et al, 1985; Tung and Fritz, 1986). Until recently, it was believed that the PTM cells were among the migrating cells that enter the gonad from the mesonephros (Buehr et al, 1993; Merchant-Larios et al, 1993; Martineau et al, 1997; Campagnolo et al, 2001). Recent findings, however, suggest that the migrating mesonephric cells do not contribute to the PTM population and that this cell type probably originates from within the gonad itself (Cool et al, 2008; Combes et al, 2009). Studying the origin and development of PTMs has been challenging because no PTM-specific marker currently exists (Jeanes et al, 2005).

Interstitialium

Leydig cells

Leydig cells produce hormones that are essential for spermatogenesis and virilization. The expression of steroid biosynthesis pathway enzymes can be detected by E13.0 in the mouse testis (Baker et al, 1999). Leydig cells are located in the interstitium between testis cords and are in close association with blood vessels. The tissue origin of Leydig cells remains elusive, and precursor tissues have been suggested to include the genital ridge, mesonephros, coelomic epithelium and migrating neural crest cells (Griswold and Behringer, 2009). Fate mapping studies will be required to clarify the tissue origin of Leydig cells.

Vasculature

Testis cord formation is preceded and dependent upon sex-specific mesenchymal cell migration from the mesonephros (Buehr et al, 1993; Merchant-Larios et al, 1993; Martineau et al, 1997; Tilmann and Capel, 1999). Although the identity and fate of the entire migrating cell population is questionable, one of the cell types that migrate into the testis from the mesonephros is vascular endothelial cells (Martineau et al, 1997; Brennan et al, 2002). Indeed, a recent study suggests that the migrating mesonephric cell population is predominantly comprised of endothelial cells (Combes et al, 2009). In the fetal testis, a unique mechanism exists through which individual endothelial cells originate from the breakdown of an existing mesonephric vessel. The individual cells then migrate via specific paths to the coelomic region and remodel to form the coelomic vessel and smaller superficial vessels (Coveney et al, 2008). The coelomic vessel spans the entire length of the gonad just below the coelomic epithelium and is one of the hallmarks that distinguish the fetal testes from the ovary in the mouse.

Visualizing fetal testis differentiation

Fetal gonad culture

The XY urogenital ridge (genital ridge plus associated mesonephros) can be cultured in vitro for multiple days, resulting in the formation of testis cords and an interstitium. An early report by Byskov and Saxen described culture of embryonic mouse gonads from the eleventh day of pregnancy “glued” to Nucleopore filters using 1% agar. The filters were placed on grids to maintain the organs at the surface of Eagle’s balanced salt solution supplemented with 10% fetal calf serum in 5% CO₂. Testis cords were noted on the third day of culture so that male and female gonads could be distinguished (Byskov and Saxen, 1976). Subsequent studies have refined the culture system so that the current method of choice is to culture fetal gonads at an air-medium interface on agar blocks (Evans et al, 1982; McLaren and Buehr, 1990). Although these ex vivo cultured gonads are not identical to their in vivo counterparts (Evans et al, 1982), major developmental events such as cell differentiation, migration and testis cord formation take place to the extent that the cultured gonad’s developmental stage is more or less equivalent with in vivo controls (Buehr et al, 1993; Martineau et al, 1997; Coveney et al, 2008; Nel-Themaat et al, 2009). The ability to achieve gonad differentiation ex vivo allows for a variety of studies. Recombinant ex vivo culture experiments, for example, demonstrated the necessity of male-specific mesonephric cell migration for testis cord formation (Buehr et al, 1993; Martineau et al, 1997). The same approach was used to identify the migrating mesonephric cell type to endothelial cells, excluding peritubular myoid cells from this population (Cool et al, 2008; Combes et al, 2009). Importantly, for the purposes of this review, in vitro culture of the mouse urogenital ridge facilitates time-lapse imaging of testis differentiation and morphogenesis (Coveney et al., 2008; Nel-Themaat et al., 2009).

Transgenic mouse models for visualizing live cells in the developing testis

Histological analysis of fixed tissues combined with cell type-specific molecular markers provides important information about cell differentiation, the location of cells within the forming testis, and higher order structures like cords and the interstitium. Unfortunately, these techniques cannot be used

to follow live cell behaviors over time, including proliferation, apoptosis, cell shape changes, and migration. Transgenic mouse strains that express fluorescent proteins such as green fluorescent protein (GFP) under the control of cell-specific promoters provide important tools for visualizing live cell behaviors. Subsequently, a variety of different fluorescent protein colors and transgenic/knock-in mice that express them ubiquitously or in specific cell types have been generated (Hadjantonakis et al, 2003). Tissues from these mouse strains can be used to examine the behaviors of unique cell types or in combinations to follow potential interactions between different cell types as the testis develops. Existing transgenic mouse models that label specific testicular cells or tissues include those that express fluorescent proteins in germ cells (Anderson et al, 1999), interstitial and PTM cells (Cool et al, 2008), endothelial cells (Coveney et al, 2008; Larina et al, 2009) and Sertoli cells (Nel-Themaat et al, 2009) (**Fig. 1**), while lines that express fluorescent proteins ubiquitously are useful for tissue recombinant culture experiments (Combes et al, 2009). Fluorescent protein fusion proteins that result in their subcellular localization (e.g. nucleus or plasma membrane) provide further tools for following the behaviors of live cells (Hadjantonakis et al, 2003).

