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Development of the human prepuce and its innervation

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Abstract

Development of the human prepuce was studied over the course of 9 to 17 weeks of gestation in 30 specimens. Scanning electron microscopy revealed subtle surface features that were associated with preputial development, namely the appearance of epidermal aggregates that appeared to be associated with formation of the preputial fold. Transverse and sagittal sections revealed that the epidermis of the glans is considerably thicker than that of the penile shaft. We described a novel morphogenetic mechanism of formation of the preputial lamina, namely the splitting of the thick epidermis of the glans into the preputial lamina and the epidermis via the intrusion of mesenchyme containing red blood cells and CD31-positive blood vessels. This process begins at 10 to 11 weeks of gestation in the proximal aspect of the glans and extends distally. The process is likely to be androgen-dependent and mediated via androgen receptors strategically localized to the morphogenetic process, but signaling through estrogen receptor may play a role. Estrogen receptor alpha (ESR1) has a very limited expression in the developing human glans and prepuce, while estrogen receptor beta (ESR2) is expressed more broadly in the developing preputial lamina, epidermis and urethra. Examination of the ontogeny of innervation of the glans penis and prepuce reveals the presence of the dorsal nerve of the penis as early as 9 weeks of gestation. Nerve fibers enter the glans penis proximally and extend distally over several weeks to eventually reach the distal aspect of the glans and prepuce by 14 to 16 weeks of gestation.

Keywords

Prepuce; preputial lamina; frenulum; epidermal delamination; androgen receptor; hypospadias

I. Introduction

Schweigger-Seidel (1866) is credited with the first report on development of the human prepuce published. He stated that the human prepuce develops as a fold (of skin) that “covers the glans progressively, the epithelium covering the deep aspect of the (preputial) fold fusing with the epithelium covering the glans” (Schweigger-Seidel, 1866). Since then

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many accounts of human preputial development have appeared culminating in Glenister's report in 1956, which provides an excellent review of the subject (Glenister, 1956). Theories on development of the human prepuce fall into two opposing ideas. One idea, consistent with Schweigger-Seidel, is that development of the human prepuce involves formation and distal extension of the preputial fold to eventually completely cover the glans. This idea is exemplified by Hunter who described "folds of ectodermal tissue that appear to flow over the dorsum of the glans as the beginning of the prepuce" leaving the preputial lamina is its wake (Hunter, 1935) (Fig. 1 A, B1–D1). An alternate theory is that "the prepuce results from an ingrowth and subsequent breaking down of a cellular lamella", namely the preputial lamina. This idea was proposed by Glenister (1956) (Fig. 1 B2–D2), who incorporated the early views of Schweigger-Seidel (1866) and Hunter (1935), stating that "the preputial fold rolls over the base of the glans", but adds the idea that "basal layers of the resultant epithelial lamella proliferate to form an ingrowing shelf (preputial lamina), the depth of which is accentuated by distal extension of the preputial fold" (Glenister, 1956). Glenister's idea, illustrated in figures 1 B2–D2, is supported by several reports (Cold and Taylor, 1999; Johnson, 1920; Wood-Jones, 1910).

Since 1956 to our knowledge there is only one report on development of the human prepuce (Liu et al, 2018b), as a PubMed search using the terms "preputial/prepuce, human and development" only yielded our recent report entitled "*Human glans and preputial development*" (Liu et al, 2018b). Our brief report was based upon analysis of both transverse and sagittal sections of a limited number of human fetal specimens, which are now augmented by more detailed analysis of a wider array of specimens covering the earliest stages (8 weeks) of human preputial development to advanced preputial development at 17 weeks of gestation. Transverse sections demonstrate fusion of the bilateral halves of the preputial lamina in the mid-ventral midline during development as reported previously (Cunha et al, 2019; Liu et al, 2018b). The mid-ventral fusion of the preputial lamina is incomplete, as a layer of stroma is retained between the two sides of the preputial lamina as a component of the frenulum that attaches the prepuce with the ventral surface of the glans (Clemente, 1985). Based upon a limited number of transverse sections, we proposed the idea of a dorsal thickened patch of epidermis of the 13-week glans, which we called the "preputial placode". This structure appeared to split into two layers to form the preputial lamina and the glanular epidermis, respectively, separated by a layer of mesenchyme. Based upon a much larger sample size, it is evident the term "preputial placode" is inappropriate as the thickening of the glanular epidermis occurs globally in the glans as reported previously (Hart, 1908) and emphasized below. The process of human preputial development begins dorsally or dorsal-laterally and spreads ventral to eventually cover the glans almost in its entirety (Liu et al, 2018b). Recognizing that our earlier report on development of the human penile prepuce was incomplete, we have acquired additional specimens that span a wider age range to more fully explore human preputial development utilizing scanning electron microscopy, histology, and immunohistochemistry.

The prepuce is richly innervated and exhibits features of a specialized sensory mucosa complete with Meissner's corpuscles (Taylor et al, 1996). In addition to sensory neurons that facilitate sexual response, the penis also receives autonomic fibers that play a role in regulating blood flow, with parasympathetic and sympathetic fibers serving vasodilation and

vasoconstriction via effects on penile smooth muscle (Steers, 2000). Erection is elicited via parasympathetic neurons (Giuliano and Rampin, 2000; Nazir et al, 2004; Steers, 2000). Parasympathetic and sympathetic input to the penis is via the pelvic plexus from which emerge the cavernous nerves. The cavernous nerves exit the pelvis by two routes, but both terminating in the erectile bodies of the human penis, the corporal body and corpus spongiosum. The penis receives somatic sensory fibers from the pudendal nerve via its branch, the dorsal nerve of the penis (Clemente, 1985). Sensory input from the penile skin, prepuce, and glans conveyed via the dorsal nerve of the penis initiates and maintains erections (Steers, 2000). The development of penile innervation and specifically that of the glans and prepuce is under-represented in the literature. Yucel et al defined the input of cavernous, perineal, and the dorsal nerves of the glans penis, to the corporal body and human penile urethra using neuron specific immunohistochemical staining and 3-dimensional reconstruction on specimens 17.5 to 32 weeks of gestation (Yucel and Baskin, 2003). The present study uses S100 immunostaining to examine innervation of the prepuce and glans penis from 9 to 16 weeks of gestation, thus providing information on early stages of penile innervation. S-100 is a protein expressed in Schwann cells and some neurons (Gonzalez-Martinez et al, 2003).

II. Materials and Methods

First and second trimester lower human fetal urogenital tracts 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 was estimated using heel-toe length (Drey et al, 2005). Note, that we report age from time of fertilization and not from last menstrual period. Gender was determined using PCR to 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 16-week human fetal male specimens were processed for histologic and immunohistochemical staining.

Human fetal penile specimens were fixed in 10% buffered formalin and serially sectioned sagittally or transversely at 6 μm. Every 20th section was stained with hematoxylin and eosin to assess morphology. Intervening sections were processed for immunohistochemistry using the following primary antibodies (Table 1).

Surface details of the developing glans penis were elucidated via scanning electron microscopy (SEM). External genitalia were dissected and fixed in 2% glutaraldehyde 0.1M sodium cacodylate buffer at pH of 7.2 for 6 hours. The specimens were then fixed in 2% osmium tetroxide for 2 hours, subsequently dehydrated in serial alcohol solutions and critical point dried in a Tousimis AutoSamdri 815 Critical Point Dryer (Tousimis, Rockville, MD). The specimens were then mounted on a stub with carbon tape, and images were obtained using a Hitachi TM-1000 Scanning Electron Microscope (Hitachi High Technologies America, Inc. Pleasanton, CA). The SEM analysis is based upon 8 specimens ranging in age from 8 to 17 weeks of gestation.

III. Results

The adult human prepuce is a circumferential flap of highly vascular skin largely covering the glans penis. Its outer surface is continuous with skin of the penile shaft and is covered by a glabrous stratified squamous keratinized epithelium. Its inner mucosal surface is lined by variably-keratinized squamous epithelium dependent upon friction (Taylor et al, 1996) and defines the preputial space (Fig. 2). A frenulum attaches the ventral inner surface of the prepuce to the surface of the glans in the depth of the preputial space (Fig. 2).

A. Surface features of the developing penis.

By SEM analysis the developing human prepuce cannot be discerned until 10–11 weeks of gestation when subtle surface features can be observed on the glans near the coronal sulcus (the groove separating the glans from the shaft) (Fig. 3). For example, beginning at 10–11 weeks epidermal aggregates project from the lateral and dorsal surfaces of the glans at the glans/shaft interface (Fig. 3A–D & A1 & D1). These small epidermal aggregates become more numerous in the ensuing weeks and by 13 weeks can be seen at the edge of a fold of skin which we interpret as the preputial fold (Fig. 3D & D1, asterisks). By 15 to 16 weeks the preputial folds have completely covered the glans, the ventral margins of the prepuce have fused in the ventral midline, and the epidermal aggregates are no longer present having apparently been sloughed (Fig. 3E–F).

B. Morphogenesis of the preputial lamina

Histologic sections are particularly informative and provide the first clue to preputial development at 9.5 weeks when it is evident that the epidermis covering the penile glans is thicker than the more proximal epidermis of the penile shaft (Figs. 4 & 6) as reported previously (Hart, 1908; Liu et al, 2018b). Analysis of mid-sagittal sections of the human penile glans at latter stages (≥10 weeks) reveals that the thickened epidermis of the glans splits due by intrusion of mesenchyme containing patches of red blood cells. This red blood cell enriched mesenchyme divides the thickened epidermis of the glans into two layers: (a) the epithelium of the preputial lamina and (b) the outer surface epidermis (Figs. 5–6). This process begins dorsally (Fig. 9E–F) or dorsal-laterally (Fig. 5 & 9B) and extends distally and ventrally. In a 10-week specimen the splitting and remodeling of the thickened glans epidermis began laterally (Fig. 5, arrowheads).

At 11.5 weeks (Fig. 6A & B), formation of the preputial lamina has extended only part way to the distal tip of the glans. The preputial lamina forms via the intrusion of a blood-vessel-rich mesenchyme which splits the thick epidermis of the glans into two layers: (a) the thin epidermis of the outer surface of the glans and (b) the preputial lamina. With developmental progression the red blood cell rich mesenchyme is consistently seen at/near the point of splitting of the glanular epidermis. The preputial lamina has two basal surfaces, one facing the mesenchyme of the glans (glanular basal layer, Gbl) and the other basal layer facing the surface epidermis (Ebl). The basal epithelial layer of the preputial lamina facing the mesenchyme of the glans (Gbl) (Fig. 6D) is composed of epithelial cells that are basophilic and cuboidal/columnar. In contrast, the basal epithelial layer of the preputial lamina facing

the epidermis (Ebl) is composed of flattened (squamous) cells (Fig. 6D), similar to that of the epidermis of the penile shaft (Fig. 6D).

By 14.5 weeks splitting and remodeling of the epidermis of the glans has progressed distally in the dorsal midline to the distal tip of the glans indicating that formation of the preputial lamina has progressed nearly to its final distal extent. Accordingly, the density of red blood cell patches within the developing prepuce becomes reduced dorsally (Fig. 7). However, a red blood cell rich mesenchyme (Fig. 7, BV) was seen ventral to the urethra, perhaps indicative of continued morphogenetic activity to define the urethral meatus.

To further explore the nature of the red blood cell rich mesenchyme, sagittal sections were immunostained with antibodies to CD31 (endothelial marker) and hemoglobin (Fig. 6E). Our identification of red blood cells was confirmed by immunostaining with anti-hemoglobin (Fig. 6E). CD31 immunostaining indicated that the red blood cells are associated with blood vessels lined by endothelium (Fig. 6E). Thus, the thick epidermis of the glans is split into two layers by mesenchyme containing red blood cells and blood vessels. The process of preputial lamina formation involving epidermal splitting and epithelial remodeling typically initiates dorsally (Fig. 6) or dorsal-laterally (Fig. 5 & 9B) but progresses ventrally and distally. The ventral aspect of the preputial lamina is seen in favorable sagittal sections cut lateral to the midline (Fig. 8). It is notable that at 14–16 weeks of gestation when preputial development has extended to the distal tip of the glans that the density of red blood cells and CD31-positive vessels becomes reduced dorsally where the process is complete, but is still seen ventrally, perhaps indicative of an ongoing process of preputial lamina formation or epithelial remodeling (Fig. 8).

Figure 9 summarizes the process of formation of the preputial lamina from the earliest stage. Before appearance of the preputial lamina (8–10 weeks) transverse sections of the distal aspect of the glans penis reveal a dense mesenchyme surrounded by a thick epidermis from which the urethral plate extends into the mesenchyme (Fig. 9A). At this stage and at this proximal-distal position the preputial lamina has not yet formed. The process of preputial lamina formation is initiated dorsally or dorsal-laterally in the proximal aspect of the glans at 11 to 12.5 weeks (Figs. 9B–C) as the thickened epidermis in the proximal portion of the glans undergoes splitting/remodeling to form the preputial lamina (Figs. 9B & C). Figure 9E (a section published in our previous paper) (Liu et al, 2018b) shows that the epidermis is thickened in the mid-dorsum and appears to be splitting/remodeling to give rise to the preputial laminae (PPL) and the overlying epidermis (Fig. 9F). We previously designated this thickened mid-dorsal epidermis as the preputial placode (Liu et al, 2018b), but now realize that the epidermal thickening occurs globally throughout the glans as reported previously (Hart, 1908) and seen in figures 4–6 & 9. In some specimens the process of epidermal splitting/remodeling appears to occur asymmetrically, beginning in a dorsal-lateral region on one side (Figs. 5C & 9B), but eventually extending to the ventral midline on both sides (Fig. 9D) leaving a mid-ventral zone of mesenchyme between the bilateral preputial laminae destined to become the stroma of the frenulum (* in Fig. 9D). As a cautionary note, the asymmetry described above could be due to orientation of the specimen within the paraffin block. Note the density of red blood cells/vessels (BV) in the advancing edge of epidermal splitting/remodeling (Figs. 6 & 9B–C).

