Minireview

Testis Cord Maintenance in Mouse Embryos: Genes and Signaling[1]

Su-Ren Chen[2] and Yi-Xun Liu[3]

State Key Laboratory of Stem Cell and Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing, China

ABSTRACT

Testis cords, embryonic precursors of the seminiferous tubules, are fundamental for testis structure and function. Delay or disruption of testis cord formation could result in gonadal dysgenesis. Although mechanisms regulating testis cord formation during sex determination have been well-studied, the genes and signaling pathways involving in testis cord maintenance after the cords have formed are not well characterized. It is now clear that the maintenance of cord structure is an active process. In this review, we summarize the recent findings regarding the regulation of testis cord integrity by a series of Sertoli cell transcription factors, including the WT1-SOX8/SOX9-beta-CAT-ENIN-DHH network, GPR56, STIM1, and NR0B1 (also known as DAX1). In particularly, we emphasize the underappreciated role of peritubular myoid cells in testis cord maintenance and their cooperation with Sertoli cells. The regulation of the size, shape, and number of testis cords by Sertoli cell proliferation (e.g., SMAD4, GATA4, and TGF-beta signaling), Leydig cell products (e.g., ACTIVIN A), vascular development (a lesson learned from PDGF signaling), and available gonad space (as observed in *Ift144* mutant mice) is also addressed. Further efforts and new genetic models are needed to unveil the gene networks and underlying mechanisms regulating testis cord integrity and morphology after sex determination.

maintenance, PTM cells, Sertoli cells, testis cord, Wt1

INTRODUCTION

The key structural components in mammalian testes are seminiferous tubules, which provide physical barriers and supply nutrients required for the survival and maturation of germ cells. Seminiferous tubules begin to form during the embryonic stage as testis cords, and the formation of seminiferous tubules relies on cord remodeling during development. Testis cords are formed from epitheliallike Sertoli cells along with the associated myofibroblastlike peritubular myoid (PTM) cells at the periphery. Sertoli cells and PTM cells secrete specific extracellular matrix (ECM) proteins (mainly laminin and collagen) and assemble a layer of basement membrane to separate the testis cords from the interstitial space [1]. Abnormalities in testis cord formation and maintenance are associated with various diseases, including infertility, disorders of sexual development, and a predisposition to testicular dysgenesis syndrome disorders [2].

The developmental process of testis cord occurs via two main steps. The first step includes sex determination and testis cord formation, and the second step involves testis maturation through cord maintenance and remodeling.

In mice, the genital ridge increases in size due to increased proliferation of somatic epithelial cells and interacts with primordial germ cells to form the bipotential gonad at approximately Embryonic Day 10.5 (E10.5) [3, 4]. The expression of *Sry* (from E10.5 to E12.5) and *Sox9* in the developing XY gonads results in activation of the male pathway via antagonism of female-determining genes [5–7]. DMRT1 maintains male fates by repressing multiple female-promoting genes (*Foxl2*, *Wnt4*, and *Rspo1*) and activating male-promoting genes (*Sox9* and *Sox8*), revealed by genome-wide chromatin immunoprecipitation data from postnatal mouse testes [8, 9]. Cells in XY gonads undergo de novo rearrangements over a period of approximately 24 h to form a cord structure (from E11.5 to E12.5). The prevailing view of testis cord morphogenesis by three-dimensional imaging analyses supports a model of testis cord formation in which 1) SOX9-positive pre-Sertoli cells polarize, aggregate, and assemble around germ cells to form a Sertoli-germ cell mass; 2) endothelial cells migrate in streams from the mesonephros to direct the partitioning of the XY gonad into cord domains and form the testis-specific vasculature; and 3) the XY gonad is patterned into definitive cords by PTM cells surrounding the cords and forming a basement membrane [10–13]. It follows that multiple cell types within the fetal testis play active and synergetic roles in the process of testis cord formation.

Considerable progress has been made concerning the understanding of sex determination and testis cord formation (the first step) (reviewed in [7, 14]). What interests us the most is that, even after cords are formed and stabilized, several genes are essential to maintain their integrity. Morphological analyses in mice revealed that testis cords undergo structural changes (number, size, and shape) after the cords are formed. Testis

[2]Correspondence: Su-Ren Chen, State Key Laboratory of Stem Cells and Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, 1 Beichen West Road, Chaoyang District, Beijing, 100101. E-mail: chensuren@ioz.ac.cn
[3]Correspondence: Yi-Xun Liu, State Key Laboratory of Stem Cells and Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, 1 Beichen West Road, Chaoyang District, Beijing, 100101. E-mail: liuyx@ioz.ac.cn
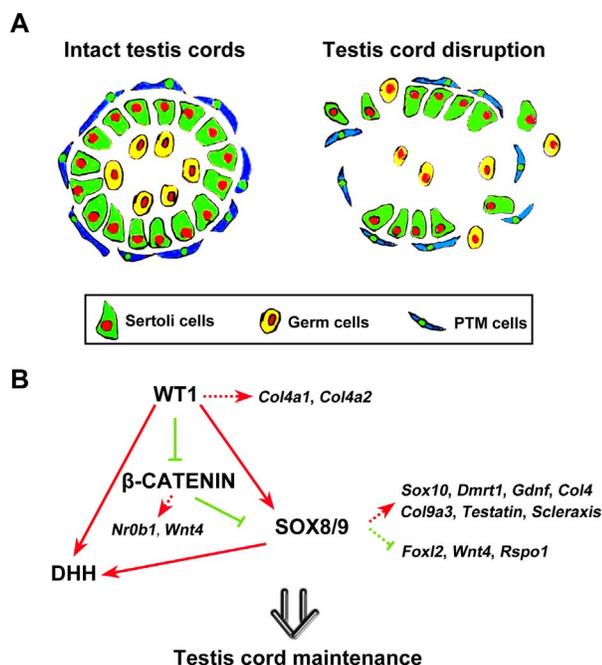
FIG. 1. WT1-SOX8/SOX9-β-CATENIN-DHH network in Sertoli cells regulates testis cord maintenance. **A**) The disruption of testis cord structures after the cord is formed results in the breakdown of the basement membrane. **B**) The WT1-SOX8/SOX9-β-CATENIN-DHH network emerges as the only well-defined signaling pathway that is critical for testis cord maintenance. WT1 is a negative regulator of β-CATENIN signaling. Loss of SOX9 expression in observed in both the *Wt1* deletion and *Ctnnb*-stabilized mutants. DHH is significantly reduced in *Wt1*-deletion and *Sox9, Sox8* double nullizygous testes, but not in the testes of *Ctnnb*-stabilized mutants.

