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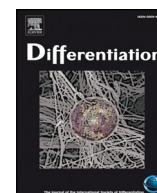
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Androgen and estrogen receptor expression in the developing human penis and clitoris



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ABSTRACT

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

Areas of reduced or absent Ki67 staining include the urethral fold epithelium that fuses to form the penile tubular urethra. In contrast, the urethral fold mesenchyme is positive for Ki67. Apoptosis was rarely noted in the developing penis and clitoris; the only area of minimal Caspase-3 localization was in the epithelium of the ventral epithelial glanular channel remodeling.

1. Introduction

The Jost Hypothesis states that androgens are necessary for male sexual differentiation (Jost, 1953, 1972). As a corollary, female sexual differentiation occurs in the absence of androgens and/or the absence of androgen action secondary to defects in, or absence of, the androgen receptor (AR) (Batista et al., 2018; Holmes et al., 2004). While human penile development is globally dependent upon androgens, many individual steps in the complex process of human penile development are androgen-independent such as development of the genital tubercle, formation of a urethral plate, canalization of the urethral plate, initial formation of human corporal bodies, formation of the glans and development of the prepuce (Cunha et al., 2019a). This interpretation is

supported by the fact that all of these individual developmental steps occur in external genitalia of both developing human males and females (Cunha et al., 2019a). Androgen-dependent steps in penile development include: specification of penile developmental identity, fusion of the urethral folds to form a penile urethra, penile growth, urethral plate canalization within the glans, preputial growth, corporal body growth, midline mesenchymal confluence ventral to the urethra and orientation of the penis to ~90° from the body wall (Baskin et al., 2018; Li et al., 2015; Liu et al., 2018). It should be noted that mouse penile anatomy and development differ from that of human, and thus mouse is not the ideal model for normal and abnormal human penile development (Cunha et al., 2015). Even though mouse penile development also involves both androgen-dependent and androgen-independent

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processes, individual developmental steps differ between these two species (Cunha et al., 2019a; Hutson et al., 2014).

Thus, androgens have clearly been shown to be necessary for normal male genital development (Cunha et al., 2019a; Baskin et al., 2018). In humans and murine transgenic models, this is best illustrated by complete androgen insensitivity in which the AR is non-functional resulting in XY individuals whose external genitalia are feminized (Yucel et al., 2004). However, close analysis of the external genitalia phenotype of such patients reveals a subtle difference between the phenotypes of human females (XX) without AR mutations compared to human females (XY) with AR mutations (Wilson et al., 2011). Such differences could be due to estrogen action (from testosterone conversion to estrogen by aromatase). The role of estrogens and the estrogen receptor (ER) during normal development of external genitalia remains to be elucidated. Recent evidence, however, has emerged that estrogens may play a role along with androgens during normal and abnormal external genitalia development (Zheng et al., 2015). Indeed, penile abnormalities may be due to alteration in the estrogen/androgen signaling balance leading specifically to induction of urethral abnormalities characterized as hypospadias in animal models treated perinatally with exogenous estrogen (Blaschko et al., 2013; Mahawong et al., 2014a, 2014b; Kim et al., 2004; Sinclair et al., 2016, 2017). In this regard, human epidemiologic data suggest that the etiology of hypospadias is based in part upon exposure to estrogenic endocrine disruptors (Baskin et al., 2001). Taken together, this body of scientific evidence supports a role of androgens in normal development of the human external genitalia and suggests that disruption of AR pathways via perturbation of the estrogen/androgen signaling balance may lead to abnormalities such as hypospadias and impaired virilization (Wilson, 2001; Baskin, 2017). The estrogen/androgen signaling balance can be perturbed in several ways: (a) acute exposure to exogenous estrogenic agents during development (Baskin et al., 2001), (b) the effect of estrogens leading to reduction in serum testosterone mediated via the pituitary/gonadal axis (Cook et al., 1998), (c) the developmental effects of exogenous estrogens leading to permanent elevation (vom Saal et al., 1997) and/or reduction in AR levels in androgen target tissues (Prins, 1992) or (d) estrogenic up-regulation of the estrogen receptor (Zheng et al., 2015).

