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Clitoral development in the mouse and human

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ABSTRACT

The goal of this report is (a) to provide the first detailed description of mouse clitoral development, and (b) to compare mouse and human clitoral development. For this purpose, external genitalia of female mice were examined by wholemount microscopy, histology and immunohistochemistry from 14 days of gestation to 10 days postnatal. Human clitoral development was examined by these techniques as well as by scanning electron microscopy and optical projection tomography from 8 to 19 weeks of gestation. The adult mouse clitoris is an internal organ defined by a U-shaped clitoral lamina whose development is associated with the prenatal medial and distal growth of the

female preputial swellings along the sides of the genital tubercle to form the circumferential preputial lamina. Regression of the ventral aspect of the preputial lamina leads to formation of the U-shaped clitoral lamina recognized as early as 17 days of gestation. While the adult U-shaped mouse clitoral lamina is closely associated with the vagina, and it appears to be completely non-responsive to estrogen as opposed to the highly estrogen-responsive vaginal epithelium. The prominent perineal appendage in adult females is prepuce, formed via fusion of the embryonic preputial swellings and is not the clitoris.

The human clitoris is in many respects a smaller anatomic version of the human penis having all of the external and internal elements except the urethra. The human clitoris (like the human penis) is derived from the genital tubercle with the clitoral glans projecting into the vaginal vestibule. Adult morphology and developmental processes are virtually non-comparable in the mouse and human clitoris.

1. Introduction

Studies on the anatomy and development of mouse external genitalia have focused primarily on penile development. Indeed, a PubMed search on “mouse/mice”, “clitoris” and “development” yielded not a single hit. Furthermore, a PubMed search on “mouse/mice” and “clitoris” reveals only one paper, in which the authors describe the adult mouse clitoris as an internal structure defined by an inverted U-shaped epithelial lamina (Martin-Alguacil et al., 2008b) (Fig. 1A), which we have confirmed in several subsequent publications (Mahawong et al., 2014; Sinclair et al., 2016; Weiss et al., 2012; Yang et al., 2010). Development of the U-shaped mouse clitoral lamina appears to be due to the absence of androgen receptor signaling, since androgen-insensitive X^{Tfm}/Y male mice exhibit clitoral development identical to that of wild-type females (Table 1) (Rodriguez et al., 2012).

The anatomical features of the adult mouse clitoris contrast substantially with the well-known anatomy of the human clitoris (Table 2). While the adult human “clitoris is in many details a smaller version of the human penis” (Warick and Williams, 1973), the adult mouse clitoris shares few anatomic homologies with the adult mouse penis (Fig. 1 and Table 3). For example, the adult human clitoris has a body and glans as well as well-defined erectile bodies (Clemente, 1985), features absent in the adult mouse clitoris (Weiss et al., 2012). In addition, the glans of the human clitoris projects (albeit minimally) from the body surface into the vaginal vestibule (Clemente, 1985), whereas the mouse clitoris is an internal organ lying deep to the surface of the body. In this regard, it is essential to recognize that the perineal appendage seen in adult female mice is not the clitoris, but instead is prepuce, having a histologic signature identical to that of the male mouse external prepuce (Sinclair et al., 2016).

The striking anatomic disparities between the adult mouse penis versus the adult mouse clitoris (Fig. 1, Table 3) as well as the morphologic disparity between the human and mouse clitoris (Table 2) reflect equally different developmental processes accounting for these adult anatomic differences. To date, there is only one brief report of mouse clitoral development (Schlomer et al., 2013), which deals su-

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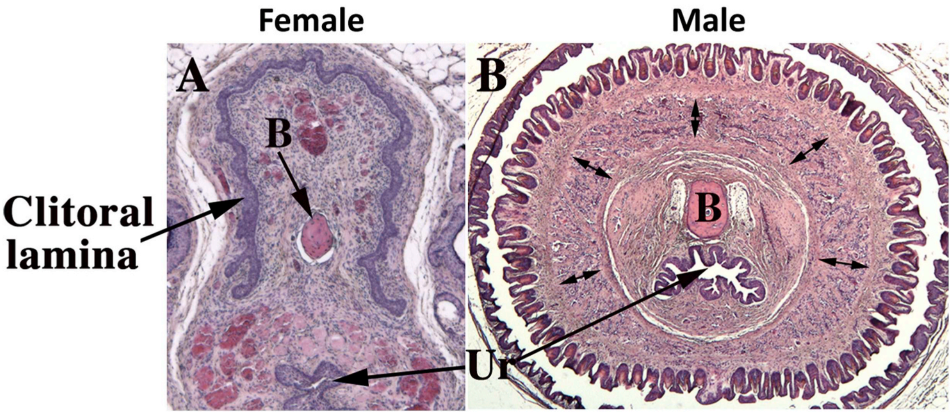

Fig. 1. Histologic sections of the adult mouse clitoris (A) and penis (B). The clitoris is defined as an inverted U-shaped clitoral lamina, whose stroma contains a small os clitoris (B), but no defined erectile bodies. Note that the urethra lies ventral to the clitoris. The adult mouse penis (B) resides within the preputial space, has spines on its surface, contains well-defined erectile bodies (corpus cavernosum glandis, double-headed arrows), a large os penis (B) and a urethra (Ur).

Table 1
Morphological features of the clitoris of adult wild-type female and androgen insensitive X^{Tfm}/Y mice.

Feature	Wild-type Clitoris	X^{Tfm}/Y Clitoris
Internal organ	Yes	Yes
U-shaped epithelial lamina	Yes	Yes
Urethra only partially within the clitoral lamina	Yes	Yes
Defined erectile bodies	No	No
Small os clitoris	Yes	Yes
Ventral tethering/mobile organ	Yes	Yes

Table 2
Comparison of the anatomic features of the adult mouse and human clitoris.

Feature	Mouse clitoris	Human Clitoris
U-shaped epithelial lamina	Yes	No
Glans	No	Yes
Os clitoris	Yes	No
Defined erectile bodies	No	Yes
Internal organ	Yes	No
Surrounded by labia minora	No	Yes
Urethra partly within organ	Yes[a]	No

[a] The position of the adult urethra relative to the clitoral lamina varies from a superficial to deep perspective (Weiss et al., 2012).

Table 3
Morphological features of penis and clitoris of wild-type mice.

Feature	Wild-type Penis	Wild-type Clitoris
Circular in transverse section	Yes	No
Adult penile and clitoral stroma derived from genital tubercle mesenchyme	Yes	Yes
U-shaped epithelial lamina	No	Yes
Epithelial spines	Yes	No
Urethra completely within organ	Yes	No
Proximal hyaline cartilage	Yes	No
Distal cartilage	Yes	No
Large organ	Yes	No
Defined erectile bodies	Yes	No
Long bone	Yes	No
Short bone	No	Yes
Ventral tethering/immobile organ	No	Yes

Table 4
Antibodies used in this study.

Antibody	Source	Catalogue #	Concentration
Keratin 6	Acris Antibodies	AM21068PU-S	1/200
Keratin 7	E.B. Lane[a]	LP1K	1/10
Keratin 8	E.B. Lane[a]	LE41	1/10
Keratin 10	Dako	M7002	1/50
Keratin 14	BioGenex	LL002	1/100
Keratin 19	E.B. Lane[a]	LP2K	1/10
Uroplakin1	T. T. Sun[b]		1/100
Foxa1	Atlas Antibodies	HPA050505	1/500
Androgen receptor	Genetex	GTX62599	1/100
Estrogen receptor α	Abcam	Ab16660	1/100

[a] Institute of Medical Biology, Singapore.
[b] New York University, New York.

perficially with postnatal developmental events. The role of prenatal rudiments of female external genitalia (genital tubercle and preputial swellings) and their relationship to the overall morphogenetic processes leading to mouse clitoral development are unknown and are the subject of this paper as well as a comparison of mouse and human clitoral development.

2. Materials and methods

The University of California, San Francisco (UCSF) Institutional Animal Care and Use Committee approved all animal protocols. Timed-pregnant CD-1 mice (Charles River Breeding Laboratories, Wilmington, MA, USA) were housed in polycarbonate cages (20 × 25 × 47 cm3) with laboratory-grade pellet bedding in the UCSF Pathogen Specific Barrier housing facility. The mice were given Purina lab diet (#5058) and tap water ad libitum and were acclimated to 20° to 23 °C and 40%–50% humidity on a schedule of 14 h light and 10 h dark. Embryos and neonates were collected at the following ages: embryonic days 14, 15, 16, 17, 18, birth. At least 3 mice were used for each time point. This paper is based upon analysis of 32 mice.

To assess possible estrogenic responsiveness of the U-shaped clitoral lamina, 6 male X^{Tfm}/Y mice were treated with diethylstilbestrol (DES) via 20 mg subcutaneous pellet for either 3 or 8 weeks. Use of X^{Tfm}/Y mice eliminates possible interfering androgenic effects. An additional 4 untreated X^{Tfm}/Y mice were used as controls. After treatment, the external genitalia containing the clitoral lamina were fixed in 10% buffered formalin, sectioned and stained as described below.

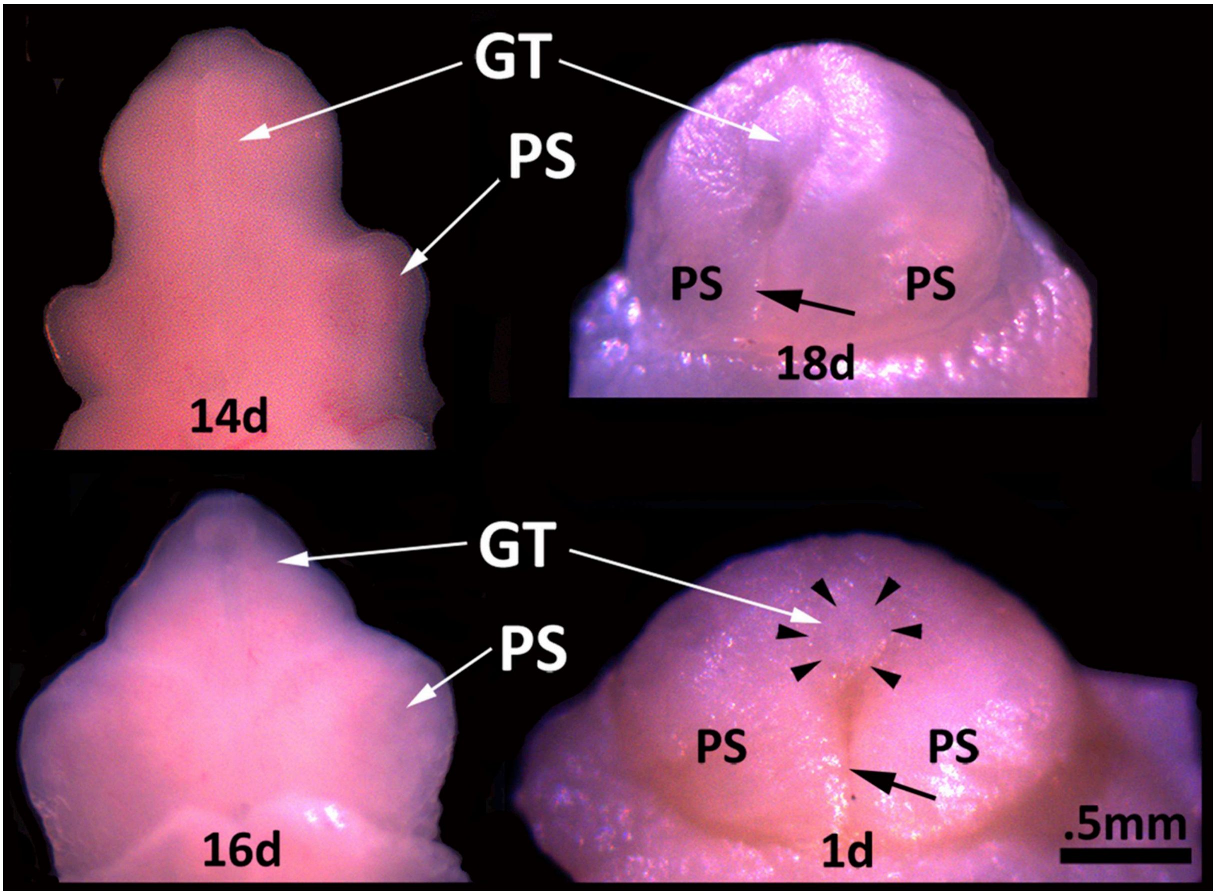

Fig. 2. Wholemount images of female mouse GTs at embryonic days 14, 16 and 18 and at birth (1d) as ventral views. The preputial swellings (PS) enlarge and fuse in the midline to form the prepuce, which extends distally to eventually completely cover the genital tubercle (GT). Arrowheads in the 1-day image outline the genital tubercle, which is almost completely covered by the preputial swellings. At embryonic day 18 and at birth, the preputial-urethral groove can be seen when the preputial swellings are fusing in the midline (large black arrows).

