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Review Article

Modeling development of genitourinary birth defects to understand disruption due to changes in gene dosage

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Abstract: Genitourinary development is a delicately orchestrated process that begins in the embryo. Once complete, the genitourinary system is a collection of functionally disparate organs spread throughout the abdominal and pelvic regions. These distinct organs are interconnected through an elaborate duct system which aggregates the organs' products to a common exit point. The complicated nature of the genitourinary system makes it highly susceptible to developmental disruptions that produce anomalies. In fact, genitourinary anomalies are among the most common class of human birth defects. Aside from congenital anomalies of the kidney and urinary tract (CAKUT), for males, these birth defects can also occur in the penis (hypospadias) and testis (cryptorchism), which impact male fertility and male mental health. As genetic technology has advanced, it has become clear that a subset of cases of genitourinary birth defects are due to gene variation causing dosage changes in critical regulatory genes. Here we first review the parallels between human and mouse genitourinary development. We then demonstrate how translational research leverages mouse models of human gene variation cases to advance mechanistic understanding of causation in genitourinary birth defects. We close with a view to the future highlighting upcoming technologies that will provide a deeper understanding of gene variation affecting regulation of genitourinary development, which should ultimately advance treatment options for patients.

Keywords: Microdeletion/microduplications, copy number variant (CNV), cryptorchidism, penile anomalies

Introduction

The genitourinary system in the adult male is dual-purpose (blood filtration, fluid regulation, reproduction) complicated network of interconnected organ hubs that span the abdominal and pelvic cavities [1]. The adult system is the product of the carefully timed and tightly regulated development of multiple progenitor cells and tissues. Further complicating the understanding of genitourinary development is the fact many of the parts must migrate during development to ultimately arrive at appropriate regions of the body plan to function. These all work to make genitourinary development stunningly intricate but also fragile. Genitourinary birth defects are among the most common class of birth defects. Although still poorly understood, in the past decade there has been a rise in identifying instances of gene variation that disrupt genitourinary development producing some of the most common birth defects. Recent advances in imaging and sequencing analysis provide hope that the coming decade will deepen our mechanistic understanding of how variation of these specific genes leads to the observed anomalies.

Development of the male genitourinary system in humans and mice

Note: Human time points are the primary listing and in days, weeks, months; while all mouse timepoints are italicized in brackets and listed by embryonic day (E) and postnatal day (P).

Embryonic and fetal development of the male genitourinary system in humans and mice can be divided into two major developmental periods: before sex determination (**Figure 1A**), and after sex determination (**Figure 2B**). The sex-dependent (reproductive tract) and sex-independent (urinary tract) developmental pathways work in concert to form a complete genito-

