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Complex epithelial remodeling underlie the fusion event in early fetal development of the human penile urethra

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Abstract

We recently described a two-step process of urethral plate canalization and urethral fold fusion to form the human penile urethra. Canalization ("opening zipper") opens the solid urethral plate into a groove, and fusion ("closing zipper") closes the urethral groove to form the penile urethra. We hypothesize that failure of canalization and/or fusion during human urethral formation can lead to hypospadias. Herein, we use scanning electron microscopy (SEM) and analysis of transverse serial sections to better characterize development of the human fetal penile urethra as contrasted to the development of the human fetal clitoris.

Eighteen 7-13 week human fetal external genitalia specimens were analyzed by SEM, and fifteen additional human fetal specimens were sectioned for histologic analysis. SEM images demonstrate canalization of the urethral/vestibular plate in the developing male and female external genitalia, respectively, followed by proximal to distal fusion of the urethral folds in males only. The fusion process during penile development occurs sequentially in multiple layers and through the interlacing of epidermal "cords". Complex epithelial organization is also noted at the site of active canalization. The demarcation between the epidermis of the shaft and the glans becomes distinct during development, and the epithelial tag at the distal tip of the penile and clitoral glans regresses as development progresses.

In summary, SEM analysis of human fetal specimens supports the two-zipper hypothesis of formation of the penile urethra. The opening zipper progresses from proximal to distal along the shaft of the penis and clitoris into the glans in identical fashion in both sexes. The closing zipper mechanism is active only in males and is not a single process but rather a series of layered fusion events, uniquely different from the simple fusion of two epithelial surfaces as occurs in formation of the palate and neural tube.

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Keywords

Penis; clitoris; development; urethra; epithelial fusion; SEM

INTRODUCTION

We recently described a two-zipper hypothesis for formation of the human penile urethra. The “opening zipper” refers to the proximal to distal canalization of the urethral plate to form the urethral groove in males and the opening of the vestibular plate into the vestibular groove in females, and is likely to be an androgen-independent event.[1,2] The “closing zipper” refers to the proximal to distal closure of the urethral groove in males to form the penile urethra, and thus is presumed to be androgen dependent.[1] This fusion of the urethral folds is the key event that distinguishes the development of male from female human external genitalia in that the closing zipper mechanism is not evident during normal female development.[2]

Fusion events occur throughout development, for example in neural tube closure and fusion of the palate and lip.[3,4] Failure of the fusion process results in neural tube defects, cleft palate and lip, and, presumably in the case of urethral fold fusion, hypospadias. In comparison to the extensive bodies of work characterizing neural tube closure and palatal shelf fusion,[3,4] very little has been published directly describing the formation of the human penile urethra.[1,5-9] In contrast, development of most (the proximal portion) of the mouse penile urethra occurs by direct canalization of the urethral plate to generate a lumen within the urethral plate (Fig. 1) but without creating an open urethral groove, a process radically different from penile urethral development in humans in which urethral plate canalization results in an open urethral groove, which subsequently closes to form the penile urethra.[10,1112] Only the most distal aspect of the mouse penile urethra forms as a result of formation of the preputial-urethral groove and its subsequent fusion (Fig. 1), a process analogous to that of human penile development.[13] By better visualizing the morphologic and histologic transitions that underlie the canalization and fusion processes during human penile and clitoral development, we aim to gain a better understanding of normal penile urethral development and thereby be better poised to dissect the pathophysiology underlying abnormal development of the male urethra, in particular hypospadias.

Previous studies of the human urethra have been limited by insufficient specimens and age ranges and by low-resolution imaging. Using optical projection tomography, we were previously able to accurately visualize the internal epithelial structures in developing human male and female external genitalia.[1,2] However, the detail in these 3D reconstructions is limited by the ability of labeled antibodies to penetrate fixed human fetal tissue and by the physical resolution limits of light microscopy. Based on immunostained sections from developing human fetal genitalia, we recognized that the mechanistic details of urethral plate canalization and urethral fold fusion were more intricate than we were able to visualize using wholemount fluorescence microscopy. To this end, we have used scanning electron microscopy (SEM) and associated histologic sections to better visualize the morphologic progression that occurs during normal development of the human fetal penis and clitoris.

High-resolution SEM images of external features of male and female human external genitalia were integrated with detailed transverse histologic sections of the developing human male and female external genitalia to generate a novel perspective for the mechanism of the closing zipper.

METHODS

First and early second trimester human fetal specimens were collected without patient identifier information after elective termination of pregnancy with approval from the Committee on Human Research at UCSF (IRB# 12-08813). Fetal age was estimated using heel-toe length.[14] Note that we report age from time of fertilization and not from last menstrual period. Gender was determined using PCR to detect X and Y- chromosomal sequences as previously described[1] and, when available, was confirmed with identification of Wolffian and Müllerian duct morphology. External genitalia were identified using a dissecting microscope, and eighteen 7-13 week specimens were processed for scanning electron microscopy and imaged using standard techniques. Fifteen additional specimens were sectioned and stained with hematoxylin and eosin.