Time-lapse imaging of fetal testes

The advantage of a live imaging culture system is that the organs can be cultured intact while data is collected at specified time intervals to generate movies. The utility of such an approach was demonstrated using a Cre-*loxP* fate mapping system to produce mice that expressed GFP in endothelial cells. The resulting movies of fetal testis and ovary development showed endothelial cell behaviors, revealing different modes of vascularization of male and female gonads (Coveney et al, 2008). In addition, time-lapse imaging of *Sox9-GFP* knock-in mice that express GFP in Sertoli cells revealed the complex behavior of the Sertoli-germ cell mass during cord formation (Nel-Themaat et al, 2009) (**Fig. 1**).

Three-dimensional modeling of testis development

Despite very detailed and precise imaging techniques, determining the overall structure of the developing gonad is a difficult task because it requires three-dimensional (3D) reconstruction. Two recent publications that focus on testis cord formation included computational analyses to create 3D models of the forming fetal testis. Combes et al. (2009) used whole mount immunostaining and confocal microscopy to obtain optical sections through the entire fetal testis at different stages of development. Cord boundaries were manually defined and processed with the tomography program IMOD to create the final 3D model. The models of the fetal testis cords were stunning, providing a 3D appreciation of testis cord complexity at different stages of development. Nel-Themaat et al. (2009) reconstructed individual cords by serial paraffin sections, hematoxylin and eosin staining, and manual outlining of individual cords. Subsequent 3D reconstruction was performed by interpolation using a custom written MATLAB program. Experiments from both groups lead to similar models, revealing the complex 3D nature of fetal testis cords. These computer models allow one to view cord structures from any perspective and movies generated of these rotating models serve as excellent visual tools. Another approach is to create 3D models based on experimental findings, using rendering software. Here, we utilized the average morphometric data obtained from *Sox9-EGFP* fetal testes at different stages of development (Nel-Themaat et al., 2009) to create a 3D model of testis cord formation (**Fig. 2**). The data demonstrated that cord formation involves a complex series of tissue remodeling events. The evenly shaped, seemingly uniform mixture of somatic and germ cells of the indifferent gonad rapidly changes morphology to first form small protrusions distally of the mesonephros. These subsequently enlarge, become defined and develop into sex cords, while the initial number of primordial cords is reduced. Complex inner cord branching is common, as well as connections with adjacent cords. These findings were incorporated into the creation of our models and provided a new perspective of overall tissue behavior during this intricate process. The models can be used to generate animations to give us additional perspectives of the organ at different developmental stages (see Fig. 2 supplemental movie).

Summary and conclusions

Organogenesis is one of the most fascinating events of developmental biology. The ability to follow cells through their specification and differentiation while they migrate and form alliances with neighboring cells to form tissues, and then to determine how different tissue types come together and interact to form a functioning organ, is truly remarkable. The ability to culture and image the urogenital ridge *ex vivo* provides an excellent model organ for studying these events. Research over the last two decades has provided knowledge of different cell type origins, genetic regulation, and interactions and cellular dynamics of how the bipotential gonad develops into the embryonic testis. Some of the cellular mechanisms that regulate testis morphogenesis may also play a role in the development of other organs, such as the lung and kidney, which are also comprised of tubular structures and can be cultured *ex vivo*. It appears that many of the imaging and computational tools required to follow and analyze live cell behaviors during organogenesis are currently in hand and will likely yield new insights into testis morphogenesis in the near future.

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Figure legends

Fig. 1. Morphology of the fetal mouse testis. Whole mount urogenital ridges from XY *Sox9-EGFP* embryos at E11.5 to E13.5 shown in bright field and imaged for GFP fluorescence. Arrows indicate individual testis cords, while arrowheads show the coelomic vessel and vascular network at the gonad and mesonephric border.

g = gonad; m = mesonephros.

Fig. 2. Model of testis cord formation. The average morphometric values of testis development described by Nel-Themaat et al (2009) were used to generate 3D models of the Sertoli-germ cell mass and testicular cords at different stages of development. Gonad borders indicated by dashed lines. Scale bar = 300 μ m. The E13.5 gonad is the still image for the supplemental movie that is available online at www.andrologyjournal.org.