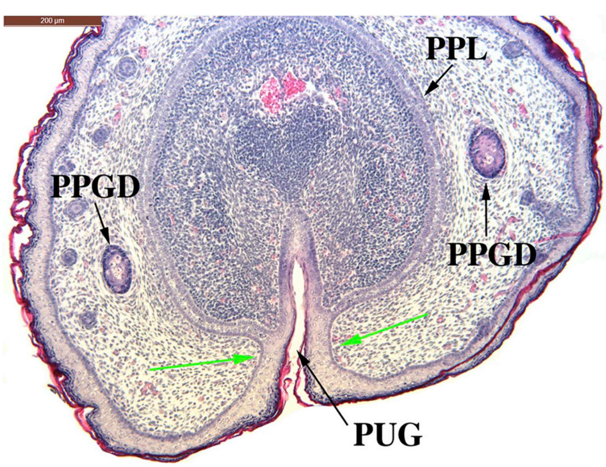

Fig. 3. Transverse section of an 18-day female mouse genital tubercle. The preputial swellings have grown ventrally and medially to define the preputial-urethral groove (PUG). Note also the preputial gland ducts (PPGD) and the preputial lamina (PPL). The green arrows indicate the growth of the preputial swellings toward the ventral midline. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this

External genitalia were dissected and photographed using a digital camera and were then fixed in 10% buffered formalin. Samples were paraffin embedded and serially sectioned transversely and coronally at 7 µm for histological staining with hematoxylin and eosin (H&E). Immunohistochemistry (IHC) was carried out as previously described (Rodriguez et al., 2012) on sections of mouse and human external genitalia using the antibodies indicated in Table 4. Signal detection was achieved using the Vector ABC System (Vector Laboratories, Foster City, CA, USA) followed by exposure to diaminobenzidine (Sigma®). Sections exposed to all steps except the application of the primary antibodies were used as negative controls.

3. Results

3.1. Mouse clitoral development

Embryonic gestation day 14 (E14) is an ideal age to begin analysis of mouse clitoral development. At 14 days of gestation the female mouse external genitalia consist of the genital tubercle and associated preputial swellings. At E14 the genital tubercle projects from the perineum and is flanked laterally by the preputial swellings (Fig. 2). Prenatally, male and female external genitalia are remarkably similar in size and shape, with a slight increase in size of the genital tubercle in

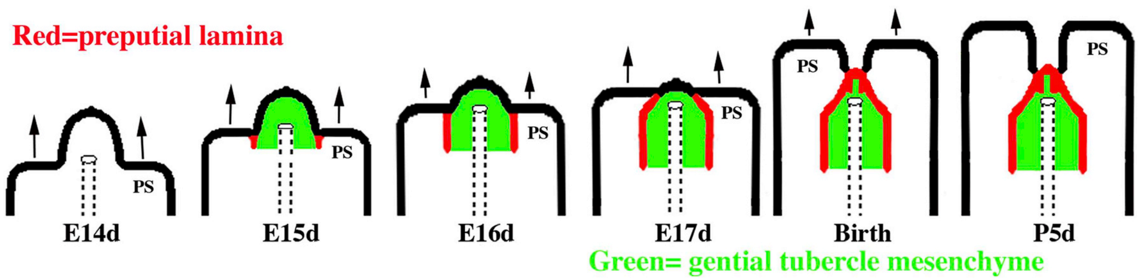

Fig. 4. Diagrammatic representation of development of the prepuce and the preputial lamina (red) from the preputial swellings (PS). Note that as the preputial swellings grow distally, the preputial lamina (red) is "left in its wake", and thus with time the length of the preputial lamina increases. Black arrows indicate distal growth of the preputial swellings. The dotted lines represent the urethra. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

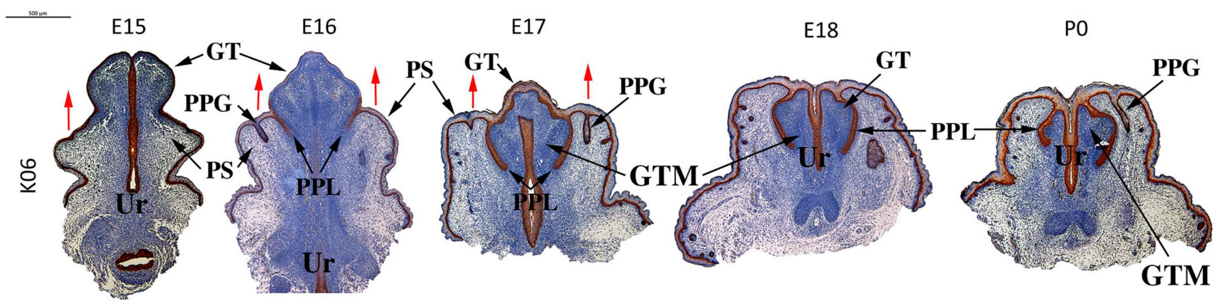

Fig. 5. Coronal sections of female mouse external genitalia at the ages indicated. The preputial swellings (PS) grow distally (red arrows) to completely cover the genital tubercle (GT). As the preputial swellings grow distally along the sides of the genital tubercle (GT), the preputial lamina (PPL) is laid down, which surrounds the dense genital tubercle mesenchyme (GTM). Note the preputial glands (PPG) and the urethra (Ur). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

males in the last few days of gestation (Liu et al., 2018; Murdaugh et al., 2018). Female preputial swellings enlarge and grow toward the ventral midline and distally (Figs. 2 and 3) to completely cover the genital tubercle by birth, a process identical to that reported for male mouse fetuses (Liu et al., 2018). As the preputial swellings converge towards the ventral midline (Fig. 3), a prominent ventral groove becomes evident called the preputial-urethral groove (PUG) seen particularly well at E18 (Fig. 3), as is also the case for developing male external genitalia at the same age (Liu et al., 2018). As the female preputial swellings grow medially and distally along the sides of the genital tubercle, the preputial lamina is laid down (Figs. 3–5). Thus, the preputial lamina is clearly ectodermal in origin. The process of distal growth of the female preputial swellings and associated formation of the preputial lamina is particularly well illustrated in coronal sections (Fig. 5). The growth and midline fusion of the preputial swellings and the formation of the preputial lamina in females is identical to that observed in male fetuses (Liu et al., 2018).

Serial transverse sections of the developing female external genitalia provide further insight into the development process. Such transverse sections are challenging to evaluate and are presented in 10 section intervals (section thickness = 7 μm) in E14-E18 female specimens (Figs. 6–8). Careful analysis of Figs. 6–8 reveal the ventral clitoral groove (VClGr, homologue of the ventral penile groove) (Liu et al., 2018), which is continuous proximally with the solid urethral plate (Fig. 6). Both the ventral clitoral groove and the solid urethral plate are composed of a non-keratinized epithelium at E14-E16 (Figs. 6 and 7). Note that at these ages the solid urethral plate does not extend to the distal aspect of the genital tubercle (Fig. 6), as the urethral plate is not seen in the most distal sections (also the case for males) (Liu et al., 2018). The first definitive hit on a preputial swelling (PS) is seen at E16 in Fig. 6 (section 60), while preputial swellings are particularly evident in the E17 and E18 specimens (Fig. 6). Note that cellular density of the genital tubercle mesenchymal (GTM) is elevated relative to the reduced mesenchymal density of the preputial swellings (PS) (see Fig. 6). This difference in H&E staining is not seen at earlier stages, and thus it is difficult/impossible to identify preputial swellings at E14 and E15 in transverse sections, even though they are evident in wholemount images (Fig. 2) and coronal sections (Fig. 5). In serial transverse section sets, the preputial swellings are initially seen in the dorsal-lateral position (Fig. 6), but as sections are followed proximally the preputial swellings expand ventral-medially to eventually define the preputial-urethral groove (PUG) in the ventral midline (Figs. 3, 6 and 7). As the preputial swellings grow towards the ventral midline, they expand ventral-medially to cover the genital tubercle. During this ventral-medial expansion of the preputial swellings, the epithelium of the preputial swellings fuses with the epidermis of the genital tubercle to

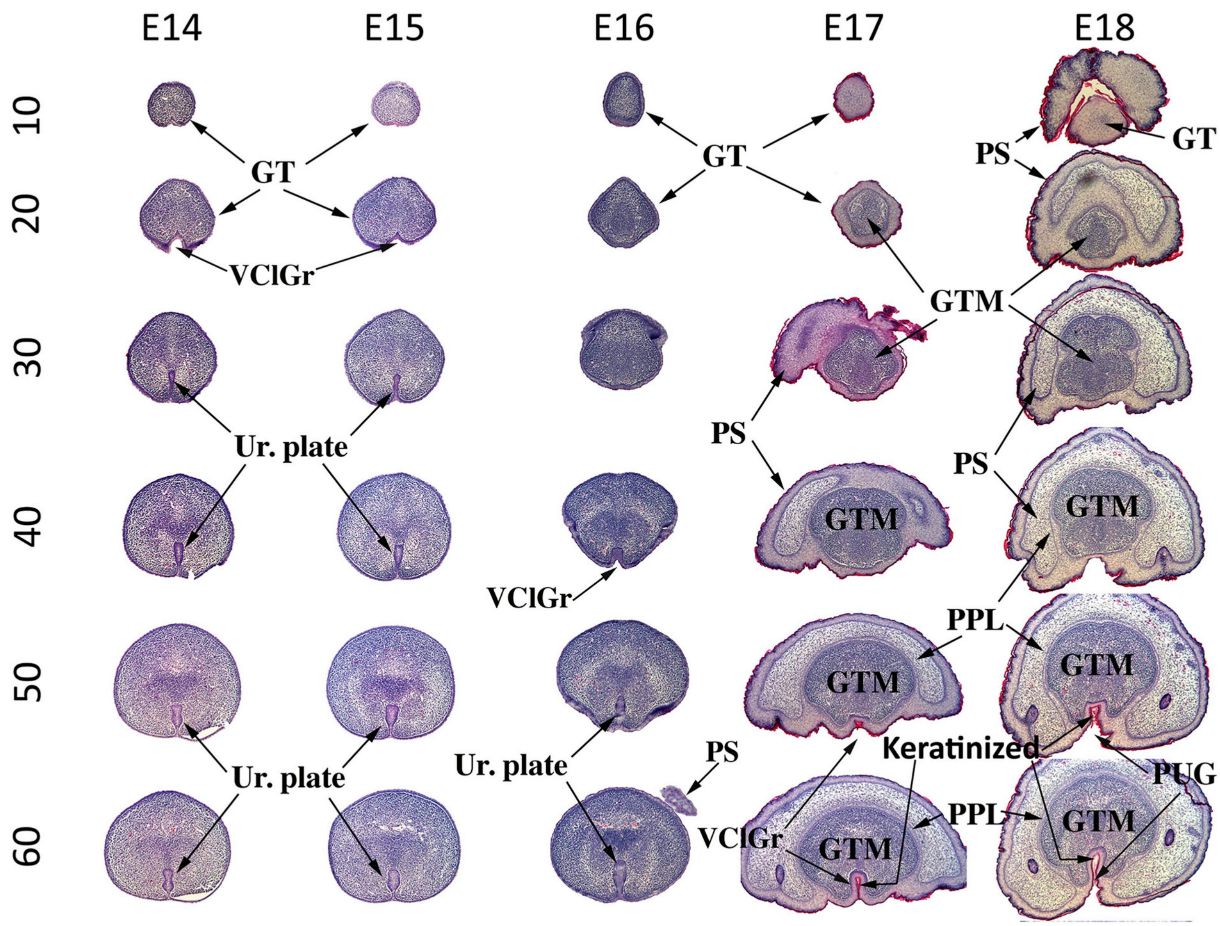

Fig. 6. A serial transverse section set (sections 10 to 60) of the female genital tubercle/preputial swellings at 10 section intervals at the ages indicated. From the distal tip of the genital tubercle the first notable structure is the ventral clitoral groove (VClGr) seen in the E14- E17 specimens. Note the solid urethral plate (Ur. plate) in the E14, E15 and E16 specimens located several sections proximal to the tip of the genital tubercle. The preputial swellings (PS) become prominent in sections of E17 and E18 specimens. As the preputial swellings fuse with the genital tubercle (GT), the circumferential preputial lamina (PPL) is laid down which encompasses the genital tubercle mesenchyme (GTM) seen in the E17 and E18 specimens. Note also the preputial-urethral groove (PUG) in the E17 and E18 specimens.

create the preputial lamina (PPL), which encloses the mesenchyme of the genital tubercle. This process is illustrated in Fig. 3, particularly well in the coronal sections (Fig. 5) and in the E17- E18 specimens sectioned transversely (Fig. 6). The process of preputial lamina formation in females is identical to that in males (Liu et al., 2018), and has no counterpart in human clitoral development.