cords appear as transverse circular loops at E14.5. Afterward, the testis cords of normal embryos began to coil at E17.5 and underwent further convolution by E19.5, the time of birth, revealing numerous small cross sections of the cords [15]. Gene knockout and inhibitor treatment experiments discovered several genes and signaling pathways that are required for testis cord morphology (see below).

Genetic background influences the number and shape of E15.5 testis cords in the fetal testis [16]. In 129T2/SvJ mice, the cords were relatively few, large in size, and packed with many germ cells. In contrast, the cords in C57Bl/6 mice were small, more numerous and contained relatively few germ cells. Accordingly, discrepancy in cord number and shape emerges in different studies, where mice of mixed genetic backgrounds were used. Although branched cord structures persist in some species, testis cords in mouse and man are continuous tubules [14].

Accordingly, this article presents an overview of the growing list of genes and gene defects that cause the breakdown of the basement membrane and/or a decreased cord number and abnormal shape of the cord structure after the cords are formed (the second step). Genes and signaling pathways are classified based on their function and relationship, and succinct information about their roles in mice is provided.

WT1-SOX8/SOX9-β-CATENIN-DHH NETWORK IN SERTOLI CELLS

Sertoli cells acting as an organizing center for testis cord maintenance has been further validated by recent Sertoli cell ablation studies. In these studies, cord structures were totally disrupted, and other cell types were severely affected [17–19]. The WT1-SOX8/SOX9-β-CATENIN-DHH network emerged as one of the well-defined signaling pathway that was critical for testis cord integrity (Fig. 1).

Active repression of the ovarian WNT signaling pathway was necessary for the male pathway [20–22], whereas, Sertoli cell-specific β-CATENIN stabilization ($Ctnnb^{\text{flox(e3)/+}}$, *Amh*-Cre) caused testis cord disruption [23]. In $Ctnnb^{\text{flox(e3)/+}}$, *Amh*-Cre testes, loss of Sertoli cell markers (*Sox9* and *Amh*) and elevated levels of *Nr0b1* and *Wnt4* were observed. However, transcript levels of several important markers of Sertoli cells, such as *Sf1, Dhh,* and *Fgf9*, were not significantly altered. The investigators proposed that WT1 is a negative regulator of β-CATENIN signaling because Sertoli cell-specific deletion of *Wt1* ($Wt1^{-/\text{flox}}$, *Amh*-Cre) resulted in upregulation and nuclear localization of β-CATENIN in Sertoli cells, and the observed defects in β-CATENIN-stabilized mutants phenocopied the abnormalities observed in the *Wt1*-deletion testes (see below) [23]. However, whether the transcriptional factor WT1 directly regulates *Ctnnb* (the gene coding β-catenin) is uncertain.

We, and others, suggest that WT1 is one of the few genes identified that is required for the full formation of the genital ridge through mitotic control of coelomic epithelial cells [4, 24]. Inactivation of *Wt1* in Sertoli cells after sex determination ($Wt1^{-/\text{flox}}$, *Amh*-Cre) causes testis cord disruption and loss of *Sox9, Sox8,* and *Amh* expression [25]. Our subsequent studies have shown that WT1, together with SOX9, directly regulates the expression of *Col4a1* and *Col4a2* (the genes coding collagen IV, which is the main component of ECMs). Therefore, loss of WT1 expression leads to a breakdown of the basement membrane [26]. Very recently, Zhang et al. [27] suggested that deletion of *Wt1* resulted in the reprogramming of Sertoli cells to Leydig-like cells, indicating that Sertoli and Leydig cells most likely originate from the same progenitor cells. Meanwhile, this intriguing study has caused researchers to re-examine the real cause of testis cord disruption in $Wt1^{-/\text{flox}}$, *Amh*-Cre embryos. We believe that the mechanisms underlying the regulation of testis cords by Sertoli cell WT1 are far more complex and dynamic than was initially thought. Because 3β-HSD, a Leydig cell marker, was expressed in Sertoli cells within the residually intact testis cords in the study of Zhang et al., the transdifferentiation of Sertoli cells into Leydig-like cells is unlikely to be the driving factor of testis cord disruption. Further studies are needed to determine the cause(s) and underlying mechanisms.

Loss of *Sox9* expression is observed in both *Wt1* deletion and *Ctnnb*-stabilized mutants. However, deleting *Sox9* after cord formation has no detrimental effect on testis cord maintenance [23, 28]. Two recent studies from Scherer's laboratory revealed functional redundancy between SOX8 and SOX9 to ensure intact testis cord structure [28, 29]. *Sox9, Sox8* double mutant testes ($Sox9^{\Delta/\Delta}$; $Sox8^{-/-}$) exhibited a reduced number of testis cords compared to controls at E17.5 and Postnatal Day 0. In addition, $Sox9^{\Delta/\Delta}$; $Sox8^{-/-}$ testes exhibited upregulation of early ovary-specific markers (*Wnt4, Rspo1*) and downregulation of Sertoli cell-specific markers (*Amh, Sf1, Dhh,* and *Gdnf*) as well as cell adhesion molecules/ECM components (*Ncam, Connexin 43, Occludin,* and *Claudin 11*) at E15.5 [28]. Their subsequent study further revealed a disruption of the basal lamina surrounding the testis cords that starts at E17.5 and, at E15.5, reduced expression levels of collagen IV, collagen IXa3, testatin, and scleraxis, structural components of basal lamina and ECMs in $Sox9^{\Delta/\Delta}$; $Sox8^{-/-}$ testes [29]. In addition, downregulation of the expression of the testis markers *Sox10* and *Dmrt1* and upregulation of the

ovarian marker *Foxl2* were observed in the mutant cords from double nullizygous testes. These two studies identified a large number of important genes under the control of SOX9 and SOX8; however, the gene(s) that are directly regulated by SOX9 and SOX8 remain largely undefined.

