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Testis cord morphogenesis: determination, establishment, and maintenance[1]

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Abstract

Since the discovery of the testis-determining gene *SRY* in the early 1990s, rapid progress has been made in illuminating the genetic pathways for testis cord morphogenesis. Research on fetal testis cord development has largely focused on two key areas – testis determination and testis cord establishment. In the first of these events, expression of *SRY* in the XY gonad triggers a signaling cascade, which establishes the Sertoli cell lineage and thus secures testis identity. The second event, testis cord establishment, relies upon expansion of the Sertoli cell population as well as the migration of cells from the mesonephros. In this review, we aim to discuss these two stages of testis cord formation as well as suggest the existence of a third phase in this fascinating process – the need for further maintenance of the testis cords during the period between testis cord establishment and birth.

Keywords: testis development, testis determination, Sertoli cells, mouse.

Determination of the testis fate

Prior to the 20th century, popular belief held that the sex of mammals, like that of many lower vertebrates, is established via environmental cues (Geddes and Thomson, 1889). The identification of the "accessory chromosome" in the early 1900s provided the first evidence that there might be a chromosomal component to sex determination (Stevens, 1905; Wilson, 1909). Despite these auspicious beginnings, it was not until the early 1990s when *SRY* (sex-determining region of the Y chromosome) was determined to be the sole gene on the Y chromosome required for testis determination (Gubbay *et al*., 1990). *SRY* is present in nearly all mammalian species, including humans and mice. The essential role of *SRY* is demonstrated by the fact that *SRY* mutations in humans result in male-to-female sex reversal (Berta *et al*., 1990; Jager *et al*., 1990). The concept of *SRY* as a "switch" controlling sex determination in the initially bipotential gonad is also supported by experiments wherein transgenic XX mice expressing ectopic *SRY* gene develop testes in place of ovaries (Lovell-Badge and Robertson, 1990; Koopman *et al*., 1991). The expression of *SRY* in the XY individual is sufficient to secure testis fate and ensure subsequent progression of the male phenotypes.

The primary action of *SRY* in testis determination is to trigger the establishment of the Sertoli cell lineage, which in turn organizes testis architecture. Although the *SRY* gene is critical for testis development in most mammals, many of the structural domains of the *SRY* genes are not conserved among species. For example, the only conserved domain between human *SRY* and mouse *Sry* is a high mobility group (HMG) box transcription factor domain (Whitfield *et al*., 1993). This HMG box domain is shared by the transcription factor *SOX9* (*SRY*-related HMG box gene 9), which is the primary target of *SRY* action in testis determination. Unlike *SRY*, the structural domains of *SOX9* have proven to be highly conserved among vertebrates (Morrish and Sinclair, 2002). The strongest evidence that *SOX9* is immediately downstream of *SRY* is the fact that changes in either *SOX9* or *SRY* expression result in similar sex reversal phenotypes. Deletion of *Sox9* in XY mice that express *Sry* in a normal fashion leads to ovary morphogenesis, whereas expression of *Sox9* in transgenic XX mouse gonads results in testis development (Bishop *et al*., 2000; Vidal *et al*., 2001; Chaboissier *et al*., 2004; Barrionuevo *et al*., 2006). The ability of *Sox9* to fully substitute for *Sry* with regard to the establishment of the Sertoli cell lineage confirms that the upregulation of *Sox9* is a critical function of *Sry*.

In mouse XY embryos, *Sry* expression begins at 10.5 dpc (days post coitum) in the Sertoli cell precursors within the gonad. As will be discussed in the second section, the end result of *Sry* expression is to establish the Sertoli cell lineage. Although the exact mechanism that stimulates *Sry* expression is not known, transcription factors SF1 (steroidogenic factor 1, also known as *NR5A1*) and WT1 (Wilms' tumor 1) can bind and activate the *SRY* promoter in humans (Shimamura *et al*., 1997; De Santa Barbara *et al*., 2001; Hossain and Saunders, 2001). In addition to initiating *Sry* expression, *Sf1* and *Wt1* both play critical roles in the initial formation of the gonadal primordium, as knocking out either of these genes results in complete gonadal agenesis (Kreidberg *et al*., 1993; Luo *et al*., 1994). In addition to *Sf1* and *Wt1*, several novel transcription factors have been characterized as being involved in male gonad establishment prior to *Sry* expression, including *Lhx9* (Lim homeobox 9), *M33* (a mouse homologue of Drosophila *Polycomb* gene), and *Emx2* (empty spiracles homolog 2). *Lhx9* and *Emx2* knockout mice experience gonad regression similar to that of *Sf1*-null and *Wt1*-null mice; *M33* mutant mice have a high incidence of male-to-female sex reversal (see review by