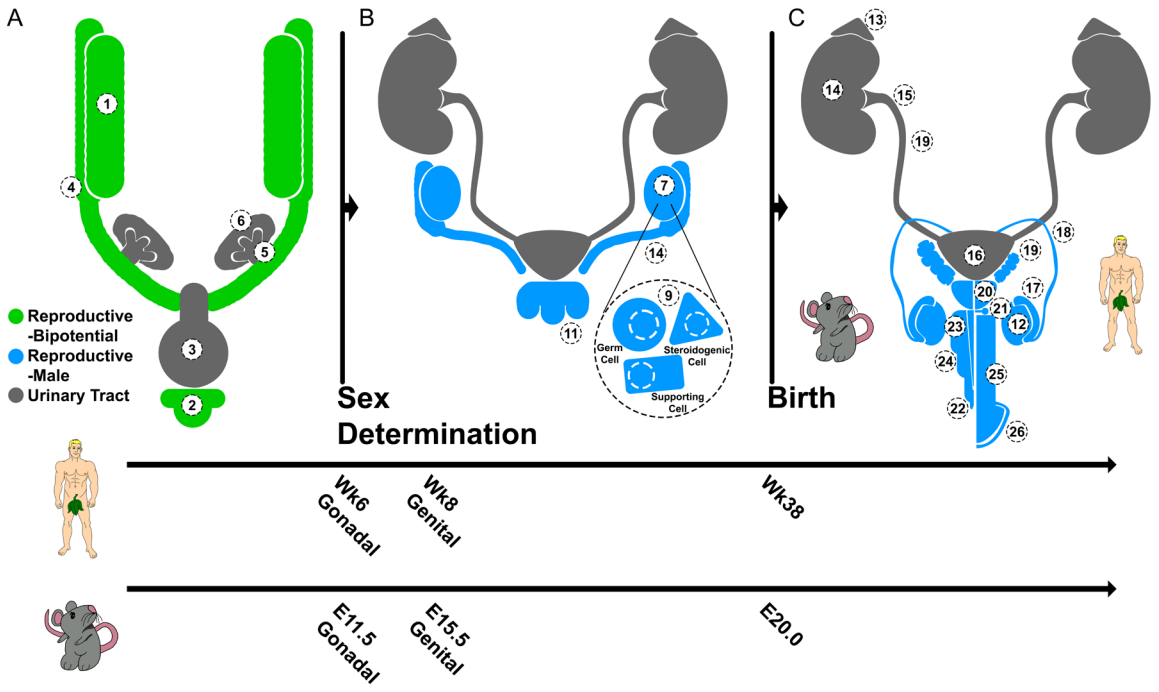

Figure 1. Genitourinary development can be divided into three major perinatal periods. A: Early in fetal development the gonad (1) and genital tubercle (2) are bipotential (green) and morphologically indistinguishable between the genetic sexes. During this period the bladder (3) starts to elaborate and the mesonephros (4) derived ureteric bud (5) invades the metanephros (6) as the first step of kidney development. B: After sex determination in XY the presence of *SRY* initiates male reproductive tract development. This begins as the gonad is directed towards a testis (7) fate with the germ, supporting and steroidogenic cells in the testis also committing to a male (9) sex specific program (see **Figure 2** for more details). Shortly after commitment to testis development testicular androgen production influences the genital tubercle towards penis (11) development. Similarly, the mesonephros and mesonephric duct (4) will complicate forming the outflow tubules that will transport sperm out of the adult testis (12, see **Figure 3** for more details). C: After birth genitourinary structures of the reproductive and urinary tracts continue to elaborate into adult morphologies: adrenal gland (13), kidney (14), ureter (15), bladder (16). The male reproductive tract (blue) also further elaborates into adult structures epididymis (17), vas deferens (18), seminal vesicle (19), prostate (20), bulbourethral gland (21) with significant gross anatomical differences between the mouse penis (22), which contains a baculum (23), and prepuce (24), and human penis (25) and prepuce (26).

urinary system after birth (**Figure 1C**). These processes begin during the first trimester in human development and the second third of mouse development. The mouse is a powerful model for understanding genitourinary development and defining genetic causation in clinical cases of genitourinary birth defects and male fertility.

The intermediate mesoderm condenses to form the mesonephros at day 25 [*E8.5*] and metanephros at day 28 [*E11.0*]. The upper tract of the human genitourinary system first begins to form around week 5 [*E10.5*] with invasion of the mesonephros-derived ureteric bud into the metanephros. Ureteric bud growth and invasion into the metanephros ultimately establishes the collecting portions of the upper tract, while the metanephros will form the excretory portions starting around week 10 [*E11.5-E15.5*]. For development of the lower tract the upper portion of the urogenital sinus will develop into the primitive bladder at week 4 [*E11.5*] and establishes a formal connection with the primitive ureters between weeks 5-8 [*E12.0*]. In the lower tract, the ventral cloaca divides to form the urogenital sinus anteriorly at week 4-7 [*E10.5*]. The urogenital sinus forms the urethra and prostate posteriorly and the primitive bladder anteriorly at week 4 [*E11.5*]. In humans the intermediate urogenital sinus will condense laterally to form paired bulbourethral (Cowper's) glands during week 10 [2]. The mesenchyme of the bladder begins to differentiate in weeks 9-10 so that by week 22 [*E14.5*]

