Canalization of the Urethral Plate Precedes Fusion of the Urethral Folds during Male Penile Urethral Development: The Double Zipper Hypothesis

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Purpose: We describe the "double zipper" mechanism of human male urethral formation, where the distal zipper opens the urethral groove through canalization of the urethral plate, and a second closing zipper follows behind and closes the urethral groove to form the tubular urethra.

Materials and Methods: Anonymous human fetal genital specimens were acquired and gender was determined by polymerase chain reaction of the Y chromosome. Specimens were processed for optical projection tomography, stained with E-cadherin, Ki67 and caspase 3, and imaged.

Results: Eight developing male fetal specimens from 6.5 to 16.5 weeks of gestation were analyzed by optical projection tomography, and an additional 5 specimens by serial sections. Phallus length ranged from 1.3 to 3.7 mm. The urethral plate canalized into a groove with 2 epithelial edges that subsequently fused. Ki67 staining was localized to the dorsal aspect of the urethral plate. In contrast, caspase 3 staining was not observed. The entire process was completed during a 10-week period.

Conclusions: The human male urethra appears to form by 2 mechanisms, an initial "opening zipper" that facilitates distal canalization of the solid urethral plate to form the urethral groove, which involves a high rate of epithelial proliferation (apoptosis not observed), and a "closing zipper" facilitating fusion of the 2 epithelial surfaces of the urethral groove, and thus extending the penile urethra distally. Improved knowledge of the molecular mechanisms of these processes is critical to understanding mechanisms of abnormal urethral development, such as hypospadias.

Key Words: growth and development, hypospadias, organogenesis, urethra

Abbreviations and Acronyms

3D = 3-dimensional

OPT = optical projection tomography

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UNDERSTANDING normal human urethral development is the first step is unraveling abnormal urethral development, of which the most common anomaly is hypospadias. Hypospadias is defined by abnormal location of the urethral meatus, impaired differentiation of the corpus spongiosum and failure of closure of the ventral foreskin.[1] The urethral defects result from failure of fusion of 2 epithelial surfaces of the urethral groove, which forms via canalization of the solid urethral plate.[2,3] Hypospadias results from events that may involve 1) formation of the urethral plate, 2) failure of canalization of the urethral plate, and thus failure of formation of the

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urethral groove, 3) failure of medial growth of the urethral fold (lateral edges of the urethral groove) or 4) failure of midline fusion of the urethral folds.[4,5]

Previous studies examining urethral development have relied on gross examination, serial sections and 3D reconstruction of images of serial sections.[5–7] With the advent of OPT, whole mount 3D imaging of the developing human urethra can now be realized.[8] We describe the "double zipper" hypothesis of urethral formation, where the "opening zipper" facilitates formation of the urethral groove distally through canalization of the urethral plate, and a "closing zipper" follows behind and closes the urethral groove to form the tubular urethra. Midline epithelial proliferation within the urethral plate is a key feature of development of the urethral groove, while apoptosis does not appear to be involved in canalization of the urethral plate.

METHODS

The study was performed after receiving approval from the committee on human research at the University of California, San Francisco. Anonymous human fetal genital specimens from elective terminations were acquired, and gestational age was estimated by heel-toe length.[9,10] Fetal gender was determined by 2 methods, ie visual inspection of internal genitalia (gonads and wolffian and müllerian ducts) using a dissecting microscope, and polymerase chain reaction for X and Y-chromosomal genes.[11] For the latter DNA was isolated from fetal tissue sample using the QIAamp® DNA Mini Kit. Isolated DNA was then amplified for specific primers of the X and Y chromosomes.

Optical Projection Tomography

OPT is a technique for volumetric visualization of transparent or slightly opaque objects on the cellular level up to small organisms.[8] The principle of OPT is similar to computerized tomography, except for the use of visible light instead of x-rays.

Dissected human fetal genitals were fixed in formalin overnight, stored in 100% methanol and prepared for OPT.[8] Samples were immunostained with anti-E-cadherin (diluted 1:100, BD Transduction Laboratories™) using the secondary antibody (goat anti-mouse diluted 1:100, Alexa Fluor® 488 nm).

Images of the specimens were captured using Bioptonics OPT scanner 3001M (Medical Research Council Technology, London, United Kingdom), as described previously.[8] The raw data (400 projected images) from each of the 2 channels were reconstructed into a pair of 3D voxel data sets using in-house reconstruction software. 3D OPT reconstructions were then loaded into PerkinElmer® Volocity software for visualization and analysis.

OPT samples were isolated from the agar blocks using the standard Bioptonics OPT protocol. Once isolated, the specimens were formalin fixed, paraffin embedded and serially sectioned at 7 microns. Immunohistochemical analysis was carried out as described previously,[5] using antibodies to Ki67 (1:100, Leica Microsystems, Inc., Buffalo Grove, Illinois) to detect cellular proliferation and caspase 3 (1:200, Cell Signaling Technology, Inc., Danvers, Massachusetts) to detect apoptosis.

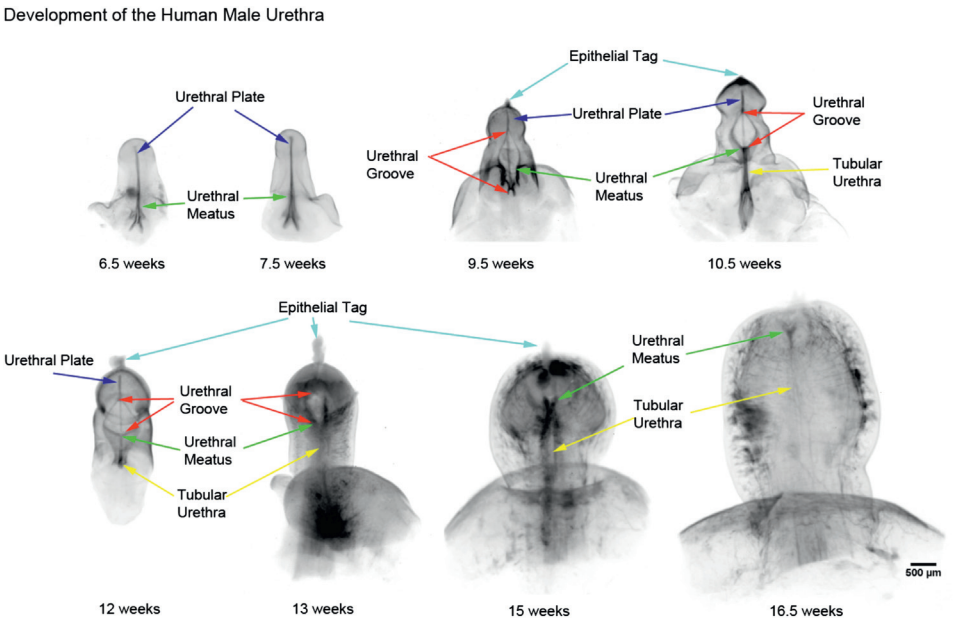

Figure 1. OPT of male urethral development from 6.5 to 16.5 weeks of gestation. Note progression of urethral meatus (green arrows) from scrotal folds at 6.5 weeks to terminal position on glans at 16.5 weeks. Wide open urethral groove (red arrows) is best seen from 9.5 to 13 weeks, with clear progression of proximal to distal fusion of edges of urethral groove to form tubular urethra (yellow arrows). At 13 weeks urethral groove is within glans penis with tubular urethra completely formed within shaft of penis, consistent with endodermal theory of urethral development. No evidence of ectodermal intrusion is evident in any specimen.