RESULTS

Definition of terms in developing male and female human external genitalia

Figure 2 gives a diagrammatic overview of the structures that constitute the external genitalia of developing human fetuses. In developing males, the glans penis is readily recognizable and contains the solid urethral plate. Canalization of the urethral plate forms the urethral groove whose edges are called the urethral folds. The scrotal folds are lateral and caudal to the genital tubercle (phallus). The urethra forms as a result of proximal to distal fusion of the urethral folds. In females, the genital tubercle is initially similar in length to that of the male and has a distinct glans. Canalization of the vestibular plate (the homologue of the urethral plate in males) generates a vestibular groove whose edges are called the vestibular folds destined to form the labia minora.

Morphologic development and epithelial transitions in the developing human penis from 7.5 to 13 weeks

SEM images of 7.5 – 13 week human fetal penises demonstrate proximal to distal “unzipping” or canalization of the urethral plate followed by proximal to distal fusion of the urethral folds (closing zipper) (Fig 3A-F), consistent with our previously published ontogeny of human fetal penis depicted by optical projection tomography.[1] At 7.5 weeks, a narrow urethral groove was observed extending to the base of the glans abutting the yet uncanalized solid urethral plate (Figs 3A, 5A & B). The groove is narrow at this stage with the distal-most portion of the groove only just beginning to canalize. The floor of the open urethral groove appears to be derived from canalization of the solid urethral plate, and thus at this stage (open urethral groove stage) the epithelium lining the floor of the urethral groove should not be construed as urethral epithelium. The epithelial cells lining the urethral groove are continuous laterally with the epidermis. The transition from the penile shaft to glans is subtle, but demarcated by a shallow circumferential groove (Fig 4A & B white arrowhead).

The epithelial tag on the glans has not yet formed as a distinct structure at 7.5 weeks gestation. Note that the epithelial cells within and immediately lateral to the urethral groove exhibit surface features distinct from the more laterally situated epidermal cells. Such epithelial cells within and bordering the edges of the urethral groove are convex with distinct cell borders, while the more laterally situated epidermal cells exhibit amorphous surface features (Fig 4A-B).

At 9 weeks, the urethral groove is noticeably wider at mid shaft but the opening zipper still has not yet progressed into the glans (Figs. 3B & 5G). In the distal-most portion of the groove, strands of epithelial cells span the groove (Fig. 6), which may represent the canalization/unzipping process illustrated in transverse histologic sections (Figs 5). The epidermis surrounding the proximal aspect of the urethral groove (closing zipper) is starting to organize into a more complex patterning as compared to the 7.5-week specimen, exhibiting subtle shallow wrinkles radiating laterally from the urethral groove (Fig 5G blue arrowheads), which may be the precursors of the interlacing epithelial cords seen later (Fig 7). Histologic sections from a specimen of equivalent age demonstrate the following stages of urethral development along the proximal-distal axis of the developing phallus at 9 weeks gestation: (A) solid urethral plate, (B) beginning of canalization process, (C) a urethral groove widely open at mid shaft, (D) initiation of fusion of the urethral folds, and (E) a fully formed tubular penile urethra (Fig 5A-F). There is a circumferential narrowing to form a neck between the penile shaft and the glans (Fig 5G white arrowhead). Near the tip of the glans a prominent epithelial tag (whose function is unknown) protrudes ventrally (Fig 5G red arrowhead).

At 9.5 weeks (Fig. 3C), the gross morphology appears similar to the 9-week specimen, with a shallow circumferential groove separating the shaft from the glans (Fig. 7A, white arrowhead) and a prominent epithelial tag (Fig. 7A red arrowhead). However, notably, the urethral groove has now extended part way into the glans. The epidermis located at the proximal aspect of the urethral groove exhibits a complex three-dimensional folded structure of interlacing epithelial “cords” in a fashion similar to aligning the teeth on a zipper (Figs 7B-C). Of note, the cords are in close approximation for a significant length without fusing (Fig 7C). Proximal to and in line with these interlacing epidermal cords is the newly formed median penile raphe (Figs 7A & B blue arrowhead). At the distal end of the urethral groove, clumps of cells project outwards, appearing to be in the process of being sloughed as the urethral plate unzips to form the urethral groove (Figs 7A-B & 8).

At 10 weeks, the opening zipper has extended into the glans and the wide-open urethral groove reveals a view to the epithelial surface of the urethral groove (Fig 3D, Fig 9). The epithelial cells lining the urethral groove at mid shaft are flattened and stretched in stark contrast to the more lateral and distal epithelial cells, which display a convex apical surface (Figs 9E,F). The epidermal cells immediately lateral to the urethral groove retain their bumpy, convex appearance but, as the urethral folds come together in the midline, the cells appear to become more flattened with an overall smoother surface appearance (Figs 9C,D). At the distal-most end of the urethral groove, clumps of epidermal cells protrude from the ventral penile surface similar to those seen at 9.5 weeks (Figs 9B,E green arrowheads). In the distal aspect of the urethral groove, the epithelial cells are densely packed and convex

apically with complex organization around a pinpoint opening that may represent the site of canalization of the urethral plate (Figs 9E-F, orange arrowhead).

At 12.5 weeks, the closing zipper has progressed nearly to the base of the glans, and the median raphe is a prominent structure along the ventral surface of the penile shaft (Fig 3E blue arrowhead). Rather than the interlacing epidermal cords previously seen in the more proximal penile shaft fusion event, the right and left epidermal edges of the urethral groove of the distal penile shaft appear to be approximating in a more simple, parallel fashion (Fig 3E).