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Brennan and Capel, 2004). Insulin receptor signaling has also been implicated in early testis establishment as XY male-to-female sex reversal is found in triple knockout mice lacking *Ir* (insulin receptor), *Irr* (insulin receptor-related receptor), and *Igf1r* (insulin-like growth factor 1 receptor) (Nef *et al*., 2003). It is thought that the sex reversal phenotype stems from a decrease in the proliferation of Sertoli cell precursors, since *Sry* is expressed normally in the insulin receptor signaling triple knockout mice. Although mutations in these genes result in deviation from the testis morphogenesis pathway, the relationship between these nonsex-linked genes and *Sry* has yet to be fully understood.

In addition to *Sf1* and *Wt1*, *Sox9* is also present in the bipotential gonad prior to expression of *Sry*. *Sox9* is expressed at low levels in the early gonads of both sexes; however, *Sry* expression at 10.5 dpc upregulates the expression of *Sox9* in the XY gonad by 11 dpc. *Sry* alone is probably not sufficient for *Sox9* upregulation, since male mice possessing a mutant *Dax1* allele demonstrated a failure in *Sox9* upregulation despite normal expression of *Sry* (Muscatelli *et al*., 1994; Meeks *et al*., 2003; Bouma *et al*., 2005). Another effect of SRY on SOX9 function is the transfer of SOX9 from the cytoplasm to the nucleus (Hanley *et al*., 2000). It has been postulated that this accumulation of SOX9 within the nucleus of Sertoli cell precursors may be all that is needed to trigger testis differentiation (Morais da Silva *et al*., 1996). In mouse XY gonads, *Sry* expression is downregulated by 12.5 dpc, but *Sox9* levels remain elevated. The continued expression of *Sox9* in the absence of *Sry* expression has been shown to be regulated by FGF9 (fibroblast growth factor 9). In *Fgf9*-null XY mice, *Sry* expression and the subsequent upregulation of *Sox9* occurred normally but *Sox9* expression was not maintained after *Sry* downregulation (Colvin *et al*., 2001; Kim *et al*., 2006). This failure to maintain *Sox9* expression resulted in abandonment of the testis pathway prior to Sertoli cell differentiation, thus the XY gonad developed as an ovary despite normal *Sry* expression (Colvin *et al*., 2001; Kim *et al*., 2006). Another signaling molecule shown to be important for proper *Sox9* expression is WNT4 (wingless-type MMTV integration site family member 4). *Wnt4* is expressed in the early gonads of both sexes but becomes ovary-specific after the timeframe of *Sry* expression in the XY gonad (Vainio *et al*., 1999). Although generally regarded as an ovary-specific factor, male mice carrying *Wnt4*-null alleles show a defect in Sertoli cell differentiation which is downstream of *Sry* but upstream of *Sox9* (Jeays-Ward *et al*., 2004). The reduced levels of *Sox9* expression in *Wnt4* knockout males result in disorganization of the testis cords. Interestingly, the testis cord phenotype in *Wnt4* knockout embryo is later resolved, suggesting the presence of a compensatory system.

The molecular pathways initiated by *Sry* are subject to control by genetic signals acting upstream and/or downstream of this male-specific factor. Although numerous genes have been shown to be essential for normal testis establishment, the exact relationships between *Sry* and these factors have yet to be elucidated. It has become clear that the expression of SRY by a threshold number of Sertoli cell precursors is crucial for the progression of testis development. Establishment of this supporting cell lineage is a prerequisite for the proper formation of testis cords.

Making testis cords

Formation of the testis cords requires a coordinate interaction between Sertoli cells and other somatic cells in the testis. Sertoli cells are the only cell type expressing *Sry* in the testis, indicating that appearance of other somatic cell lineages and organization of the testis cords must be controlled by Sertoli cells. At least two cellular events have been identified to be downstream of Sertoli cell differentiation: expansion of the Sertoli cell population and induction of mesonephric cell migration. Almost all differentiated Sertoli cells express *Sox9*; however, not all of these *Sox9*-expressing cells are positive for *Sry* at the beginning of testis determination (Albrecht and Eicher, 2000). It is therefore hypothesized that the minority population of *Sry*-expressing cells must recruit other somatic cells to turn on *Sry* and/or *Sox9* and therefore differentiate into Sertoli cells. Prostaglandin D2 (PGD2) has been considered as a possible paracrine factor produced by the *Sry*-expressing Sertoli cells for the recruitment of other Sertoli cells (Adams and McLaren, 2002; Wilhelm *et al*., 2005). PGD2 was able to induce phosphorylation of SOX9 and then importation of SOX9 to the nuclei of somatic cells in the female gonads, leading to a partial sex-reversal of the treated XX gonad (Malki *et al*., 2005).