As mentioned above, at E14-E16 day epithelia of the ventral clitoral groove and urethral plate are non-keratinized (Figs. 6 and 7). However, at E17 and later stages the epithelia of the urethral plate and ventral clitoral groove are keratinized (Figs. 6 and 7, E17-18), a process which facilitates canalization of the urethral plate. Indeed, at 18 days of gestation the distal aspect of the solid urethral plate canalizes to form the preputial-urethral groove (PUG) lined with a keratinized epithelium. Preputial-urethral groove is deepened as the preputial swellings approach the midline (Figs. 3, 6 and 7). As development proceeds the edges of the preputial-urethral groove fuse (Figs. 7 and 8) to form a canal that we have labeled "urethra", but which could also be called the preputial-urethral canal.

Initial formation of the circumferential preputial lamina is seen in the E17 specimen (Figs. 6 and 7) as the epidermis of the preputial swellings fuses with the epidermis of the genital tubercle. The fully formed preputial lamina is circumferential in transverse section and is unquestionably of ectodermal origin (Figs. 3, 6 and 7). A feature of the fully formed preputial lamina is its ventral attachment to epithelium of (or near) the preputial-urethral groove and urethral plate at E17 (Figs. 6 and 7). The inverted U-shaped clitoral lamina is revealed following detachment of the preputial lamina from the urethral plate, preputial-urethral groove and/or the urethra (Fig. 7, green double-headed arrows). The resultant formation of the U-shaped clitoral lamina establishes continuity of clitoral stroma defined within the U-shaped clitoral lamina with ventrally situated preputial stroma (Fig. 7, green double-headed arrows). The U-shaped clitoral lamina continues to be seen in proximal sections, but eventually disappears proximally (Figs. 7 and 8).

Careful examination of Figs. 7 and 8 demonstrates that the urethral plate (whether solid or canalized) extends distally a considerable distance beyond the urethral meatus. This arrangement is shown

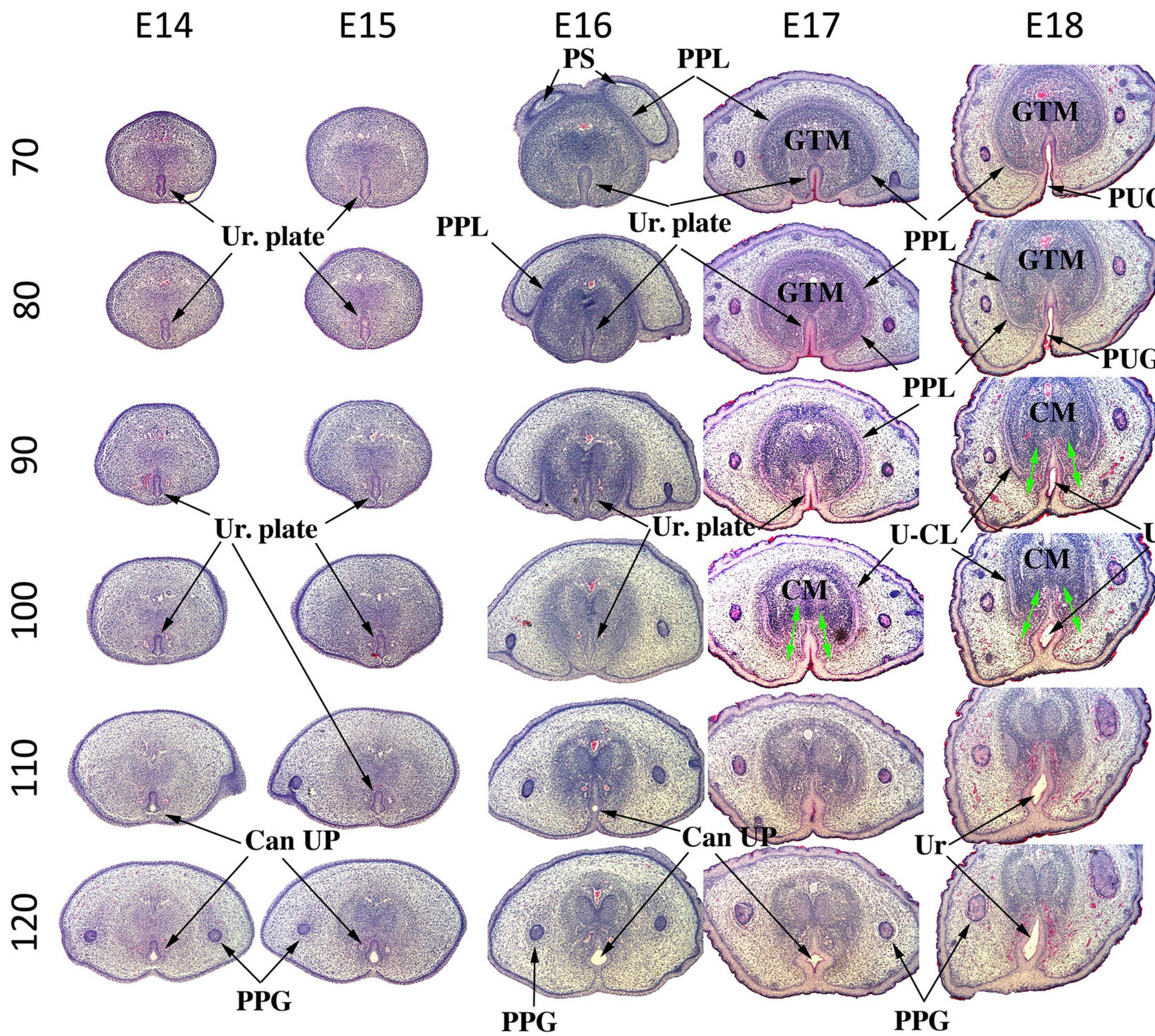

Fig. 7. Continuation of the serial transverse sections of the female genital tubercle/preputial swellings at 10 section intervals and at the ages indicated. Note the solid urethral plate (Ur. plate) in the E14, E15 and E16 specimens. The preputial swellings (PS) become prominent in the sections of the E17 and E18 specimens. As the preputial swellings fuse with the genital tubercle (GT), the circumferential preputial lamina (PPL) forms which encompasses the genital tubercle mesenchyme (GTM) seen in the E17 and E18 specimens. Note also the preputial-urethral groove in the E17 and E18 specimens. The inverted U-shaped clitoral lamina (U-CL) is indicated by double-headed green arrows and represents confluence between clitoral mesenchyme (CM) and preputial mesenchyme in the E17 and E18 specimens. Sections 110 and 120 (E17 and E18) are past the caudal extent of the clitoral lamina. PPG = preputial gland, Ur. Plate = urethral plate, Can UP = canalized urethral plate, PPL = preputial lamina. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

diagrammatically in Fig. 9A. The important point is that the solid and canalized urethral plate is located distally to the urethral meatus (Figs. 6–8 & 9A), raising the issue of the developmental fate of these distal structures in development of distal portions of the female external genitalia and how such development relates to adult female mouse external genitalia. First, it should be noted that the adult female perineal appendage (the elevation in the adult female mouse perineum) is composed of hair-bearing preputial tissues as reported previously (Sinclair et al., 2016) and is shown in Fig. 9B. Note in Fig. 9B the extensive preputial-urethral groove (PUG) that in part bisects the adult female perineal appendage, and that the actual urethral meatus is hidden from view in the depth of the preputial-urethral groove. The question is how does the morphologic pattern of the distal genital tubercle illustrated diagrammatically in Fig. 9A give rise to the distal aspect of adult female external genitalia? Our data in this regard is unfortunately incomplete, but we can infer that the following developmental events are likely involved in this process: (a) We suggest that the fate of the urethral plate is complete canalization distal to the urethral meatus to open a groove (PUG) to the exterior, and (b) perhaps more important, the distal extension of the prepuce to form the adult

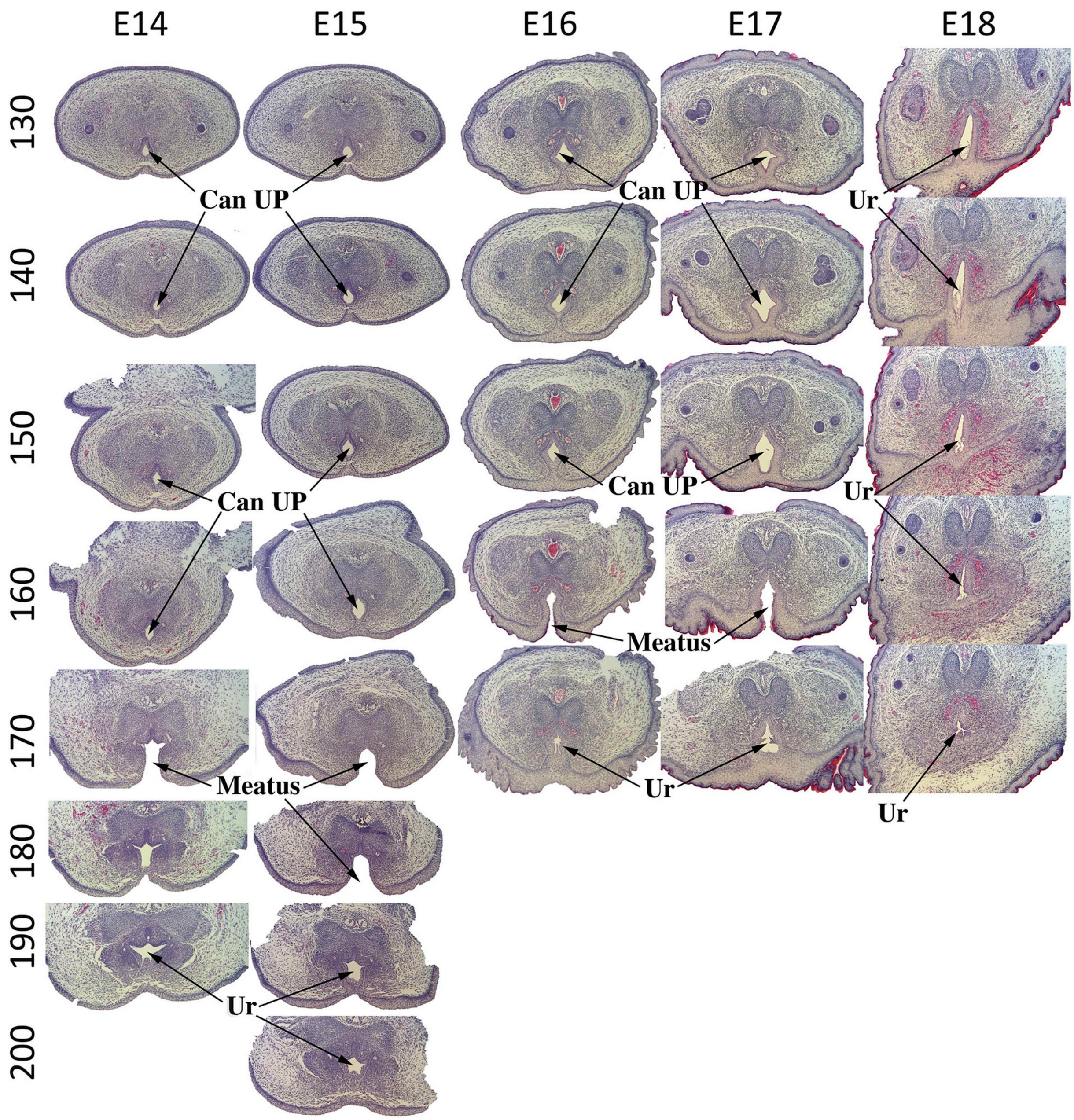

Fig. 8. Continuation of the serial transverse sections of the female genital tubercle/preputial swellings at 10 section intervals and at the ages indicated. Note urethral meatus and the canalized urethral plate (Can UP).