At 13 weeks, the closing zipper has progressed into the penile glans and a coronal ridge has formed (Fig 3F). The median raphe is a prominent structure along the ventral shaft (Figs 3F & 10A inset blue arrowheads). The epithelial projections at the distal end of the urethral groove are seen within the groove itself (Fig 10A green arrowhead). Immediately dorsal to the approximated but not yet fused epidermal edges of the urethral groove, a slightly deeper layer has already fused (Fig 10B purple arrowhead). The epithelial cells lateral to the urethral groove have flattened into a tiled, squamous pattern beneath a discontinuous layer of balled-up cells (possibly periderm cells), apparently in the process of sloughing (Fig 10C, red arrowheads and *).

Morphologic development in the developing human clitoris from 8 to 13 weeks

SEM images of the human fetal clitoris demonstrate proximal to distal “unzipping” or canalization of the vestibular plate to form a midline vestibular groove analogous to the urethral groove seen in human penile development (Fig 11). During development of the human fetal clitoris, the vestibular groove opens widely from 8 to 13 weeks and is bounded laterally by thick vestibular folds (destined to become labia minora) (Figs. 11E & 12B). There is no evidence of a closing zipper or the interlacing epithelial cords prominently seen in the 12.5-week penile specimen (Figs 11 & 12). The result is a widely open vaginal vestibule. However, analogous to development in the male, the opening zipper has extended into the glans clitoris by 12 weeks, and the glans is separated from the clitoral body by a ridge analogous to the coronal groove in the male (Figs 11 & 12 white arrowheads). Histologic sections demonstrate the following corresponding stages of vaginal vestibular development at 11 weeks gestation: (A) solid vestibular plate distally. (B) beginning of canalization of the vestibular plate, (C) a widely open vestibular groove, (D) proximal closure of the vestibule (Fig 13A-F).

Demarcation of the glans and relative regression of the epithelial tag

SEM images from a lateral view demonstrate the ontogeny of the epithelial tag in both the human fetal penis (Fig 14A-D red arrowheads) and clitoris (Fig 14E-H red arrowheads). As development progresses from approximately 10 to 13 weeks gestation, the epithelial tag becomes relatively less prominent in both male and female external genitalia. Early in both male and female development, the glans is separated from the shaft by a circumferential narrowing or “neck” but that, as development progresses, this neck disappears even though characteristic epithelial surface features are evident at the junction of shaft versus glans (Fig 14 white arrowheads).

DISCUSSION

In agreement with our previous work using optical projection tomography to visualize epithelial structures in developing human genitalia,[1,2] high resolution SEM imaging of human fetal specimens demonstrates proximal to distal “unzipping” or canalization of the urethral plate in both the developing urethral plate of the penis and the vestibular plate in the clitoris. In contrast, a subsequent proximal to distal fusion of the urethral folds is seen only in the male with no evidence of the analogous process in the female. This supports our two-zipper hypothesis of penile development and corresponding single-zipper hypothesis of clitoral development.

We further demonstrate that the “closing zipper” in human fetal penile development is not a simple epithelial fusion of right and left urethral folds, but rather a series of unique layered epithelial fusion events, in contrast to the simple fusion of two epithelial surfaces as occurs in formation of the palate and neural tube. Combining the SEM data with our analysis of serial sections, we hypothesize three distinct fusion events: 1) fusion of the epithelium of the urethral folds to form a tubular urethra, (2) fusion of the stroma ventral to the urethra to form the ventral spongiosum, and (3) fusion of the epidermis resulting in the raphe on the ventral surface of the penis (Fig, 15). Using SEM, we were able to obtain a truly novel glimpse of the mechanics of the epidermal fusion process, namely the interlacing and approximation of the epithelial cords and their subsequent fusion to form an irregular penile raphe that roughly approximates the midline. The other two fusion events (fusion of the epithelium to form a tubular urethra and fusion of the stroma ventral to the urethra) will be explored in subsequent publications.

This complex multi-stage fusion process within the penile shaft appears to be unique to penile development. There are, however, in a general sense parallels between urethral fold fusion and other well-studied developmental fusion processes. For instance, the regional and age-dependent changes we observe in epithelial cell appearance from densely packed convex cells to flattened squamous-appearing epithelial cells is paralleled by previously published SEM observations of developing rodent palatal epithelium.[15,16] The progressive rostral-caudal closure of the neural tube is analogous to the proximal to distal progression of the urethral closing zipper. However, SEM imaging of the neural tube demonstrates two grossly smooth surfaces aligning without complex interlacing epithelial cords as observed in urethral fold fusion.[17]

Using SEM, we were able to gain novel insights into the mechanics of the opening zipper as well. Interestingly, the complex epithelial organization visualized at the distal end of the urethral groove surrounding the presumed site of active canalization in the older specimens (Fig 1C-F) is absent in the 7.5-week specimen (Fig 1A), despite the fact that the opening zipper has progressed along the entire length of the shaft by this stage. This could be interpreted to mean that there is a difference in the mechanism of the opening zipper between the younger, indifferent stage and the older ages or that there is a difference in mechanism between the unzipping of the urethral plate within the shaft versus the glans.

