Vascular Influence During Patterning and Differentiation of the Gonad

by

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Fan Wang

Dissertation submitted in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy in the Department of Cell Biology in the Graduate School
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ABSTRACT

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Abstract

The gonad is a unique primordial organ that retains the ability to adopt one of two morphological fates through much of mammalian embryonic development. Previous work in our lab found that dimorphic vascular remodeling was one of the earliest steps during sex-specific morphogenesis. In particular, vessels in XY gonads display highly ordered behavior that coincides with testis cord formation. It was unknown how the vasculature may influence testis cord morphogenesis and, if so, how this was mechanistically related to sex determination. The work in this thesis addresses a single over-arching hypothesis: Male-specific vascular remodeling is required for testis morphogenesis and orchestrates differentiation of the XY gonad.

To address this question we have modified and developed techniques that allow us to isolate aspects of vascular behavior, gene expression, and endothelial influence on surrounding cells. In particular, the application of live imaging was instrumental to understanding the behavior of various gonadal cell-types in relation to remodeling vessels. It is difficult to grasp the complexity of an organ without understanding the dynamics of its constituents. A critical aim of my work was to identify specific inhibitors of the vasculature that do not affect the early stages of sex determination. Combining inhibitors, live imaging, cell sorting, qRT-PCR, mouse models, and whole organ culture has led to a far richer understanding of how the vasculature behaves and the cell-types that mediate its influence on organ morphogenesis. The beauty of our system is that we do not have to settle for a snapshot of the fate of cells in vivo, but can document their journeys and their acquaintances along the way.

Vascular migration is required for testis cord morphogenesis. Specific inhibitors revealed that in the absence of vessels, testis cords do not form. The work below shows that vessels establish a feedback loop with mesenchymal cells that results in both
endothelial migration and subsequent mesenchymal proliferation. Interstitial control of testis morphogenesis is a new model within the field. The mechanisms regulating this process include Vegf mediated vascular remodeling, Pdgf induced proliferation, and Wnt repression of coordinated endothelial-mesenchymal dynamics. Our work also suggests that vascular patterning underlies testis patterning and, again, is mediated by signals within the interstitial space not within testis cords themselves.

A final aspect of my work has been focused on how vessels continue to influence morphology of the testis and the fate of surrounding cells. Jennifer Brennan, a graduate student in our lab, previously showed that loss of Pdgfra antagonizes cord formation and development of male-specific lineages. The mechanisms and cell-types related to this defect were not clear. I began to reanalyze Pdgfra mutants after finding remarkable similarity to gonads after vascular inhibition. This work is providing data suggesting that vessels are not simply responsible for testis morphology but also for the fate of specialized cells within the testis. On the whole, this thesis describes specific roles for endothelial cells during gonad development and mechanisms by which they are regulated.
Dedication

I would like to dedicate this dissertation to Dr. Robert Morris. He, among other faculty members at Wheaton College, was instrumental in exciting my curiosity and love of scientific research. Betsey Dyer, Mark LeBlanc, and Dr. Bob encouraged my inclination to go about things in a very non-linear way. They celebrated my wanderings and encouraged my curiosity and along the way, I fell in love with science. As a senior honors student with Dr. Bob he pushed me toward historical scientific literature, introduced me to a community of scientists, and helped me attend my first conferences. These were all eye-opening experiences that left lasting impressions.

After I left Wheaton my love of science persisted but I rarely considered applying to PhD programs. I felt it was overly ambitious and perceived that the myopic world of a PhD would collide with my general approach of painting with broad strokes. After a year working in litigation, it became clear to me that I wanted to get back to science. However, I thought it best to apply for masters programs. I remember clearly the day that I asked Dr. Bob for a recommendation - he was of course was happy to help, but insisted that I first explain the reasons that this was the right degree for me. He then went on to highlight his view of my general curiosity, love of the scientific community, and desire to explore in unstructured ways. He suggested that without doubts a PhD was the right choice. And I knew he was right.

As I wrote this dissertation, I reflected on how I got here and how much my view of science has changed. I never expected that I would become this passionate. I don’t know if Dr. Bob had a sense for my yet to be discovered excitement, although something tells me he did. For that I am forever grateful and dedicate this document to him.
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My family is amazing. Jesse Cool and Robert Cool, my mom and dad, are two of the most passionate people I know. Throughout their life they have worked hard at the things that they love. They taught me, frequently by force, the value of hard work and the importance of community. Both of them encouraged me to go about life in a way that I loved as opposed to one that made sense. The rest of my family follows a similar lead. It is fair to say that my brother always figured something was a little wrong with his younger brother. I can’t refute these claims, but I respect him so much and know that it is mutual.

Friends and colleagues make the graduate process tolerable. The Capel lab is an amazing place to live. All of the members of the Capel lab generate an environment that is clearly the exception and not the rule. It is fun to come to work and even more fun to stay late and talk science. Most of my time in the lab overlapped with Matt Cook,
Lindsey Barske (now Mork), Jordan Batchvarov, and Steve Munger. They put up with me, which is more than I could have asked. I overlapped with Doug Coveney for only six months but it was an influential period of time – I fondly remember many of our conversations. Additionally, all the time with Leo, Yuna, Hao, Danielle, Tony, and Samantha have been wonderful. The newest batch, including Anirudh, Jason, and Mike were a shot of life and I can’t wait to see how the lab continues to grow. Outside of lab, my classmate Josh W. Ziel is an inspiration. He is one of the sharpest men I know and the years of running, drinking bourbon, and cooking inspired many conversations and helped me grow as a scientist.

Luckily my life did not stop at the lab. Wenny Wiggly, Kevin Magill, Schuyler Brown, and Eliza Maclean consistently helped me take breaths and enjoy so many wonderful events. Countless bike rides, shared meals, time at farmers markets, and concerts gave Durham life. The last 6 years of my life were not simply academically rich but rife with good times and this group made it consistently possible. I will be sad to part with North Carolina because of them.

My final paragraph has to be for Steve. He somehow has managed to sit back-to-back with me for 6 years and still maintain a friendship. Steve is one of the most loyal, attentive, and levelheaded men I know. He has been a constant source of sound advice and encouragement in and out of lab, although I have to be careful to take his advice out of lab. Steve inspires me to maintain balance and reminds what it means to be there for people. He opened his house and family to me in a way that I will always remember and will never fully repay.
1. Introduction

1.1 Primary Sex Determination and the Initiation of Divergent Morphogenesis

In the mouse, the gonads arise at E10.0 as a thickening of the epithelium on the ventro-medial surfaces of the paired tissues on either side of the midline known as the mesonephroi. The bipotential primordium continues to thicken until it is approximately six cell layers thick at embryonic day (E)11.0. An epithelium lines the coelomic surface of the gonad while the remaining cells reside below the surface without apparent organization or orientation. Primordial germ cells (PGCs), perhaps the most studied cell type in the gonad, do not originate in the mammalian gonad. PGCs are specified in the epiblast as early as E6.25 and migrate through the hindgut into the mesonephros. The PGCs first arrive in the gonad around E10.0 where they intermingle with undifferentiated somatic cells (McLaren, 2003).

 Shortly after the arrival of the PGCs, sex determination occurs. The SOX family transcription factor Sry is first detected around E10.5 in somatic cells of the XY gonad. The pattern of Sry expression resembles a wave emanating from the center of the gonad and extending toward the poles (Bullejos and Koopman, 2001; Koopman et al., 1990). Sry expression peaks at E11.5 and is no longer detectable by E12.5 (Hacker et al., 1995). The so-called “bipotential window” extends from E10.0 to E12.5 and denotes the period of mouse development during which the fate of the gonad is determined and sex-specific morphogenesis occurs. Sry lies upstream of all known male factors and morphogenetic programs, indicating that it is a master regulator of testis development in mammals. Transgenic expression of Sry in XX primordia results in full female-to-male sex reversal (Koopman et al., 1991).
Sry initiates Sertoli cell differentiation and male morphogenesis. Attempts to determine the binding and functional interactions of SRY with DNA have met little success (Canning and Lovell-Badge, 2002). Our current understanding is limited to the observation that SRY bends DNA in vitro and fulfills a strict requirement for male development in vivo through activation of its only known target, Sox9. SRY interacts with DNA through a high mobility group (HMG) domain that recognizes sharp bends in DNA and a consensus sequence found in many promoter regions throughout the genome (Ferrari et al., 1992). This is a common feature of all Sox family transcription factors. Mutations in the HMG box of Sry are frequently the cause of human male-to-female sex reversal (Harley et al., 1992), reflecting the importance of SRY’s ability to bind DNA (Pontiggia et al., 1994). SRY binds the Sox9 promoter directly and many believe that Sox9 is the sole target of SRY (Lovell-Badge et al., 2002; Sekido and Lovell-Badge, 2008).

SOX9 localizes to the nuclei of Sertoli cells and is absent from XX gonads by E12.5 (Kent et al., 1996; Morais da Silva et al., 1996; Sekido et al., 2004). Like Sry, Sox9 is also required for testis morphogenesis (Wagner et al., 1994). Induced expression of Sox9 rescues testis development when Sry is mutated, and Sox9 is sufficient for complete female-to-male sex reversal when misexpressed in XX gonads (Bishop et al., 2000; Qin and Bishop, 2005; Vidal et al., 2001). Mutation of Sox9 derails the male program regardless of Sry expression. Expression of Sry and Sox9 in Sertoli progenitors is the foundation of a cell autonomous program required for male development.

Although Sry and Sox9 expression is restricted to Sertoli precursors, sex determination is not purely an autonomous program within a subset of somatic cells. Chimeric mice composed of XX and XY cells demonstrated that paracrine signaling is capable of reinforcing either the male or female fate in the gonad. Many experiments
suggest that a threshold level of male signals or a threshold number of Sertoli cells is necessary to establish the testis fate. When the somatic cells of a chimeric gonad are predominantly XY cells, XX cells are recruited to express Sertoli markers (Palmer and Burgoyne, 1991). Reciprocally, when the gonad contains >70% XX cells, XY cells fail to maintain Sertoli markers and instead begin to express markers of the female pathway.

By E12.5, Sry expression is no longer detected. However, subsets of genes, transcribed at basal levels in the bipotential gonad, undergo a monumental shift in expression profiles. Expression of male pathway genes, such as Sox9 and Fgf9, persists in male primordia. Female pathway genes that are basally expressed at E11.5, such as Wnt4, persist only in female primordia that have not seen Sry. Mutation of Wnt4 or another associated gene, R-spondin1 (Rspo1) results in partial female-to-male sex reversal. The common phenotype of these mutants is the development of a coelomic vessel and ectopic expression of critical male factors such as Sox9 and Fgf9. Female-to-male sex reversal suggests that active repression of male signaling is critical for ovarian development. Both Wnt4 and Rspo1 encode secreted proteins that are thought to function through canonical beta-catenin and T-cell factor (Tcf) signaling. The preponderance of secreted growth factors raises the question of how various signals converge on the various gonadal cell-types and collectively direct differentiation.

Between E11.5 and E12.5, expression profiles shift and reflect a developmental and morphological decision. In females, WNT4- and RSPO1-mediated beta-catenin signaling appears necessary to repress male signaling and development (Chassot et al., 2008; Maatouk et al., 2008; Parma et al., 2006). Reciprocally, in the male, FGF9 and SOX9 repress a commitment to the female fate (Kim et al., 2006). The decision to commit to one fate at the expense of the other is reflected by a shift from nonspecific expression of key factors to their sex-specific expression and the upregulation of downstream testis or
ovarian pathways. Mutual antagonism appears to create a situation in which the fate decision is reinforced by feed-forward signaling that drives rapid commitment of the once plastic organ.

Factors expressed early during primary sex determination control subsequent morphogenesis of the respective organs, but little is known about the discrete steps or cell-cell interactions that are ultimately involved in morphogenesis. This thesis will focus specifically on male development and the de novo formation of testis cords. In the following introduction, I will attempt to familiarize the reader with what is known about the early events of testis cord morphogenesis including cellular interactions and pathways known to influence the process.

1.2 De novo Testis Cord Morphogenesis

1.2.1 Cell lineages within the XY gonad

Commitment to the testis pathway extends much further than the simple expression of Sry and Sox9. More important for my work, morphogenesis of the testis involves various cell-types with different identities and origins. The gonad originates as a thickening of the intermediate mesoderm along the coelomic surface of the mesonephros. This nascent field of cells expands by somatic proliferation. By E10.5 the growth of the gonad yields a population of somatic mesenchyme that resides under the coelomic epithelium. Currently it is thought that the epithelium undergoes some rate of asymmetric division and leaves non-epithelial cells behind, as it grows outward. All of these early somatic cells are positive for progenitor markers such as WT1, SF1, POD1 (TCF21), and LHX9. Mutation of these early somatic markers blocks formation of the gonad primordium (Luo et al., 1994; Pelletier et al., 1991; Birk et al., 2000; Cui et al., 2004). Between E10.0 and E10.5 microvascular sprouts invade this field of cells and
provide circulation from a large vascular bed that sits along the gonad/meseonephros border. The possibility that somatic cells enter from the mesonephros very early (before E11.0) has not been addressed.

Starting around E10.0, germ cells begin to populate the gonad after their migration from the hindgut. Within the gonad, the germ-line proliferates prior to sex determination. In mammals, no portion of the germ line originates from cells within the gonad or neighboring mesonephros. Unlike some organisms, germ cells do not influence morphogenesis of the gonad (DeFalco and Capel, 2009). Ablation of the germ line does not disrupt testis cord morphogenesis. Interestingly, one experiment showed that meiotic XX germ cells antagonize testis cord formation when mixed with XY somatic cells (Yao et al., 2003). Germ cells are no doubt amazing but in the context of how testis cord morphogenesis is regulated, they are dispensable and I will largely omit additional reference to them.

As noted above, the tipping point for testis morphogenesis is the expression of Sry in a subset of the early gonadal somatic cells. Sertoli precursors are those somatic cells that express Sry and subsequently Sox9. Sertoli cells are believed to arise exclusively from the coelomic epithelium (Schmahl et al., 2000). Dye labeling experiments revealed that divisions within the coelomic epithelium prior to 18 tail somites (E11.5) give rise to Sertoli cells (Karl and Capel, 1998). These cells then exit the coelomic domain and are scattered throughout XY gonads until they epithelialize and form testis cord structures.

XY somatic cells that are excluded from testis cords constitute the interstitium. Non-Sry expressing gonadal cells are one source of interstitial mesenchyme. After 18ts, divisions in the coelomic domain exclusively give rise to mesenchymal cells (Schmahl et al., 2000). Once in the gonad, interstitial mesenchyme is highly proliferative and remains
largely undifferentiated through development. It is possible that a subpopulation of mesenchymal progenitors enters the gonad from the mesonephros or another source. Various ideas on the origin of the gonadal mesenchyme constitute a topic of increasing debate and will require definitive lineage tracing experiments.

After Sertoli and germ cells segregate from the interstitium during cord formation, several specialized cell types arise within the mesenchyme. Fetal Leydig cells, the male-specific neuroendocrine cell, arise at least in part from divisions in the coelomic domain. Steroidogenically active mature fetal Leydig cells can be detected after E12.5. Vascular cell adhesion molecule (VCAM1)-positive cells initially associated with vascular sprouts may also be a source of Leydig cells. However, origins for Leydig cells remain highly controversial within the field. The progenitors of Leydig cells are also believed to reside in the interstitium, although specifics of their identity and regulation are poorly understood. Peritubular myoid cells also develop from the mesenchyme and are morphologically distinct by E13.5. In the adult these cells act to enable constriction of the seminiferous tubules.

Other than mesenchymal cells, the major cellular component of the fetal interstitium is endothelial cells. Before E11.5, endothelial cells form small vascular sprouts that branch into the gonad from a large vascular bed in the mesonephros. In males, this vasculature remodels shortly after sex-determination and adopts a highly dimorphic structure (Brennan et al., 2002). One major hallmark of early male morphogenesis is the development of a large artery that sits just below the coelomic surface and is thus termed the coelomic vessel (Brennan et al., 2002). In the XY gonad, vessels run throughout the interstitial space and separate adjacent testis cords. Endothelial cells do not arise from the coelomic domain and exclusively migrate from the mesonephros.
1.2.2 Influence of various cell-types on cord morphogenesis

Seminiferous tubules do not form by budding or the invagination of an existing epithelium, as is typical during tube formation. Instead testis cords form de novo from somatic cells within the gonad. In less than 24 hours in the mouse, interspersed Sertoli cells form an epithelium around germ cells and become a segregated compartment distinct from the interstitium. Many of the initial experiments aimed at understanding testis cord morphogenesis utilized an in vitro model of Sertoli cell aggregation. This in vitro system, developed in Irving Fritz’s lab, used dissociated adult rat testes in order to isolate and expand individual fractions of gonadal cells (Fig. 1). Density gradient centrifugation of dissociated cells separated the various cell-types based on physical properties. Most importantly, this approach generated a fairly pure Sertoli cell fraction (Tung and Fritz, 1980). Interestingly, once in culture Sertoli cells expand as a monolayer and do not aggregate or exhibit behavior reminiscent of testis cord formation. This simple experiment clearly demonstrates that non-autonomous influences are required to initiate epithelialization into tubules. Co-culture experiments introduced various cellular fractions and hormones from the gonad back to pure Sertoli cells in order to determine the conditions that induced aggregation. When interstitial cells were added to Sertoli cells, “nodules” formed and mimicked several morphological hallmarks of testis cord formation (Fig. 1). In particular, interstitial cells surrounded epithelial balls of Sertoli cells with an intervening layer of extracellular matrix (Tung and Fritz, 1980). When the two fractions of cells were cultured together but separated by a filter, aggregation failed, suggesting that cell-cell communication is critical (Skinner et al., 1985). Based on the morphology and localization of the interstitial cells, the
prevailing model posited that peritubular myoid ("PTM") cells contact Sertoli cells and induce testis cord aggregation.

Using slight variations on the in vitro aggregation model, several groups began to test mechanisms that regulate PTM and Sertoli cell cooperation. Extracellular matrix (ECM) became a focal point of cord formation based on these early experiments. A functional role for ECM deposition remains intriguing due to several pieces of data. First, the general characterization of the testis reveals a thick basement membrane that separates Sertoli cells from the interstitial space. Second, Sertoli cells localize on the inner face of this matrix and polarize in relation to it. Lastly, either cell-type alone fails to deposit ECM in monoculture. ECM deposition becomes robust when the two cell types are allowed to interact, highlighting the correlation between ECM deposition and cord formation (Skinner et al., 1985; Tung and Fritz, 1986).

One illustrative study combined basement membrane from seminiferous tubules with Sertoli cells in suspension culture (Enders et al., 1986). Amazingly, Sertoli cells bound the cut surface or the inner tubule, but not regions of the ECM that would normally be exposed to the interstitium. Furthermore, Sertoli cells displayed no preference for vascular derived matrix, which was periodically captured during ECM isolation (Enders et al., 1986). In both cases, fibroblasts associate with ECM promiscuously. Thus, the ECM surrounding testis cords is functionally asymmetric and may be capable of providing a spatially instructive scaffold during de novo testis cord morphogenesis. These experiments do not, however, provide any evidence for how asymmetric assembly of the structure occurs or whether this is a primary mechanism in vivo.

ECM constituents are preferentially expressed by specific cell-types. Sertoli cells express multiple ECM components, including high levels of Collagen IV and Laminin
(Skinner et al., 1985; Tung et al., 1984). In addition to Collagen IV, interstitial mesenchyme produces high levels of Collagen I and Fibronectin. However, the cells used in these experiments were from adults and while retaining the capacity to undergo de novo reaggregation, they may not utilize the same transcriptional programs or mechanisms as the fetal testis. Microarray analysis on sorted fetal gonadal cells confirmed the expression of the above factors but also identified a large number of additional candidates.
Whole adult testes were dissociated and centrifuged to separate various cellular fractions. Various fractions were then recombined to determine cells and conditions that promoted the aggregation of Sertoli cells (Tung and Fritz, 1980; Skinner et al., 1985).
However, gene expression alone will not clarify the composition of the ECM or its specialized functional attributes. When Sertoli or PTM cells are cultured separately, ECM constituents are expressed but remain intracellular. Only after the cells are allowed to interact in co-culture do ECM components localize to the extracellular space and form fibrils (Skinner et al., 1985). Further, the ratio of Sertoli/PTM cells modulates ECM modification through variable secretion of MMPs, proteases, and collagenases (Sang et al., 1990). Thus, we must understand the interactions among various cell lineages that enable the construction of functionally heterogeneous basement membrane along with how that matrix is subsequently modified by additional cell types.

To clarify how ECM may influence gonad development and testis cord morphogenesis, in vitro cultures and eventually organ explants were treated with a variety of inhibitors. Comparing the morphology of cells before and after testis cord formation reflects the development and specialization of the tissue as a whole: Sertoli cells become polarized and a subset of interstitial cells adopt a squamous and adherent morphology along the ECM. Addition of ECM to gonads with reduced Sox9 expression enhances testis cord morphogenesis and suggests that matrix interactions reinforce male morphology (Matoba et al., 2008). In culture, these morphological changes are inhibited either by treatment with excess cAMP analogs or inhibitors of the cytoskeleton (Taketo et al., 1984). Blocking morphological changes correlates with a failure during ECM deposition and cord formation (Tung and Fritz, 1987). In peritubular monocultures, the addition of btcAMP induces a rounded morphology. Recent work from various fields shows that cell shape can be a primary factor influencing cell-fate and gene expression (Connelly et al., 2010; Nelson et al., 2008). Whether the changes in cell shape are primary or secondary to cord formation is unclear. Although cell shape was not implicated, persistent Sox9 expression is enhanced by ECM interactions [Matoba, 2008]. Analysis of
cells in the fetal gonad may reveal that adhesion between cell types induces cell shape changes and, as a direct consequence, induces differentiation of these cells and deposition of the ECM. Preliminary work on this topic will be presented in chapter 6.

