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The road to maleness: from testis to Wolffian duct

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The establishment of the male internal reproductive system involves two crucial events: the formation of the testis and the maintenance and differentiation of the Wolffian duct. Testis formation, particularly the specification of Sertoli cell and Leydig cell lineages, is controlled strictly by genetic components initiated by the testis-determining gene *SRY* (*sex-determining region of the Y chromosome*). Conversely, Wolffian duct differentiation is not directly mediated via the composition of the sex chromosome or *SRY*; instead, it relies on androgens derived from the Leydig cells. Leydig cells do not express *SRY*, indicating that a crosstalk must be present between the SRY-positive Sertoli and Leydig cells to ensure normal androgen production. Recent advancement of genetic and genomic approaches has unveiled the molecular pathways for differentiation of Sertoli cells and Leydig cells as well as development of the Wolffian duct.

Introduction

Sex determination in eutherian mammals is thought to equate to testis determination as active genetic components drive the gonadal primordium to testis differentiation; by contrast, the absence of these active components leads to ovary development. As early as the 1940s and 1950s, Alfred Jost established the paradigm that composition of the sex chromosomes (or chromosomal sex) controls the sex of the gonad, which eventually determines the phenotypic sex of the embryo (internal and external sexual characteristics) [1,2]. The advancement of genetic analysis and genomic research further validates the Jost paradigm, particularly regarding the establishment of maleness. The presence of the Y chromosome or the *SRY* gene (*sex-determining region of the Y chromosome*) activates a cascade of molecular and cellular events leading to differentiation of various somatic cell types (i.e. Sertoli and Leydig cells) and organization of testis structure. The establishment of these somatic cells ensures production of anti-Müllerian hormone (AMH) and androgens, which are responsible for removing the Müllerian duct (the precursor of the female reproductive tract) and maintaining the Wolffian duct (also known as the mesonephric duct; the precursor of the male reproductive tract), respectively. The focus of this review is to summarize the current knowledge on the molecular and cellular mechanisms for the establishment of maleness – particularly the specification of testicular cell types and subsequent differentiation of the Wolffian duct. Other aspects of male development, such as testis descent and development of the external genitalia, have been discussed extensively in other current reviews [3,4].

Is it all about the Y chromosome?

The essential role of the Y chromosome for testis formation was first confirmed by Welshons and Russell in 1959 [5]. However, the testis-determining gene *SRY* was not identified until the early 1990s [6]. *SRY*, at least in mice [7] and humans [8], is the only gene on the Y chromosome required for testis determination. Introduction of *SRY* to the XX individual resulted in a complete ovary-to-testis sex reversal [9,10]. Conversely, mutation in the *SRY* gene in human patients caused a male-to-female sex reversal [11,12]. Other nonsex-linked genes such as *M33*, a mouse homolog of the *Drosophila Polycomb* gene, *Empty spiracles homolog 2* gene (*Emx2*) and *lens intrinsic membrane (Lim) homeobox 9* gene (*Lhx9*) have been shown to be essential for early formation of male gonad because null mutations of any of these genes resulted in male-to-female sex reversal [13]. However, the exact connection of these genes to *SRY* is yet to be defined. The most crucial function of *SRY* is to trigger the differentiation of Sertoli cells, the somatic cell type responsible for testis morphogenesis and the specification of other somatic cells.

The mechanism for the triggering of Sertoli cell differentiation by SRY has gradually come to light. Sertoli cells are the only somatic cell type in the testis that exhibits a bias toward the Y chromosome [14]. Indeed, *SRY* expression in the gonad is restricted to the precursors of Sertoli cells [15,16]. Increasing evidence indicates that SRY induces Sertoli cell differentiation via another transcription factor, *SRY-box containing* gene 9 (SOX9). *Sox9* alone is sufficient to induce testis formation in mice [17–22]. Conversely, a null mutation in *SOX9* prevents testis differentiation in XY mice and humans [23–25]. *Sox9* upregulation is the earliest event in pre-Sertoli cells after *Sry* expression [26] and SOX9 is colocalized with SRY in the nuclei of pre-Sertoli cells [27–29]. Additionally, an SRY-binding site has been identified on the *Sox9* promoter, suggesting transcriptional regulation of *Sox9* by SRY [30]. However, although all Sertoli cells become SOX9-positive, only a portion of them express SRY initially [27,31]. This unique pattern of *Sry* and *Sox9* expression suggests that some SRY-negative somatic cells are recruited to become SOX9-positive and differentiate into Sertoli cells. Prostaglandin D2 (PGD2) has been implicated as a