To better understand the mechanism of action of androgens and estrogens in human genital development, we explored the ontogeny and precise location of the androgen receptor (AR), estrogen receptors alpha (ER α) and beta (ER β) and the progesterone receptor (PR) in the developing human penis and clitoris from the indifferent stage at 8 weeks gestation to the fully differentiated penis and clitoris at 16–17 weeks of gestation (Baskin et al., 2018; Li et al., 2015; Overland et al., 2016; Shen et al., 2016).

2. Methods

Human fetal lower urogenital tracts were collected without patient identifiers after elective termination of pregnancy with approval from the Committee on Human Research at UCSF (IRB# 16–19909). Fetal age was estimated using heel-toe length (Drey et al., 2005). Age is reported from the time of fertilization and not from last menstrual period. Chromosomal sex was determined by PCR to detect the presence of a Y-chromosomal gene (Li et al., 2015) and, when possible, was confirmed by identification of Wolffian and Müllerian duct morphology using a dissecting microscope. Eight - to 17-week human fetal male and female

specimens were processed for scanning electron microscopy, optical projection tomography and immunohistochemical staining for the AR, ER α , ER β and PR as previously described using the antibodies in Table 1 (Li et al., 2015; Shen et al., 2018; Isaacson et al., 2018). A total of 36 human fetal specimens were analyzed, split equally between male and female with ~4 specimens analyzed at each time point. Transverse or sagittal sections of male and female external genitalia were cut at 6 μ m and mounted on glass slides for histologic and immunohistochemical staining.

3. Results

Scanning electron microscopy (SEM) of the developing human fetal penis and human clitoris reveals a divergence in genital morphology after the indifferent stage (~8 weeks gestation) in which males and females are indistinguishable (Fig. 1). Canalization of the urethral plate in males and vestibular plate in females occurs in both sexes, but in males (a) the urethral folds subsequently fuse to form a penile urethra, (b) the glanular urethra forms by direct canalization of the urethral plate and (c) a complete circumferential prepuce forms that covers the glans (Liu et al., 2018; Shen et al., 2016; Cunha et al., 2019b). In females the vestibular groove remains open with the vestibular folds forming the labia minora, while the prepuce of the clitoris only forms dorsally on the clitoral glans (Fig. 1). A lateral SEM view of the ontogeny of the developing penis and clitoris illustrates the difference in orientation of the penis which remains ~90° to the body wall in contrast to the clitoris whose orientation remains angled into the body wall (Fig. 2). In both the developing penis and clitoris an epithelial tag of unknown significance is transiently present on the glans (blue arrowheads) (~9–13 weeks gestation) (Figs. 1 and 2). On the ventral surface of the glans penis extensive tissue remodeling is noted (Figs. 1 and 2) as aggregates of epidermal cells form and “ball up” (red arrowheads) on the surface of the penis (Fig. 1 N & O). These epidermal aggregates appear to be sloughed/jettisoned as development advances as a circumferential prepuce covers the glans (Fig. 1 N & O inserts).

Optical projection tomography of the developing penis illustrates several androgen-dependent events in penile development (Fig. 3). Note the progressive proximal to distal fusion of the urethral folds to form the penile urethra, thus advancing the urethral meatus distally (orange arrows) (Fig. 3). The transient epithelial tag (blue arrows) and sloughed/jettisoned epidermal tissue on the ventral aspect of the glans (red arrows) are also well visualized (Fig. 3), both of which disappear by 15 weeks.

At 8 weeks of gestation, the indifferent stage, the human genital tubercle expresses AR in mesenchyme adjacent to the urethral plate with ER β localizing preferentially to the urethral plate and the surface epithelium (Fig. 4). ER α was not detected at this stage (data not shown), and PR was not detected at any stage of human penile development.