The adult human penile prepuce is an extensive flap of skin that almost completely covers the glans (Clemente, 1985). In females, a prepuce of sorts covers the dorsal aspect of the glans clitoris, formally called *prepuce of the clitoris*, which is absent on the ventral aspect of the clitoris (Clemente, 1985). It is likely that ventral preputial development may be dependent on formation of the glanular urethra which is androgen-dependent and only occurs in males (Baskin et al, 2018). Accordingly, we examined the expression of the androgen receptor (AR) as well as estrogen receptors alpha and beta (ESR1 and ESR2) and the progesterone receptor in the developing human prepuce.

At 9.5 weeks the corporal body is intensely AR-positive as is also the mesenchyme ventral to the urethra (Figs. 10A–B), while AR is undetectable in the glanular epidermis (B). In contrast, the mesenchyme of the glans is a mixture of AR-positive and AR-negative cells (Figs. 10B–C). At 12.5 weeks of gestation AR expression increases generally within the mesenchyme of the glans, but the glanular epidermis remains AR-negative (Fig. 11A–B). The brown staining of the blood vessels (BV) seen at low magnification (Fig. 11A) is an artifact due presumably to hemoglobin in red blood cells, since at higher magnification (Fig. 11b) such staining is not seen. The blood-vessel-rich mesenchyme at the distally-advancing zone of epidermal splitting/remodeling contains AR-positive cells (Fig. 11B, half circle of dotted lines). AR are generally undetectable in the epidermis and in the preputial lamina with the exception that the proximal tip of the preputial lamina which contains a few AR-positive epithelial cells (Fig. 11C, boxed area), suggesting that this area is an active site of androgen-regulated development. The preputial lamina is surrounded on all sides by AR-positive mesenchymal cells (Fig. 11B–C). At 14 to 16 weeks when development of the preputial lamina is nearly complete, sagittal sections lateral to the midline reveal the preputial lamina extending from dorsal to ventral (Fig. 12A). The preputial lamina is for the most part devoid of AR staining (Fig. 12A & C) with the exception of AR-positive cells at its free proximal tips (Fig. 12B). In addition, rare AR-positive epithelial cells are seen in the distal aspect of the preputial lamina (Fig. 12C, inset). The skin of the glans was AR-negative (Fig. 12A & C).

Estrogen receptor alpha (ESR1) was examined in developing penises of 4 specimens at the following ages: 9, 12, 12.5 and 13 weeks. ESR1 staining was consistently absent in the developing prepuce with one exception. At 13 weeks ESR1 was detected in mesenchymal cells associated with the proximal tip of the preputial lamina (Fig. 13).

Estrogen receptor beta (ESR2) was not detected in any of the cells/tissues of the developing glans penis at 9.5 days of gestation (not illustrated). At 12.5 weeks ESR2 was detected in the preputial lamina (Fig 14A & B), in the epidermis of the glans (Fig. 14C) and in the epithelium of the urethral meatus (Fig. 14C). The corporal body appeared weakly ESR2-positive (Fig. 14A & C), while the mesenchyme of the glans was ESR2-negative. Blood vessels were ESR2-positive (Fig. 14A & C). The progesterone receptor was examined in the developing glans at 9.5, 12.5 and 13 weeks. All tissues/cells were devoid of progesterone receptor staining at all ages (not illustrated).

C. Innervation of the glans penis and prepuce

Due the importance of sensory and autonomic innervation of the glans penis, immunostaining for S100 was carried out. S100 is a protein expressed in Schwann cells and in a subset of neurons. Accordingly, S100 immunostaining gives a global picture of nerve distribution (Gonzalez-Martinez et al, 2003). To obtain a precise distribution of nerves entering the glans and prepuce, we immunostained both sagittal and transverse penile sections. At 9 weeks of gestation, before the emergence of the preputial lamina, nerves/nerve bundles were observed dorsal to the corporal body. This anatomical position, confirmed in transverse sections (Figs. 15A–C), is consistent with the location of the dorsal nerve of the penis (Fig. 15A) (Lue, 2000). Nerve fibers were also seen at the glans/shaft interface (Fig. 15A), but not in the distal portion of the glans (Fig. 15A).

By 11.5 to 12.5 weeks of gestation the thick epidermis of the glans is partially split and thus remodeled to form the preputial lamina. Sagittal sections at this stage demonstrate thick nerve bundles dorsal to the corporal body interpreted as the dorsal nerve of the penis (DNP) and its branches (Fig. 16). Nerve fibers have entered the proximal aspect of the glans, but the distal portion of the glans is still devoid of nerve fibers (Fig. 16, double-headed arrows). Nerve fibers are also seen within the mesenchyme of the dorsal aspect of the prepuce (Asterisk, Fig. 16B).

The penile glans and prepuce at 14 to 16 weeks of gestation show a consistent pattern and thus only the 14-week specimen is illustrated (Fig. 17). Branches of the dorsal nerve of the penis were seen dorsal to the corporal body (Fig. 17A & E). Large nerve bundles were also observed at the glans/shaft interface (Fig. 17A, large arrow). Fine nerve fibers have now arrived at the distal aspect of the glans and are associated with mesenchyme of the preputial lamina and the uncanalized urethral plate within the glans (Fig. 17B–D). Nerve fibers were also observed in the mesenchyme of the prepuce (PPM) (Figs. 17B–C & F). More proximally where the two sides of the preputial lamina approach each other in the midline, a high density of fine nerve fibers are seen in the gap between the bilateral preputial laminae (mesenchyme of the frenulum) and in the preputial mesenchyme ventral to the gap between the preputial laminae (Fig. 17E–F).

IV. Discussion

A. Preputial ontogeny

Our observations suggest a novel mechanism of human preputial development that differs significantly from previous theories, especially in regard to the formation of the preputial lamina (Fig. 18). A key finding is the fact that the epidermis covering the glans is substantially thicker than the epidermis of the penile shaft (Fig. 18A), and that this difference in epidermal thickness occurs precisely at the shaft-glans boundary, an observation reported previously (Hart, 1908). The thick epidermal layer covering the glans is split by the influx/invasion of mesenchyme containing CD31-positive blood vessel and red blood cells that divides the thick epidermal layer in two: (1) the outer layer becoming the definitive epidermis and (2) the inner layer becoming the preputial lamina. Other mechanisms may also be at play in the formation of the prepuce such as those proposed

earlier: (a) distal growth of a fold “fusing with the epithelium covering the glans” (Hunter, 1935; Schweigger-Seidel, 1866), (b) proximal ingrowth of the preputial lamina (Glenister, 1956). The proximal extension of the preputial lamina proposed by Glenister (1956) is compatible with our view of human preputial development. As an aside, it should be noted that development of the mouse external and internal prepuces occur via entirely different morphogenetic mechanisms (Liu et al, 2018a).

Our study of human preputial development raises the more general question of the mechanism of epithelial and mesenchymal remodeling involved in division of a single structure into two separate entities. There are several examples of such processes in both mouse and human development, namely (a) division of the neonatal female mouse urogenital sinus into the urethra and vagina, (b) separation of the urethral plate/urethra from the epidermis with the establishment of midline mesenchymal confluence, (c) division of the cloaca into the urogenital sinus and the rectum/anal canal, (d) remodeling of the embryonic trachea-esophageal orifice to form the upper trachea and esophagus, (Fig. 19). In all of these cases a septum of mesenchyme insinuates in such a way to separate two epithelial structures. Similar to human preputial development described above, separation of the human urethral plate/urethra from the epidermis also involves invasion of a blood vessel rich mesenchyme (Sinclair, unpublished observations). Whether the other examples illustrated in figure 19 also involve an invasive blood vessel rich mesenchyme remains to be determined, even though the common feature for all of the above examples is insinuation of mesenchyme to separate two epithelial structures. Another common feature of all 4 examples is an association with congenital malformations or experimentally induced anomalies such as hypospadias, tracheal-esophageal fistula, urethral-vaginal fistula and a variety of cloacal anomalies. Given the extensive epithelial and mesenchymal remodeling associated with all of the above examples, it is likely that matrix metalloproteinases and their regulators are involved.

Splitting/remodeling of the thick epidermis of the glans by the influx of a blood vessel rich mesenchyme is surely an androgen-dependent event in humans. This conclusion is supported by 2 observations: (a) Formation of a complete circumferential prepuce only occurs in males, while in females the *prepuce of the clitoris* only forms dorsally. (b) Androgen receptors are expressed in glanular mesenchyme prior to and during the process of splitting/remodeling of the glanular epidermis and thus formation of the preputial lamina. For the most part androgen receptors are expressed in the mesenchyme associated with the preputial lamina, which is consistent with a paracrine developmental mechanism in which mesenchyme is the primary target of androgen action, in turn directing epithelial morphogenesis. Epithelium of the preputial lamina is mostly AR-negative, with the notable exception of consistent prominent AR expression in the proximal tip of the preputial lamina (Figs. 11–12). Perhaps AR expression in the proximal tips of the preputial laminae is involved into proximal growth of the preputial lamina.

Experimental studies in mice and epidemiologic studies in human demonstrate/suggest a role of estrogenic endocrine disruptors in hypospadias, one feature of which are preputial malformations (Cunha et al, 2015; Kalfa et al, 2011; Yiee and Baskin, 2010). Accordingly, ESR1 was detected in the mesenchyme associated with the developing human preputial lamina. ESR2 was more broadly expressed in glanular epidermis, in the preputial lamina and

in the mesenchyme associated with the human preputial lamina. Such expression of ESR1 and ESR2 may be indicative of a role of estrogens in normal human preputial development and also may provide the substrate from which penile/preputial malformations are elicited by developmental exposure to estrogenic agents (Kim et al, 2004; Mahawong et al, 2014a, b; Sinclair et al, 2016; Zheng et al, 2015). Developmental mechanisms reported herein for human preputial development are unlikely to be relevant to mouse preputial development as the morphogenesis of the mouse prepuce differs significantly from human preputial development (Liu et al, 2018a), suggesting that the mouse is an unacceptable model of human preputial development. Indeed, the splitting and remodeling of glanular epidermis to form the human preputial lamina does not occur in mouse preputial development (Liu et al, 2018a; Liu et al, 2018b). The key feature of human preputial development is extensive epithelial and mesenchymal remodeling involved in the splitting of the thick glanular epidermis into two layers, one being the preputial lamina. Accordingly, it is likely that matrix metalloproteineases and their regulators play an important role in human preputial development as well as is human and mouse penile urethral development (example B in figure 19).

Preputial malformations are one of the features of human hypospadias (Baskin et al, 1998; Cunha et al, 2015), which is characterized by an ectopic urethral meatus on the ventral shaft of the penis and arrest in ventral preputial development with excess foreskin on the dorsal aspect of penis (Baskin, 2017). In mild forms of hypospadias the urethral meatus is not affected, residing in its normal position on the glans with the only abnormality being the ventral preputial defect (Fig. 20). If the urethral plate does not canalize within the glans, the urethral meatus will be located proximally on the ventral aspect of the proximal glans or coronal margin instead of in its normal position at the distal tip of the glans (Fig. 20B–D), the prepuce will be deficient ventrally and the frenulum will be absent. Severe hypospadias with a urethral meatus located along the penile shaft is thought to be due to disruption of fusion of the urethra folds to form the penile urethra. Such urethral malformations are associated with a dorsal hooded foreskin with deficit in ventral foreskin formation. For all but the most-mild forms of hypospadias, we hypothesize that failure of formation of the urethra within the glans is linked to a ventral deficit in the prepuce. Additional data linking anomalies in human urethral development with deficits in preputial development/anatomy are the rare congenital anomalies, epispadias and classic bladder exstrophy (Fig. 21). In epispadias the dorsal wall of the urethra is absent, and the prepuce only forms on the ventral aspect of the epispadiac penis (Ebert et al, 2009). A rare form of hypospadias, megameatus intact prepuce (Fig. 22) is a condition in which the prepuce forms in a normal circumferential fashion and the unexpected urethral defect only becomes apparent after neonatal circumcision or in the first few years of life when the physiologic preputial adhesions spontaneously resolve (Baskin and Ebbers, 2006). This is apparently an isolated malformation of the glanular urethra that involves complex remodeling of the canalized urethra plate and midline mesenchymal confluence (Baskin, 2017; Liu et al, 2018b). The result is a large caliber urethral meatus (megameatus) with normal preputial formation.