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paracrine factor derived from *Sry*-expressing Sertoli cells [31]. PGD2 was able to induce phosphorylation of SOX9, thus enhancing nuclear import of SOX9 [32] and partially sex-reversing the XX gonad [33]. These data indicate that SRY utilizes a cell-autonomous as well as a paracrine mechanism to induce differentiation and expansion of the Sertoli cell population (Figure 1).

Several factors act parallel to, or downstream of, SRY to either activate SOX9 expression or promote the nuclear localization of SOX9 in Sertoli cell precursors. Nuclear receptor 0B1 (DAX1), a known SRY antagonist in B6 mice carrying the Y chromosome of *Mus domesticus poschiavinus* origin [34], is essential for normal *Sox9* expression [35]. It has also been suggested that upregulation of *Sox9* expression in pre-Sertoli cells depends upon the coordinated interactions of SRY, DAX1 and testis-determining autosomal 1, the product of an autosomal gene located on 4q in mice [36]. It was found that *Sry* transcript levels were

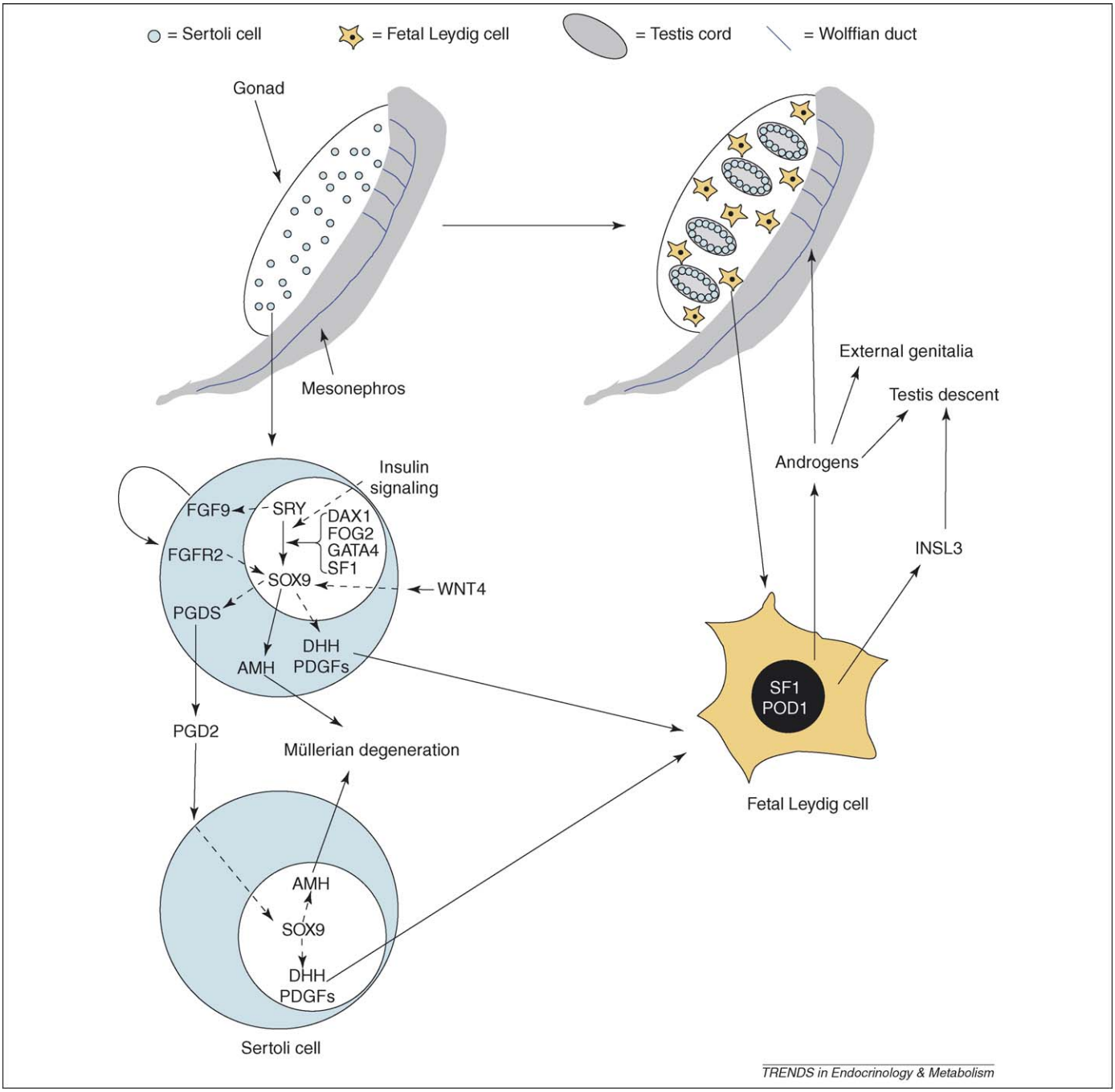

Figure 1. Molecular and cellular pathways that connect Sertoli cells, Leydig cells and Wolffian duct development based on mouse genetic models. Solid lines represent pathways supported by genetic or biochemical evidence. Dotted lines represent putative pathways responsible for the corresponding events. At the time of sex determination in mouse embryos (E10.5–11.5), *Sry* is expressed in pre-Sertoli cells (light blue cell; only one is shown here). SRY, along with other transcription regulators (DAX1, FOG2, GATA4, SF1 and other, unidentified factors), upregulates the expression of *Sox9*, which putatively controls the production of AMH, DHH, PDGFs and prostaglandin D synthetase (PGDS). PGDS leads to the production of PGD2, which recruits *Sry*-negative Sertoli cell precursors by inducing *Sox9* expression. DHH