female perineal appendage, thus extending and deepening the preputial-urethral groove to the distal tip of the adult female perineal appendage (Fig. 9B). The distal aspect of the adult female perineal appendage is unquestionably prepuce as it is covered by a hair-bearing epidermis (Fig. 9B) and is filled with hair follicles and associated sebaceous glands (Fig. 9B, inset). This interpretation is supported by transverse sections from the tip of the 10-day female perineal appendage inward. Conclusive proof of contribution of the preputial swellings to the distal surface of the female perineal appendage is demonstrated by the presence of preputial gland ducts in the distal aspect of the female mouse perineal appendage (Fig. 9C and D). Preputial gland ducts arise at E14-E15 from the distal surface of the preputial swellings (Cunha, 1975) (see also Fig. 5). The first few sections of the 10-day postnatal specimen shown in Fig. 9C and D shows separate bilateral preputial appendages (not illustrated). Section 39 from the tip (Fig. 9C) shows the paired preputial swellings fusing and thus creating the preputial-urethral groove (Fig. 9C). Section 44 (Fig. 9D) shows fusion of the bilateral preputial swellings and formation of the preputial-urethral

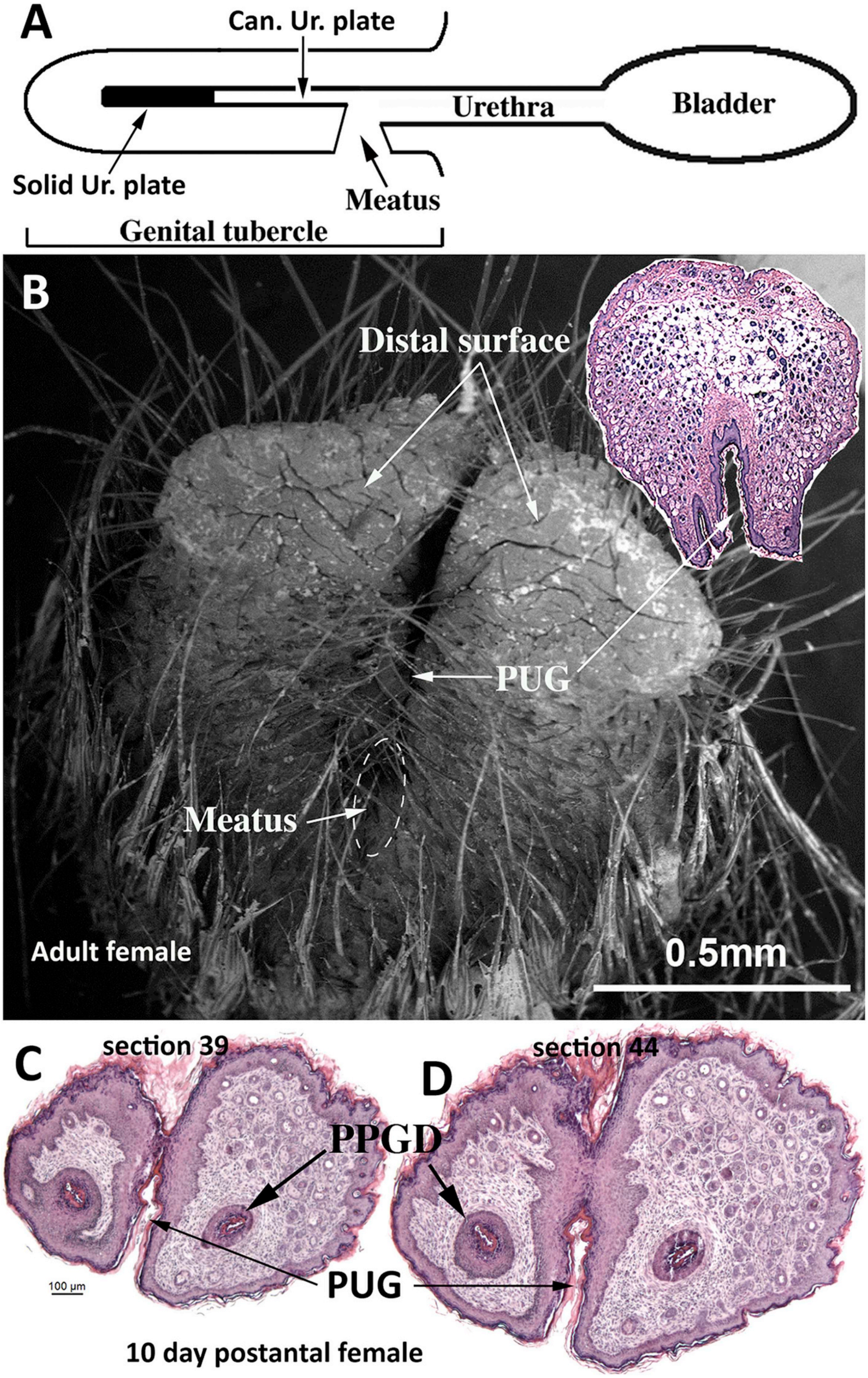

Fig. 9. (A) Diagrammatic representation of the relationship of the embryonic female urethral meatus and the solid and canalized urethral plate (Can. Ur. Plate), which extends distally beyond the urethral meatus at E14-E17. Refer to Figs. 5–7 to see histologic sections on which this diagram is based. (B) Scanning electron micrograph of the adult hair-bearing mouse perineal appendage (ventral view). Note its blunt distal surface and the preputial-urethral groove (PUG). The urethral meatus is in the depth of the preputial-urethral groove and is located as indicated. The inset in (B) is a section near the distal surface of the female perineal appendage whose interior is filled with hair follicles and sebaceous glands. (C & D) are transverse sections of a 10-day postnatal female perineal appendage illustrating the fusion of the paired preputial swellings (C) to form the preputial-urethral groove (D). Note preputial gland ducts (PPGD).

groove. Fig. 11A is section 61 of this serial section set showing complete dorsal fusion of the preputial swellings and the preputial-urethral groove. During this process the position of the female urethral meatus extends distally to its final position in the depth of the preputial-urethral groove (Fig. 9B). Considerable epithelial remodeling is certainly involved encompassing: (a) canalization of the urethral plate, (b) distal extension of the preputial-urethral groove (deepened by medial growth of the preputial swellings) and (c) fusion of the edges of the preputial-urethral groove to distally extend the urethra and the urethral meatus.

To summarize, mouse clitoral development involves prenatal

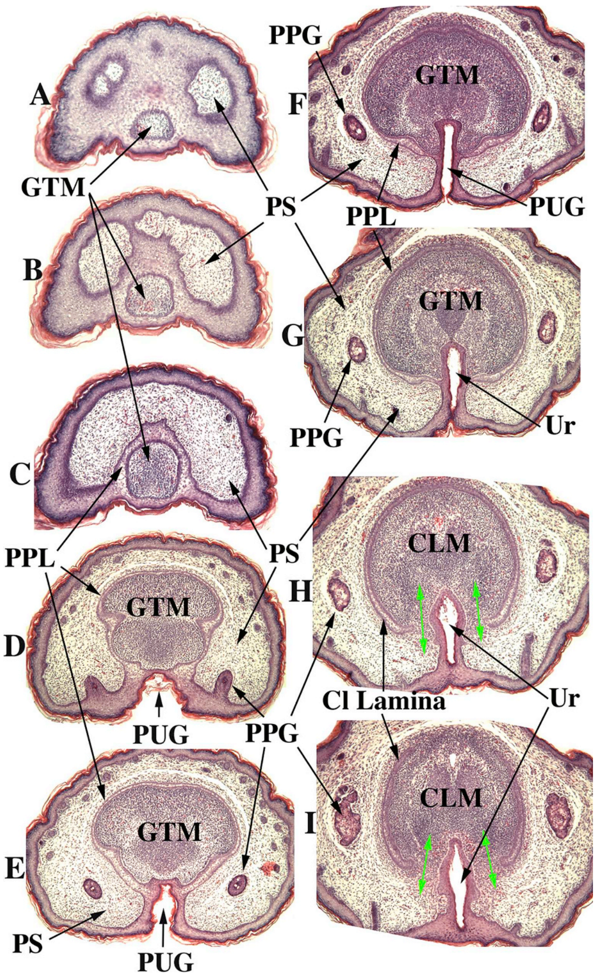

Fig. 10. Selected transverse sections of the newborn female external genitalia in distal (A) to proximal (I) sequence. Sections (A & B) mostly contain epidermis, surrounding 3 "islands" of mesenchyme: (genital tubercle mesenchyme [GTM] ventrally and mesenchyme of the two preputial swellings [PS] dorsally). Mesenchyme of the preputial swellings coalesce in (C), and the combined preputial swellings grow ventrally to form the preputial-urethral groove (PUG) (D–F). In section (G) the edges of the PUG are fusing to form the urethra. Note that as the preputial swellings grow ventral-laterally around the genital tubercle mesenchyme, the circumferential preputial lamina is laid down which initially is connected ventrally to the epithelium of the PUG and urethra (D–G). The U-shaped clitoral lamina is formed when this attachment disappears, and clitoral mesenchyme becomes confluent ventrally with preputial mesenchyme (double-headed green arrows, H and I). Note the small round hair follicles in the preputial mesenchyme. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

formation of the U-shaped clitoral lamina characteristic of the adult mouse clitoris. The origin of the U-shaped clitoral lamina can be directly traced to initial formation of the circumferential preputial lamina which owes its development to the ventral-medial and distal growth of the preputial swellings and their fusion with the genital tubercle.

Postnatal mouse clitoral development was examined at birth, 5 (not illustrated) and 10 days postnatal. At birth the most distal transverse sections of the perineal appendage contain epidermis only (not illustrated). A few sections into the specimen three masses of mesenchyme are encountered surrounded by epithelium (Fig. 10A) as reported previously (Schlomer et al., 2013). The two dorsal masses of mesenchyme are preputial mesenchyme, and these coalesce in more proximal sections (Fig. 10A–E). Hair follicles, a characteristic feature within preputial mesenchyme, invade the preputial mesenchyme (Fig. 10C–F). The centrally placed mesenchymal mass (Fig. 10A) is genital tubercle mesenchyme (GTM), which is surrounded by the preputial lamina (Fig. 10C–E). As the bilateral preputial swellings grow toward the ventral midline, the preputial-urethral groove is defined (Fig. 10D–F), and ducts of the preputial glands invade into the preputial mesenchyme as the preputial-urethral groove is forming (Fig. 10D). Progressing sequentially from section 10A to section 10G, note that the genital tubercle mesenchyme (GTM) expands and is surrounded by the preputial lamina (PPL) which is attached ventral-medially to the epithelium of the preputial-urethral groove (Fig. 10D–F) or more proximally to the epithelium of the urethra once the edges of the preputial-urethral groove have fused in the midline (Fig. 10G). Finally, in even more proximal sections the attachment of the preputial lamina to the urethra disappears, thus defining the U-shaped clitoral lamina, and establishing confluence between clitoral mesenchyme and preputial mesenchyme (Fig. 10H and I, green double-headed arrows).

At postnatal day 10, transverse sections through female external genitalia reveal the ventral preputial-urethral groove (PUG) distally whose proximal region defines the urethral meatus through which urine is transmitted (Fig. 11A). In more proximal sections the edges of the PUG fuse in the ventral midline to from the urethra (Fig. 11B). Initially the epithelium of the urethra is attached to the epidermis by a transient epithelial cord (Fig. 11B, large arrowhead) which disappears in more proximal sections (Fig. 11C). Dorsal to the PUG and the urethra, islands of genital tubercle mesenchyme appear within the epithelium (Fig. 11A–C). The bilateral islands of genital tubercle mesenchyme seen in the most distal section (Fig. 11A) fuse in the midline to form a single mesenchymal island of genital tubercle mesenchyme (GTM) (Fig. 11B and C). Additional mesenchymal islands appear and fuse with the central mesenchymal island (GTM) to eventually partially surround the epithelium of the urethra (Fig. 11D–F). Finally, the original connections between the circumferential preputial lamina and the urethra disappear thus forming the U-shaped clitoral lamina and establishing ventral confluence between clitoral mesenchyme (within the U-shaped clitoral lamina) and preputial mesenchyme (Fig. 11E–I, green double-headed arrows). Clearly, the overall process involves extensive epithelia remodeling.