B. Ontogeny of penile and preputial innervation

Because Schwann cells wrap around most peripheral the nerve fibers, S100 immunostaining was chosen to globally define the ontogeny of innervation of the glans penis and the prepuce knowing that there are four neural inputs to the penis: somatosensory, somatomotor, parasympathetic and sympathetic. The latter two regulate vasodilation and vasoconstriction respectively via effects on intracorporal smooth muscle (Steers, 2000). Erection is elicited via parasympathetic, nitric oxide synthase-containing neurons (Giuliano and Rampin, 2000; Nazir et al, 2004; Steers, 2000). Both parasympathetic and sympathetic fibers reach the penis via the cavernous nerves that emanate from the pelvic plexus (Steers, 2000). Some of the branches of the cavernous nerves penetrate the tunica albuginea of the crura and at the hilum of penis, while the remaining fibers merge with the dorsal nerves to innervate the mid- and distal penis and the glans (Lue, 2000). Somatic sensory fibers reach the penis via a branch of the pudendal nerve, namely the bilateral *dorsal nerves of the penis*, which contains nerve fibers responsible for sensation and motor responses for micturition and procreation (Weech and Ashurst, 2019). As the name implies these nerves extend distally along the dorsal aspect of the corporal body sending fine branches ventrally around the corporal body to terminate in the ventral midline where some fibers innervate the corpus spongiosum (Lue, 2000). The *dorsal nerve of the penis* gives off efferent fibers to the corpus cavernosum and corpus spongiosum of the penis, as well as afferent fibers that innervate the penile skin and the glans penis. These afferent fibers are critical to achieve reflex erections and sexual function (Weech and Ashurst, 2019). The parasympathetic and sympathetic fibers of the cavernous nerves penetrate the tunica albuginea of the corpora cavernosa and corpus spongiosum to innervate smooth muscle within these erectile bodies. Thus, the anatomical positions of nerve fibers in immunostained sections provides clues as to the fiber type.

One nerve bundle for which identification is most certain is the *dorsal nerve of the penis*, which can be identified as early as 9 weeks of gestation and is prominently displayed dorsal to the corporal body. Small fibers forming a circumferential constellation around the large *dorsal nerve of the penis* extending into the penile dermis are likely afferent fibers. Those fibers more closely associated with the mesenchymal condensations, which define the corporal body and corpus spongiosum, are likely to be sympathetic and/or parasympathetic efferent fibers.

The 12.5-week specimen (Fig. 16***) represents an early stage in preputial development in which the preputial lamina is present but not yet fully formed. The preputial lamina has an inner surface facing mesenchyme of the glans and an outer surface facing preputial mesenchyme. Nerve fibers, presumably branches of the *dorsal nerve of the penis*, are already present within the preputial mesenchyme as well as in close approximation to the inner (glanular) surface of the preputial lamina. Thus, nerves have entered these positions shortly after initiation of morphogenesis of the preputial lamina.

A striking feature of the ontogeny of innervation of the penis in general and the glans specifically, is that nerve fibers appear to extend into the penis from proximal to distal. This interpretation is derived from examination of innervation to the glans. In the youngest specimen (9.5 weeks), nerve fibers were not seen in the distal aspect of the glans, but in the older specimens there was a progressive distal extension of nerve fibers that reached the

distal tip of the glans at 14 to 16 weeks of gestation. Finally, we note a very dense array of nerve fibers at 14 weeks in the mesenchyme of the frenulum and in the mid-ventral aspect of the prepuce. These latter fibers were closely associated with the overlying skin and thus are likely to be afferent fibers.

C. Comparative ontogeny

Finally, the morphogenetic process of preputial development in mice differs substantially from that seen in human as reported recently (Liu et al, 2018a). In fact mice have two prepuces, an external prepuce which creates the preputial space housing the mouse penis and an internal prepuce that is integral to the penis and thus appears to be homologous with the human penis (Blaschko et al, 2013). The external prepuce of the mouse forms the prominent hair-bearing elevation in the perineum and is derived from the preputial swelling for which there is no human counterpart. Nonetheless, the formation of the mouse external prepuce does involve midline fusion of the bilateral preputial swellings and establishment of midline mesenchymal confluence (Liu et al, 2018a), thus perhaps sharing certain developmental mechanisms with human. Formation of the mouse internal prepuce has not been examined adequately.

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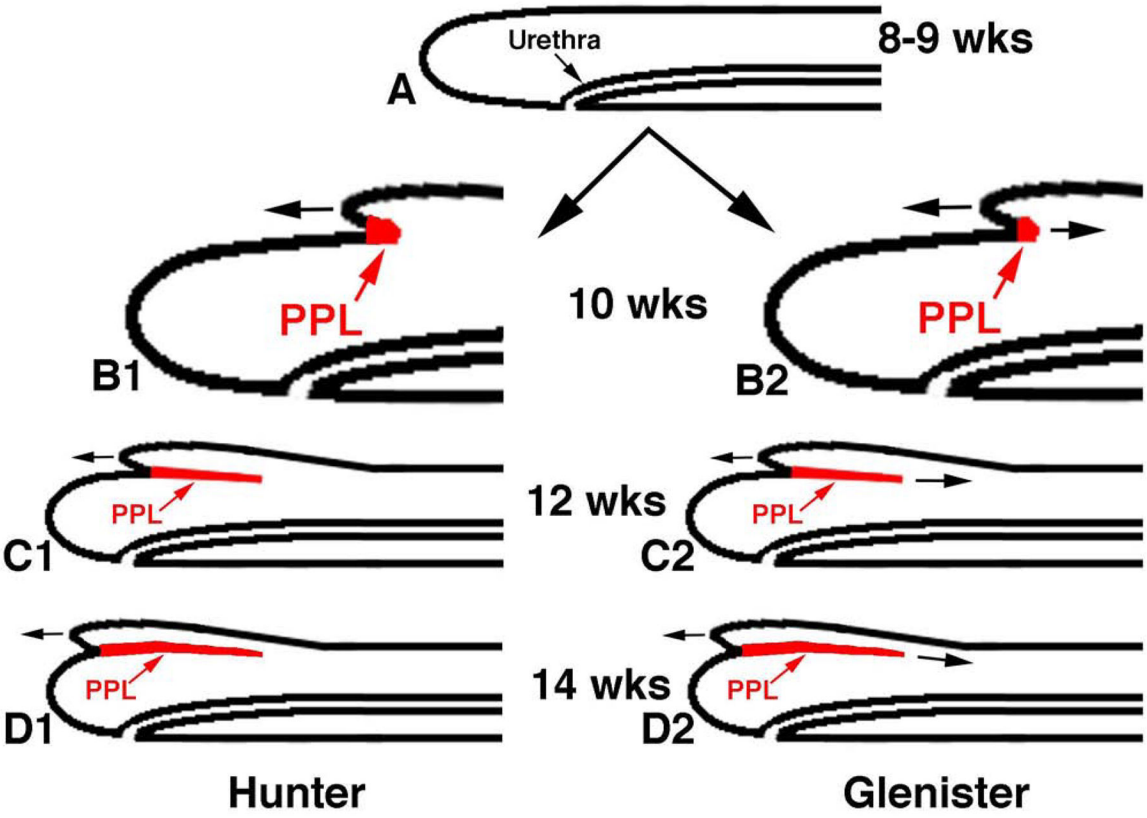

Figure 1.

Models of human preputial development consistent with the reports of Schweigger-Seidel (1866) and Hunter (1935) (B1–D1) versus Glenister (1956) (B2–D2)). According to Schweigger-Seidel/Hunter (B1–D1), the human prepuce develops as a result of formation of a dorsal skin fold (B1) (preputial fold) that extends or “rolls” distally to cover the glans and in so doing creates the preputial lamina in its wake (B1–D1). The model of human preputial development proposed by Glenister (1956) (B2–D2)) suggests that a dorsal skin fold (preputial fold) forms and extends distally to cover the glans, coincident with an epithelial ingrowth to form the preputial lamina as indicated by the distal- and proximal-pointing black arrows in (B2, C2 & D2). (B1 & B2) represent the distal aspect of the glans penis drawn at higher magnification.

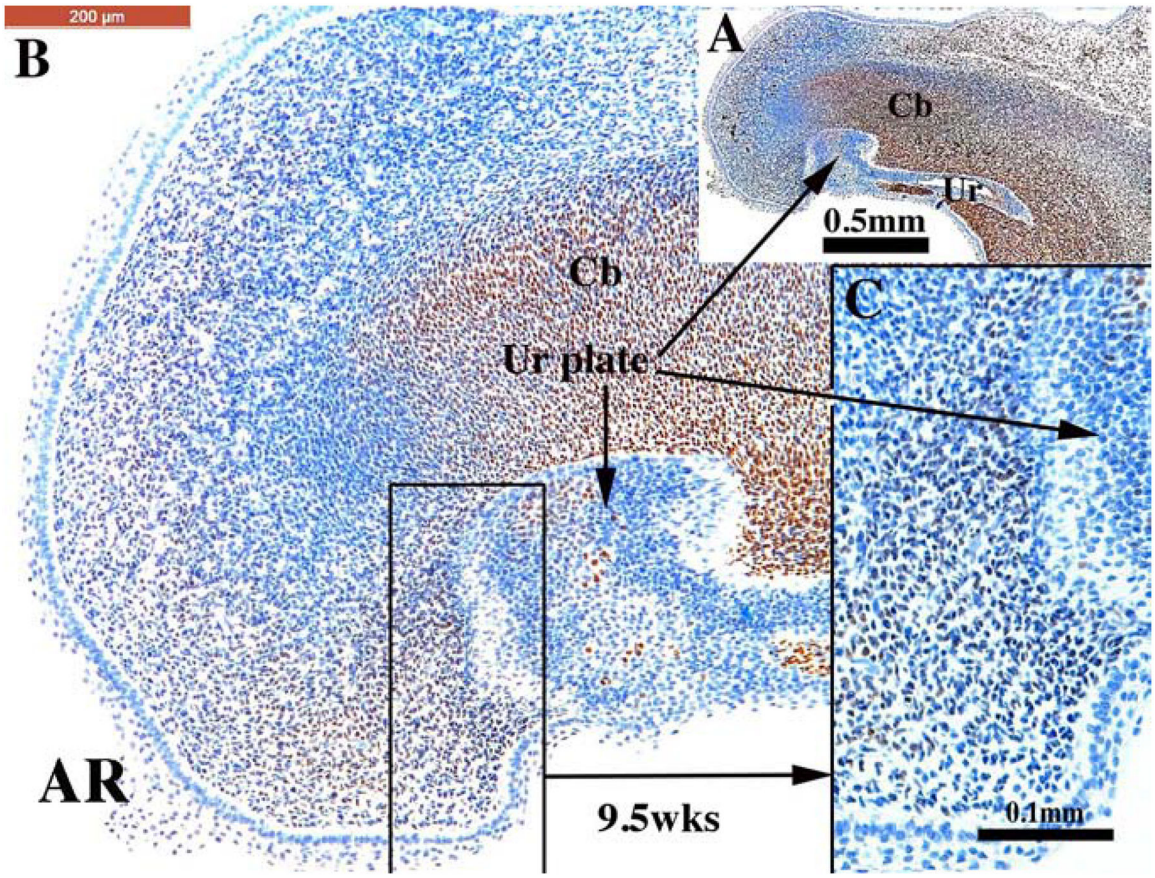

Figure 2.

Diagrams of the adult human penis in mid-sagittal (left) and transverse sections (right). The transverse section is at the level indicated with the large green arrow (left). Note the frenulum (green area) connecting the glans to the inner surface of the prepuce.

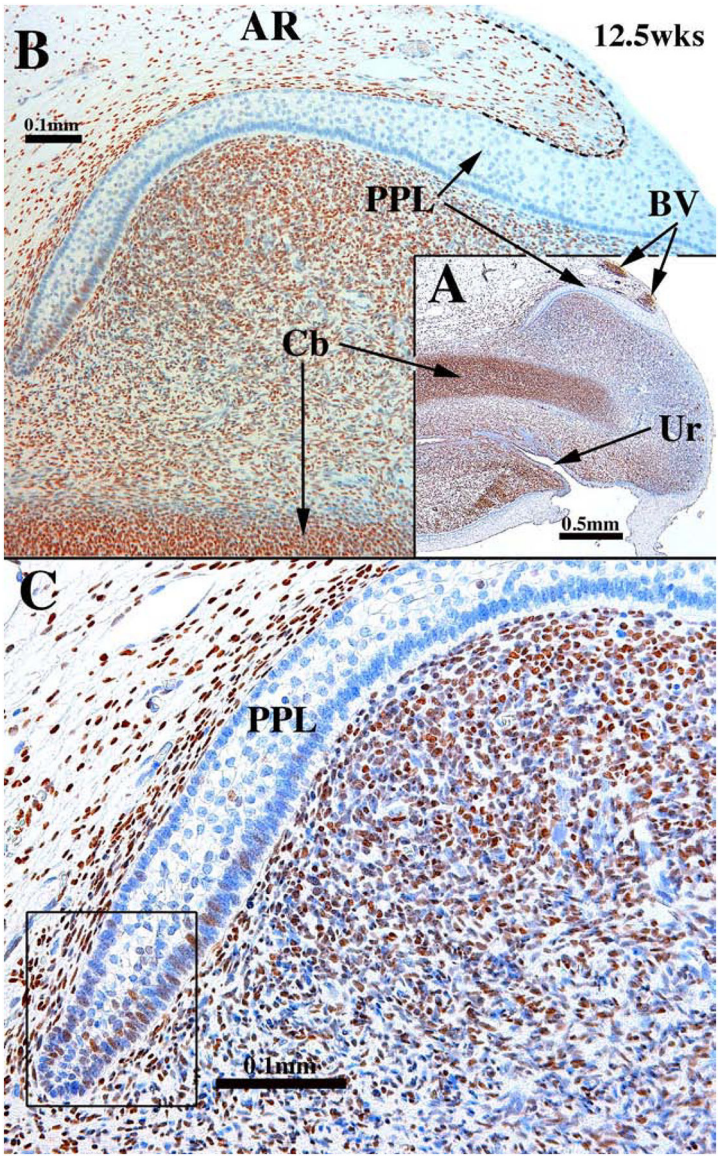

Figure 3.