In addition to recruitment, differentiating Sertoli cells also produce FGF9 to stimulate proliferation of Sertoli cell pool. In the *Ffg9-/-* male gonads, Sertoli cell proliferation was significantly reduced, leading to male to female sex reversal (Colvin *et al*., 2001; Schmahl *et al*., 2004). FGF9 probably interacts with FGFR2 (FGF receptor 2), which is co-localized with SOX9 in Sertoli cell nuclei (Schmahl *et al*., 2004). These observations suggest that FGF9 acts downstream of *Sry* to initiate testis development by inducing cell proliferation and localization of FGFR2 in the nucleus of Sertoli precursors (Schmahl *et al*., 2004). Defects in Sertoli cell proliferation were also found in mouse embryos without a functional platelet-derived growth factor receptor alpha gene (Brennan *et al*. 2003). However the exact mechanisms of PDGF signaling are yet to be identified.

Another event downstream of SRY and subsequent Sertoli cell differentiation is the induction of mesonephric cell migration. It was postulated that the mesonephros contributes to other somatic cell lineages in the testis, specifically peritubular myoid cells (PTM). The PTM cells are a layer of squamous smooth muscle

cells which enclose the testis cords. PTM cells interact with Sertoli cells to deposit components of the basement membrane which delineate the boundary between the testis cords and the interstitial compartment. Classic recombination experiments, which produce recombinants of wild type gonads and LacZ-tagged mesonephroi, revealed that migration of mesonephric cells occurs in a testis-specific manner. Some of the migrating mesonephric cells become integrated proximal to the PTM cells (Magre *et al*., 1980; Buehr *et al*., 1993). Although it remains to be determined whether the migration of mesonephric cells contributes to the PTM cell population, there is little doubt that this migration is essential for testis cord formation. When a barrier was placed between male gonads and mesonephroi, mesonephric cell migration was blocked and testis cord formation did not occur. Similarly, when early-stage male gonads were cultured without mesonephroi, testis cords did not form properly (Buehr *et al*., 1993; Merchant-Larios *et al*., 1993; Capel *et al*, 1999).

Induction of mesonephric cell migration appears to be mediated via a chemotactic signal(s) produced by the differentiating Sertoli cells (McLaren, 1991; Buehr *et al*., 1993). Growth factors such as neurotropin 3(NT3), PDGF, nerve growth factors (NGFs), anti-Müllerian Hormone (AMH), and activins have been implicated in the process of mesonephric cell migration. NT3 is expressed in Sertoli cells and treatment with NT3 inhibitors has been found to block both mesonephric cell migration and testis cord formation *in vitro* (Cupp *et al*., 2000). PDGF ligands and receptors are also expressed in the embryonic testes at the time of testis cord formation. Inhibitors specific to PDGF receptors cause abnormal testis cord formation in the fetal rat (Uzumcu *et al*., 2002). Recombinant experiments performed using *Pdgfr*α null gonads and wild-type mesonephroi demonstrated that *Pdgfr*α is required in the gonads for proper migration to occur (Brennan *et al*., 2003). The potential involvement of NGF in mesonephric cell migration is first indicated by receptor inhibitor treatment *in vitro*, suggesting that NGF signaling is essential for testis cord formation. However, deletion of the NGF receptor only causes minor defects in both testis cord and interstitial development (Cupp *et al*., 2002). Similarly, AMH and activin induces migration *in vitro* but no apparent testis cord defects are found in *Amh*-null or *Activin* knockout mice (Ross *et al*., 2003; Yao *et al*., 2006). Together, this evidence demonstrates that mesonephric cell migration is induced via redundant pathways derived from the Sertoli cells.