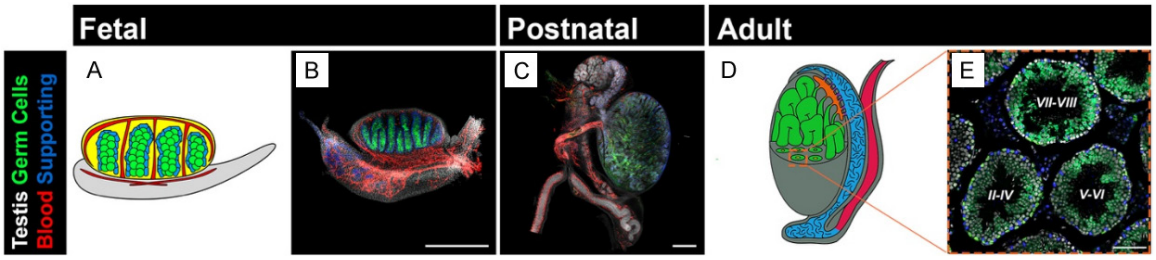

Figure 2. After gonadal sex determination in the male, cell populations of the testis drive morphogenesis. (A) Diagram of the fetal testis with showing male germ cells (green), supporting cells (blue), area of steroidogenic cells (yellow), an vasculature (red, blood), which invade from the mesonephros (grey). (B) Whole mount immunofluorescent imaging of a E14.5 mouse testis with germ cells (green), supporting cells (blue), and blood supplying vasculature (red). (C) Whole mount immunofluorescent imaging of a P2 mouse testis with germ cells (green), supporting cells (blue), and blood supplying vasculature (red). (D) Cartoon diagram of the adult mouse testis (see **Figure 3** for details). (E) Cross section immunofluorescent imaging of adult mouse seminiferous tubules showing different stages of active spermatogenesis (noted in Roman numerals) with developing germ cells (green) facilitated by supporting cells (blue). Scales are: 500 μm in (B and C), and 75 μm in (E).

there are three clear layers: urothelium, lamina propria, muscle (from apical to basal) [3-5].

The gonad initially forms around week 4-5 [*E9.5*] as the genital ridge, a thin layer of cells sandwiched between the mesonephros and coelomic epithelium. Initially the gonad is bipotential, able to produce either an ovary or testis under the direction of the sex chromosomes (XX female/ovary, XY male/testis). The early gonad will undergo a series of morphological changes (thickening, rotation, shortening, etc.) to prepare for the migratory germ cells which begin to arrive at week 6 [*E10.5*] [6].

The purpose of the gonad is to house germ cells which will eventually enter meiosis to produce the haploid gametes (oocytes, spermatozoa), which confer fertility to the individual and ensure continuation of the species. Primordial germ cells originate extra-embryonically being specified near the allantois at week 3 [*E7.5*]. After specification primordial germ cells will enter the embryo proper to migrate along the hindgut to reach the bipotential gonad. Primordial germ cells enter the developing gonad at week 6 [*E10.5*] [7, 8]. By day 41-44 [*E11.5*] the presence of the Y chromosome, specifically the testis determining gene *SRY* (Sex Determining Region Y), initiates the male fate program of sex determination that will commit the bipotential gonad to form a testis [6, 9-12] (**Figure 1B**). The somatic cells (supporting and steroidogenic) of the developing testis environment influence the newly arrived primordial germ cells which transition to pro-spermatogonia as they commit to the male fate (**Figure 2A**). Pro-spermatogonia are highly pluripotent germ cells that undergo a rapid mitotic burst and then asynchronously transition into cell cycle arrest during week 16-20 [*E14.5*] [13-17] (**Figure 2B**). The cell cycle arrest is a critical period when pro-spermatogonia undergo extensive epigenetic regulation and differentiate to form spermatogonial stem cells by birth (**Figure 2C**). Spermatogonial stem cells of the adult testis will supply the pool of germ cells that continuously enter meiosis to produce sperm though a process called spermatogenesis (**Figure 2D**, **2E**).

During this period of fetal male germ cell differentiation the testis and mesonephros also continue to develop. Morphological changes continue, starting at week 7 [*E11.5*] the testis chords elaborate within the interstitium of the testis, which is simultaneously growing [18] (**Figure 2A-C**). The mesonephros also elaborates and forms mesonephric tubules at the cranial end of the mesonephric duct (Wolffian Duct, nephric duct). Mesonephric tubules are the precursors of the rete testis and efferent ducts which subsequently will allow passage of spermatozoa through the epididymis and vas deferens both of which are produced by mesonephric tissue. The mesonephros will also form the seminal vesicles which supply semen with energy components in the form of fructose during ejaculation (**Figure 3**). Concurrent with tissue morphogenesis, the entire structure also actively migrates from the abdominal cavity to the scrotum. Testis migration is divided into a

