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Human glans and preputial development☆

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ABSTRACT

The urethra within the human penile shaft develops via (1) an "Opening Zipper" that facilitates distal canalization of the solid urethral plate to form a wide urethral groove and (2) a "Closing Zipper" that facilitates fusion of the epithelial surfaces of the urethral folds. Herein, we extend our knowledge by describing formation of the human urethra within the glans penis as well as development of the prepuce. Forty-eight normal human fetal penile specimens were examined using scanning electron microscopy and optical projection tomography. Serial histologic sections were evaluated for morphology and immunohistochemical localization for epithelial differentiation markers: Cytokeratins 6, 7, 10, FoxA1, uroplakin and the androgen receptor. As the closing zipper completes fusion of the urethral folds within the penile shaft to form a tubular urethra (~ 13 weeks), canalization of the urethral plate continues in proximal to distal fashion into the glans penis to directly form the urethra within the glans without forming an open urethral groove. Initially, the urethral plate is attached ventrally to the epidermis via an epithelial seam, which is remodeled and eliminated, thus establishing mesenchymal confluence ventral to the glanular urethra. The morphogenetic remodeling involves the strategic expression of cytokeratin 7, FoxA1 and uroplakin in endodermal epithelial cells as the tubular glanular urethra forms. The most ventral epithelial cells of the urethral plate are pinched off from the glanular urethra and are reabsorbed into the epidermis ultimately losing expression of their markers, a process undoubtedly regulated by androgens. The prepuce initially forms on the dorsal aspect of the glans at approximately 12 weeks of gestation. After sequential proximal to distal remodeling of the ventral urethral plate along the ventral aspect of glans, the prepuce of epidermal origin fuses in the ventral midline.

1. Introduction

The human penis develops from the ambisexual genital tubercle between eight and eighteen weeks of gestation under the influence of androgens (Li et al., 2015; Shen et al., 2016; Baskin et al., 2018). In the absence of androgens, the female genital tubercle develops into the clitoris, which despite its smaller size is anatomically homologous with the penis except for the absence of a "clitoral urethra" (Overland et al., 2016). Previously, we have shown that in the human, the urethra of the penile shaft develops via an "Opening Zipper", that is, by canalization of the solid urethral plate to form the open urethral groove (Li et al., 2015). In females the same canalization process occurs forming the open vestibular groove (Overland et al., 2016). In males, the "Closing Zipper" or fusion of the epithelial surfaces of the urethral folds, occurs within the penile shaft to form the penile urethra (Shen et al., 2016; Li et al., 2015), while in females the "Closing Zipper" does not occur due to lack androgenic stimulation secondary to the absence of testes.

In humans, penile urethral development within the glans occurs via an entirely different mechanism from that in the penile shaft, namely direct canalization of the endodermal urethral plate without formation of an open urethral groove (Liu et al., 2018a). An endodermal origin of the penile urethra has been considered for many years and is based upon classic morphological studies performed in the late 1800's and early part of the 1900's (Felix, 1912; Herzog, 1904; Spaulding, 1921; Tourneux, 1889). However, Tourneux proposed an ectodermal origin of the urethra with the formation of the lacuna magna as an ingrowth of an ectodermal urethral plate (Tourneux, 1889). Many of these earlier investigators recognized that the bladder was of endodermal urogenital sinus origin with the urethral plate being an extension of the urogenital sinus extending into the genital tubercle to meet an "ectodermal

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ingrowth" from the penile skin (Herzog, 1904; Felix, 1912; Johnson, 1920). Van den Broek (1909) and Siddiqi (1937) proposed that the penile urethra was formed of an endodermal roof and ectodermal floor. It is evident that an ectodermal-endodermal interface must exist somewhere near the urethral meatus. Glenister proposed that the glanular urethra formed by ingrowth of ectoderm (skin) inward to meet the endodermal urethra at the junction of the penile shaft and glans, the so called Glenister hypothesis (Glenister, 1954). More recently, this has come into question based on gross and histologic observations of Altemus and Hutchins who suggested that the glanular urethra is derived not from skin intrusion but from an extension of bladder urothelium. Our and other investigators studies suggest that the entire human urethra is derived from the endodermal urethral plate (Altemus and Hutchins, 1991; Shen et al., 2016; Li et al., 2015; Baskin et al., 2018; Kurzrock et al., 1999; Liu et al., 2018a, 2018b), an idea further supported by immunohistochemical analysis of FoxA1, cytokeratin 7 and uroplakin (all endodermal urothelial markers) which extend within the glanular urethra up to the urethral meatus (Shen et al., 2018b). An important caveat to this conclusion is that it is based upon observations derived from the developing human urethra during the 1st and 2nd trimesters. Thus, it will be important in the future to confirm these findings through examination of the adult human penile urethra.

The problem of assigning germ layer origin of the penile urethra based solely on observations made during the embryonic period (and not adulthood) can be also be found in a study of the mouse penile urethral development. In a study based upon analysis of E15.5 and E17.5-day mouse embryos Seifert et al. (2008) asserted that the "urethral plate is derived from endoderm that gives rise to the entire urethra". Whether the adult mouse penile urethra is derived entirely from endoderm remains to be determined.

A more important question is whether mouse studies on penile urethral development have relevance to human urethral development. Our recent multi-technique paper has demonstrated vast differences in mouse versus human penile urethral development based upon scanning electron microscopy, optical projection tomography, morphometry, and analysis of serial histologic sections (Liu et al., 2018b). Two disparate morphogenetic mechanisms are involved in penile urethral development in mouse and human: (A) canalization of the urethral plate to directly form the urethral lumen and (B) epithelial fusion events (Liu et al., 2018a). In humans, direct canalization of the urethral plate without formation of an open urethral groove occurs distally within the glans, whereas in mice direct canalization of the urethral plate occurs within the proximal portion of the penis. Fusions events are also common to both mice and humans. In mice, fusion events account for the development of the distal portion of the penile urethra and the urethral meatus, whereas in humans fusion events are involved in formation of the urethra within the penile shaft (Liu et al., 2018a; Sinclair et al., 2016; Li et al., 2015).

Cell lineage studies in mice (based upon ShhGFPcre;R26R mice and Msc2cre;R26R mice) have been used to address the germ layer derivation of mouse penile urethral development (Seifert et al., 2008). The ShhGFPcre;R26R mice used to identify endodermal lineage cells unfortunately has a major flaw. In this model Shh-directed LacZ expression was observed in hair follicles and preputial glands known to be derived from ectoderm (Seifert et al., 2008), even though the endodermal interpretation of the mouse urethral plate is consistent with comparable observations in which Osr1 was used to target LacZ (Grieshammer et al., 2008). The Msc2cre;R26R mice used by Seifert et al. to identify ectodermal lineage cells was not properly validated. Taking a more traditional approach, the epithelial cells involved in the epithelial fusion events occurring in the distal aspect of the mouse penile urethra clearly have the histologic and immunohistochemical signature of epidermis (ectoderm) (Liu et al., 2018a). Thus, our studies suggest that the distal aspect of the mouse penile urethra is derived from ectoderm. The relative contribution of endoderm versus ectoderm to the human penile urethra is a subject of this paper.

At ~11 weeks of gestation the human glans contains a solid urethral plate that lacks a lumen (See Fig. 4 (Baskin et al., 2018). We believe that 5 processes must act in synchrony for successful formation of the glanular urethra: 1) Extension of the urethral plate distally to the tip of the glans to meet surface ectodermal epithelium. 2) Canalization of the urethral plate to form the urethral lumen. 3) Lateral to midline mesenchymal fusion (resulting in mesenchymal confluence) to separate the tubular glanular urethra from the skin. 4) Reabsorption of discarded endodermal cells ventral to the mesenchymal confluence. 5) Remodeling of endodermally derived epithelial channels in the distal glans to form a "stand alone" distal glanular urethra. Our proposal modifies and extends the work of Altemus and Hutchins (Altemus and Hutchins, 1991).

The goal of this study is to apply a multi-technical approach to development of the urethra within the human glans penis using state of the art imaging techniques including optical projection tomography and scanning electron microscopy along with gross wholemount imaging, histology and immunohistochemistry (Li et al., 2015; Overland et al., 2016; Shen et al., 2018a, 2016, 2018b; Isaacson et al., 2018). In addition, we will provide a detailed description of preputial formation that occurs in conjunction with formation of the glanular urethra.

2. Methods

Forty-eight human fetal penile specimens were collected without patient identifier information with IRB approval (UCSF 16-19909 #167670). Fetal age was estimated using heel-toe length based on macroscopic photography. Male sex was confirmed by the presence of testis and Wolffian structures and/or PCR to detect the X and Y chromosome. The gestation ages of the human fetal specimens ranged from 9 to 18 weeks gestation. None of the specimens collected, exhibited any evidence of congenital malformation which we have previously detected at a rate of ~ 1:250 (Shen et al., 2018a). The specimens were prepared for scanning electron microscopy, optical projection microscopy, histology and immunohistochemistry as previously described (Li et al., 2015; Shen et al., 2016). Serial paraffin sections were evaluated for histology (hematoxylin and eosin) and immunohistochemical localization for epithelial differentiation markers: cytokeratins 6, 7, 10, FoxA1, uroplakin, Ki67 and androgen receptor (AR) (Li et al., 2015; Shen et al., 2018b).

3. Results

3.1. Morphogenesis of the glanular urethra

Fig. 1 is a scanning electron microscopic ontogeny of the developing human fetal penis from 10 to 16.5 weeks of gestation. The 10-week specimen shows the open urethral groove within the penile shaft (Fig. 1A) and the previously described fusion process (closing zipper) that forms the penile urethra within the shaft (Fig. 1B–E) (Li et al., 2015; Shen et al., 2016). Note that urethral plate canalization terminates distally at the proximal aspect of the glans (Fig. 1A). Fig. 1B–E show the ontogeny of surface morphology from 12 to 16.5 weeks of gestation. Note the formation of the prepuce beginning dorsally at 12 weeks of gestation with ventral completion of the prepuce at ~16 weeks of gestation. Formation of the prepuce occurs after formation of the urethra in the penile shaft. The penile raphe within the penile shaft is a manifestation of fusion of the urethral folds within the shaft, and it is notable that the penile raphe is not always located in the midline (Compare Fig. 1E with Figs. 1C and D). The epithelial tag can be seen in the 10-week specimen and is especially prominent in the 12- and 13-week specimens (Fig. 1A–C) disappearing after that time point.