3.1. AR, ER β and ER α expression in the developing penis

At 9 weeks of gestation, AR was expressed globally throughout the fetal penis with particularly high density of the AR staining in the corporal body and in ventral mesenchyme adjacent to the urethral groove and tubular urethra (Fig. 5).

Table 1
Antibodies used in this study.

Antibody	Source	Catalogue #	Concentration	Reacts with:
Androgen receptor	Genetex	GTX62599	1/100	Androgen receptor
Estrogen receptor alpha (ER α)	Abcam	Ab16660	1/100	Estrogen receptor alpha
Estrogen receptor beta (ER β)	Leica	NA	1/50	Estrogen receptor beta
Progesterone receptor	Abcam	Ab16661	1/100	Progesterone receptor

Ontogeny of the Male and Female Fetal Penis and Clitoris

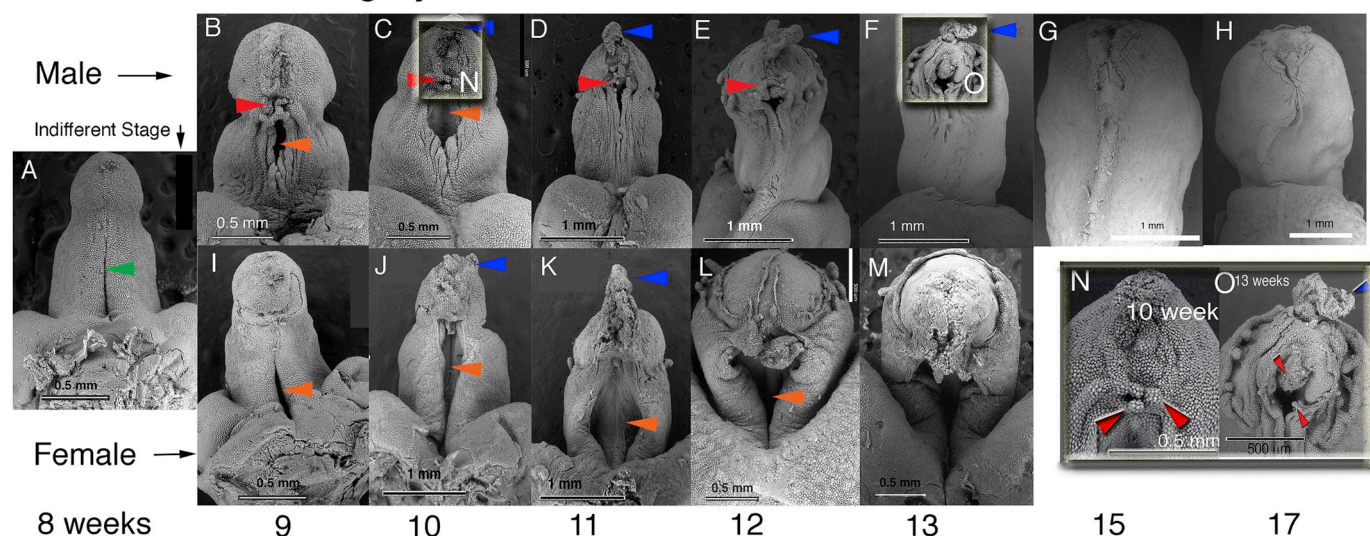


Fig. 1. Scanning electron microscopy (ventral views) of the developing human fetal penis (A–H) from 8 to 17 weeks of gestation and of the developing human clitoris (I–M) 8–13 weeks of gestation. Note the indifferent stage at 8 weeks of gestation (A). The green arrowhead highlights the urethral plate, blue arrowheads indicate the epithelial tag, red arrowheads denote sloughing epithelial tissue and orange arrowheads the urethral and vestibular groove, respectively. Note the progressive fusion of the urethral folds in the penile specimens to form the male urethra (B–H) in contrast to the wide open vestibular groove. Note the aggregates of epidermal cells that appear to “ball up” and slough during development and remodeling of glanular urethra (higher power inserts, N & O). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Lateral View: Ontogeny of the Developing Penis and Clitoris