The overall process of mouse clitoral development is associated with development of the distal portion of the female urethra, whose epithelium is initially attached to the preputial lamina. Accordingly, the urethra initially develops in association with clitoral stroma, that is, mesenchyme located within the confines of the U-shaped clitoral lamina. With postnatal development the female urethra comes to be located partially within clitoral mesenchyme and partially ventral to the clitoral lamina (Weiss et al., 2012). Clitoral stroma arises from the mesenchyme of the genital tubercle, one of the few features that the mouse clitoris shares with the mouse penis.

In summary, the signature feature of the adult mouse clitoris, the U-shaped clitoral lamina, arises via a process in which the distal growth of the preputial swellings plays a central role in generating the preputial

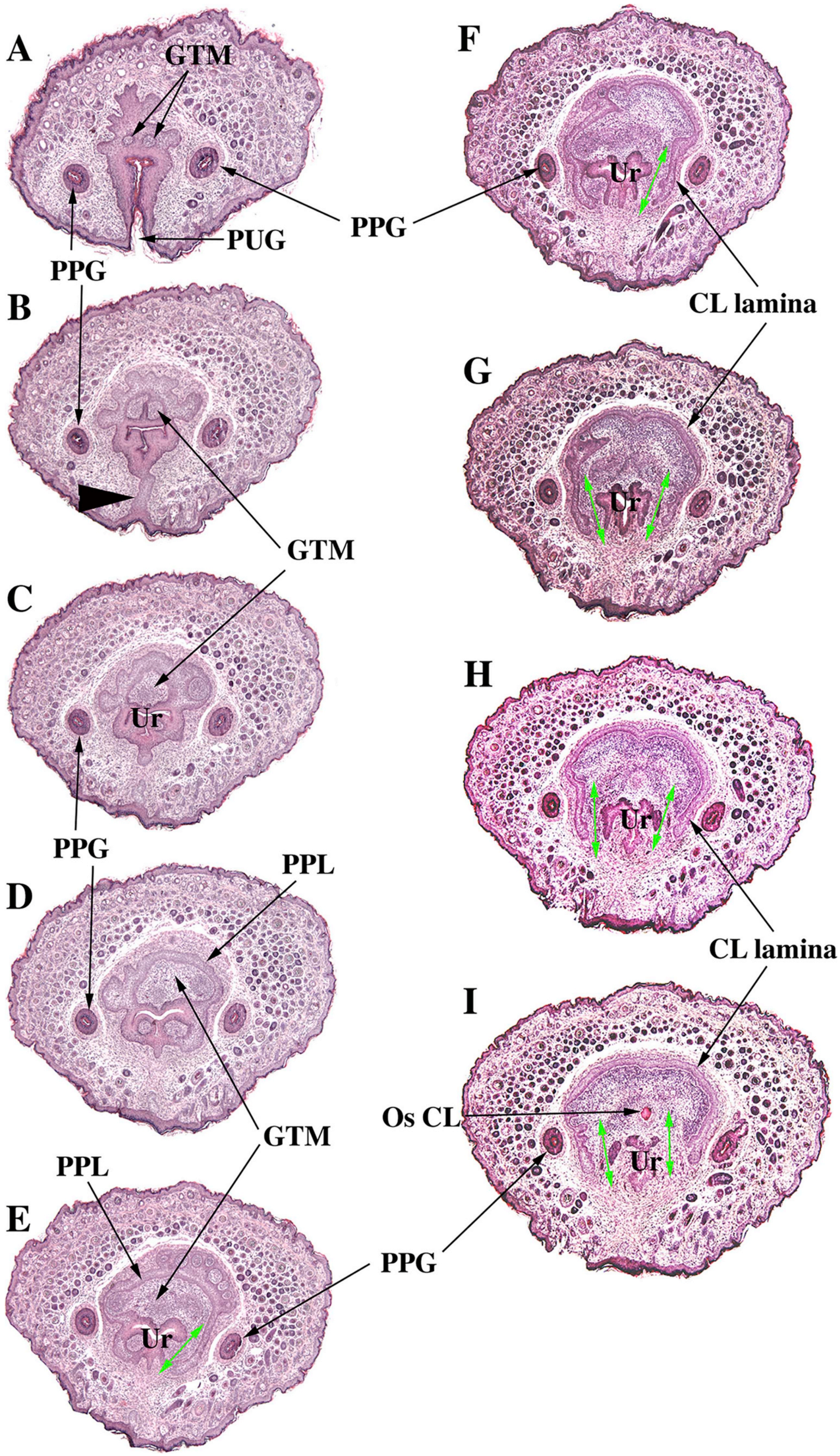

Fig. 11. Transverse sections of GD 10 female external genitalia. In (A) note the preputial-urethral groove (PUG) and the two "islands" of genital tubercle mesenchyme (GTM). Between (A) and (B) the edges of the PUG have fused to form the urethra and a transient epithelial cord (large arrowhead). In (B–E) note coalescent of "islands" of genital tubercle mesenchyme surrounded by the preputial lamina. In (E–I) the attachment of the preputial lamina (PPL) to the urethra (Ur) disappears, thus creating the U-shaped clitoral lamina (CL lamina) and establishing confluence between clitoral stroma and preputial stroma (green double-headed arrows). PPG = preputial gland duct. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 5
Rationale the immunohistochemical markers.

Protein	Rationale	Reference
Estrogen receptor α	Previously reported in mouse genital tubercle which is known to be sensitive to estrogen	(Rodriguez et al., 2012; Sinclair et al., 2016a)
Androgen receptor	Previously reported in mouse genital tubercle	(Baskin et al., 2018;Blaschko et al., 2013 ; Cunha et al., 2005; Liu et al., 2018b; Rodriguez et al., 2012; Shen et al., 2018; Shen et al., 2016; Zheng et al., 2015)
Foxa1	Known endodermal marker expressed in urethral plate and urethra	(Cunha et al., 2018; Robboy et al., 2017)
Uroplakin1	Known marker of mature urothelium	(Yu et al., 1990)
Keratin 6	Known marker of stratified epithelium and epidermis	(Moll et al, 1982, 2008)
Keratin 7	Known marker of developing urothelium	Cunha et al. (2017)
Keratin 8	Known marker of developing urothelium	Cunha et al. (2017)
Keratin 10	Known marker of stratified epithelium and epidermis	(Moll et al, 1982, 2008)
Keratin 14	Known marker of stratified epithelium and epidermis	(Moll et al, 1982, 2008)
Keratin 19	Known marker of developing urothelium	Cunha et al. (2017)

Table 6
Expression of immunohistochemical markers in 16- and 17-day female mouse genital tubercles.

Protein	Epidermis	Ventral Clitoral groove	Urethral plate	Urethral plate mesenchyme	Canalized urethral plate	Preputial-urethral groove	Preputial lamina[a]	Urethra
Estrogen receptor α	No	No	No	No	No	ND	No	No
Androgen receptor	No	No	No	Yes	No	No	No	No
Foxa1	No	No	Yes	No	Yes	Yes	No	Yes
Keratin 7	No	No	Yes	No	Yes	Yes	No	Yes
Uroplakin1	No	No	No	No	No	No	No	No
Keratin 8	No	No	No	No	No	No	No	No
Keratin 19	No	No	No	No	No	No	No	No
Keratin 6	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes
Keratin 10	Yes	Yes	Yes	No	Yes	Yes	No	Yes
Keratin 14	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes

[a] Examined at 17 days only.

lamina from which the clitoral lamina is derived. As an aside the preputial swellings in mice have no counterpart in humans. Also the preputial lamina of males has an entirely different fate. Postnatally the male preputial lamina delaminates to create the preputial space, thus forming the inner lining of the external prepuce and penile surface epithelium (Mahawong et al., 2014; Rodriguez et al., 2011). Thus, the preputial lamina undergoes completely different developmental processes in male versus female mice.

Immunohistochemical markers known to be associated with urogenital tract organs/tissues were examined across the distal to proximal zones of E16 and E17 mouse genital tubercles. The rationale for examining these markers is given in Table 5. At E16-E17 days estrogen receptor α, uroplakin 1, keratin 8 and keratin 19 were undetectable in all female genital tubercle tissues (not illustrated) (Table 6). The androgen receptor was not detected in epithelia within the female genital tubercle but was expressed in the mesenchyme associated with the solid and canalized urethral plate and urethra (Fig. 12A and B). Foxa1 (an endodermal marker) and keratin 7 had identical patterns of expression in epithelium of the solid and canalized urethral plate, preputial urethral groove and urethra (Fig. 12C–G), but not in the ventral clitoral groove (not illustrated) (Table 6). Keratins 10 and 14 exhibited complimentary expression in the epidermis, and in epithelia of the ventral clitoral groove, solid and canalized urethral plate and urethra. Keratin 10 was expressed in suprabasal and apical epithelial cells (Fig. 13D–F), while keratin 14 was expressed in basal cells and immediately adjacent suprabasal cells (Fig. 13G–I). Keratin 6, also a marker of stratified epithelia, exhibited a more extensive expression frequently in both basal and supra-basal layers of the solid and canalized urethral plate, preputial-urethral groove, urethra and the preputial lamina (Fig. 13A–C). In the epidermis keratin 6 was confined to basal layers. The keratinization process within the urethral plate documented via keratin 6, 10 and 14 immunohistochemistry was associated with canalization of the urethral plate to the exterior (Fig. 13A, D, G).

Consistent with the apparent absence of estrogen receptor alpha immunostaining, sections of clitori of DES-treated adult X^{Tfm}/y mice showed no evidence of estrogenic stimulation in the U-shaped clitoral lamina, while the nearby vaginal epithelium was thick and cornified, consistent with intense estrogenic stimulation (Fig. 14A). Indeed the histologic phenotype of the U-shaped clitoral lamina was identical in the DES-treated (Fig. 14A) and the untreated controls (Fig. 14B).

3.2. Human clitoral anatomy and development

The adult human clitoris is an erectile structure that is anatomically homologous with the penis. The adult clitoral glans projects into the vaginal vestibule, which is enclosed between the cranial aspect of the

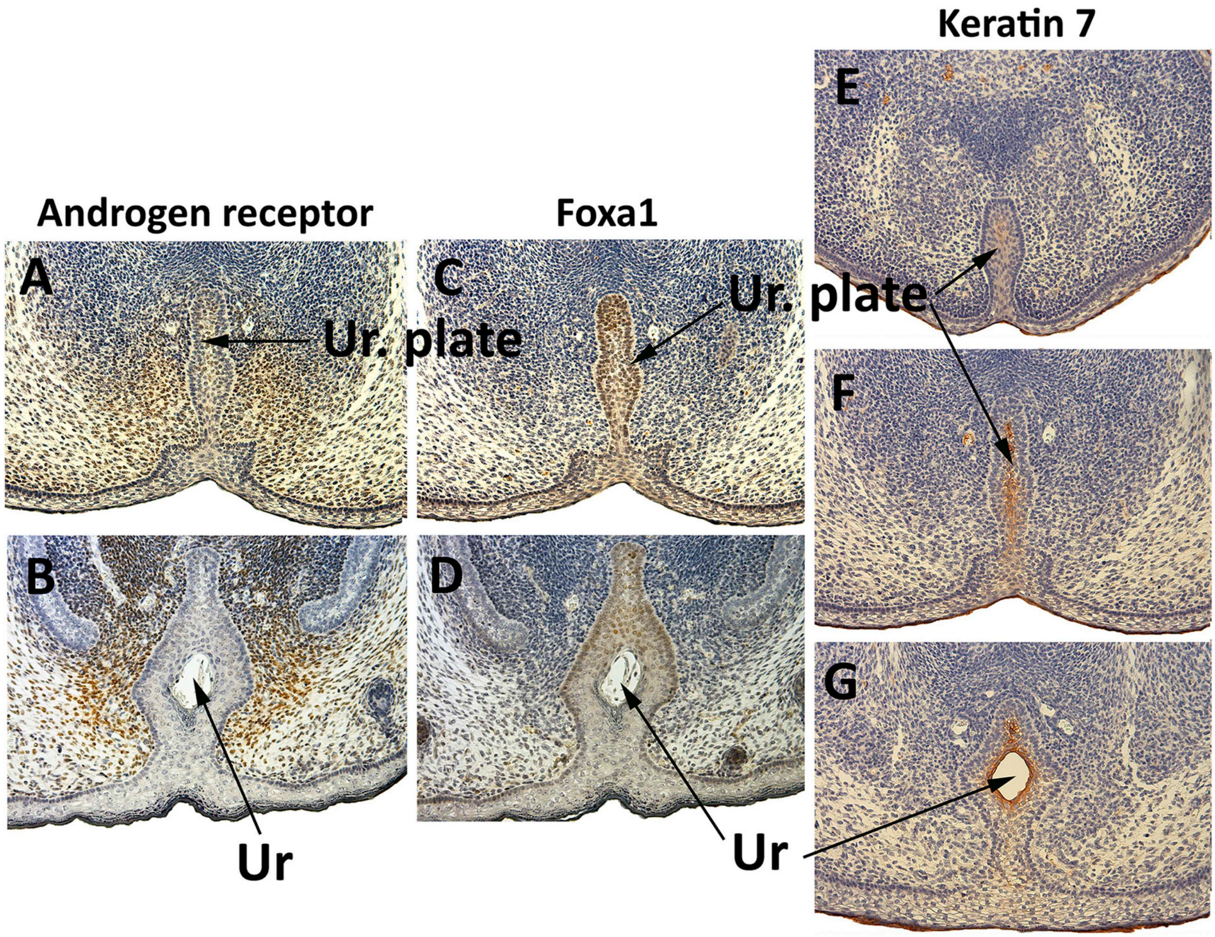

Fig. 12. Immunohistochemistry of E17 female mouse genital tubercles. Note androgen receptor expression in mesenchyme associated with the urethral plate (Ur. Plate) and urethra (Ur). Note Foxa1 and keratin 7 expression in epithelium of the urethral plate (Ur. Plate) and urethra (Ur).