Serial hemoxylin and eosin stained sections (Fig. 2A–F) and a scanning electron microscopic image (Fig. 2G) of a 9-week developing penis (Li et al., 2015) verify the absence of a urethral groove within the glans, and also demonstrate extension of the urethral plate into the

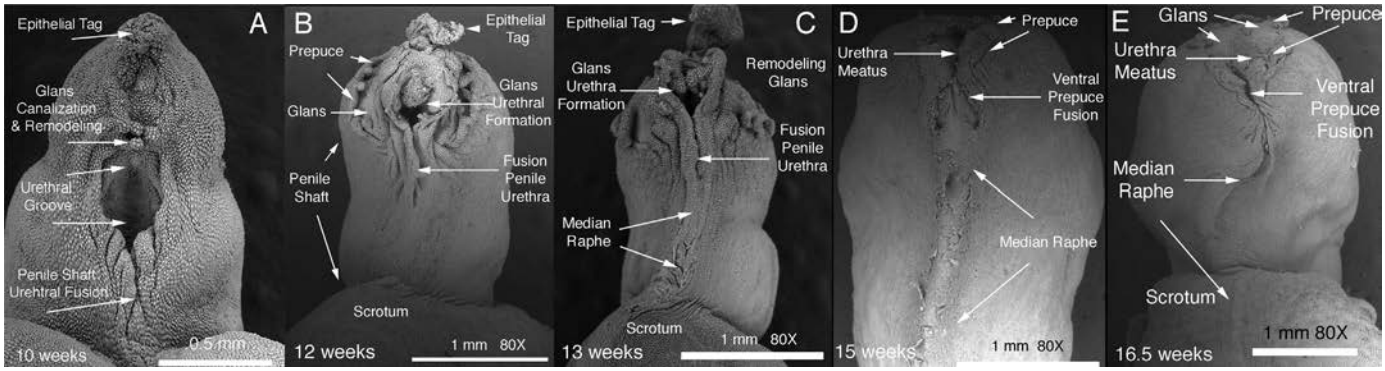

Fig. 1. Scanning electron microscopic ontogeny of the developing human fetal penis and urethra from 10 to 16.5 weeks of gestation (A–E). Formation of the prepuce begins on the dorsal aspect of the glans penis (B), and after glandular urethral formation extends ventrally. In (B) note the prepuce dorso-laterally beginning to cover the glans. In (C–E) the preputial folds are approaching and fusing in the midline. The epithelial tag of unknown significance is present in A–C. Note also the curvature of the penile raphe in (E).

glans (Fig. 2A). Previously published optical projection studies (Li et al., 2015) demonstrate the progressive extension of the solid urethral plate distally into the developing glans to eventually reach the tip of the glans. The transverse section in this paper closest to the tip of the glans is shown in Fig. 3A.

The urethral plate within the glans (Fig. 3A) canalizes directly to form the glanular urethra (Fig. 3B–E). The process of canalization is asymmetric and begins within the ventral portion of the urethral plate, but progressively expands dorsally to canalize the urethral plate (Fig. 3B–E). This dorsal-ventral asymmetry was consistently observed within the urethral plate of the glans, which presumably is based upon dorsal-ventral differences in gene expression.

Thus, entirely different morphogenetic mechanisms of penile urethral development occur within the human penile shaft versus the glans. Direct canalization of the urethral plate without formation of an open urethral groove occurs within the human glans (Fig. 3). In contrast, within the human penile shaft, the urethral plate canalizes to form a wide-open urethral groove whose edges (urethral folds) fuse in the midline to form the urethral within the penile shaft (Fig. 2). Linkage of the urethral tubes formed by these two disparate mechanisms occurs in the region of the coronal margin thus completing a urine channel to the exterior at the urethral meatus. This process surely requires considerable morphogenetic remodeling where the epithelial cells of the skin (ectoderm) meet the endodermally derived cells of the glanular urethra (Fig. 4) defined by immunohistochemical markers as described below.

4. Immunochemical analysis of the glanular urethral development

Immunohistochemical studies were undertaken to explore the question of the germ layer origin of the human glanular urethra. For this purpose, the expression of cytokeratins 6, 7, and 10, FoxA1 and

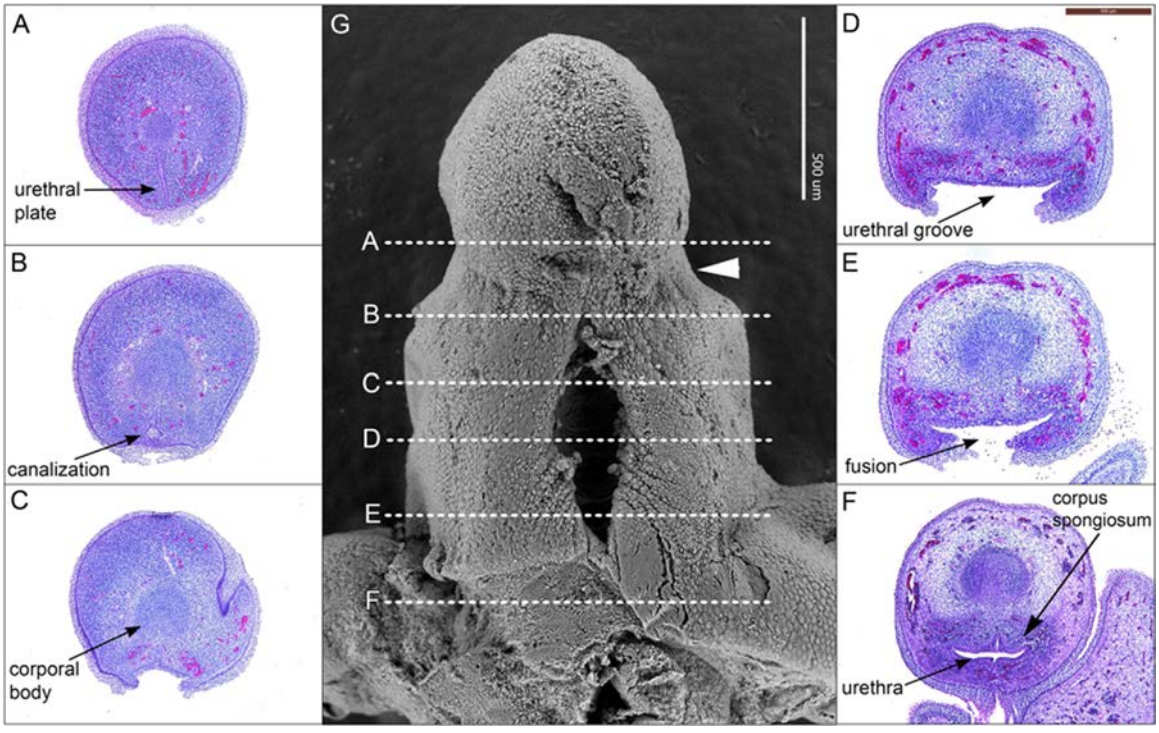

Fig. 2. Scanning electron micrograph (G) and transverse sections of a 9-week human fetal penis stained with hematoxylin and eosin demonstrating (A) the solid urethral plate, (B) the beginning of canalization of the urethral plate, (C) the urethral plate has canalized to from an open urethral groove in the distal penile shaft, (D) mid-shaft showing a widely open urethral groove, (E) beginning of the process of fusion of the urethral folds, and (F) fully formed urethra at the levels indicated in (G). White arrowhead in (G) indicates the transition from penile shaft to glans. Modified from (Shen et al., 2016) with permission.

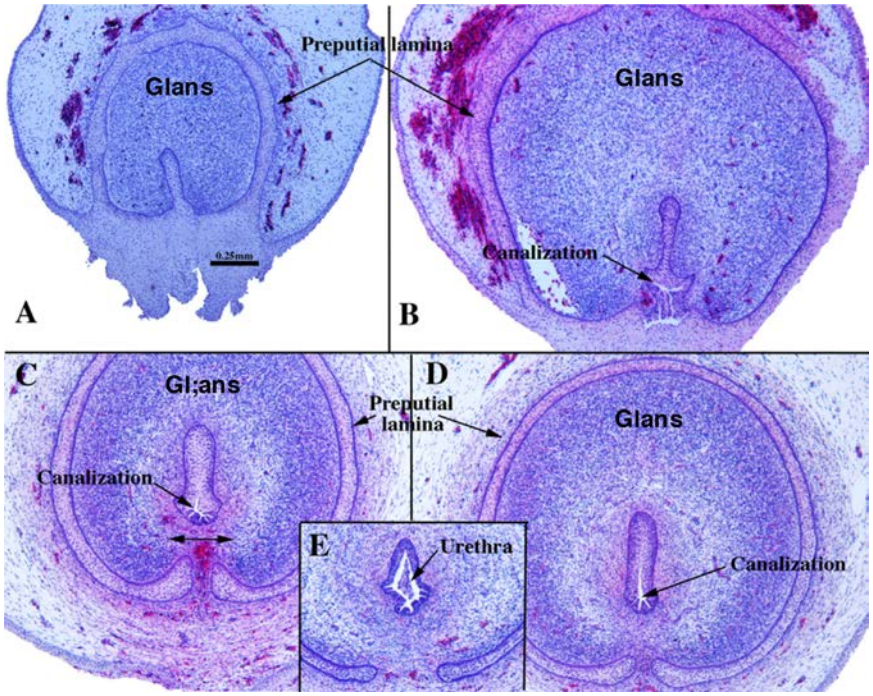

Fig. 3. Sections through the human penile glans at 14 (A, C–E) and 15 weeks (B) gestation arranged in distal (A) to proximal (E) order. In (A) note the solid urethral plate located near the tip of the glans, which is surrounded by the preputial lamina. In (B) the urethral plate is canalizing at its junction with the epidermis. In (C) mesenchymal confluence (double-headed arrow) has been established ventral to the canalizing urethral plate whose dorsum is still solid. In (D) the canalization process is extending dorsally. In (E) a fully canalized urethra has developed. From (Liu et al., 2018a) with permission.

uroplakin are particularly informative. Cytokeratin 7 is a known marker of developing and mature urothelium of the urethra and bladder (Kurzrock et al., 1999; Moll et al., 2008, 1982; Shen et al., 2018b). In contrast, cytokeratin 10 is a known marker of developing and mature epidermis (ectodermal associated marker) (Moll et al., 2008, 1982; Shen et al., 2018b). Cytokeratin 6 is a useful marker of a variety of stratified epithelia (including the solid urethral plate and the epidermis during urogenital development (Shen et al., 2018b; Moll et al., 2008, 1982). Foxa1 is a known endodermal marker within pelvic/perineal organs (Diez-Roux et al., 2011; Robboy et al., 2017; Besnard et al., 2004). Uroplakin is a unique marker for urothelium (Sun et al., 1999).