and PDGFs derived from the Sertoli cells act as paracrine factors to induce specification and differentiation of fetal Leydig cells (yellow cells; only one is shown here). The developing fetal Leydig cells then produce androgens and INSL3. Androgens facilitate the survival and differentiation of the Wolffian duct and male external genitalia. INSL3, along with androgens, ensure the occurrence of testis descent.

significantly reduced in XY Friend of GATA$^{-/-}$ (*Fog2*$^{-/-}$) mouse gonads at embryonic day 11.5 (E11.5), and Sertoli cell differentiation was blocked in *Fog2*$^{-/-}$ and *Gata4*$^{ki/ki}$ XY gonads* [37]. In addition to transcription factors, secreted factors and downstream signaling pathways have been implicated in *Sox9* expression in Sertoli cells. For example, insulin signaling has crucial roles in Sertoli cell differentiation; a triple knockout of insulin growth factor I receptor, insulin receptor and insulin receptor related-receptor in mice leads to female-to-male sex reversal with normal expression of *Sry* but defective expression of Sertoli cell markers including *Sox9* [38]. Another factor, wingless-related MMTV integration site 4 (WNT4), previously known to be involved in ovary development [39], has been shown to regulate Sertoli differentiation through a mechanism upstream of SOX9 but downstream of SRY [40]. Recently, it has been shown that *Wnt4* and the *fibroblast growth factor 9* gene (*Fgf9*), which both work downstream of SRY, antagonize each other to control *Sox9* expression [41]. Pathways induced by mitogen-activated protein kinase (MAPK) [42,43], RHO–ROCK [44] and vinexin γ–MAPK have been shown to regulate *Sox9* expression both in cell culture and *in vivo* [45]. SOX9 nuclear translocation was also shown to be regulated via importin β1 [46] and calmodulin [47], which further indicates that SRY and/or SOX9 are regulated at the post-translational level.