Scanning electron microscopic images of developing human penis (ventral view). Note the epidermal aggregates on the surface of the glans that appear initially at or near the glans/shaft interface (A, A1 & B) and become more numerous as development proceeds (C-D and D1). By 13 weeks a ridge (asterisks) adorned with these epidermal aggregates is seen apparently extending towards the ventral midline (D & D1). At 15–17 weeks the developing prepuce has reached and fused in the ventral midline to cover the glans (E-F). (A1 & D1) are higher magnifications of (A & D). From Baskin et al (2018) with permission.

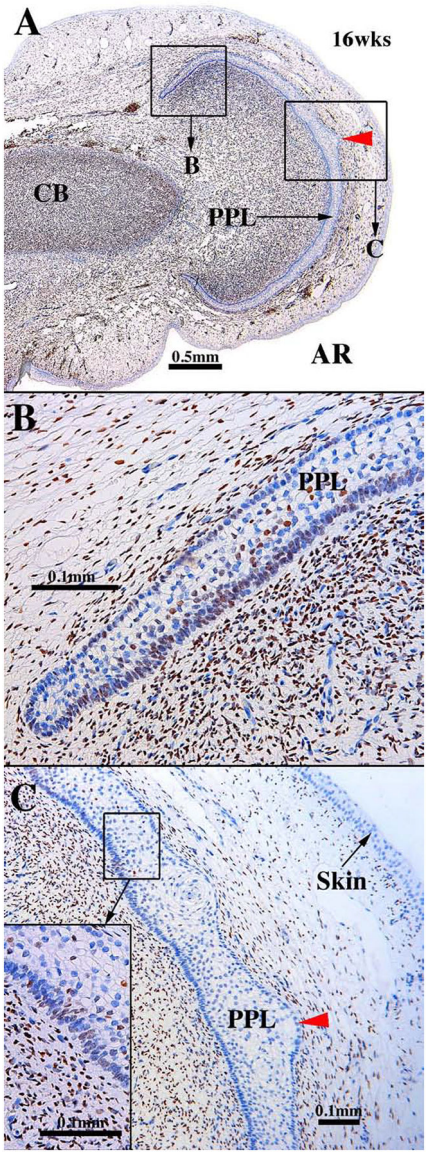

Figure 4.

Mid-sagittal sections of male external genitalia at 9.5 weeks of gestation stained with hematoxylin and eosin. (A) is a low magnification image giving overall orientation. At higher magnifications (B-D) note that thickness of the epidermis is greater on the glans versus that of the more proximal shaft (see apposed arrowheads). (B & D) are higher magnification of the boxed area in (C) and are at the same magnification. Difference in epithelial thickness is shown in the red and green bars in (B & D). In (C) note the glans/shaft boundary (dashed line). See also Figs. 6C–D for transition from thick epidermis on the glans and thin epidermis proximally.

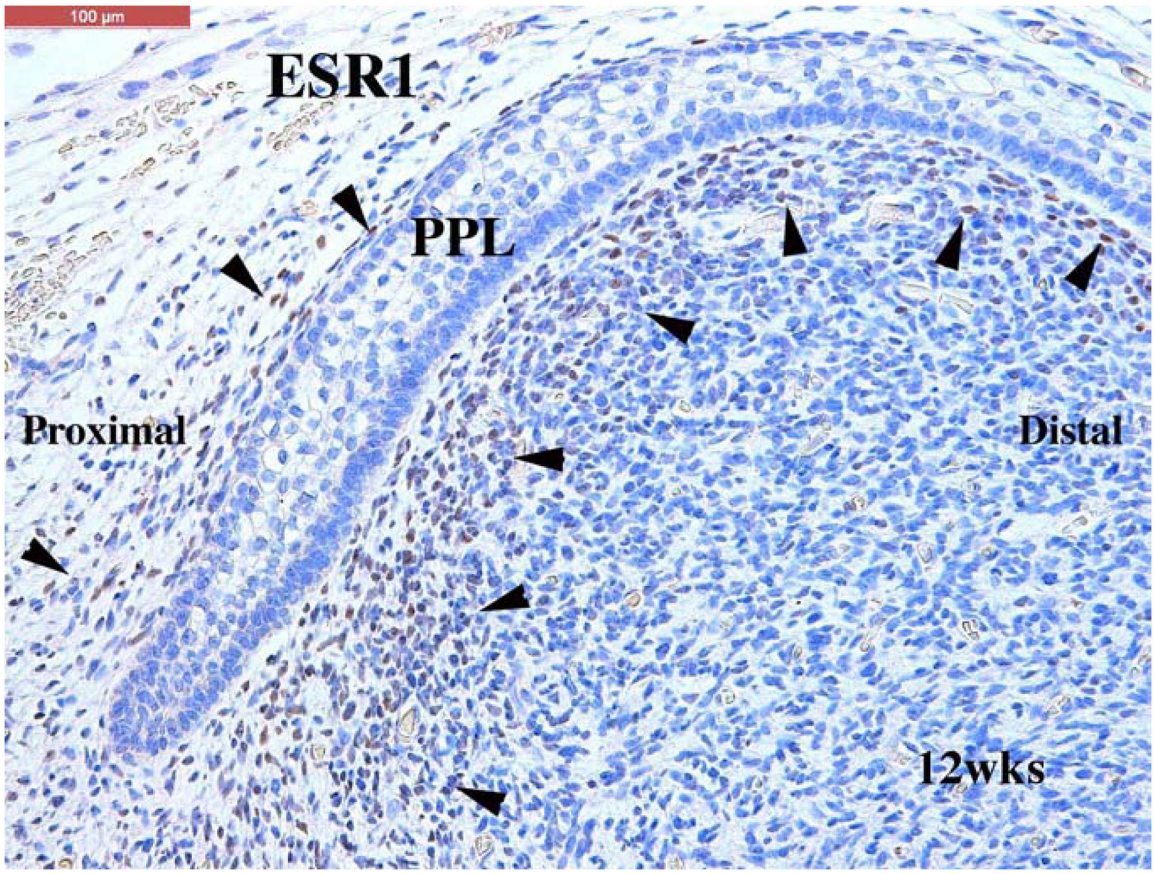

Figure 5.

Transverse serial sections of a 10-week human fetal penis immunostained for E-cadherin to reveal epithelium. Non-specific staining reveals blood vessels scattered throughout the interior of the glans. Sections A-L are in order from distal to proximal. Sections (K) is separated by several sections from section (J). Note the thickened glanular epidermis in (A-B, double-headed arrows). Splitting and remodeling of the epidermis is seen in sections (C-F, white arrowheads) (See also figure 6). Preputial laminae (PPL) are seen in sections (G-K). Section (L) is proximal to preputial lamina formation.

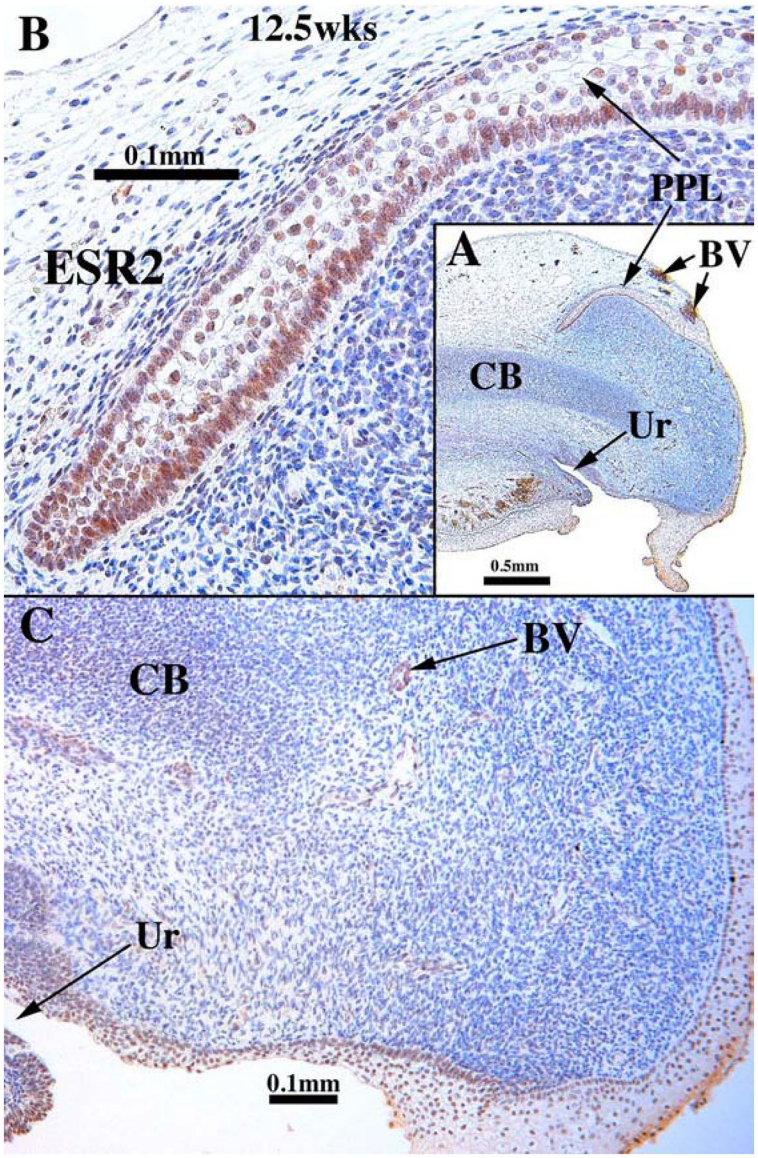

Figure 6.

Mid-sagittal sections of the external genitalia at 11.5 weeks of gestation stained with hematoxylin and eosin (A-D) at several magnifications. (A) is a low magnification image giving overall orientation. At higher magnifications (B-D) note that thickness of the epidermis is greater on the glans versus the more proximal epidermis of the penile shaft (in C and D, compare the distance between the tips of the red arrowheads versus the tips of the green arrowheads). The thick epidermis of the glans splits by the intrusion of a red blood cell rich mesenchyme to create the preputial lamina (PPL) deep to the thin epidermis (BV=blood vessels). Note in (D) that the basal epithelial cells on the glanular surface of the preputial lamina (Gbl) are basophilic and cuboidal/columnar, while basal cells of the preputial lamina facing the epidermis (Ebl) are squamous and similar to that of the epidermis (see double-headed arrow). Epi. Tag= Epithelial tag, a transitory structure that eventually disappears (Baskin et al, 2018). Section (E) is a combined immunostain for CD31

(green) to reveal blood vessels and hemoglobin (red) to reveal red blood cells. HB=hemoglobin, BV=blood vessels.

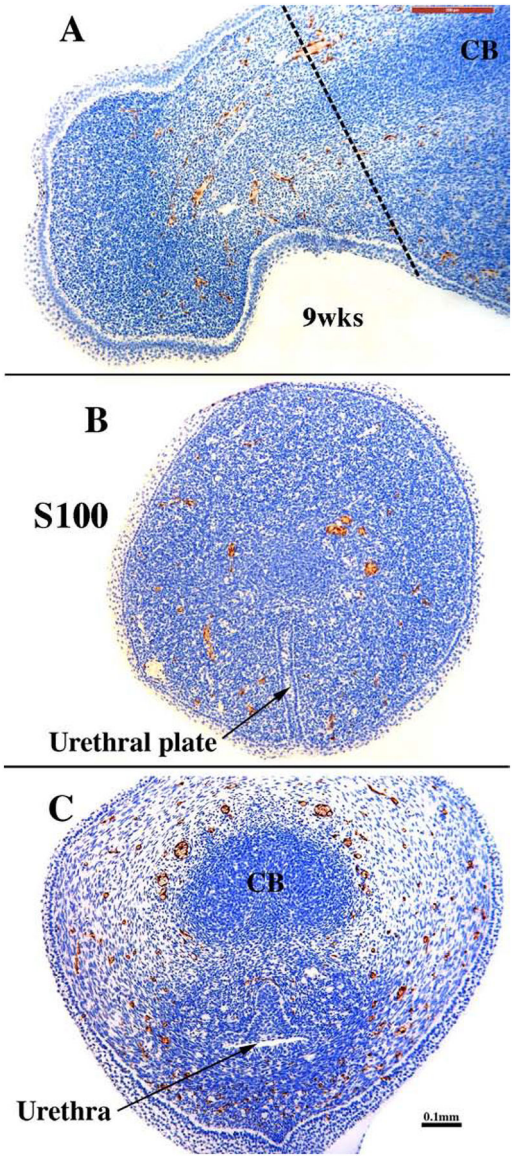

Figure 7.

Mid-sagittal section of the penile glans at 14.5 weeks of gestation stained with hematoxylin and eosin. Splitting of the epidermis of the glans has progressed in the dorsal midline to the distal aspect of the glans, and the density of blood vessels is now reduced dorsally. High density of blood vessels (BV) is seen ventral to the distal aspect of the urethra, perhaps indicative of active morphogenesis of the urethral meatus.

