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INVITED REVIEW

Sperm Biology

Understanding normal and abnormal development of the Wolffian/epididymal duct by using transgenic mice

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The development of the Wolffian/epididymal duct is crucial for proper function and, therefore, male fertility. The development of the epididymis is complex; the initial stages form as a transient embryonic kidney; then the mesonephros is formed, which in turn undergoes extensive morphogenesis under the influence of androgens and growth factors. Thus, understanding of its full development requires a wide and multidisciplinary view. This review focuses on mouse models that display abnormalities of the Wolffian duct and mesonephric development, the importance of these mouse models toward understanding male reproductive tract development, and how these models contribute to our understanding of clinical abnormalities in humans such as congenital anomalies of the kidney and urinary tract (CAKUT).

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INTRODUCTION

Understanding the mechanisms that regulate the development of the Wolffian duct (WD) is important because disruption of epididymal function may arise as a consequence of its abnormal development. Very little is known of either the process of WD development or the nature and causes of congenital defects that lead to male infertility. For example, it is clear that an undeveloped initial segment of the epididymis leads to male infertility[1,2] and considering that the human epididymis has an initial segment-like epithelium,[3] it is important to at least understand the development of this region. There are three developmental processes that are considered to be important during the development of the WD: (1) mesonephros formation, (2) stabilization of the ductal system and further growth, (3) postnatal differentiation (**Figure 1**). Each process is dependent upon developmental factors as shown by WD phenotypic mice carrying mutations of each factor.

This review focuses on mouse models that display abnormalities in WD or mesonephric development, the importance of these mouse models toward understanding male reproductive tract development, and how these models contribute to understanding clinical abnormalities in humans. **Table 1** shows mutations of genes in mice that display Wolffian/epididymal duct phenotypes.

DEVELOPMENT OF WOLFFIAN/EPIDIDYMAL DUCT AND MOUSE MODELS

Mesonephros formation

During development, the nephric duct/Wolffian duct (WD) arises from the anterior, intermediate mesoderm, and extends caudally.[4] In the case of mouse, WD formation begins approximately on embryonic day (E) 8.5 and is completed by reaching the cloaca at E9.5[5] (**Figure 1a** and **1b**). As the WD elongates, it induces the formation of nephric tubules through a mesenchymal-epithelial transition process. The tubules form three kidney primordia: pronephros, mesonephros and metanephros[6] (**Figure 1c**). The pronephros and mesonephros are transient kidneys and degenerate soon after their formation. However, in the mesonephros, the WD and cranial mesonephric tubules (MT) are retained and give rise to the male reproductive tract including the epididymis and efferent ducts, respectively.

Because WD formation is crucial for kidney development in mammals, many mouse models that show abnormal WD or mesonephric development also display urogenital abnormalities. The paired domain transcription factors Pax2 and Pax8 are well-known inducers of the initial formation of the WD.[7,8] The LIM-class homeobox gene *Lim1* is required for the extension of the WD.[9,10] Mice carrying a null mutation of *Emx2*, a mouse homologue of the *Drosophila* head gap gene *empty spiracles* (*ems*), display normal WD development until E10.5, but at later time points the duct degenerates, resulting in lack of a kidney and a failure of the reproductive tract to develop. [11] Mice carrying a null mutation of *Gata3*, which is a transcriptional target of Pax2 and Pax8, also show defects in WD initiation.[12]

Growth factors can differentially regulate gene expression especially through epithelial-mesenchymal interactions. Fibroblast growth factor (FGF) signaling is one of the well analyzed growth factor signaling events during mesonephric formation. *Fgf8* encodes an FGF ligand, which is expressed in the intermediate mesoderm, and lack of its expression results in the absence of the cranial mesonephros and MTs.[13]