The phenotypes of the *Wt1* deletion and *Ctnnb*-stabilized mutants were more severe than the phenotypes of the *Sox9, Sox8* double nullizygous mutants, with disruption of testis cords and a scatter of germ cells already observable at E15.5 (these phenotypes were only observed in the *Sox9, Sox8* double nullizygous mutants after E17.5). Considering that loss of *Sox9* and *Sox8* expression occurs in both the *Wt1* deletion and the *Ctnnb*-stabilized mutants, we conclude that SOX9 and SOX8 act as the downstream targets of WT1 and β-CATENIN.

Strikingly, gene expression analysis revealed that *Dhh* is significantly reduced in *Wt1*-deletion and *Sox9, Sox8* double nullizygous testes, but not in the testes of *Ctnnb*-stabilized mutants (Fig. 1). In addition, the phenotype of *Dhh*-null mice is somewhat similar to that of the $Wt1^{-/flox}$; *Amh*-Cre and $Sox9^{\Delta/\Delta}$; $Sox8^{-/-}$ mice. DHH is a secreted signaling factor that acts via its receptor PTCH1 on interstitial compartment (PTM cells [30] and Leydig cells [31]). *Dhh*-null and control testes are generally similar during the period of early cord formation (E11.5–E12.5). By E13.5, the basal lamina delimiting the cords is lacking in some regions and is disorganized in *Dhh*-null testes, and occasional germ cells are observed outside the cords [32]. Disruption of testis cords was also observed in DHH signaling inhibitor-treated testes from the marsupial species the tammar wallaby [33]. Additionally, a recent study showed that mutations in the DHH palmitoyl-transferase (*Hhat*) gene (palmitoylation is required for efficient DHH signaling) in both mice and humans caused both decreases in testis cord numbers and alterations of the size and shape of the testis cords [34]. *Dhh* knockout mice display compromised PTM cells and compromised fetal Leydig cell differentiation [32, 35], leading to the hypothesis that the interstitium regulates the maintenance of the fetal testis cord in vivo. This idea was further supported by a study of *Hes1* mutants that showed that the number of progenitor mesenchymal cells in the interstitium was reduced and that testis cords were irregular [36]. Additional studies are needed to address the following questions: 1) whether *Dhh* is a direct target of WT1, SOX9, and SOX8, 2) how DHH induces distinct cellular responses in PTM and Leydig cell populations [37], 3) whether PTM cell-specific or Leydig cell-specific *Ptch1* knockout embryos exhibit disrupted testis cord structure, 4) whether DHH stimulates the small GTPase RhoA (noncanonical Hedgehog signaling) in endothelial cells to regulate tubulogenesis [38], and 5) does noncanonical Hedgehog signaling exist in Sertoli cells, and does it play a role in testis cord maintenance?

GPR56 AND STIM1 IN SERTOLI CELLS

GPR56 is expressed in Sertoli cells, and, in its absence, testis cords are partially disrupted between E14.5 and E18.5, resulting in reduced male fertility [39]. Sertoli cells are scattered and the basement membrane is fragmented in the defective areas of $Gpr56^{-/-}$ testes. Morphometric analyses by both the Koopman and Behringer laboratories [40, 41] suggest that cord remodeling is distinct in different regions of the testis. Cord size is wider in the distal part of the loops and narrower in the proximal segments that connect to the rete testis near the mesonephric border. Interestingly, disruptions of testis cords were only observed on the mesonephric side of $Gpr56^{-/-}$ gonads, indicating that remodeling of the testis cords on the mesonephric side is dependent on the activity of GPR56. However, neither GPR56 mRNA nor protein exhibited

asymmetric distributions in the testes. This study did not investigate how these distinct remodeling processes are regulated.

One recent study presents the possibility that reactive oxygen species (ROS) in Sertoli cells may be important for testis cord maintenance. Zheng et al. [42] showed that suppression of STIM1 in the cultured fetal testis in vitro increased ROS production and disorganized testis cord structure. When the knockdown group was treated with the antioxidant scavenger N-acetylcysteine, the defects in the testicular morphology were partially rescued. The investigators proposed that STIM1 could regulate β-CATENIN by ROS signaling and could control testis cord structure. In support of this proposed mechanism, β-CATENIN was shown to be upregulated after *Stim1* inhibition. However, the relationship between β-CATENIN and ROS remains undefined, and β-CATENIN in Sertoli cells disrupts testis cords (using *Amh*-Cre) [23] or causes male-to-female sex-reversal (using *Sf1*-Cre) [22], which is not identical to the phenotype observed in *Stim1* knockdown testes. It will be interesting to generate Sertoli cell-specific *Stim1* knockout mice and to examine whether STIM1 regulates testis cord maintenance via ROS signaling and/or other mechanisms in vivo.

NR0B1 IN PTM CELLS

In adult testes, PTMs have been suggested to influence spermatogenesis and male fertility in PTM-specific *Ar* knockout [43] and *Lgr4*-null mice [44]. In fetal testes, Sertoli cells work in concert with PTM cells to deposit an intervening layer of ECMs (also known as the basement membrane) to contribute to testis cords. In vitro coculture experiments clearly demonstrated that ECM deposition was robust only when both Sertoli cells and PTM cells were allowed to interact [45]. When Sertoli cells or PTM cells were cultured separately, ECM constituents were expressed but remained intracellular [46]. Thus, PTM cells are required for the functional development of the basement membrane in vitro. However, it will be important to confirm the indispensable role of PTM cells in testis cord maintenance in vivo using cell ablation strategies to permit the deletion of PTM cells.