Cell culture models of testis cord morphogenesis offered clues as to how the mesenchyme induces Sertoli cells to encase the germ line. A critical question that remained was how the process was initiated in vivo. Reconstitution of adult cells does not explain the mechanisms that initiate cord formation and may not even be reflective of the cells present during the precipitating event.

A seminal experiment, nearly 15 years after the in vitro experiments, found that cell migration into the gonad was sex-specific. In an elegant series of experiments, transgenic mesonephroi were recombined with unlabeled gonads of various genotypes just prior to sex determination (E11.5). In wild-type tissue, cells entered XY gonads and were completely absent from XX primordia (Martineau et al., 1997). The sex of the mesonephros does not affect migration. Subsequent experiments showed that Sry expression in XX gonads induces cell migration coincident with testis cord formation. More convincingly, when a filter was placed between an XY gonad and mesonephros, migration and cord formation were blocked (Tilmann and Capel, 1999). Similar to in vitro experiments in which Sertoli and mesenchymal cells were separated, this experiment established the requirement for cell-cell interactions as opposed to a diffusible factor. Fetal gonad recombinations clarified that cell migration from the mesonephros initiates testis cord formation in vivo.

The identity of migrating cells was not immediately clear. Histological sections of recombination cultures found that migrating cells reside within the interstitium and never in testis cords (Tilmann and Capel, 1999). Labeled cells were scattered throughout the interstitium and frequently close to the border of testis cords. Moreover, they had a
squamous and elongated morphology. Morphologically, these migrating cells appeared to be PTM cells. Subsequent recombinations with GFP-labeled mesonephroi used immunocytochemistry to show that many migrating cells were PECAM1 (CD31) positive endothelial cells. Migrating GFP-positive cells that were PECAM1-negative were presumed to be PTM cells, although a positive identification was not possible and PECAM1-negative cells were scarce. Clarifying the identity of cells that enter the XY gonad remained a pivotal question for several years and is the focus of chapter 2.
1.3 The Relationship of Vessels to Testis Cords

Could the requirement for, and perhaps identity of, the squamous cells shown to be required in culture, be fulfilled by endothelial cells? This question is the starting point for this thesis. Prior to my joining the lab, Doug Coveney adapted our organ culture protocol to include real-time confocal microscopy. Live-imaging opened a door allowing us to directly observe how cells behave during cord formation. Live-imaging of endothelial cell migration revealed that individual cells flood into XY gonads. Although this was known from recombination experiments, the dynamics of cell migration were well beyond the imaginable. XY gonads specifically induce the breakdown of the large vascular bed in the mesonephros. After vessel dissolution, individual endothelial cells migrate in a highly directed fashion toward the coelomic epithelium. Individual cells then coalesce into a large vessel that runs below the coelomic surface of the XY gonad. These observations established a novel mechanism of vascular remodeling distinct from vasculogenesis and angiogenesis (Coveney et al., 2008). By E12.5, flow is completely diverted to the large arterial coelomic vessel and microvascular branches extend from it into the gonad to separate newly formed testis cords. Essentially, we proposed that this process occurs in three steps: (1) the male-specific breakdown of the mesonephric vascular bed leading to (2) recruitment of endothelial cells to the coelomic domain and finally (3) branching of the coelomic vessel to establish vessels between testis cords (Fig. 2). All of these events are repressed in XX gonads.
Characterization suggests that at least 3 critical events occur that promote male-specific remodeling and artery formation. First, the large vascular bed dissociates specifically in XY gonads. Second, endothelial cells enter XY gonads and appear to respond to a strong chemoattractant in the coelomic domain. Third, branches form from the newly formed coelomic vessel and appear to pattern the aggregation of testis cords. Adapted from Coveney et al., 2008.
Endothelial migration from the mesonephros is confined to tracts within the gonad (Coveney et al., 2008). Live-imaging revealed that shortly after E11.5 a small number of endothelial cells enter the gonad and extend long cellular processes toward the coelomic domain (Fig. 3, top row). The cellular protrusions of these initial cells appear to sense something that directs the cells into the gonad in a highly organized manner. Subsequent cells follow the paths pioneered by prior cells. It is possible that the initial pioneer cells define the tracts, but plenty of alternatives exist. It is a provocative, (but unfounded), idea that migration tracks are molecularly distinct prior to migration and initial cells are following a preordained route. Regardless, endothelial migration demarcates presumptive testis cord domains prior to the aggregation of Sertoli cells. Two-color imaging of Sertoli and endothelial cells further clarified this point by integrating the real-time behavior among the cells types (Fig. 3). Two color movies confirm that Sertoli cells are initially spread throughout the gonad and separate at points where vessels reside (Fig. 3). Characterizing endothelial migration lead to the hypothesis that endothelial cells may contact or directly influence Sertoli aggregation and cord formation.
Figure 3: Two color live imaging of Flk1-mCHERRY and Sox9-ECFP.

Live markers of both endothelial cells (Flk1) and Sertoli cells (Sox9) confirmed that endothelial migration and vessel formation precedes Sertoli cell aggregation and looping.
1.4 Growth Factor Families Implicated in Cord Formation and Cell Migration

1.4.1 Fgf

Male sex determination requires Fgf9. At E11.5, Fgf9 is expressed in both XX and XY gonads, but Fgf9 expression is lost in the XX and enriched in the XY gonad shortly after Sry is expressed (Kim et al., 2006). Loss of Fgf9 in XY gonads results in phenotypic male-to-female sex reversal (Colvin et al., 2001). Surprisingly, sex reversal was not 100% penetrant on all genetic backgrounds, with a portion of the XY Fgf9 mutants developing some testicular structures. Characterization of Fgf9-null mice showed that Fgf9 is required for Sertoli cell differentiation, male-specific proliferation, and cell migration (Colvin et al., 2001; Schmahl et al., 2004). Based on this data, Fgf9 is generally thought to operate near the top of the sex determination cascade.

To achieve its diverse functions during sex determination, FGF9 may act both as a paracrine and autocrine factor. Nonautonomous signals promoting the adoption of Sertoli identity were previously based on using XX/XY chimera experiments. Consistent with this prediction, XX cells or intact organs that are treated with exogenous FGF9 fail to maintain female gene expression profiles and upregulate Sox9 (Kim et al., 2006). In treated organs, cells migrate from the mesonephros into the gonad, an early hallmark of the testis pathway and the most obvious evidence for nonautonomous signaling in the testis (Colvin et al., 2001). These culture experiments demonstrate that FGF9 can act nonautonomously to reinforce the commitment of somatic cells to the Sertoli cell fate and initiate male morphogenesis in other cell types.

Fgf9 is required for Sertoli cell commitment in the XY gonad. Fgf9 and Sox9 are expressed at low levels in the bipotential gonad until after Sry expression, when they become male-specific and enriched specifically in Sertoli progenitors (Kent et al., 1996;
Kim et al., 2006; Sekido et al., 2004). Loss of Fgf9 does not affect the initial expression of Sox9 or Sry in Sertoli precursors, although, without Fgf9, SOX9 levels gradually disappear. However, the relationship is not linear. SOX9 is required to initiate Fgf9 expression, suggesting that the two genes engage in an Fgf9/Sox9 feed-forward loop to drive the onset of the male pathway (Colvin et al., 2001; Kim et al., 2006). It is still unclear how FGF9 and SOX9 are mechanistically related, although their genetic relationship clearly lies at the heart of Sertoli cell specification (Kim and Capel, 2006).

Although Fgf9 is important for promoting the male pathway, it also represses the female fate and is itself repressed by a number of female factors. Somatic cells specified to become Sertoli cells do so at the expense of adopting the female granulosa cell fate (Albrecht and Eicher, 2001). Male versus female somatic identity is achieved through both positive and negative signals. Wnt4 is one of a few known factors that promote ovarian development and, when mutated, results in partial sex reversal (Jeays-Ward et al., 2003; Vainio et al., 1999). Male development in XX Wnt4−/− mice is likely a consequence of depressing Sox9 and Fgf9 (Kim et al., 2006). Similarly, XY Fgf9−/− mice fail to repress Wnt4, causing male to female sex reversal. Similar results in culture showed that treatment of XX cells and gonads with exogenous FGF9 represses Wnt4 expression. Collectively these results revealed that Fgf9 and Sox9 form a genetic feed-forward loop that stabilizes male development at the expense of the Wnt4-mediated female pathway (Kim et al., 2006).

FGFR1, FGFR2, FGFR3, and FGFR4 are present in both XX and XY gonadal cells. Fgfr1, Fgfr3, and Fgfr4 are expressed in a non-sex-specific pattern in somatic cells as well as germ cells (Schmahl et al., 2004). Fgfr2 is expressed in the cells of the coelomic epithelium of both XX and XY gonads. However, FGFR2 also localizes to the nucleus of a subset of XY, but not XX, cells beginning soon after E10.0 (Kim et al., 2007). These cells
are found within the gonad and are likely Sertoli progenitors that migrate from the coelomic epithelium into the gonad as they transition to differentiated Sertoli cells [Karl et al., 2004]. Nuclear accumulation of growth factor receptors is a documented but poorly characterized process. In other systems nuclear receptors modulate gene expression, participate in splicing, or function as nuclear kinases (Wells and Marti, 2002). Accordingly, FGFR2 nuclear localization is a possible mechanism by which this receptor could mediate Sertoli cell specification while performing different roles in other cell types in the gonad (Kim et al., 2007). However, the mechanics of nuclear translocation and the pathways activated by FGFR2 nuclear accumulation have not been clarified.

FGFR2 appears to be the receptor through which FGF9 acts in the gonad because loss of Fgfr2 phenocopies gonadal defects in Fgf9 mutants. When Fgfr2 was conditionally deleted in the gonad, using two Cre lines, the sex-reversing phenotypes were variable but recapitulated the major hallmarks of Fgf9 mutants (Bagheri-Fam et al., 2008; Kim et al., 2007). Specific deletion in Sertoli progenitors abrogates their proliferation and differentiation, but some aspects of the testis program, including limited mesonephric cell migration into XY gonads and formation of the XY coelomic vessel, still occur (Kim et al., 2007). In contrast, a complete temporal deletion of Fgfr2 at E11.0 blocks migration as well as all other aspects of testis development. This begs the question of whether Fgf signaling is responsible for vascular recruitment.

Presently, intracellular effectors downstream of FGF9 signaling in the gonad are unknown. Some evidence indicates that Sprouty proteins are active in the gonad and could participate in regulated cell migration (Chi et al., 2006). The Sprouty family was identified in Drosophila and subsequently shown to negatively regulate RTK pathways mediated by factors such as Egf, Fgf, Vegf, and Pdgf (Hacohen et al., 1998; Reich et al., 1999; Sasaki et al., 2003). Mechanistically, Sprouty’s antagonize RAF-mediated
extracellular signal-regulated kinase (ERK) activation (Sasaki et al., 2003). Sprouty2 is expressed in the coelomic epithelium, the destination of many migrating endothelial cells that enter the gonad. Overexpression of Sprouty2 reduces cell migration into the gonad (Chi et al., 2006). Although Chi et al. conclude this is an effect on Fgf signaling, other RTK pathways influence migration into the testis (Brennan et al., 2003). A function for Sprouty proteins is difficult to reconcile because their expression seems to be specific to the male gonad, whereas their function inhibits male migration. Additionally, the levels of Sertoli markers is reduced, raising the possibility that migration is decreased because Sertoli differentiation – as well as the entire male program - is disrupted (Chi et al., 2006). This work does suggest that ERK signaling acts downstream of RTKs during Sertoli specification and cell migration in the testis. Unpublished data in our lab confirms that a general ERK inhibitor robustly antagonizes vascular migration and Sertoli differentiation, although neither of these experiments provide increased insight into the cell types or pathways that are being targeted.

1.4.2 Pdgf

Platelet derived growth factors (Pdgf) are diffusible extracellular ligands. In mammals, four PDGF ligands undergo either hetero- or homodimerization, and they interact with one of the two PDGF receptors (PDGFRα and PDGFRβ). Both Pdgfra and Pdgfrb are expressed in the XY gonad in cells throughout the interstitial mesenchyme. When PDGF-AA, PDGF-BB, or the heterodimer PDGF-AB are added to XX gonads, cell migration is induced and gonads display partial XX to XY sex reversal. Despite gain of function results, neither Pdgfa nor Pdgfb single mutants result in obvious embryonic dysgenesis (Brennan et al., 2003; Gnessi et al., 2000). However, XY Pdgfra mutants have defects in testis cord formation and interstitial development.
Analysis of Pdgfra mutants revealed defects in specification and patterning of interstitial cells and in formation of the testis vasculature (Brennan et al., 2003). The hallmark male coelomic vessel still forms, although its branching is disrupted. In Pdgfra mutants, large irregular testis cords coincide with mispatterned vasculature. Although the simple prediction from these results was that PDGF ligands signal to mesonephric cells to enter the gonad, tissue recombination experiments indicate that the requirement for the receptor is in gonadal and not mesonephric cells (Brennan et al., 2003). Despite the broad expression of Pdgfra in the mesonephros, mutant mesonephroi are still able to contribute cells to a wilde-type male gonad, whereas the converse experiment revealed mutant gonads are unable to recruit robust migration. These results suggest that Pdgfra in gonadal cells promotes a receptive environment for migrating cells and/or participates in signal transduction downstream of migration (Brennan et al., 2003).

The ligand that stimulates Pdgfra is unknown. Expression analysis of Pdgf ligands does not clarify how Pdgfra is activated. Pdgfa is specific to Sertoli cells. Pdgfb is generally expressed by endothelial cells. Lastly, Pdgfc is expressed by a minor population of cells at the gonad/mesonephros border (Brennan et al., 2003). As noted above, XY Pdgfa mutants are viable and have reduced levels of adult Leydig cells (Gnessi et al., 2000). However, the testis still forms and mutation of this ligand does not phenocopy the morphogenesis defects seen in Pdgfra mutants. Pdgfa/c double mutants have variable gonad phenotypes, although these mutants again do not fully recapitulate XY Pdgfra mutants (B.C, unpublished results). This raises the possibility that either the receptors (co-expressed by interstitial cells) are redundant or that various ligands converge on these cells and are responsible for eliciting discrete aspects of the Pdgfra mutant phenotype. I will revisit this topic in chapter 3.
In addition to vessel and cord patterning defects, *Pdgfra* mutants lack fetal Leydig cells (Brennan et al., 2003). This steroidogenic cell type is found in the interstitium of the testis and differentiates between E12.5 and E13.5 based on detection of steroidogenic enzymes. Leydig cells are known for their important role in testosterone secretion, which masculinizes the male reproductive tract and genitalia. However, they may have other active and unappreciated roles during morphogenesis of the fetal testis.

Proliferation of the mesenchyme is reduced in *Pdgfra* mutants, which may explain cord formation and Leydig cell development defects. However, whether the receptor is required for the development of specific interstitial lineages or general proliferation is unresolved.

The defects in PDGF mutants highlight the dynamic interactions among cord formation, interstitial patterning, and migration, but they do not provide a clear indication of which process is directly controlled by Pdgf signaling. Like most interstitial markers, *Pdgfra* expression is detected throughout the interstitium and is not specific to a particular lineage. However, disrupted morphogenesis and a lack of fetal Leydig cells frequently track together raising the possibility that continued signals between testis cords and interstitium are required for the differentiation and maintenance of the two compartments. It is likely that one phenotype is secondary to the other. Identifying additional cell specific markers and patterning mutants will establish direct relationships among cell-types and clarify how the interstitium is partitioned and how it generates the distinct lineages critical for testis physiology and function.

1.4.3 Wnt

Wnt proteins are a large class of small, secreted factors that signal through two primary pathways. The first, frequently termed “canonical Wnt signaling”, involves Wnt
binding to frizzled receptors at the membrane, which represses the intracellular degradation of cytoplasmic beta-catenin. Upon activation, and in the absence of its degradation, beta-catenin translocates from the cytoplasm to the nucleus to activate transcription via interactions with TCF/LEF transcription factors. Wnt signaling that excludes beta-catenin has been coined “non-canonical Wnt signaling” and frequently involves the intracellular effector, disheveled. Non-canonical Wnt signaling typically regulates cytoskeletal dynamics and polarity of responsive cells.

In the gonad, Wnt4 is critical for the early commitment to the female program (Jeays-Ward et al., 2003). When Wnt4 is mutated, XX gonads develop several structures that are normally observed only in XY gonads. In particular, XX Wnt4−/− gonads induce cell migration into the gonad, develop a vessel in the coelomic domain, and contain neuroendocrine cells that resemble Leydig cells (Jeays-Ward et al., 2003). Recent work showed that Wnt4 utilizes the canonical pathway, through beta-catenin, to exert its influence during sex determination (Maatouk et al., 2008).

Based on the presence of male-like vasculature in the mutants, Wnt4 is critical for actively repressing vascular migration. It is not clear whether Wnts actively signal to vessels to promote their stability, or if another cell-type is involved. Sertoli cells are not persistent in XX Wnt4 mutants, suggesting that the effect of Wnt signaling on migration may be direct. However, we do not know which cells are Wnt responsive, and this is a major roadblock toward understanding how Wnt4 influences gonad development. In the absence of these data, it is difficult to pin-point a clear model of how Wnt signaling directly or indirectly regulates the vascular bed at the gonad-mesonephros border. This is a great question for future work.
1.5 Vascular Influence During Organ Growth & Regeneration

A pair of seminal papers uncovered the role of vasculature during organ growth. Both papers were elegant experiments in which vascular development was genetically blocked and found to inhibit pancreatic and liver development respectively (Lammert et al., 2001; Matsumoto et al., 2001). Perhaps the most surprising aspect of this work was that the requirement for vessels was independent of circulation. These amazing results presented a paradigm shift from the view that blood vessels were little more than conduits for systemic nutrient delivery. While oxygen delivery remains a fascinating area of organ growth, the Zaret and Melton labs showed that endothelial cells provide signals required for tissue growth, which has been confirmed in other tissues. However, both studies failed to clarify the vascular-derived signal(s) responsible for mediating this effect.

Subsequent studies found that tissue-specific stem cells are associated with vessels in many tissues. Sally Temple’s lab demonstrated that neural stem cells remain undifferentiated when co-cultured with endothelial cells (Shen et al., 2004). Vascular smooth muscle cells do not maintain neural stem cells. Neural stem cell maintenance requires direct interaction with blood vessels to promote Notch signaling. Work in the pancreas found a slightly different “vascular niche” required specifically for beta-cells. Beta-cell differentiation is rescued when precursors are cultured with specific ECM components deposited by endothelial cells (Nikolova et al., 2006).

Work describing the vascular influence on progenitor cells shows that endothelial cells are critical during the growth of organs as well as the continued maintenance of tissue specific progenitor cells. Signals that mediate this effect remain very poorly understood. Subsequent studies have deleted vasculature to show that organ growth and regeneration are affected. However, very few of these reports have
gone on to describe a mechanism through which endothelial cells communicate with the tissue to exert their influence. Vascular stem cell niches in solid organs are even less well understood. How progenitors cells home to, or are retained, within a physical niche is a significant question with major therapeutic consequences. Chemokine signaling is one possibility that has been recently proposed, although additional studies are needed to determine how common this mechanism is (Kokovay et al., 2010). The neural stem cell and beta-cell niche are two carefully characterized and yet common aspects are tenuous. Significant work is being done with the hematopoietic niche, although extrapolating coherent models into potentially less dynamic solid tissue remains difficult. Future work will need to clarify endothelial-derived signals during organ development but also their influence on mature tissue maintenance and homeostasis. Experimental approaches to these questions are complicated by the importance of the circulatory system for oxygen and nutrient delivery during both development and adult life. Perturbation of the vasculature without significant secondary consequences requires careful consideration.
2. General Materials and Methods

2.1 Summary

**Live Imaging:** All imaging experiments were performed on a Zeiss LSM510 or LSM710 as previously described (Coveney et al., 2008). Z-stacks were collected every 10-15 minutes. All movies are maximum intensity projections at each time, unless otherwise noted.

**Organ Culture:** E11.5 genital ridges were cultured in 1.5% agar blocks at 37°C with 5% CO₂/95% air. Organs were cultured in Dulbecco’s Minimal Eagle Medium (DMEM) supplemented with 10% FBS and 50µg/ml of ampicillin (Martineau et al., 1997). BV13 (Kind gift of Elisabetta Dejana, Milan) was used at 24-48µg/ml as previously described (Combes et al., 2009; Corada et al., 1999). PDGF-BB, PDGF-AB, and PDGF-AA (R&D Biosystems, USA) was added to medium with a final concentration of 50ng/ml as previously described (Brennan et al., 2003). AMD3100 was used at a final concentration of 15ug/ml, including the volume of the agar block. When culturing, I habitually switch medium in the morning and evening, including flushing wells with fresh medium before draining them.

**Animals:** Vegfa<sup>LacZ</sup> mice, in which lacZ was inserted into the 3’ UTR of the Vegfa locus (Miquerol et al., 1999), were kindly provided by Victoria Bautch. Flk1-mCherry mice were generated by expressing the myristylated Cherry fluorophore with a fragment of the Flk1 promotor, and were generously provided by Dr. Mary Dickinson (Poche et al., 2009). αSma-EYFP mice (provided by Dr. James Lessard, Cincinnati) express EYFP controlled by a fragment of the αSma promotor. Sox9-ECFP mice were generated in the transgenic facility at DUMC and express CFP under the control of a
characterized Sox9 promoter fragment provided by Robin Lovell-Badge (Sekido and Lovell-Badge, 2008). Pdgfra-H2B mice were maintained on a C57Bl/6J background and crossed to CD-1 animals. All timed matings were between two F1 Gfp/+ parents. PlxnD1 mice were maintained on a mixed background and were generated in David Ginty’s lab (Gu et al., 2005). Timed matings were set-up between PlxnD1+/− males and females to generate homozygous embryos. Embryos were collected at E12.5 in the Ginty lab and then shipped after over-night fixation. Sema3E mice were maintained on a C57Bl/6J at Genentech by Marc Tessier-Lavigne’s lab. Timed matings were collected at E12.5, fixed, and then transported to Duke prior to antibody staining. All expression analysis and inhibitor injections were performed on outbred CD-1 mice (Charles River). Timed matings were checked daily for the presence of a vaginal plug. When plugs were detected in the morning, embryos were considered to be E0.5 at 12 noon. All experiments were conducted in accordance with the principles and procedures outlined in the NIH Guidelines for the Care and Use of Experimental Animals.