Immunohistochemical localization of cytokeratins 6, 7, 10 and FoxA1 is shown in the human glans penis at 14 and 17 weeks gestation (Fig. 5). Note at both the 14- and 17-week time points (Fig. 5A and D) expression of cytokeratin 7 and FoxA1 (both endodermal urothelial markers) in the forming urethra within the glans, whereas cytokeratin 6 was detected in the epidermis and the preputial lamina (Fig. 5A and D). Cytokeratin 6 was also detected in the urethral plate (Fig. 6A and D). In contrast, cytokeratin 10 localized to the epidermis and the preputial lamina (Figs. 5B–C, E–F and 6B). Significantly, the interface between Foxa1 and cytokeratin 7 versus cytokeratin 10 immunostaining coincided precisely at the distal urethral meatus consistent with our previous observation that the entire penile urethra is derived from endoderm in humans (Kurzrock et al., 1999).

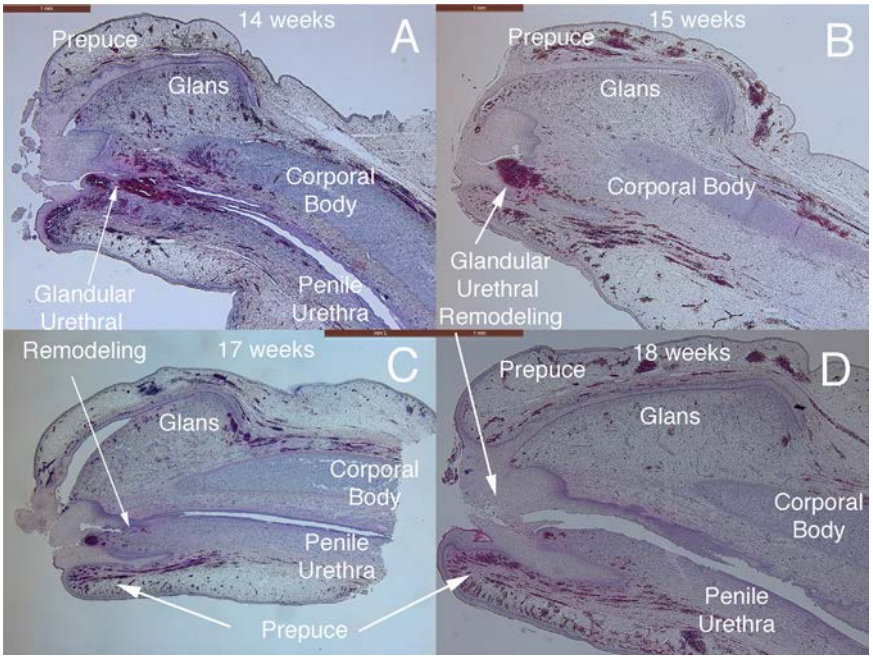

Fig. 4. Mid-sagittal sections of the human fetal glandular urethra. (A) 14 weeks gestation, (B) 15 weeks, (C) 17 weeks and (D) 18 weeks. Note the progressive remodeling and formation of glandular urethra. The prepuce is present dorsally in each image with progressive ventral extension of the foreskin (C and D).

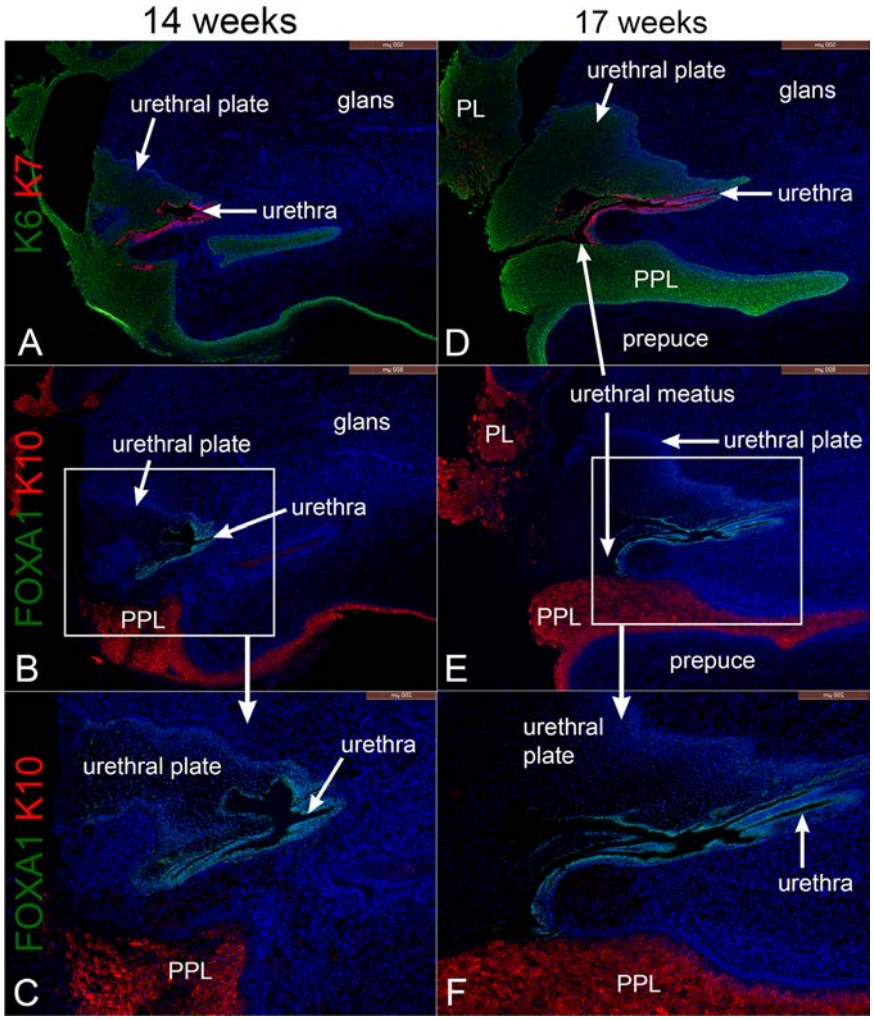

Fig. 5. Immunochemical localization of cytokeratins 6, 7, 10 and FoxA1 in "mid" sagittal sections of the human glans penis at 14- and 17-weeks of gestation. Note at both the 14- and 17-week time points (A and D) cytokeratin 7 (red) localized to the terminal urethra and cytokeratin 6 to the skin and urethral plate (green). The endodermal marker FoxA1 (green) also localized to the terminal urethra (B, C, E and F) with K10 (red) localizing to the skin, and preputial lamina.

Expression of cytokeratins 6 and 10 differ in several ways. While cytokeratins 6 and 10 were both expressed in the epidermis (Figs. 5 and 6B), the urethral plate was cytokeratin 6 reactive (Fig. 6A) but not cytokeratin 10 reactive (Fig. 6). The epithelial tag was reactive to both cytokeratins 6 and 10. Another difference between cytokeratin 6 and 10 expression is that all epidermal layers (basal to apical) are cytokeratin 6 positive, while cytokeratin 10 is expressed only in supra-basal layers as described previously (Shen et al., 2018b; Moll et al., 2008, 1982).

The transition from a solid urethral plate attached to ventral epidermis to a "stand alone" glandular urethra completely surrounded by penile stroma involves considerable remodeling and alteration in protein expression in both the epithelium and mesenchyme. Figs. 7–11 show composite images of serial sections of human fetal glans penis at 13, 14, 15, 16 and 17 weeks of gestation, with representative low power histologic images through the glans penis, as well as macroscopic gross images for orientation. Over the time period studied, canalization of the urethral plate begins in the ventral portion of the solid urethral plate (Figs. 3B–D and 7–10). Subsequently the canalization extends into the dorsal aspect of the urethral plate (Figs. 3E and 11M–R light blue arrows). The corresponding serial sections in the three areas within the

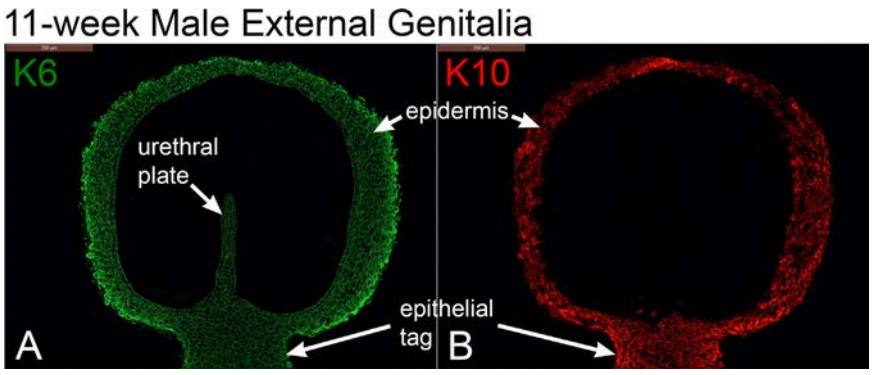

Fig. 6. A single section of an 11-week human fetal penis immunostained for cytokeratin 6 and 10. In (A) note cytokeratin 6 (green) localized to the full epidermal thickness, the epithelial tag and the urethral plate. In (B) note cytokeratin 10 localized to supra-basal layers of the epidermis and the epithelial tag but not the urethral plate.