Canalization of the urethral plate in males and of the vestibular plate in females occurs in a similar fashion (Figs 1 & 7) and is presumably an androgen-independent event. In contrast, fusion of the urethral folds to form the tubular urethra occurs only in males (Fig 1), presumably under the influence of androgens. This observation is supported by the external phenotype of patients born with congenital adrenal hyperplasia, who have inborn errors of adrenal steroid metabolism resulting in exposure to endogenous androgens in utero.[18] Depending on the severity and timing of this metabolic defect, the closing zipper may be activated, and thus these XX females undergo formation of a tubular urethra exactly analogous to penile development. These patents are born with atypical genitalia, which in the most severe form is characterized by a normally formed phallic structure with a common urogenital channel for passing urine, but with normal internal female anatomy consisting of a vagina, cervix, uterus, Fallopian tubes and ovaries.[19]

Subtle differences in the progress of the proximal to distal zippers from week to week between this and previous studies may represent normal variations in development or, alternatively, may be due to the less than perfect accuracy of heel-toe measurements and their correlation to fetal age. In this study, we were limited to detailed SEM imaging of surface features, namely the urethral fold fusion event and were only able to catch occasional glimpses of the canalization and deeper fusion processes. Our analysis of serial transverse sections reveals that the urethral fold fusion event is equally mechanistically rich. Future studies aimed at reconstructing a three-dimensional high-resolution view of the deeper fusion events will be revealing.

The three sequential fusion events (Fig. 15) we describe in human penile development have analogous counterparts in development of the distal aspect of the mouse penile urethra and urethral meatus (Fig. 1),[20] though it should be noted that this does not apply to the proximal aspect of the mouse urethra, which forms by direct canalization of the urethral plate.[10,11,13,21] Moreover, murine penile morphology and the process of urethral meatus formation in the mouse are substantially different from that observed in human fetal genitalia. Most of the mouse penile urethral develops as a result of canalization of the urethral plate to form a urethral lumen without formation of an open urethral groove. In contrast, the human urethra forms as a result of canalization of the urethral plate to form an open urethral groove whose edges (urethral folds) fuse in the midline to form the penile urethra. Only the distal part of the mouse urethra (including the urethral meatus) forms as a result of fusion of the edges of the preputial-urethral groove that appears late in gestation. [12] Thus, the mouse is appropriate model for only certain aspects of human penile development.[22]

In conclusion, high-resolution SEM imaging supports our two-zipper hypothesis of penile development and corresponding single-zipper hypothesis of development of female external genitalia. The androgen-dependent “closing zipper” in human fetal penile development turns out to be a series of developmentally unique layered epithelial fusion events, including the interlacing of epithelial cords to form the median penile raphe. Complex epithelial organization also occurs at the site of canalization (androgen-independent “opening zipper”), which is conserved in both penile and clitoral development.

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Abbreviations

SEM	scanning electron microscopy

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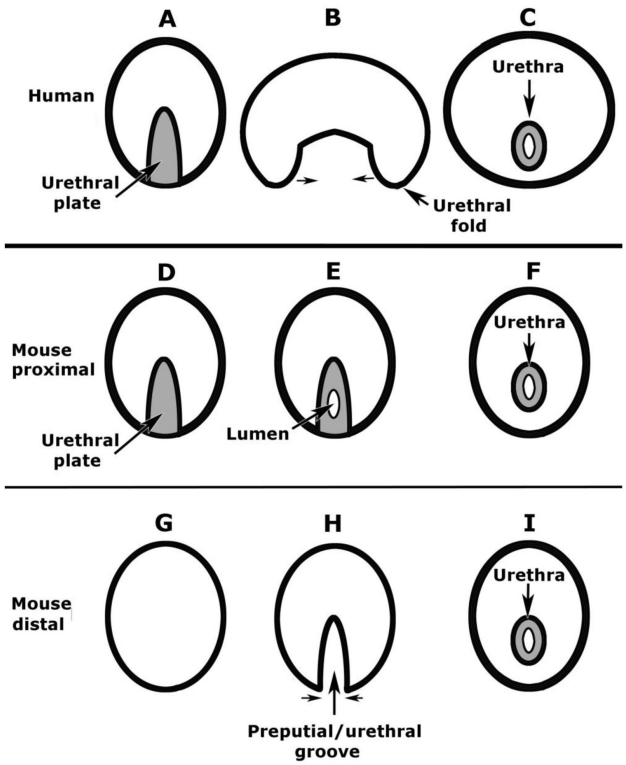

Figure 1.

Diagrammatic representation of penile urethral development in mouse and human. In human penile urethral development (A-B) the solid urethral plate canalizes to form an open urethral groove whose edges (urethral folds) fuse in the midline to form the penile urethra. Most of the mouse penile urethra (proximal portion) forms via direct canalization of the urethral plate to form the urethral lumen (D-F). The distal portion of the mouse penile urethra (including the urethral meatus) forms as a result of formation of the preputial/urethral groove, which subsequently fuses to complete the distal aspect of the penile urethra (G-I). Opposed arrows in (B & H) indicate fusion of the human urethral folds (B) or the mouse preputial/urethral folds (H), which do not extend to the distal aspect of the mouse genital tubercle (G).