RT-PCR: Gonads were separated from the mesonephros and total RNA was extracted using TRIzol (Invitrogen, USA). Reverse transcription reactions were performed using the iScript cDNA kit (Biorad, USA). All reactions were run with gene specific primers and the following conditions: 30 cycles of 95°C for 30s, 62°C for 30s, and 72°C for 30s. Vegfa isoforms were analyzed using nested primers as previously described (Zhang et al., 2002). qRT PCR was performed on an ABI StepOnePlus instrument using Quantace mastermix (Bioline, USA). Cycling conditions were 40 cycles at 95°C for 30s, 59°C for 30s, and 72°C for 30s. Ct values were calculated by the ABI software with any signal over 35 cycles being disregarded. All primer sequences are included as Appendix A.
β-gal staining and Immunohistochemistry: All gonads were dissected and fixed in 4% paraformaldehyde-phosphate-buffered saline (PFA-PBS). Embryonic stages were determined by daily timed matings, with E0.5 being noon on the day that a vaginal plug was detected. Whole-mount β-gal staining was performed as previously described (Yao et al., 2002). Immunohistochemistry was performed on cryosections and whole-mount tissue as previously described (Brennan et al., 2002). Antibodies used were Rat anti PECAM-1 (BD-Pharmingen, USA - 1:500), Goat anti NRPI (R&D Biosystems, USA - 1:350), Rabbit anti SOX9 (Chemicon/Millipore, USA - 1:750), Rabbit anti 3β-HSD (kind gift of Ken-ichirou Morohashi, 1:500), Rabbit anti pHH3 (Cell Signaling, USA - 1:300), Rabbit anti Laminin (gift of Harold Erickson -1:1000), Goat anti VCAM1 (R&D Biosystems, USA, 1:1000). Cy2-, Cy3-, and Cy5- conjugated secondary antibodies (Jackson ImmunoResearch, USA) were used at a dilution of 1:500. Samples were mounted in DABCO as previously described (Karl and Capel, 1998) and imaged on a Zeiss LSM510 or LSM710 scanning confocal microscope. All images represent multiple planes from no fewer than 4 independent organs.

Statistics: All charts represent manual counts of somatic proliferation in the coelomic domain. We defined the coelomic domain as the region extending from the top of testis cords to the coelomic surface. Error bars indicate +/- SEM and significance was calculated using an independent student t-test with significance considered (p<=0.05).

FACS Sorting: Gonads were dissected at E12.5 and separated from the mesonephros. Organs from each litter were pooled and treated with 1X Trypsin-EDTA for 15 minutes. Trypsinized cells were further dissociated by manual pipetting before
being pelleted and washed in PBS. Prior to sorting, all samples were resuspended in PBS and passed through a cell strainer cap (BD Falcon, USA). Cells were sorted on a Cytomation MoFlo sorter (Duke Comprehensive Cancer Center FCSR). RNA was extracted from sorted cells as described above.

**Heart Injections:** 13-18 tail somite embryos were removed from the uterus and placed in PBS containing ions (Ca/Mg). The yolk sac of each embryo was carefully removed in an effort to avoid disrupting major blood vessels. Glass capillaries were either hand-pulled or pulled using an automated machine. Needles were front-loaded with rhodamine-lectin or a mixture of rhodamine-lectin and VEGF Trap (38ug/ul, aflibercept, Regeneron Pharmaceutical, Tarrytown, NY). Embryos were injected in the left ventricle while the heart was still beating. Injected embryos were allowed to rest for 15-20 minutes before dissection and whole gonad culture.

**Tissue Recombinations:** WT and aSma-EYFP mice were maintained on an outbred CD-1 background while EGFP (Tg(GFPU)5Nagy) mice were maintained on FVB. Mice were checked daily for vaginal plugs as described above. Amnion stains were used to determine the sex of the embryo by the detection of condensed sex chromatin bodies (Barr bodies) in XX individuals as described (Palmer and Burgoyne, 1991). All genotypes were confirmed by PCR. Whole genital ridges were removed and separated from sexed embryos. Once gonad-mesonephroi complexes were dissected out, the gonad was separated from the mesonephros and tissue was transferred using capillaries and a mouth pipette. After separation, WT XY gonads and transgenic meseonephroi were recombined on agar blocks as previously described (Martineau et al., 1997). All cultures were then incubated under previously described conditions for 24-48 hours.
In situ probes:

*Sema3E* and *PlxnD1* probes were provided by Fan Wang. *Sema3E* was generated in the Cheng lab and binds between nucleotide 2752-3138 of the coding sequence. The probe for PlxnD1 was generated in Marc Tessier-Lavigne’s lab.

Measurements:

Measurements were made in ImageJ by drawing lines between adjacent vessels. Line lengths were measured in pixels and compared as raw values. The resolution of all images was identical. Lines were drawn perpendicular to vessels to account for variation in gonad orientation or mounting.

Measurements of cells relative to vessels:

Single optical sections were used in which *Pdgfra* -H2B was labeled in addition to a vascular marker. A threshold of expression was set for the vascular channel such that the morphological structure of vessels was accurately reflected in a mask of the gonadal vessels. Masks were then dilated in Metamorph to include a desired distance from vessels. Binary masks were generated from the dilated distance and were used as an overlay for *Pdgfra*-H2b expression from the same optical section in order to determine which cells were within or outside the desired distance.

Cell shape analysis:

40X Z-stacks were collected for 2-3 pairs of *Pdgfra* mutant and littermate controls. From each individual, 4 non-overlapping optical sections were analyzed. Each section was imported into Metamorph and subjected to a 3x3x1 median filter. Images were then converted into 16-bit tiff files and analyzed using the count nuclei function.
All images were analyzed using identical parameters, which included a min/max nuclear diameter of 4/25 micros respectively. To help accurately identify individual nuclei, we utilized a detection algorithm that did not rely on threshold but relative changes in fluorescent intensity across the field. This value was set at 25% change for segmentation. After nuclei were segmented, each shape was manually checked. Manual correction was made, in the form of splitting or joining two shapes, when multiple nuclei were included in a single shape or several shapes comprised single nucleus. To the extent that nuclei detection did not reflect confocal images, the nucleus was excluded from analysis. After inspection, a binary image was generated for all shapes. Binary images were quantified in ImageJ using the “analyze particle” function. Cell measurements included the area of each object as well as the Ferets diameter, which is the longest possible line through the object. From original images, a small nucleus was selected and its area was deemed to be the minimum actual shape in the field. Any object that had an area smaller than this minimum value was discarded to prevent the inclusion of small specks or single pixels.

Cell shape was calculated assuming an elliptical shape. To determine the minor radius, we used ImageJ measurements of major radius (.5 x Ferets Diameter) and the area. Spherical index was then determined by dividing the major radius by the minor. Violin plots were generated in R and basic statistical analysis was performed in R.

3.1 Summary

Cell migration is one of the earliest events required for development of the testis. Migration occurs only in XY gonads downstream of Sry expression and is required for the subsequent epithelialization of testis cords. Based on organ culture and tissue recombination experiments detailed above, we and others speculated that peritubular myoid (PTM) cells were among the migratory cells and were likely the cell type required for cord formation. However, because no unique marker was found for PTM cells, their positive identification during or after migration remained unclear. \( \alpha \)-Smooth Muscle Actin (\( \alpha Sma \), a.k.a \( \text{Acta2} \)), a classic marker of adult PTM cells, is expressed broadly in testis interstitial cells at E12.5, and becomes highly enriched in PTM cells by E15.5–16.5. We used a novel transgenic line expressing EYFP under the control of an \( \alpha Sma \) promoter fragment to determine whether \( \alpha Sma \)-EYFP positive cells migrate into the gonad. Surprisingly, mesonephroi expressing \( \alpha Sma \)-EYFP do not contribute any EYFP positive cells to XY gonads when used as donors in recombination cultures. These results indicate that \( \alpha Sma \)-EYFP positive cells do not migrate into the gonad during the critical window of sex determination and cannot be the migrating cell type required for testis cord formation. Our results suggest that PTM cells, and most other interstitial lineages, with the exception of endothelial cells, are induced within the gonad or arrive at an earlier time. These experiments suggest that endothelial cells are the sole migrating cell type required for epithelialization of testis cords.
3.2 Introduction

The epithelialization of Sertoli cells and morphogenesis of seminiferous tubules has been a primary focus of the sex determination field for many years. Classic experiments initiated by Skinner et al. (1985) sought to identify any factors, hormones, or cell interactions that are required for morphogenesis of testis cords. A Sertoli cell fraction was isolated by dissociation and centrifugation of adult rat testes. Additional fractions and factors were added to the Sertoli cultures to identify conditions that increased epithelialization of Sertoli cells. Interestingly, the addition of purified stromal cells to cultures increased matrix deposition and the appearance of cord-like epithelial structures (Tung and Fritz, 1980; Skinner et al., 1985). The squamous shape of the stromal fraction suggested a myoid identity, although a positive identification was never made. A role for peritubular myoid cells (PTM) during Sertoli cell epithelialization was attractive because these cells line the outside of seminiferous tubules and could partner with Sertoli cells during testis cord formation.

Subsequently, organ culture tissue recombination experiments revealed a wave of sex specific cell migration from the mesonephros into the XY gonad, and established that cells entering the gonad are required for morphogenesis of seminiferous tubules (Buehr et al., 1993; Merchant-Larios et al., 1993; Tilmann and Capel, 1999). Histological sections of these organ recombinations showed that cells entering the gonad were squamous cells immediately adjacent to Sertoli cells (Martineau et al., 1997). In the context of the previous studies on adult and neonatal rodents, these studies led to the conclusion that PTM cells migrate into the male gonad where they are required for Sertoli cell epithelialization.

At the time of the initial migration experiments, few cell-specific markers were available to identify migrating populations. It was shown that most cells that enter the
testis are positive for the endothelial marker PECAM-1, consistent with the establishment of a de novo arterial network by E12.5 in the testis (Brennan et al., 2002). PECAM-1 negative cells were rare and were not identified by any specific marker. Most of these unidentified cells were located adjacent to cord boundaries and were assumed to be PTM cells based on earlier work (Martineau et al., 1997). Another group sorted migrating cells, and after a period of culture in vitro, reported that cultured cells gave rise to several lineages including PTM cells (Nishino et al., 2001). Nonetheless, previous efforts to identify a specific marker for PTM cells in vivo were unsuccessful (Jeanes et al., 2005), thus the question of whether these cells were actually part of the migrating population remained open.

In adult muscle, various actin isoforms are frequently specific to particular populations of myoid cells. However, during development many myoid markers are expressed across cell types and become specific subsequently. Antibody staining of αSMA (Acta2) showed that 2-3 days after sex determination, it is expressed throughout the interstitium and enriched in PTM cells in rodent testes (Palombi et al., 1992). However, during the fetal stages when migration can be assayed (E11.5--E13.5) αSma-EYFP is broadly expressed in the mesonephros and most interstitial cells of the testis. For this reason, antibodies were not informative when used on recombination assays to specifically identify PTM cells from among the migrating population.

Endothelial cells represented a large proportion of the migrating cell population. However, at the time of the original experiments, no function other than nutrient and gas exchange had been ascribed to the endothelium. Subsequently endothelial cells were shown to affect the development and differentiation of surrounding tissues independent of circulation (Lammert et al., 2001; Matsumoto et al., 2001). This raised the possibility that endothelial cells entering the testis from the mesonephros, rather than PTM cells,
are responsible for the induction of testis cords, which led us to readdress the question of whether PTM cells are part of the migrating population.

To address this question, we utilized the advantages of in vivo analysis with a new transgenic mouse line, which expresses EYFP under the control of the αSma promoter (αSma-EYFP). In this transgenic, EYFP is expressed brightly and broadly in the mesonephros and in most interstitial cells of the gonad, including PTM cells, from ~E12.5. We reasoned that the broad expression of αSma-EYFP could be advantageous. Even though αSma-EYFP is not specific to PTM cells, recombination cultures using a αSma-EYFP mesonephros and a wild type gonad would reveal whether any EYFP-positive interstitial cells (including PTM cells) migrate from the mesonephros into the gonad. We show that no αSma-EYFP positive cells migrate from the mesonephros during the period when migration is required for testis cord formation to occur.
3.3 Results

3.3.1 $\alpha$Sma-EYFP is expressed by peritubular myoid cells

To characterize the expression of the $\alpha$Sma-EYFP transgene in the testis, we examined gonads at stages between E11.5--E18.5 using confocal microscopy. $\alpha$Sma-EYFP was never seen within testis cords but was expressed in many interstitial cells of the testes (Fig. 4A). Expression was never seen in SOX9 positive cells (Fig. 4A'). Expression of the transgene did not result in developmental delays or altered fertility. Importantly for the purpose of this work, $\alpha$Sma-EYFP was not expressed in endothelial cells (Fig. 4A''), but was enriched in the squamous PTM cells adjacent to Sertoli cells at both E12.5 and E15.5 (Fig. 4B, arrowheads), and at later stages. Higher magnification images show EYFP enriched cells are immediately adjacent to the laminin rich ECM (extracellular matrix) surrounding testis cords confirming proper PTM localization (Fig. 4C).

$\alpha$Sma-EYFP shows clear expression in PTM and other interstitial cells and therefore serves as an effective marker for many cells in the interstitial compartment of the testis with the exception of endothelial cells. Expression of the EYFP protein in this transgenic model was robust and stable such that no antibodies were needed for its detection after fixation or during prolonged live imaging. This preliminary data suggested that $\alpha$Sma-EYFP would represent a robust marker for tissue recombination experiments designed to distinguish between the migration of endothelial cells and the migration of other interstitial cell types, including PTM cells.
Figure 4 (Page 38): αSma-EYFP labels a subset of interstitial cells including peritubular myoid cells.

(A) Z-section of E12.5 αSma-EYFP testes. (A’) EYFP is not expressed by SOX9-positive Sertoli cells (blue arrowhead). (A”) αSma-EYFP is specific to interstitial mesenchyme and is not expressed by PECAM1-positive endothelial cells (red arrowhead). (B) At early (E12.5) and late (E15.5) stages of testis development, EYFP is enriched in cells immediately adjacent to the laminin (red) rich extracellular matrix (arrowheads). (C) Magnification of a small portion of figure 4B, as outlined by the white box, shows αSma-EYFP (green) expressed in presumptive peritubular cells immediately adjacent to the ECM (red).
3.3.2 \( \alpha \text{Sma-EYFP} \) positive cells do not migrate from the mesonephros into XY gonads

Migration from the mesonephros into the XY gonad was originally detected using tissue recombinations. Recombinations are composed of wild-type gonads that are removed from the adjacent mesonephros and recombined with a mesonephros expressing a transgenic label (\( \beta \)-gal or EGFP). To assay general migration of mesonephric cells to the gonad, recombinations with a mesonephros expressing a ubiquitous marker were performed. To assay the migration of a single lineage, recombinations with mesonephroi expressing cell-specific markers were performed. Assays designed to detect endothelial migration revealed robust vascular migration into the gonad and confirmed the arterial identity of migrating cells (Brennan et al., 2002). To determine whether myoid cells migrate into the testis, we recombined \( \alpha \text{Sma-EYFP} \) mesonephroi with wild type CD-1 XY gonads. Recombinations were initiated at or prior to E11.5 and cultured for 24--36 hours.

In parallel with mesonephroi expressing \( \alpha \text{Sma-EYFP} \), a ubiquitous EGFP expressing transgenic line was used as a positive control. When recombined with XY gonads, cells from EGFP mesonephroi migrated into XY gonads as previously described (Fig. 5A-B). In contrast, when using \( \alpha \text{Sma-EYFP} \) mesonephroi as a donor, EYFP positive cells were never found in recombinations with CD-1 XY gonads (Fig. 5C-D). In \( \alpha \text{Sma-EYFP} \) recombinations, testis cords formed and PECAM-1 positive endothelial cells were still detected (Fig. 5C-D, arrowheads) suggesting that endothelial migration and cord formation occurred normally. Although many recombinations were initiated as early as E11.25, we never observed \( \alpha \text{Sma-EYFP} \) expressing cells within the gonad.
A possible explanation for the observed lack of migration is that the PTM cells reside only in XY mesonephroi. Previous work did not detect a difference based on the XX or XY genotype of the mesonephros. However, we could not rule out the migration of a smaller population of cells present only in XY mesonephroi and masked by the robust migration of endothelial cells. To address this possibility, we assembled recombinations in which both αSma-EYFP mesonephroi and CD-1 gonads came from XY embryos. These experiments confirmed our earlier results: αSma-EYFP expressing cells do not migrate from the mesonephros into the testis between E11.5-E12.5.

The absence of migrating EYFP positive cells in the E11.5 αSma-EYFP recombination experiments indicates that PTM cells do not migrate during the bipotential window and are unlikely to be the migrating population required for testis cord formation. However, the possibility remained that PTM cells entered the gonad after the bipotential window and were found in previous recombinations due to their migration after testis cords had formed. Accordingly, we initiated recombination cultures at stages between E12.5-E13.5. Recombinations initiated at E12.5 with mesonephroi expressing ubiquitous EGFP showed robust migration restricted to interstitial cells of the testis (Fig. 5B). In contrast, αSma-EYFP mesonephroi did not contribute EYFP positive cells to CD-1 gonads despite normal development of the testis over the culture period (Fig. 5D). Subsequent staining indicated that cells entering the testis between E12.5--E13.5 were PECAM-1 positive endothelial cells, consistent with earlier studies of endothelial migration between E11.5--E13.5 (Fig. 5B, arrowhead).

Overall, we never observed EYFP positive cells entering XY gonads after recombination with αSma-EYFP mesonephroi at E11.5 or E12.5 (0/19). This was in stark contrast to migration observed in nearly all recombinations using ubiquitous EGFP mesonephroi and XY gonads (21/23, Fig. 5E).
Figure 5: \(\alpha Sma\)-EYFP positive cells do not migrate into the testis.

Control experiments confirmed robust migration of cells from the mesonephros (m) into the gonad (g) at E11.5 (A) and E12.5 (B). As previously reported, the vast majority of migrating cells (green) co-labeled with the endothelial marker PECAM1 (red). Identical recombinations using \(\alpha Sma\)-EYFP mesonephroi resulted in no migration when initiated at E11.5 (C) and E12.5 (D). In these experiments, migration and testis cord formation occurred normally as indicated by PECAM1 endothelial cells and germ cell aggregation (arrowheads). All images are maximum intensity projections of multiple optical sections. (E) Quantification of recombination experiments.
3.3.3 Live imaging of GFP-recombinations

To further characterize cell migration after gonad recombination, we utilized live imaging to visualize the behavior of cells as they enter the gonad. Consistent with our analysis of fixed tissue, cells that entered XY gonads displayed behavior indicative of an endothelial identity (Fig. 6). GFP-positive cells traversed the gonad before aggregating in the coelomic domain and contributing to the male-specific coelomic vessel. We had previously characterized this behavior using a number of endothelial-specific reporters. Moreover, cells behaved identically regardless of whether recombinations were performed at E11.5 and E12.5. This clearly demonstrated in both fixed tissue and cultured organs that endothelial cells are the sole migratory population that enters the gonad. Moreover, it appears that these cells continue to enter the gonad and contribute to the establishment and expansion of the male arterial system. In contrast to this, but consistent with fixed samples, live imaging of αSma-EYFP mesonephroi recombinations never indicated that EYFP-positive cells entered the gonad. Limited EYFP positive regions appeared to extend from the mesonephros at the poles of the gonad, but was most likely an artifact of damage to the tissue during recombination.

Live imaging of EGFP recombinations showed migration of endothelial-like cells to the coelomic domain of the testis. Cells entering the gonad extended long filopodial extensions that appeared to direct their migration toward the coelomic epithelium where they established the presumptive male vasculature (Fig. 6).
Figure 6: Live imaging of GFP recombination.

A CD-1 gonad recombined with a mesonephros expressing EGFP ubiquitously. Live imaging revealed that cells entering the gonad exhibited endothelial behavior. At 8hrs after recombination, GFP cells were seen entering the gonad and extending long protrusions toward the coelomic surface (8hr, arrowhead). Cells were highly directed to the coelomic domain and after 16 hours aggregate below the coelomic epithelium to form a presumptive coelomic vessel (16 hr, arrowheads).
3.4 Discussion

In vitro culture of adult Sertoli cells demonstrated that squamous stromal cells induced epithelialization of cord like structures and establishment of a basal lamina. Furthermore, mesonephric migration into the gonad between E11.5--E12.5 was shown to be required during epithelial morphogenesis of testis cords (Buehr et al., 1993; Tilmann and Capel, 1999). A subset of migrating cells were found to be squamous cells located near testis cord boundaries. Combined, these findings gave rise to the idea that PTM cells migrate into the testis from the mesonephros and actively participate in testis cord formation. However, in both of these studies PTM cells were never positively identified using molecular markers. Conclusions were based on stromal localization and squamous morphology of the migrating cells.