A previous study suggested that NR0B1 (also known as DAX1) plays a crucial role in the development of intact testis cords by regulating the development of PTM cells [47]. In $Nr0b1^{-/Y}$ males, the gonad develops normally until E12.5. However, by E13.5, the testis cords are disorganized and incompletely formed, as indicated by disruption of the basement membrane. Bromodeoxyuridine (BrdU) labeling of PTM cells is low and consistent with decreased proliferation. Sertoli cell proliferation is normal, as assessed by BrdU labeling, and expression of the Sertoli-specific genes *Sox9, Dhh,* and *Amh* is relatively unaffected in *Nr0b1*-deficient testis. Because NR0B1 is mainly expressed in Sertoli cells, it is curious that PTM cells were specifically affected and accounted for testis cord disruption. The role of PTM in testis cord in vivo maintenance is unclear.

MORPHOLOGY OF TESTIS CORDS-TGF-β

The results from several reports supported the role of the TGF-β superfamily in the full development of testis cords. When E12.5 testes were treated with inhibitors of TGF-β signaling receptors, ALK4/5/7, for 72 h, testis cord growth was affected, with the cords exhibiting a stunted and wider appearance [48]. The researchers proposed that a significant reduction in Sertoli cell proliferation might account for the stunted testis cord growth. In addition, the basement membrane of *Tgfbr3* (a coreceptor for TGF-β) knockout testes is thin and discontinuous

[49]. However, mice lacking individual *Tgfb* genes (*Tgfβ1-3*) exhibit normal testis cord formation, indicating that functional redundancy may obscure testicular phenotypes in these models [50]. Recently, Sarraj et al. [51] showed that exogenous TGFβ2 (a high-affinity ligand for TGFBR3) partially rescues the dysgenic cord phenotype in *Tgfbr3* knockout explants. Accordingly, the compromised cord development in *Tgfbr3*-null fetal testis is due, at least in part, to disrupted TGFβ2 function. Importantly, how TGFβ1/2/3-TGFBR3-ALK4/5/7 signaling modulates Sertoli cell proliferation is still unknown.

Furthermore, Nie and Arend [52] suggested that polycystic kidney disease 1 (PKD1) and PKD2 regulate testis cord development likely via TGF-β signaling. Testis cord growth of $Pkd1^{-/-}$ and $Pkd2^{-/-}$ testes is inhibited, as indicated by a decrease in the average number of testis cords per section at E15.5 and E16.5. There is evidence that PKD1 and PKD2 are linked to TGF-β signaling. The investigators showed that the level of the TGF-β signaling member phospho-SMAD2 was significantly reduced in $Pkd1^{-/-}$ and $Pkd2^{-/-}$ testes. However, the mechanism by which PKD1/2 regulates TGF-β signaling is still not completely clear.

MORPHOLOGY OF TESTIS CORDS-ACTIVIN A-SMAD4 SIGNALING

ACTIVIN A-SMAD4 signaling, also belonging to the TGF-β superfamily, is thought to regulate testis cord coiling. Specific disruption of ACTIVIN A in fetal Leydig cells (*Inhba*$^{-/flox}$, *Amhr2*-Cre) results in a failure of fetal testis cord elongation and expansion due to decreased Sertoli cell proliferation [15]. Before this study, fetal Leydig cells and their products were not known to influence testis cord morphogenesis. Interestingly, the postnatal coiling results in the recovery of seminiferous tubule coiling, and grossly normal spermatogenesis is observed in *Inhba*$^{-/flox}$, *Amhr2*-Cre mice [15]. The question of why and how testis cord coiling is ensured by fetal and postnatal coiling is not clear. Finally, the conditional inactivation of SMAD4, the central component of TGF-β signaling, in Sertoli cells (*Smad4*$^{-/flox}$, *Amh*-Cre) eliminates ACTIVIN A signaling to the Sertoli cells and leads to both a reduction in the number of testis cords and abnormal shape of the testis cords, which is similar to those of Leydig cell-specific *Inhba* knockout testes [15, 53].

MORPHOLOGY OF TESTIS CORDS-PDGF-BB-PDGFR SIGNALING

A previous study suggest that inhibition of platelet-derived growth factor (PDGF) actions by a PDGFR-specific tyrosine phosphorylation inhibitor does not inhibit cord formation but does alter normal cord development and morphology in rat fetal testes [54]. A significant decrease in the number of cords per testis area and increased cord diameter were noted in the treated samples. PDGFR-α was expressed on cells of the mesonephros and interstitial cells of the gonad. *Pdgfr-α*$^{-/-}$ XY gonads displayed disruptions in the vasculature organization, testis cord development, and fetal Leydig cell differentiation [55]. A recent study suggests that endothelial-derived PDGF-BB, rather than Sertoli-derived PDGF-AA, is the ligand that drives proliferation of mesenchymal cells to form wedgelike structures that subdivide the gonad [56]. Addition of recombinant PDGF-BB rescued mesenchymal proliferation in the absence of the vasculature or when adhesion of endothelial cells was blocked.
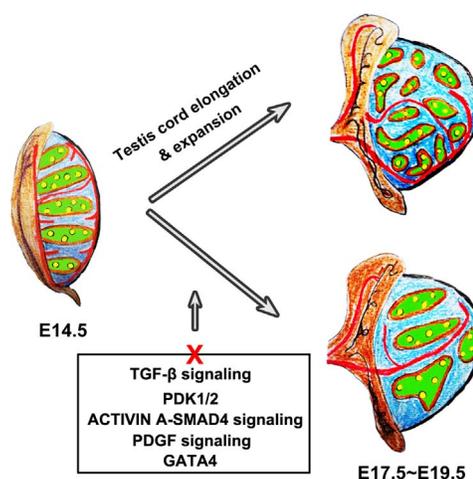

FIG. 2. Genes and signaling required for testis cord elongation and expansion. Testis cords appear as transverse circular loops at E14.5. Afterward, the testis cords of control embryos begin to coil and undergo further convolution by E17.5 to ~E19.5, revealing numerous small cross sections of the cords. The number, size, and shape of the testis cords are regulated by a variety of factors, including Sertoli cell proliferation (e.g., SMAD4, GATA4, TGF-β signaling, and PDK1/2), Leydig cell products (e.g., ACTIVIN A), vascular development (a lesson learned from PDGF signaling), and available space (as observed in *Ift144* mutant mice).