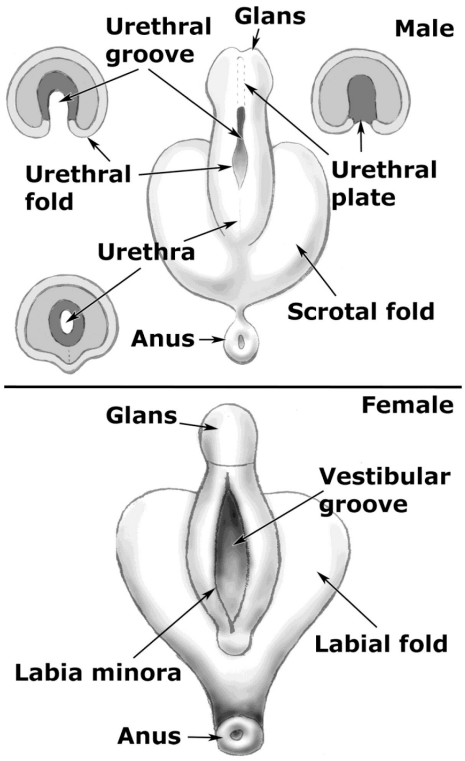

Figure 2.
Pictorial summary of the structures and terms involved in development of human male and female external genitalia.

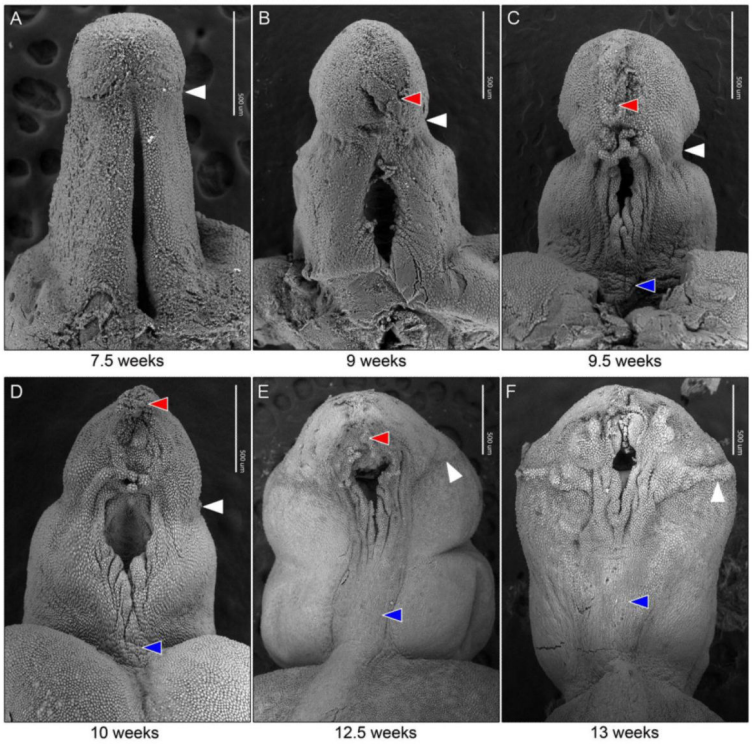

Figure 3.

SEM ontogeny of the developing human fetal penis from 7.5 weeks to 13 weeks of gestation in ventral view. White arrowheads indicate the junction of the penile shaft to glans, red arrowheads indicate the distal epithelial tag, and blue arrowheads indicate the median penile raphe.

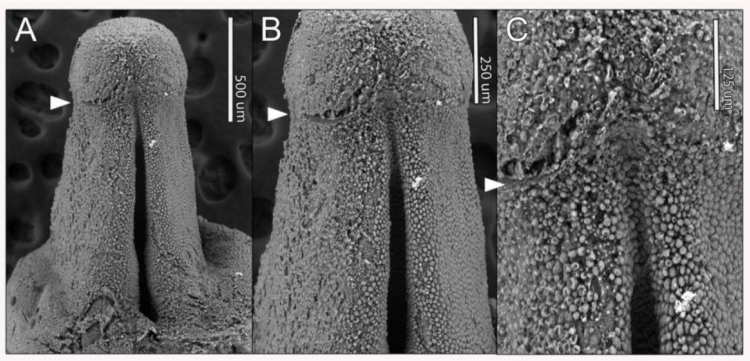

Figure 4.

High-resolution ventral SEM views of 7.5 weeks gestation human fetal penis. White arrowheads indicate the junction of the penile shaft to glans.

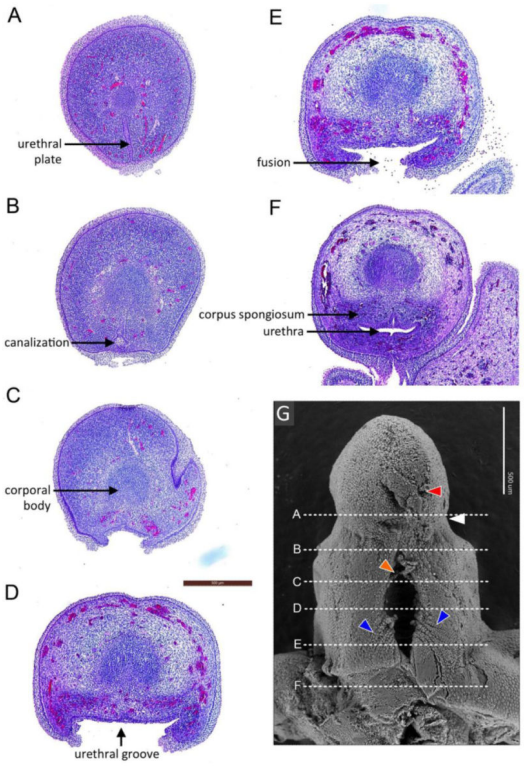

Figure 5.