In an effort to positively identify migrating cells and confirm previous conclusions, we utilized a αSma-EYFP transgene that efficiently labels both PTM cells and other interstitial populations. Expression of the transgene, albeit non-specific, is nonetheless an effective label for PTM cells at early stages of testis development. As αSma-EYFP was not expressed in endothelial cells, their migration could be separated from PTM and other interstitial cells. Thus, we concluded that αSma-EYFP mesonephroi would clarify previous recombination experiments by distinguishing endothelial and PTM cell migration. Recombinations using αSma-EYFP and EGFP positive controls showed that under culture conditions where robust migration occurs, neither PTM cells nor any other EYFP positive cell type enters the gonad from the mesonephros. Although recombinations were performed throughout and beyond the bipotential window, αSma-EYFP expressing cells were never observed in the XY gonad, despite the fact that testis cord formation occurred normally. While it is conceivable that these αSma-EYFP transgenic cells are disadvantaged in some way, it seems unlikely based on the normal
development of $\alpha Sma$-EYFP transgenic testes. In any case, this experiment proves that migration of PTM cells is not required for male morphogenesis, as this process occurred normally in the absence of PTM migration. It is important to note that these results do not refute a requirement for PTM cells during testis cord formation, but suggest that PTM cells are induced within the testis, at least in the mouse.

Based on these results, PECAM-1 positive endothelial cells are the only identified population that enters the testis. Previously, a small population of PECAM-1 negative cells was detected after recombination with mesonephroi expressing a ubiquitous marker (Martineau et al., 1997). At present we cannot account for these cells, but they were a rare minority population relative to the numbers of endothelial cells detected in these assays.

Membrane barriers placed between the mesonephros and gonad during recombination culture resulted in disruption of testis cord formation (Buehr et al., 1993; Tilmann and Capel, 1999). These assays confirmed the requirement for cell migration but did not clarify which cell population is required. Our present results suggest that reassessing endothelial migration may clarify the influence of the endothelium during testis cord morphogenesis. Endothelial cells are responsible for cell differentiation and patterning in both the liver and pancreas raising the possibility of a similar function in the testis (Lammert et al., 2001; Matsumoto et al., 2001). Future work must selectively inhibit individual populations of cells to determine which cell type(s) are required for the epithelialization of testis cords.
4. Evaluation of candidates from Wnt4 microarray screen

4.1 Introduction

Several pathways are known to influence vascularization of the gonad. Of these, none are as instructive as mutation of Wnt4. XX Wnt4 mutants exhibit partial sex-reversal characterized by the development of male-specific vasculature in XX organs. The mechanism by which Wnt signaling affects vascular migration and remodeling remains unknown. As noted above, XX vasculature remains fairly quiescent and undergoes modest angiogenic expansion within the ovary between E11.5-E13.5. This is in stark contrast to vascular remodeling in the testis in which established vessels dissociate and establish a de novo arterial network. In XX Wnt4 mutants, a vessel develops in the coelomic domain during the bipotential window suggesting that Wnt4 is critical within the female pathway for repressing male-specific vascular remodeling.

Wnt4 mutants do not undergo full sex-reversal. Despite the development of phenotypically male vasculature, XX Wnt4 mutants do not sustain Sertoli cells. Wnt4 mutants do contain steroidogenic cells that resemble fetal Leydig cells, although the source of these cells is controversial. Many debate whether neuroendocrine cells are ectopically specified in XX mutants or are present as a secondary consequence of misappropriateion of progenitor cells from the adrenal. Regardless, it is clear that Wnt4 mutant gonads are permissive for both male vascularization and neuroendocrine cell differentiation. Moreover, these mutants separate the development and maintenance of fetal Leydig cells and vasculature from Sertoli cell specification.

The finding that male-specific vascularization is able to proceed in the absence of Sertoli cells led to the hypothesis that XX Wnt4 gonads express factors regulating vascularization but not the core male pathway. To identify mis-expressed genes, microarrays were run on whole XX Wnt4-/- gonads and compared to both XY and XX
controls (Coveney et al., 2007). To identify misexpressed genes enriched in XX mutants and XY gonads relative to wild type XX gonads, RNA from whole gonads was analyzed by microarray. To our surprise, very few obvious candidates came out of the screen although trends emerge. Morphogens and growth factors were noticeably sparse while factors involved in ECM assembly or modification were abundant. The two major caveats of this data are that (1) total RNA was extracted from whole gonads, which naturally dampens the signal intensity due to the presence of cells uninvolved in vascularization; (2) the arrays were run on custom spotted arrays, which are both difficult to analyze and extremely insensitive compared to current array platforms. Despite these concerns, the arrays did identify attractive candidates with expression profiles correlating with an influence during vascular remodeling.

This chapter summarizes various candidates that were misexpressed in XX Wnt4 mutant gonads. Genes with known functions in vascular remodeling or morphogenesis were of particular interest. However, over the years we have referenced these arrays as a convenient model of gonadal tissue in which vasculature is ectopically recruited. In addition to the preliminary data included in this chapter, I will return to various candidates identified in XX Wnt4 mutants in subsequent chapters.
4.2 Results

Preliminary analysis of XX Wnt4 mutant microarrays revealed that numerous genes associated with male development were misregulated in XX gonads. The original hypothesis was that Wnt signaling antagonized a male-specific morphogen that induced male-specific vasculature and that we would identify candidates through a differential expression screen between mutants and wild type. Although the arrays did not identify a clear candidate, several clues deepened our understanding of how vascular remodeling influences testis morphogenesis. Many trends were not initially apparent but, in retrospect, confirm findings presented in subsequent chapters of this thesis showing that morphogenesis is separable from Sertoli cell differentiation and mediated by vascular-mesenchyme interactions. Gene ontology (GO) analysis reveals that genes enriched in XY and XX Wnt4 mutants fall into categories related to ECM, morphogenesis, and cell adhesion (Fig. 7). Over the years many candidates from this list have been examined at various levels and we now know that many of the factors within the GO categories are factors expressed by the interstitium. Thus, a global perspective of the Wnt4 screen indicates that the mesenchyme responds to vascular remodeling and is central to male morphogenesis. One interesting note is that very few genes associated with blood vessels themselves were found to be mis-regulated in the Wnt4 arrays. The dearth of vascular factors indicates either that array sensitivity was unable to detect quantifiable changes in this minority population or that the endothelium is similar between mutants and wild type but is induced by other lineages to undergo sex-specific remodeling.
Figure 7: Screen shot from DAVID GO analysis of Wnt4 microarrays.

Analysis shows significant categories of genes over-represented in XY and XX *Wnt4*\(^{+/−}\) relative to XX gonads. Representative genes from several GO categories are listed.
4.2.1 Pitx2

Expression of Pitx2 is highly dimorphic at E12.5 with levels in the XY gonad being approximately 4-fold higher than whole XX gonads (Coveney et al., 2007). In XX Wnt4 mutants, Pitx2 expression is roughly 4-fold higher than XX control gonads. Whole mount in situ hybridization (WISH) suggested that Pitx2 is expressed throughout the XY gonad (Coveney et al., 2007). To resolve the poor cellular resolution of WISH, we referenced a panel of transgenic lines that are expressed in subsets of gonadal cells. Expression of Pitx2 in sorted cells was very clearly enriched in male germ cells, which had we known, would have excluded it as a candidate for regulating vascular migration. However, it may suggest an unknown role of the endothelium or interstitium in regulating sex-specific germ cell behavior.

To characterize the functional role of Pitx2 during gonad vascularization, we obtained mutant embryos through collaboration with Sally Camper (University of Michigan). XY mutant and control gonads were stained with PECAM1 to visualize vessels and germ cells. Upon dissection it was clear that the Pitx2 mutant embryos were significantly affected and displayed the well-characterized left-right defects. Defective left-right patterning affected gonad development where we observed that the length of the gonads was disproportionate, coinciding with the extended axis. However, at the light microscope level the basic structure of the testis did not appear morphologically disrupted. Development was clearly delayed, but our analysis was limited to a very cursory examination.

Confocal analysis of stained XY Pitx2<sup>−/−</sup> gonads confirmed the presence of testis cords and vascular development (Fig. 8A-C). Amazingly, even in the extremely elongated gonads, coelomic vessel formation appeared grossly normal (Fig. 8A, arrowhead). Consistent with normal morphogenesis, PECAM1-positive germ cells
aggregate and are not distended or misshapen (Fig. 8B). Mutants also deposited laminin around presumptive testis cords suggesting that Sertoli and germ cells were segregating away from the interstitial space (Fig. 8C). We did not analyze germ cells at later stages to determine if they entered mitotic arrest normally. Control littermates had no apparent defects including robust formation of the coelomic vessel and deposition of laminin around testis cords (Fig. 8D-F).
Figure 8: Analysis of Pitx2 mutant XY gonads.

(A-C) XY Pitx2 gonads were stained with PECAM1 (B) and Laminin (C) to characterize defects in vascular remodeling and cord formation. (B) Endothelial cells were present in the coelomic domain of Pitx2 mutants and appeared to aggregate into a normal coelomic vessel (arrowhead). (C) Laminin was also deposited indicating that testis cord aggregate in mutants (arrowheads). (D-F) Littermate controls were more developed at E12.5 suggesting a delay or subtle defect in Pitx2 mutants.
4.2.2 Dact1

Dapper homolog 1, antagonist of beta-catenin (Dact1) is a poorly characterized member of the Wnt/PCP pathway that controls polarity and other non-cannonical Wnt processes. Dact1 mutants are known to suffer from neuronal and posterior defects during embryogenesis (Okerlund et al., 2010; Suriben et al., 2009). In XY and XX Wnt4−/− gonads, Dact1 is upregulated relative to whole XX gonads by roughly 1.5-fold. Q-PCR confirmed that E13.5 XY whole gonads have roughly 2-fold more Dact1 transcription than XX gonads (Fig. 9A). Microarray analysis of SF1+ progenitor cells also found enriched expression of Dact1 in XY cells between E11.5-E12.5 (Nef et al., 2005). A complication of using SF1 as a marker is that it labels progenitors of both Sertoli and mesenchymal cells. Thus, it was not possible to distinguish whether Dact1 acts in Sertoli or mesenchymal progenitors during the initiation of the male pathway. Combined, these empirical microarray results indicate that Dact1 expression is male-specific and correlates with vascular remodeling.

We next sought to clarify the population of XY cells that express Dact1. RNA from E12.5 and E13.5 FACS sorted αSma-EYFP positive mesenchymal cells was used to compare Dact1 expression relative to the negative fraction. Expression of Dact1 was significantly enriched in the interstitium and was expressed at low levels in the negative fraction, which includes vasculature, germ cells, and Sertoli cells (Fig. 9B). This result is consistent with the absence of Sertoli cells in Wnt4 mutants. The presence of Dact1 in αSma-positive mesenchymal cells led us to hypothesize that non-cannonical Wnt signaling regulates interstitial response to vascular migration.
Figure 9: QRT-PCR analysis of Dact1 expression.

(A) Dact1 is expressed ~2-fold higher in XY vs. XX whole gonads. (B) Dact1 is expressed by αSma-positive interstitial cells in XY gonads.
Expression analysis clarified that \textit{Dact1} is expressed within the interstitium and appears to act downstream of male-specific vascular remodeling. In collaboration with Ben Cheyette’s lab at UCSF, we began to analyze the functional requirement for \textit{Dact1} during gonad morphogenesis. Based on expression, we hypothesized that the primary defect would be in XY \textit{Dact1}\textsuperscript{−/−} interstitial cells. Within the interstitium, we were most interested in analyzing the presence of fetal Leydig cells given that many of these cells arise from SF1+ progenitors and are tightly associated with blood vessels.

\textit{Dact1}\textsuperscript{−/−} embryos are viable until shortly after birth (Suriben et al., 2009). Mutant pups are easily identified by their truncated and looped tails, which is typical of non-canonical Wnt mutants. Morphological analysis of mutant gonads did not reveal overt phenotypes. XY genotyping confirmed that phenotypic and genotypic sexes were consistent. Despite their normal morphology, mutant testes were noticeably smaller than littermate controls.

Staining of P0 testes confirmed that testis morphogenesis is normal in XY \textit{Dact1} mutants relative to stage-matched controls (Fig. 10A-D). AMH-positive Sertoli cells aggregate into well-formed testis cords (Fig. 10C). E-cadherin staining suggested that the number of germ cells within testis cords was significantly reduced (Fig. 10D). After birth germ cells are sparsely distributed within testis cords, but are consistently found within each tubules. In \textit{Dact1} mutants seminiferous tubules without germ cells were prevalent (Fig. 10D, arrowheads). Loss of germ cells is consistent with the overall decrease in testis size.
Figure 10: Analysis of P0 XY Dact1 mutants.

(A) P0 testes are filled with testis cords that contain AMH-positive Sertoli cells (blue). (B) Large E-Cadherin-positive germ cells are present throughout tubules, although not as dense as during embryonic development. (C) Dact1 mutant testes are morphologically normal and contain AMH-positive testis cords. (B') Zoom of single testis cord containing two germ E-cad positive germ cells. (D) Germ cell numbers are reduced in Dact1+/− testes, based on E-Cadherin staining and many testis cords are empty (arrowheads). (D') Zoom of mutant cord showing a lack of germ cells, despite the otherwise normal morphology.
P0 analysis revealed that loss of Dact1 reduced the number of germ cells and resulted in many empty cords. However, germ cell loss was confounded by expression data showing that Dact1 is expressed in mesenchymal cells. Disruptions in fetal Leydig cell development frequently correlate with the loss of germ cells. We sought to clarify whether loss of Dact1 affected fetal Leydig development resulting in the observed germ cell loss as a secondary consequence. Mutant gonads and littermate controls were collected from E13.5, E14.5 and E16.5 embryos. Major hallmarks of male development occur between E11.5 and E16.5. Testis cord morphogenesis occurs between E11.5 and E12.5. By E13.5 cords are further remodeled and a subpopulation of the interstitial mesenchyme differentiates giving rise to 3β-HSD-positive fetal Leydig cells. Between E14.5 and E16.5 XY germ cells undergo mitotic arrest while the XX germ line continues into meiosis.

To assess the role of Dact1 during gonad morphogenesis, we stained mutant embryos with markers of each respective male-specific process. At all stages, XY Dact1 mutant gonads were smaller than controls, consistent with our analysis at P0 (Fig 11A-B, 13.5 shown). Vascular structure was not overtly affected by loss of Dact1. Control E13.5 males develop a large coelomic vessel with microvascular sprouts that branch into the gonad and between adjacent testis cords (Fig. 11C, arrowhead). A coelomic vessel was always present in Dact1 mutants (Fig. 11C). The structure of the coelomic vessel also appeared normal and showed no signs of disrupted vacular adhesion or branching. Testis cord formation occurred normally in mutants and AMH-positive Sertoli cells all aggregate without defect (Fig 11C’). Consistent with normal vascular branching, the number of testis cords was similar between mutants and controls despite the reduced size of the gonads. E13.5 Dact1 mutants lacked germ cells and contained many testis cords with only AMH-positive Sertoli cells, similar to our observations at P0 (Fig. 11C’-
D’), arrowhead). However, despite the male-specific pattern of Dact1 expression in the gonad, our analysis revealed that the effect on germ cells is not sex-specific. XX Dact1 mutants also had a significant reduction in germ cell numbers at E13.5 (Fig. 11E-F). E13.5 is prior to the sex-specific behavior of germ cells, suggesting that the absence of germ cells in mutants is probably due to earlier defect in germ cell migration or proliferation. Future work may seek to identify how Dact1 regulates germ cell survival or migration in the early embryo.

Dact1 is not required for Leydig cell differentiation. E13.5 XY Dact1 mutant gonads retained a subset of interstitial cells positive for the steroidogenic enzyme 3β-HSD (Fig. 12A-B). Localization of these cells was unaffected by loss of Dact1. Staining did suggest that mutants had fewer 3β-HSD-positive cells, although a quantitative analysis is required to confirm this observation. The observed decrease in Leydig cell number may be proportionate to the size of the organ and, again, requires confirmation by other means. General markers of non-Leydig interstitial cells including ITGA9 (Fig. 12C-D) as well as the vascular responsive gene VCAM1 were also unaffected (Fig. 12E-F). Accumulation of VCAM1 requires vascular development (Defalco et al., 2011) suggesting that Dact1 is not strictly required for mesenchymal-vascular interactions during testis development.

Based on these markers, it appears that Dact1 mutants are smaller and lack germ cells, which may be a secondary consequence of earlier developmental defects. At E13.5 Dact1 mutants specified Leydig cells as well as other interstitial cells. These populations may be quantitatively reduced. Analysis of RNA levels in the interstitium may clarify whether mutation of Dact1 regulates the initial differentiation of the interstitium.
Figure 11: Analysis of E13.5 XY *Dact1* gonads.

(A-B) *Dact1* mutant gonads were morphologically normal but much smaller. (C) Testis cords formed normally in *Dact1* mutants but contained many fewer, if any, germ cells (C'). (D) Littermate controls had large testis cords filled with germ cells (D'). (E-F) E13.5 XX mutants were also depleted of germ cells.
To address whether the male germ line is misregulated in *Dact1* mutants, we analyzed E14.5 and E16.5 XY mutants. At E14.5, XY germ cells begin to enter mitotic arrest. By E16.5 all male germ cells are arrested in G0, in contrast to meiotic XX germ cells in which the cell cycle remains active. Ki67 labels all cells actively in the cell cycle and thus can distinguish between properly arrested XY germ cells or those that erroneously escape mitotic arrest. Consistent with this progression, we found that both XY control and *Dact1* mutant germ cells were nearly all Ki67-positive at E13.5. By E14.5 some mutant germ cells were still Ki67 positive, although the vast majority of germ cells had exited the cell cycle and appear to arrest normally (Fig. 12G-J). Our data suggests that XY *Dact1*−/− somatic cells are able to initiate mitotic arrest in the male germ line.

Development of the interstitium was not affected in *Dact1* mutants. However, it remained possible that non-canonical Wnts control maintenance of this population after the initial specification. In particular, fetal Leydig cells are believed to exist in the interstitium and differentiate independent of proliferation. According to this model, *Dact1* may would not regulate the initial specification of fetal Leydig cells but affect the subsequent replenishment of these cells within the interstitium. We stained E14.5 and E16.5 *Dact1* mutants and controls with a panel of interstitial markers including VCAM1, ITGA9, and 3β-HSD to visualize mature Leydig cells and the surrounding non-Leydig interstitium. Mutation of *Dact1* did not affect the presence of any marker at either E14.5 or E16.5 (Fig. 13A-B). Fetal Leydig cell numbers appeared normal in *Dact1* mutants, in agreement with analysis at E13.5. Analysis at E14.5 and E16.5 does not preclude a quantitative difference. This issue is complicated by the smaller gonad in the mutant and analysis would require careful counts relative to other cell types.
Figure 12: Analysis of E13.5 XY *Dact1* mutant interstitium and germ line.

(A-B) *Dact1* is not required for the development of mature Leydig cells. (C-F) Non-Leydig interstitial cells were present in XY mutants. VCAM1 (F) and ITGA9 (D) expression was indistinguishable from controls (C,E). (G-H) Germ cells present in XY *Dact1* mutants and controls are actively dividing at E13.5. (I-J) Loss of *Dact1* does not affect the ability of XY germ cells to enter mitotic arrest by E14.5.
Figure 13: Dact1 does not control maintenance of fetal interstitial lineages.

(A) At E14.5 Leydig cells are found throughout the interstitium and surrounded by ITGA9 mesenchyme. Leydig cells are present in XY Dact1+ gonads along with non-Leydig ITGA9-positive interstitium. Analysis of E16.5 gonads confirm that Dact1 is not required for the maintenance of mature Leydig cells or non-Leydig interstitial mesenchyme.
4.3 Discussion

Analysis of XX Wnt4 mutant microarrays identified factors involved in cell migration, ECM remodeling, and cell-cell adhesion. In addition to those presented above, genes expressed by the XY mesenchyme, including Itga9, Vcam1, and Cxcl12 (SDF-1α), are all over-expressed in XX Wnt4 mutants. Antibodies against two of these candidates, VCAM1 and ITGA9, have developed into very effective markers of the male interstitium after vascular migration. The final factor, SDF-1α, is perhaps the best candidate to come out of this screen but was overlooked for several years. Hindsight is 20/20. Recent work indicated a functional role for SDF-1α in vascular remodeling and testis morphogenesis independent of its requirement during germ cell migration. This preliminary data is briefly discussed in chapter 7. Interestingly, CXCR4 the receptor for SDF-1α is not misexpressed, suggesting that this factor may serve as a sex-specific trigger. These experiments are on-going.

Functional analysis of Pitx2+/− and Dact1+/− embryos showed that both develop normal vasculature and undergo testis morphogenesis. Recent expression data revealed that Pitx2 is highly specific to male germ cells. This result may suggest an undefined role of Wnt4 on female germ cell development, or (a more interesting idea from our perspective) that vascular remodeling influences sex-specific behavior of the germ line and ectopic migration induces XX germ cells to express certain male factors. This is an intriguing possibility that warrants future examination. Further, it is clear that many interstitial genes are misregulated in Wnt4 mutants. Future work will continue to explore the mechanisms by which endothelial remodeling influences mesenchymal development. Dact1 is one example of a gene expressed within the interstitium that may regulate the proportion of the various interstitial lineages. However, a quantitative analysis of interstitial lineages in Dact1 mutants must be performed while taking into
account the reduced size of mutant testes. Germ cell loss in XX and XY *Dact1* mutants may result from an influence of *Dact1* prior to their arrival in the gonad and not the result of defects during somatic gonad development. Analyzing germ cell migration at early stages would resolve this issue.
5. Regulation of Vascular Remodeling by Vegfa

5.1 Summary

As suggested by the data presented in chapter 3, endothelial cell migration into XY gonads initiates testis morphogenesis. However, neither the signals that regulate vascularization of the gonad, nor the mechanisms through which vessels affect tissue morphogenesis are known. In this chapter, we show that Vegf signaling is required for gonad vascularization and cord morphogenesis. We establish that interstitial cells express Vegfa and respond, by proliferation, to endothelial migration. In the absence of vasculature, 4D imaging of whole organs revealed that interstitial proliferation is reduced and prevents formation of wedge-like structures that partition the gonad into cord-forming domains. Antagonizing vessel maturation also reduced proliferation. However, proliferation of mesenchymal cells was rescued by the addition of PDGF-BB. These results establish the first pathway integrating initiation of vascular development and testis cord morphogenesis, and lead to a model in which undifferentiated mesenchyme recruits blood vessels, proliferates in response, and performs a primary function in the morphogenesis and patterning of the developing organ.
5.2 Introduction

Endothelium-derived signals are required for the development and maintenance of many vertebrate organs. Information about vascular influences on organ budding, tissue-specific cell-type specification, and generation of progenitor niches have come from seminal work on the liver, pancreas, and nervous system (Lammert et al., 2001; Matsumoto et al., 2001; Shen et al., 2004). Despite the broad implications of this research, the mechanisms through which endothelial cells influence tissues have been difficult to identify. In several organs, specialized progenitor cells associate with the vasculature and maintain their proliferative status through contact with the extracellular matrix that shrouds vessels (Shen et al., 2008). However, the dynamics and cellular response of less specialized cells that mediate organ morphogenesis are not understood with the same clarity.