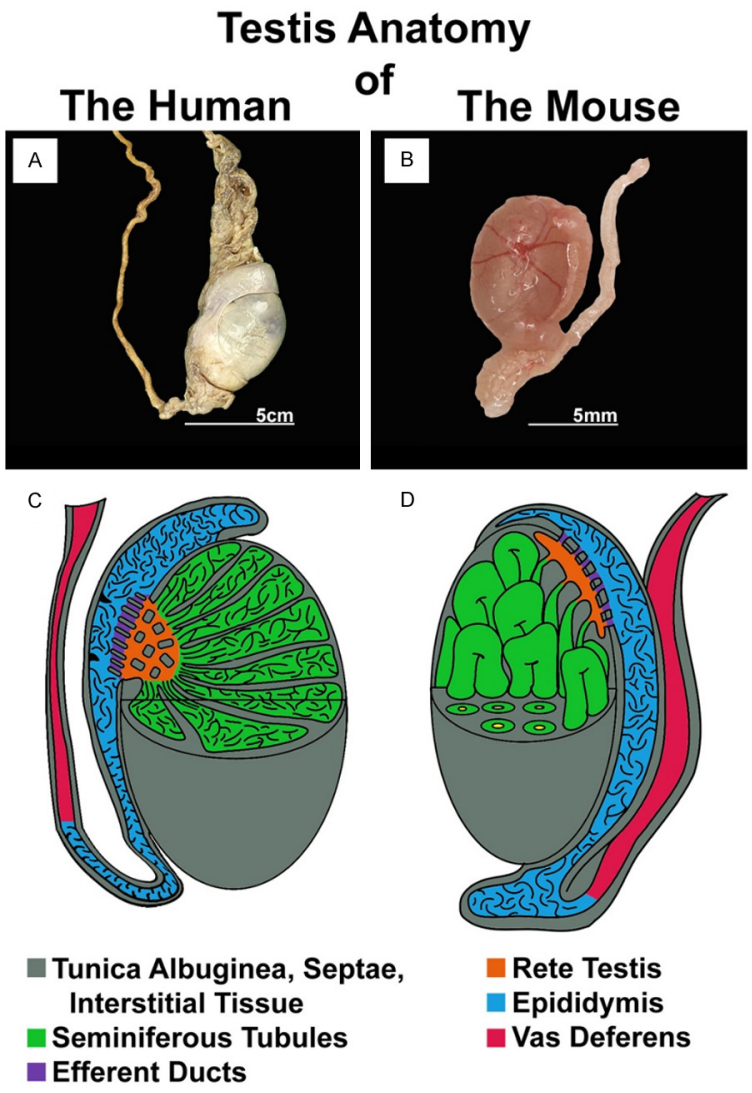

Figure 3. Mouse and human testis anatomy. (A, B) Testis, epididymis and vas deferens from human (A) and mouse (B); (A) is planstinated tissue and (B) is fresh tissue. (C, D) Diagrammatic representation of human (C) and mouse (D) testis interior. The human testis is divided into a series of radiating septa that each contain a highly coiled loop of seminiferous tubule (C). The seminiferous tubules of the mouse have a pole-to-pole looping swooping structure (D). Scale is 5 cm in (A) and 5 mm in (B). Image in (A) is part of a larger dissection published in Ruthig et al. 2016 Anatomy [1] in (C) was inspired by work of Netter [111].

transabdominal phase from weeks 8-15 [*E13.0-E17.0*] and inguinoscrotal phase from weeks 25-35 [*P21-P28*]. The phases are separated by a pause in migration.

By the end of the first month the cloacal folds have united cranially to form the bipotential genital tubercle, and subdivided caudally to form urethral folds. Just lateral to the urethral folds are the genital swellings. The bipotential structures that will form the external genitalia have appeared as the genital tubercle and paired genital swellings [*E10.5*] [19]. At around 8 weeks gonadal sex determination in favor of the male pathway commits the genital tubercle to forming a penis during the next 8 weeks of development [*E15.5*] [20]. This is under the influence of testosterone, the masculinizing hormone produced by Leydig cells of the fetal testis starting at week 8 in the human [*E15.5* in the mouse]. Initially the penile urethra is formed by elongation and canalization of the urethral groove which is flanked laterally by paired urethral folds. By week 12, the urethral folds will fuse medially on the ventral aspect of penis. This fusion encloses the tubular epithelial lumen derived from endoderm, called the urethral plate, and forms the penile urethra. However, the most distal portion, the glanular region of the penis, is invaded by a solid urethral plate which must then canalize while the mesenchyme ventral to the canalization fuses to form a confluence that will fully tubularize the region [21]. This process also requires endodermal reabsorption and epithelial remodeling to finalize the glanular region of the urethra [22]. It is worth noting two major differences in human and mouse penis development. The mouse penis contains a bone within, the baculum (Os Penis), while the human does not (**Figure 3C**). Also, in humans the urethral plate forms the urethral meatus and penile urethra, while in the mouse the urethral plate forms only the penile urethra [23-26].