Transverse sections from 9 weeks gestation human fetal penis stained with hematoxylin and eosin demonstrating (A) solid urethral plate, (B) beginning of canalization process, (C) distal open urethral groove, (D) mid-shaft open urethral groove, (E) fusion process, and (F) formed urethra at the levels indicated in (G). White arrowhead indicates the transition from penile shaft to glans, red arrowhead indicates the distal epithelial tag, orange arrowhead indicates strands of epithelial cells spanning the distal urethral groove, blue arrowheads indicate wrinkles lateral to proximal urethral groove, which may be the precursors of the interlaced epithelial cords seen later.

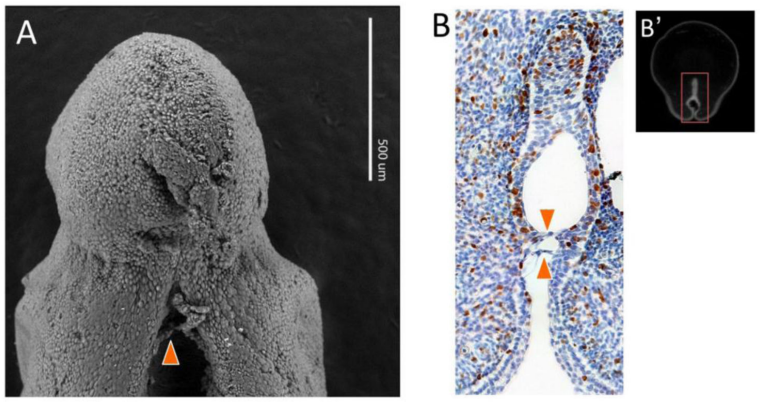

Figure 6.

Epithelial stranding at canalization site as seen in (A) SEM image of 9-week fetal penis and (B) distal transverse section from 8 week fetal clitoris immunostained for Ki67 at site of active canalization (B' schematic shows location of section). Orange arrowheads indicate epithelial strands.

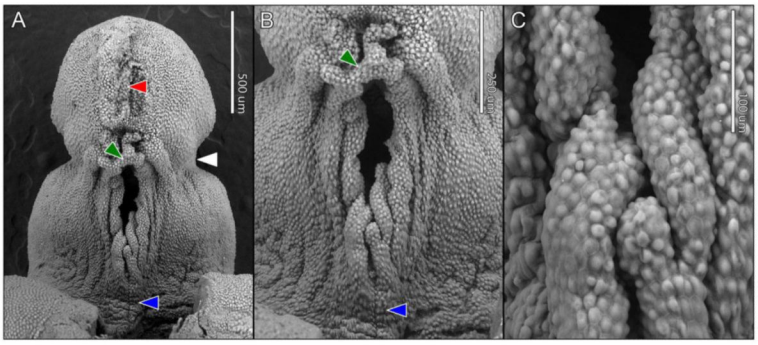

Figure 7.

High-resolution ventral SEM views of a 9.5-week gestation human fetal penis. White arrowhead indicates the transition from penile shaft to glans, red arrowhead indicates the distal epithelial tag, blue arrowhead indicates the median raphe, and green arrowheads indicate ventrally projecting clumps of cells at the distal aspect of the urethral groove.

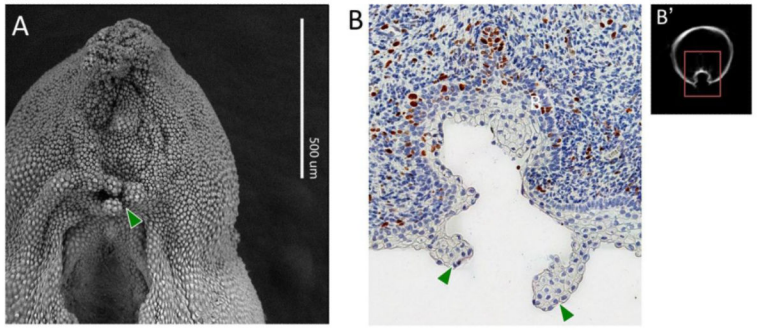

Figure 8.

Sloughing clumps of epithelial cells at the canalization site as seen in (A) SEM image of 10 week fetal penis and (B) distal transverse section from 10.5 week fetal clitoris immunostained for Ki67 proliferative marker at site of active canalization (B' schematic shows location of section). Green arrowheads indicate ventrally protruding clumps of epithelial cells.