Endothelial cells influence testis cord morphogenesis in the embryonic mouse gonad (Combes et al., 2009; Cool et al., 2008). The gonad is a uniquely powerful model for studying the role of vasculature during organ morphogenesis due to the ability to culture and image whole organs during the coincident processes of vascularization and epithelial morphogenesis. In the lung, the endothelium is reported to interact with the airway epithelium to induce septae formation in the distal airways (Yamamoto et al., 2007). However, the potential importance of the mesenchyme was not investigated in this study, despite the extensive literature supporting mesenchymal-epithelial interactions as a primary force during lung morphogenesis. In the gonad, Sertoli cells were assumed to attract migrating endothelial cells and initiate the hallmark patterning of testis cords, although there is no direct evidence that this is the case.

In this chapter, I detail work that proposes cross-talk between the endothelium and the non-specialized mesenchymal cells as a driving force during testis
morphogenesis. Vegfa is expressed specifically by the undifferentiated mesenchyme. Neutralizing antibodies against Vegf reveal a requirement during the initial steps of testis vascularization. Using real-time imaging of whole organs, we demonstrate that the primary cell-type affected by endothelial migration is the mesenchyme itself. In the absence of vasculature, interstitial proliferation is reduced and wedge-like structures of mesenchyme that partition the gonad into cord-forming domains do not form. We propose that the endothelium does not directly regulate epithelialization, but promotes mesenchyme protrusion as a primary morphogenetic force. When the endothelial cell adhesion molecule, VE-Cadherin, was blocked, a less-severe effect on mesenchymal proliferation was observed. However, mesenchymal proliferation was rescued by the addition of PDGF-BB to XY gonads treated with either Vegf Trap or BV13. This leads to a model in which undifferentiated mesenchyme recruits blood vessels, proliferates in response, and performs a primary function in the morphogenesis and patterning of the developing organ. Importantly, this was the first demonstration that specifically removing endothelial cells from the gonad blocked testis cord formation. It confirmed our conclusions in chapter 3 that PTM cells do not migrate into the testis and provided a mechanism by which the requirement for cell migration during testis cord formation is fulfilled by endothelial cells.
5.3 Results

5.3.1 Vegfa expression in the XY gonad

Endothelial migration into XY gonads begins by E11.5. Major vascular remodeling of the XY circulation occurs around E12.0 and continues over the next 12-24 hours (Brennan et al., 2002). Expression of Vegfa was visualized using Vegfa-lacz mice at E12.0 (Fig. 14A-B). LacZ was widely expressed throughout XX and XY gonads, but with subtle differences (Fig. 14A'-B'). In XX organs, Vegfa was expressed throughout much of the gonad and parts of the mesonephros. Interestingly, expression was absent in the coelomic domain (Fig. 14B', brackets) and enriched along the gonad-mesonephros border (Fig. 14B', arrowhead). In XY gonads, Vegfa was expressed strongly in the coelomic domain, but appeared at low levels in cells along the mesonephric border (Fig. 14A', brackets & arrowhead). Domains of Vegfa expression are consistent with the sexually dimorphic vascular stability along the mesonephric border. In XX organs this vascular bed remains intact, while the same vessels in XY urogenital ridges dissociate, giving rise to individual endothelial cells that migrate to the coelomic surface of the gonad (Coveney et al., 2008).
Figure 14: Whole mount E12.0 Vegfa-lacZ gonads stained with X-gal.

(A) X-gal staining (blue) of XY E12.0 gonads shows broad expression throughout the gonad including the coelomic domain. Expression of Vegfa extends to the surface of the gonad (A’, brackets) and is reduced at the gonad/mesonephros border (A’, arrowhead). (B) XX gonads also express Vegfa, although expression is absent from the coelomic domain (B’, brackets) and is enriched along the gonad/mesonephros border (B’, arrowhead).
5.3.2 VEGF receptors are endothelial cell specific

VEGFA is a secreted ligand and signals primarily through three receptor tyrosine kinases, VEGFR1 (Flt-1), VEGFR2 (Flk1), and NRP1. Of these receptors, FLK1 is the most critical for activation of downstream signaling, and mutation of this receptor prevents endothelial specification and patterning by VEGFA (Shalaby et al., 1995). In the E12.5 XY gonad, the vascular marker CD31 (PECAM-1) labels both endothelial cells and the germ line (Fig. A). These cell-types are easily distinguished based on their morphology and localization within the gonad. Antibodies against NRP1 and a Flk1-mCherry reporter line reveal robust expression of both of these receptors in the microvasculature of the gonad (Fig. 15B-C). Flk1 expression and NRP1 staining colocalized with PECAM-1 specifically on cells comprising the microvasculature of the gonad, and in particular the large male-specific coelomic vessel (Fig. 15E). We never found Sertoli or germ cells that were positive for NRP1 or Flk1. Our findings are consistent with widespread expression of these receptors on endothelial cells.
Figure 15: Vegfa receptors are expressed specifically on endothelial cells.

(A) VEGFA receptor expression was examined relative to PECAM-1, which labels both germ and endothelial cells in the gonad. Both NRP1 (B) and Flk1-mCherry (C) localize specifically to PECAM-1 positive endothelial cells (D).
5.3.3 Vegfa expression is differentially regulated in the XY gonad

VEGFA was previously reported in Sertoli cell cytoplasm with only faint expression in germ and interstitial cells (Bott et al., 2006). However, Vegfa-lacZ did not appear to be enriched in testis cords based on whole mount staining (Fig. 14A). To determine which cells expressed Vegfa, we isolated individual populations of cells from the embryonic testis (Fig. 16A). By E12.5, the XY gonad is segregated into two major cellular compartments, the testis cords, containing Sertoli cells and germ cells, and the interstitium, which is comprised of a heterogeneous population of mesenchyme that surrounds endothelial cells. Using transgenic mice expressing specific fluorescent tags for each cell type and Fluorescence Activated Cell Sorting (FACS), we isolated Sox9-ECFP positive Sertoli cells, Oct4-EGFP-positive germ cells, αSma-EYFP positive interstitial cells, and Flk1-mCherry positive endothelial cells (Fig. 16A). RNA was extracted from each positive fraction, and expression of Vegfa was measured using qRT-PCR normalized to whole XY gonad cDNA to identify gonadal populations enriched for Vegfa expression. Surprisingly, at E12.5 Vegfa was not detected in Sertoli cells (Sox9-ECFP positive). Instead, we found that interstitial cells (αSma-EYFP positive) were enriched for Vegfa. Comparison of XX and XY whole gonads also revealed an approximately two-fold enrichment of Vegfa transcripts in males (Fig. 16B).

Post-transcriptional modifications of Vegfa transcripts constitute an additional possibility for sex specific regulation. In the Vegfa-lacZ reporter line, lacZ was inserted into the 3’ UTR of Vegfa and does not provide information about the numerous post-transcriptional Vegfa splice variants. Previous studies examined post-transcriptional regulation of Vegfa in the gonad but never compared XX and XY gonads for sexually dimorphic isoforms (Bott et al., 2006). To determine whether sex-specific isoforms of Vegfa are present in XX vs. XY gonads, we utilized nested RT-PCR. Predominant
isoforms of Vegfa-164 and Vegfa-120 were detected in both sexes, although a rare variant, Vegfa-144, was specific to XY gonads at all time points between E11.5 and E13.5 (Fig. 16C, white arrowhead, E12.5 shown). Consistent with qRT-PCR results, all isoforms of Vegfa were detected in αSma-EYFP positive interstitial cells and not Sox9-ECFP positive cells (Fig. 16D). The 144kDa isoform is thought to behave similarly to VEGFA-164 due to its intermediate affinity for ECM and potent signaling interactions with FLK1. Expression analysis of Vegfa suggested that expression domain variation, expression level differences, and sex specific splicing may all contribute to the differential regulation of vascular recruitment between the XX and XY gonad.
Figure 16: Cell-type specific analysis of Vegfa expression.

(A) Schematic representation of the early gonad showing the expression domain of various transgenic reporter lines expressed by gonadal sub-populations at E12.5. Key regions of the XY gonad including the coelomic domain (CD, brackets) and coelomic vessel (CV, arrow) are also indicated. (B) mRNA was extracted from FACS sorted populations and expression of Vegfa was compared across XY cell-types after normalizing to expression in whole E12.5 XY gonads. Bar colors represent analysis of specific populations as indicated in schematic (A). Pink bar indicates Vegfa expression levels of whole XX gonads relative to whole XY gonads (*=p<0.05, **=p<0.005, ***=p<0.0005). (C) Using nested RT-PCR, individual isoforms of Vegfa were assayed in E11.5-E13.5 XX and XY gonads. Vegfa-144 was found only in XY gonads whereas major isoforms (164 & 120) were detected in both male and female organs (C, white arrowhead, E12.5 shown). (D) RT-PCR on individual sorted populations revealed very low Vegfa expression in Sox9-ECFP positive Sertoli cells but robust expression in αSma-EYFP positive interstitial cells including specific expression of Vegfa-144.
5.3.4 Inhibiting Vegfa with VEGF Trap

Traditional genetic ablation of Vegfa is complicated by the severe, systemic, and early phenotypes resulting from the loss of this growth factor (Ferrara et al., 1996). To overcome these obstacles, we took advantage of a well-characterized and pharmacologically specific intervention, VEGF Trap (aflibercept, Regeneron), which contains critical domains of VEGFR1 and VEGFR2 that bind and inhibit secreted VEGF (Holash et al., 2002) (Fig. 17A). Importantly, VEGF Trap does not broadly affect RTK signaling, and distinguishes the role of VEGF from FGF and PDGF, which also have critical functions during sex determination and the initiation of testis development.

To deliver VEGF Trap to the gonad, we developed a method to introduce inhibitors into the systemic circulation of the embryo and then explanted the organ for prolonged culture (Fig. 17B). The success of each injection was monitored in individual organs by co-injecting fluorescent lectins and imaging immediately after dissection (Fig. 17B'). Strong fluorescence in the vasculature at the border of the gonad and mesonephros suggested that drug delivery was efficient. Control organs were uninjected or injected with fluorescent dye without VEGF Trap.
Figure 17: Inhibition of Vegf using Vegf Trap.

(A) Vegf Trap is a soluble recombinant antibody containing regions of VEGFR1 and VEGFR2 with high affinity for secreted Vegf (Holash, et al., 2002). (B) Vegf Trap was injected into the hearts of E11.5 embryos along with fluorescent dyes. After injection, gonads were explanted to culture and visually checked to determine the quality of injection (B'). High fluorescence within the vascular bed at the gonad (G)-mesonephros (M) border was indicative of efficient delivery.
Injection with VEGF Trap followed by in vitro culture for 24-36 hours resulted in a strong blockade of male-specific vascular development in most gonads (Fig. 18B-C). Coelomic vessel formation and endothelial migration were robust in control organs injected with lectin alone (Fig. 18A, arrowhead). When all vasculature was eliminated from XY gonads, there were no regions of germ cell aggregation and male morphogenesis failed completely (Fig. 18C). A limited number of PECAM-1 positive endothelial cells were observed in some XY gonads after injection with VEGF Trap (Fig. 18B, arrowhead). Interestingly, the sporadic presence of these endothelial cells correlated with limited regions of germ cell aggregation and presumptive testis cord formation (Fig. 18B, dotted lines). Together these data conclusively demonstrate the requirement for endothelial migration into the XY gonad and the tight relationship between endothelial cells and male-specific morphogenesis.
Figure 18 (Page 79): Inhibition of VEGF blocks endothelial remodeling in the gonad.

(A-C) Dotted line indicates gonad/mesonephros boundary. (A) After 24-36 hours of culture, control gonads, injected only with rhodamine-lectins, showed normal development of a coelomic vessel (arrowhead) and germ cell aggregation inside testis cords. (B-C) In XY organs injected with VEGF Trap, male-specific vasculature was very limited or absent. (B) The presence of some endothelial cells in injected samples correlated with sporadic cord-like structures (arrowhead/dotted lines). (C) Robust delivery of VEGF Trap completely blocked male-specific vascular development and testis morphogenesis.
5.3.5 Endothelial migration does not control Sertoli or Leydig cell specification, but patterns testis cords morphogenesis

The failure of cord formation after VEGF Trap injection suggested that vascular migration may be required for promoting development of Sertoli cells, which are believed to be the primary cell-type regulating cord formation. To address this possibility, we first compared the differentiation of Sertoli cells before and after blocking the vasculature. After injections with lectin alone, control organs developed normal testis cords containing SOX9 and AMH-positive Sertoli cells within, surrounded by unlabeled interstitial space. Injection of VEGF Trap by E11.25 resulted in robust inhibition of cord formation. However, AMH/SOX9 positive Sertoli cells were specified in the absence of the vasculature (Fig. 19A-B).

The male-specific Leydig cell lineage (3β-HSD positive) was also specified in the absence of the vasculature, although these cells were found throughout XY gonads instead of concentrated in a defined interstitial space (Fig. 19C-D). Fetal Leydig cells are typically located in close proximity to the vasculature, as seen in control XY gonads (Fig. 19C’, arrowhead). Interestingly, both Sertoli and interstitial progenitor cells arise from early (prior to E11.5) divisions of Steroidogenic Factor 1 (SF1) positive cells in the coelomic epithelium (Schmahl et al., 2000). Subsequently (after E11.5), the coelomic domain gives rise to a population of SF1-negative somatic cells, which are uncharacterized and have not been suggested to play a primary role during early morphogenesis of the testis. Although blocking vascular development did not inhibit Sertoli or Leydig cell specification, the coelomic domain of the gonad (A-F, below dotted lines), which typically consists of several layers of mesenchymal cells above the condensing Sertoli cells (See schematic in Fig. 16A, brackets), was significantly reduced in injected samples (Fig. 19B,D,F).
The reduced size of the coelomic domain and the failure of testis cords to form in the absence of blood vessels, indicated that a primary function of the vasculature was to promote somatic development and patterning of the gonad. To investigate the dynamics of the interstitial population more carefully, we injected VEGF Trap or lectin alone into \( \alpha Sma^{+} \)EYFP positive embryos, which express EYFP throughout the interstitial mesenchyme. Gonads from embryos that received control injections developed a clear interstitial space marked by the dense accumulation of EYFP-positive cells and a thick layer of somatic cells surrounding the coelomic vessel (Fig. 19E, bar). However, in Trap-injected samples, interstitial cells were severely reduced and did not coalesce into distinct compartments between testis cords. Consistent with our previous observations, EYFP-positive cells were mostly restricted to the shrunken coelomic domain (Fig. 19F, bar).
Figure 19 (Page 83): Vascular remodeling does not affect specification of sex-specific lineages.

Markers of distinct gonadal cell-types were specified but mislocalized after vascular inhibition (right column) compared to controls injected with lectins alone (left column). (A-F) Dotted lines define the surface epithelium and bars indicate the coelomic domain in E and F. (A,B) SOX9 (red) and AMH (green) positive Sertoli cells did not aggregate into testis cords. (C,D) 3ß-HSD positive Leydig cell (red) localization was severely disrupted after Vegf inhibition. (C’) In controls, Leydig cells (red) are in close proximity to PECAM-1 positive (green) endothelial cells (arrow). (D’) Injection of Vegf Trap randomized Leydig localization. (E) αSma-EYFP positive (green) interstitial protrusions typically extend into the gonad at regular intervals and surround testis cords. (F) Inhibiting Vegf blocked extension of interstitial protrusions between testis cords.
5.3.6 Live imaging of interstitial development

To further characterize somatic cell development in the gonad, 4D confocal microscopy was used to visualize how αSma-EYFP positive interstitial cells respond in both the presence and absence of VEGF-mediated vascular migration. At E11.5, cells throughout the coelomic epithelium express heterogeneous levels of αSma-EYFP in both XX and XY gonads. Live imaging of XY αSma-EYFP gonads showed that interstitial cells rapidly expand between E11.5-E12.5 (Fig. 20A-D). After ~6 hours of culture αSma-EYFP is expressed by several layers of cells in the coelomic domain (Fig. 20A). Between 12-18 hours after initiation of the cultures, imaging revealed that the interstitium extends throughout the gonad and a clear distinction between testis cords and the interstitial space can be made (Fig. 20B-C). Live imaging showed that during this time period, cells initiate EYFP expression as well as migrate into the interior of the gonad from the coelomic domain. Testis cord domains (Fig. 20D, asterisk) occupy dark areas in stark contrast to the brightly labeled αSma-EYFP positive interstitium after 24 hours of culture.

VEGF Trap injection into αSma-EYFP XY embryos revealed a severe defect in the expansion of the interstitium (Fig. 20E-H). At the outset of culture, treated and untreated gonads appear nearly identical (Fig. 20A, E). Expression of EYFP in the coelomic domain of both organs is abundant prior to the initiation of testis cord morphogenesis. Twelve hours after injection with VEGF Trap, it is evident that expansion of the interstitium is defective compared to controls (Fig. 20F, B). Within the 24-hr period of culture, in the absence of vasculature, interstitial cells expand only modestly and do not penetrate into the gonad (Fig. 20H compared to D).
Figure 20: Interstitial expansion fails after VEGF block.

(A-D) αSma-EYFP positive gonads were imaged in real-time to characterize interstitial dynamics. (E-H) Littermates were injected with Vegf Trap and imaged in parallel. (A-B) After 6-12 hours of culture, control organs display increased αSma-EYFP expression. (C-D) Within 18-24 hours, controls develop clear testis cord domains that are segregated from the interstitium (asterisk). (E-H) Cord structures never appear and αSma-EYFP positive cells do not expand into the interior of the gonad in the absence of robust vascular remodeling.
To clarify the dynamics of endothelial and mesenchymal cell interactions during organ patterning, we performed live imaging on embryos positive for Flk1-mCherry and αSma-EYFP. These movies revealed that the presence of endothelial cells preceded expansion of the mesenchyme into the interior of the testis from both the coelomic epithelium and gonad/mesonephros border. Higher resolution imaging focused on the coelomic surface in order to characterize how vessels entered this domain and interacted with the mesenchyme that induced migration (Fig. 21). Endothelial cells enter the gonad, form a coherent vessel (Fig. 21top, red arrowhead) and then give rise to microvascular branches. Mesenchymal cells surround the large coelomic vessel and then expand during the initial stages of vascular migration. As vascular branching proceeds, the mesenchyme responds and EYFP-positive cells accumulate around microvascular branches and begin to expand into the interior of the gonad (Fig. 21, green arrowheads). The initiation of this dynamic vascular-mesenchyme relationship may represent the initial steps of de novo testis cord formation.
Figure 21: Two-color imaging of vascular-mesenchyme interactions.

Flk1-positive endothelial cells migrate toward the coelomic domain (top) and form microvascular sprouts attached to the coelomic vessel (red arrowhead). In subsequent hours, αSma-EYFP-positive mesenchymal cells appear to aggregate around and migrate into the gonad along existing vessels (green arrowheads). Movies were taken from a single optical section collected at 10-minute intervals.
5.3.7 Endothelial remodeling is required for male-specific proliferation

5.3.7.1 Vascular migration promotes mesenchymal proliferation

Live imaging indicated that expansion and penetration of αSma-EYFP positive cells from the coelomic domain into the gonad occurred in part by migration away from the coelomic domain. However, another likely explanation for the failure of the expansion of the coelomic domain is that interstitial cells failed to proliferate in the absence of the vasculature. Proliferation is required for morphogenesis of XY gonads, although the direct regulatory mechanisms are not well understood (Schmahl and Capel, 2003).

To compare active proliferation of gonadal cells in the coelomic domain in the presence or absence of the coelomic vasculature (Fig. 22A-B, boxed areas), we stained with antibodies against phosphorylated histone H3 (pHH3). Confocal Z-stacks of individual organs were condensed into maximum intensity projections and dividing cells within the indicated domain were manually counted. This eliminated variation in focal plane and assured that the observed decrease in proliferation was reflective of the entire coelomic domain. Proliferation in XY gonads is enriched in somatic cells throughout the coelomic domain after 24 hours of culture (~E12.5) (Fig. 22A). In addition to divisions in the coelomic epithelium, somatic cells throughout the domain surrounding the coelomic vessel are highly proliferative (Fig. 22A, arrowheads). These results are consistent with previously published data carefully characterizing sex specific proliferation patterns (Schmahl et al., 2000). In the absence of endothelial cells after Vegf Trap injection, fewer cells were dividing throughout XY gonads based on pHH3 staining (Fig. 22B).
Figure 22: Vascular remodeling is required for male-specific proliferation.