Collectively all of these processes in genitourinary development rely heavily on canonical pathways responsible for: tubularization (SOX9/FGF, BMP), mesenchymal to epithelial transi-

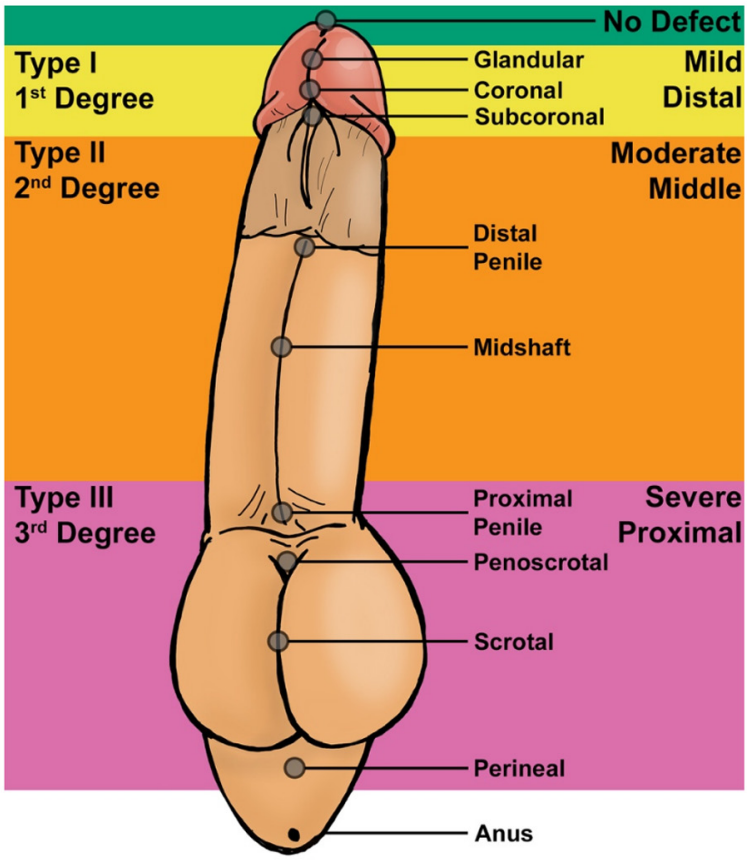

Figure 4. Classifications of hypospadias severity by urethral opening location. Hypospadias is characterized by ectopic placement of the urethral opening, which can occur distally to proximally on the penis as the severity increases (denoted by colored bars [112]). Ectopic urethra can occur in conjunction with a foreskin that has failed to fuse ventrally "hooded foreskin", and varying degrees of chordee, curvature, of the shaft of the penis (figure inspired by Piñeyro-Ruiz 2020 Frontiers Pediatrics and informed by [19, 20]).

tions (WT1 via Wnt/β-catenin), outgrowth/branching (GDNF/RET) growth/patterning (Hedgehog/TGFβ) [27-32]. Proper regulation of the sex hormones (androgens and estrogens) is also critical after sex determination for masculinization of the male reproductive tract [33, 34].

In general, the following reviews and textbook chapters served as references throughout this section [35-40].