Although the molecular mechanisms by which SRY and SOX9 induce testis formation remain unknown, the cellular process for testis morphogenesis downstream of the SRY–SOX9 pathway has been characterized. SRY and SOX9 are known to stimulate the proliferation of different testis somatic cell lineages [48–50], to induce migration of somatic cell precursors from the mesonephros and to establish testis-specific vasculature [13,51]. In the following section, we discuss in detail how Leydig cells, the androgen-producing somatic cells, emerge under the control of the SRY–SOX9 pathway.

From SRY to androgens

SRY does not directly control the production of androgens, the hormones that sculpt male phenotypes. Fetal Leydig cells, the source of androgens, start to differentiate in the mouse testis interstitium ~24 hours after Sertoli cell appearance [52]. In contrast to Sertoli cells, fetal Leydig cells do not express *Sry* or *Sox9*, suggesting that their differentiation is regulated indirectly by paracrine factors secreted from Sertoli cells. Two signaling molecules from Sertoli cells are involved in fetal Leydig cell differentiation: desert hedgehog (DHH) and platelet-derived growth factors (PDGFs). In the absence of *Dhh*, fetal Leydig cell numbers were dramatically decreased owing to failure to initiate normal differentiation [53]. When one receptor for PDGF was inactivated (*Pdgfrα*), XY gonads displayed disruptions in proliferation and mesonephric cell migration, as well as fetal Leydig cell differentiation [54].

In addition to the signaling proteins produced by Sertoli cells, transcriptional factors including X-linked aristaless-related homeobox gene (ARX), podocyte-expressed 1 (POD1, also known as transcription factor 21, capsulin and epicardin) and steroidogenic factor 1 (SF1, also known as nuclear receptor 5A1) are involved in fetal Leydig cell differentiation. In *Arx*-null mice, the expression of the Leydig cell marker HSD3β1 was severely diminished. At E14.5, *Arx* was strongly expressed in several interstitial cell types, such as in peritubular myoid cells, endothelia cells and fibroblasts, but expression was not detected in Leydig cells. This suggests that ARX indirectly regulates fetal Leydig cell differentiation [55]. The nuclear receptor SF1, which is known to be essential for somatic cell lineage establishment during testis development [56], also has a role in Leydig cell differentiation. In gonad-specific *Sf1* knockout mice, expression of Cyp11a and the steroidogenic acute regulatory protein in fetal Leydig cells was decreased [57]. In *Sf1* heterozygous embryos, *Dhh* expression was temporarily reduced at E11.5, and fetal Leydig cell markers Cyp17 and Cyp11a1 were reduced at E13.5 [58], suggesting an indirect role for SF1 in initial fetal Leydig differentiation via DHH. Additionally, a null mutation of *Pod1*, a helix-turn-helix transcription factor expressed in the gonadal interstitium, increased the number of steroidogenic cells in both male and female gonads but reduced androgen production [59]. These results indicate that fetal Leydig cell differentiation is controlled externally through pathways elicited by Sertoli cells and intrinsically by transcription factors.

Androgens and Wolffian duct development

The sexually dimorphic development of the reproductive tract in the male is established by androgens and AMH. Sertoli cells produce AMH, which induces regression of the Müllerian duct, the precursor of the female reproductive tract, whereas Leydig cells produce androgens and insulin-like factor 3 (INSL3; Figure 1). Androgens are important for differentiation of various parts of the Wolffian duct and accessory glands (discussed below), and both androgens and INSL3 are required for testicular descent [3]. The Wolffian duct is originally derived from the pronephros, whose ductal derivative elongates posteriorly through the mesonephros and extends to the cloaca [60]. The pronephros eventually degenerates but its ductal derivative remains in the mesonephros and becomes the Wolffian duct between E9–10 in mice. The epithelium of the Wolffian duct is derived from the mesonephric mesenchyme, which undergoes transformation to differentiate into the epithelial tubes of the ducts. Initial formation of the Wolffian duct is independent of the sex of the animal or hormones derived from the gonads, and is instead controlled by a network of transcription factors and signaling molecules. Several genes encoding transcription factors, such as *paired box* gene 2 (*Pax2*), *Pax8*, *lens intrinsic membrane 1* (*Lim1*) and *Emx2*, are expressed in the epithelium of the Wolffian duct. Null mutations of these genes affect different aspects of epithelial differentiation but eventually lead to a similar phenotype: degeneration of the Wolffian duct by E13.5 [61–64]. The molecular interactions among these transcription factors are still unclear. Once the Wolffian duct structure is stabilized, its further differentiation into epididymis, vas deferens, seminal vesicle

* GATA is a zinc-finger transcription factor that recognizes the consensus target sequence (T/A)GATA(A/G), whereas the *Gata*$^{K}_{i}$ allele is a targeted mutation in Gata4 that abrogates its interaction with its cofactor FOG.

and ejaculatory duct becomes dependent on androgens from fetal Leydig cells [65].