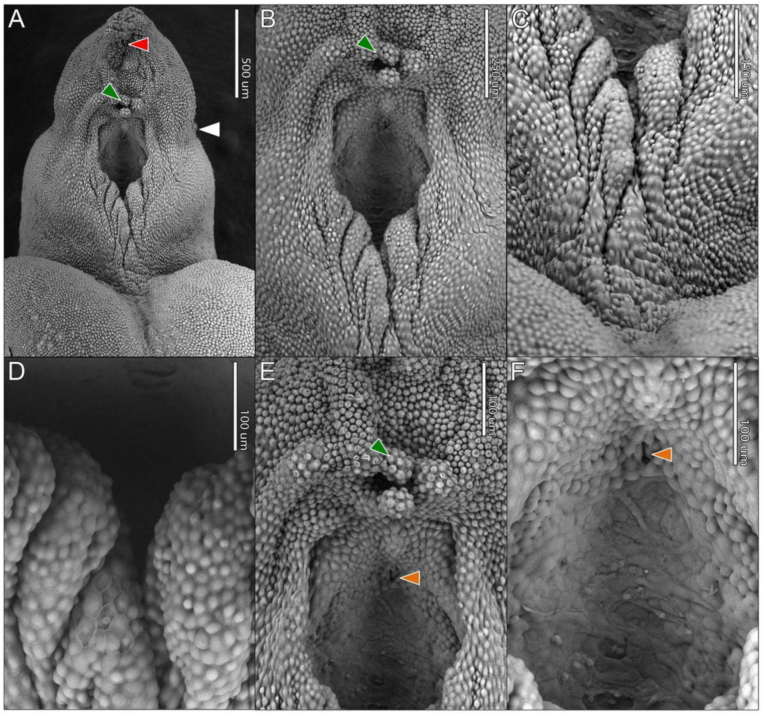

Figure 9.

High-resolution ventral SEM views of a 10-week gestation human fetal penis. White arrowhead indicates the transition from penile shaft to glans, red arrowhead indicates the distal epithelial tag, green arrowhead indicates ventrally projecting clumps of cells at the distal aspect of the urethral groove, orange arrowheads indicate pinpoint opening representing likely site of canalization.

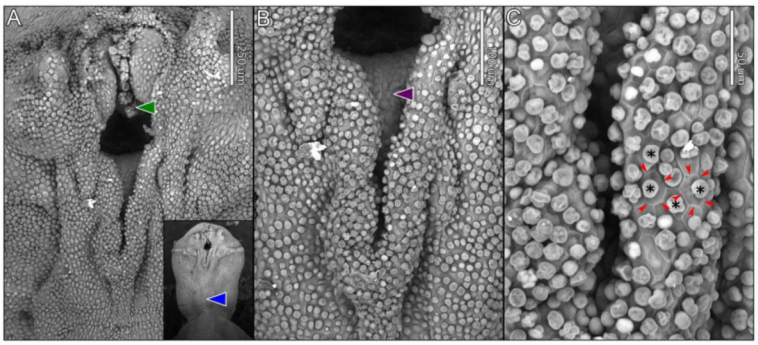

Figure 10.

High-resolution ventral SEM views of a 13-week gestation human fetal penis. Blue arrowhead in (A) indicates the median raphe, green arrowhead (A) indicates inward projecting clumps of cells at the distal aspect of the urethral groove. Purple arrowhead (B) indicates fused epithelial layer immediately dorsal to unfused epidermal cords. In (C) note the “balled up” surface cells rarely in contact with each other (*) that sit upon tightly opposed squamous cells (red arrowheads).

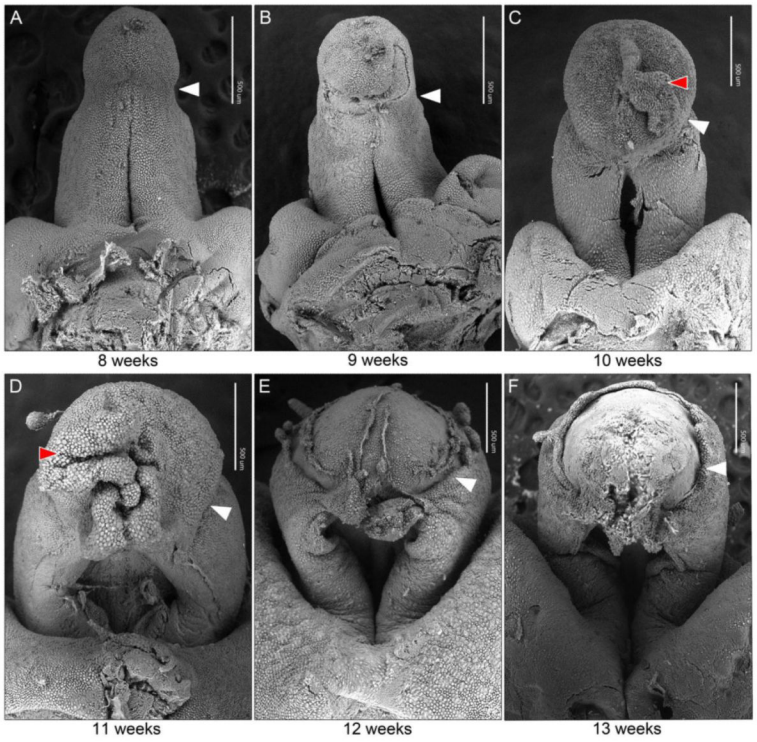

Figure 11.

SEM ontogeny of the developing human fetal clitoris from 8 weeks to 13 weeks of gestation in ventral view. White arrowheads indicate the transition from clitoral shaft to glans; red arrowheads indicate the distal epithelial tag.