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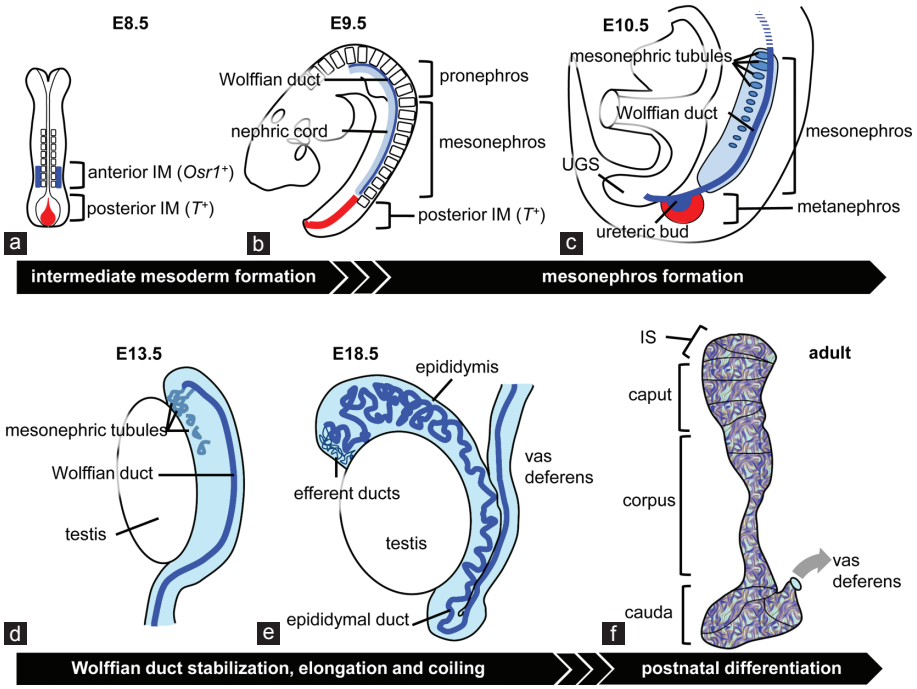

Figure 1: Schematic diagram of mouse Wolffian/epididymal duct development. (**a–c**) The origin of the epididymis is the intermediate mesoderm. Spatiotemporally distinct intermediate mesoderm at E8.5 gives rise to the WD and metanephric mesenchyme.[37] The anterior intermediate mesoderm, which gives rise to the pronephros and the whole WD, is composed of *Osr1*-positive cells at E8.5. The posterior intermediate mesoderm, which gives rise to the metanephric mesenchyme, is positive for *T* at E9.5. The posterior intermediate mesoderm may correspond to axial progenitor cells, which serve as the source of the caudal body trunk.[96,97] The WD begins to form from the anterior intermediate mesoderm at E8.5 and grows posteriorly reaching the urogenital sinus at E9.5.[98] Meanwhile, the pronephros regresses through apoptosis.[99] The WD induces the formation of mesonephric tubules from the mesenchyme (nephric cord) adjacent to the WD in a cranio-caudal manner. At the caudal end of the WD, the metanephros is initiated by ureteric bud formation through the interaction between WD epithelia and the metanephric mesenchyme at E10.5. (**d**) After gonadal sexual differentiation begins, the WD in the female embryo regresses from cranial to caudal while the WD in the male embryo is stabilized. The cranial set of mesonephric tubules connected to the WD is stabilized while the caudal set of mesonephric tubules regresses *via* apoptosis. (**e**) In the male embryo, the stabilized WD begins to coil from the cranial portion at E15.5. The duct continues to elongate and coil throughout development. (**f**) Ductal elongation and coiling continue after birth. The single-layered ductal epithelia undergo differentiation between P15 and P44. At the same time, the regions of the epididymis, initial segment, caput, corpus and cauda, become morphologically distinct. Sperm transport through the duct begins at approximately P35.[59,68] IM: intermediate mesoderm; UGS: urogenital sinus; IS: initial segment.

FGF ligands bind and activate alternatively-spliced forms of four tyrosine kinase FGF receptors (FGFRs 1–4).[14] During mesonephric development, *Fgfr1* is expressed in the mesenchyme while *Fgfr2* is in the epithelium, maintaining the WD and mesonephric mesenchyme.[15] The function of FGFR2 in the WD epithelia is suggested to maintain the caudal part of the WD in the mesonephros by regulating cell proliferation.[16]

Wnt genes encode a family of secreted glycolipoproteins regulating multiple processes during development, including cell proliferation and cell polarity. Among the *Wnt* genes, *Wnt9b* is mainly expressed in the WD epithelium while *Wnt7b* is faintly expressed from E9.5 onward. In animals devoid of *Wnt9b* their MTs are absent, and the epididymis is lacking at birth despite the normal formation of the WD at E10.5.[17] β-catenin-dependent canonical WNT signaling, which mainly regulates cell proliferation and differentiation, is sufficient to rescue MT induction in *Wnt9b* null mice. On the other hand, during metanephric kidney development, attenuation of *Wnt9b* affects the planar cell polarity of the epithelium and lead to tubules with an increased diameter.[18] Further spatiotemporal analyses of epididymal development in this mutant would contribute to our understanding of this molecule in tubulogenesis and its maintenance.