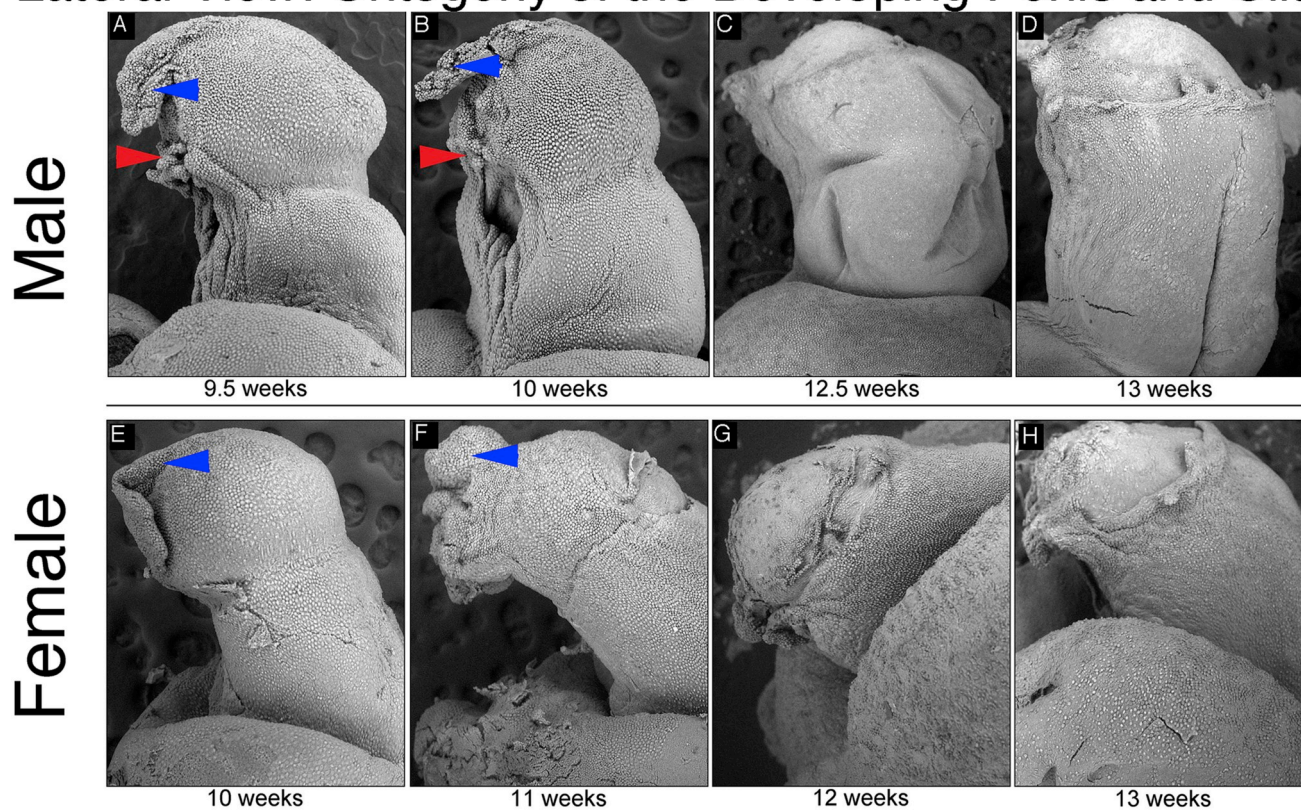


Fig. 2. Scanning electron microscopy (lateral views) of the ontogeny of the developing human fetal penis (A–D) from 9.5 to 13 weeks of gestation and developing human clitoris (E–H) 10–13 weeks of gestation. The blue arrowheads highlight the epithelial tag and red arrowheads the sloughed epithelial tissue. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Human Fetal Penis Ontogeny: Optical Projection Tomography Showing Urethral Plate, Epithelial Tag, Urethral Groove and Extruded Tissue