(A-G) Male-specific proliferation was quantified by counting pHH3-positive dividing cells (green) in control and cases where vasculature is blocked or misregulated. (A-C) E11.25-E11.5 gonads were cultured for 24 hours. Endothelial cells were visualized using PECAM-1 (red). (A-B) Proliferation in the coelomic domain (CD) (boxes designated with dotted lines) was compared between WT and VEGF-Trap injected embryos. (A) In WT XY gonads, proliferation is abundant and up-regulated in somatic cells within the epithelium and surrounding vessels (arrowheads). (B) Blocking vascular development inhibits male-specific somatic cell proliferation in the CD.
5.3.7.2 Vascular adhesion promotes mesenchymal proliferation

Endothelial cells migrate into the XY gonad as individual cells, then coalesce to form the coherent coelomic vessel (Coveney et al., 2008). In previous reports, disruption of endothelial adhesion disrupted cord formation (Combes et al., 2009). However, the mechanism mediating the effect on morphogenesis was unclear. We investigated whether endothelial cell adhesion and vessel maturation are required to stimulate proliferation of mesenchymal cells. Blocking endothelial cell adhesion by treating E11.5 cultured gonads with an antibody against VE-Cadherin (BV13) resulted in a significant decrease in proliferation in the coelomic domain after 24 hours of culture, suggesting that vessel coalescence is required after migration of individual endothelial cells, but prior to the induction of interstitial proliferation (Fig. 23A-B). Proliferation was quantified after Vegf Trap injection (n=13, p=.0001) and BV13 treatment (n=13, p=.0005) to confirm that reduced proliferation caused by both treatments was significant (Fig. 24D).
Figure 23: Vascular adhesion is required for male-specific proliferation.

Proliferation was quantified in the coelomic domain as in Figure 22. Actively dividing cells were stained with pHH3 (green) and endothelial and germ cells were labeled with PECAM1 (red). (A-B) BV13 treated gonads develop disorganized vasculature with reduced proliferation in the CD compared to controls.
5.3.7.3 Ectopic vascular migration is sufficient to induce mesenchymal proliferation

Proliferation analysis after inhibition of vascular migration and disrupted endothelial adhesion demonstrated that both of these processes were critical to induce male-specific expansion of the coelomic domain. To test whether a male-specific coelomic vessel was sufficient to induce somatic proliferation, XX Wnt4/- gonads were analyzed. In XX Wnt4/- gonads, an ectopic vessel reminiscent of the male-specific coelomic vessel forms (Jeays-Ward et al., 2003). Comparison of XY Wnt4+/-, XX Wnt4+/-, and XX Wnt4/- littermates revealed increased proliferation in the coelomic domain of XX Wnt4/- mutants with a particular increase in cells adjacent to the ectopic vasculature (Fig. 24A-C). Proliferating germ cells found in the XX coelomic domain were identified as PECAM-1/pHH3 positive cells (blue cells) and were not counted in our analysis. XX Wnt4/- gonads had similar levels of somatic proliferation to XY Wnt4+/- controls, which is significantly more than XX controls (n=5, p=.001) (Fig. 24A-D). This result suggests that ectopic endothelial cell migration is sufficient to induce proliferation of nearby mesenchymal cells.

Modulation of vascular remodeling revealed that inhibiting vascular migration or adhesion reduced XY specific proliferation. Induced vascularization into XX gonads increased proliferation to levels comparable to XY littermates. Quantification of all the proliferation results are summarized in Figure 24D.
Figure 24 (Page 94): Ectopic endothelial migration is sufficient to induce mesenchymal proliferation.

(A-C) To determine whether vasculature was sufficient to induce proliferation, XX Wnt4−/− mutants were compared to littermate controls. Blue nuclei are false colored, and indicate PECAM-1/pHH3 double-positive proliferating germ cells, not included in this analysis. In XY Wnt4+/− gonads (A), proliferation is higher than XX Wnt4−/− littermates (B). (C) However, XX Wnt4−/− gonads develop male-specific vasculature and exhibit proliferation levels similar to XY littermates and significantly higher than XX gonads, suggesting the presence of vasculature is sufficient to drive proliferation. (D) Quantification of these results, and results from vascular inhibition presented in Figure 22-23, are presented in panel D (***=p<0.0005).
5.3.8 PDGF as a candidate pathway downstream of vascular remodeling

XY *Pdgfra* mutants resemble BV13 treated gonads: despite their ability to recruit endothelial cells to the coelomic domain, they show defects in the later stage of male-specific somatic proliferation (Brennan et al., 2003). Moreover, expression analysis of XX *Wnt4*/*-/- gonads showed upregulation of several Pdgf pathway components in the presence of ectopic vasculature, although Sertoli cells do not persist (Coveney et al., 2007). This suggested that *Pdgfraβ*, which are both expressed in the interstitium, are activated by vascular migration in the absence of Sertoli cells. Mutation of *Pdgfrβ* does not result in overt gonad phenotypes. It is possible that *Pdgfrα* compensates for the loss of *Pdgfrβ* given co-expression on interstitial cells (Brennan et al., 2003). *Pdgfa* is expressed by Sertoli cells and originally proposed to activate *Pdgfra* and induce XY-specific proliferation (Brennan et al., 2003). However, gonad morphogenesis is normal in *Pdgfa*/*-/-* mutants, suggesting the involvement of an alternate ligand (Gnessi et al., 2000b).

We utilized qRT-PCR to measure changes in *Pdgfa* and *Pdgfb* expression in XY gonads after BV13 or Vegf Trap treatment. Under both conditions the expression of *Pdgfa* was unchanged compared to controls (Fig. 25A). Stable expression of *Pdgfa* after disrupted vascular development is consistent with our observation that Sertoli specification is unaffected as shown by normal levels of *Sox9* and *Fgf9*. In contrast, we found that *Pdgfb* expression was significantly reduced after injection with Vegf Trap (Fig. 25A). Interestingly, treatment with BV13 resulted in a slight reduction in *Pdgfb* expression despite constant expression of the endothelial cell receptor *Tie2*. Although *Fgf9* was another attractive candidate given its well-characterized role in promoting testis development (Colvin et al., 2001; Schmahl et al., 2004), we found that expression of *Fgf9* was invariant in the presence of both inhibitors (Fig. 25A). Thus *Fgf9* is unlikely to
be mediating this effect. Using our panel of gonadal populations isolated by FACS, we confirmed that \textit{Pdgfa} is expressed predominantly by Sertoli cells and \textit{Pdgfb} is specific to endothelial cells (Fig. 16A, Fig. 25B-D). This expression analysis suggests that \textit{Pdgfb} expression reflects the presence of mature vessels in the gonad and correlates with the ability of endothelial cells to induce mesenchyme proliferation.
Figure 25 (Page 98): Evaluation of Pdgf ligand expression.

(A) Expression of Pdgfb, but not Pdgfa or Fgf9, was reduced after Vegf Trap and BV13 treatment. FACS analysis of gonadal populations was performed using an array of transgenic mice (Fig. 16A). (B-D) Using RNA extracted from individual populations of gonadal cells, we measured expression of Pdgfa, Fgf9, and Pdgfb. (B) Pdgfa expression was enriched in Sox9-ECFP positive Sertoli cells. (C) Fgf9 was also highly enriched in Sox9-ECFP positive cells. (D) Pdgfb was detected only in Flk1-mCHERRY positive endothelial cells. Enrichment was calculated based on normalization to XY whole gonad expression levels.
5.3.9 Addition of recombinant PDGF rescues proliferation in the absence of the vasculature

Expression analysis suggested that Pdgf ligands were likely candidates to communicate vascular migration to the mesenchyme. To test this, we treated both BV13 and VEGF-Trap treated gonads with recombinant PDGF-AB. This heterodimer is an extremely stable and frequently used ligand that can interact with both PDGFRα and PDGFRβ. Addition of PDGF-AB rescued proliferation after vascular disruption to wild-type levels (Fig. 26A-B, E). However, based on our expression analysis it appeared that PDGF-AB was not a physiological target given that no single population of cells produced both Pdgfa and Pdgfb. Interestingly, addition of rPDGF-AA did not have a robust affect on proliferation after treatment with BV13 (Fig. 26C). This result is consistent with the finding that expression of this ligand is not changed by blocking vascular invasion or adhesion (Fig. 25B-D), but it is contrary to previous hypotheses that Sertoli derived PDGFA is the primary stimuli of PDGFRα. Based on this result, we tested whether PDGF-BB can compensate for disrupted vasculature. We added recombinant PDGF-BB (rPDGF-BB) to Vegf Trap and BV13 cultures (Fig. 26D,G). Addition of rPDGF-BB to culture media increased the number of pHH3 positive cells in the coelomic domain after Vegf Trap treatment compared to organs cultured in unsupplemented media (n=6, p=.004) (Fig. 26E-H). Addition of PDGF-BB to BV13 cultures resulted in a modest but significant increase in proliferation that was commensurate with the milder proliferative phenotype (Figure 26A, D). These results clarify the context of Pdgf signaling in the fetal testis and also establish PDGF-BB as a critical signal downstream of vascular development during organ morphogenesis.
Figure 26: PDGF-BB rescues proliferation after vascular inhibition.

(A-D) Pdgf ligands had variable affects on proliferation after BV14 treatment. (A-B) rPDGF-AB rescued proliferation when vascular adhesion was disrupted. (C) rPDGF-AA did not rescue proliferation after BV13 culture. (D) rPDGFBB had an was able to partially rescue proliferation after BV13 treatment. rPDGF-BB increased somatic proliferation in cells adjacent to vessels (arrowheads). (E-G) Reduced proliferation after Vegf Trap injection was rescued by addition of rPDGF-BB to cultures. (E-F) Injected cultures showed a loss of proliferation relative to controls. (G) Proliferation was restored in injected cultures treated with rPDGF-BB. (E) Although addition of rPDGF-BB to wild type gonads had no significant effect on proliferation, quantification of pH3-positive cells confirmed that the significant decrease in proliferation after Vegf Trap injection, and to a lesser extent BV13, was rescued by the addition of rPDGF-BB (*=p<0.05, ***=p<0.0005).
5.4 Discussion

Dimorphic development of the gonad is a paradigm for organ growth and patterning. Shortly after the bipotential window, the XY mouse gonad begins a remarkable 24 hours in which male-specific vascular remodeling and de novo cord morphogenesis occur. Like other internal mammalian organs, the rapid developmental timeframes combined with inaccessibility of organs during morphogenesis have made integrating concurrent events difficult. However, the inaccessibility and temporal limitations have been overcome in the gonad by the advent of whole organ culture and simultaneous live-imaging, which allows us to integrate cell behavior and molecular mechanisms in real time. We have used these tools to investigate vessel dynamics in an organ undergoing morphogenesis. Experiments perturbing vascular development demonstrate a link between Vegfa-guided endothelial migration and interstitial proliferation, which suggests a novel endothelial-mesenchymal feedback loop necessary for organ morphogenesis (Fig. 27).

Endothelial cells have emerged as a critical influence on developing tissues. Endothelial-progenitor cell interactions are a mechanism by which vessels promote tissue development and maintenance. In the few characterized cases, vascular derived ECM establishes a niche for progenitor cells (Nikolova et al., 2006). In the adult testis several populations of specialized cells are closely associated with blood vessels, although mechanisms underlying putative functional relationships are unknown (Yoshida et al., 2007). In these cases, disruption of vessel-progenitor cell interactions prevented the differentiation of specific cell types, but did not affect gross morphology of the organs. Our data does not support a requirement for the vasculature during early specification of either Sertoli or fetal Leydig cells, although we cannot preclude homeostatic relationships at later stages of development.
In addition to progenitor cell interactions, vessels have a less well-characterized role during organ formation and recovery after injury. In endodermal organs, mature vessels influence surrounding pancreatic tissue by providing growth and survival signals (Jacquemin et al., 2006). Although previous work established vessels as a source of mitogens, relating this to a mechanism that affects mammalian organogenesis and patterning has proven difficult.

The gonad is an ideal model system in which to investigate how the initial steps of vascularization occur in a developing organ. In this study we show that vascular recruitment and interstitial proliferation are interdependent during tissue morphogenesis. We show that the Vegf pathway is a primary mediator of gonad vascular remodeling and acts upstream of VE-Cadherin mediated vascular adhesion. We previously showed that PDGFRα activation is required for proliferation but not initial vascularization of the XY gonad (Brennan et al., 2003). Here we provide evidence that Pdgf signaling lies downstream of the establishment of endothelial migration and adhesion during testis morphogenesis. Specifically, addition of rPDGF-BB rescued reduced proliferation after vascular perturbation, which suggests that this endothelial-derived signal plays a critical role during PDGFRα activation. The addition of recombinant growth factors can give misleading results. However, both the similar phenotype of the Pdgfra mutants, and the fact that only levels of Pdgfb are altered in the absence of the vasculature, supports the hypothesis that endothelial cells promote proliferation by secreting PDGF-BB (Fig. 27).

Although interactions between remodeling endothelial cells and PDGFR-positive mesenchyme are common in the cardiovascular system, they are not well understood as a primary morphogenetic force during tissue growth and patterning. Our results support a model in which endothelial cells and αSMA-positive mesenchyme maintain a
dynamic relationship central to testis morphogenesis. Our results suggest that Vegf and Pdgf initiate a feed-forward relationship among endothelial cells and gonadal mesenchyme. However, it remains unclear what mechanisms coordinate ingression of the interstitium into the gonad. Two-color live imaging supports a model in which the mesenchyme expands along vessels to form physical “wedges” that divide the nascent tissue into testis cords and suggests that adhesion between the cell types is an important factor. However, these observations do not preclude the existence of a morphogen or secreted factor that may coordinate the dynamics of the various cell types. Clarifying mechanisms of endothelial-mesenchymal interaction and their cumulative effect on Sertoli epithelialization will be an important area of investigation moving forward.

The field of sex determination has assumed that the Sertoli cells mediate the formation of testis cords. As in many tissues, the idea that a highly specific cell type orchestrates development is appealing. Our work promotes a different model, and suggests that the segregation of epithelial cells into testis cord structures is driven by changes in the mesenchymal population rather than by cell autonomous forces within epithelial cells. The gonadal mesenchyme uniformly expresses many smooth muscle markers including PDGFRα/β, αSMA, and various ECM constituents. We suggest that this cell-type, common to most organs and tumors, plays a much more critical role during tissue development and repair than we have previously appreciated. Understanding the dynamic relationship between these cells and the vasculature will further our understanding of normal tissue growth and patterning in addition to identifying effective targets for control of vascularization during neoplastic growth.
Figure 27: Model summarizing vascular regulation of sex-specific proliferation.

Vegf signaling promotes migration of endothelial cells (red) toward the coelomic domain. After establishment of coherent vasculature in the coelomic domain, vascular derived PDGF-BB promotes proliferation of surrounding mesenchymal cells (stars). These vascular-mesenchyme interactions promote the formation of “wedges” into the tissue and subdivide the field of Sertoli (aqua) and germ cells (green).
6. Semaphorin3E and PlexinD1 Influence Testis Vascular Development and Consequent Patterning of Testis Cords

6.1 Summary

The morphology and patterning of the embryonic gonad is an exquisite result of highly ordered interactions. Previous chapters clarify the role of the vasculature during the initiation of testis cord formation. In particular, it appears that endothelial migration promotes the proliferation of mesenchymal cells leading to the formation of physical wedges that subdivide the XY gonad. However, our initial work does not account for the regularity and patterning that is critical during testis cord formation. How are testis cords formed at such regular intervals? Characterization of vascular migration found tracks of migrating cells, which appear to sense the surrounding environment and remain confined to discrete paths. Given the importance of vascular migration, we hypothesized that vascular patterning was an underlying determinant of cord spacing. To address this problem, we performed a candidate in situ screen of several guidance cue families including Netrins, Robos, and Semaphorins. We focused our analysis on PlxnD1 and Sema3E, a signaling pair with known roles during vascular patterning. Consistent with other systems in which they have been investigated, we find mild defects in both mutants that are likely to underlie endothelial behavior and migration.
6.2 Introduction

Vascularization of the XY gonad is one of the first sexually dimorphic events following the expression of the dominant male transcription factor, Sry. Development of the male-specific arterial vasculature and its periodic branching pattern coincides with the aggregation of Sertoli and germ cells into loops that constitute the primitive testis cords. Disruption of endothelial cell migration blocks cord formation. Recent reports proposed that feedback between endothelial cells and interstitial mesenchyme drive testis cord aggregation (Cool et al., 2011). However, whether the vasculature exerts a patterning effect on the number and spacing of cords is not known.

Testis cord aggregation is a dynamic process that occurs over the 24 hours between E11.5-E12.5. In this brief temporal window, Sertoli and germ cells aggregate into testis cords coincident with cell migration from the adjacent mesonephros and a burst of mesenchymal proliferation. Both cell migration and somatic proliferation in the gonad are required for testis cord formation (Martineau et al., 1997). Inhibition of Vegf blocked endothelial migration and prevented all of the hallmark events leading to formation of testis cords. Specifically, when vascular development was prevented, mesenchymal cells did not proliferate and failed to form an interstitial compartment of mesenchyme between testis cords (Cool et al., 2011). Based on this work, we hypothesized that the pattern of endothelial migration and/or branching might play a critical role in patterning testis cord number and/or spacing.

Live-imaging of endothelial remodeling clearly demonstrated that XY gonad vascularization occurred in at least three sequential steps (Coveney et al., 2008). (1) shortly after E11, cells in a large vascular bed running along the gonad/mesonephros border dissociated specifically in the male. (2) Individual endothelial cells that arose from the breakdown of the mesonephric vasculature migrated into the XY gonad.
displaying highly directed behavior, as evidenced by large cellular protrusions that appeared to sense extracellular cues. (3) Endothelial migration was restricted to “tracks” that appeared to be initiated by pioneer cells and then followed by subsequent cells (Fig. 2). Migratory tracks appeared at regular intervals that coincided with formation and expansion of the interstitial compartments between condensing testis cords. This final observation suggested that vascular tracks might partition the gonad into cord-forming domains and might be an early influence on testis cord condensation, spacing, and size (Coveney et al., 2008).

Receptors for neural guidance cues are expressed by endothelial cells and have characterized roles during blood vessel patterning in a variety of embryonic and adult tissues (Gu et al., 2005; Lu et al., 2004). Several families of neural guidance cues including Netrin, Slit/Robo, and Semaphorins have well characterized roles during embryonic vascular patterning. Much like axonal growth cones, endothelial cells express receptors that sense signals and adjust cell behavior based on secretion of guidance cues expressed by surrounding cell-types (Eichmann et al., 2005). Interestingly, numerous screens for sex-specific expression have identified guidance cues among the pathways that are enriched in male gonads (Nef et al., 2005; Coveney et al., 2007) This data suggested that neural guidance cues may influence male-specific vascular patterning of the testis.

To investigate this possibility, we performed an expression analysis and preliminary analysis of mutant phenotypes of the neural guidance cue Sema3E and its receptor PlxnD1. Our results show that these guidance molecules are expressed in a pattern consistent with a role during vascular patterning of the XY gonad. Functional analysis of both XY Sema3E−/− and PlxnD1−/− mutants suggested a delay in vascular development as well as increased vascular branching that coincides with an increase in
testis cord number, and a reduction in testis cord size. Generating this preliminary data was made possible through collaboration with Marc Tessier-Lavigne (Sema3E mutants), David Ginty (PlxnD1 mutants), and Fan Wang (more in situ probes than I would like to admit).
6.3 Results

6.3.1 Expression of Candidate Guidance Cues

Our first experiments focused widely on several families of neural guidance cues. First, I was interested in the role of Netrin signaling in the gonad. Netrin is a secreted ligand that typically interacts with either Unc5b or Unc40 (DCC). How netrin mediates axon guidance and, specially, the role of the receptors remains unclear but it is generally thought that netrin interactions with UNC5b results in repulsive behavior as opposed to growth cone attraction stimulated by UNC40. Both in situ and a reporter allele suggested that Netrin was expressed within testis cords while Unc-5b was specific to endothelial cells (J.C., unpublished data).

Semaphorins are a large family of neural guidance cues that interact with the Plexin and Neuropilin families of transmembrane receptors. Doug Coveney, a rotation student Uma Newton, and I performed a preliminary whole mount in situ (WISH) screen for Sema3 genes to determine if their expression patterns correlated with vascular migration. PlxnD1 and Sema3E were particularly intriguing candidates given their apparent expression on vessels and mesenchyme, respectively. To determine which cells express individual guidance molecules with greater resolution, we sorted cells from a panel of transgenic mice and then performed quantitative real-time PCR (qRT-PCR) on RNA extracted from each specific gonadal population. This panel of transgenic lines includes Sox9-ECFP (Sertoli cells), Oct4-EGFP (germ cells), aSma-EYFP (interstitial mesenchyme), and Flk1-myr-mCherry (endothelial cells), representing each cellular component of the E12.5 gonad. Our lab has subsequently performed microarrays on each cell type furthering our ability to resolve the expression of these genes to specific compartments of the XY gonad.
6.3.2 Expression of *PlxnD1* in the Embryonic Gonad

Outside of the nervous system, *PlxnD1* is widely expressed by endothelial cells (Gu et al., 2005). To determine the pattern of *PlxnD1* expression in the gonad, we used both WISH and qRT-PCR. In situ analysis of *PlxnD1* expression revealed a pattern throughout the interstitial space and excluded from testis cords (Fig. 28A). The signal appeared in clear tracks of cells that ran between presumptive testis cords. Based on in situ results alone, we were unable to determine if *PlxnD1* expression was endothelial cell specific. However, sorted cell qRT-PCR confirmed that *PlxnD1* expression was highly enriched in *Flk1*-positive endothelial cells and not *aSma-EYFP*-positive mesenchymal cells (Fig. 28B). Our results confirm that *PlxnD1* is expressed by the microvasculature found throughout the developing testis.
Figure 28: Analysis of PlxnD1 expression in XY gonads.