The number of MTs differs between species, and their function as a secretory organ is observed in pigs and humans but not in mice.[19–21] The number of efferent ducts reaching the testis also differs between species.[22,23] It is unclear whether there is a correlation between early MT number and the final number of efferent ducts observed in the adult. MT formation may resemble the formation of the renal nephron; both have the characteristic 'J' or 'S' shape during early development. The nephric tubule is formed through a mesenchymal-to-epithelial transition, and this cellular process is shared between mesonephric and metanephric tubules. *Pax2/8*, *Emx2* and *Lim1* are expressed in the condensed nephric cord and are required for tubulogenesis in addition to WD development.[7–11,24] The Wilms' tumor suppressor gene *Wt-1* and the homeobox gene *Six1* are also expressed in the nephrogenic mesenchymal condensation throughout the nephrogenic cord. Mice lacking *Wt-1* or *Six1* lack caudal MTs while cranial MTs are intact. These observations indicate that the regulation of the cranial and caudal set of MTs is distinct.[25–27] Conversely, lack of the forkhead transcription factors *Foxc1* and *Foxc2*, as well as *Sonic hedgehog* (*Shh*) expressed in the notochord or floor plate, results in supernumerary MT formation, suggesting suppressive effects of these genes on MT formation.[28,29] It is important to uncover how the differential regulation of tubule formation and stabilization along the anterior-posterior axis of the nephrogenic cord is established.

The connection between the rete testis and efferent ducts is observed at E13.5, and testicular fluid transport is detected at the corresponding stage of the rat embryo.[30] The patterning of efferent duct formation is intriguing, but the manner by which they reach the testis is not clear. There are at least two hypotheses on how the efferent ducts could be formed: (1) that a subset of MTs branch and fuse with each other forming the characteristic network of ductules, (2) that branching morphogenesis does not occur and the characteristic

Table 1: Mouse models which show defects in WD/epididymal duct development

Gene	*Type of mutation, Cre driver*	*Phenotype of the mutant*	*References*
Defect in mesonephros formation			
Pax2	KO	Dysgenesis of WD and MD, absence of MT	7
Pax8	KO	Normal	24
Pax2/Pax8	dKO	Dysgenesis of WD and MD, absence of MT	8
Lim1	KO	Dysgenesis of WD	10
	Pax2-Cre	Defect in caudal WD extension	9
Gata3	KO	Dysgenesis of WD and MD, absence of MT	12
Wt-1	KO	Absence of caudal MT	26
Six1	KO	Absence of caudal MT	27
Osr1	KO	Defect in WD extension, absence of MT	100
Emx2	KO	Regression of whole WD	11
Wnt9b	KO	Absence of MT, absence of epididymis	17
Fgf8	*T-Cre*	Regression of cranial mesonephros	13
Fgfr1/2	*T-Cre*	Dysgenesis of WD and MT	13
	Pax3-Cre	Absence of MT	15
Fgfr2	*Hoxb7-Cre*	Regression of caudal WD	16
Shh	KO	Numerous ectopic MT, ectopic UB	29
Foxc1/2	*Foxc1/Mf1*^{ch}, KO	Numerous ectopic MT, ectopic UB	28, 101
c-ret	*ret-k*$^{-}$	Reduced number of MT	102
Raldh2	KO	Absence of WD	103
Lfng	KO	Blockage of the connection between efferent duct and rete testis	36
Defects in WD stabilization, elongation and coiling			
Ar	*Tfm*, KO	WD regression	40,41
Inhba	KO	Failed to develop ductal coiling in epididymis	53
Sfrp1/2	dKO	Shortened vas deferens	56
Vagl2	*Vangl2*$^{lp/lp}$	Shortened vas deferens	56
Wnt5a	KO	Shortened vas deferens	56
Pkd1	KO, *Pax2-Cre*	Coiling defect, cystic dilation of efferent ducts	54
Defects in postnatal differentiation			
Pten	*Rnase10-Cre*	Dedifferentiation of IS	2
Ros1	KO	Undifferentiated IS	1
Dusp6	KO	Large caput and corpus	67
Frs2	*Hoxb7-Cre*	Morphologically normal	68
	Rnase10-Cre	Abnormal shape of epididymis	68
Ar	*Ap2a-Cre*	Defective epithelial cell differentiation	47
	Rnase10-Cre	Absence of IS, defective epithelial cell differentiation	70
	FoxG-Cre	Absence of IS, defective epithelial cell differentiation	71
	Probasin-Cre	Small epididymis and seminal vesicle	69
Dicer	*Defb4-Cre*	Epithelial cell dedifferentiation	75
miR-29a	*miR-29b1*^{UBC} transgene	Hypoplastic epididymis	77
Lgr4	*Lgr4*$^{Gt/Gt}$	Short, dilated and much less convoluted epididymal ducts	104
	KO	Blockage of efferent duct	105
Shp1	*mev/mev*	Aberrant epididymal region	66
Hoxa11	KO	Transformation of vas deferens to epididymis	79
Hoxa10	KO	Transformation of vas deferens to epididymis	80