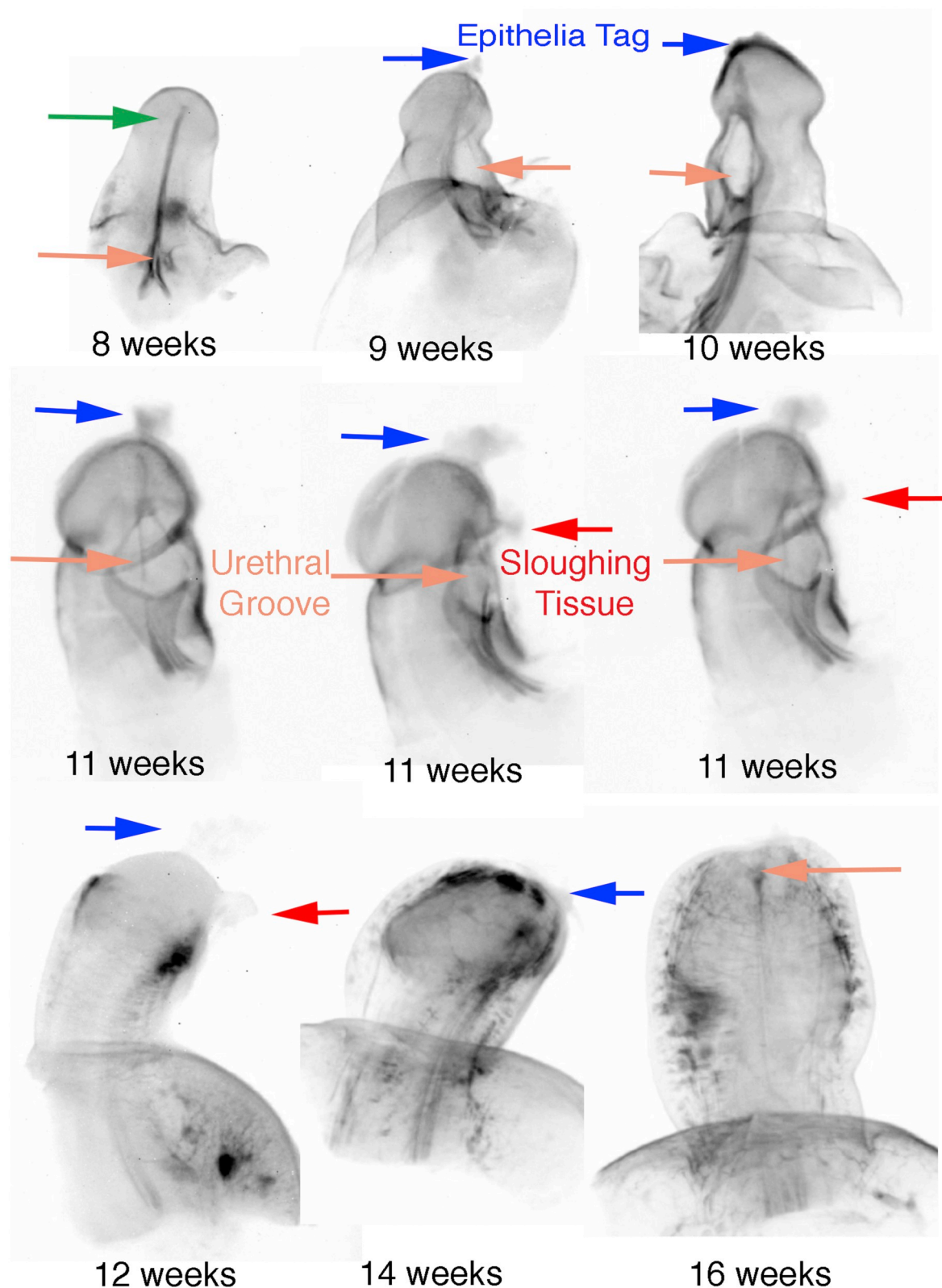


Fig. 3. Optical Projection Tomography of the developing human fetal penis from 8 to 16 weeks of gestation. The epithelial tag is seen from 9 to 12 weeks of gestation (blue arrows). Note the progression of the urethral meatus from the penoscrotal junction to the tip of the penis (orange arrows). Sloughing epidermal tissue from the glans can be seen in the 11–14-week specimens (red arrows). Note the urethral plate in the 8-week specimen extending almost to the tip of glans (green arrow). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Human Genital Tubercle: Indifferent Stage 8 Weeks Gestation