(A) Whole mount in situ hybridization for PlxnD1. (B) Q-PCR for PlxnD1 in each cell-type of the gonad relative to whole gonad cDNA.
6.3.3 Expression of Sema3E in the Embryonic Gonad

Using WISH, Sema3E expression appeared within the interstitium and was enriched along the coelomic surface of the E12.5 XY gonad (Fig. 29A). Cells within the coelomic domain are believed to have an intimate relationship with the vasculature that includes recruitment of endothelial cells and proliferation in response to male-specific coelomic vessel development (Cool et al., 2011). No signal was detected in the adjacent mesonephros or within presumptive testis cords. In situ analysis was confirmed by qRT-PCR for Sema3E, which was significantly enriched in aSma-EYFP cells (Fig. 29B). Low expression of Sema3E in the Flk1-positive fraction is likely to result from contamination of mesenchymal cells that adhere to vessels. Expression of the aSma-EYFP transgene is restricted to interstitial cells within the gonad, and is not found in the Sertoli or germ cell populations. Both methods of expression analysis revealed that Sema3E expression was specific to the testis interstitium and was excluded from testis cords. Expression was also absent along the gonad/mesonephros border and in the mesonephros itself.

The expression of these guidance cues in adjacent cell-types that comprise the interstitial space between testis cords was interesting in light of recent work showing that signaling within the interstitium plays an active role during testis cord condensation and patterning (Cool et al., 2011; Defalco et al., 2011). The patterns of Sema3E and PlxnD1 expression suggested they could have a role in testis cord patterning through their effects on guidance of the vasculature.
Figure 29: Analysis of *Sema3E* expression in XY gonads.

(A) Whole mount in situ hybridization for Sema3E. (B) Q-PCR for *Sema3E* in each cell-type of the XY gonad relative to whole gonad cDNA.
6.3.4 Analysis of XY PlxnD1<sup>−/−</sup> Gonads

Previous studies established that PlxnD1 and Sema3E are required during vascular patterning around condensed somitic mesoderm (Gu et al., 2005). However, in that example, where somites have already condensed, neural guidance cues provide a mechanism by which migrating endothelial cells are restricted to particular domains. Deletion of Sema3E or PlxnD1 results in vessels that are not confined to vascular regions and instead appear overly arborized. It is important to note that disruption of this signaling pair does not affect the presence or specification of vessels but rather their patterning and organization. In contrast to the somites, vascularization of the testis coincides with, and perhaps even precedes, testis cord aggregation and morphogenesis. Accordingly, we reasoned that signals regulating vessel patterning and organization would coincidently pattern testis cord aggregation.

We performed a preliminary analysis of two pairs of PlxnD1<sup>−/−</sup> E12.5 XY gonads to determine if loss of this guidance cues had a functional effect on endothelial patterning. In control XY gonads, PECAM-1 staining revealed normal vascular development including the hallmark microvascular sprouts from the coelomic vessel that separate adjacent testis cords (Fig. 30A). Effects in the mutant gonads were subtle, similar to the effects described in the somitic vessels (Gu et al., 2005). Although PlxnD1<sup>−/−</sup> gonads developed a coelomic vessel, the branches extending from the vessel were disorganized and more numerous than in wild type gonads (Fig. 30B). Coincident with the increased number of vascular branches, germ cells were aggregated into smaller clusters within testis cords that were smaller and more numerous (Fig. 30A-B, asterisk).
Figure 30: Analysis of vascular remodeling in XY \textit{PlxnD1} mutant gonads.

(A) Wild-type testis cords (asterisks) are characterized by large condensations of germ cells separated by regular intercord microvasculature branching from the coelomic vessel. (B) Testis cords still form in XY \textit{PlxnD1}^{\text{+/−}} gonads, although they were smaller and more numerous in parallel with a more highly arborized vascular pattern.
6.3.5 Analysis of XY Sema3E<sup>−/−</sup> Gonads

SEMA3E is the cognate ligand required for PLXND1 dependent vascular patterning of the somites (Gu et al., 2005). We analyzed two pairs of E12.5 XY Sema3E<sup>−/−</sup> gonads to determine if mutant testes had defects similar to those observed in PlxnD1<sup>−/−</sup> gonads. Examination of littermate controls revealed normal coelomic vessel formation and showed typical condensation of testis cords (Fig. 31A). However, like PlxnD1<sup>−/−</sup> embryos, Sema3E<sup>−/−</sup> gonads had many more vascular branches extending from the coelomic vessel (Fig. 31A-B, arrowheads). Increased vascular branching again correlated with increased testis cord number.

To quantify how the loss of PlxnD1 or Sema3E affects vascular development in the gonad, we measured the horizontal distance between adjacent vascular sprouts (Fig. 31C). We used this measurement as an indirect measure of testis cord size. Consistent with visual inspection, all PlxnD1<sup>−/−</sup> and Sema3E<sup>−/−</sup> gonads had shorter distances between adjacent vessels (Fig. 31D). Decreased inter-vessel distance was inversely correlated with the number of testis cords per image. Combined, our preliminary analysis of XY PlxnD1<sup>−/−</sup> and Sema3E<sup>−/−</sup> gonads revealed subtle effects in the mutants. While testis cord morphogenesis was not grossly disrupted, the number of vascular branches from the coelomic vessel increased (similar to results in the somitic mesoderm) and affected cord number and size.
Figure 31: Analysis of Sema3E mutants.

(A-B) Sema3E mutants had more vascular branches descending from the coelomic vessel when compared to WT controls (arrowheads). (C) Variation in branching and cord-size was quantified by measuring the distance between adjacent blood vessels that separate cords. (C) Measurements were normalized to littermate controls and showed a ~25% decrease in cord size. (D) Testis cord number was inversely related to the decrease in vascular distance.
6.4 Discussion

Recent work demonstrated the requirement for migrating endothelial cells during morphogenesis of the embryonic testis. However, it remained unclear whether the patterning of vasculature actively influences the spacing and number of testis cords. The current preliminary results suggest that neural guidance cues are one mechanism by which vascular development in the XY gonad is patterned. Interestingly, differences in vascular patterning affected testis cord number and spacing.

This study provides additional evidence that signaling between vessels and the interstitial mesenchyme is of central importance during testis morphogenesis. Multiple expression assays showed that interstitial cells express Sema3E while endothelial cells express its receptor, PlxnD1. Our limited mutant analysis provides support for a subtle functional role of Sema3E and PlxnD1 during vascular patterning of the testis. Both Sema3E-/- and PlxnD1-/- XY testes displayed identical phenotypes. Mutant organs had highly branched microvasculature that was associated with decreased testis cord size. We propose that neural guidance cues provide patterning signals from the interstitium to endothelial cells as they are attracted into the XY gonad by signals such as Vegfa. It remains unclear whether guidance cues affect the initial pathways of migration of endothelial cells or only the subsequent branching of microvasculature from the coelomic vessel. In recent work, we showed that the endothelium induces proliferation in the surrounding mesenchymal cells and patterns the interstitium of the testis (Cool et al., 2011). In mutants where vascular branching is increased, this mechanism could lead to subdivision of the gonadal field into more numerous testis cords. Interactions between these cells types and mechanisms that regulate their collective dynamics remain poorly understood. A higher resolution analysis, such as live imaging of endothelial and interstitial cells in mutant gonads, might allow us to directly observe
differences in cell behavior and further our understanding of the roles of these guidance molecules in organ morphogenesis.
7. Vascular control of mesenchymal differentiation

7.1 Introduction

In work described in previous chapters, we have examined the influence of endothelial cell migration and patterning on morphogenesis of the testis. Functional analysis led to the finding that endothelial migration is required for mesenchyme proliferation. Coincident with proliferation, vessels and mesenchyme establish a tight relationship that leads to the formation of wedges that subdivide the tissue. Live imaging of vessels and mesenchyme revealed that wedges extend into the XY gonad along the preexisting vascular tracks. Wedge formation may be explained by differential adhesion among the cells within testis cords and the emerging interstitium.

The mesenchymal component of the interstitium is a poorly understood heterogeneous population. Despite the various sex-specific cell-types that arise from this population, early markers specific to subsets of the population have been extremely difficult to identify. Recent work described a number of markers that label discrete populations of the interstitium (Defalco et al., 2011). Between E11.5 and E12.5, testis cord morphogenesis proceeds and the interstitium expands in response to vascular migration. During this stage, molecular markers of both mature and progenitor cells are expressed throughout the interstitium, suggesting a single pool of mesenchymal progenitors capable of adopting a variety of fates. Between E12.5-E13.5 sex-specific cell-types begin to appear in the interstitial space. As specialized cells arise, the uniformly expressed markers begin to diverge and label specific groups of cells.

Development of interstitial markers correlates with vascular remodeling. Mesenchymal proliferation in response to vascular migration established a clear role for vessels during expansion of the multi-potent pool of cells within the XY gonad. However, live imaging suggested that the coordinated behavior of endothelial and
mesenchymal cells persisted after proliferation. This lead to the hypothesis that vascular derived signals regulate differentiation of the interstitium from E12.5 onwards.

Endothelial derived PDGF-BB is sufficient to initiate proliferation in the absence of vessels. Genetic evidence strongly suggests that the vasculature signals through Pdgfra expressed by the mesenchyme. Mesenchymal proliferation is reduced in Pdgfra mutants despite the presence of vascular migration, supporting its function downstream of vascular migration. In addition to a defect in proliferation. Leydig cells, the male neuroendocrine cells, are significantly reduced in Pdgfra mutants (Brennan et al., 2003). Reduced numbers of Leydig cells in XY Pdgfra mutants may be proportional to the reduced proliferation and reflective of a hypocellular tissue. However, an alternate hypothesis is that Pdgfra regulates mesenchyme differentiation after proliferation.

To address how PDGFRα mediates mesenchymal response to vascular remodeling, we revisited mutants lacking this receptor. Utilizing a new H2B-EGFP knock-in to the Pdgfra (PdgfraGfp) locus, we were able directly observe the behavior of cells with time lapse microscopy. Two-color live imaging enabled analysis of Pdgfra-positive cells and their response to endothelial cells migration. Imaging confirmed that mutant PdgfraGfpGfp cells were not only less abundant but also displayed behavioral defects when compared to heterozygous PdgfraGfpGfp cells. Mutant Pdgfra cells did not aggregate around vessels and remained significantly more rounded than controls. Consistent with this, mutants did not activate subsets of interstitial genes that are vascular responsive. This data showed that while Pdgfra is critical for mediating the proliferative response to vascular migration, it is further required for promoting differentiation of specialized cell-types in response to vascular remodeling.
7.2 Results

7.2.1 Analysis of *Pdgfra*-H2B-EGFP

Our lab previously showed that *Pdgfra* is required for male-morphogenesis. This work characterized multiple male-specific events that require *Pdgfra*, including expansion of the interstitial mesenchyme, cell migration, testis cord morphogenesis, and fetal Leydig cell development. However, the mechanisms by which *Pdgfra* mediates these aspects of testis morphogenesis have remained unexplored. A difficult aspect of the initial study was the early embryonic lethality of mutant litters. The original *Pdgfra* mutant allele (Soriano, 1997) was backcrossed to several inbreed backgrounds, but litter sizes remained small and lethality was not significantly rescued.

*Pdgfra*-H2B mice have significantly better viability and exhibit previously described defects in testis development. To visualize *Pdgfra*-expressing cells, Phillipe Soriano’s lab generated a knock-in line that inserted EGFP tagged histone 2B (H2B) into the coding region of *Pdgfra* (Hamilton et al., 2003). Accordingly, embryos homozygous for the H2B allele lack a functional *Pdgfra*. This mutant line was reported to have better viability than the original targeted mutation. It is unclear whether this is a background effect (outcross to CD-1 to enhance viability) or the allele is in fact a lowly expressing hypomorph.

Expression of H2b-EGFP in heterozygous animals recapitulated previously reported in situ patterns and was found throughout the XY interstitium. Expression of the transgene was heterogeneous, although the extremely stable nature of the H2b-EGFP protein confounds our ability to make definitive conclusions as to which cells are actively expressing *Pdgfra*. Interestingly, a subset of Sertoli cells were faintly positive. Given protein stability, this could be indicative that some Sertoli cells arose from a common *Pdgfra* positive progenitor or, more likely, that some Sertoli cells express the
receptor at low levels that were not detectable by in situ (Fig. 32A, arrowheads). To address this, early XY gonads were stained with antibodies specific to early Sertoli progenitors. At 16 tail somites, Pdgfra is enriched along the border of the gonad and mesonephros and also found within the interior of the gonad (Fig. 32A). SOX9-positive Sertoli progenitors are found through much of the gonad but appear to be either negative for Pdgfra or are only faintly EGFP-positive (Fig. 32A, arrowheads). Staining with DMRT1 also partially overlapped with Pdgfra-low cells. The expression domain of Pdgfra expands as development proceeds and by E11.75 includes cells throughout the coelomic domain (Fig. 32B).

Despite the enhanced viability of the Pdgfra-H2B line, XY PdgfraαCfp/Cfp gonads recapitulated every aspect of the previously described null phenotype. At E14.5 fetal Leydig cell numbers were severely reduced (Fig. 32C). Testis cord morphogenesis was disrupted and resulted in large looping cords at all stages examined (Fig. 32E). Additionally, a detailed analysis of proliferation showed that proliferation throughout the gonad was reduced, although Pdgfra-positive cells were more severely affected (Fig. 32D). Divisions within the coelomic epithelium were not strongly affected, whereas mesenchymal cells around vessels failed to divide at wild-type rates. This finding is consistent with the ability of Pdgf to rescue reduced proliferation in the absence of vascular development.
Figure 32 (Page 125): Analysis of Pdgfra-H2B gonads.

(A) In the early XY gonad (16ts), Pdgfra is expressed in many somatic cells. Pdgfra-high cells are not SOX9 positive, although a subset of SOX9 cells express low levels of Pdgfra (arrowheads). (B) Expression of Pdgfra in XY gonads during morphogenesis. Reporter expression is faintly detected in a subset of Sertoli cells (arrowheads). (C) Proliferation is reduced in Pdgfra<sup>Gfp/Gfp</sup> gonads. Staining with pH3 (green) shows reduced proliferation specifically in somatic cells below the PECAM1-positive coelomic vessel (red). (D) Leydig cell differentiation was significantly reduced in Pdgfra mutants, as previously described. Dotted lines outline the gonad. (E) Pdgfra<sup>Gfp/Gfp</sup> mutants displayed severely disrupted cord formation at all staged examined. All scale bars are set to 50um.
7.3.2 Time-lapse imaging of $Pdgfra^{Gfp/Gfp}$

$Pdgfra$ regulates mesenchymal behavior during vascularization. Quantification of proliferation revealed a significant defect associated with the loss of $Pdgfra$. However, this result did not clarify the mechanisms by which $Pdgfra$ regulates testis cord morphogenesis. We reasoned that if gonads were simply hypo-cellular, we would expect mutant organs to be smaller but still capable of developing limited structures and differentiated cell-types. Original characterization of $Pdgfra$ mutants showed the formation of primitive wedge-like structures of interstitium at branch points along the coelomic vessel that failed to force Sertoli cells into testis cords (Brennan et al., 2003).

To characterize vascular-mesenchyme interactions in the absence of $Pdgfra$, we utilized live imaging of Flk1-myr-mCherry; $Pdgfra$-H2B XY gonads. Double transgenics allowed us to simultaneously image $Pdgfra$-positive mesenchyme and its behavior in regard to endothelial cells. Imaging initiated at E11.75 revealed that heterozygous $Pdgfra^{Gfp/+}$ gonads quickly form a coelomic vessel and induce the aggregation of GFP-positive mesenchyme around microvasculature (Fig. 33A). $Pdgfra^{Gfp/Gfp}$ mutants still recruit endothelial cells and form a coelomic vessel, although formation of the vessel was not apparent until the end of the imaging timeframes suggesting a slight delay (Fig. 33B, arrowhead). The finding that $Pdgfra^{Gfp/Gfp}$ gonads recruit endothelial cells from the mesonephros is contrary to previous reports. However, live-imaging is far more sensitive than previous analysis and does confirm that the process is less robust than in wild-type XY gonads. The more striking finding is that although vasculature enters the gonad, EGFP-positive cells fail to respond. $Pdgfra^{Gfp/Gfp}$ positive cells are found throughout the interior of the gonad as well as the coelomic domain. The behavior of mutant cells is erratic and random in contrast to the $Gfp/+ controls in which cells quickly associate with the vasculature (Fig. 33A-B). Live imaging suggests that mutant cells not
only proliferate at a slower rate but also fail to respond to and associate with remodeling vasculature.
Figure 33 (Page 129): Live imaging of $Pdgf\alpha^{Gfp/Gfp}$ behavior in relation to endothelial remodeling.

$Flk1$-mcherry mice were crossed onto the $Pdgf\alpha$ background to generate double heterozygous animals in which endothelial cells (red) were labeled along with the $Pdgf\alpha$-positive interstitium. Littermates were imaged starting at E11.75. $Pdgf\alpha^{Gfp^+}$ gonads developed a coelomic vessel by E12.0. $Pdgf\alpha$-positive cells aggregated around developing vasculature to form wedges into the gonad. $Pdgf\alpha^{Gfp/Gfp}$ gonads recruit endothelial cells, although coelomic vessel formation is delayed. $Pdgf\alpha$-positive cells do not respond to vascular migration and fail to aggregate around developing vessels. In real-time, mutant cells migrate erratically and appear oblivious to the presence of endothelial cells.
7.3.3 \textit{Pdgfra} cells do not cluster around vessels

To confirm live-imaging results, we analyzed fixed tissue and quantified the association of \textit{Pdgfra}-positive cells with blood vessels. Fixed tissue was stained with PECAM1 to label endothelial cells and confocal Z-stacks were collected. After imaging, we identified individual in-plane vessels and determined a threshold fluorescence to accurately reflect their location. Multiple in-plane vessels were used within each gonad to determine the proportion of \textit{Pdgfra}-positive cells that lay within, or outside, of a 15\textmu m range from the vessel. In \textit{Pdgfra}^{Gfp/+} controls, the vast majority of cells lay within 15\textmu m (Fig. 34A). The few cells that lay outside this range appear to be a small population of the interstitium or the subpopulation of Sertoli cells that label weakly. Consistent with live imaging results, \textit{Pdgfra}^{Gfp/Gfp} cells do not accumulate around vessels and are found distributed throughout the tissue (Fig. 34B). Even though some of these cells are likely Sertoli cells, it is clear that the population of interstitial cells normally associated with vessels is absent.
Figure 34: Pdgfrα promotes mesenchymal response to vascular remodeling.

To measure the proximity of Pdgfrα-H2B-positive nuclei to blood vessels, we generated masks of vasculature structure in E12.5 Pdgfrα<sup>Gfp/+</sup> (A) and Pdgfrα<sup>Gfp/Gfp</sup> (B) XY gonads. Masks were then dilated to extend 15um from their original surface. (A-B) Positive nuclei were pseudocolored to indicate whether they were greater than (aqua) or less than (red) 15um from a vessel.
7.3.4 \textit{Pdgfra} cells remain spherical

Another observation from fixed analysis was that in the absence of \textit{Pdgfra}, GFP-positive cells remained round and morphologically unspecialized. Three-dimensional reconstruction of \textit{Pdgfra}^{Gfp/+} gonads showed that GFP-positive nuclei became elongated and tightly associated with blood vessels by E12.5 (Fig. 35A). Further, they were clearly partitioned into the interstitial space and excluded from the negative space in which testis cords formed. In light of live imaging and two-dimensional static analysis, it appeared that \textit{Pdgfra}-positive cells were attracted to vasculature where many cells adopt a specialized shape in which they elongate and are closely associated with vessels. Analysis of \textit{Pdgfra}^{Gfp/Gfp} mutant gonads showed that GFP-positive cells were not vascular associated and remained rounded (Fig. 35B, arrowhead). Mutant interstitial cells were found throughout the tissue and, consistent with live-imaging, did not aggregate into a distinct interstitial compartment along with endothelial cells. Although endothelial branching is reduced in \textit{Pdgfra} mutants, the vessels that are present fail to induce \textit{Pdgfra}-positive cells to acquire the distinct morphology observed in controls.

Three-dimensional analysis revealed that mutant cells remained rounded and failed to acquire specialized morphology associated with many interstitial cells. To address this on a more global scale, we developed a method to measure cell-shape more systematically. Initial attempts to perform this analysis in three-dimensions were confounded by the poor Z-resolution of confocal microscopy. Instead, we used high-magnification Z stacks and set parameters that recognized and segmented the nuclei in a given optical section. \textit{Pdgfra}-H2B gonads were used such that analysis focused on cells that are poised to respond to Pdgf signaling. Although the signal varied from litter to litter, the parameters for segmenting nuclei was kept constant (see materials and methods).
To analyze the 3D orientation of $Pdgfr\alpha$-positive cells and blood vessels, we generated surface models of dense confocal Z-sections. $Pdgfr\alpha$-positive nuclei (green) were imaged in relation to Flk1-positive (red) endothelial cells. (A) $Pdgfr\alpha^{Gfp/+}$ interstitial cells developed a squamous morphology that was tightly associated with blood vessels. (B) $Pdgfr\alpha^{Gfp/Gfp}$ mutant cells were scattered throughout the gonads, were not closely associated with vessels, and retained a globular and rounded morphology (arrowheads).
Nuclear shape is highly correlated with cell shape. Although this is not initially apparent, staining for cell surface markers and interstitial nuclei revealed that the morphology of the nucleus accurately reflects the overall shape of individual cells. Subsequent EM analysis confirmed this observation in much greater detail. At the EM level the heterogeneous shape of nuclei is clearly correlated with slight variations in cell-shape (Fig. 36A). Recent reports suggest that cell shape and nuclear shape have a correlation coefficient of \( \sim 0.9 \), consistent with our analysis (Minc et al., 2011). Thus, segmenting nuclei and measuring shape is a reliable proxy for observing overall shape of the cell (Fig. 36B). In complex tissue this methodology presents significant advantages since accurately segmenting cells with adjacent walls is nearly impossible.