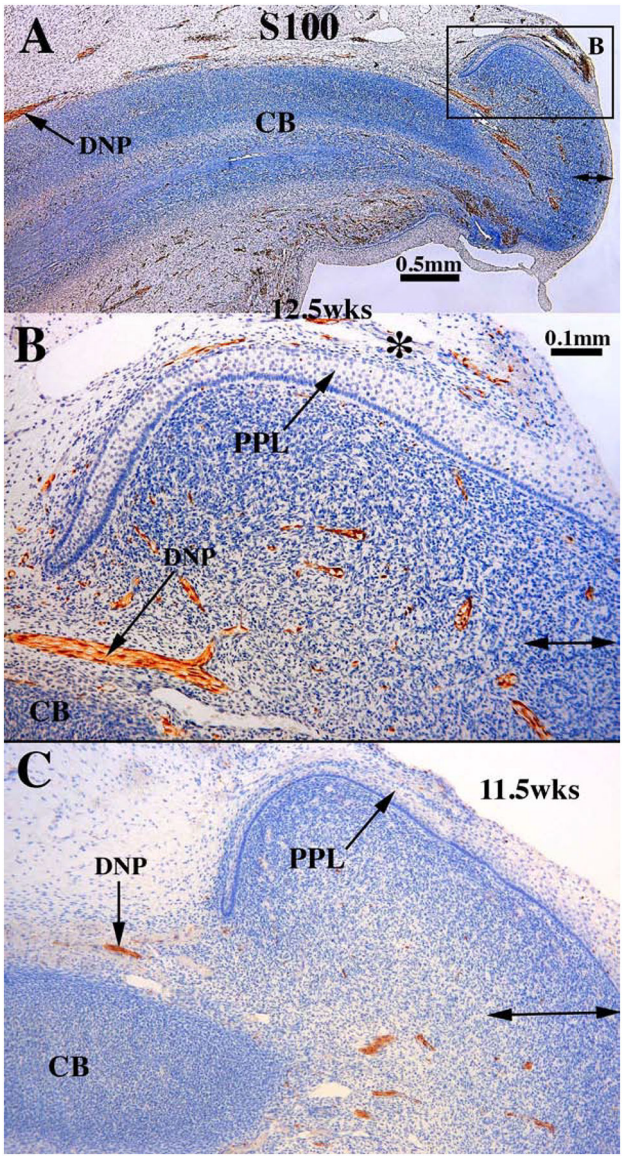

Figure. 8.

Mid-sagittal sections of the penile glans at 14 (A) and 16 (B) weeks of gestation stained for androgen receptor (A) and hematoxylin and eosin (B). Both sections are lateral to the midline to display the ventral aspect of the preputial lamina. Mesenchyme of the prepuce (double-headed arrows) has extended to the distal aspect of the glans, where the density of blood vessels is reduced dorsally, but red blood cell enriched vessels (BV) are prominent ventrally where preputial development is may still be in progress (A).

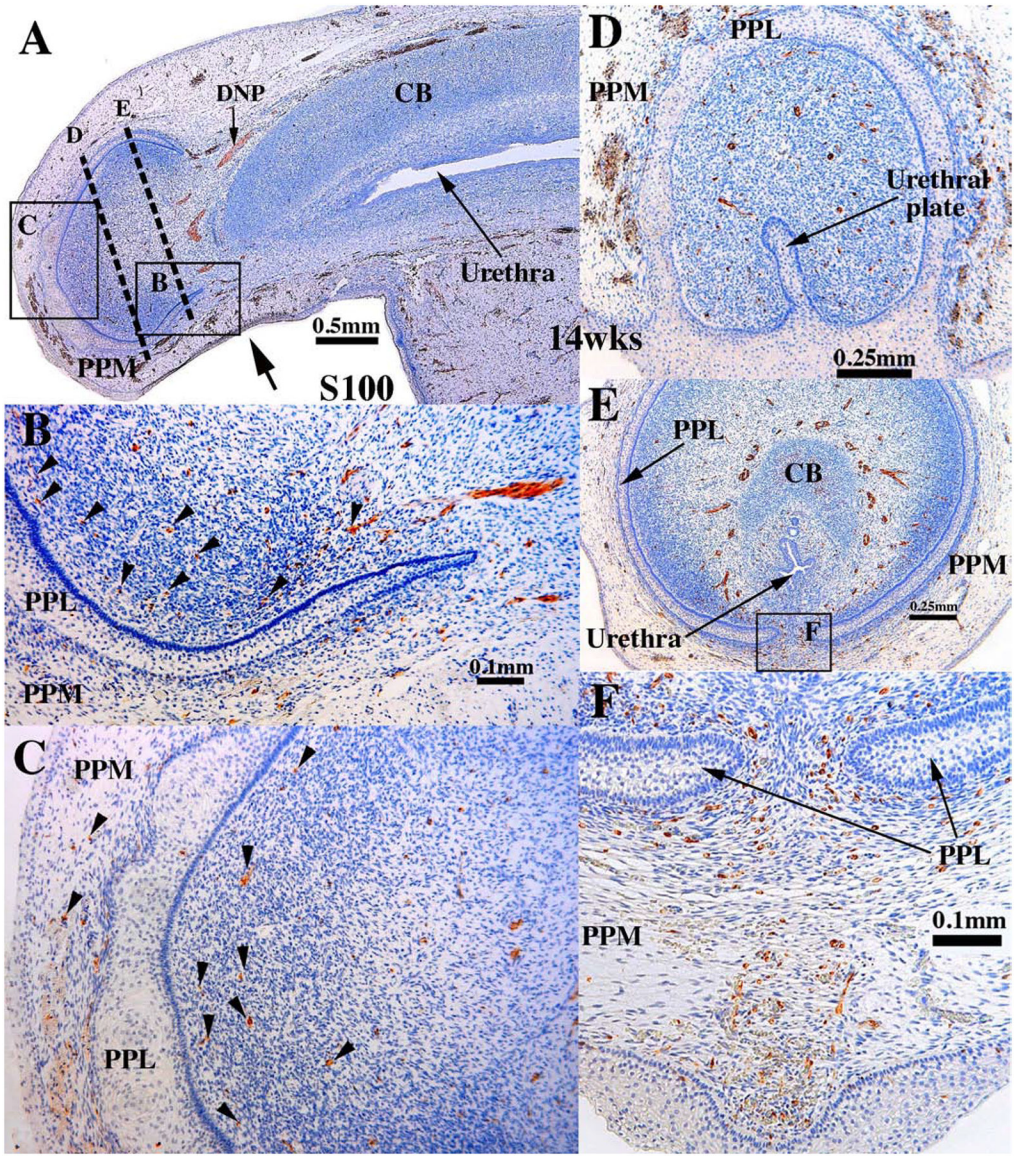

Figure 9.

Transverse sections of a human fetal glans penis at 9.5 to 13 weeks. (A) The distal aspect of the glans only contains the urethral plate (Ur. Plate) at 9.5 weeks. (B-C) Splitting/remodeling of the glanular epidermis begins dorsal-laterally (B) to generate the preputial lamina (PPL). In some specimens (B) splitting/remodeling of the thick glanular epidermis by a blood-vessel-rich mesenchyme occurs asymmetrically and thus is seen only on one side at 12.5 weeks (BV=blood vessels). (C) shows nearly complete splitting/remodeling of the glanular epidermis to the ventral midline. (D) Shows retention of mesenchyme between the bilateral halves of the preputial lamina (PPL), destined to form the stroma of the frenulum (*) (12.5 weeks). (E) has been immunostained for cytokeratin 6 (green) and cytokeratin 10 (red). Ur=urethra. In (E) it appears that the splitting/remodeling of the epidermis has been initiated in the mid-dorsal sector. (F) Preputial lamina in the dorsal midline. The scale bar

between (B & C) applies to (D). (A & C)=E-cadherin stain, (B)=H&E stain (E)=keratin 6-green and keratin 10-red stain. (F)=keratin 6 stain, (D)=keratin 14 stain.

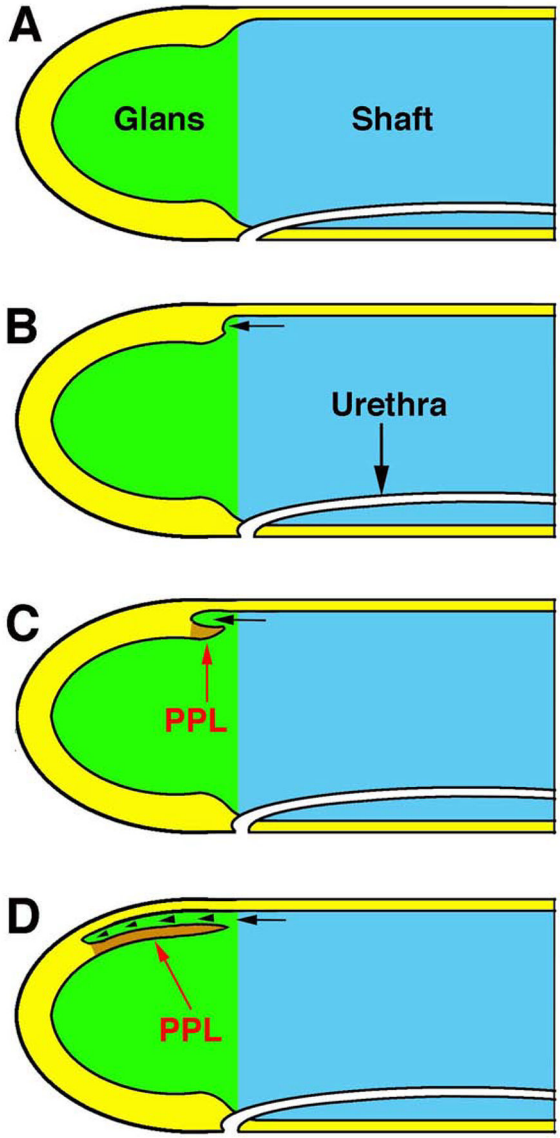

Figure 10.

Mid-sagittal sections of male genital tubercle at 9.5 weeks of gestation stained for the androgen receptor (AR). The corporal body (Cb) and the mesenchyme ventral to the urethra (Ur) prominently express AR. The mesenchyme of the glans is a mixture of AR-positive and AR-negative cells (B-C). Ur plate=urethral plate.

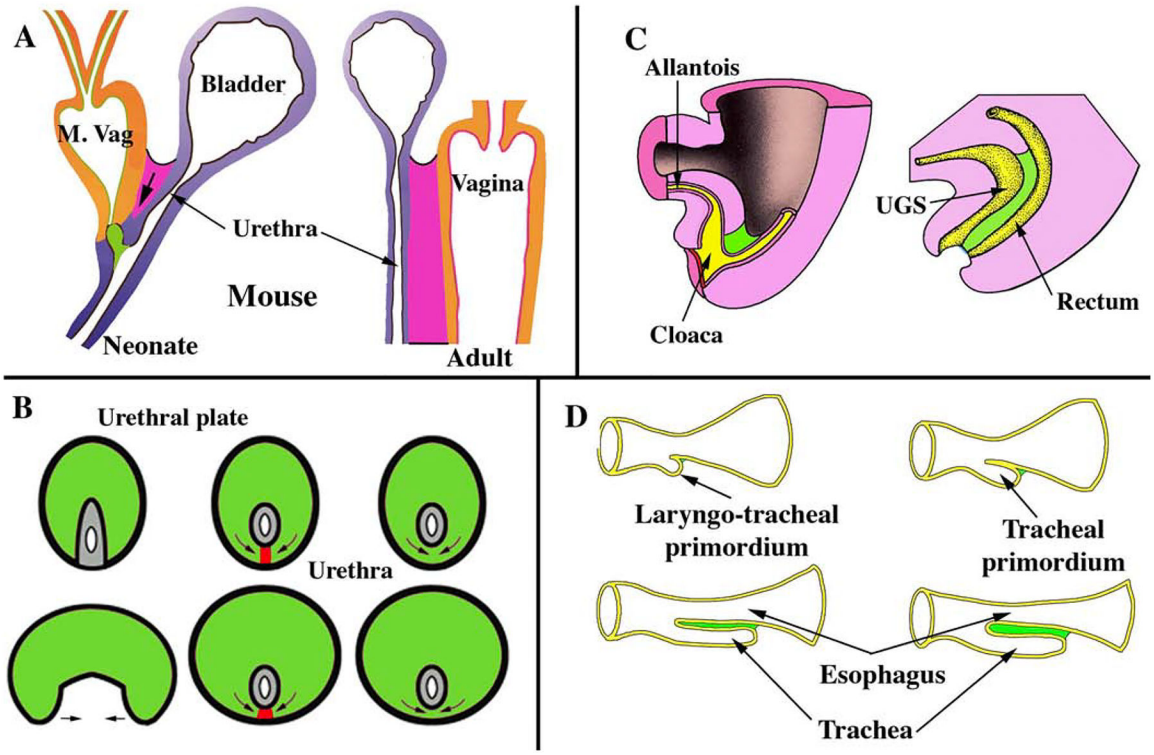

Figure 11.

Sagittal sections of human male glans at 12.5 weeks of gestation stained for androgen receptor (AR). Note AR in the corporal body (Cb), in the mesenchyme of the glans especially surrounding the preputial lamina (PPL), in the zone of distally advancing mesenchyme splitting/remodeling the epidermis (dotted lined in B), in the proximal tip of the preputial lamina (boxed area in C) and in mesenchyme surrounding the urethra (Ur) (A). The glanular epidermis appears AR-negative as is most of the preputial lamina.