WD: wolffian duct; MT: mesonephric tubules; UB: ureteric bud; IS: initial segment; MD: mullerian duct

network of ductules is formed by simple fusion of a subset of MTs. The latter hypothesis would seem more feasible than the first because of the presence of blind-ended tubules. These MTs only fuse to one other MT, leaving one end sealed, hence becoming blind-ended. Obviously, there must be considerable coordination between the fusion events that limit the number of MTs that can fuse[4,5] resulting in the conus (2–3 fused MTs) and the single common ductule.[22] Identification of the genes and processes by which the formation and patterning of the efferent ducts occur is crucial, and the GUDMAP *in situ* hybridization database (http://www.gudmap.org/index.html)[31,32] clearly shows some potential genes that may regulate their formation, e.g., collagen triple helix repeat containing 1*(Cthrc1),* cortexin 3 *(Ctxn3)* and laminin, alpha1 *(Lama1)*. *Lunatic fringe* (*Lfng*) is one of the mammalian *fringe* genes encoding a modifier of the notch receptor expressed in the developing WD, MTs and testis.[33–35] *Lfng*-null mice show partial bilateral blockage of the connection between the rete testis and the efferent ducts, indicating the involvement of notch signaling in establishing the rete testis-efferent duct boundary.[36]

The origins of nephron progenitor cells are suggested to differ between mesonephros and metanephros.[37] Metanephric mesenchyme is

derived from a posterior immature caudal population, which is positive for *Brachyury* (*T*) expression, and persists in the posterior end of the embryo until body axis extension is complete (**Figure 1a**). On the other hand, the WD and at least part of the mesonephric mesenchyme arise from the anterior intermediate mesoderm, which is defined by *Osr1* expression at E9.5 (**Figure 1b**). These recent studies may indicate that abnormal body axis extension affects the intermediate mesodermal cell fate. It is possible that disruption of the A-P body axis extension affects not only the metanephric mesenchyme but also the mesonephric mesenchymal distribution, and subsequently further male reproductive tract development. Conditionally-induced mutations of the planar cell polarity (PCP) pathway-related genes, *Wnt5a*, *Ror2* and *Vangl2*, which are important for A-P body axis extension, demonstrate that insufficient A-P axis extension of the posterior intermediate mesoderm is correlated with urogenital tract abnormalities.[38] It is clear that more studies are needed to examine the early formation of the intermediate mesoderm and how this translates into development of the WD.

Stabilization of the ductal system and further growth: elongation and coiling

During embryogenesis, the mesonephros gives rise to a stable male reproductive tract whereas the mesonephros in the female regresses (**Figure 1d** and **1e**). Androgens produced in the testis are a major factor regulating this stabilization.[39–42] Following gonadal sex differentiation, the testis begins to produce the androgen, testosterone, at approximately E12.5.[43,44] Unlike for other androgen-dependent organs, such as the prostate and seminal vesicle, it has been suggested that locally-produced, and not systemic androgen, from the testis is necessary for WD stabilization.[45] Indeed, fluorescence labeling of an androgen ligand shows that androgen is transported within the luminal fluid.[30] However, there are studies showing that testicular androgen delivered via the systemic circulation is sufficient to prevent WD regression. Subcutaneous testicular grafts stabilize the WD in female marsupial embryos.[46] Androgens act through the androgen receptor (AR), a member of the nuclear receptor superfamily. The expression of AR is mainly detected in the mesenchyme surrounding WD epithelia at E13.5 in the mouse. Tissue-specific *Ar* knockout (KO) analyses demonstrate that WD stabilization and coiling is induced in the absence of epithelial-expressed *Ar*, demonstrating the importance of *Ar* in the mesenchyme.[47] This finding is consistent with the observation from tissue recombination experiments on androgen-insensitive *Testicular feminized* (*Tfm*) mice.[48,49] Several growth factors, including FGF and Epidermal growth factor (EGF), are suggested to mediate androgen functions in the prostate and WD.[50–52] However, the molecular mechanisms by which androgens regulate these genes *in vivo* are not known.