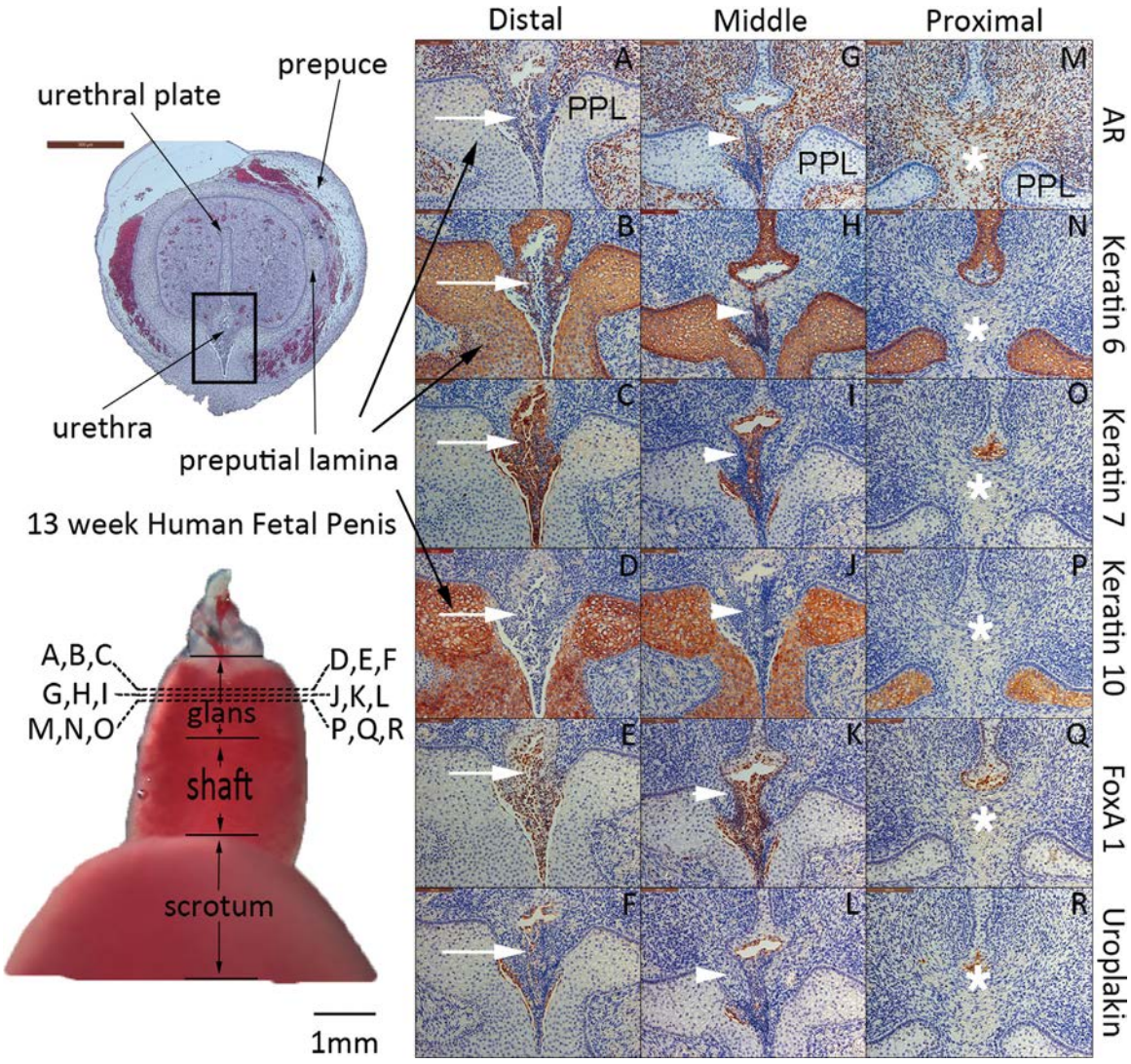

Fig. 7. 13-Week gestation human fetal penis with a representative low power histologic image through the glans, a macroscopic gross image, and corresponding serial sections in the distal, mid and proximal glans immunostained with androgen receptor (AR), cytokeratins 6, 7 and 10, FoxA1 and uroplakin. PPL = preputial lamina. Note the mesenchymal confluence ventral to the formed tubular urethra in the proximal glans (white asterisks) (proximal column M–R) and mesenchymal confluence in progress in the mid glans (white arrowheads) (middle column G–L). Note the open channels in the distal glans (white arrows) (distal column A–F) that will remodel into the urethra.

glans from distal to proximal are immunostained for androgen receptor, cytokeratin 6, 7 and 10, FoxA1 and uroplakin.

Critical evaluation of Figs. 7–11 requires that two important points are kept in mind: (a) That age is a factor in these 5 figures spanning 13–17 weeks of gestation, and (b) that positional factors on a proximal to distal basis also come into play. This latter factor means that morphogenesis is more advanced proximally than distally at all ages. Accordingly, we will present data on age-related changes in the proximal zone first as this sets the stage for evaluating the more distal zones.

At all ages the proximal zone within the glans contains a “stand alone urethra” with mesenchymal confluence ventral to the urethra. FoxA1, K7 and uroplakin (markers indicative of an endodermal urothelium) are expressed in parallel fashion in the epithelial cells bordering the ventrally situated canalized urethral lumen (Figs. 7–11, proximal column). Note that FoxA1, K7 and uroplakin are expressed only in the epithelial cells bordering the ventrally situated canalized urethral lumen and not in the uncanalized urethral plate dorsal to the urethral lumen. Another point to be emphasized is that canalization of the urethral plate begins ventrally and progresses dorsally (compare proximal columns Fig. 7 at 13 weeks with Fig. 11 at 17 weeks). Cytokeratin 10, a marker of maturing and mature epidermis, is not expressed in epithelial cells bordering the urethral lumen or in the uncanalized urethral plate (Figs. 6 and 7–11, proximal column). Cytokeratin 6 is known to be expressed in the urethral plate as early as 11 weeks of gestation (Fig. 6) (Shen et al., 2018b) as well as in the epidermis and is so expressed from 13 to 17 weeks of gestation (Figs. 7–11). Cytokeratin 6 is also expressed in the basal cells of the canalized urethra (Figs. 7–11, proximal column). The androgen receptor is expressed in the luminal cells of the canalized glanular urethra (Figs. 7–11, A, G & M), most prominently at 14 weeks, but not in the uncanalized urethral plate. Androgen receptor is also strongly expressed in mesenchymal cells surrounding the urethral plate (canalized or solid) and especially in mesenchyme ventral to the glanular urethra where lateral to midline mesenchymal fusion occurs in a proximal to distal fashion within the glans. Thus, the fully formed tubular urethra expresses cytokeratin 7, FoxA1 and uroplakin (endodermal cell markers) as well as the androgen receptor indicative of a role of androgens in glanular morphogenesis (panels A, G & M Figs. 7–11). During urethral development extremely, narrow channels between epithelial cells (through which urine can pass) are visible distally, but disappear with

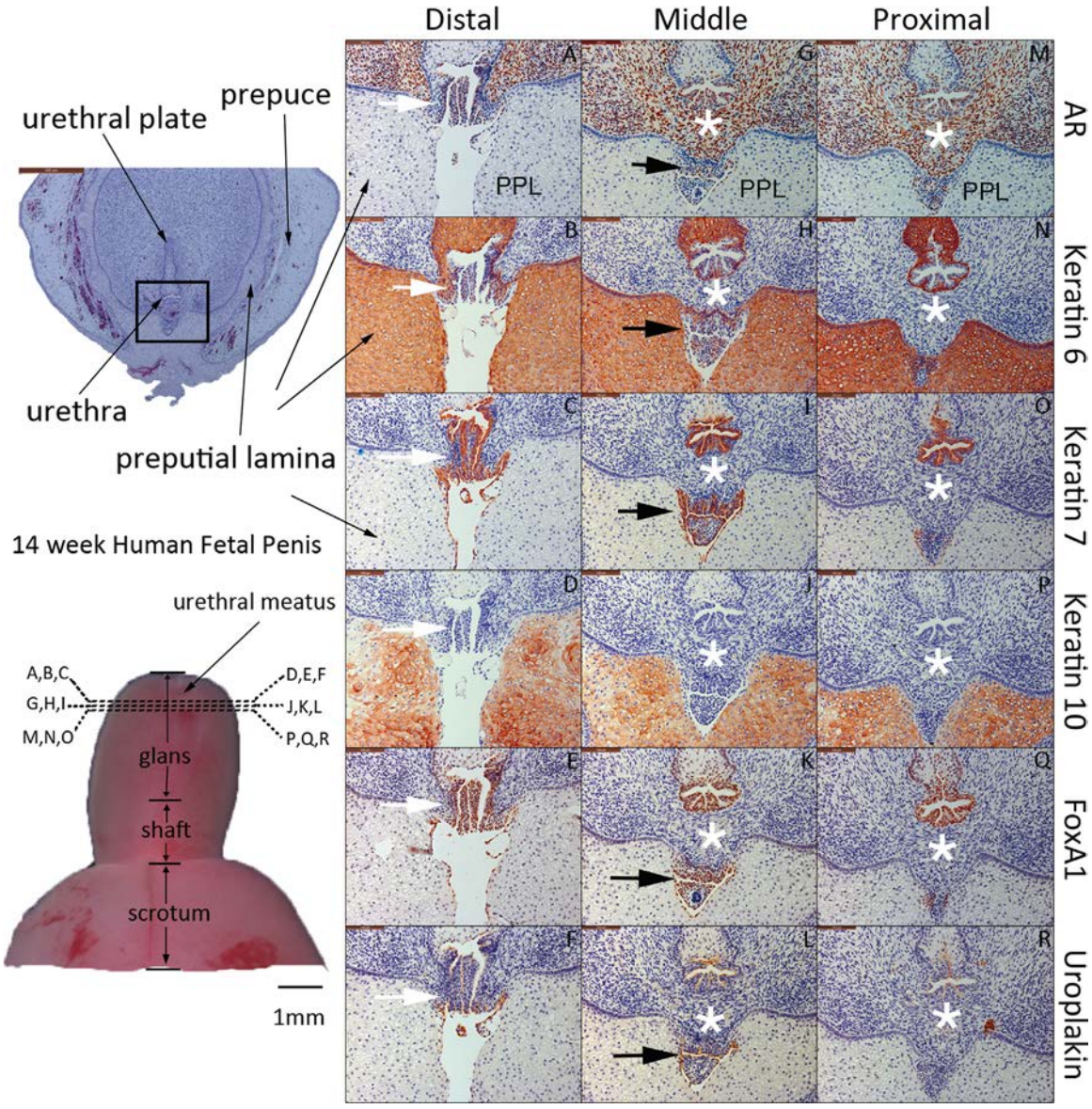

Fig. 8. 14-Week gestation human fetal penis with a representative low power histologic image through the glans, a macroscopic gross image, and corresponding serial sections in the distal, mid and proximal glans immunostained with androgen receptor (AR), cytokeratins 6, 7 and 10, FoxA1 and uroplakin. PPL= Preputial lamina. Note the completed mesenchymal confluence ventral to the formed tubular urethra in the proximal (M–R) and mid glans (G–L) (white asterisks). Note the open channels through which urine can pass in the distal glans (A–F) (white arrows). The endodermally derived epithelial cells that have pinched off, remodeled and ultimately resorbed are well visualized below the tubular urethra in the mid glans (G–L) (black arrows).

time (Figs. 7–11). Cytokeratin 7-, FoxA1- and uroplakin-positive epithelial cells of the ventral aspect of the urethral plate are pinched off and resorbed during the process of mesenchymal confluence (best seen at gestational days 14–16 in Figs. 8–10G–L).

In summary, the proximal zone at all ages is noted for having a canalized urethra whose luminal cells express cytokeratin 7, FoxA1, uroplakin and androgen receptor, while the uncanalized urethral plate is unstained for these markers as well as for cytokeratin 10. Cytokeratin 6 is expressed in the uncanalized urethral plate, in the basal cells of the urethra and in the preputial lamina and epidermis.