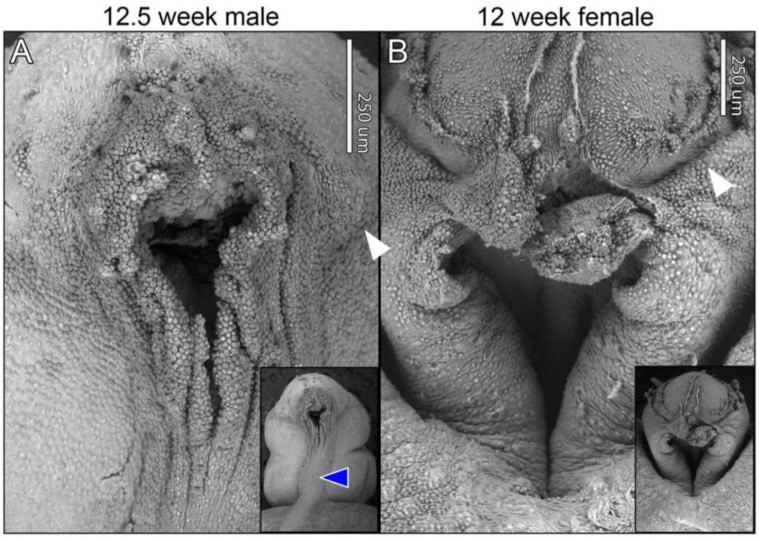

Figure 12.

High-resolution ventral SEM views of 12.5-week gestation human fetal penis and 12 weeks gestation human fetal clitoris. White arrowheads indicate the transition from penile/clitoral shaft to glans, blue arrowhead indicates the median raphe in the male.

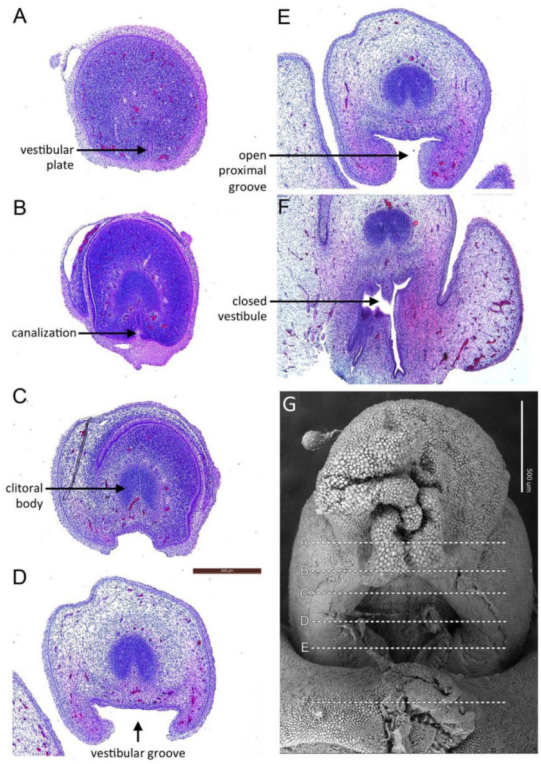

Figure 13.

Transverse sections from a 11-week gestation human fetal clitoris stained with hematoxylin and eosin demonstrating (A) solid vestibular plate, (B) beginning of canalization process, (C) distal open vestibular groove, (D) mid-shaft open vestibular groove, (E) proximal open vestibular groove and (F) closed vestibule at the levels indicated in (G).

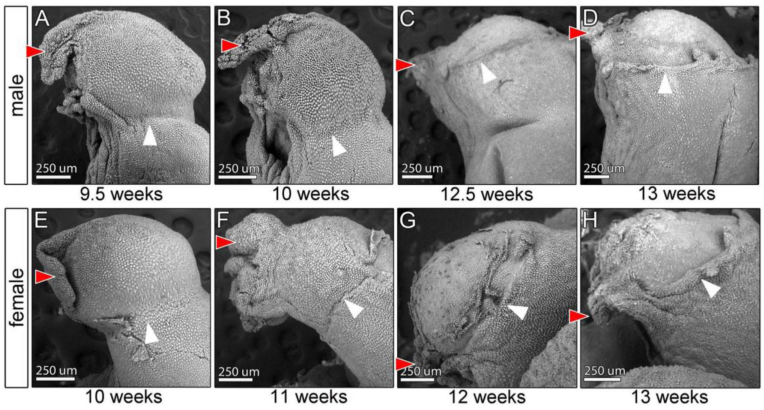

Figure 14.

SEM ontogeny of the glans and distal epithelial tag of the developing human fetal penis and clitoris from 9.5 weeks to 13 weeks of gestation in lateral view. White arrowheads indicate the transition from clitoral/penile shaft to glans and red arrowheads indicate the epithelial tag.

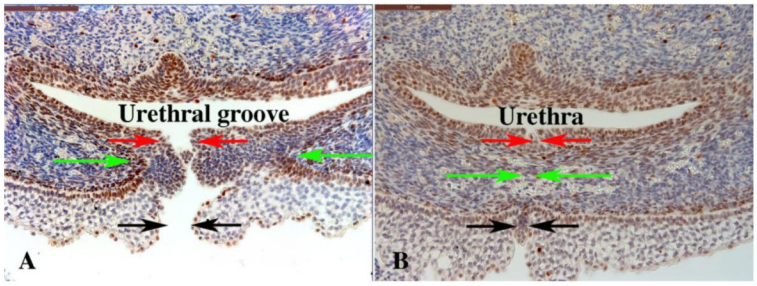

Figure 15.

Transverse sections of the 12 week human fetal penis at the site of the closing zipper in which three fusion events occur: In (A) note (1) Epithelial fusion to form the tubular urethra (red arrows); (2) Epithelial fusion to complete formation of the ventral epidermal surface (black arrows); (3) Midline fusion of right and left mesenchymes ventral to the urethra (green arrows). In (B) the corresponding arrows indicate the completed fusion processes.