Key genitourinary anomalies presenting a challenge to human health at birth

Genitourinary anomalies are a very common class of birth defects. With externally recognizable anomalies accounting for (11:1,000) live births [41]. Among the externally recognizable anomalies, 1:125 live male births occur with hypospadias [NBDPN 2019 Annual Report]. Hypospadias is a failure in urethral closure during development. Urethral closure is most dependent on two key events: preputial swelling with ventrolateral growth, and bilaminar urethral plate remodeling into a tube. When these events do fail and cause hypospadias, there is a range of severity that can occur (**Figure 4**) [42, 43]. Failure of the testis to descend fully into the scrotum, cryptorchidism, affects 1:30 full-term live male births [44, 45] and increases the chance of infertility and testicular cancer risk [46, 47]. Congenital anomalies of the kidney and urinary tract (CAKUT) include conditions such as (renal agenesis/dysplasia/hypoplasia, obstruction sometimes causing hydronephrosis, vesicoureteric reflux) [48, 49]. CAKUTs represent 20-30% of all prenatally diagnosed general anomalies [41]. CAKUT pathologies can arise during any part of fetal development. Often severity of the CAKUT is typically directly related to how early genitourinary development was initially derailed [41]. One major cause, among others, for male genitourinary birth defects are variations in genes responsible for regulating part(s) of the closely orchestrated pathways that collectively produce a functional genitourinary system.

Gene dosage anomalies underlie a significant number of birth defects and syndromes

CRKL

The 22q11.2 region is the site of gene deletions causing diGeorge Syndrome. Multiple studies established that a subset of patients with diGeorge Syndrome have genitourinary anomalies [50-52]. Follow up studies narrowed

down many of these microdeletions encompassing *CRKL* (CRK Like Proto-Oncogene, Adaptor Protein) [52, 53]. Clinical cases of *CRKL* deletions have genitourinary anomalies that include bladder exstrophy, cryptorchidism, hypospadias, micropenis, and CAKUT occurring in 1.4% of non-syndromic males with genitourinary birth defects [52, 53]. CRKL functions downstream of tyrosine kinase transducing intercellular signals as an adaptor protein [54, 55]. Regulatory targets of CRKL include multiple growth factors most notably fibroblast growth factors (FGFs) [56-59]. Genitourinary anomalies associated with *CRKL* loss are thought to be primarily due to an impaired development of the metanephric mesenchyme.

MAZ

MAZ (MYC Associated Zinc Finger Protein, aka *SAF-1*) is located within the 16p11.2 microdeletion/microduplication syndrome region and is important for genitourinary development [60]. Clinical genitourinary anomalies reported for cases of *MAZ* copy number variation include CAKUT, hypospadias, cryptorchidism, micropenis [51, 60]. About 6.2% of non-syndromic males with genitourinary anomalies have copy number variants that encompass *MAZ* [60]. MAZ can act as a transcriptional initiator or terminator; among MAZ targets are the promotor regions of WT1, MYC and Sp1 [61, 62]. *MAZ* regulation of *Wnt* morphogens is implicated as required for normal genitourinary development [60]. The Wnt/β-catenin pathway is critical to normal genitourinary development [30, 63-65].

KCTD13

KCTD13 (Potassium Channel Tetramerization Domain Containing 13) is yet another gene in the 16p11.2 minimal region identified by Tannour-Louet, et al., (2010) [51] which shows a link between gene variation and genitourinary anomalies. Patients with *KCTD13* microdeletion and microduplication have hypospadias and cryptorchidism, though rarely just one defect [66]. KCTD13 is a binding partner adapter for the E3 ubiquitin ligase complex (BTB domain Cullin3 complex RING protein Rbx1 (BTB-CUL3-RBX1) aka BCR E3 ubiquitin ligase complex) and confers substrate specificity to the BCR E3 ubiquitin ligase complex [67, 68]. Dosage changes in KCTD13 expression were shown to dysregulate androgen receptor localization vitiating masculinization of the penis and testis development [66].

TBX6

In a study of 2,824 CAKUT cases a reductive approach was used to ultimately identify a new gene microdeletion in the larger common microdeletion region within 16p11.2 region in *TBX6* (T-Box Transcription Factor 6). Inactivation of *TBX6* was correlated with a subset of the CAKUT cases with kidney anomalies [69]. A follow up study with increased clinical cases from two centers and multiple racial backgrounds (Chinese and Caucasian/Hispanic) further validated 16p11.2 microdeletion specifically in *TBX6* as causative in kidney anomalies of CAKUT [70]. A second group recently corroborated these findings in a study that linked TBX6 variation to vertebral and rib malformations and CAKUT [71]. TBX6 is a DNA-binding transcription factor with targets that include *SOX2* and *WNT3A* [72, 73]. Dosage changes in TBX6 are implicated in causing dysregulation of the developmental pathways controlling ureteric bud invasion of the metanephros and ureteric bud branching [69-71].