The middle zone has a similar protein expression pattern for cytokeratins 6, 7, 10, FoxA1, uroplakin and the androgen receptor, but is a zone of considerable morphogenetic remodeling. The principal event seen in the middle zone is the process of midline lateral to midline mesenchymal confluence ventral to the canalizing urethral plate, which is particularly well illustrated at 13 weeks of gestation in Fig. 7G–L (middle column). Most of these sections (Fig. 7G–L middle column) show an attenuated epithelial connection between the canalizing urethral plate and the preputial lamina (PPL) (Figs. 5, 7–11). This attenuated epithelial connection expresses cytokeratins 6 and 7, FoxA1 and AR, but not cytokeratin 10 or uroplakin at 13 weeks of gestation (Fig. 7G–L middle column). It is important to emphasize that at all stages and in all zones examined, cytokeratin 10 is not expressed in the urethral plate or the canalized urethra (Figs. 6–11). Note that as the attenuated epithelial connection breaks down, some of the epithelial cells of this connection are retained in association with the preputial lamina (Fig. 7G–L, K and L middle column) at 13 weeks and to various degrees at 14–17 weeks (Figs. 8–11 middle column). The mesenchyme associated with developing mesenchymal confluence is strongly androgen receptor-reactive at 13 weeks of gestation (Fig. 7G and M) and thereafter (Fig. 8G and M). At all later stages (14–17 weeks of gestation) the middle columns exhibit completed mesenchymal confluence (Figs. 8–11).

The distal zone is the least mature from a morphogenetic

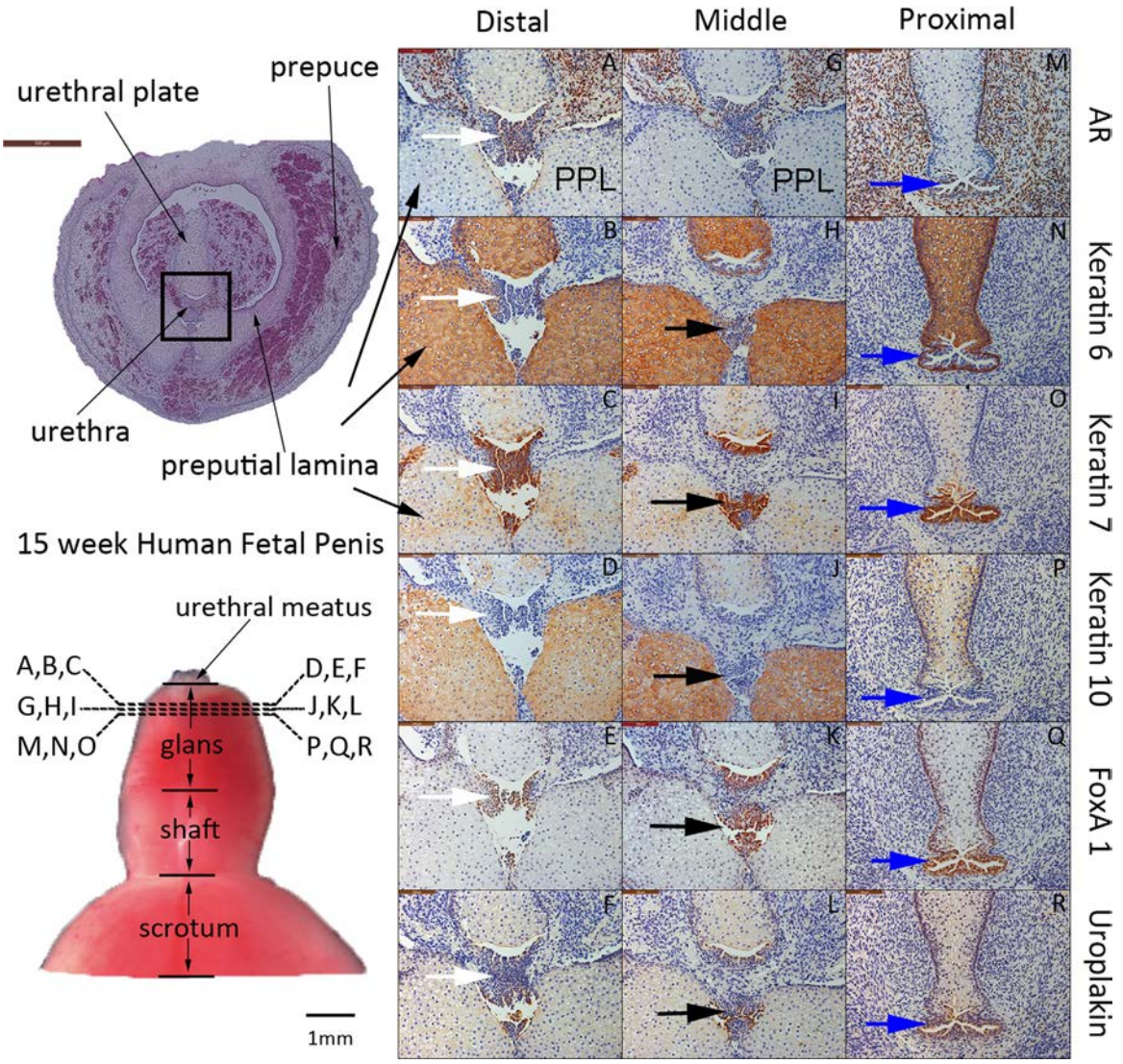

Fig. 9. 15-Week gestation human fetal penis with a representative low power histologic image through the glans, a macroscopic gross image, and corresponding serial sections in the distal, mid and proximal glans immunostained with androgen receptor (AR), cytokeratins 6, 7 and 10, FoxA1 and uroplakin. PPL = Preputial lamina. Note the tubular urethra in the proximal glans (M–R) with dorsal canalization still to occur (blue arrows). Mesenchymal confluence and pinched off, remodeled and ultimately resorbed epithelial cells (black arrows) are well visualized below the tubular urethra in the mid glans (G–L). Note the open channels in the distal glans (white arrows)(distal column A–F) that will remodel into the urethra.

standpoint. A common feature of the distal zone is a broad connection between the canalizing urethral plate and the preputial lamina/epidermis (Figs. 7–11 distal column). The protein expression patterns seen in the distal zone are an extension of the middle and proximal zones. Cytokeratins 6 and 7, FoxA1, uroplakin and AR are expressed in the urethral epithelium of canalizing urethral plate, while keratin 10 is exclusively expressed in the preputial lamina and the epidermis (Figs. 7–11 distal column). In the 14–16 week specimens (Figs. 8–10, distal column) (but not the 17 week specimen), the canalization process has opened narrow channels (white arrows) continuous externally to a space associated with the prepuce. These narrow channels are not seen in the distal zone at 17 weeks of gestation. In summary the distal zone is notable for epithelial remodeling as the endodermally urethra meets the skin of ectodermal origin.

At 9 weeks gestation the ventral aspect of the glans is relatively smooth (Fig. 12A), while at 10 weeks of gestation balled up epithelial cells are seen on the ventral aspect of the glans where glanular urethral remodeling is occurring (Figs. 1 and 12B). These “epithelial balls” are also seen in histologic sections and in sections derived from optical projection tomography (Fig. 12C–D). These epithelial balls (green arrowheads in Fig. 12B and C) appear to exfoliate as they are only seen at early stages of urethral development (Fig. 1). Cellular proliferation based on Ki67 staining appears to be involved in epithelial remodeling (Fig. 12C).

Optical projection tomography also reveals epithelial projections from the ventral surface of the developing penis in the region of the coronal margin (Fig. 13 red arrows). Also note the prominent epithelial tag at 9–12 weeks gestation (blue arrows). The formation of the tubular urethra from the penoscrotal junction to the tip of the glans penis is denoted by orange arrows in Fig. 13. Note the urethral plate in the 8-week specimen extends almost to the tip of glans (Fig. 13, green arrow). Subsequently, the urethral plate extends to a terminal position on the glans meeting the skin epithelium to form the urethral meatus.

5. Development of the human prepuce

Human preputial development begins at ~11 weeks of gestation (Figs. 1, 14–16) when the epithelium thickens on the dorsal aspect of the glans penis and forms the preputial placode (Figs. 15B and 16A1 and A2) from which bilateral preputial laminar processes extend