MORPHOLOGY OF TESTIS CORDS-GATA4

We, and others, find that the enlarged volume and decreased number of testis cords in Sertoli cell-specific *Gata4* knockout testes (*Gata4*$^{flox/flox}$; *Amh*-Cre or *Gata4*$^{flox/flox}$; *Sf1*-Cre) are remarkable [57, 58]. We observed that the number of BrdU-positive Sertoli cells was significantly reduced in *Gata4*$^{flox/flox}$; *Amh*-Cre testes after E14.5, which might explain why the reduced number and abnormal shape of testis cords were observed (our unpublished data). However, the exact mechanism of abnormal shape of the testis cords in *Gata4*$^{flox/flox}$; *Amh*-Cre embryos need further investigation. Morphological analysis revealed a similar phenotype (failure of fetal testis cord elongation and expansion) and possible mechanism (decreased Sertoli cell proliferation) between *Smad4* and *Gata4* conditional knockout testes, leading to the hypothesis that GATA4 and SMAD4 may genetically interact in the reproductive system. This idea was consistent with a report that double heterozygous *Gata4* and *Smad4* embryos (*Gata4*$^{flox/+}$*Smad4*$^{flox/+}$; *Tie2*-Cre) displayed severe atrioventricular defects [59]. However, our preliminary data suggested that both the number and shape of the testis cords were normal in *Gata4*$^{flox/+}$*Smad4*$^{flox/+}$; *Amh*-Cre embryos and adults, indicating this genetic and functional interaction is not ubiquitous between the cardiac system and the reproductive system.

MORPHOLOGY OF TESTIS CORDS-IFT144

An interesting recent study suggested that the number of testis cords was also determined by the gonad size because extension of the developing gonads in *Ift144* mutant mice resulted in a significant increase in testis cord number [60]. It is now clear that the gonad is partitioned into a number of testis cords that depends on the available space, rather than an innate determination to generate a fixed number of cords, although the pathways that mediate this effect require further study.

PERSPECTIVES

This review synthesizes the current knowledge of how the architecture of the testis cord is maintained and highlights the questions that remain to be explored. The disruption of testis cord structures after the cord is formed results in the breakdown of the basement membrane (Fig. 1) or a decreased number of testis cords and abnormal shape of the cord structure (Fig. 2). Several transcription factors in Sertoli cells (such as WT1, β-CATENIN, SOX9, SOX8, DHH, GPR56, and NR0B1) have been suggested to play significant roles in the stabilization of the basement membrane. Therefore, deficiency of these genes causes a scatter of germ cells in the interstitial region and is detrimental to spermatogenesis. The number, size, and shape of the testis cords are regulated by a variety of factors, including Sertoli cell proliferation (e.g., SMAD4, GATA4, and TGF-β signaling), Leydig cell products (e.g., ACTIVIN A), vascular development (a lesson learned from PDGF signaling), and available space (as observed in *Ift144* mutant mice) (Fig. 2).

Although Sertoli cells have traditionally been considered the key cell type responsible for maintaining, elongating, and expanding the testis cord structures, other somatic cells (Leydig cells, PTM cells, and endothelial cells) also contribute to testis cord integrity and normal morphogenesis. ACTIVIN A, a product of fetal Leydig cells, is a unique paracrine regulator of testis cord expansion [15]. It is interesting to identify other Leydig cell products and study their roles in testis cord maintenance. Meeks et al. [47] suggest that the reduced proliferation of PTM cells in *Nr0b1*-deficient testes accounted for testis cord disruption. Further observation of PTM cell-specific markers and the generation of PTM cell-specific Cre transgenes could be helpful for investigating the function of genes in PTM cells and for certifying the contribution of PTM cells to basal lamina integrity. Vascular-mesenchymal cross talk through VEGF and PDGF has been shown to drive vascular development and testis cord morphogenesis [56].

We should note that fetal testis development is a continuous and complex process. It will be problematic to view testis cord maintenance as an independent process. Moreover, the specific contribution of testis cord disorganization to functional development of the testis needs further investigation.

The reconstruction of testis cord structure both in vitro and in xenografts from immature, dissociated testicular cells during neonatal periods was reported in several mammalian species, including mice [61–63], rats [64, 65], pigs [66, 67], and monkeys [68, 69]. Furthermore, Matoba and Ogura [70] suggested that the cells of fetal gonads reconstructed the testis cord structure, and even functional gametes, in the renal subcapsular space. Most importantly, human fetal testis tissue xenografts demonstrated normal structure, function, and development after xenografting [71, 72]. In addition, human fetal testes could be cultured on membranes [73, 74] or under ex vivo hanging-drop conditions [75]. These methods provide an in vivo system to study testis cord maintenance in normal human fetal testes and to determine its susceptibility to disruption by gene silencing and by the addition of exogenous factors.

Further studies will be needed for a more detailed understanding of the role of Sertoli and PTM cells (or other cell types) in testis cord maintenance after sex determination. A deeper analysis of the molecular mechanisms regarding the breakdown of the basement membrane and the decreased number and abnormal shape of testis cords will greatly improve our understanding of the genetic causes underlying the pathophysiological conditions of infertility and sexual development and testicular dysgenesis syndrome disorders.