Maintenance of testis cords: emerging new players

In the interest of studying fetal testis cord morphogenesis in the mouse, our laboratory conducted *in situ* hybridization screening for signaling molecules involved in this process. Our screening procedure identifies that inhibin βA (*Inhba*) and βB (*Inhbb*) subunits are expressed in a testis-specific manner at the time of testis formation in the mouse (Yao *et al*., 2006). In the mouse, both of the inhibin β subunits are found in the developing testes starting at 12.5 dpc with distinct expression domains; *Inhba* is localized to the interstitium whereas *Inhbb* is expressed by the Sertoli cells. *Inhbb* appears to be dispensable for normal testis development as *Inhbb* knockout males develop and reproduce normally (Vassalli *et al*., 1994). We therefore examined whether *Inhba* has a functional role in testis morphogenesis.

We found that the early stages of testis cord morphogenesis (before 15.5 dpc) seemed to occur normally in *Inhba* knockout embryos. However, after 15.5 dpc obvious abnormalities in testis cord architecture arose in the absence of *Inhba*. In wild-type littermates, the testis cords became highly convoluted, folded structures at birth (Fig. 1A). In contrast, *Inhba* knockout testes failed to elongate and remained as a straight tube at birth (Fig. 1B). In addition to the failure in elongation and/or convolution in testis cords, some Sertoli cells of the *Inhba* knockout males were found within the lumen of the testis cords, apparently sloughed off from their usual position encircling the germ cells. Although further experiments need to be conducted to ascertain the mechanism of *Inhba* action in the fetal testis, our current hypothesis is that interstitium-derived *Inhba* is essential for testis cord maintenance and differentiation by regulating the proliferation of Sertoli cells after testis cord formation.

Perhaps the most exciting and novel aspect of the testis cord phenotype observed in *Inhba* knockout mice is the fact that the defect does not occur during the process of testis cord establishment. As discussed earlier, numerous genes have been identified which if mutated or knocked out result in abnormal morphogenesis of the testis cords. In the instance of the *Inhba* knockout mice, initial testis cord formation appears normal and the defect occurs in the subsequent growth of the cords between 15.5 dpc and birth. The *Inhba* knockout phenotypes suggest that proper testis cord establishment is not sufficient to ensure normal testis cord maintenance; additional signaling molecules are necessary for maintenance of the testis cords after their initial formation.

In contrast to the *Inhba* knockout embryos, loss of *Inhbb* had no effects on normal testis development, suggesting that *Inhbb* is dispensable or its functions are compensated by other factors. As mentioned earlier, *Inhbb* is expressed in Sertoli cells, which also produce *Amh* (anti-Müllerian hormone), another member of the transforming growth factor beta family (Vigier *et al*., 1984). Similar to the loss of *Inhbb*, loss of *Amh* has no effects on testis formation. To test the possibility that Sertoli cell-derived *Inhbb* and *Amh* function redundantly during testis formation, we generated *Amh;Inhbb* double knockout mice. At birth, the *Amh;Inhbb* double knockout testis contained less testis cords with signs of

degeneration. This finding revealed that the combination of *Amh* and *Inhbb* is required for maintenance of the testis cords, despite the fact that testis cords form normally in the absence of one or the other.