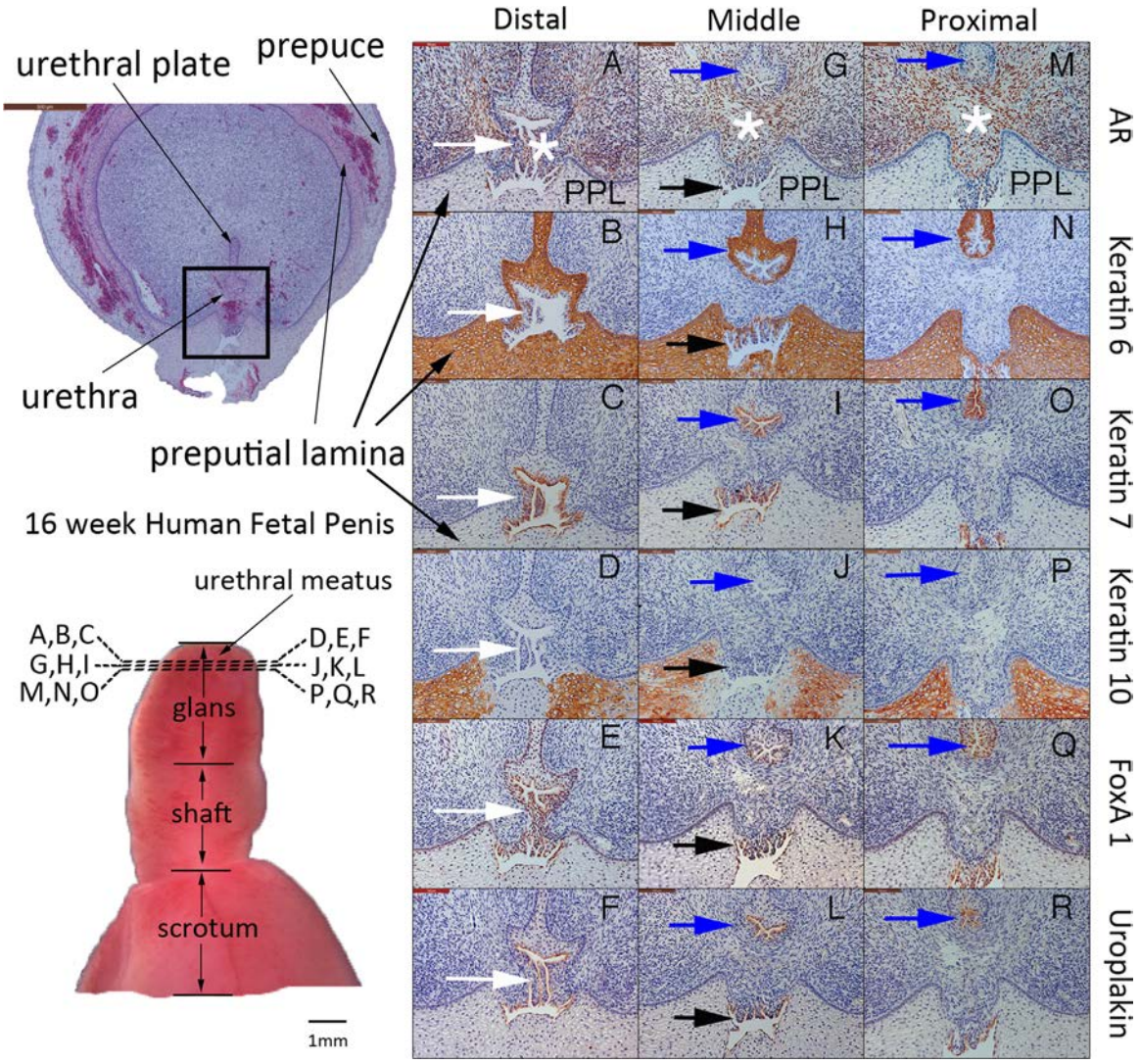

Fig. 10. 16-Week gestation human fetal penis with a representative low power histologic image through the glans, a macroscopic gross image, and corresponding serial sections in the distal, mid and proximal glans immunostained with androgen receptor (AR), cytokeratins 6, 7 and 10, FoxA1 and uroplakin. Note the formed tubular urethra in the mid (G–L) and proximal glans (M–R) (blue arrows) completed mesenchymal confluence and pinched off, remodeled and ultimately resorbed epithelial cells that are well visualized below the tubular urethra (black arrows). Note the AR positive cells at the site of the completed urethral formation (G and M) and in process mesenchymal confluence (A) (white asterisk). Note the open channels in the distal glans (white arrows) (distal column A–F) that will remodel into the urethra.

ventrally into the glanular mesenchyme (Figs. 15B and 16B2). Prior to 11 weeks of gestation there is no evidence of preputial development (Figs. 14A and 15A1–A3). Shortly after the preputial placode forms it delaminates from the epidermis with appearance of a layer of mesenchymal cells between the preputial lamina and the epidermis (Figs. 14B–D, 15C). With time the preputial lamina expands ventrally to almost completely cover the glans (Fig. 1). This dorsal-ventral expansion of the preputial lamina can be appreciated over the period of 12–16 weeks of gestation in Fig. 14B–D (black arrows = dorsal preputial lamina, white arrows = ventral preputial lamina), Fig. 15B, diagrammatically in Fig. 16 and in scanning electron micrographs (Fig. 1B–C). As development proceeds the lateral ventral edges of the preputial lamina approach the ventral midline. The ventral edges of the preputial lamina do not fuse in the midline but remain separated by a thin septum of mesenchyme (Figs. 15C and 16D2) destined to form the preputial frenulum. The preputial lamina, once formed, has a basement membrane on both its superficial and deep faces. Near birth and continuing postnatally the preputial lamina delaminates to create the preputial space. Postnatally the prepuce has an epidermis externally and is lined on its inner surface by a stratified epithelium.

6. Discussion

We have previously shown that the urethra within the human penile shaft develops via an “opening zipper”, that facilitates distal canalization of the solid urethral plate to form the urethral groove and “closing zipper, that facilitates complex fusion of the two epithelial surfaces of the urethral folds (Li et al., 2015; Shen et al., 2016). Herein, we extend our knowledge of human urethral development by describing formation of urethra within the glans penis along with formation of the prepuce.

7. Development of the glanular urethra

Using a multimodal technical approach including optical projection tomography and scanning electron microscopy along with gross wholemounts, histology and immunohistochemistry, we have advanced understanding of urethral development in the human glans penis. The key finding in this study is based on an immunohistochemical ontogeny during human glanular urethral development, which occurs from 13 to 17 weeks of gestation. The analysis focuses on known markers of endodermal urothelial differentiation: cytokeratin 7, FoxA1 and

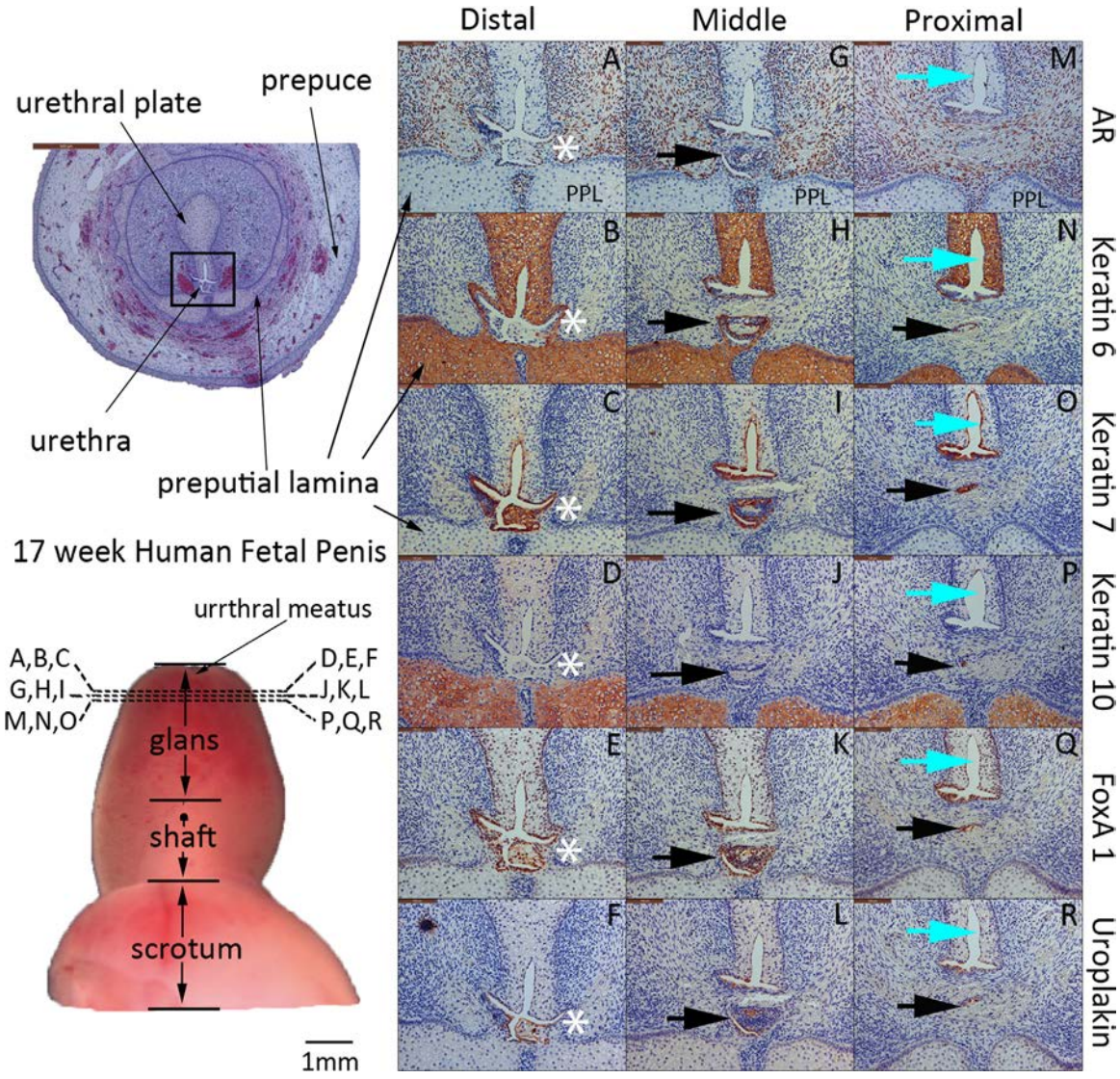

Fig. 11. 17-Week gestation human fetal penis with a representative low power histologic image through the glans, a macroscopic gross image, and corresponding serial sections in the distal, mid and proximal glans immunostained with Androgen Receptor (AR), cytokeratin 6, 7 and 10, FoxA1 and uroplakin. PPL = Preputial lamina. Note the formed tubular urethra in all the panels. The canalization process is now visible in the dorsal (light blue arrows) as well as the ventral aspect of the urethral and is at a more advanced stage in the proximal glans (M–R). Mesenchymal confluence and pinched off, remodeled and ultimately resorbed epithelial cells are well visualized below the tubular urethra in the mid glans (G–L) (black arrows) with the last remnants of reabsorbed cells still visible below the tubular urethra in the proximal glans (M–R) (black arrows). Mesenchymal confluence is not quite complete in the distal glans (A–F) (white asterisk).

uroplakin. Cytokeratin 7 (Moll et al., 2008, 1982) is known to be expressed in fetal and adult bladder epithelium (Baskin et al., 1996) and the embryonic urogenital sinus and the human fetal urethra (Cunha et al., 2017) showing a similar pattern of staining to the urothelial marker, uroplakin. Uroplakin is a unique terminal urothelial marker composed of an asymmetric unit membrane with four major integral proteins that is only expressed on the apical aspect of the urothelial cells of the bladder, urethra and ureter (Sun et al., 1999, 1996). Foxa1

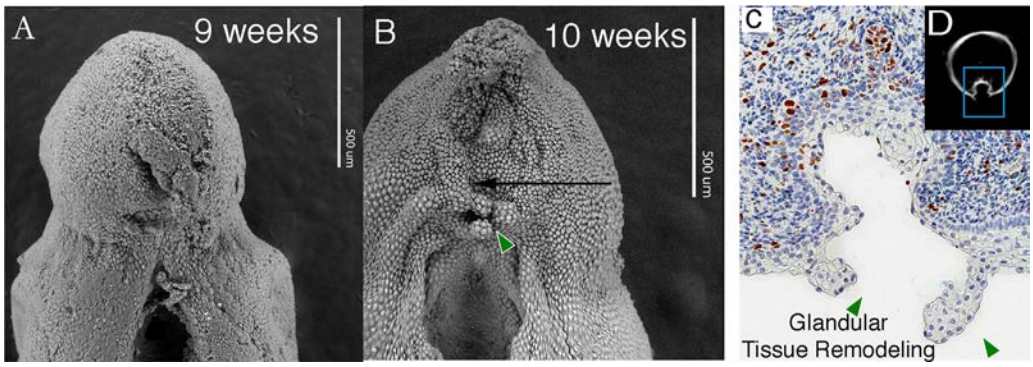

Fig. 12. Scanning electron microscopy, Ki67 Immunohistochemistry and optical projection tomography of 9 (A) and 10 (B) week human fetal penis. A and B: Note the progressive glandular tissue remodeling (green arrowhead) seen on the surface of the glans shown histologically in C (green arrowhead) and by optical projection tomography in D.

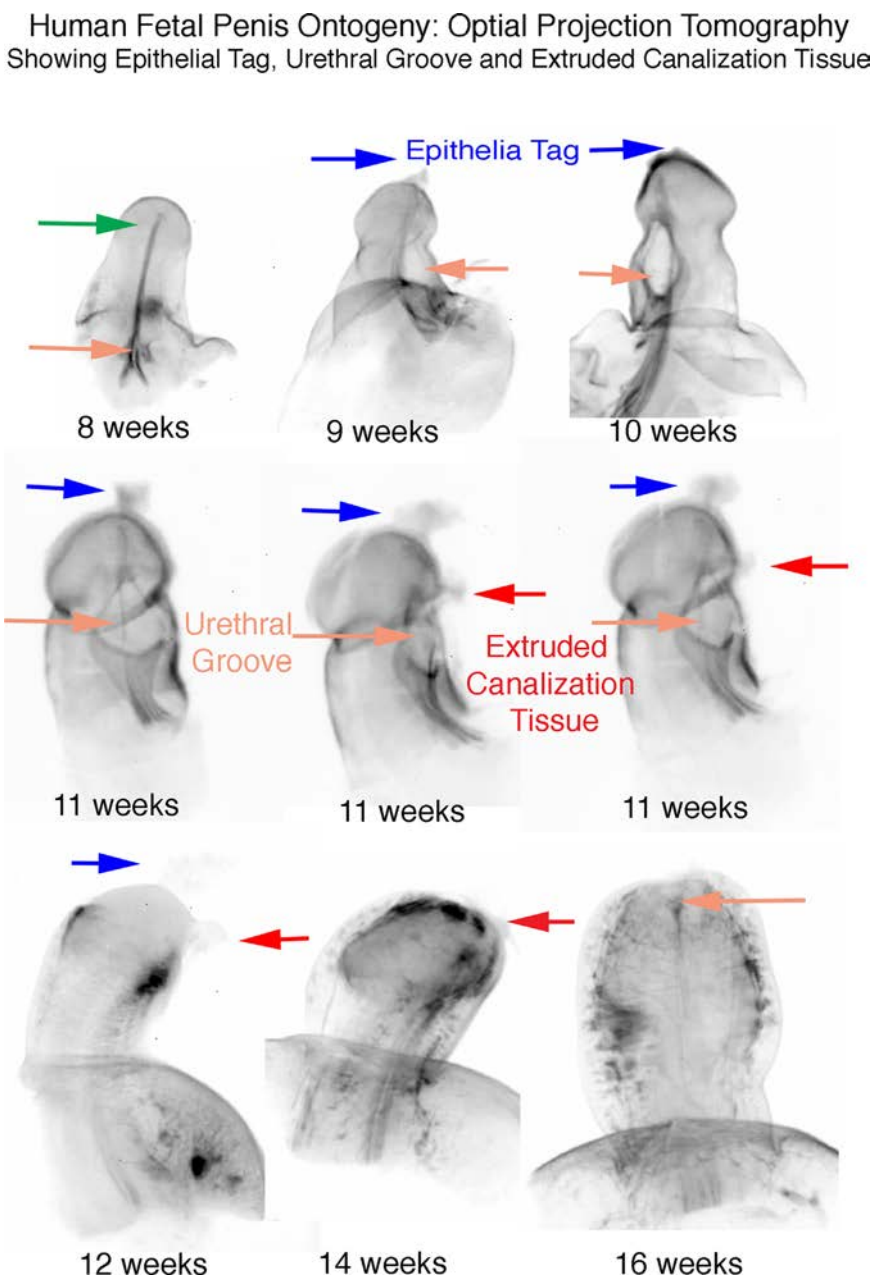

Fig. 13. Optical Projection Ontogeny of the human fetal penis from 8–16 weeks gestation. The epithelial tag is seen from 9–12 weeks gestation (blue arrows). Note the progression of the urethral meatus from the penoscrotal junction to the tip of the penis (orange arrows). Extruded glandular canalization tissue can be seen from 11–14 weeks gestation (red arrows). Note the urethral plate in the 8 week specimen extending almost to the tip of glans (green arrow).

is a marker of endodermal lineage cells (Diez-Roux et al., 2011; Robboy et al., 2017; Besnard et al., 2004) known to be expressed in human fetal bladder and urethral epithelium (Cunha et al., 2018b, 2018a; Robboy et al., 2017). Thus, taken together cytokeratin 7, uroplakin and FoxA1 are all proteins of epithelial cells of endodermal origin and are expressed in the urethral plate and developing urethra of the penile glans (Figs. 5, 7–11).

Cytokeratin 6 and 10 are frequently co-expressed in certain stratified epithelia. Cytokeratin 10 is a known marker of terminal differentiation in epidermis and is expressed in supra-basal layers but not the basal layer of epidermis (Moll et al., 2008, 1982). In contrast, cytokeratin 6 is expressed in the full epidermal thickness in both basal and supra-basal layers of epidermis. However, the major difference in the expression of cytokeratins 6 and 10 is that cytokeratin 6 is expressed from as early as 11 weeks in the solid urethral plate, while cytokeratin 10 is not expressed in the human urethral plate or the urethra (Fig. 6).

Our immunohistochemical data of the endodermal markers in the urethral plate and urethra (cytokeratin 7, uroplakin and FoxA1), in skin, (cytokeratins 6 & 10), as well as the androgen receptor in the developing human glanular urethra was observed from 13 to 17 weeks of gestation (Figs. 7–11), and thus covers the critical periods of penile urethral development. In the most proximal sections of the 13-week glans penis (see Figs. 7–11), there is clear evidence of endodermal expression markers cytokeratin 7, FoxA1 and uroplakin in the developing urethra. Cytokeratin 10 expression is absent in the urethra but present in the preputial lamina and epidermis. In contrast, cytokeratin 6 was expressed in the urethral plate (Fig. 7N) as well as the preputial lamina. The same pattern holds at 14–17 weeks of gestation. Canalization of the urethral plate within the glans penis initially occurs in the ventral portion of the solid urethral plate within the glans from 10 to 16 weeks gestation (Figs. 7–10). By 17 weeks the canalization of the urethral plate has extended dorsally (Fig. 11). This consistent ventral-dorsal

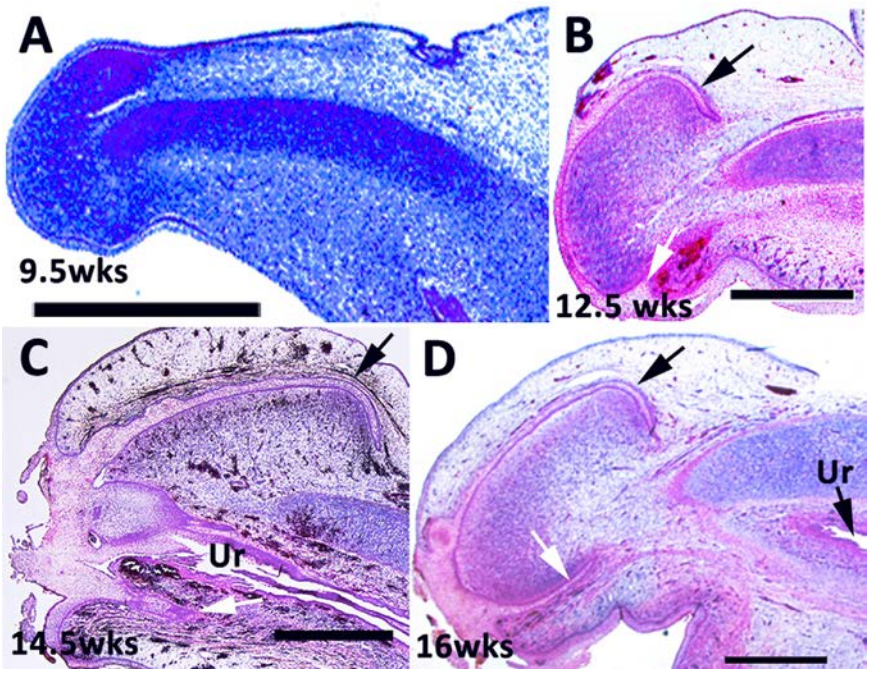

Fig. 14. Near sagittal sections of human fetal penis at the ages indicated. At 9.5 weeks (A) the glans penis can be recognized, but there is no evidence of preputial development. Sections B–D are lateral to the midline. At 12.5 weeks the preputial lamina is seen dorsally (black arrow), but is absent ventrally (white arrow). At 14 (C) and 16 weeks (D) the preputial lamina is extensive dorsally (black arrows) and reduced in size ventrally (white arrows).

canalization of the urethral plate within the glans is associated with differential protein expression, as cytokeratin 7, FoxA1 and uroplakin were detected only in epithelial cells bordering the forming urethral lumen and not dorsally in the solid urethral plate.