REFERENCES

1. Tung PS, Skinner MK, Fritz IB. Cooperativity between Sertoli cells and peritubular myoid cells in the formation of the basal lamina in the seminiferous tubule. Ann N Y Acad Sci 1984; 438:435–446.
2. Das DK, Sanghavi D, Gawde H, Idicula-Thomas S, Vasudevan L. Novel homozygous mutations in Desert hedgehog gene in patients with 46,XY complete gonadal dysgenesis and prediction of its structural and functional implications by computational methods. Eur J Med Genet 2011; 54: e529–e534.
3. Schmahl J, Eicher EM, Washburn LL, Capel B. Sry induces cell proliferation in the mouse gonad. Development 2000; 127:65–73.
4. Chen SR, Zheng QS, Zhang Y, Gao F, Liu YX. Disruption of genital ridge development causes aberrant primordial germ cell proliferation but does not affect their directional migration. BMC Biol 2013; 11:22.
5. Koopman P, Gubbay J, Vivian N, Goodfellow P, Lovell-Badge R. Male development of chromosomally female mice transgenic for Sry. Nature 1991; 351:117–121.
6. Sekido R, Lovell-Badge R. Sex determination involves synergistic action of SRY and SF1 on a specific Sox9 enhancer. Nature 2008; 453:930–934.
7. Quinn A, Koopman P. The molecular genetics of sex determination and sex reversal in mammals. Semin Reprod Med 2012; 30:351–363.
8. Matson CK, Murphy MW, Sarver AL, Griswold MD, Bardwell VJ, Zarkower D. DMRT1 prevents female reprogramming in the postnatal mammalian testis. Nature 2011; 476:101–104.
9. Lindeman RE, Gearhart MD, Minkina A, Krentz AD, Bardwell VJ, Zarkower D. Sexual cell-fate reprogramming in the ovary by DMRT1. Curr Biol 2015; 25:764–771.
10. Combes AN, Wilhelm D, Davidson T, Dejana E, Harley V, Sinclair A, Koopman P. Endothelial cell migration directs testis cord formation. Dev Biol 2009; 326:112–120.
11. Cool J, Carmona FD, Szucsik JC, Capel B. Peritubular myoid cells are not the migrating population required for testis cord formation in the XY gonad. Sex Dev 2008; 2:128–133.
12. Coveney D, Cool J, Oliver T, Capel B. Four-dimensional analysis of vascularization during primary development of an organ, the gonad. Proc Natl Acad Sci U S A 2008; 105:7212–7217.
13. Svingen T, Koopman P. Building the mammalian testis: origins, differentiation, and assembly of the component cell populations. Genes Dev 2013; 27:2409–2426.
14. Cool J, DeFalco T, Capel B. Testis formation in the fetal mouse: dynamic and complex de novo tubulogenesis. Wiley Interdiscip Rev Dev Biol 2012; 1:847–859.
15. Archambeault DR, Yao HH. Activin A, a product of fetal Leydig cells, is a unique paracrine regulator of Sertoli cell proliferation and fetal testis cord expansion. Proc Natl Acad Sci U S A 2010; 107:10526–10531.
16. Western PS, Ralli RA, Wakeling SI, Lo C, van den Bergen JA, Miles DC, Sinclair AH. Mitotic arrest in teratoma susceptible fetal male germ cells. PLoS One 2011; 6:e20736.
17. Fouchecourt S, Livera G, Messiaen S, Fumel B, Parent AS, Marine JC, Monget P. Apoptosis of Sertoli cells after conditional ablation of murine double minute 2 (Mdm2) gene is p53-dependent and results in male sterility. Cell Death Differ (in press). Published online ahead of print 16 October 2015 as DOI: 10.1038/cdd.2015.120.
18. Rebourcet D, O'Shaughnessy PJ, Monteiro A, Milne L, Cruickshanks L, Jeffrey N, Guillou F, Freeman TC, Mitchell RT, Smith LB. Sertoli cells maintain Leydig cell number and peritubular myoid cell activity in the adult mouse testis. PLoS One 2014; 9:e105687.
19. Rebourcet D, O'Shaughnessy PJ, Pitetti JL, Monteiro A, O'Hara L, Milne L, Tsai YT, Cruickshanks L, Riethmacher D, Guillou F, Mitchell RT, van't Hof R, et al. Sertoli cells control peritubular myoid cell fate and support adult Leydig cell development in the prepubertal testis. Development 2014; 141:2139–2149.
20. Jameson SA, Lin YT, Capel B. Testis development requires the repression of Wnt4 by Fgf signaling. Dev Biol 2012; 370:24–32.
21. Nicol B, Yao HH. Gonadal identity in the absence of pro-testis factor SOX9 and pro-ovary factor beta-catenin in mice. Biol Reprod 2015; 93:35.
22. Maatouk DM, DiNapoli L, Alvers A, Parker KL, Taketo MM, Capel B. Stabilization of beta-catenin in XY gonads causes male-to-female sex-reversal. Hum Mol Genet 2008; 17:2949–2955.

23. Chang H, Gao F, Guillou F, Taketo MM, Huff V, Behringer RR. Wt1 negatively regulates beta-catenin signaling during testis development. Development 2008; 135:1875–1885.

24. Kreidberg JA, Sariola H, Loring JM, Maeda M, Pelletier J, Housman D, Jaenisch R. WT-1 is required for early kidney development. Cell 1993; 74: 679–691.

25. Gao F, Maiti S, Alam N, Zhang Z, Deng JM, Behringer RR, Lecureuil C, Guillou F, Huff V. The Wilms tumor gene, Wt1, is required for Sox9 expression and maintenance of tubular architecture in the developing testis. Proc Natl Acad Sci U S A 2006; 103:11987–11992.

26. Chen SR, Chen M, Wang XN, Zhang J, Wen Q, Ji SY, Zheng QS, Gao F, Liu YX. The Wilms tumor gene, Wt1, maintains testicular cord integrity by regulating the expression of Col4a1 and Col4a2. Biol Reprod 2013; 88: 56.

27. Zhang L, Chen M, Wen Q, Li Y, Wang Y, Wang Y, Qin Y, Cui X, Yang L, Huff V, Gao F. Reprogramming of Sertoli cells to fetal-like Leydig cells by Wt1 ablation. Proc Natl Acad Sci U S A 2015; 112:4003–4008.