labia minora, part of which forms the prepuce of the clitoris (Clemente, 1985; Cold and Taylor, 1999; Warick and Williams, 1973). Mice do not have labia (minora or majora). The body of the clitoris consists of corpus cavernosum whose crura are attached proximally to the ischio-pubic rami and are covered by the ischiocavernosus muscles (Clemente, 1985; Warick and Williams, 1973). Thus, the clitoris is basically a smaller version of the penis with the exception that the urethra is separate from the clitoris. The anatomy of the human clitoris is in stark contrast to that of the mouse (Table 2), and accordingly human clitoral development is significantly different from that of the mouse. The human clitoris and penis develop from the genital tubercle, which forms at 6 weeks (Grumbach and Ducharme, 1960) and at 8 weeks is a prominent perineal projection (Fig. 15). In humans the genital tubercle is not associated with preputial swellings comparable to those of the mouse in morphology, location or developmental fate. Proximally the human genital tubercle is associated with bilateral labial-scrotal swellings that form the scrotum in males and labia majora in females (Moore and Persaud, 2003), which are absent in mice. Wholemount images of developing human male and female external genitalia emphasize the remarkable similarity in the shape of the developing male and female genital tubercles (precursors of the penis and clitoris) from the ambisexual stage (7–9 weeks) to about 10 weeks (Fig. 15). Beyond 10 weeks clitoral length lags behind that of the developing penis, and the clitoris becomes surrounded by the labia minor and labia majora (Fig. 15). In males the urethra forms within the penile shaft and glans (Li et al., 2015), whereas in females the urethra opens to the exterior at the "base" of the clitoris (Baskin et al., 2018; Overland et al., 2016). Note also that as development proceeds the clitoris retracts towards the body wall and becomes embraced by the labia minora and labia majora. Both male and female genital tubercles develop epithelial tags visible near the tip of the glans from 10 to 13 weeks of gestation (Figs. 15–17). These epithelial tags subsequently disappear after ~13 weeks of gestation (Fig. 15). At ~13 weeks of gestation a prepuce forms dorsally and extends ventrally around the glans of both the male and female genital tubercles. The prepuce becomes circumferential in males by 14–15 weeks of gestation, in contrast to the female where the ventral aspect of the prepuce does not fuse in the midline (Fig. 15, yellow arrows).

From the onset, female (and male) genital tubercles develop a solid urethral plate (Fig. 16) which beginning at ~9 weeks canalizes to form a wide urethral (males) or vestibular (females) groove, which is particularly evident at 10 weeks and thereafter (Figs. 17–19). The more

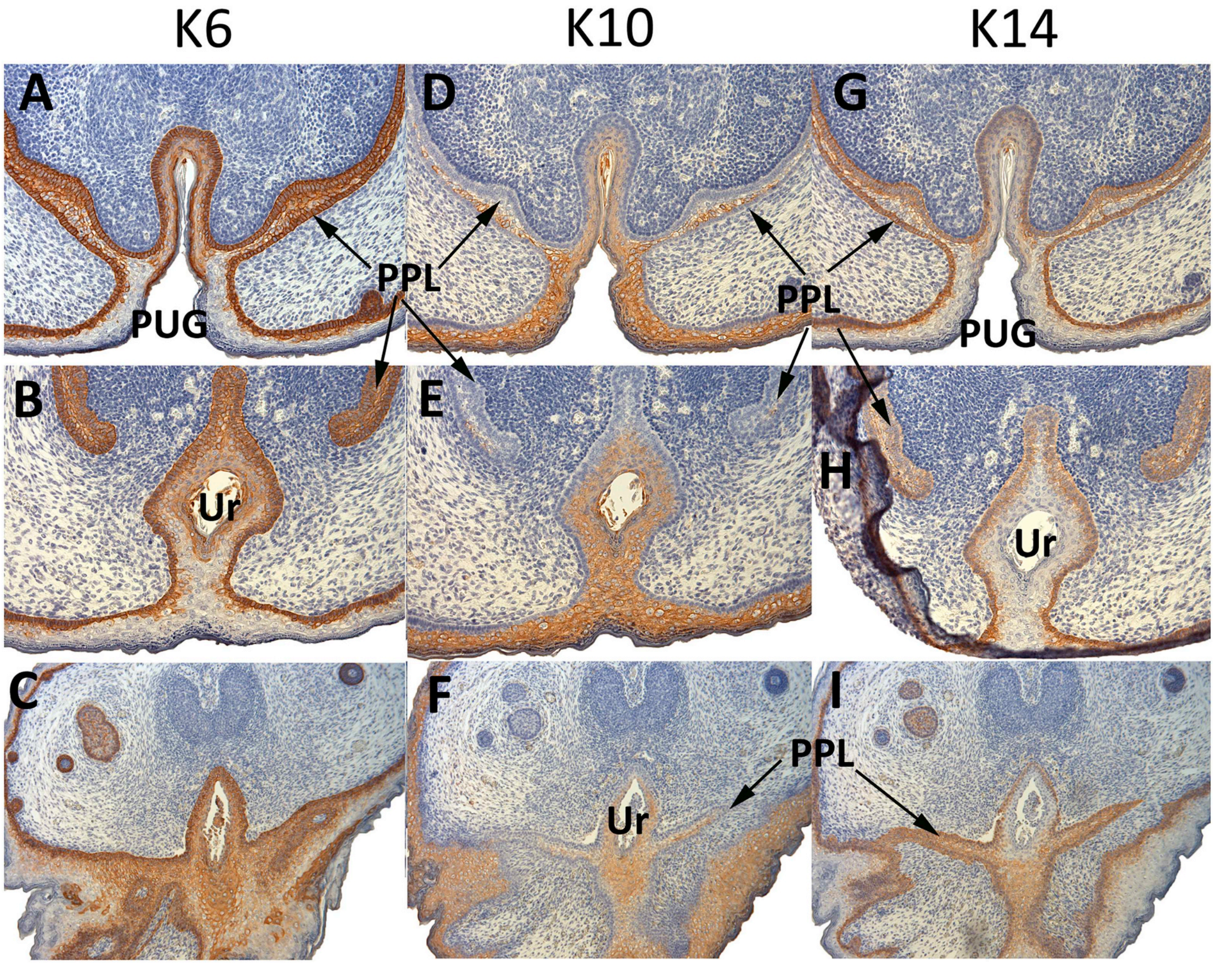

Fig. 13. Immunohistochemistry of E17 mouse genital tubercle for keratins 6, 10 and 14. All 3 keratins are expressed in the epidermis but differ in which layers they are expressed. Notably keratin 10 was weakly expressed or not at all in the preputial lamina. PUG = preputial-urethra groove, Ur = urethra, PPL = preputial lamina.

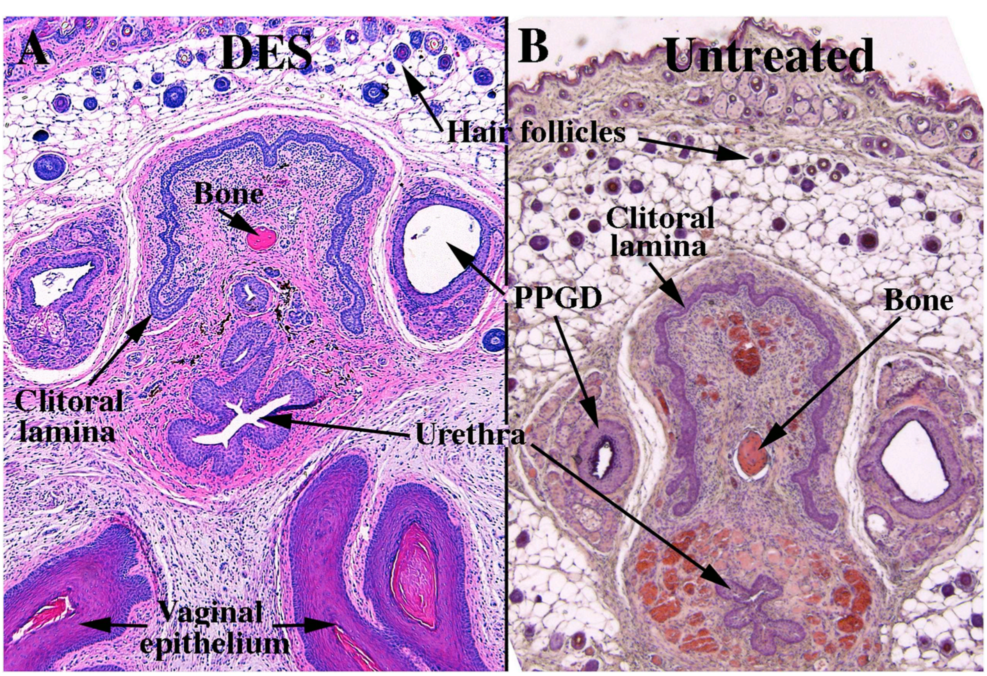

Fig. 14. Section of external genitalia of adult X^{Tfm}/y mice treated for 3 weeks via subcutaneous pellet of diethylstilbestrol (DES) or untreated. In the DES-treated specimen (A) the vaginal epithelium is thick and cornified consistent with estrogenic stimulation, while the U-shaped clitoral lamina of the DES-treated specimen is identical histologically to that of the untreated control (B). PPGD = preputial gland duct, Ur = urethra.