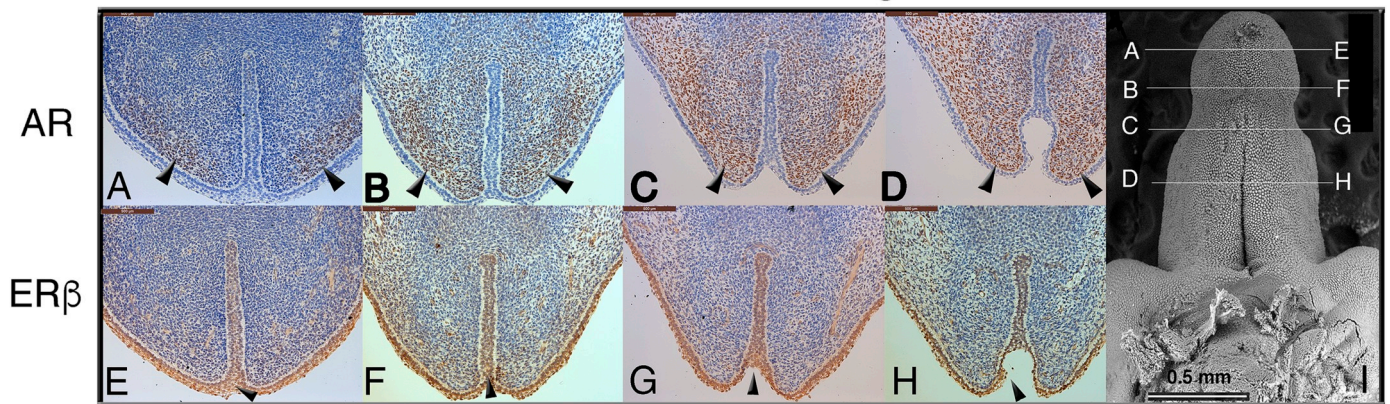


Fig. 4. Transverse sections of human genital tubercle at the indifferent stage (8 weeks of gestation) immunostained for: AR (A–D) and ER β (E–H). Representative scanning electron microscopic image (I) showing relative location of each histologic section. Note the relative high density of the AR staining in the mesenchyme associated with the urethral plate (arrowheads, A–D). ER β preferentially localizes to the urethral plate (arrowheads, E–H) and the surface epithelium.

At 11.5 weeks of gestation AR, ER α and ER β were all expressed in the human fetal penis (Fig. 6). AR was prominently expressed in the corporal body, in the mesenchyme of the glans, in mesenchyme ventral to the penile corporal body (Fig. 6A) and in mesenchyme associated with the developing prepuce. Epithelium of the preputial lamina was AR-negative (Fig. 6B).

ER α expression was relatively sparse at 11.5 weeks but was clearly seen in the basal epithelial cells of the penile preputial lamina and ventral epidermis (Fig. 6D, arrowheads). In contrast, ER β expression was prominent in (a) the penile corporal body, (b) epithelium of the urethral groove, (c) peripheral mesenchyme associated with the epidermis of the glans penis, (d) in basal and supra-basal cells of the preputial lamina and (e) and in penile epidermis (Fig. 6 E&F).

Similarly, at 12.5 weeks gestation AR, ER α and ER β continued to be expressed in the human fetal penis (Fig. 7). AR was prominently expressed in the corporal body and in epithelium and mesenchyme at the site of urethral plate canalization and formation of the glanular urethra and the urethral meatus (Fig. 7B). ER α expression was again relatively sparse but was clearly seen in the basal epithelial cells of the epidermis near the urethral meatus (Fig. 7D). ER β expression was prominent in the corporal body, in epithelium and mesenchyme of the urethral meatus, and in basal and supra-basal cells of the preputial lamina (Fig. 7 F&G).