Calculating spherical index across many cells revealed a general decrease in mutant cells compared to heterozygous littermates. To systematically compare cells, we calculated the individual spherical index ("SI") (major axis/minor axis). Calculations assumed that nuclei are roughly elliptical and accurately reflects even slight variation in SI (Fig. 36B). At E12.5 \( Pdgfr\alpha^{Gfp/Gfp} \) nuclei had a mean SI of \( \sim 1.87 \). Control \( Pdgfr\alpha^{Gfp/+} \) nuclei had a much broader distribution, although the mean was centered at \( \sim 1.95 \) (Fig. 36C). SI of individual cells was mapped back to segmented nuclei and showed that even subtle changes in SI were reflected in visually dramatic differences in cell shape (Fig. 36B). Although the difference of the mean is significant, the more striking feature of the analysis was that only 2% of mutant cells attained a SI of greater than 3.0 compared to 5% of heterozygous cells (Fig. 36D). At E13.5 the same analysis showed that 6% of mutant cells attained a SI of \( \geq 3.0 \) whereas nearly 15% of the control interstitium adopted a highly elongated and specialized morphology (Fig. 36D).
Figure 36 (Page 136): Nuclear shape is disrupted by loss of \textit{Pdgfra}.

(A) Electron micrograph of E12.5 gonad mesenchyme indicating nuclei (red) and cytoplasm (blue) confirming that nuclei shape is highly reflective of cell shape. (B) Individual nuclei were systematically detected in non-overlapping Z-stacks of \textit{Pdgfra} mutants and littermate controls. (B) Spherical index (SI) was calculated using individual measurements of nuclear shape. Small changes in SI reflect morphological heterogeneity within \textit{Pdgfra}-positive nuclei. (C) SI of \textit{Pdgfra} mutant cells are clustered toward a more spherical shape. To determine the ratio of highly specialized and elongated cells within each sample, we measured the percentage of cells with a SI greater than or equal to 3.0. (D) At both E12.5 and E13.5 significantly fewer \textit{Pdgfra} mutant cells adopted an elongated morphology.
7.3.5 Pathways downstream of *Pdgfra*

Live imaging and cell shape analysis suggest that *Pdgfra* mediates not only cell number, but also cellular morphogenesis. We next sought to identify pathways mediating this effect. To address this question, we used FACS to isolate *Pdgfra*<sup>Gfp/+</sup> and *Pdgfra*<sup>Gfp/Gfp</sup> gonads and extracted RNA from the GFP positive fraction. By sorting GFP-positive cells, analysis was restricted to cells competent to receive Pdgf signals. Expression from mutant cells was compared to littermate controls using qRT-PCR for a panel of candidate genes. Among the candidates were early markers of the interstitium, vascular associated cells, and factors involved in recruiting vasculature into the XY gonad.

Among the candidates, several showed reduced expression in *Pdgfra*<sup>Gfp/Gfp</sup> cells. Previous expression results, including the Wnt4 microarray screen had identified *Sdf1a* as a gene associated with male vascular remodeling. Additionally, cell sorting confirmed that expression of *Sdf1a* is enriched within the mesenchyme. *Sdf1a* expression was two-fold lower in *Pdgfra*<sup>Gfp/Gfp</sup> cells. The function of SDF1α is well characterized within the immune system and specifically attracts CXCR4-positive cells to regions of inflammation (Kucia et al., 2004). Further, it is a therapeutic target for AIDS treatment and stem cell mobilization. AMD3100 is a specific small molecule that inhibits CXCR4 interactions with SDF1α, preventing the attraction among positive cells (for review see, De Clercq, 2003).

AMD3100 inhibits vascular development in XY gonads. In E11.5 gonads treated with AMD3100, coelomic vessel formation was disrupted, although some vessels were still observed in the interior of the gonad (Fig. 37A-B). The effects of AMD3100 treatment were variable, although ranging from the formation of a thin coelomic vessel to total inhibition of the coelomic vessel formation. Proliferation and mesenchymal expansion
are also reduced after AMD3100 treatment, consistent with experiments using VEGF Trap. Unlike VEGF-Trap, treatment with AMD3100 could block vascular development in gonads treated after E11.5. The role of SDF1α/CXCR4 signaling in the early gonad remains unclear, although this preliminary data suggests that it is an early factor required for initiating feedback between endothelial cells and remodeling vasculature.

Vascular-mesenchyme interactions are required for the maintenance of testis cord shape and structure. Experiments using VEGF Trap established that recruitment of vessels into the gonad is required to initiate morphogenesis of testis cords. However, after the initial de novo formation of cords, cords continue to remodel. Treatment with AMD3100 at E11.5 suggested that this signaling pathway is downstream of VEGF and promotes mesenchyme-vascular interactions. To characterize CXCR4-SDF1α function in greater detail, we treated E12.5 XY gonads with AMD3100. By E12.5 the male-specific arterial network is formed and testis cords have aggregated. Amazingly, E12.5 gonads cultured with AMD3100 developed extreme vascular dilation and irregularly shaped cords (Fig. 37D-E). Thus, vascular-mesenchyme interactions are required for the maintenance of testis cord morphology.

Dilated vessels coincide with relaxation of the mesenchymal compartment. E12.5 Pdgfrα<sup>Gfp</sup> + gonads were cultured with AMD3100 to determine if nuclear shape was affected by SDF1α-CXCR4 signaling. Inhibitor treatment resulted in a widening of the interstitial space and fewer elongated vascular associated cells (Fig. 37C'-D'). In light of the Pdgfrα phenotype and VEGF Trap experiments, SDF1α appears to mediate attraction between vessels and mesenchymal cells, which then respond to additional cues to drive cell shape changes and promote microvascular development. Like many inhibitors, the effect of AMD3100 was variable and ranged from mild disruption (Fig. 37D) of interstitial aggregation to severe cases in which the interstitium became extremely
distended (Fig. 37E). Our results suggest that \textit{Pdgfra} promotes cell-shape change through SDF1α and CXCR4. This interaction requires in vivo validation.
Figure 37 (Page 141): Inhibiting CXCR4-SDF1α partially phenocopies loss of \( Pdgfra \).

AMD3100 was used to specifically inhibit SDF1α/CXCR4 signaling at various time-points of XY gonad development. (A-B) Treatment at E11.5 inhibits formation of the coelomic vessel (A, arrow) in gonads treated with AMD3100. (A) Interstitial development is typically highly ordered and tightly associated with vessels. (B) AMD3100 results in defective vascular remodeling but also disorganized and poorly formed interstitial space. E12.5 \( Pdgfra\)-Gfp/+ (green) gonads were cultured with AMD3100 for 48 hours. (C-C') Control XY gonads maintained vasculature with closely associated \( Pdgfra\)-positive cells. (C') Higher magnification of boxed region shows elongated morphology of nuclei adjacent to vessels. (D) Gonads treated with AMD3100 show regression of the coelomic vessel (yellow arrow) and dilated interstitial space (white arrows). (D') \( Pdgfra\)-positive cells that are adjacent to the remaining vasculature become rounded and loosely associated with vessels. (E) AMD3100 treatment is variable and in some cases results in extreme relaxation of the interstitial space (arrows).
7.3.6 Expression analysis of Pdgfrα-H2B-EGFP

We next asked whether Pdgfrα inhibited the expression of vascular induced markers. Cell shape analysis suggested that when vascular-mesenchyme interactions are perturbed, mesenchymal cells fail to adopt specialized morphologies. However, it remained unclear whether these cell shape changes were reflective of changes in the expression profile of these cells. To address whether specialized interstitial cells were developing within the interstitium we measured expression of GFP-positive cells isolated from XY PdgfrαGfp/Gfp and PdgfrαGfp/+ gonads. Preliminary analysis focused on genes associated with two cell-types of interest: (1) Perivascular cells, based on our finding that Pdgfrα blocks the formation of elongated cells associated with the vasculature and (2) Leydig cells, because their development is affected by loss of Pdgfrα by an unknown mechanism (Fig. 38A).

VCAM1 is expressed broadly in the interstitium and its expression requires vascular development. In sorted Pdgfrα-positive cells, VCAM1 expression is reduced to ~30% of heterozygote levels at E13.5 (Fig. 38A). Antibody staining at several stages confirms the inefficient expression of VCAM1 in Pdgfrα mutants. At E12.5, vasculature invades the XY Pdgfrα mutant gonad, but VCAM1 is expressed in very few cells (Fig. 38B,E). Cells that persist in expressing VCAM1 are scattered throughout the tissue and appear disorganized. In E13.5 mutants, VCAM1-positive cells expand and can be found within the coelomic domain (Fig. 38B,E). Consistent with results at E12.5, staining indicates that cells remain disorganized and rounded. Also, mutants appear to have fewer VCAM1-high cells compared to controls. This is particularly clear a day later at E14.5 (Fig. 38C,F). At E14.5, WT gonads have a very dense layer of mesenchyme that surrounds the testis and extends through the interstitial space between testis cords (Fig.
However, in Pdgfra mutants VCAM1 is sporadically expressed at low levels (Fig. 38F).

Many vascular smooth-muscle genes are also downregulated in Pdgfra mutants. Notably, Pdgfrβ and Jag1 were significantly reduced in XY mutant cells (Fig. 38A). Of these Jag1 is an intriguing ligand given the importance of Notch signaling for the maintenance of fetal Leydig progenitor cells. The notch ligand responsible for stimulating notch signaling in progenitors is unknown. Markers of Leydig progenitor cells are also reduced in Pdgfra mutants raising the possibility that fetal progenitor cells overlap, or are functionally reliant on, perivascular cells. We are currently attempting to clarify vascular regulation of Leydig progenitor cell specification and maintenance.
Figure 38: Pdgfrα mutant cells fail to commit to specialized fates.

(A) Pdgfrα<sup>Gfp/Gfp</sup> and Pdgfrα<sup>Gfp/+</sup> cells were sorted from individual gonads. RNA was extracted from sorted cells and expression was analyzed by qRT-PCR. All values are relative to Pdgfrα<sup>Gfp/+</sup> sorted cells. (B) E12.5 WT gonads contain many cells throughout the interstitium are positive for the cell adhesion molecule VCAM1 (purple). At E13.5 (C) and E14.5 (D) expression restricts slightly and is enriched on cells proximal to blood vessels and within the coelomic domain. (E) In Pdgfrα mutants, expression is nearly absent at E12.5 despite the presence of vascular migration. (F) E13.5 mutants contain more VCAM1 positive cell, although expression is reduced and VCAM-1 high cells are very rare. (G) Reduced VCAM1 expression remains reduced at E14.5.
7.4 Discussion

Morphology meets function at the vascular wall – or so it appears. Our systematic analysis of cell shape revealed that Pdgfra promotes the acquisition of mesenchymal cell-shape and attraction to vessels. The extent to which these two are related remains under investigation. Electron microscopy has revealed that a subset of interstitial cells form physical junctions with endothelial cells that may play a critical role in mediating endothelial-mesenchymal interactions.

Previous experiments presented in chapter 5 clarified the requirement for vascular migration to initiate proliferation of the mesenchyme. In addition, live-imaging of vascular-mesenchyme interactions showed that endothelial cells and interstitial mesenchyme show coordinate behavior. Concurrent with proliferation, mesenchyme aggregated around vascular sprouts. Collective behavior of endothelial and mesenchymal cells is akin to Sertoli-germ cell aggregation and may be the primary physical force that drives testis cord aggregation.

Pdgfra is a critical signal to promote vascular-mesenchyme interactions. In chapter 5 we suggest that PDGF-BB promotes mesenchymal proliferation through Pdgfra. Beyond proliferation, Pdgf signaling is also critical during vascular attraction of mesenchyme. Live imaging of Pdgfra-H2B mutants revealed that mutant cells do not efficiently respond to vascular remodeling. Mesenchymal cells do not aggregate around vessels and “wedges” are rarely observed. Mutant mesenchymal cells remain dispersed throughout the gonad, resulting in the formation of large poorly formed testis cords.

Pdgfra regulates cell shape. Live imaging and static analysis found that mesenchymal cells fail to associate with vessels. However, we also observed that mutant cells lacked the morphological heterogeneity normally observed within the interstitium. In particular, specialized cells that become elongated were sparse in Pdgfra mutant
gonads. Systematic analysis of nuclear shape at E12.5 and E13.5 confirmed this observation. Expression analysis of mutant gonads identified SDF-1/CXCR4 signaling as a candidate Pdgfra-dependent pathway regulating vascular-mesenchyme attraction and retention. Inhibition of CXCR4 disrupted this relationship and phenocopied disrupted cell shape in Pdgfra mutants. Thus, SDF1α/CXCR4 likely function downstream of Pdgfra and promote vascular attraction and acquisition of specialized cell shapes.

Development of perivascular cells is affected in Pdgfra mutants. Based on the observation that perivascular morphology was lost, we found that molecular markers of perivascular cells were also reduced. This data argues that Pdgfra is required for differentiation of specific perivascular cell-types, although the specific mechanisms involved require further investigation. We continue to be very interested in clarifying how fetal Leydig progenitor cells establish, or land in, a niche that supports their undifferentiated state. We are hopeful that additional experiments will clarify the role that vascular attraction and the resulting gene expression changes plays in this process.

Ongoing experiments are focused on the relationship between perivascular fate and cell shape. Analysis of Pdgfra mutants showed that cell shape and commitment to specific lineages are both disrupted. However, once cells are associated to the vascular wall, it is unclear what the primary determinant of cell fate is. One possibility is that cells begin to adopt a characteristic elongated shape and signaling communicated by the cytoskeleton influences the expression profile of perivascular genes. At this point, the converse hypothesis is also possible. It also remains unclear whether physical association with the endothelium is required for changes in expression profile and cell shape. Experiments to specifically address these questions are ongoing.
8. Conclusion

When this work began, there were several experiments suggesting that endothelial cells influenced development of the gonad. First, it was well known that vascular development was closely related to testis cord aggregation. Second, while it was thought that migrating PTM cells initiated cord formation, these cells were never positively labeled and specific molecular markers were unknown. Third, it was reported in several mammalian organs that endothelial cells were required for development. Finally, detailed characterization of endothelial migration into XY gonads revealed that endothelial cells were excluded from domains where testis cords form.

Our first experiments clarified that PTM cells do not migrate into XY gonads during the critical window of sex-determination. This simple, but critical, set of experiments clarified the hypothesis that followed: Endothelial cells provide critical signals required for male-specific morphogenesis. Characterization of vascular development was consistent with our hypothesis, but there was no mechanistic data supporting this view. Inhibition of Vegf signaling blocked vascular remodeling and, as a consequence, prevented male morphogenesis. To our surprise, vascular remodeling is not directly regulated by Sertoli cells. Vegfa is expressed by mesenchyme and vessels promote cord formation by initiating proliferation of the same population. This positive feedback loop between vessels and mesenchyme shed a new light on how we think about testis cord formation. Our results also highlight the importance of undifferentiated mesenchymal cells during morphogenesis. Cord formation is not all about Sertoli cells. As a matter of opinion, I think it is all about the interstitium and the Sertoli cells are not the dominant force in the school yard that many believed them to be.

After clarifying a clear functional role for endothelial cells in the gonad, I became fascinated by the cell biology and behavior of endothelial-mesenchymal interactions.
Endothelial derived PDGF-BB appeared to be one mechanisms by which mature vessels influenced mesenchyme. However, the phenotype of \( Pdgfr \alpha \) mutants is far more complex than a simple defect in proliferation. Inhibitor treatment followed by short-term organ culture constrained our ability to examine later stages of development, including questions related to continued endothelial-vascular interactions. To better address vascular-mesenchyme interactions, we revisited \( Pdgfr \alpha \) mutants.

Analysis of \( Pdgfr \alpha \) mutants is ongoing, but promises to clarify mechanisms of interstitial development. Preliminary data shows that \( Pdgfr \alpha \) mutant mesenchyme does not aggregate around blood vessels. Further, cells do not express markers of specialized lineages and remain morphologically round. Given these observations, it appears that the vasculature influences development of the interstitium beyond proliferation. Vascular regulation of mesenchymal progenitors during development and tissue homeostasis is a poorly understood field with very general implications. I am specifically excited about clarifying how the two cell-types interact to direct differentiation.

The influence of cell shape on differentiation is an emerging field of study and may clarify how a large pool of mesenchymal progenitors differentiates. Interstitial mesenchyme is morphologically heterogenous well before molecular markers distinguish sub-populations. Could morphology induce or bias fate within these undifferentiated cells? What influence does physical association with vasculature have?

What we can conclude at this point is that vascular remodeling is required for male morphogenesis and is regulated by Vegf signaling. Vessels fulfill this requirement by initiating a relationship with undifferentiated mesenchymal cells and promote their proliferation through the Pdgf pathway. The mesenchyme proliferates and aggregates around vessels. We cannot exclude a diffusible signal and favor a model in which
aggregation forms physical wedges that separate testis cords. We found that \textit{Pdgfra} is required for aggregation of mesenchyme around vessels and may promote this process through regulation of \textit{Sdf1a}. Further, aggregation and perhaps adhesion to vessels may direct the differentiation of the undifferentiated mesenchyme.
9. Future Directions

This project had its moments, not the least of which is excluded from this thesis: for nearly two years we attempted to delete Vegfa in vivo only to find that we had been given a mouse lacking a critical loxp site. However, I am pleased with the fact that we WERE able to clarify a definitive role for vascular remodeling. Our progress also includes clarifying the first mechanisms directly regulating vascular development in the gonad. The identification of vascular-mesenchyme interactions were a surprising result of this work but pose perhaps the most interesting directions moving forward.

There are so many questions that remain or were generated by this work that this section feels oddly unfair. We now have some concept of why endothelial cells enter the XY gonad but no idea why they do so in a limited temporal window. The signals that destabilize the large vascular bed along the gonad/mesonephros border are totally unknown. Wnt4 is thought to be involved, but nothing is known about the cell-types or mechanisms at play.

Endothelial migration is downstream of Sry. Given this, there is presumably a signal from Sertoli cells that promotes early mesenchyme development and indirectly promotes migration. Current efforts in the lab are utilizing genome-wide analysis to characterize expression in specific lineages. This systems level analysis is likely to uncover key changes during gonad development and signals among various cell-types. We would predict that early signals that partition the Sertoli and mesenchymal cell populations exist within this network and may clarify this gap in our understanding.

The final direction that I am currently pursuing is how endothelial cells influence mesenchymal development. Multiple lines of evidence suggest that perturbation of vascular remodeling affects certain mesenchymal lineages more than others. The mechanisms underlying this are unclear. Before we started this work the mesenchyme
was poorly characterized and largely overlooked. Given our newly developed tools and increased ability to isolate, image, and distinguish mesenchymal cells I believe we are poised for a breakthrough. Careful characterization of the mesenchymal compartment reveals an amazingly dynamic group of cells that encapsulate questions directly related to progenitor maintenance, vascular biology, and hormonal signaling.

A particular interest of mine moving forward is the environments that cells reside in. How does a vascular niche form? What are critical characteristics of a niche? Vascular niches in the brain and pancreas have propagated the idea that vessels maintain progenitor populations. However, we do not know how these niches are assembled. Our recent work suggests that a vascular niche may exist in the gonad and promote Notch mediated maintenance of Leydig progenitor cells. Given our ability to image the various cell-types in real time it may be possible to describe how vascular niches are generated. We are currently developing in vitro co-culture assays that seeks to mimic in vivo vascular-mesenchymal interactions and allow us to test individual questions in a focused and isolated environment.

Lastly, our observation that cell shape correlates with cell fate has me very excited. Seminal work has established that cell shape and physical environment are strong determinant of cell identity. Mechanisms mediating this effect remain unclear, but the fact remains that the morphology of divergent cells is not merely an artifact of their fate – it can determine fate. The gonadal interstitium is morphologically heterogeneous from an early stage but specific markers have not been found. This suggests that cell shape changes anticipate molecular changes in cells. We hope to directly test whether cell shape is capable of influencing commitment to various mesenchymal lineages. There are many mechanistic questions underlying this observation. How is the cytoskeleton involved in specific gene regulation programs? Is
adhesion between various cell-types required for morphological changes? Extracellular matrix deposition is also a little understood influence during gonad development that has the potential to explain both morphogenesis at the level of cells and how large aggregations of mesenchyme are able to “force” their way into the gonad.

In conclusion, my work has made significant in-roads toward understanding testis morphogenesis. We now know that the vasculature and mesenchyme play a significant role that was previously unappreciated. Further, the initial mechanisms and integration with known pathways provides a nucleating point for future work to build upon. We have a glimmer of understanding about how various cell-types in the gonad behave. What we still do not understand is the underlying cell biology of individual cells and their contribution to the whole.
## Appendix A: QPCR Primers

Table 1: Table listing of QRT-PCR primers and sequences.

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VEGFA  F  CAGGCTGCATGAAAGCATGAA
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References


Parma, P., Radi, O., Vidal, V., Chaboissier, M.C., Dellambra, E., Valentini, S., Guerra, L.,


Biography

Jonah Cool was born in Menlo Park, California on April 28th, 1982 to Jesse and Robert Cool. He was raised as a good hippie child in Palo Alto, California prior to attending Ohlone Elementary where he learned to garden, Jane Lathrop Stanford Middle School where he learned to eat junk food, and finally graduated in 2000 from Henry M. Gunn high school where he learned there is much to learn out of class. Jonah then attended Wheaton College (MA) where he was intoxicated by biology after starting as a classics major. Throughout the summers, and after graduation, he worked in IP litigation but missed science and the ability to synthesize new ideas. In 2005 Jonah joined the Developmental Biology Training Program at Duke University and quickly joined Blanche Capel’s lab.

While at Duke Jonah received several presentation and research awards from the Society for Developmental Biology (SDB) as well as the Duke Cardiovascular working group. Jonah was chosen to participate in the 2008 SDB-LASDB short course in Buenos Aires, Argentina - an experience that refocused his graduate career in many ways. From 2009-2011 he received an American Heart Association Predoctoral Fellowship.
Publications:


