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Hot spots in fetal human penile and clitoral development☆

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ABSTRACT

To better understand how the human fetal penis and clitoris grows and remodels, we undertook an investigation to define active areas of cellular proliferation and programmed cell death spatially and temporally during development of human fetal external genitalia from the indifferent stage (8 weeks) to 18 weeks of gestation. Fifty normal human fetal penile and clitoral specimens were examined using macroscopic imaging, scanning electron microscopy and immunohistochemical localization for the cellular proliferation and apoptotic markers, Ki67 and Caspase-3, respectively. A number of hot spots of cellular proliferation characterized by Ki67 localization are present in the penis and clitoris especially early in development, most notably in the corporal body, glans, remodeling glanular urethra, the urethral plate, the roof of the urethral groove and the fully formed penile urethra. The 12-fold increase in penile length over 10 weeks of growth from 8 to 18 weeks of gestation based on Ki67 labelling appears to be driven by cellular proliferation in the corporal body and glans. Throughout all ages in both the developing penis and clitoris Ki67 labeling was consistently elevated in the ventral epidermis and ventral mesenchyme relative to the dorsal counterparts. This finding is consistent with the intense morphogenetic activity/remodeling in the ventral half of the genital tubercle in both sexes involving formation of the urethral/vestibular plates, canalization of the urethral/vestibular plates and fusion of the urethral folds to form the penile urethra. Areas of reduced or absent Ki67 staining include the urethral fold epithelium that fuses to form the penile tubular urethra. In contrast, the urethral fold mesenchyme is positive for Ki67. Apoptosis was rarely noted in the developing penis and clitoris; the only area of minimal Caspase-3 localization was in the epithelium of the ventral epithelial glanular channel remodeling.

1. Introduction

Indifferent stage human male and female fetal external genitalia consist of indistinguishable genital tubercles at 8–9 weeks of gestation. Ambisexual genital tubercles subsequently differentiates into a penis under the influence of androgens (Li et al., 2015; Baskin et al., 2018; Shen et al., 2018) or a clitoris in the absence of androgens (Baskin et al., 1999; Overland et al., 2016). The human fetal penis becomes fully formed by 18 weeks of gestation with a tubular urethra that exits on the terminal aspect of the glans and formation of a symmetric circumferential prepuce (Shen et al., 2016; Baskin et al., 2018; Liu et al., 2018). The human fetal penis increases 12-fold in length from ~0.5 mm to 6 mm from 8 to 18 weeks of gestation (Shen et al., 2018). This fetal time period from ~8 to 18 weeks of gestation corresponds to production of the testosterone from the fetal testis and increased levels of serum testosterone in the male fetus (150–400 ng/dl) (Wartenberg, 1989).

In females, the urethra does not form within the clitoral body or glans. Unlike the male, the female prepuce does not fully cover the glans clitoris, but instead the prepuce of the clitoris is located dorsal and lateral to the glans (Overland et al., 2016) (Clemente, 1985). The clitoris also remains closer to the body wall unlike the penis which protrudes at nearly a right angle from the body wall. The size of the clitoris increases substantially over this early gestational time period (8–16 weeks), presumably reflecting androgen-independent growth (Cunha et al., 2019) since the fetal testis and hence testosterone are not present. Precise measurements of clitoral length are difficult to make, as the proximal end of the clitoris is difficult to define as compared to the distinct penoscrotal junction in males (Baskin et al., 2018).

In newborn males flaccid penile length is ~3 cm implying that between 22 weeks of gestation (when the penis is close to 1 cm) and birth the penis grows an additional 2 cm (Shen et al., 2018). After birth penile size increases to ~4 cm by 6 months of age presumable do to the documented postnatal rise in testosterone at 3 months of age (so called mini puberty) (Boas et al., 2006; Wylie and Eardley, 2007; Wang et al., 2018). The size of the penis in humans from one year of age until puberty changes very little growing ~1 cm after 6 months of age to a mean length of 5 cm at 11–12 years before the onset of puberty, presumably an androgen-independent event since serum testosterone is undetectable during this time period (Wang et al., 2018). This is in contrast to an overall increase in body length (~2 inches per year) and weight (~6.5 pounds per

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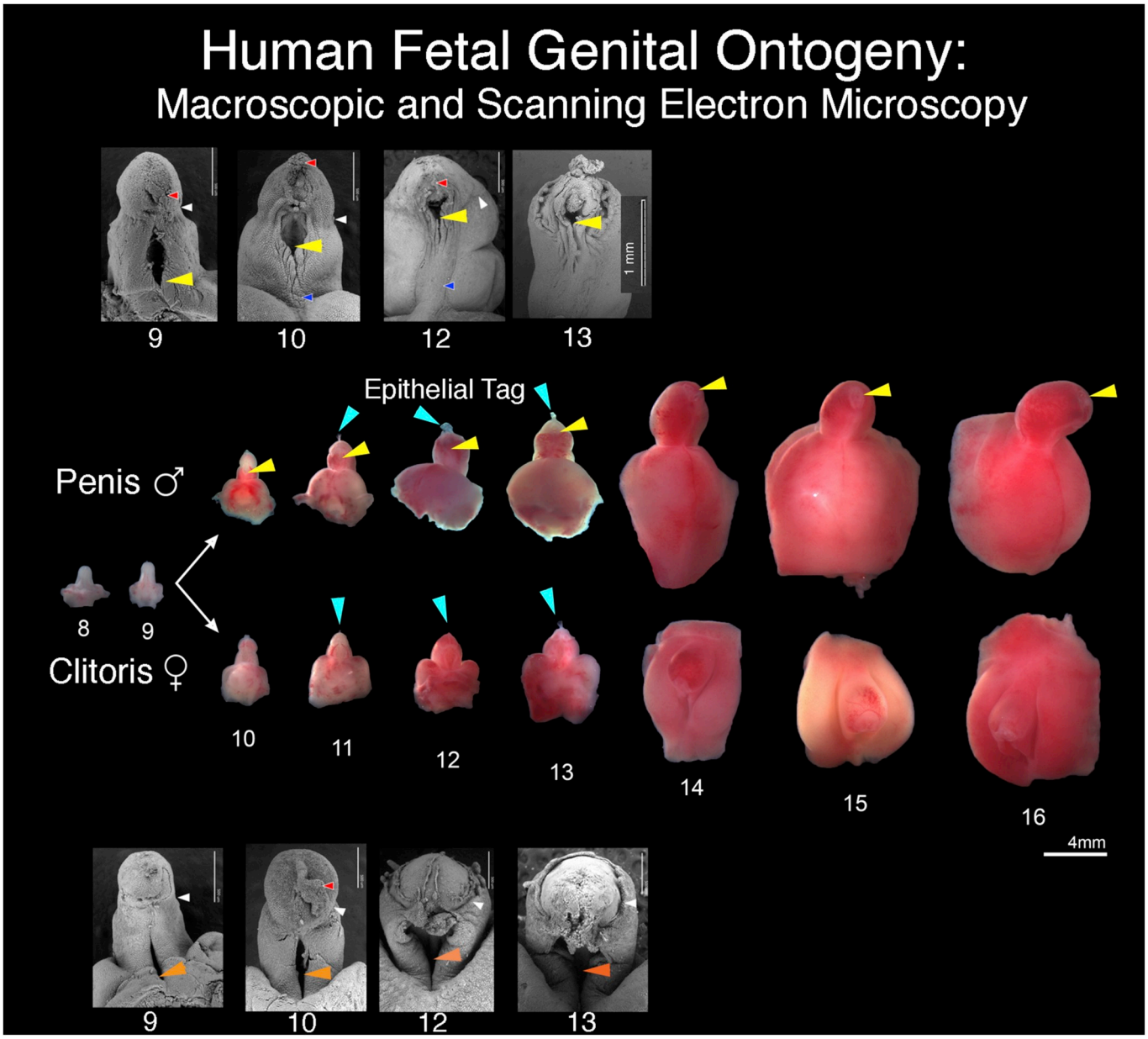

Fig. 1. Human Fetal Genital Ontogeny: Macroscopic (middle rows) and Scanning Electron Microscopy(SEM) (top and bottom row) of the developing human fetal penis from 8 to 16 weeks of gestation (top two rows) and of the developing human clitoris from 8 to 16 weeks of gestation (bottom two rows). Note the indifferent stage at 8 weeks of gestation. The light blue arrowheads indicate the epithelial tag (small red arrowheads in the SEM images) and the yellow arrowheads indicate the advancing urethral meatus in males. Note, the progressive fusion of the urethral folds in the penile specimens to form the male urethra (top rows, yellow arrowheads) in contrast to the wide open vestibular groove in females (bottom row, orange arrowheads). The small white arrowheads in the SEM images denote the junction between the glans the penile/clitoral shaft and the small blue arrowheads the median raphe. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

year) in boys (and girls) which also occurs in the absence of androgens. At puberty penile growth is marked in humans (mean length ~9 cm at 16–18 years) coinciding with a rise in testosterone level and an increase in the size of the testes (Wang et al., 2018). Thus, phallic growth in human is androgen-dependent during early fetal development (Jost, 1972; Baskin et al., 2018), just after birth at the time of mini puberty (~ 3–6 months of age) and during puberty, but is androgen independent from ~20 weeks of gestation until mini puberty at ~3 months of age and from ~6 months of age until puberty (Wartenberg, 1989; Wang et al., 2018; Cunha et al., 2019).

To better understand how the fetal human penis and clitoris grows, we undertook a study to define areas of cellular proliferation spatially and temporally during fetal development of external genitalia from the indifferent stage (8 weeks) to 18 weeks of gestation when the penile urethra is completely formed. Cellular proliferation was determined by immunohistochemical expression of Ki67, a nuclear protein associated with ribosomal RNA transcription (De Souza and Osmani, 2007). Ki67 protein is present and detectable during all active phases of the cell cycle (G_1 phase = post mitotic gap phase, S phase = DNA replication, G_2 = growth and mitotic phase = chromosomal separation) but absent in resting or quiescent cells (G_o phase) (Nigg, 1995). Cellular content of Ki-67 protein markedly increases during cell progression through S phase of the cell cycle (Lilly and Duronio, 2005). We also investigated programmed cell death using caspase-3 as a marker for apoptosis (McIlwain et al., 2013; Li et al., 2015; Lossi et al., 2018). Herein we define the hot spots of cellular proliferation based on Ki67 to better define areas of human fetal penile and clitoral growth and development.

2. Materials and methods

Human fetal external genitalia from the first and early second trimester were collected without patient identifiers after elective termination of pregnancy with approval from the Committee on Human Research at UCSF (IRB#12–08813). Fetal age reported from time of fertilization and not from last menstrual period was estimated using heel-toe length (Drey et al., 2005). Chromosomal sex was determined using PCR to detect Y-chromosomal sequences as previously described (Li et al., 2015) and, when possible, was confirmed by identification of Wolffian and Müllerian duct morphology using a dissecting microscope. Eight to 18-week human fetal male and female specimens (N = 35) were photographed and processed for immunohistochemical staining and scanning electron microscopy (N = 15) as

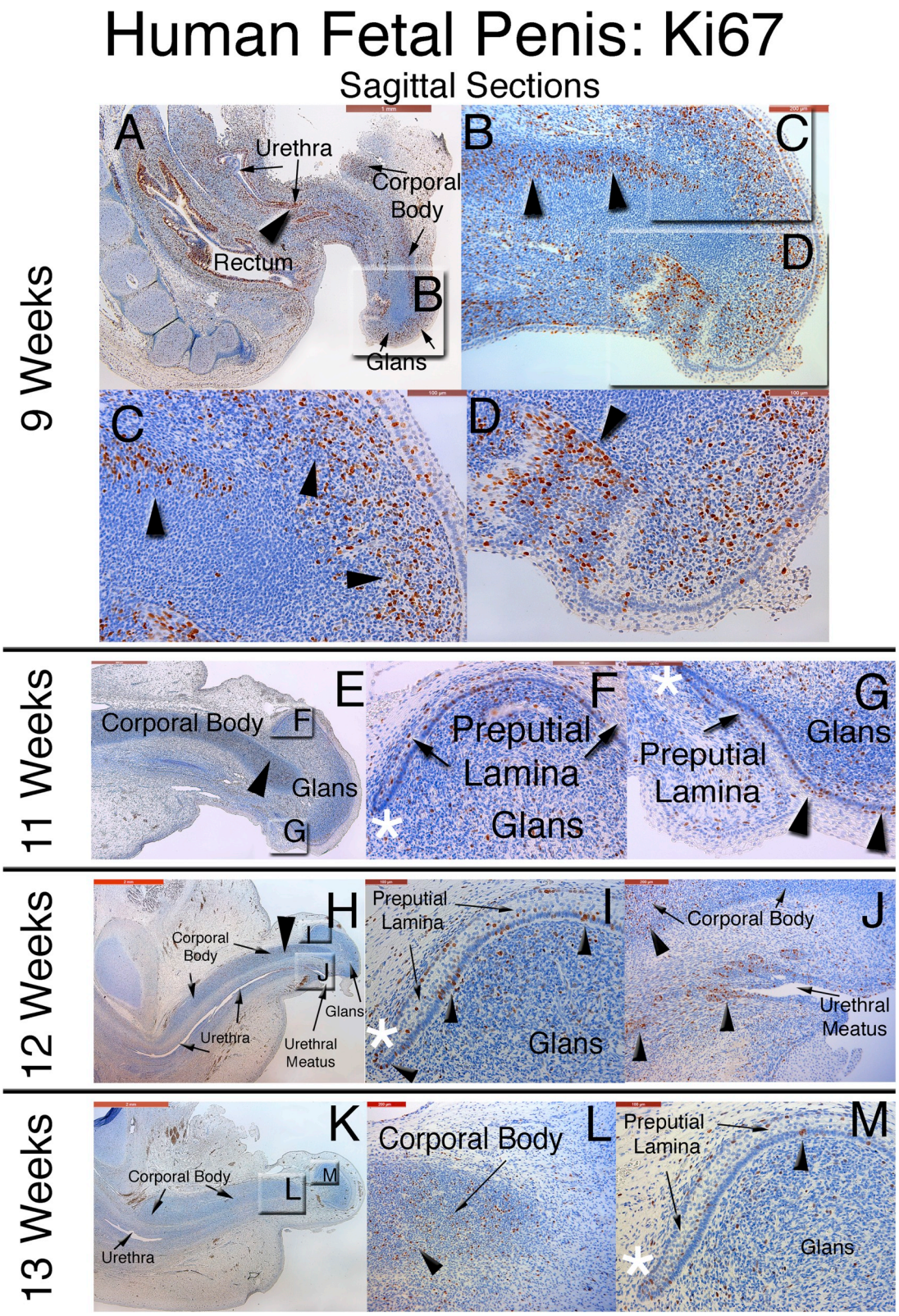

Fig. 2. Human Fetal Penis immunostained for the proliferation marker Ki67 (Sagittal sections): 9 weeks of gestation A-D, 11 weeks of gestation E-G, 12 weeks of gestation H-J and 13 weeks of gestation K-M. Areas of increased Ki67 staining are labeled with a black arrowheads. At 9 weeks of gestation, note five areas of increased Ki67 staining: the urethra (1), rectum (2), the corporal body (3), the glans (4) and the urethral plate (5). At 12 weeks of gestation prominent Ki67 staining is seen in the remodeling glanular urethra near the meatus (J). At 13 weeks of gestation Ki67 staining remains in the corporal body, glans and preputial lamina (M) but is less prominent. white asterisk = proximal edge of the preputial lamina.

previously described (Li et al., 2015; Shen et al., 2016; Shen et al., 2018).

The specimens were serially sectioned sagittally and/or transversely at 7 μm. Every 20th section was stained with hematoxylin and eosin to assess morphology. Intervening sections were processed for immunohistochemical analysis 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.

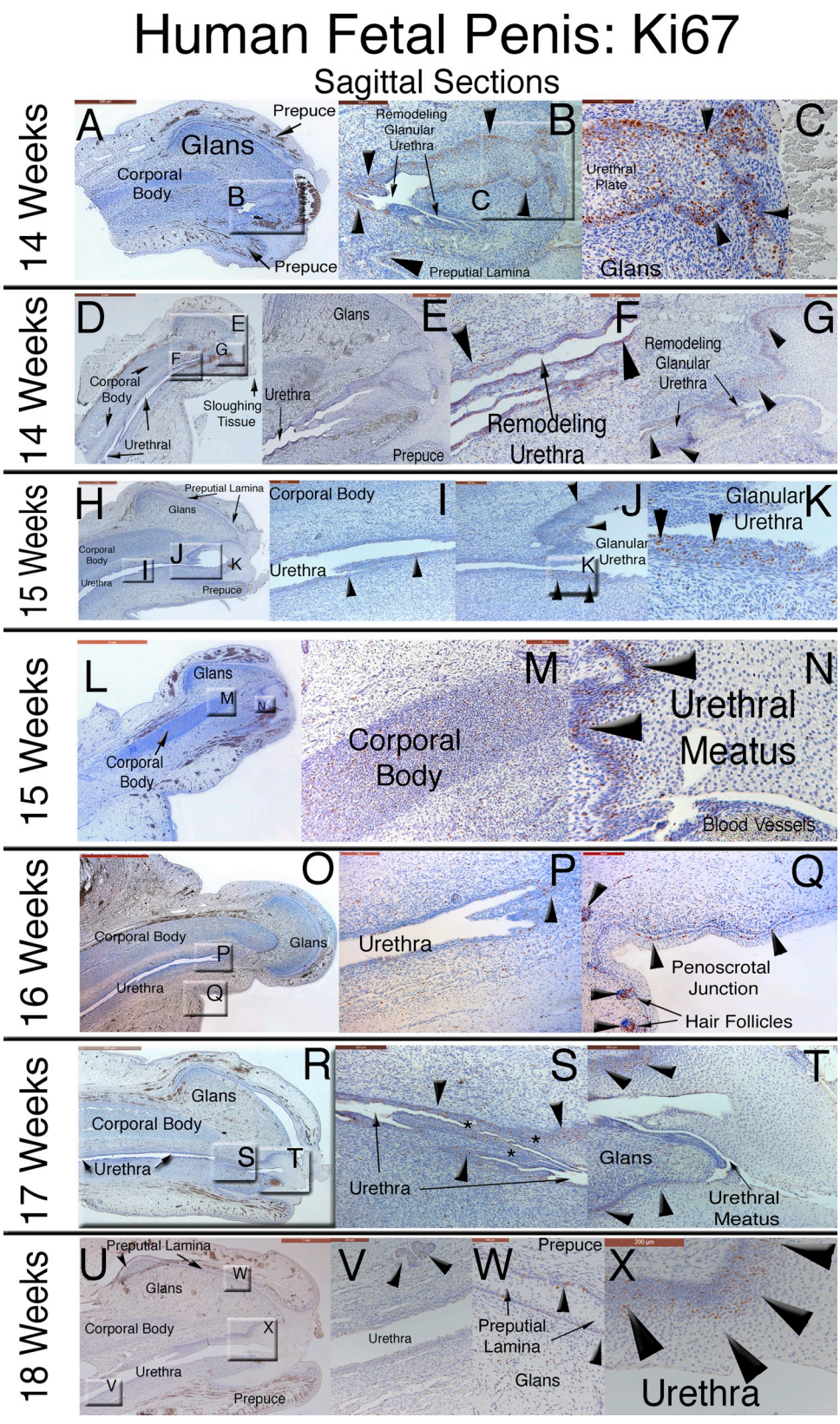

Fig. 3. Human Fetal Penis immunostained for the proliferation marker Ki67 (Sagittal sections): 14 weeks of gestation A-C, 14 weeks of gestation D-G (different specimen), 15 weeks of gestation H–K, 15 weeks of gestation L-N (different specimen), 16 weeks of gestation O-Q, 17 weeks of gestation R-T and 18 weeks of gestation U-X. Areas of prominent Ki67 staining are labeled with black arrowheads. Note in the 14–18 week specimens localization of Ki67 staining in the remodeling glanular urethra (B, C, F, G, I, J, K, N, P, S, T and X) that is especially prominent in the 14 week specimens (B, C, F, G). The corporal body continued to express Ki67 (M) but to a lesser extent than the younger fetal specimens (9–11 weeks (Fig. 2)). The glans (T and X) and preputial lamina (W) also had less Ki67 staining compared to the younger specimens (Fig. 2). Note the formation of hair follicles (Q) (black arrowheads) at 16 weeks of gestation with intense Ki67 staining at the penoscrotal junction.

3. Results

Development of male and female external genitalia diverges after the indifferent stage (8–9 weeks of gestation) with clear morphologic differences seen in whole-mounts and scanning electron micrographs at 10 weeks of gestation (Fig. 1). In males the urethra forms within the penile shaft via canalization of the urethral plate to form an open urethral groove and subsequent fusion of the urethral folds. The urethra within the penile glans forms via direct canalization of the urethral plate with the meatus reaching the terminal aspect of the ventral glans along with formation of a circumferential prepuce (Fig. 1, see second row with yellow arrowhead depicting the advancing urethral meatus) (Shen et al., 2016; Liu et al., 2018). In contrast, in the clitoris the vestibular plate undergoes canalization, without fusion of the vestibular folds, which remain as the labia minora (Overland et al., 2016). During the course of development, the clitoris remains closer to the body wall in

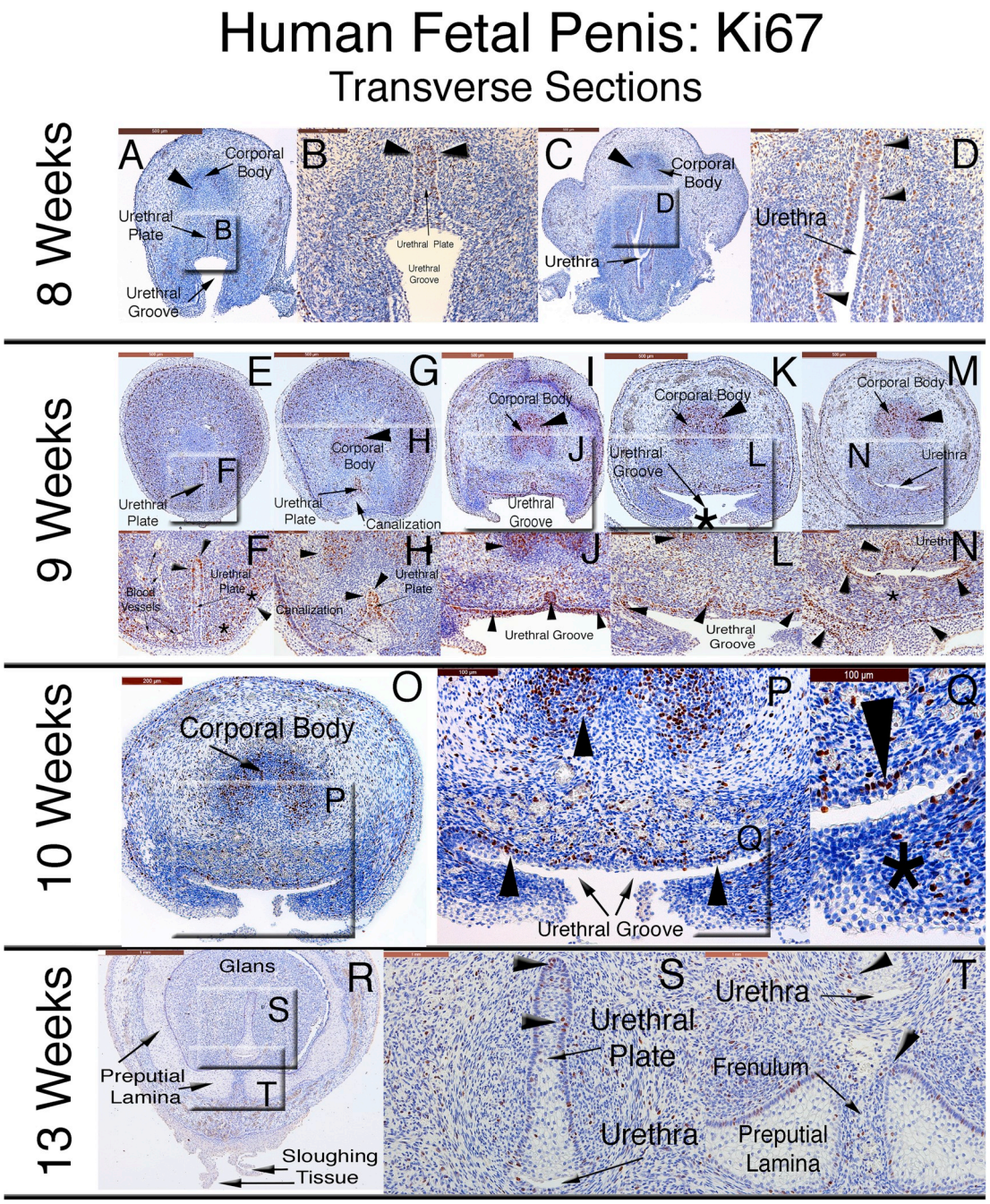

Fig. 4. Human Fetal Penis immunostained for the proliferation marker Ki67 (Transverse sections): 8 weeks of gestation A-D, 9 weeks of gestation E-N, 10 weeks of gestation O- Q and 13 weeks of gestation R-T. Note sections from left to right are oriented from the distal to proximal aspect of the penis. Areas of increased Ki67 staining are labeled with black arrowheads. Note, at the indifferent stage of 8 weeks of gestation Ki67 staining is prominent in the urethral plate (B) and proximally in the urethra (D). Ki67 is also present in the developing corporal bodies (A and C). At 9 weeks of gestation note the prominent expression of Ki67 in the corporal bodies (G–M) (see Fig. 2 for sagittal sections), in the urethral plate (F and H), in the urethral groove (J and L) and urethra (N). The asterisk in K is in the middle of the two epithelial edges that will subsequently fuse to form the penile urethra.

contrast to the penis which extends outward at an approximately 90-degree angle (Fig. 1, compare 2nd to 3rd rows). Note the subsequent ten-fold increase in penile length (and clitoral length) from 8 to 16 weeks of gestation during the first and second trimesters (twelve-fold from 8 to 18 weeks) (Fig. 1) (Shen et al., 2018).

3.1. Ki67 in the developing penis and clitoris

3.1.1. Ki67 labeling: dorsal versus ventral

In both the developing penis and clitoris Ki67 labeling was consistently elevated in the ventral epidermis and ventral mesenchyme (Figs. 2–10) relative to the dorsal counterparts. This finding is consistent with the intense morphogenetic activity/morphogenetic remodeling in the ventral half of these developing organs. Morphogenetic activity in the ventral half of the genital tubercle of males and females involves formation of the urethral/vestibular plates, canalization of the urethral/vestibular plates and fusion of the urethral folds to form the penile urethra in males.

3.2. Ki67 in the developing penis

3.2.1. Ki67 labeling in the urethral plate, urethral groove and tubular urethra within the penile shaft

Ki67 staining was detected from 8 to 18 weeks of gestation in the developing human fetal penis (Figs. 2–5) (Table 1). At 8 weeks the urethral plate within the penile shaft is partially canalized with the presence of the urethral groove as well as a solid (uncanalized) urethral plate extending from the roof of the urethral groove dorsally into penile mesenchyme (Fig. 4A–B, G, I, K). The dorsal aspect of the urethral plate exhibits substantial Ki67 labeling versus the ventral aspect of the urethral plate (Fig. 4A and B) at 8–9 weeks of gestation. The residual dorsal tip of the urethral plate, when present, exhibited a consistently high intensity of Ki67 labeling

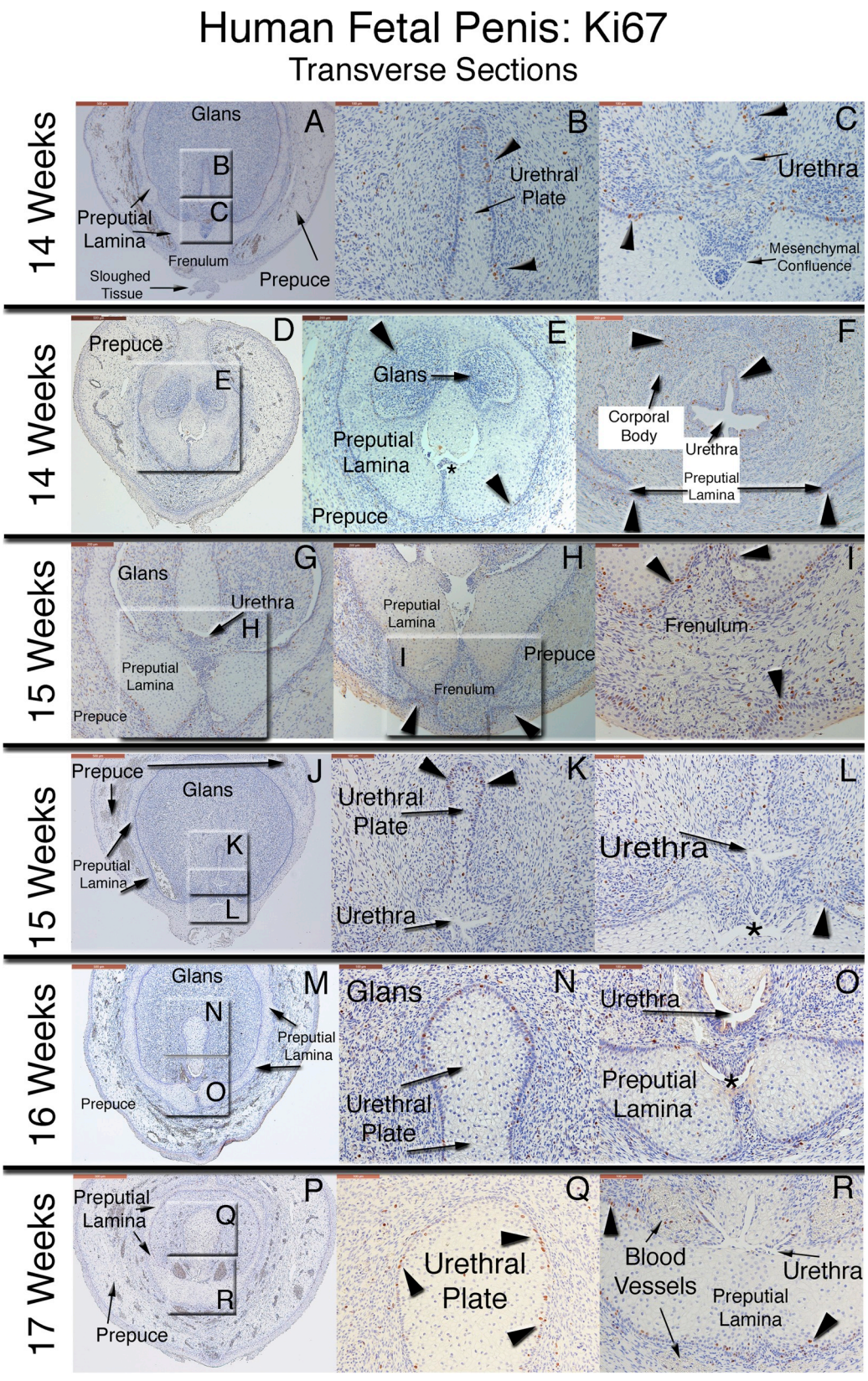

Fig. 5. Human Fetal Penis Glandular area immunostained for the proliferation marker Ki67 (Transverse sections): 14 weeks of gestation A-C, 14 weeks of gestation D-F (different specimen), 15 weeks of gestation G-I, 15 weeks of gestation J-L (different specimen), 16 weeks of gestation M-O and 17 weeks of gestation P-R. Note sections from left to right are oriented from the distal to proximal aspect of the penis. Note these sections all depict the formed or forming glanular urethra (except F which is more proximal in the distal penile shaft based on the presence of the corporal body). Note the localization of Ki67 staining to the dorsal aspect of the urethral plate (B, K, N and Q). Also note the increased expression Ki67 in the area of frenular formation (I).

versus the ventral aspect of the urethral plate as well as the epithelium of the roof of the urethral groove (Fig. 4A-N). By 10 weeks of gestation, the residual dorsal tip of the urethral plate has been incorporated into the wide urethral groove (Fig. 4J, L, O, P). At 9 weeks of gestation, the epithelium of the roof of the urethral groove is intensely Ki67-positive (Fig. 4J and L) with a distinct reduction in Ki67 labeling of the epithelial roof of the urethral groove at 10 weeks of gestation (Fig. 4O–Q). The

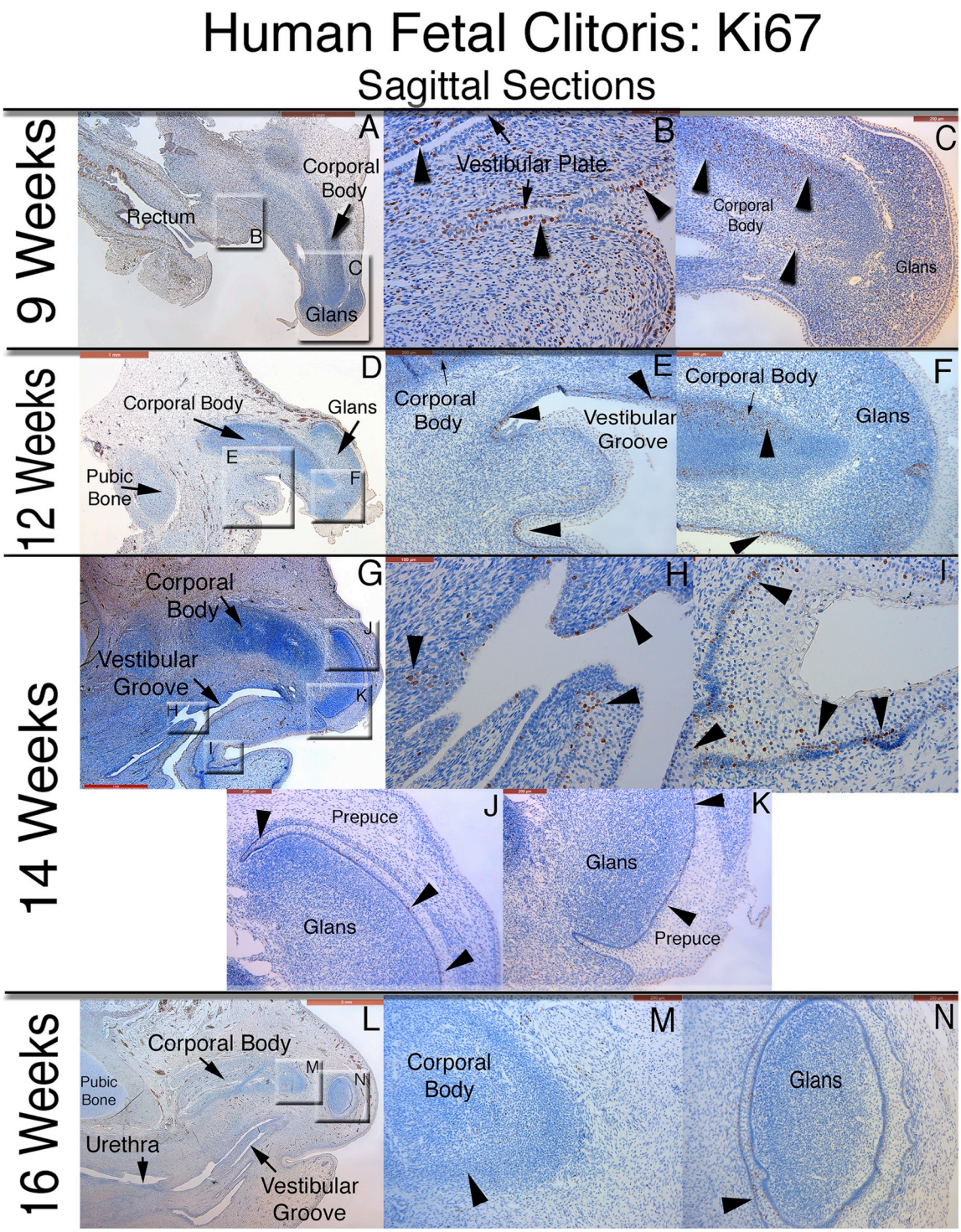

Fig. 6. Human Fetal Clitoris immunostained for the proliferation marker Ki67 (Sagittal sections): 9 weeks of gestation A-C, 12 weeks of gestation D-F, 14 weeks of gestation G-K and 16 weeks of gestation L-M. Areas of increased Ki67 staining are labeled with a black arrowheads. Note localization of Ki67 in the corporal body (C, E and M) and glans (C, F, J, K and N) although less intense compared to the fetal penis (Figs. 2 and 3). In the area of the vestibular plate analogous to the urethral plate and groove (Figs. 2–5) note the increased Ki67 localization (B, E, H and J). At the site of the female urethral meatus Ki67 staining was especially prominent compared to the surrounding area (H).

epithelium of the fully formed tubular urethra consistently exhibited prominent Ki67 staining from 8 to 14 weeks of gestation (Fig. 2A, H, 3D, I, 4D) with slightly less Ki67 urethral epithelial labeling after 15 weeks of gestation (3P, 3S, 3W). Notably, Ki67 staining was absent/reduced in the penile epidermis at all stages and reduced/absent at the edges of the urethral folds at 9–10 weeks of gestation (Fig. 4I–J and O-Q). Mesenchyme of the urethral folds (Fig. 4Q, asterisk) was modestly Ki67-positive.

3.2.2. Ki67 labeling in the urethral plate, urethral groove and tubular urethra within the penile glans

The solid urethral plate within the glans penis canalizes directly to form the urethral lumen and undergoes considerable remodeling to generate the glanular urethra and the urethral meatus. Fortuitous sagittal sections through the urethral plate within the glans illustrates intense Ki67 staining (Figs. 2B and D, 3A-C). (At 13–18 weeks of gestation the solid urethral plate is only present within the glans in close anatomic association with the forming preputial lamina (Figs. 4R–T, 5B, K, N, Q)). The ventral aspect of the urethral plate canalizes to form the glanular urethra, and in older specimens the canalization extends into the dorsal portion of the glanular urethral plate (Fig. 5F (Liu et al., 2018)). At all ages examined (13–18 weeks), Ki67 labeling was consistently higher in the dorsal versus the ventral aspect of the glanular urethral plate (Figs. 4R–T, 5A-C, K-L, M-O, Q-R). The urethral plate within

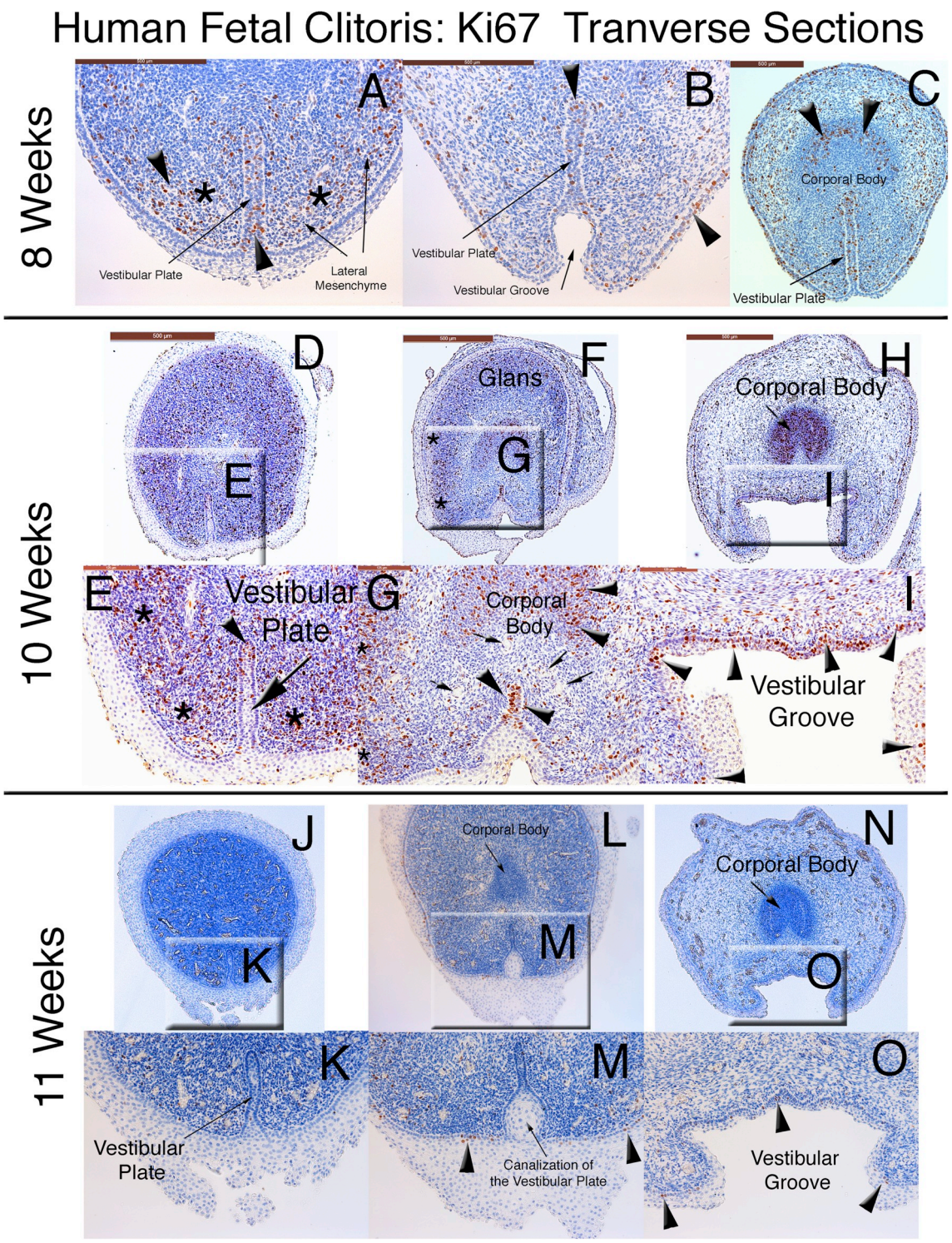

Fig. 7. Human Fetal Clitoris immunostained for the proliferation marker Ki67 (Transverse sections): 8 weeks of gestation A-C, 10 weeks of gestation D-I and 11 weeks of gestation J-O. Areas of increased Ki67 staining are labeled with black arrowheads. Note prominent staining of the corporal body (C, H, G and N) and vestibular plate (A, B E, and G) and groove (I and) that becomes less intense after 10 weeks of gestation. Note the asterisk (A,E and G) at the site of increased localization of Ki67 in the lateral mesenchyme, to the corporal body (H), the vestibular plate (B and F) and the vestibular groove (H).

the glans undergoes considerable remodeling to form the glanular urethra, and during this process prominent epithelial Ki67 was strongly present in the remodeling glanular urethral epithelium from 12 to 16 weeks of gestation (Fig. 3B, C, F, G, I, J, K, N, P, S, T, X). Especially note the 14-week specimen with prominent Ki67 staining in the remodeling epithelium of the glanular urethra (3C, 3F, 3G).

3.2.3. Ki67 labeling within the epidermis of the penile glans and the developing preputial lamina

The development of the preputial lamina and prepuce are described in detail in an accompanying paper (Cunha et al., 2019), and the role of proliferation in this process will not be presented in detail herein. The glanular epidermis of the developing penis is modestly Ki67-positive from 8 to 11 weeks of gestation (Figs. 2B, C, G, 4E) after which Ki67 staining was sparsely present in the epidermis (note that at 11–12 weeks of gestation prepuce formation begins with the prepuce starting to cover the glans penis) (Figs. 2H, K, 3A, D, H, L, O, R, U). During human male preputial development, the thick epidermis of the glans delaminates from proximal to distal. During this process the forming preputial lamina is attached to glanular epidermis distally, but has a “free edge” proximally (Cunha et al., 2019). At the time of

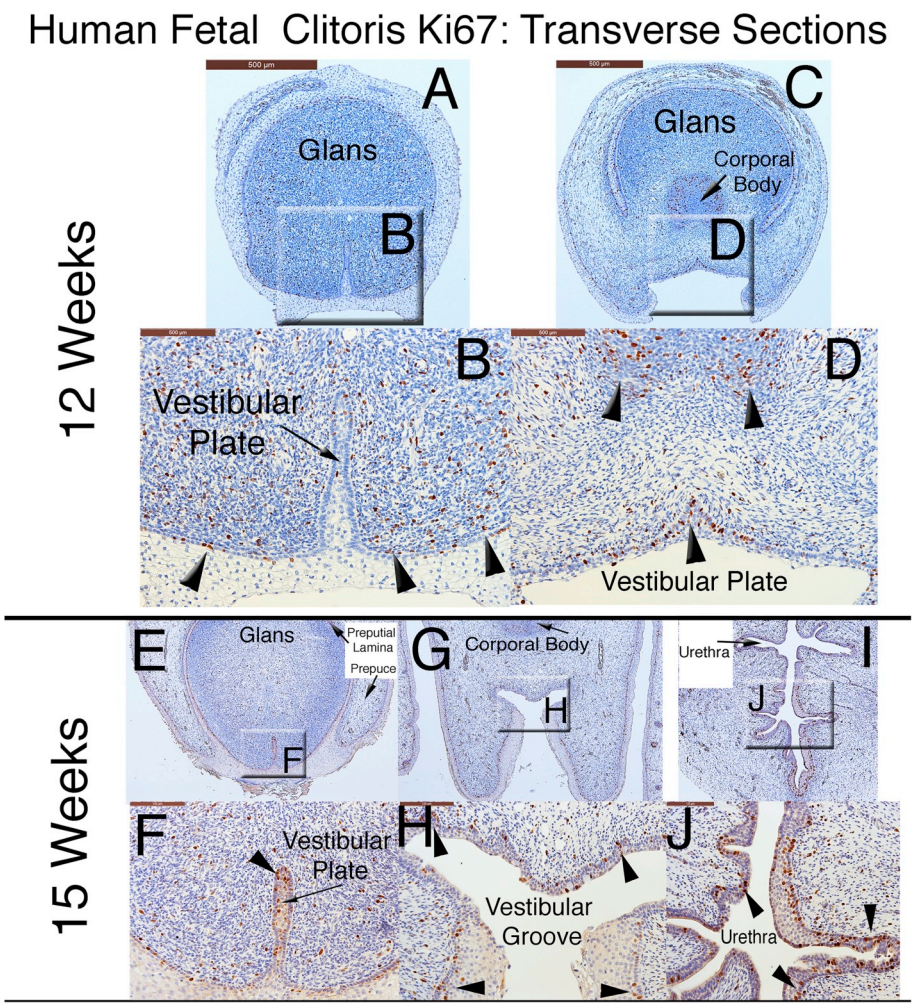

Fig. 8. Human Fetal Clitoris immunostained for the proliferation marker Ki67 (Transverse sections): 12 weeks of gestation A-D, and 15 weeks of gestation E-J. Areas of increased Ki67 staining are labeled with a black arrowhead. Note localization of Ki67 to the corporal body (D) and vestibular plate (B and F) and groove (D, H). Ki67 localization is well seen in the basal layer of the proximal urethra (J).

formation of the preputial lamina at 11–12 weeks of gestation Ki67 staining was sparsely but consistently present, particularly at its proximal edge where a subset of epithelial cells are androgen receptor positive (Cunha et al., 2019) (Fig. 2F, G, I, M). The preputial mesenchyme itself was mostly devoid of Ki67 staining (Figs. 2K, 3H, R, U). Also note the increased expression Ki67 in the area of frenular formation (Fig. 5I).

3.2.4. Ki67 labeling in the within the developing corporal body and in glanular mesenchyme

Penile corporal bodies can be recognized as early as 8 weeks of gestation as prominent mesenchymal condensations, which is prior to production of testosterone by the testes (Cunha et al., 2019). Penile corporal bodies exhibit strong expression of Ki67 from 8 to 12 weeks of gestation (Fig. 2A, E, H), but thereafter (13–18 weeks) Ki67 staining was present but much reduced (Figs. 2K, 3A, D, H, L, O, R, U). Subepidermal mesenchyme of the glans penis was strongly Ki67-positive at 8 and 9 weeks (Figs. 2C and 4E), and reduced at 10 weeks of gestation and at later stages (2F, 2I, 2M, 3C, 3T, 4R, 5J). At 15 weeks of gestation when the penoscrotal junction was first noted, Ki67 staining was prominent in forming hair follicles (Fig. 3Q).

3.2.5. Ki67 in the vestibular plate, vestibular groove of the developing clitoris

Ki67 staining was detected from 8 to 16 weeks of gestation in the developing human fetal clitoris (Figs. 6–8). Ki67 staining in the clitoris was globally less intense than that of the penis (Table 1). Ki67 was expressed in the vestibular plate in the human fetal clitoris in an analogous fashion to the human fetal penis although with less intensity at all stages of development studied (8–16 weeks of gestation (Figs. 6B, H, 7A, G, 8F, B). As in the penis, Ki67 labeling was elevated in the dorsal half of the vestibular plate versus that in the ventral half (Fig. 7). This was particularly evident at 10 weeks of gestation (Fig. 7D–G). Once the vestibular plate begins to canalize to form the vestibular groove, residual vestibular plate is seen attached to the mid-point of the roof of the vestibular groove (Figs. 7B, F, G, 8C-D). The residual vestibular plate consistently exhibited elevated Ki67 labeling (Figs. 7G and 8D). Ki67 was also expressed in an analogous fashion in the roof of the vestibular groove in the human female clitoris compared to the urethral groove of the penis although with less intensity (Figs. 6B, 7H, I, 8H). At 10 weeks of gestation the epithelium of the roof of the vestibular groove was intensely Ki67-positive (Fig. 7I), while at later stages Ki67 labeling was sparse (Fig. 7I, O, 8D, H). Ki67 labeling was reduced/absent in the vestibular folds (edges of the vestibular groove) (Figs. 7B, I, O, 8C-D, G-H), again a pattern similar to that of the male counterparts. Within the perineum Ki67 was also expressed in the female urethral epithelium as in the penis at all stages development (Fig. 8J). At/near the site of the female urethral meatus Ki67 staining was especially prominent compared to surrounding area (Fig. 6H, black arrowheads).

3.2.6. Ki67 in the clitoral corporal body

Ki67 staining of the human clitoral corporal body was markedly less intense of that of the penile corporal body (Table 1). However, at early developmental stages (8–10 weeks) Ki67 staining intensity was similar in male and female corporal bodies Ki67 (compare Fig. 4K, 4O[male] to Fig. 7C and H [female]). At later time points (10 weeks of gestation) the intensity in Ki67 staining was reduced in the female corporal body compared to that of the male corporal body (compare Fig. 3M[male] to Fig. 6M[female]).

3.2.7. Ki67 in the glans clitoris and preputial lamina

Ki67 staining in the glans clitoris was also less intense compared to the penile glans, with rare Ki67 staining in the glans clitoris and preputial lamina after 12 weeks of gestation (compare male Fig. 3W to female Fig. 6J, M, N, K). As in the developing human penis Ki67 was consistently but sparsely expressed in the both the glanular and preputial epidermal layer of the preputial lamina (Fig. 6J) but not in the preputial mesenchyme or prepuce (Fig. 6J). As in the male, the epidermis of the clitoral glans in early stages of development expressed Ki67 (Fig. 6C) but at later stages after ~11 weeks little Ki67 expression was noted (Fig. 6J).

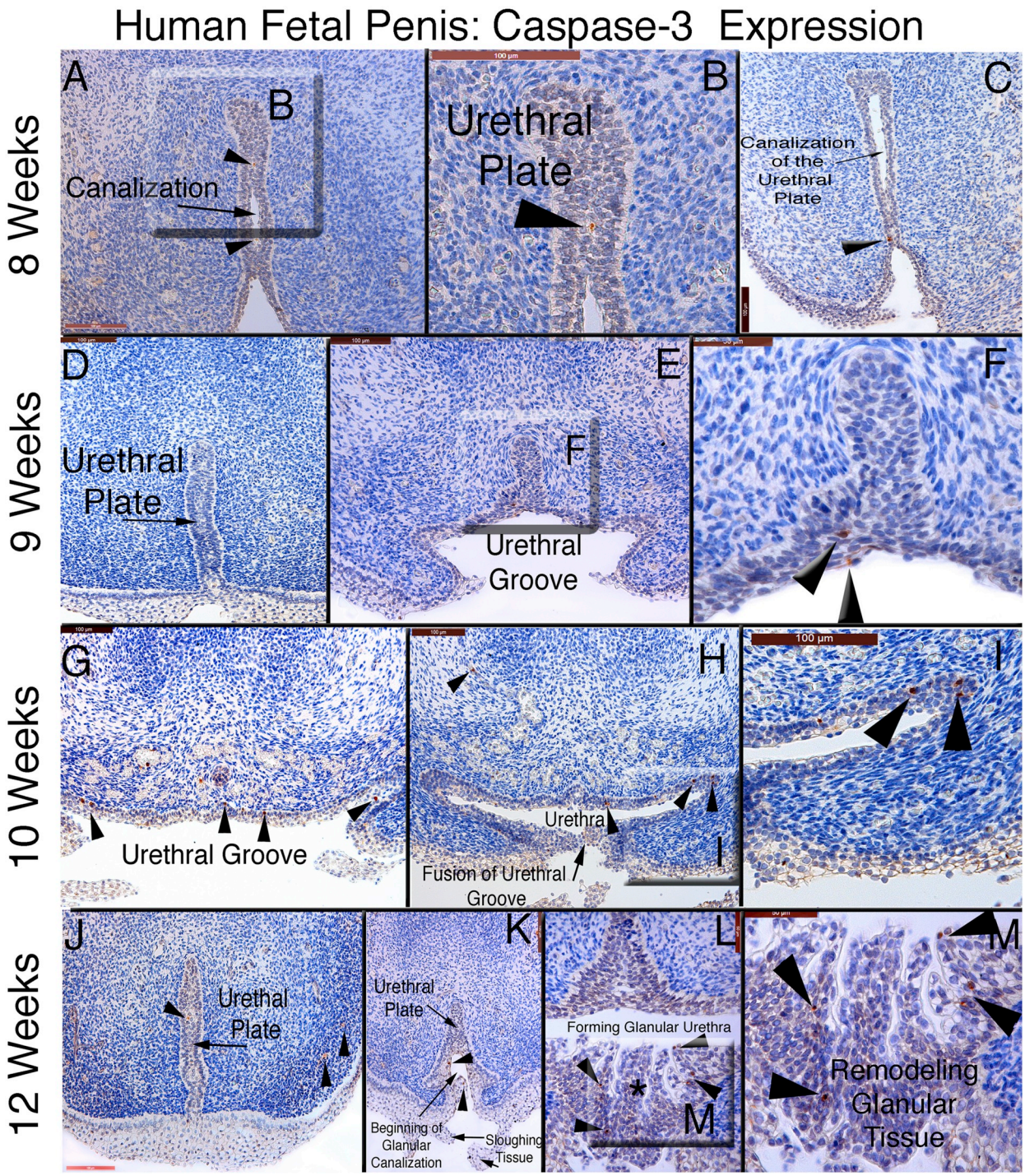

Fig. 9. Human Fetal Penis immunostained for the apoptotic marker caspase 3 (Transverse sections): 8 weeks of gestation A-C, 9 weeks of gestation D-F, 10 weeks of gestation G-I and 12 weeks of gestation J-M. Areas of Capsase-3 staining are labeled with black arrowheads. Note the occasional positive cell for caspase 3 in the area of the urethral plate (B), urethral groove (F and I) and area of remodeling glanular urethra (M).

3.3. Caspase- 3 in the developing penis and clitoris

Caspase-3 staining was present but quite sparse in the developing penis (Fig. 9). Note rare caspase 3-positive epithelial cells in the urethral plate (Fig. 9B) and urethral groove (Fig. 9F and I). Caspase 3-positive cells were rare/absent in penile and clitoral mesenchyme and corporal bodies (Figs. 9 and 10). The remodeling penile glanular urethra with its narrow ventral epithelial channels (Fig. 9M) contained a small number of caspase 3-positive epithelial cells. In contrast, caspase 3-positive epithelial cells were rarely seen in the human fetal clitoris (Fig. 10C).

4. Discussion

The human fetal penis and clitoris have evolved into different organs with both reproductive and in the case of the male urinary function (Baskin et al., 2018). Androgens induce the indifferent human genital tubercle to form a penis (Fig. 1). In the absence of androgens and/or a functioning androgen receptor, the indifferent genital tubercle forms a clitoris without an enclosed tubular urethra and a circumferential prepuce. It is well known that exogenous or endogenous prenatal exposure to androgens can virilize the XX female genital tubercle. In the most severe cases a normal penis can be induced to form. In less severe cases penis-like structures form with various degrees of hypospadias (Baskin, 2017; Speiser et al., 2018).

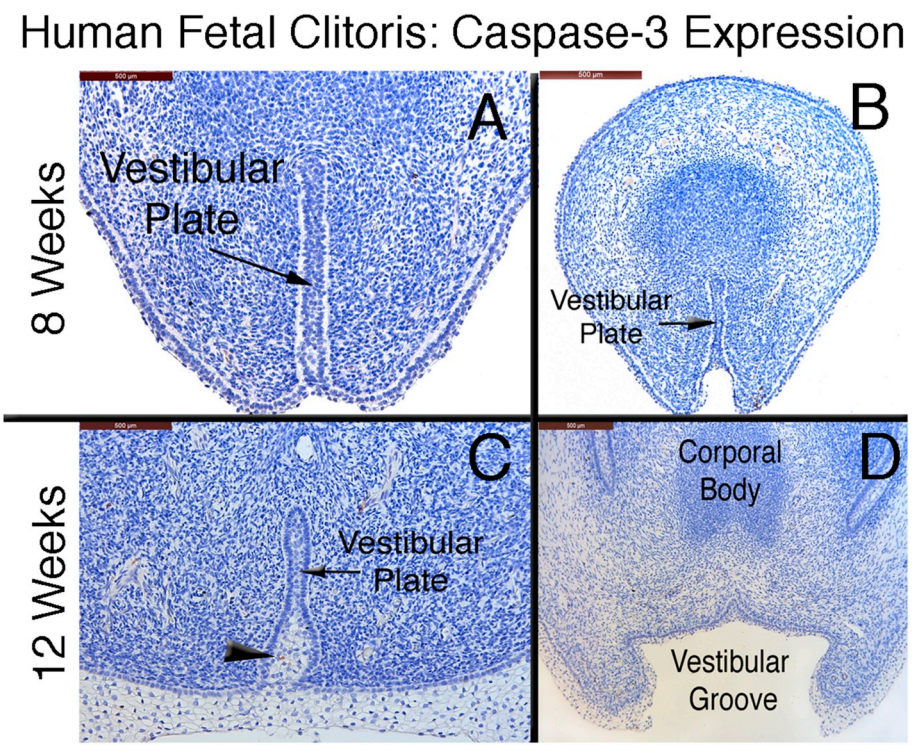

Fig. 10. Human fetal clitoris immunostained for the apoptotic marker caspase 3 (Transverse sections): 8 weeks of gestation (A–B), and 12 weeks of gestation (C–D). No evidence of caspase 3 staining was seen in any of the sections that included the vestibular plate (A–C) and groove (D).

The consistent trend seen in Ki67 labeling is that it is highest in the indifferent stage (8–9 weeks of gestation) of the penile and clitoral development in early stages, which coincides with divergence of the ambisexual genital tubercle into male and female genital anatomy (Baskin et al., 2018; Liu et al., 2018). Thereafter (10–18 weeks), Ki67 labeling was reduced globally in penile and clitoral development and specifically in the glans, corporal body epidermis and urethral plate.

To further delineate the growth of the penis and clitoris during early fetal development, we compared Ki67 staining from 8 to 9 weeks of gestation, the indifferent stage to the fully formed penis and clitoris at 18 and 16 weeks of gestation respectively. We found a number of areas of increased Ki67 expression during penile and clitoral development as well as differences in Ki67 staining between the clitoris and penis.

In the indifferent stage (8–9 weeks of gestation) the morphology of male and female genital tubercles is indistinguishable (Fig. 1). Not unexpectedly, Ki67 staining was analogous in ambisexual male and female specimens (Table 1) (compare Figs. 2 and 4 to 6 & 7). In contrast, after 9 weeks of gestation many penile structures/tissues exhibited higher Ki67 labeling compared to homologous clitoral counterparts. Penile structures/tissues exhibiting elevated Ki67 labeling included the corporal body, the urethral epithelium, urethral plate, urethral groove epithelium and glans mesenchyme. Consistently high Ki67 labeling in the epithelium of the roof of the urethral/vestibular grooves may be involved in widening these grooves. The human fetal penis grows from ~0.5 mm to ~6 mm in length from 8 to 18 weeks of gestation (Shen et al., 2018). This 12-fold increase in penile length over 10 weeks of growth appears to be driven by cellular proliferation (Ki67) in the corporal body and mesenchyme of the glans (Figs. 2–5).

Another impressive hot spot of Ki67 localization is at the site of glanular urethral remodeling (Fig. 3). Note in multiple panels in Fig. 3, highlighted by the black arrowheads, Ki67 staining in the urethral epithelium (urothelium) and the epithelium of the skin (epidermis). This is consistent with the mechanism of the glanular urethral remodeling involving extensive cellular growth and proliferation as described by Hadidi in 2014 and Shen in 2018 (Hadidi et al., 2014) (Shen et al., 2018). As noted, there was an overall paucity of programmed cell death as indicated by caspase-3 localization in the developing penis and clitoris. The one "slightly" warm spot of caspase-3 expression was in this area of glanular urethral remodeling, specifically in the area of the ventral epithelial urethral channel remodeling (Fig. 9M) (Liu et al., 2018).

Another hot spot of Ki67 expression was seen in the formed urethra in both the male and female especially early in development. We hypothesize that Ki67 staining in the formed urethra is left over cellular proliferation and growth from the remodeling urethra. Ki67 staining was also prominent in the urethral plate, urethral groove and vestibular groove areas of active remodeling. This is consistent with the hot spots of Ki67 expression that we see in the urethral plate (and vestibular plate) whose epithelial cells presumably flow into the roof of the urethral groove (and vestibular groove) (4B, 4J, 7A, 7C, 7G, 7I).

Interestingly, Ki67 staining was not seen in the epithelium of the urethral folds at/near the time of urethral fold fusion (Fig. 4P) but was seen in urethral fold mesenchyme (Fig. 4Q). The mechanism of urethral fold fusion into a tubular urethra does not appear to involve epithelial proliferation. In contrast, intense Ki67 staining was seen in the mesenchyme associated with the epithelium of the urethral folds, which is consistent with the idea that mesenchymal proliferation may play a role in pushing together the urethral folds (Fig. 4Q) that will subsequently fuse to form the penile urethra (Shen et al., 2016).

As noted, especially early in the development of the human penis and clitoris, Ki67 expression localized strongly to the corporal body and glans. The Ki67 staining intensity reduced or disappeared overtime (especially in the clitoris) consistent with the hypothesis that androgens induce cellular proliferation in penile versus clitoral development (Baskin et al., 1997).

Light but consistent Ki67 staining was seen in the epithelial cells, especially in the proximal free edge of the preputial lamina (Fig. 2F, G, I, M) in both the developing male and female human penis and clitoris (Figs. 2I, 3W, 6J). In contrast, mesenchyme of the prepuce in males and females (dorsal prepuce in females) contained rare Ki67-positive cells. Cellular proliferation proximal edge of the preputial lamina coincides with the presence of a subset of androgen receptor-positive epithelial cells and is likely to be a site of active area of growth and remodeling.

Interestingly, Ki67 staining was substantial in basal epithelial cells in and around the penoscrotal junction (Fig. 3Q) and in hair follicles located at the penoscrotal junction.

The morphology and function of the developing human penis and clitoris diverges after the indifferent stage at 8–9 weeks of gestation. Androgens are necessary for this process. Abnormalities in androgen metabolism and/or defects in the androgen receptor in XY patients results in impaired virilization of the external genitalia. Human examples, include XY gonadal dysgenesis (King and Conway, 2014) and complete androgen insensitivity (Batista et al., 2018). In addition, in-born errors of steroid metabolism such as in patients with congenital adrenal hyperplasia with chromosomal XX genotypes elicit virilization of the external genitalia (Speiser et al., 2018). Herein, we have shown that Ki67 staining as a surrogate for cellular proliferation is responsive to androgens with less Ki67 expression as noted in the developing human female versus the male specimens (Table 1).

In conclusion, Ki67 staining is present in the human fetal developing penis and clitoris. A number of hot spots of cellular proliferation characterized by Ki67

Table 1

Ki67 in the Developing Penis

	Urethra Epithelium	Urethral Plate	Urethral Groove Epithelium	Urethral Folds Epi.	Urethral Folds Mes	Remodeling Glanular Urethral	Epidermis	Corpora Body	Glans	Preputia Lamina	Prepuce	Peno-Scrotal Junction
8 weeks	+ +	+ +					+	+ +	+ +			
9 “	+ +	+ +	+ +	–	+		+	+ +	+ +			
10 “	+ +	+	+ +	–	+		+	+ +	+			
11 “	+ +	+	+ +	–	+		+	+ +	+	+		
12 “	+ +	+				+ +	+/-	+ +	+	+	–	
13 “	+ +	+				+ +	+/-	+	+	+	–	
14 “	+ +	+				+ +	–	+	+	+	–	
15 “	+ +	+				+ +	–	+	+	+	–	+ +
16 “	+	+				+ +	–	+	+	+	–	+ +
17 “	+	+				+	–	+	+	+	–	+ +
18 “	+	+					–	+	+	+	–	

Ki67 in the Developing Clitoris

	Urethra Epithelium	Vestibular Plate	Vestibular Groove Epithelium	Epidermis	Corporal Body	Glans	Preputia Lamina	Dorsal Prepuce
8 weeks	+	+		+	+ +	+		
9 “	+	+		+	+ +	+		
10 “	+	+	+	+	+	+		
11 “	+	+	+	–	+	–	+	
12 “	+	+	+	–	+	–	+	–
13 “	+	+		–	+	–	+	–
14 “	+	+		–	+	–	+	–
15 “	+	+		–	+	–	+	–
16 “		+		–	+	–	+	–

Note + + = strongly expressed. + = expressed - = not expressed. Blank = the anatomical structure is not present at this stage.

localization are present in the penis and clitoris especially early in development. Most notable, the male corporal body and glans are active sites of cellular proliferation and may drive the increase in penile length. In addition, morphogenetic remodeling in the glandular urethra, the urethral plate, the ventral urethral groove and the fully formed urethra are also active areas of cellular proliferation. Areas of rare/absent Ki67 staining include the urethral fold epithelium that fuses to form the penile tubular urethra. In contrast, mesenchyme associated with the epithelium of the urethral folds is Ki67-positive. The preputial lamina exhibits sparse but consistent Ki67 expression in contrast to absent staining in preputial mesenchyme. Apoptosis was rarely noted in the developing penis and clitoris with the only area of minimally increased localization in the epithelium of the ventral epithelial glanular channel remodeling.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.diff.2019.11.001.

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