To create a long, highly-convoluted epididymal duct, the WD begins to elongate and coil from E15.5, following stabilization (**Figure 1e**). This process is also androgen-dependent, but growth factor signaling has been reported to regulate this elongation event. Tomaszewski *et al.* reported that *Inhba*, a subunit of both inhibins and activins, is a regional paracrine factor in mouse mesonephroi that controls coiling of the epithelium in the anterior WD.[53] *Pkd1*, whose mutation accounts for 85% of autosomal dominant polycystic kidney disease, and is a membrane-spanning glycoprotein involved in growth factor signaling transduction and cytoskeleton dynamics. Epithelial coiling is absent from the *Pkd1* mutant.[54] In both mutations, epithelial cell proliferation is attenuated. Recently, mathematical modeling has suggested that epididymal tubule morphogenesis is dependent upon the cell proliferation area in the tubule and mechanical resistance from the tissues surrounding the tubule.[55]

The secreted frizzled-related proteins (SFRPs) antagonize WNT ligand protein binding to its receptor FZD. The double KO (dKO) of *Sfrp1* and *Sfrp2* genes results in a shortened WD and vas deferens.[56] Androgen administration to these animals never rescues this phenotype, indicating that the abnormalities in *Sfrp1/2* dKO mutant male embryos are not caused by insufficient production of testosterone from the testes, but may reflect insensitivity of some target tissues to androgens.[56] It is also possible to consider that these phenotypes are, at least partially, a secondary consequence of the A-P extension defect of intermediate mesoderm formation described above. Although recent analyses have partially revealed the molecular mechanisms of ductal morphogenesis, further analyses should be performed including how androgen signaling regulates these molecules.

Postnatal differentiation: regional differentiation and epithelial cell differentiation

The epididymis consists of distinct anatomical regions that vary between species. However, in the mouse four regions can be defined: initial segment and caput, corpus and cauda epididymidis (**Figure 1f**). Each region is further divided into many segments characterized by expression of specific mRNAs, proteins and a repertoire of cell types.[57,58] The segments, divided by septa, are observed after birth and are distinct during puberty, postnatal (P) days 14–35. Impaired epididymal regionalization or epithelial cell differentiation results in male infertility. For example, if the initial segment does not develop, then male infertility results. Data from efferent duct ligation (EDL) experiments suggested that luminal fluid coming from testis is responsible for the maintenance of initial segment cell survival, proliferation and differentiation.[59,60]

Several growth factors, including FGFs 2,4 and 8, are detected in testicular fluid, and *Fgfr*s are expressed in the epithelium of the initial segment.[61,62] During normal development, high activity of the MAPK pathway, especially p-MAPK1/3 (p-ERK1/2), is detected in the initial segment.[60] EDL abolishes their activities, emphasizing the importance of lumicrine factors regulating their activity.[60] *Ros1* encodes an orphan receptor tyrosine kinase that is expressed in few epithelia, among them the WD and its derivatives.[63–65] Loss of *Ros1* expression or a naturally-occurring mutation of *Shp1* (*me*^{v}), a negative regulator of ROS1, results in abnormal differentiation of the initial segment.[1,66] *RNase10-Cre* drives gene recombination in the initial segment epithelia from P17 onward. *RNase-Cre*-mediated mutation in *Pten*, a negative regulator of PIP3/AKT signaling, induces dedifferentiation of the initial segment.[2] In these animals, abnormal differentiation results in an abnormally shaped initial segment. MAPK signaling regulators such as DUSP6 and FRS2 play important roles in epididymal cell proliferation and survival during postnatal development.[67,68]

Androgens are important regulators of epididymal development from embryonic to adult stages. From later stages of development to the adult stage, *Ar* expression in the epithelia is greater than that in the mesenchyme. Several *Ar* KO mice have been reported, and the majority show a hypoplastic epididymis and defective epithelial cell differentiation.[47,69–71] A differentiated epididymal epithelium is pseudostratified and comprises principal, clear, narrow, basal and recently-identified dendritic cells throughout the duct.[72,73] Similar to other pseudostratified epithelia, for example the trachea, the epididymal luminal environment regulates secretion and absorption of ions, water, organic solutes and proteins.[74] The molecular mechanisms of epididymal epithelial differentiation are not clear. Chimeric mutation of the *Ar* indicates that defective epithelial cell differentiation is cell-autonomous.[47] Dicer and small RNAs also regulate epididymal

development and epithelial cell differentiation partially through androgen action.[75–77]