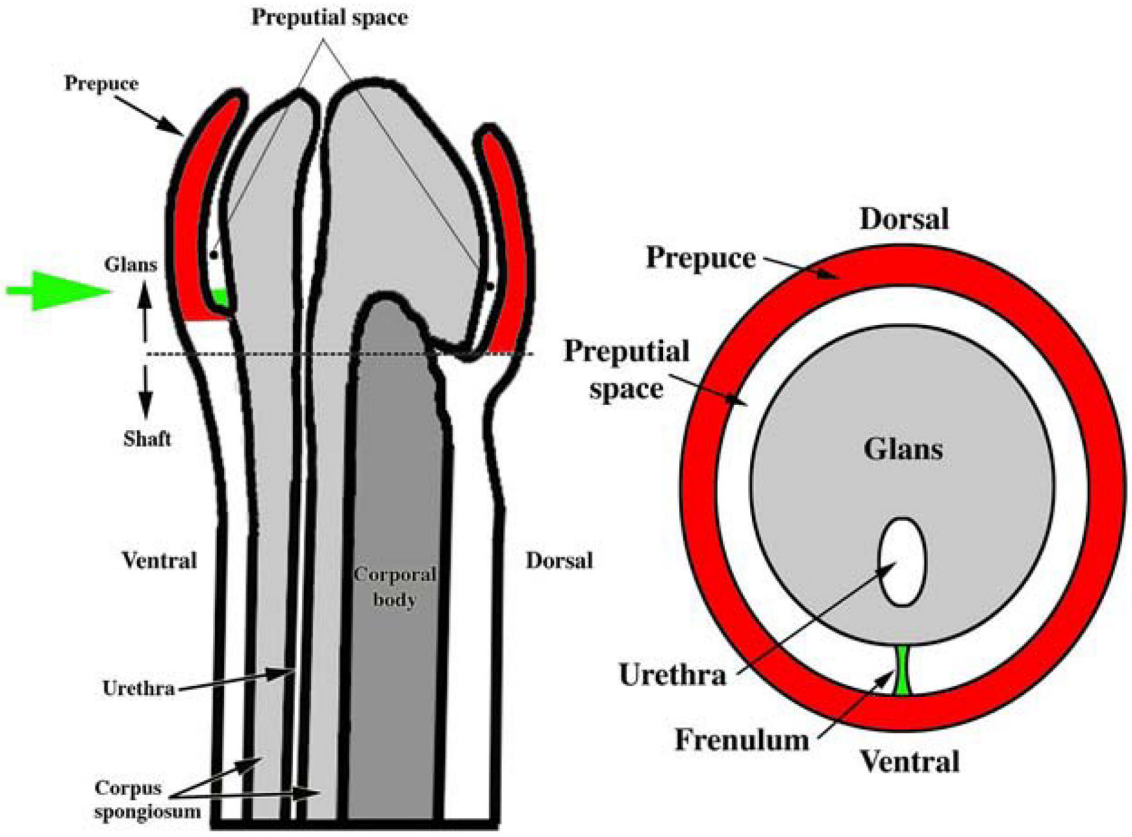

Figure 12.

Sagittal sections of human male glans at 16 weeks of gestation stained for androgen receptor (AR). (A) Overview showing the preputial lamina (PPL) extending from dorsal to ventral. Boxed areas indicate the positions of images (B and C). Note the AR-positive corporal body (CB) and the mostly AR-negative preputial lamina (PPL). (B) Higher magnification of the proximal free tip of the preputial lamina shows some AR-positive epithelial cells surrounded by AR-positive mesenchymal cells. (C) Central portion of the preputial lamina is mostly AR-negative with the exception of rare AR-positive epithelial cells (see inset). AR were not detected in the glanular epidermis (A & C). Red arrowheads in (A & C) point to the same region of the preputial lamina.

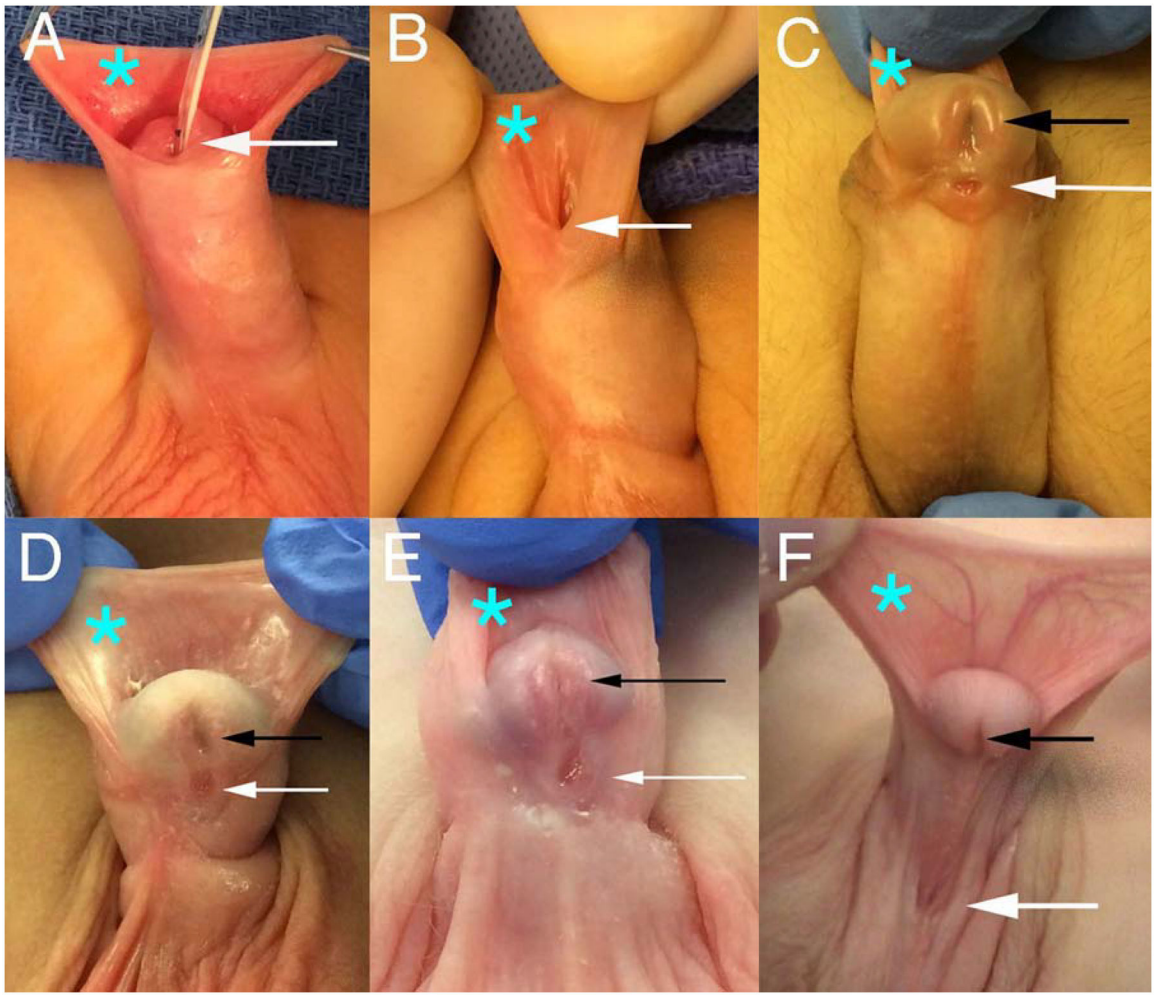

Figure 13.

Sagittal sections of human male glans at 12 weeks of gestation stained for estrogen receptor alpha (ESR1). Note ESR1 staining in mesenchymal cells associated with the preputial lamina.

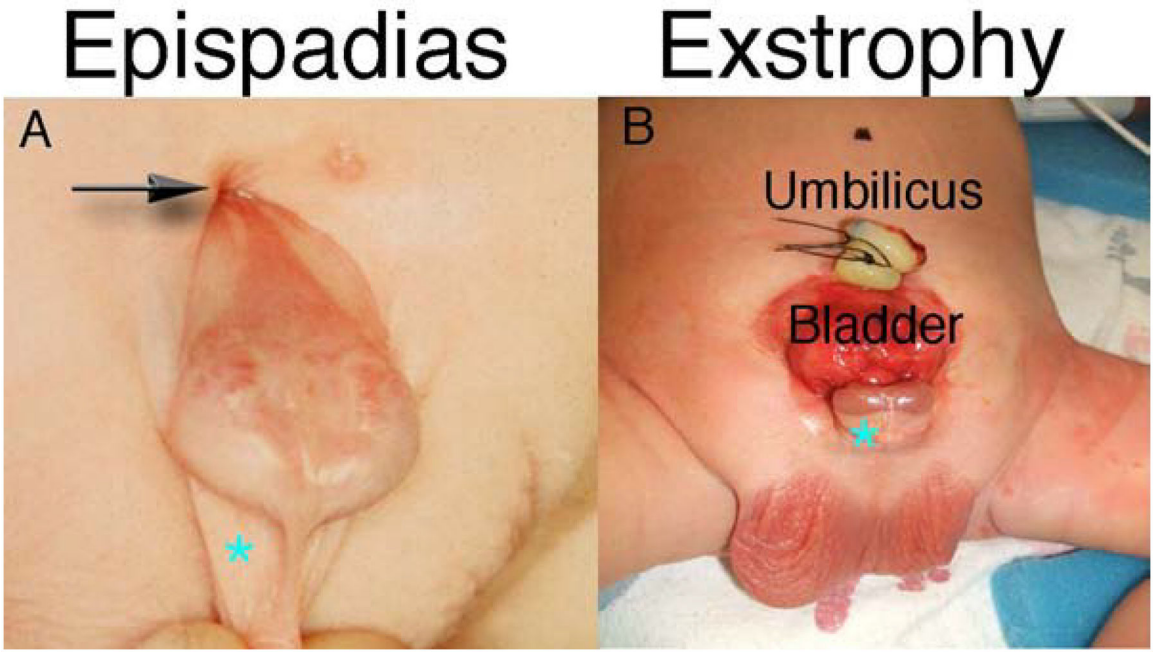

Figure 14.

Sagittal sections of human male glans at 12.5 weeks of gestation stained for estrogen receptor beta (ESR2). ESR2 is expressed in the preputial lamina (PPL) (A & B), weakly in the corporal body (CB), in the glanular epidermis (C), in the epithelium of the urethral meatus (A & C), and in blood vessels (BV) (A & C). The mesenchyme of the glans was generally ESR2-negative.

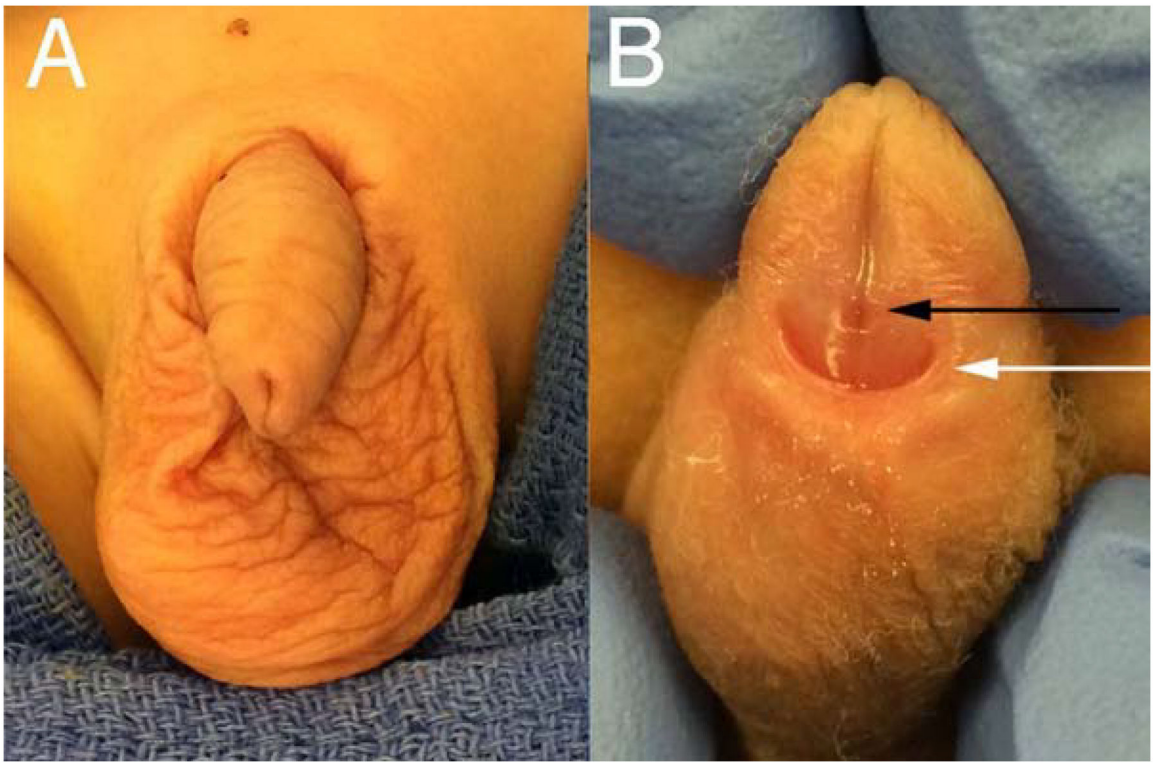

Figure 15.

Sagittal (A) and transverse (B-C) sections of the glans at 9 weeks of gestation immunostained for S100. Brown stained nerve fibers were observed dorsal to the corporal body (CB) in sagittal (A) and transverse (C) sections, and thus appear to be branches of the dorsal nerve of the penis. Transverse section (B) is at the distal extremity of the corporal body as indicated by the dotted lines in (A). Note nerve fibers in the distal aspect of the shaft (B) in association with the urethral plate. The density of nerve fibers in highest proximally (C) relative to more distal areas (A & B).