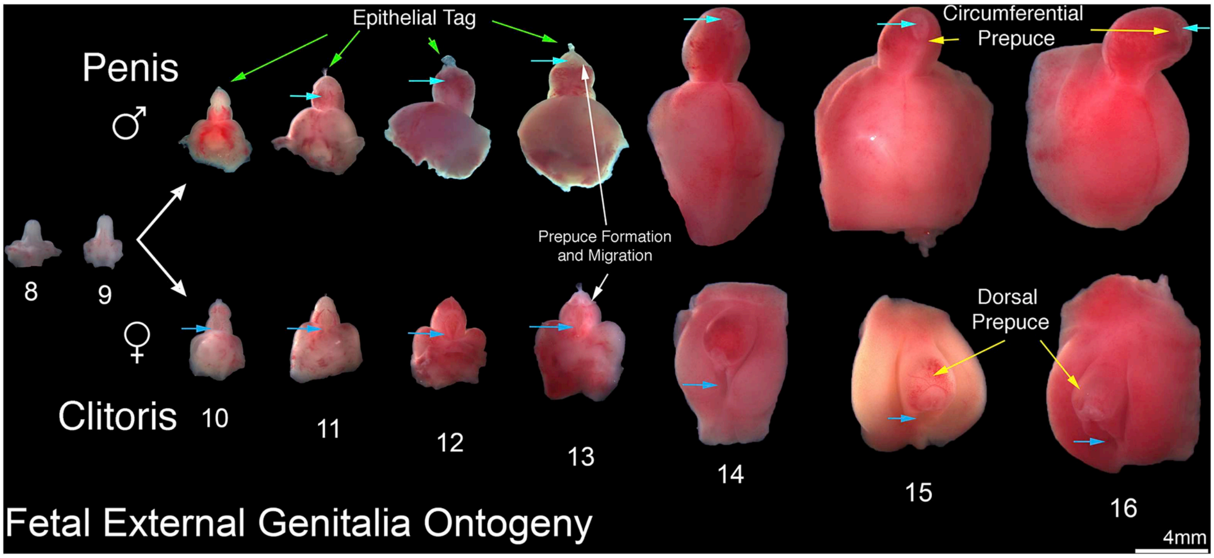

Fig. 15. Fetal External Genitalia Ontogeny: Representative ventral views of external genitalia of the human males (top row) and females (bottom row) at 8–16 weeks of gestation. Note the morphologic differences that emerge in male and female specimens after the indifferent stage (8–9 weeks of gestation) with formation of the penile urethra within the shaft due to urethral fold fusion in the penis and lack of urethral (vestibular) fold fusion in female specimens (light blue arrows depict the location of the urethra meatus in both the male and female specimens). Note the divergent evolution of the male and female prepuce (yellow arrows) with complete circumferential formation of the prepuce at 14–16 weeks of gestation in males and a resulting dorsal prepuce in females. The epithelial tag is seen in both male (green arrows) and female (clearly visible without arrows) specimens from 10 to 13 weeks of gestation disappearing after this time point. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

appropriate terminology for the "urethral plate" and "urethral groove" in females is vestibular plate and vestibular groove as these structures are involved in the development of the vaginal vestibule (Overland et al., 2016). The lateral edges of the vestibular groove (vestibular folds) do not fuse (as is the case in males) and remain separate to form the labia minora (Figs. 17 and 18), which cranially are associated with the glans clitoris. Consequently, as the clitoris appears to "retract" towards the body wall and is embraced by the labia minora and labia majora, the tip of the clitoris (glans clitoris) continues to project freely into the forming vaginal vestibule, thus defining the definitive mature morphology (Figs. 15 and 18) (Clemente, 1985). "The (human) clitoris is in many details a smaller version of the penis, but it differs basically in being entirely separate from the urethra" (Warick and Williams, 1973). Thus, both the human penis and clitoris develop a glans and a body (shaft in males) containing a corporal body whose crura attach to the inferior pubic rami (Warick and Williams, 1973).

Transverse sections of the developing human clitoris further our understanding of the morphogenetic process (Fig. 19). A vestibular plate is seen distally in transverse sections of the female genital tubercle (Figs. 15 and 19A and), which more proximally begins to canalize (Fig. 19B). The canalization process generates a wide vestibular groove (Fig. 19C–E) which can be traced proximally to the closed vestibule (Fig. 19F).

4. Discussion

We have undertaken this study to fill a striking void in developmental biology of the mammalian genital tract, namely, development of the mouse clitoris. While there are several reports on development of male mouse external genitalia, development of the mouse clitoris and its comparison to human clitoral development is almost completely non-existent in the literature. Indeed the only publication dedicated to this subject is a brief report by Schlomer et al. (2013) on postnatal mouse clitoral development. In mouse embryos, male and female external genitalia are composed of the genital tubercle and preputial swellings. Development of the genital tubercle can be recognized at ~ E11.75 in the mouse (Perriton et al., 2002), and shortly thereafter the urethral plate can be observed in both male and female genital tubercles (Hynes and Fraher, 2004a, b; Petiot et al., 2005; Seifert et al., 2008; Yamada et al., 2006). Bilateral preputial swellings can be recognized extending laterally from the mouse genital tubercle at E14 (Cunha, 1975; Perriton et al., 2002; Petiot et al., 2005). It is perhaps worth noting that the preputial swellings of the mouse do not have a counterpart in humans. The labial-scrotal swellings of human fetuses are destined to form the scrotum in males and the labia majora in females. Mice do not have labia majora or labia minora, and mouse preputial swellings do not form the scrotum. These adult anatomic differences are, of course, reflective of dramatic species differences in development.

Mouse clitoral morphogenesis is a function of the developmental fate of the preputial swellings which grow to the ventral midline where they fuse and eventually completely cover the genital tubercle (Perriton et al., 2002; Petiot et al., 2005), a process that occurs identically in male (Liu et al., 2018) and female mouse fetuses. As the preputial swellings grow distally, the preputial lamina is laid down, presumably as a result of fusion of epidermis of the preputial swellings with the epidermis of the genital tubercle, again a process identical to that in males (Liu et al., 2018) and without a counterpart in humans.

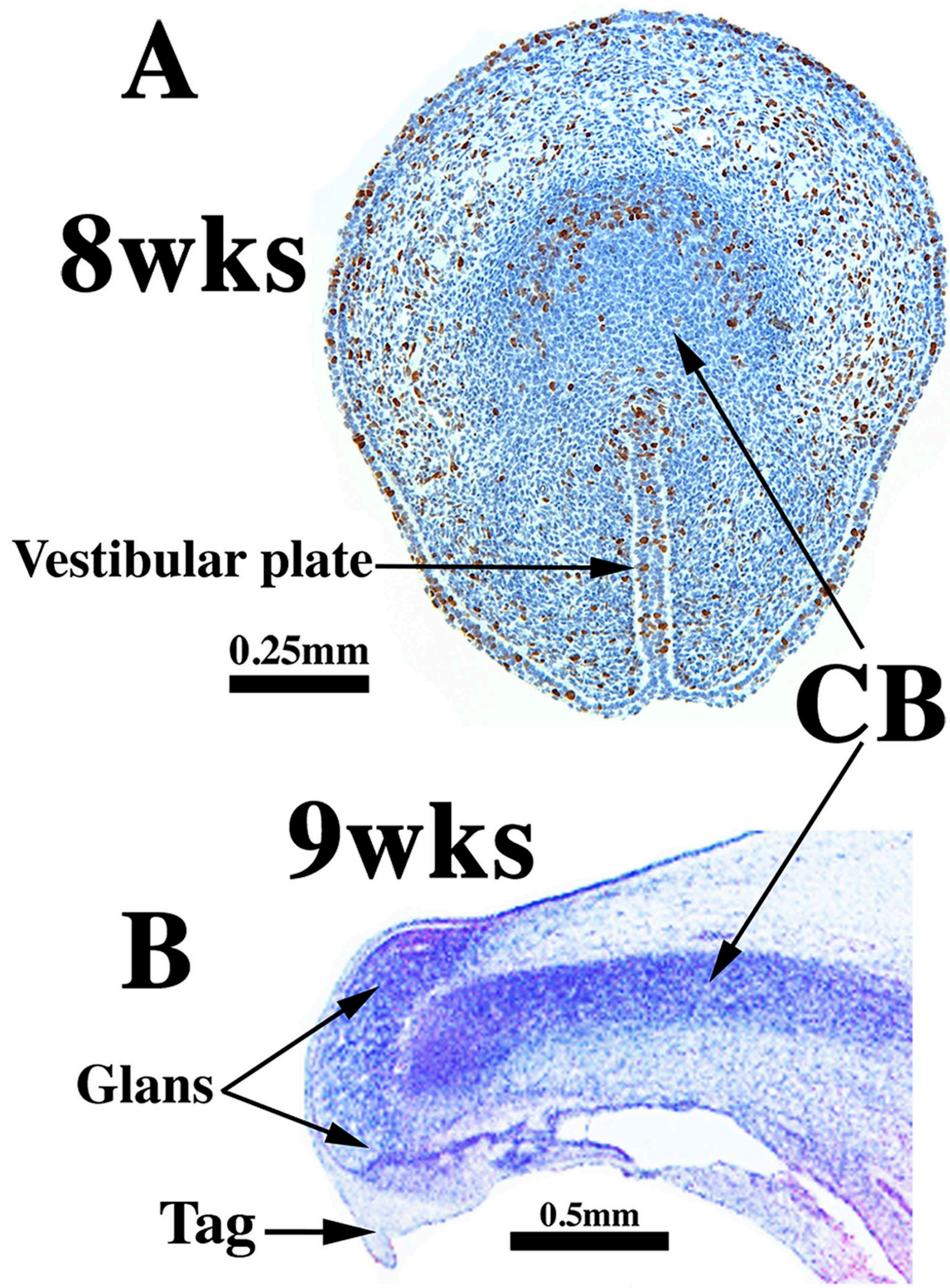

Fig. 16. Transverse (A) and mid-sagittal sections of human female genital tubercles at the ages specified (ambisexual stage). Note the vestibular plate in (A), the corporal bodies (CB) in (A & B) and the glans in (B). (A) has been stained for Ki67 and (B) has been stained with H&E.

Embryonic, neonatal and adult female mice have a perineal appendage. Prenatally the perineal appendage is properly called the genital tubercle. At birth and thereafter, the female genital tubercle is covered by the preputial swellings, and thus the term "perineal appendage" is more appropriate. In adulthood, the female perineal appendage is prepuce as judged by histologic criteria and is not the clitoris. The mouse clitoris is an internal structure defined by a U-shaped clitoral lamina (Martin-Alguacil et al., 2008a; Weiss et al., 2012). One feature particularly unique to development of female mouse external genitalia is an extensive preputial-urethral groove in the perineal appendage which is evident from fetal to adulthood stages (Figs. 3 and 9B). Presence of the preputial-urethral groove on the distal

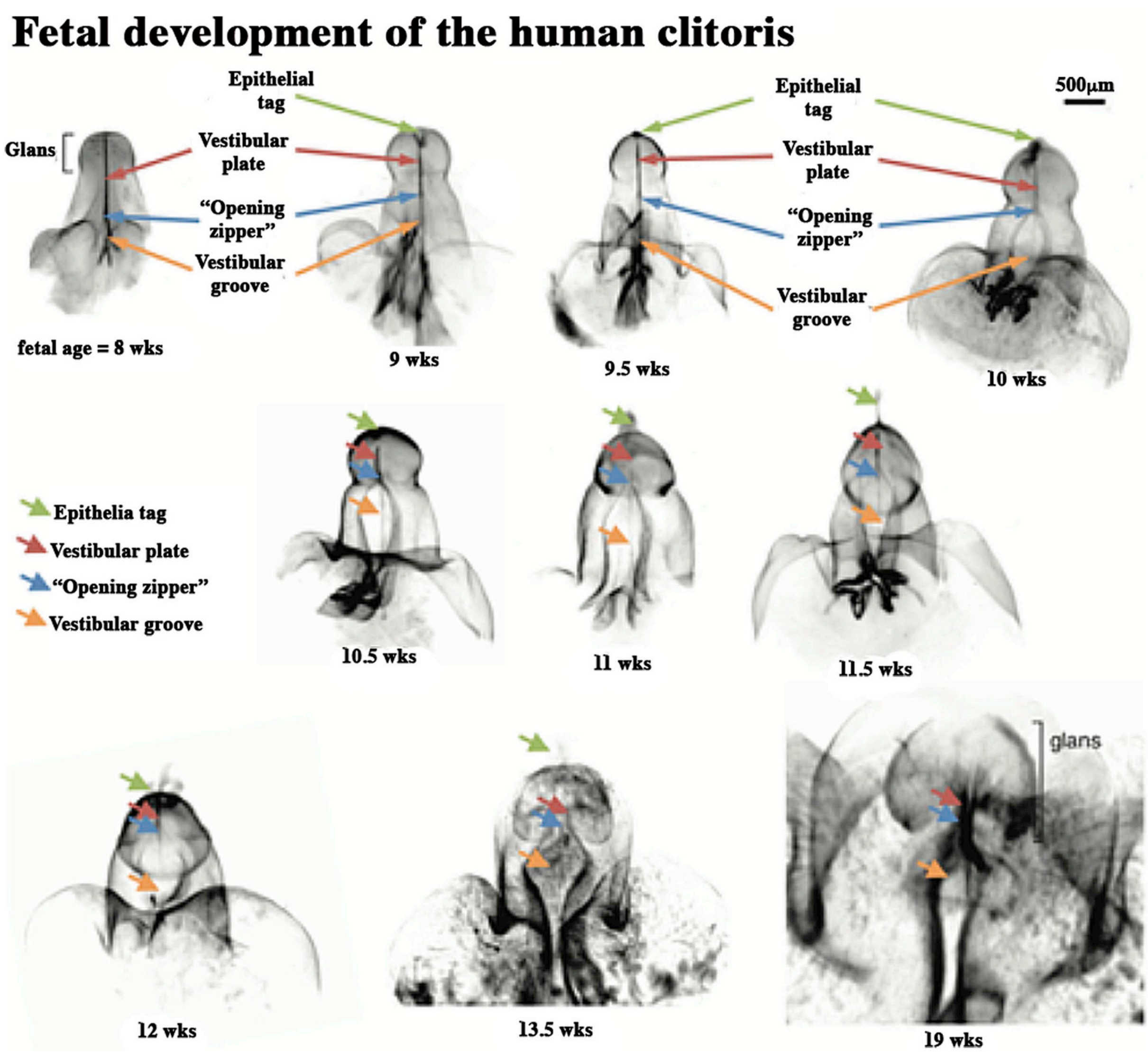

Fig. 17. Optical Projection Tomography of the developing human clitoris from 8 to 19 weeks of gestation. Note the opening zipper that facilitates canalization of the vestibular plate to form the wide vestibular groove. Also note the lack of closing zipper with the vestibular groove remaining open.

surface of the adult perineal appendage raises the question of how the preputial-urethral groove extends to the distal terminus of the female perineal appendage. The two likely scenarios are: (a) Complete canalization of the urethral plate distal to the urethral meatus (Fig. 9A) to extend the groove distally, and/or (b) distal extension of the hair-bearing preputial swellings, thus extending the preputial-urethral groove to the distal surface of the adult mouse female perineal appendage (Fig. 9). Clearly, considerable epithelial remodeling is involved.