28. Barrionuevo F, Georg I, Scherthan H, Lecureuil C, Guillou F, Wegner M, Scherer G. Testis cord differentiation after the sex determination stage is independent of Sox9 but fails in the combined absence of Sox9 and Sox8. Dev Biol 2009; 327:301–312.

29. Georg I, Barrionuevo F, Wiech T, Scherer G. Sox9 and Sox8 are required for basal lamina integrity of testis cords and for suppression of FOXL2 during embryonic testis development in mice. Biol Reprod 2012; 87:99.

30. Clark AM, Garland KK, Russell LD. Desert hedgehog (Dhh) gene is required in the mouse testis for formation of adult-type Leydig cells and normal development of peritubular cells and seminiferous tubules. Biol Reprod 2000; 63:1825–1838.

31. Bitgood MJ, Shen L, McMahon AP. Sertoli cell signaling by Desert hedgehog regulates the male germline. Curr Biol 1996; 6:298–304.

32. Pierucci-Alves F, Clark AM, Russell LD. A developmental study of the Desert hedgehog-null mouse testis. Biol Reprod 2001; 65:1392–1402.

33. Chung JW, Pask AJ, Renfree MB. Seminiferous cord formation is regulated by hedgehog signaling in the marsupial. Biol Reprod 2012; 86: 80.

34. Callier P, Calvel P, Matevossian A, Makrythanasis P, Bernard P, Kurosaka H, Vannier A, Thauvin-Robinet C, Borel C, Mazaud-Guittot S, Rolland A, Desdoits-Lethimonier C, et al. Loss of function mutation in the palmitoyl-transferase HHAT leads to syndromic 46,XY disorder of sex development by impeding Hedgehog protein palmitoylation and signaling. PLoS Genet 2014; 10:e1004340.

35. Yao HH, Whoriskey W, Capel B. Desert Hedgehog/Patched 1 signaling specifies fetal Leydig cell fate in testis organogenesis. Genes Dev 2002; 16:1433–1440.

36. Tang H, Brennan J, Karl J, Hamada Y, Raetzman L, Capel B. Notch signaling maintains Leydig progenitor cells in the mouse testis. Development 2008; 135:3745–3753.

37. Barsoum IB, Bingham NC, Parker KL, Jorgensen JS, Yao HH. Activation of the Hedgehog pathway in the mouse fetal ovary leads to ectopic appearance of fetal Leydig cells and female pseudohermaphroditism. Dev Biol 2009; 329:96–103.

38. Chinchilla P, Xiao L, Kazanietz MG, Riobo NA. Hedgehog proteins activate pro-angiogenic responses in endothelial cells through non-canonical signaling pathways. Cell Cycle 2010; 9:570–579.

39. Chen G, Yang L, Begum S, Xu L. GPR56 is essential for testis development and male fertility in mice. Dev Dyn 2010; 239:3358–3367.

40. Nel-Themaat L, Vadakkan TJ, Wang Y, Dickinson ME, Akiyama H, Behringer RR. Morphometric analysis of testis cord formation in Sox9-EGFP mice. Dev Dyn 2009; 238:1100–1110.

41. Combes AN, Lesieur E, Harley VR, Sinclair AH, Little MH, Wilhelm D, Koopman P. Three-dimensional visualization of testis cord morphogenesis, a novel tubulogenic mechanism in development. Dev Dyn 2009; 238: 1033–1041.

42. Zheng B, Zhao D, Zhang P, Shen C, Guo Y, Zhou T, Guo X, Zhou Z, Sha J. Quantitative proteomics reveals the essential roles of stromal interaction molecule 1 (STIM1) in the testicular cord formation in mouse testis. Mol Cell Proteomics 2015; 14:2682–2691.

43. Welsh M, Saunders PT, Atanassova N, Sharpe RM, Smith LB. Androgen action via testicular peritubular myoid cells is essential for male fertility. FASEB J 2009; 23:4218–4230.

44. Qian Y, Liu S, Guan Y, Pan H, Guan X, Qiu Z, Li L, Gao N, Zhao Y, Li X, Lu Y, Liu M, et al. Lgr4-mediated Wnt/beta-catenin signaling in

45. Skinner MK, Fritz IB. Androgen stimulation of Sertoli cell function is enhanced by peritubular cells. Mol Cell Endocrinol 1985; 40:115–122.

46. Skinner MK, Tung PS, Fritz IB. Cooperativity between Sertoli cells and testicular peritubular cells in the production and deposition of extracellular matrix components. J Cell Biol 1985; 100:1941–1947.

47. Meeks JJ, Crawford SE, Russell TA, Morohashi K, Weiss J, Jameson JL. Dax1 regulates testis cord organization during gonadal differentiation. Development 2003; 130:1029–1036.

48. Miles DC, Wakeling SI, Stringer JM, van den Bergen JA, Wilhelm D, Sinclair AH, Western PS. Signaling through the TGF beta-activin receptors ALK4/5/7 regulates testis formation and male germ cell development. PLoS One 2013; 8:e54606.

49. Sarraj MA, Escalona RM, Umbers A, Chua HK, Small C, Griswold M, Loveland K, Findlay JK, Stenvers KL. Fetal testis dysgenesis and compromised Leydig cell function in Tgfbr3 (beta glycan) knockout mice. Biol Reprod 2010; 82:153–162.

50. Memon MA, Anway MD, Covert TR, Uzumcu M, Skinner MK. Transforming growth factor beta (TGFbeta1, TGFbeta2 and TGFbeta3) null-mutant phenotypes in embryonic gonadal development. Mol Cell Endocrinol 2008; 294:70–80.

51. Sarraj MA, Escalona RM, Western P, Findlay JK, Stenvers KL. Effects of TGFbeta2 on wild-type and Tgfbr3 knockout mouse fetal testis. Biol Reprod 2013; 88:66.