Hox genes are evolutionarily-conserved transcriptional regulators that determine body patterning.[78] As found for body plan formation, vertebrae and the gut, *Hox* genes, *Hoxa10* and *Hoxa11* are suggested to determine the boundary between the epididymis and vas deferens.[79–81] Later studies by Snyder *et al*.[82] showed that there were additional region-specific (efferent ducts, epididymis and vas deferens) *Hox* transcripts that may define boundaries along the reproductive tract during development.

POSSIBLE CONTRIBUTION OF MOUSE MODELS TO UNDERSTAND HUMAN CINICAL ABNORMALITIES

One of the most well-known congenital anomalies of the epididymis or vas deferens is congenital bilateral absence of the vas deferens (CBAVD). It occurs in 1%–2% of men with infertility.[83] 60%–90% of the CBAVD men harbor at least one associated *cystic fibrosis transmembrane conductance regulator* (*CFTR*) gene mutation.[84] 10%–40% of CBAVD men do not have recognizable *CFTR* gene abnormalities accompanied by unilateral renal agenesis (URA).[85] Presumably, CBAVD patients have disrupted morphogenesis of the early mesonephros owing to the mutation of genes.[86] Those genes involved in mesonephros formation, e.g., *Pax2*, *Wt-1* and *Fgfs*, may be viable candidate genes responsible for CBAVD with renal malformation.

Conversely, congenital anomalies of kidney and urinary tract (CAKUT) often carry mutations in genes, such as *PAX2* and *WT-1*, and male mice carrying mutations of these genes also exhibit reproductive tract malformations.[87] Syndromes with renal tract abnormalities also carry mutations in the genes described above. Branchio-Oto-Renal (BOR) syndrome is a genetic condition that typically disrupts the development of tissues in the neck and causes malformations of the ears and kidneys. *EYA1*, the human homolog of the *Drosophila eyes absent* gene, is the most common gene responsible for BOR.[88] Further, *Foxc1* regulates *Eya1* expression.[28] Mutations in the *SIX1* gene can be detected in 2% of individuals with the clinical diagnosis of BOR.[89] Mutations in both *ROR2*[90] and *WNT5A*[91] have been implicated in a rare genetic disease, Robinow syndrome, which exhibits several defects such as dwarfism, hydronephrosis and genital abnormalities. Because these syndromes often exhibit lethal abnormalities, it is still unclear if these mutations affect male fertility in humans.

Epididymal disjunction is the failure of the efferent ducts to reach the testis, which may reflect the failure of the efferent ducts to elongate, and presumably coil, during their development.[92–95] Interestingly, one study[95] has shown that 30%–79% of boys with an undescended testis also have Wolffian duct abnormalities, of which 25% display epididymal disjunction. Therefore, it is important that epididymal abnormalities be detected at orchidopexy, or other male infertility, which may be classified as idiopathic, will result. As mentioned above, it is not clear how the efferent ducts form, elongate, are directed toward the testis and then fuse with the rete testis. Obviously, mouse models that display epididymal disjunction will greatly aid our understanding of this abnormality.

SUMMARY

One of the striking characteristics of the epididymis is its complex developmental process. The primordium of the epididymis, the mesonephros, arises as a part of the transient kidney, and its stability and differentiation are regulated by hormonal signaling including by androgens and growth factors. In human, it transforms its morphology to form a 6 m duct that is coiled and packed into a three-dimensional organ of approximately 10 cm in length. Recent studies utilizing a variety of transgenic mice have revealed the molecular contribution of numerous factors at each stage of epididymal development. The molecular dissection of the developmental mechanisms of the epididymis has just begun. Integrative understanding of the hierarchy and interaction of each factor will provide new directions in this field. Considering that the epididymis shares its origin with the urinary tract, it is noteworthy that the molecular mechanisms which lead to kidney mal-development, such as CAKUT, may provide significant insight for the mesonephros derivative mal-development, such as CBAVD and *vice versa*.

COMPETING FINANCIAL INTERESTS

Neither author declares a competing interest.

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