A requisite event in formation of the glanular urethra is separation of the forming urethra from its attachment to the ventral epidermis/prepuce, a process dependent upon the establishment of mesenchymal confluence ventral to the urethra. This process is surely androgen-dependent as the mesenchymal cells involved in this process are androgen receptor positive (Figs. 7–11). The process of mesenchymal confluence separates the K7, FoxA1- and uroplakin-expressing endodermal epithelial cells from the preputial lamina and epidermis. During this

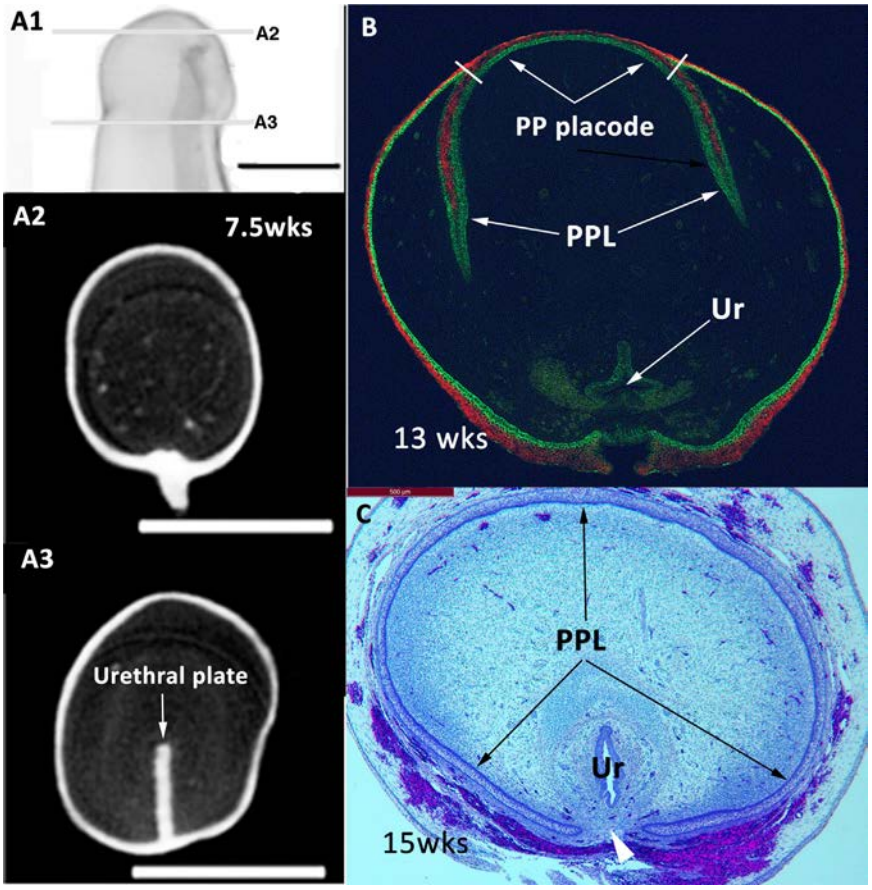

Fig. 15. (A1–A3) are images of a 7.5-week human genital tubercle. A1 is a surface rendering generated by optical transmission tomography. Lines (A2) and (A3) indicate where the optical projection tomographic transverse sections were taken. At this stage there is no evidence of preputial development. (B) is a transverse section through the glans penis at 13 weeks immunostained for cytokeratin 6 (green) and cytokeratin 10 (red). Note the preputial placode (PP) dorsally between the white lines and the preputial laminae (PPL). (C) is a transverse section through the glans penis at 15 weeks. Note that the preputial lamina (PPL) has grown ventrally leaving a gap of mesenchyme destined to form the frenulum (white arrowhead). Ur = urethra.

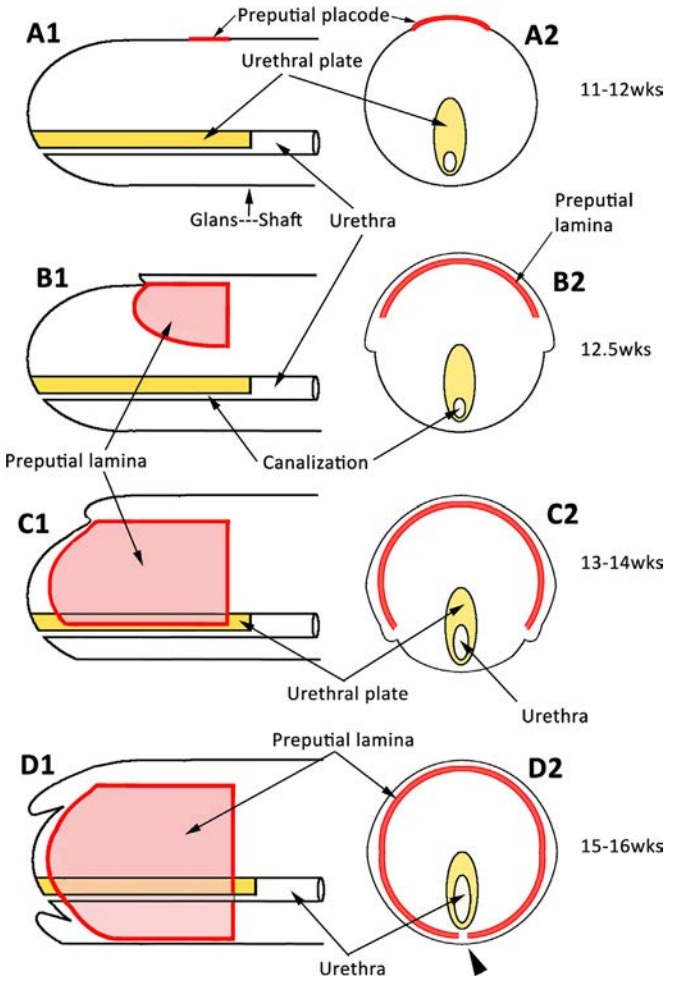

Fig. 16. Diagrammatic representation of human preputial development. Note the preputial placode in (A1 and A2). Separation of the preputial placode from the epidermis is seen in (B–D). Ventral expansion of the right and left preputial laminae is seen in (B–D). Note the ventral gap in the preputial lamina in (black arrowhead) (D2). Canalization of the urethral plate begins ventrally and extends dorsally (A2–D2).

process attenuated strands of K7, FoxA1 and uroplakin-expressing epithelial cells can be seen that eventually disappear and are presumably resorbed. Thus, the overall process required to establish a "stand alone" glanular urethra involves considerable epithelial and mesenchymal remodeling of tissues and extracellular matrix and likely to involve the action of matrix metalloproteinases.

A constant theme in penile development is the requisite action of androgens during development of the urethra within the penile shaft and the glans. Development of a urethra within the genital tubercle only occurs in males and not in females. Impaired androgen action in humans due to mutations in the androgen receptor or type 2 5α-reductase leads to varying degrees of feminization of the external genitalia (Wilson, 2001). Female patients with congenital adrenal hyperplasia (and thus in utero production of androgens) exhibit varying degrees of virilization of the external genitalia, which in the most severe cases can result in development of normal penile morphology (Speiser et al., 2010). The role of androgens in penile urethral development is well established in both humans and animal models. Consistent with this concept is the localization of androgen receptors in the developing human penile shaft and glans. The fusing edges of urethral fold epithelium (closing zipper) is androgen receptor positive (Baskin et al., 2018). The process of mesenchymal confluence within the penile shaft is associated with the strategic localization of androgen receptors in mesenchyme fusing in the midline ventral to the urethra. A comparable process occurs during formation of the urethra within the glans with a virtually identical pattern of androgen receptor expression in the mesenchyme (Figs. 7–11). Thus, androgen receptors are expressed in epithelial and mesenchymal cells precisely in regions of profound epithelial and mesenchymal remodeling. The molecular events associated with tissue and extracellular remodeling down stream of androgen action are yet to be revealed.

The fundament difference between development of the human urethra within the shaft versus the glans is complete canalization of the urethral plate to form an open urethral groove in the shaft versus direct canalization of the urethral plate without formation of an open urethral groove in the glans. We described narrow channels between epithelial cells the forming urethra and the exterior in the most distal sections of the glans (Figs. 7–10). These extremely narrow channels approximately 1–2 cell diameters in width are in no way comparable to the wide diamond-shaped urethral groove seen within the forming penile shaft (Fig. 1A and 2G) and represent transitory openings presumably conveying urine to the exterior.

Five processes act in synchrony for successful completion of the glanular urethra: 1) Extension of the urethral plate distally to the tip of the glans to meet surface ectodermal epithelium. 2) Canalization of the glanular urethral plate (ventral initially followed by dorsal canalization) to form the urethral lumen. 3) Lateral to lateral mesenchymal fusion (mesenchymal confluence) to form the tubular glanular urethra. 4) Reabsorption of pinched off endodermally derived cells ventral to the mesenchymal confluence 5) Remodeling of endodermally derived epithelial channels in the distal glans from mesenchymal confluence to form the distal glanular urethra. The molecular mechanisms underlying each of these processes are yet to be revealed with the downstream mediators of androgen action being of prime importance.

8. Development of the prepuce

Development of the prepuce is initiated by ~12 weeks with the appearance of a novel structure, the preputial placode, which is a dorsal thickening of the epidermis on the dorsal aspect of the developing glans penis. From the lateral aspect of the preputial placode the bilateral preputial laminae expand ventrally until the preputial folds (foreskin) covers all of the glans, fusing in the ventral midline at ~16 weeks gestation. An important early process in development of the prepuce is delamination of the preputial placode which results in dorsal mesenchymal confluence between the preputial lamina and the overlying epidermis, yet another remodeling event. We look forward to the examination of additional closely timed specimens to more fully describe the initial appearance of the preputial placode and its separation from the epidermis to establish a preputial lamina deep to the epidermis (see Figs. 15–16). Subsequent ventral expansion of the right and left edges of the preputial lamina almost completely covers the glans. However, the right and left ventral edges of the preputial lamina do not fuse in the ventral midline and remain separated by a thin layer of mesenchyme, which forms the frenulum of the prepuce. The fully formed preputial lamina is an epithelial structure (undoubtedly of ectodermal origin), which has a basement membrane on its inner and outer faces. At birth the solid preputial lamina is intact and thus "physiologically adherent" to the glans. Thereafter, the preputial lamina will canalize creating the preputial space that "houses" the glans. In this way the prepuce eventually develops an outer epidermal surface and an inner surface lined by a stratified epithelium. Preputial development is in some way linked to urethral development within the glans since in hypospadias, preputial tissue is absent ventrally, and excessive dorsally (classic dorsal hooded foreskin) (Baskin, 2017).

The most common location of hypospadias is at the junction of the glans and coronal margin defined as the junction of glans with the penile shaft (Baskin, 2017). This position corresponds to interface between the two disparate mechanisms of urethral development (urethral

fold fusion events in the shaft and direct urethral plate canalization in the glans). Clearly, the tubular elements generated by these two disparate morphogenetic mechanisms must join at or near the glans/shaft interface, which presumably accounts for the high incidence of hypospadias at this location. In contrast, the less prevalent but more severe forms of hypospadias occur in the penile shaft where disruption of urethral fold fusion occurs. In patients with hypospadias often a urethral pit (or secondary epithelial lined channel) is present within the glans. This extra channel is consistent with a disruption of ventral mesenchymal confluence and cellular tissue remodeling during normal glanular urethra development. It is also possible that an abnormality in ventral mesenchymal confluence with normal formation of the prepuce could explain the relatively rare variation of hypospadias, megameatus intact prepuce (Baskin, 2017). In this unusual form of hypospadias it may be that an isolated defect in mesenchymal bridge formation with normal glanular canalization and prepuce formation leads to the large caliber urethra.

9. Conclusion

The human male urethral forms within the glans penis between 12 and 18 weeks gestation by a unique mechanism distinctly different from urethral development within the penile shaft. The process involves a sequential canalization of the urethral plate followed by mesenchymal confluence and epithelial remodeling to form a “stand alone” tubular urethra. The presence of androgen receptors in both morphogenetic processes in the shaft and the glans is an important mechanistic theme requiring further investigation during normal penile development and in the etiology of hypospadias.

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