52. Nie X, Arend LJ. Novel roles of Pkd2 in male reproductive system development. Differentiation 2014; 87:161–171.

53. Archambeault DR, Yao HH. Loss of smad4 in Sertoli and Leydig cells leads to testicular dysgenesis and hemorrhagic tumor formation in mice. Biol Reprod 2014; 90:62.

54. Uzumcu M, Dirks KA, Skinner MK. Inhibition of platelet-derived growth factor actions in the embryonic testis influences normal cord development and morphology. Biol Reprod 2002; 66:745–753.

55. Brennan J, Tilmann C, Capel B. Pdgfr-alpha mediates testis cord organization and fetal Leydig cell development in the XY gonad. Genes Dev 2003; 17:800–810.

56. Cool J, DeFalco TJ, Capel B. Vascular-mesenchymal cross-talk through Vegf and Pdgf drives organ patterning. Proc Natl Acad Sci U S A 2011; 108:167–172.

57. Manuylov NL, Zhou B, Ma Q, Fox SC, Pu WT, Tevosian SG. Conditional ablation of Gata4 and Fog2 genes in mice reveals their distinct roles in mammalian sexual differentiation. Dev Biol 2011; 353:229–241.

58. Chen SR, Tang JX, Cheng JM, Li J, Jin C, Li XY, Deng SL, Zhang Y, Wang XX, Liu YX. Loss of Gata4 in Sertoli cells impairs the spermatogonial stem cell niche and causes germ cell exhaustion by attenuating chemokine signaling. Oncotarget 2015; 6:37012–37027.

59. Moskowitz IP, Wang J, Peterson MA, Pu WT, Mackinnon AC, Oxburgh L, Chu GC, Sarkar M, Berul C, Smoot L, Robertson EJ, Schwartz R, et al. Transcription factor genes Smad4 and Gata4 cooperatively regulate cardiac valve development. Proc Natl Acad Sci U S A 2011; 108: 4006–4011.

60. Wainwright EN, Svingen T, Ng ET, Wicking C, Koopman P. Primary cilia function regulates the length of the embryonic trunk axis and urogenital field in mice. Dev Biol 2014; 395:342–354.

61. Zhang J, Hatakeyama J, Eto K, Abe S. Reconstruction of a seminiferous tubule-like structure in a 3 dimensional culture system of re-aggregated mouse neonatal testicular cells within a collagen matrix. Gen Comp Endocrinol 2014; 205:121–132.

62. Kita K, Watanabe T, Ohsaka K, Hayashi H, Kubota Y, Nagashima Y, Aoki I, Taniguchi H, Noce T, Inoue K, Miki H, Ogonuki N, et al. Production of functional spermatids from mouse germline stem cells in ectopically reconstituted seminiferous tubules. Biol Reprod 2007; 76: 211–217.

63. Yokonishi T, Sato T, Katagiri K, Komeya M, Kubota Y, Ogawa T. In vitro reconstruction of mouse seminiferous tubules supporting germ cell differentiation. Biol Reprod 2013; 89:15.

64. Gassei K, Schlatt S, Ehmcke J. De novo morphogenesis of seminiferous tubules from dissociated immature rat testicular cells in xenografts. J Androl 2006; 27:611–618.

65. Gassei K, Ehmcke J, Wood MA, Walker WH, Schlatt S. Immature rat seminiferous tubules reconstructed in vitro express markers of Sertoli cell maturation after xenografting into nude mouse hosts. Mol Hum Reprod 2010; 16:97–110.

Article 42

66. Dufour JM, Rajotte RV, Korbutt GS. Development of an in vivo model to study testicular morphogenesis. J Androl 2002; 23:635–644.

67. Honaramooz A, Megee SO, Rathi R, Dobrinski I. Building a testis: formation of functional testis tissue after transplantation of isolated porcine (Sus scrofa) testis cells. Biol Reprod 2007; 76:43–47.

68. Wistuba J, Mundry M, Luetjens CM, Schlatt S. Cografting of hamster (Phodopus sungorus) and marmoset (Callithrix jacchus) testicular tissues into nude mice does not overcome blockade of early spermatogenic differentiation in primate grafts. Biol Reprod 2004; 71:2087–2091.

69. Aeckerle N, Dressel R, Behr R. Grafting of neonatal marmoset monkey testicular single-cell suspensions into immunodeficient mice leads to ex situ testicular cord neomorphogenesis. Cells Tissues Organs 2013; 198: 209–220.

70. Matoba S, Ogura A. Generation of functional oocytes and spermatids from fetal primordial germ cells after ectopic transplantation in adult mice. Biol Reprod 2011; 84:631–638.

71. Yu J, Cai ZM, Wan HJ, Zhang FT, Ye J, Fang JZ, Gui YT, Ye JX. Development of neonatal mouse and fetal human testicular tissue as ectopic grafts in immunodeficient mice. Asian J Androl 2006; 8:393–403.

72. Mitchell RT, Saunders PT, Childs AJ, Cassidy-Kojima C, Anderson RA, Wallace WH, Kelnar CJ, Sharpe RM. Xenografting of human fetal testis tissue: a new approach to study fetal testis development and germ cell differentiation. Hum Reprod 2010; 25:2405–2414.

73. Lambrot R, Coffigny H, Pairault C, Donnadieu AC, Frydman R, Habert R, Rouiller-Fabre V. Use of organ culture to study the human fetal testis development: effect of retinoic acid. J Clin Endocrinol Metab 2006; 91: 2696–2703.

74. Bendsen E, Laursen S, Olesen C, Westergaard L, Andersen C, Byskov A. Effect of 4-octylphenol on germ cell number in cultured human fetal gonads. Hum Reprod 2001; 16:236–243.

75. Jorgensen A, Nielsen JE, Perlman S, Lundvall L, Mitchell RT, Juul A, Rajpert-De Meyts E. Ex vivo culture of human fetal gonads: manipulation of meiosis signalling by retinoic acid treatment disrupts testis development. Hum Reprod 2015; 30:2351–2363.