Morphological transformation of the Wolffian duct into specific regions of the male reproductive tract occurs as early as E15 in mice. The anterior or upper portion of the Wolffian duct adjacent to the testis elongates and folds into the epididymis. Meanwhile, the mesonephric tubules differentiate into efferent ducts that eventually connect the rete testis and epididymis. The middle portion of the Wolffian duct remains a simple tube, to form the vas deferens. The posterior or caudal portion of the Wolffian duct dilates, elongates cranially and eventually forms a distinct diverticulum [66].

The importance of androgens on Wolffian duct development was first identified by Alfred Jost, who determined that testosterone replacement was able to restore male differentiation in castrated rabbit embryos [1,2]. It is clear that androgens are not essential for initial Wolffian duct formation, as evident by the fact that the Wolffian duct is present in both sexes before the onset of dimorphic production of androgens. Androgens are steroid hormones which are crucial for the maintenance and elaboration of the Wolffian duct later in development. Their action is mediated via their receptor [the androgen receptor (AR)] inside target cells. Androgens enter their target cells and bind to AR to regulate the transcription of specific genes. Molecular evidence for the role of androgens is well documented in both mice and humans. In mice, loss of AR in both testis feminization (*Tfm*) mice and AR knockouts led to the degeneration of the Wolffian duct [67–69]. In humans, androgen insensitivity syndrome owing to null mutations of AR [70,71] resulted in the same phenotypes as in mice. Furthermore, when females were exposed to excessive androgens by testis transplantation during fetal development, the Wolffian duct persisted [2,72,73].

The essential role of androgens in male differentiation is apparent but it remains unclear how androgens alone facilitate the complex patterning of the Wolffian duct into the various functionally and structurally distinct regions of the male reproductive tract. Homeobox transcription factors (*Hox*) are appealing candidates for the spatial differentiation of reproductive tracts because of their roles in the patterning of embryos and other organs. Homeotic transformation of the anterior vas deferens into epididymis was found in single mutant $Hoxa11^{-/-}$ and double mutant $Hoxa11^{-/-}$; $Hoxd11^{-/-}$ male newborns [74,75]. Also, in $Hoxa10^{-/-}$ males, the distal epididymis and the proximal ductus deferens have acquired some morphological features of anterior epididymal segments [76]. In addition to genetic analysis, many *in vitro* experiments have been performed to examine the involvement of various growth factors on Wolffian duct development. However, the functional roles of these factors in relation to androgens remain to be determined.

Concluding remarks

Organization of the male reproductive system in mammals requires a coordinated interaction between genetic (SRY–SOX9) and hormonal (androgens) pathways. Sertoli cells, where the genetic components act, control the differentiation of Leydig cells. Androgens derived from Leydig cells, along with imprinted regulation via various transcription factors, mold the structure of the male reproductive tract. Over the past ten years, tremendous progress has been made to uncover the molecular mechanisms for the development of Sertoli and Leydig cells, as well as the patterning of the Wolffian duct. However, the exact relationship and functional connection among different regulatory components requires further investigation. How does SRY regulate the production of factors such as DHH and PDGFs to trigger Leydig cell differentiation? What is the origin of fetal Leydig cells? Which regulators, other than DHH and PDGFs, are responsible for Leydig cell differentiation? Are androgens the only factors required for normal Wolffian duct development? How does the Wolffian duct differentiate into the complex segments of the male reproductive tract? The development of new molecular, genetic and genomic techniques will be crucial to answer these questions and hopefully provide substance to the paradigm proposed by Jost 50 years ago.

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