VAMP7

Tannour-Louet, et al., (2014) was the first to identify a dosage-sensitive gene microduplication in the pseudo-autosomal region 2 of the sex chromosomes that specifically duplicated a single gene, *VAMP7* [74]. Which encodes a vesicle-trafficking protein that is part of the SNARE complex that was present in 3.6% of non-syndromic males with masculinization disorders of the male external genitalia (cryptorchidism and/or hypospadias), but was not present in 8951 individuals with no genitourinary anomalies. The studies were important because they showed that up-regulation of *VAMP7* enhanced estrogen receptor action and to a lesser extent blunted androgen receptor action affecting virilization of the male genital tract during development resulting in an increased incidence of genitourinary birth defects. The over-expression of *VAMP7* impacted steroid hormone actions in ways not previously recognized.

Exciting new frontiers

Although there are a growing number of identified genes that are linked to causing genitouri-

nary anomalies, there is still limited mechanistic understanding of how these genes usually regulate the complicated process of genitourinary development and how this is aberrated with changes in gene dosage. However, a few recent advances in technology and developmental biology show promise to help more deeply define mechanisms. Recent publications applying light sheet imaging to the developing genitourinary system have demonstrated how apt this technology is at capturing the interconnectivity between regions of the system. Light sheet imaging also offers a lot of promise for advancing how genitourinary anomalies due to gene variation are imaged [75-79].

There was a recent surge in scRNA-seq datasets representing many different tissue types in the developing and adult genitourinary tract including penis [80-82], germ cells [83-86], testis [87-91], kidney [92-95], prostate [96-98]. Some of these datasets were paired with some of the new spatial transcriptomic methods to validate scRNA-seq findings in tissue samples [80, 98]. The plethora of scRNA-seq datasets being produced can also serve as references to deconvolute bulk RNA-seq datasets [99, 100]. There is a great benefit of using deconvolution to disentangle some of the muted transcriptomic signal that is hindered by the heterogeneity inherent in bulk RNA-seq samples when it is not be feasible to process the samples as scRNA-seq. This benefit has already begun to be utilized in genitourinary research [101-104].

In conclusion, with the advent of genomic technologies as well as next generation sequencing in recent years our understanding of the genetic and genomic defects causing genitourinary birth defects in humans has rapidly expanded. The additional extensive studies of in vitro and in vivo models (mainly in mouse models), together with the use of advanced bioinformatic analyses of complex single-cell RNA seq data has allowed researchers to not only precisely time and identify specific cell types critical to key developmental processes, but to also dissect the complex interactions of multiple signaling pathways critical for normal genitourinary development. Of note, many of these developmental pathways critical to normal GU development are also needed for development of other organs, tissues or cells in the body. An example of this observation is the realization that individuals with gene-dosage changes in *Maz* not only have genitourinary birth defects but also may have a plethora of eye defects, including microphthalmia, anophthalmia and coloboma resulting from dysregulation of the Wnt/β-catenin pathway and disruptions in the network controlling ciliary margin patterning due to *MAZ* deficiency [105]. This phenotype together with microdeletions encompassing *MAZ* or damaging mutations in *MAZ* was identified in humans by both copy number variant assessment, as well as whole exome sequencing analysis [105]. CNVs in 16p11.2 in general are associated with microphthalmia, anophthalmia and coloboma ocular malformations in humans [106, 107]. Importantly, deletions and duplications of chromosome 16p11.2 is reported as one of the most frequent genetic causes for autism spectrum disorders, schizophrenia and other neurodevelopmental disorders [108, 109] and suggests that a subset of patients seen by the urologist for surgical correction of genitourinary birth defects are syndromic (undiagnosed) and who might benefit from an evaluation by a medical geneticist specializing in this area.

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Disclosure of conflict of interest

DJL previously served on the scientific advisory board of Fellow Health (equity), and serves on the American Board of Bioanalysts as secretary-treasurer (Honorarium), the American Association of Bioanalysts as Section Representative (Honorarium), as a Consultant and member of the Advisory Board of Roman, Inc (Equity and Compensation). None of these are relevant for the studies presented herein. The authors state explicitly that they have no conflicts of interest relevant to the studies presented in this article.

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