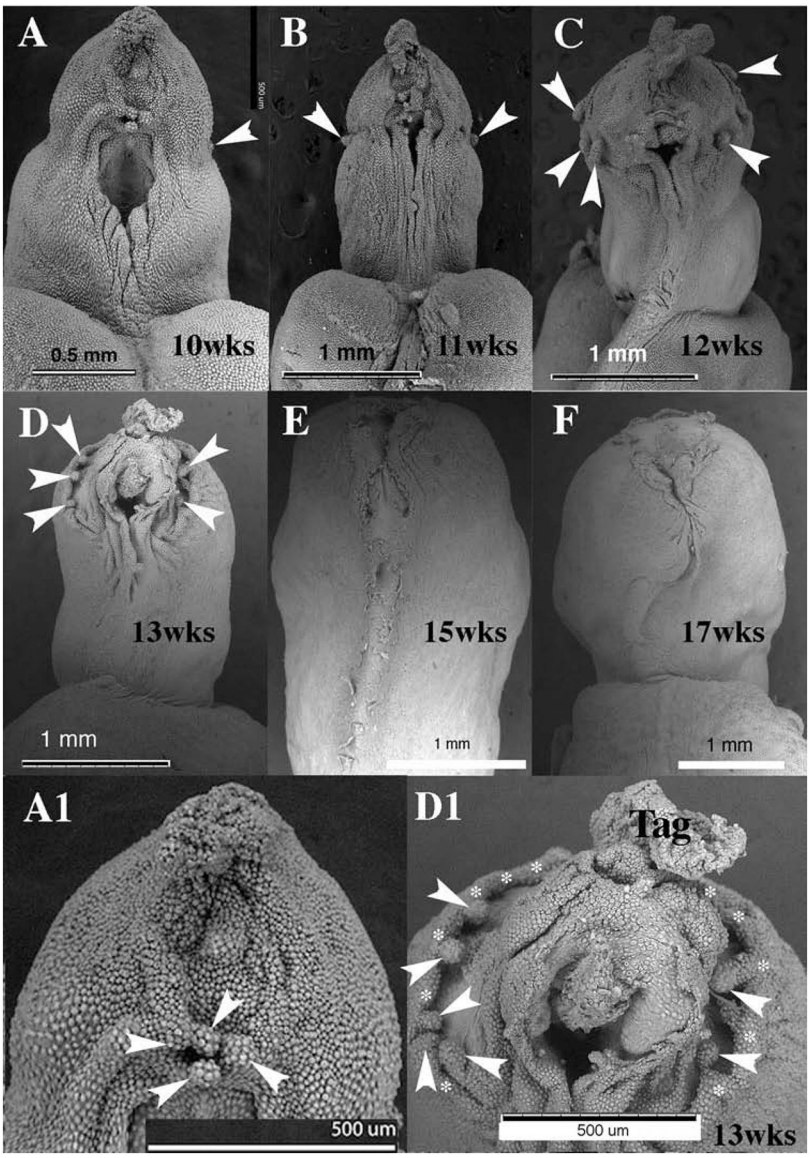

Figure 16.

Sagittal sections of developing human penis at 12.5 weeks (A & B) and 11.5 weeks (C) of gestation immunostained for S100. DNP=dorsal nerve of penis, PPL=preputial lamina, CB=corporal body. Note absence of nerve fibers from distal aspect of the glans (double headed arrows). Scale bar in (B) applies to (C).

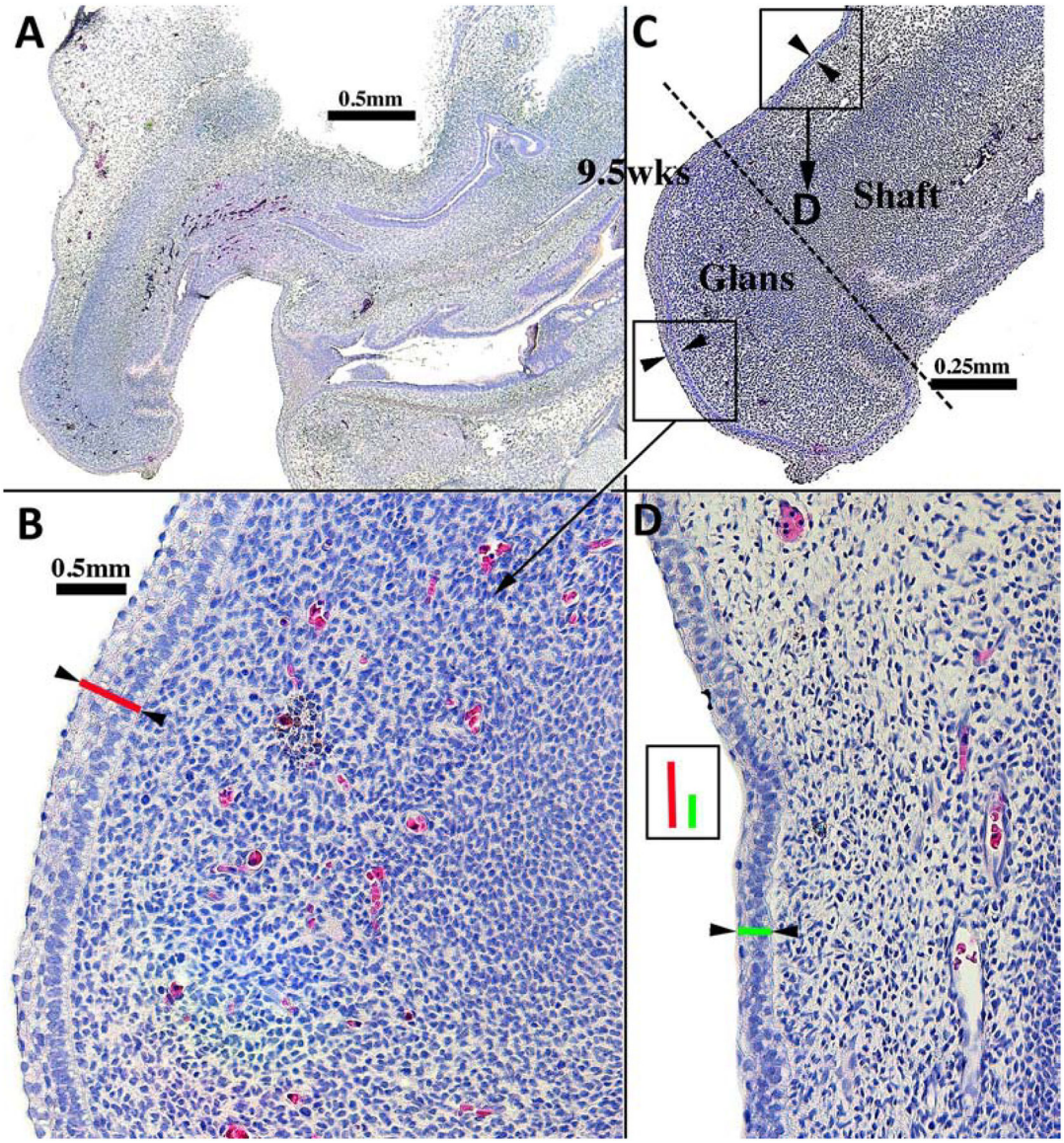

Figure 17.

Sagittal (A-C) and transverse (D-F) sections of the 14-week glans penis immunostained for S100. (A) gives an overview and shows large nerve bundles dorsal to the corporal body (CB) that are the dorsal nerve of penis (DNP). (B) is a higher magnification of the ventral portion of the preputial lamina (PPL) as indicated by the box showing numerous fine nerve fibers within the distal aspect of the glans as well as nerve fibers in the preputial mesenchyme (PPM). (C) is a higher magnification of the distal aspect of the glans and the preputial lamina (PPL) in sagittal section as indicated by the box in (A). Note the fine nerve fibers in this distal position. (D & E) are transverse sections at the levels indicated by the dotted lines in (A). (D) is a transverse section of a 14-week fetal glans penis at the level of the uncanalized urethral plate. Fine nerve fibers are seen in this distal portion of the glans. Branches of the dorsal nerve of the penis are seen dorsal to the corporal body (CB) in (E). In

(F) a density of nerve fibers is seen in the gap between the preputial laminae (frenulum) and in the preputial mesenchyme ventral to the gap.

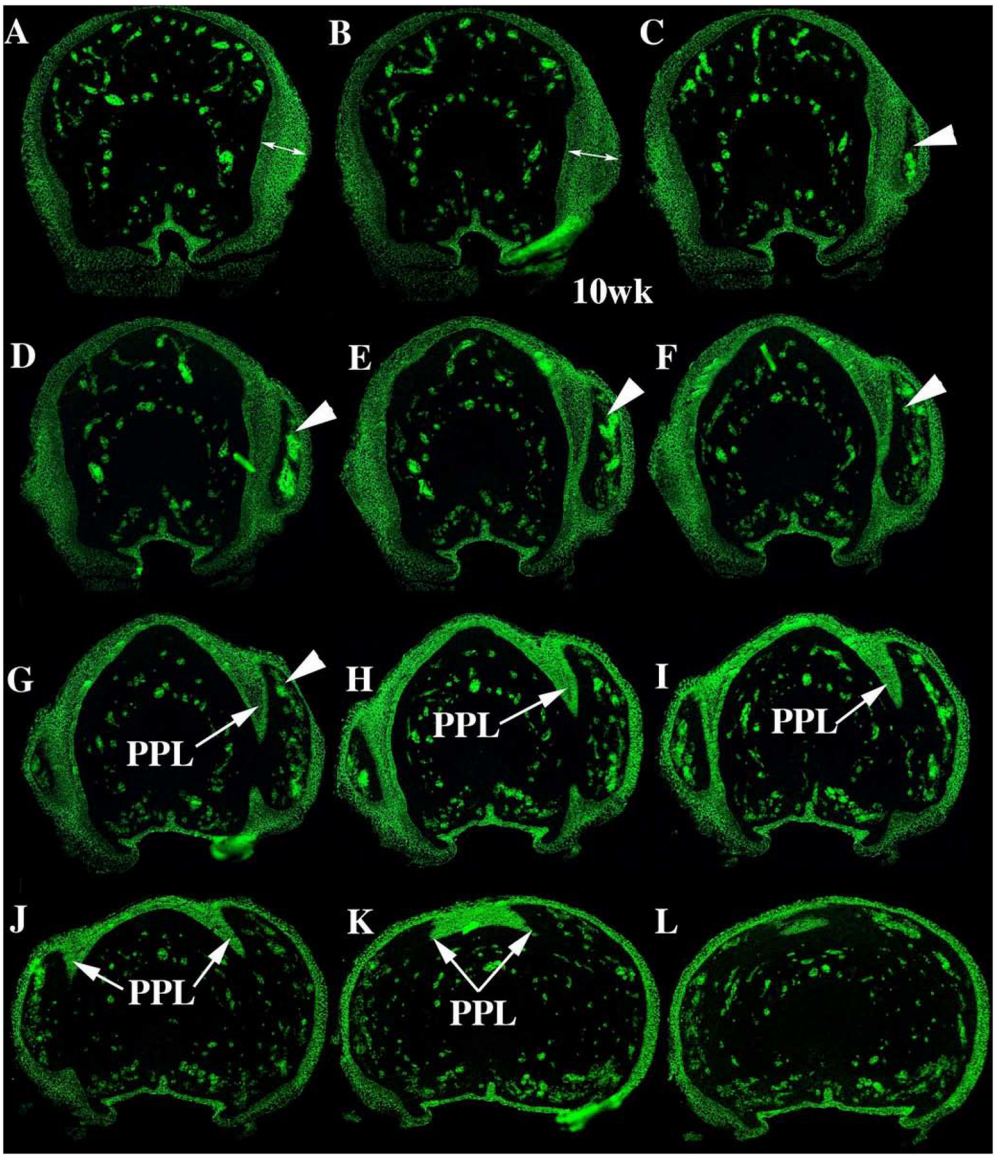

Figure 18.

Our model of development of the preputial lamina begins with the observation that the epidermis of the glans is substantially thicker than that of the penile shaft (A). The thick epidermis of the glans is split and remodeled by influx/invasion of a blood-vessel-rich mesenchyme to form the preputial lamina (brown) and a thin surface epidermis (yellow). The process begins in mid-dorsal or dorsal-lateral positions and spreads ventral-laterally. Eventually the glans is almost entirely covered by the prepuce.