One epithelial differentiation event observed prenatally involves the ventral clitoral groove (VClGr) and the urethral plate, which initially (E14-16) are non-keratinized, but that subsequently become keratinized and thus express the characteristic keratins. These differences in epithelial differentiation can be recognized in the H&E stained sections (Figs. 6–8) and has been verified by keratin immunohistochemistry (Fig. 13). Thus, as development proceeds the urethral plate, the preputial-urethral groove and the urethra becomes positive for keratins 6, 10 and 14 all known to be expressed in keratinized epithelia (Moll et al, 1982, 2008). The keratinization process is associated (and likely causally) with canalization of the urethral plate (Fig. 13).

Immunohistochemistry of Foxa1 and keratin 7 reveal a likely contribution of endodermal epithelial cells to the distal aspect of the developing female urethra. Foxa1 is a known marker of endoderm (Besnard et al., 2004; Diez-Roux et al., 2011; Robboy et al., 2017) and is expressed in epithelia of the urogenital sinus and its male and female derivatives, bladder, prostate and urethra, in which keratin 7 is

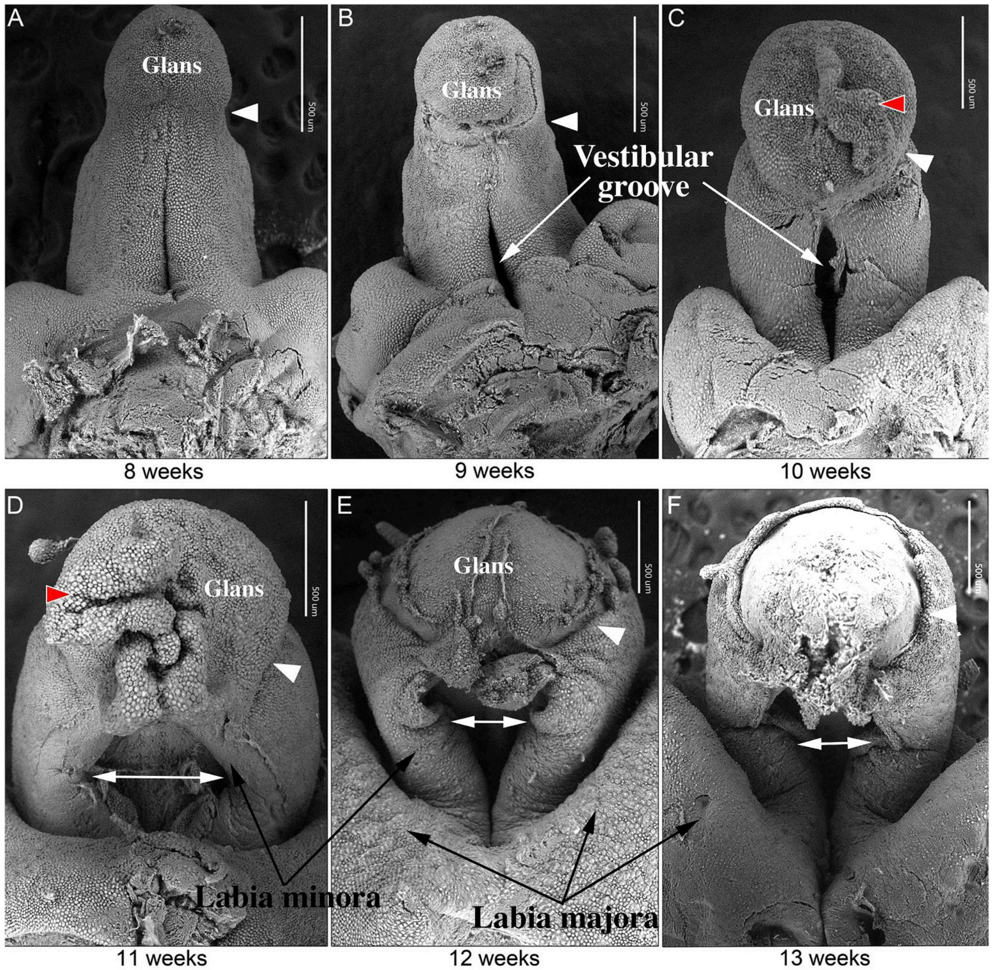

Fig. 18. SEM ontogeny of the developing human fetal clitoris from 8 to 13 weeks of gestation in ventral view. White arrowheads indicate the transition from clitoral shaft to glans; red arrowheads indicate the distal epithelial tag. Double-headed white arrows = vestibular groove. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

typically co-expressed (Cunha et al, 2017, 2018). Both of these markers were co-expressed in the female urethral plate and urethra suggesting a contribution of endoderm to the distal aspect of the female urethra, an observation that compliments similar findings for the distal aspect of the mouse penile urethra (Liu et al., 2018). Finally, androgen receptors were detected in the mesenchyme surrounding the prenatal female mouse urethral plate and urethra. This observation in and of itself does not imply that androgens are involved in development of female external genitalia, as androgen levels are thought to be non-existent in female fetuses and evidence of androgen action is lacking globally in female fetuses.

Finally, morphology of adult external genitalia is the endpoint of developmental processes, and thus vast differences in adult clitoral morphology in mice versus humans is indicative of vast differences in developmental processes. Now that we have described the salient steps in development of the mouse clitoris, it is possible to compare human and mouse clitoral anatomy and development, which are substantially different (Table 7). The human clitoris is known to be an anatomic homologue of the penis (Clemente, 1985; Warick and Williams, 1973), and both the human penis and clitoris develop directly from the genital tubercle (Baskin et al., 2018; Li et al., 2015; Overland et al., 2016). In contrast, the mouse clitoris is defined by the U-shaped clitoral lamina which is derived from the preputial lamina formed via fusion of the mouse preputial swellings with the genital tubercle. Humans do not have preputial swellings. Thus, there is scant justification for using the mouse as a model of human clitoral development.

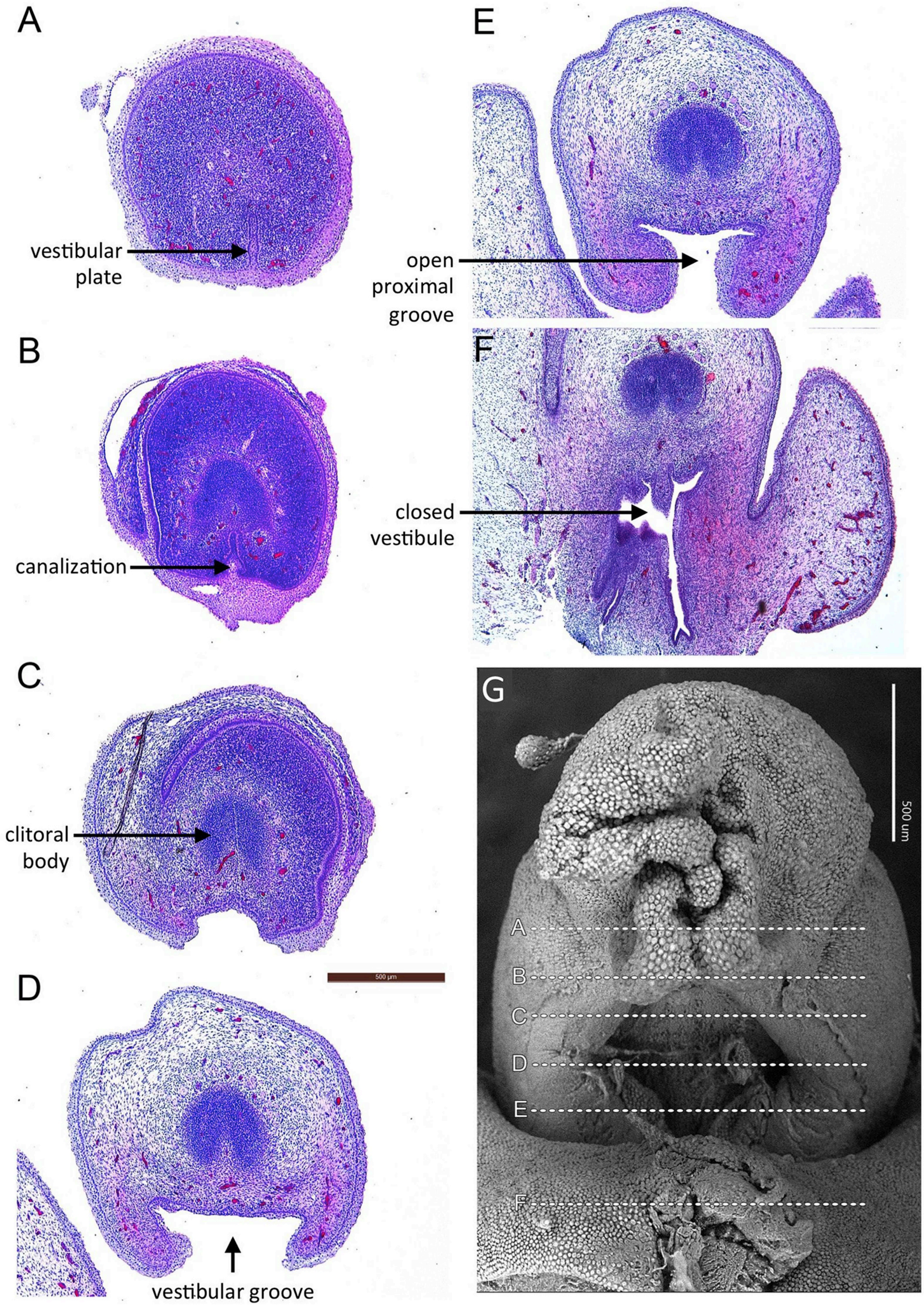

Fig. 19. Transverse sections from human fetal clitoris at 11 weeks of gestation stained with hematoxylin and eosin demonstrating (A) the solid vestibular plate, (B) beginning of canalization process, (C) distal open vestibular groove, (D) mid-shaft wide vestibular groove, (E) proximal open vestibular groove and (F) closed vestibule at the levels indicated in (G).

Table 7
Anatomic and developmental features of the human versus mouse clitoris.

Feature	Human	Mouse	Comment
Preputial swellings	Absent	Present	Central role in mouse clitoral development
Genital tubercle	Present	Present	Human genital tubercle is the direct precursor of the clitoris. Mouse genital tubercle forms clitoral stroma.
Stromal derivative of genital tubercle mesenchyme	Present	Present	Mouse clitoral stroma confluent with preputial stroma. Not the case for human.
Urethral/vestibular plate	Present	Present	Canalizes to form the vestibule in humans. Canalizes to form the preputial-urethral groove in mice.
Labial-scrotal swelling	Present	Absent	Forms labia majora in humans
Preputial lamina	Present	Present	Precursor to mouse clitoral lamina
U-shaped clitoral lamina	Absent	Present	Defining feature of mouse clitoris
Clitoral glans	Present	Absent	Projects into vestibule in humans
Preputial-urethral groove	Absent	Present	Present from embryonic to adult stages in mice
Prepuce of the clitoris	Present	Absent	
Labia minora	Present	Absent	
Labia majora	Present	Absent	
Clitoral crura	Present	Absent	Covered by ischiocavernosa muscles in humans
Clitoral corporal body	Present	Absent	
Ischiocavernosa muscles	Present	Absent	

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