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\textsuperscript{1,2} and considering that the human epididymis has an initial segment-like epithelium,\textsuperscript{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.\textsuperscript{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\textsuperscript{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 (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 effenter 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.\textsuperscript{6-8} The LIM-class homeobox gene Lim1 is required for the extension of the WD.\textsuperscript{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.\textsuperscript{11} Mice carrying a null mutation of Gata3, which is a transcriptional target of Pax2 and Pax8, also show defects in WD initiation.\textsuperscript{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.\textsuperscript{13}
FGF ligands bind and activate alternatively-spliced forms of four tyrosine kinase FGF receptors (FGFRs 1–4). During mesonephric development, Fgfr1 is expressed in the mesenchyme while Fgfr2 is in the epithelium, maintaining the WD and mesonephric mesenchyme. 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. Wnt genes encode a family of secreted glycoproteins 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. 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. 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. The number of efferent ducts reaching the testis also differs between species. 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 nphrogenic cord and are required for tubulogenesis in addition to WD development. 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.

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. 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. 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 development in this mutant would contribute to our understanding of this molecule in tubulogenesis and its maintenance.
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, resulting in the conus (2–3 fused MTs) and the single common ductule.

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) 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. 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.

The origins of nephron progenitor cells are suggested to differ between mesonephros and metanephros.

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

| Gene        | Type of mutation, Cre driver | Phenotype of the mutant                                                  | References |
|-------------|------------------------------|--------------------------------------------------------------------------|------------|
| 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         |
| Osf1        | 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 mesonephrons                                         | 13         |
| Fgrl1/2     | T-Cre                        | Dysgenesis of WD and MT                                                   | 13         |
|            | Paf3-Cre                     | Absence of MT                                                             | 15         |
| Fgr2        | Hoxb7-Cre                    | Regression of caudal WD                                                   | 16         |
| Ssh         | KO                           | Numerous ectopic MT, ectopic UB                                           | 29         |
| Foxc1/2     | Foxc1/Mf1c, 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         |
| Ar          | Tfmr, KO                     | WD regression                                                             | 40,41      |
| Inhba       | KO                           | Failed to develop ductal coiling in epididymis                            | 53         |
| Sfrp1/2     | dKO                          | Shortened vas deferens                                                   | 56         |
| Vang/2      | Vangl2<sup>2<sub>op</sub></sup>| Shortened vas deferens                                                   | 56         |
| Wnt5a       | KO                           | Shortened vas deferens                                                   | 56         |
| Pkd1        | KO, Pax2-Cre                 | Coiling defect, cystic dilation of efferent ducts                         | 54         |
| 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 | KO                           | Absence of IS, defective epithelial cell differentiation                  | 70         |
| Fox3-Cre    | KO                           | Absence of IS, defective epithelial cell differentiation                  | 71         |
| Probasin-Cre| KO                           | Small epididymis and seminal vesicle                                      | 69         |
| Dicer       | Defb4-Cre                    | Epithelial cell dedifferentiation                                         | 75         |
| miR-29a     | miR-29b<sup>1<sup>op</sup></sup>transgene | Hyoplastic epididymis                                                      | 77         |
| Lgr4        | Lgr4<sup>op</sup>             | Short, dilated and much less convoluted epididymal ducts                 | 104        |
| Shp1        | mev/mev                      | Aherent epidymal 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
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. 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. Following gonadal sex differentiation, the testis begins to produce the androgen, testosterone, at approximately E12.5. 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. Indeed, fluorescence labeling of an androgen ligand shows that androgen is transported within the luminal fluid. 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. 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 epithelium 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. This finding is consistent with the observation from tissue recombination experiments on androgen-insensitive Testicular feminized (Tfm) mice. Several growth factors, including FGF and Epidermal growth factor (EGF), are suggested to mediate androgen functions in the prostate and WD. 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. 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. Epididymal coiling is absent from the Pkd1 mutant. 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.

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. 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. 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. 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.

Several growth factors, including FGFs 2, 4 and 8, are detected in testicular fluid, and Fgfrs are expressed in the epithelium of the initial segment. During normal development, high activity of the MAPK pathway, especially p-MAPK1/3 (p-ERK1/2), is detected in the initial segment. EDL abolishes their activities, emphasizing the importance of lumicrine factors regulating their activity. Ros1 encodes an orphan receptor tyrosine kinase that is expressed in few epithelia, among them the WD and its derivatives. Loss of Ros1 expression or a naturally-occurring mutation of Shp1 (mce), a negative regulator of ROS1, results in abnormal differentiation of the initial segment. 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. 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.

Androgens are important regulators of epididymal development from embryonic to adult stages. From later stages of development to the adult stage, 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. A differentiated epididymal epithelium is pseudostratified and comprises principal, clear, narrow and recently-identified dendritic cells throughout the duct. Similar to other pseudostratified epithelia, for example the trachea, the epididymal luminal environment regulates secretion and absorption of ions, water, organic solutes and proteins. The molecular mechanisms of epididymal epithelial differentiation are not clear. Chimeric mutation of the Ar indicates that defective epithelial cell differentiation is cell-autonomous. Dicer and small RNAs also regulate epididymal
Hox genes are evolutionarily-conserved transcriptional regulators that determine body patterning. 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. Later studies by Snyder et al. 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. 60%–90% of the CBAVD men harbor at least one associated cystic fibrosis transmembrane conductance regulator (CFTR) gene mutation. 10%–40% of CBAVD men do not have recognizable CFTR gene abnormalities accompanied by unilateral renal agenesis (URA). Presumably, CBAVD patients have disrupted morphogenesis of the early mesonephros owing to the mutation of genes. 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. 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. Further, Foxc1 regulates Eya1 expression. Mutations in the SIX1 gene can be detected in 2% of individuals with the clinical diagnosis of BOR. Mutations in both ROR2 and WNT5A have been implicated in a rare genetic disease, Robinow syndrome, which exhibits several defects such as dwarfism, hydrencephalus 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. Interestingly, one study has shown that 30%–79% of boys with an undescended testis also have Wolfian 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 CUBKUT, 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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