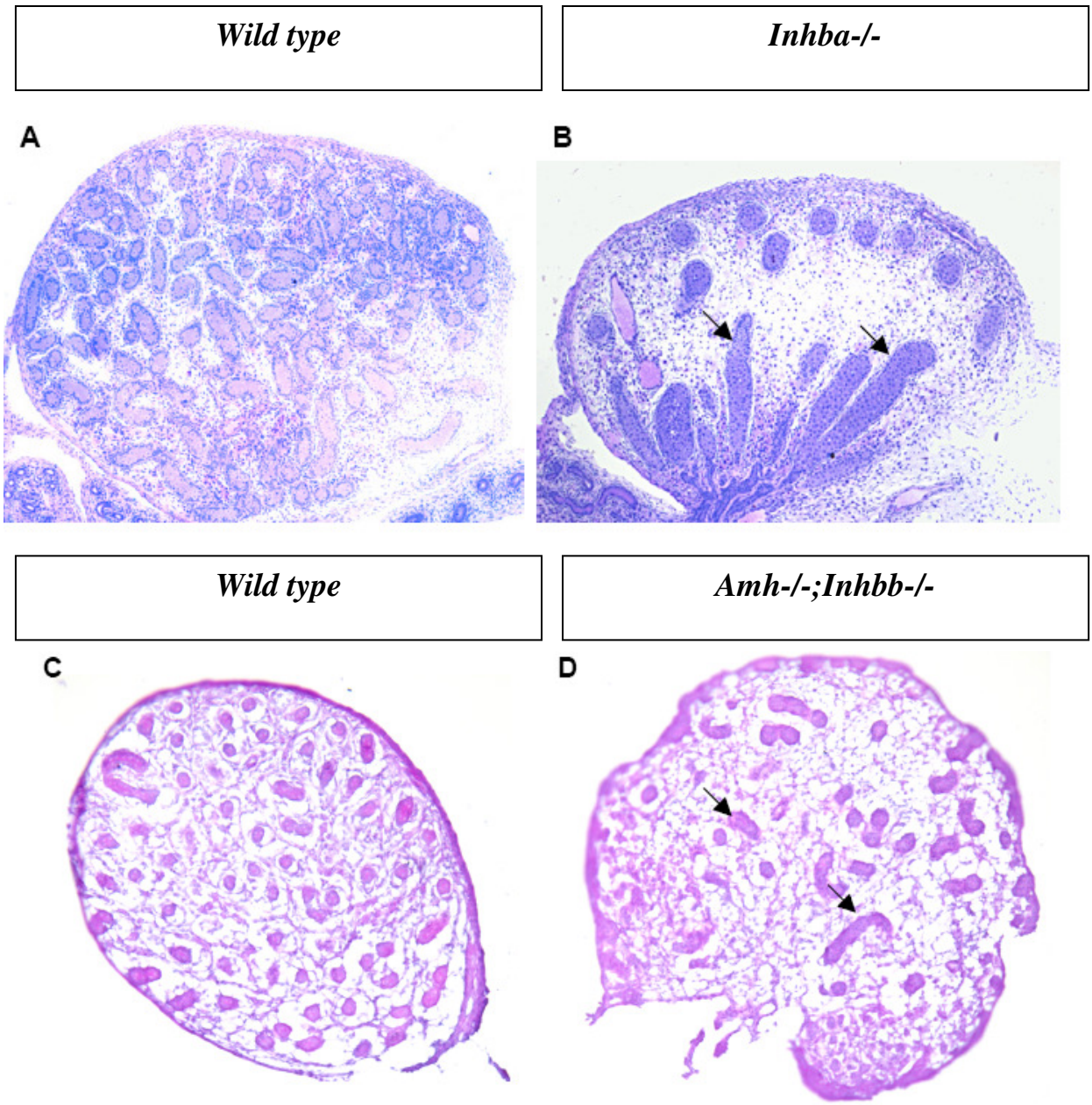

Figure 1. Histological analysis of testis cord structures in newborn male. Cross sections of testes were stained with either Periodic Acid-Schiff (PAS) for (A) wild-type and (B) *Inhba-/-* animals or H&E for (C) wild-type and (D) *Amh-/-; Inhbb-/-* double knockout. Magnification was 4X and the arrows indicate testis cords that fail to undergo further development.

Concluding remarks and future directions

Our discovery of novel signaling molecules involved in the maintenance of fetal testis cords adds to the increasing evidence that testis cord morphogenesis is not an "all-or-nothing" process. Proper testis development hinges upon three key events – testis determination, testis cord establishment, and testis cord maintenance. The first of these events is dependent upon *SRY* expression, *Sox9* upregulation and/or nuclearization, and the subsequent differentiation of Sertoli cells. The second event involves the recruitment and determination of the remainder of the testis cell lineages, as well as their organization into the testis cord architecture. The third and final event, testis cord maintenance, requires the production of additional

factors in order to increase the size and complexity of the testis cords. Evidence for these three phases of testis development comes from the distinct phenotypes arising from defects in a particular stage as discussed in the review.

The evolutionary logic behind the need for different signaling inputs at each stage of testis growth has yet to be fully understood. In particular, the requirement of apparently distinct factors to maintain the testis cords during the later part of embryonic development is intriguing. Further work is needed to determine whether testis cord maintenance is achieved primarily through factors that seem dispensable during the earlier stages of testis formation, such as *Inhba* or the combination of *Amh* and *Inhbb*, or whether the continued expression of genes important for initial testis development is also needed to maintain the cords. The fact that a late-stage fetal testis cord phenotype is observed in the *Amh;Inhbb* double knockout mouse but not in single knockouts of either of these genes indicates that redundant signaling is an important component of testis cord maintenance. It also remains to be determined whether defects in fetal testis cord maintenance can be overcome by the rapid amount of growth that occurs during the perinatal period. Conversely, a failure to maintain testis cords during the later stages of fetal development could result in subfertility or even infertility of the adult animal. A relationship between defects in fetal testis cord maintenance and infertility in adulthood could provide new molecular targets for understanding and perhaps even treating male infertility.

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