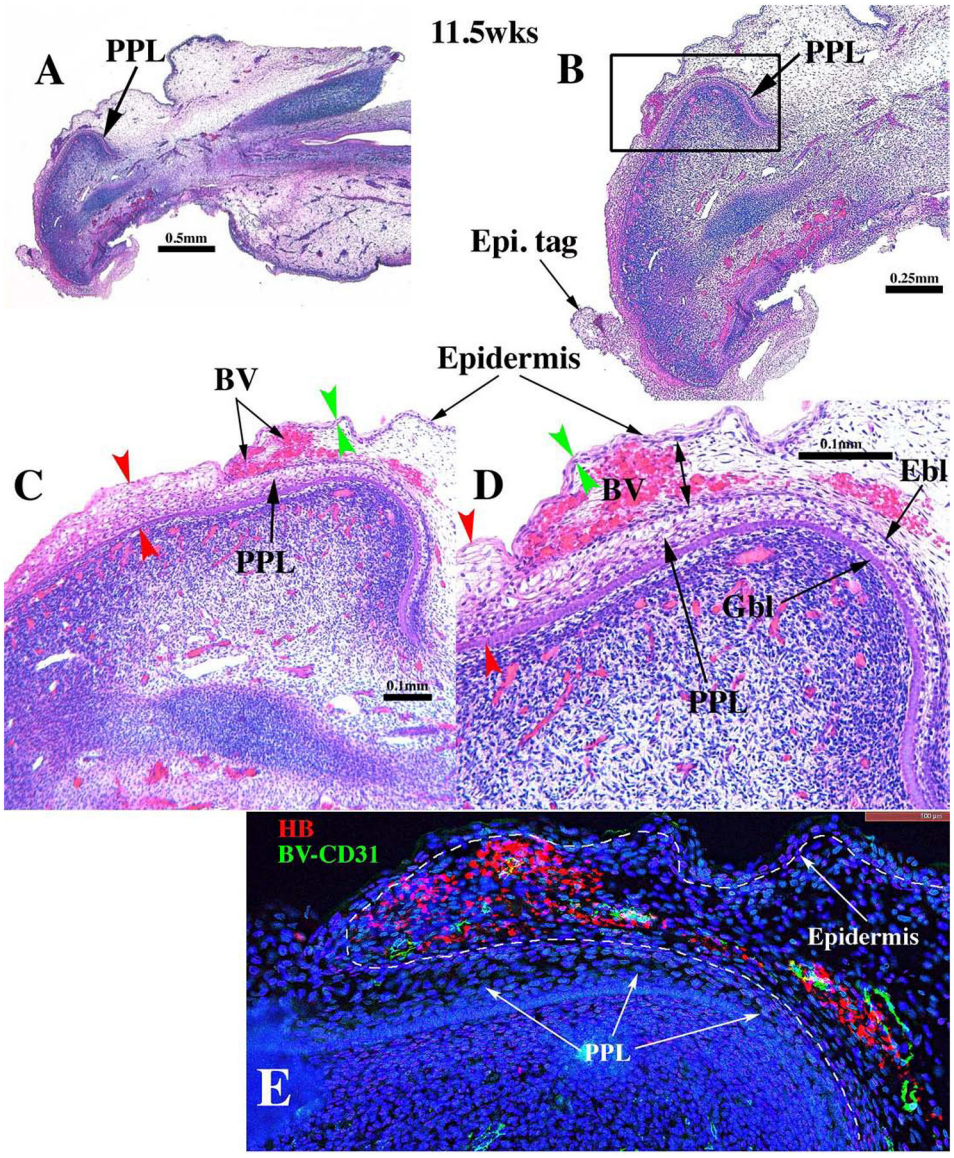

Figure 19.

Examples of developmental processes that involve division of a single morphological entity into 2 separate entities. (A) In the neonatal mouse the Mullerian vagina (M. Vag) inserts into the urethra just below the bladder and is separated from the urethra by a mesenchymal septum (pink). Accordingly, at birth the only urogenital orifice to the exterior is the urethral meatus. In the course of postnatal development the mesenchymal septum descends to separate the urethra from the vagina each having its own meatus to the exterior. (B) In human and mouse penile urethral development an epithelial seam (red) is removed as mesenchyme insinuates itself between the canalized urethral plate and the epidermis (top row) or between the urethra and the epidermis (bottom row) to establish midline mesenchymal confluence. (C) Division of the cloaca is accomplished by the urorectal septum (green) that divides the cloaca into the urogenital sinus ventrally and the rectum and

anal canal dorsally. (D) During remodeling of the laryngo-tracheal primordium, the trachea is separated from the esophagus by a mesenchymal septum (green).

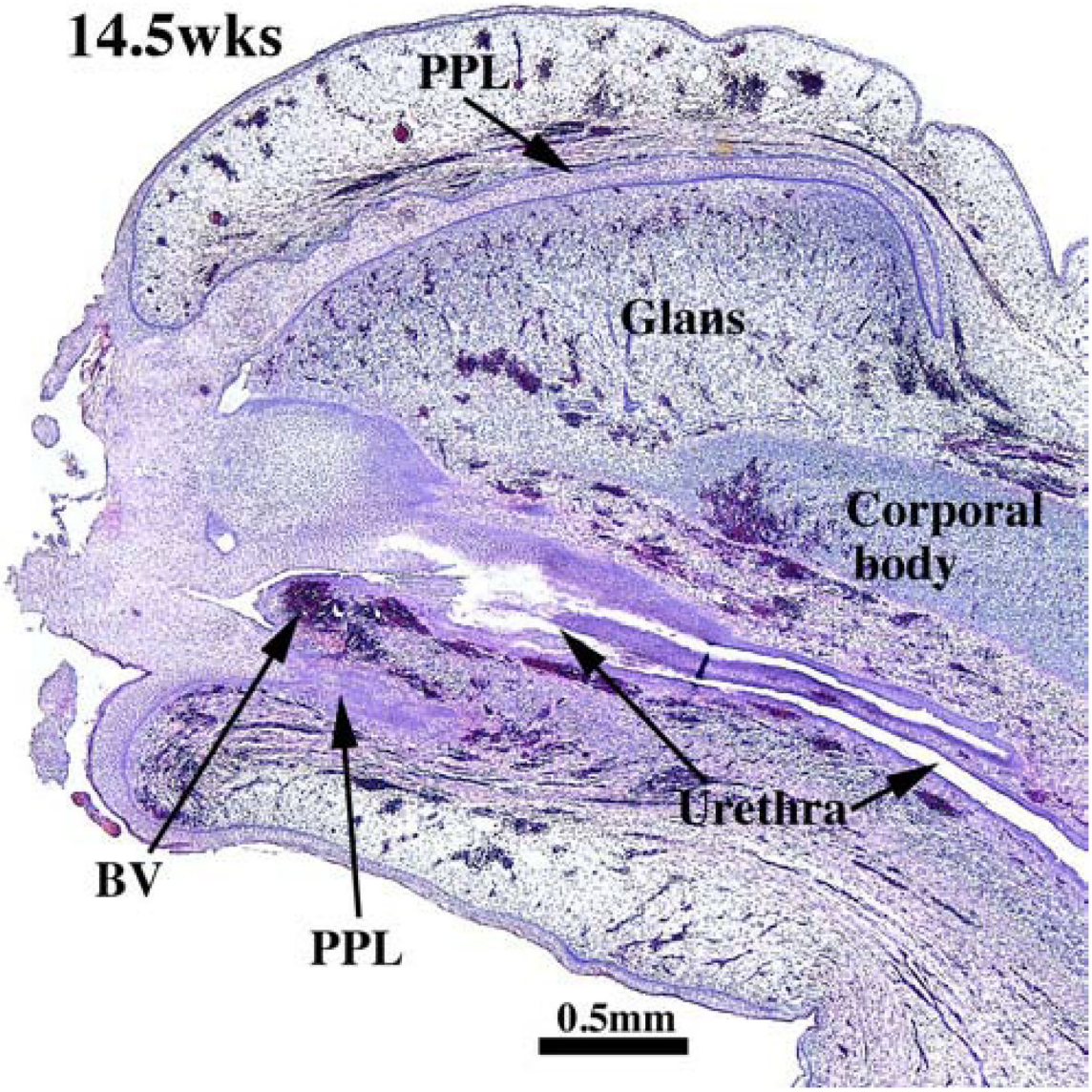

Figure 20.

Hypospadias: Mild to Severe A-F. Note as the hypospadias is more severe, there is an increase in the amount of the dorsal foreskin. Light blue asterisk = dorsal hooded foreskin. White arrow= urethral meatus (ectopic in B-F). Black arrow = open glanular urethra groove.

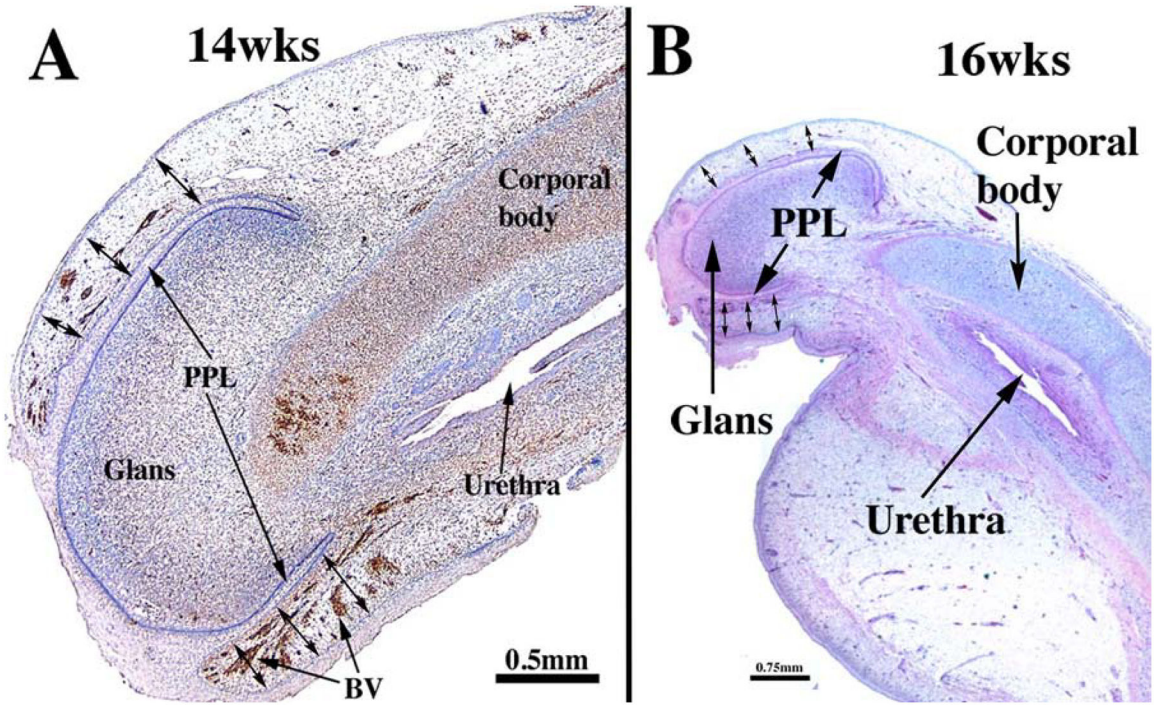

Figure 21.

A. Epispadias. B. Classic Bladder exstrophy. Note that in both of these congenital anomalies there is a severe defect in urethral formation on the dorsal aspect of the penis with a ventral hooded foreskin (light blue asterisk). The black arrow is the opening from which urine exits in A. The bladder is B is open to the skin and what is visualized is bladder mucosa.

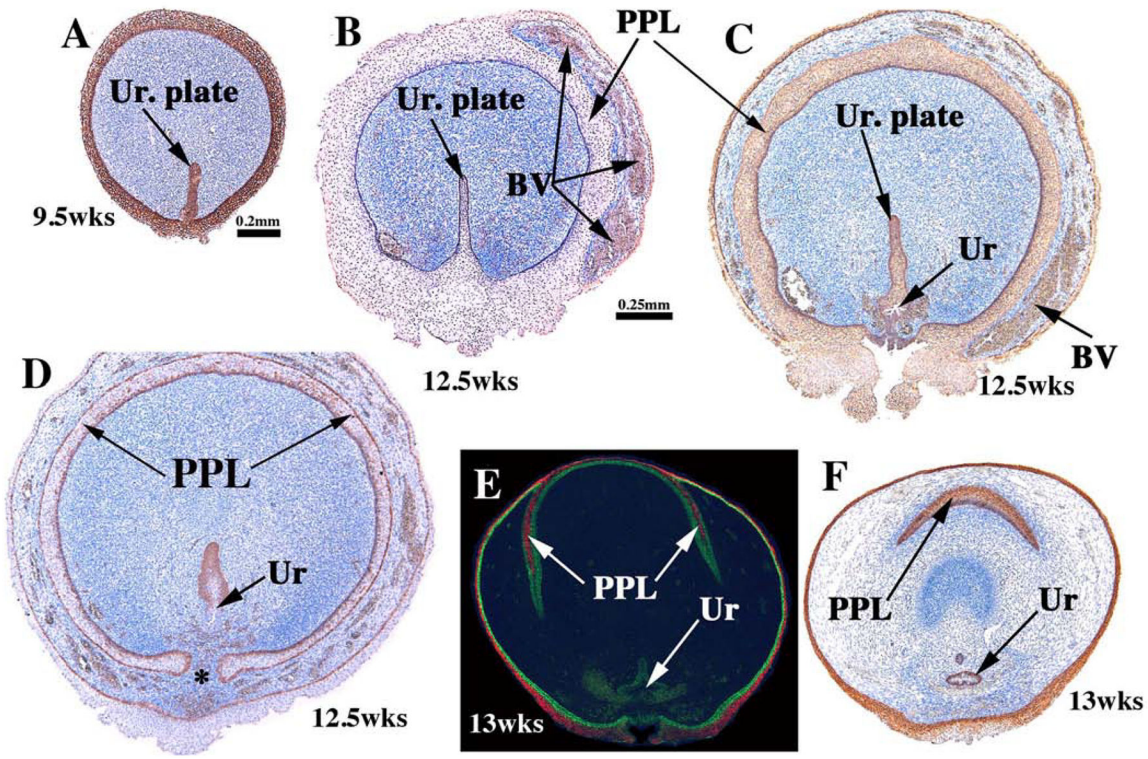

Figure 22.

Megameatus intact Prepuce. (A) Normal appearing penis with circumferential foreskin. (B) After retracting the normal prepuce, the megameatus is revealed with an opening in the proximal glans (white arrowhead) and the urethral pit (black arrow) consistent with a failure in establishing midline mesenchymal confluence (See figure 19B).

Table 1.

Antibodies used in this study

Antibody	Source	Catalogue #	Concentration
E-cadherin	BD Transduction Laboratories	610181	1/100
Keratin 6	Acris Antibodies	AM21068PU-S	1/200
Keratin 10	BioGenex	LL002	1/100
Keratin 14	E.B. Lane *	LP2K	1/10
Estrogen receptor alpha (ESR1)	Abcam	Ab16660	1/100
Estrogen receptor beta (ESR2)	Leica	NCL-ER-beta	1/50
Androgen receptor	Genetex	GTX62599	1/100
S100	Abcam	ab52642	1/1000

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