At 14 weeks of gestation AR, ER α and ER β were also detected in the human fetal penis (Fig. 8). AR expression was prominent in the corporal body and in the mesenchyme surrounding the urethra (Fig. 8A). AR was also detected in mesenchyme associated with the preputial lamina, while epithelium of the preputial lamina was generally AR-negative

(Fig. 8B) with the exception of rare AR-positive cells (Cunha et al., 2019b). ER α was seen only in the basal epithelial cells of the preputial lamina (Fig. 8 D&E, arrowheads). ER β was prominently expressed in the basal and supra-basal epithelial layers of the preputial lamina (Fig. 8 G&H, arrowheads).

At 15 weeks of gestation AR, ER α and ER β continued to be expressed in the human fetal penis (Fig. 13). The corporal body was intensely AR-positive (Fig. 9A). AR was also expressed in epithelium and mesenchyme at the site of urethral plate canalization within the glans and sites of formation of the glanular urethra and the urethral meatus (Fig. 9B). ER α expression was focally expressed in the basal epithelial cells of the preputial lamina and in the urethral plate (Fig. 9 D&E). ER β expression was prominently expressed in the canalizing urethral plate and in association with the forming urethral meatus (Fig. 9 G&H (arrow)). PR was not detected in the developing penis.

Transverse sections of the human fetal penis at 15 weeks gestation reveal AR expression in the glans mesenchyme, in the basal epithelial layer of the urethral plate within the glans (Fig. 10 B&E) and in regions of urethral plate canalization and remodeling (Fig. 10C). Also note the preferential AR expression in the epithelium lining the ventral aspect of the newly formed glanular urethra and within the forming frenulum (Fig. 10E–F).

At 16 weeks of gestation AR, ER α and ER β continued to be prominently expressed in the human fetal penis (Fig. 11). AR was detected in the corporal body, in mesenchyme of the glans and in the basal epithelial layer of the preputial lamina (Fig. 11A–C). The preputial lamina has a basal layer facing the glans as well as a basal layer associated with preputial mesenchyme. ER α was expressed in the basal

AR Expression Human Penis: 9 Weeks Gestation

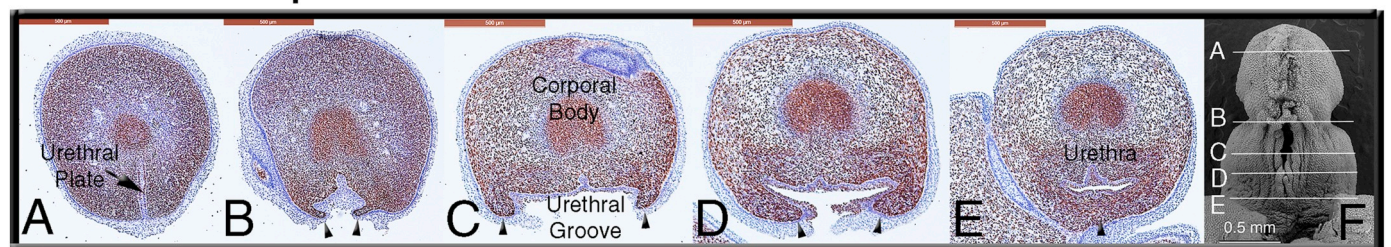


Fig. 5. Transverse sections of 9-week human penis immunostained for AR (A–E). Representative scanning electron microscopic image (F) showing relative location of each histologic section. AR is expressed in penile mesenchyme from the distal to proximal in association with the urethral plate (A), the canalizing urethral plate (B), the open urethral groove (C, D) and the tubular urethra (E). Note the high density of the AR staining in the corporal body and in the mesenchyme adjacent to the urethral groove and tubular urethra (arrowheads, D–E).

Human Fetal Penis: 11.5 Weeks Gestation

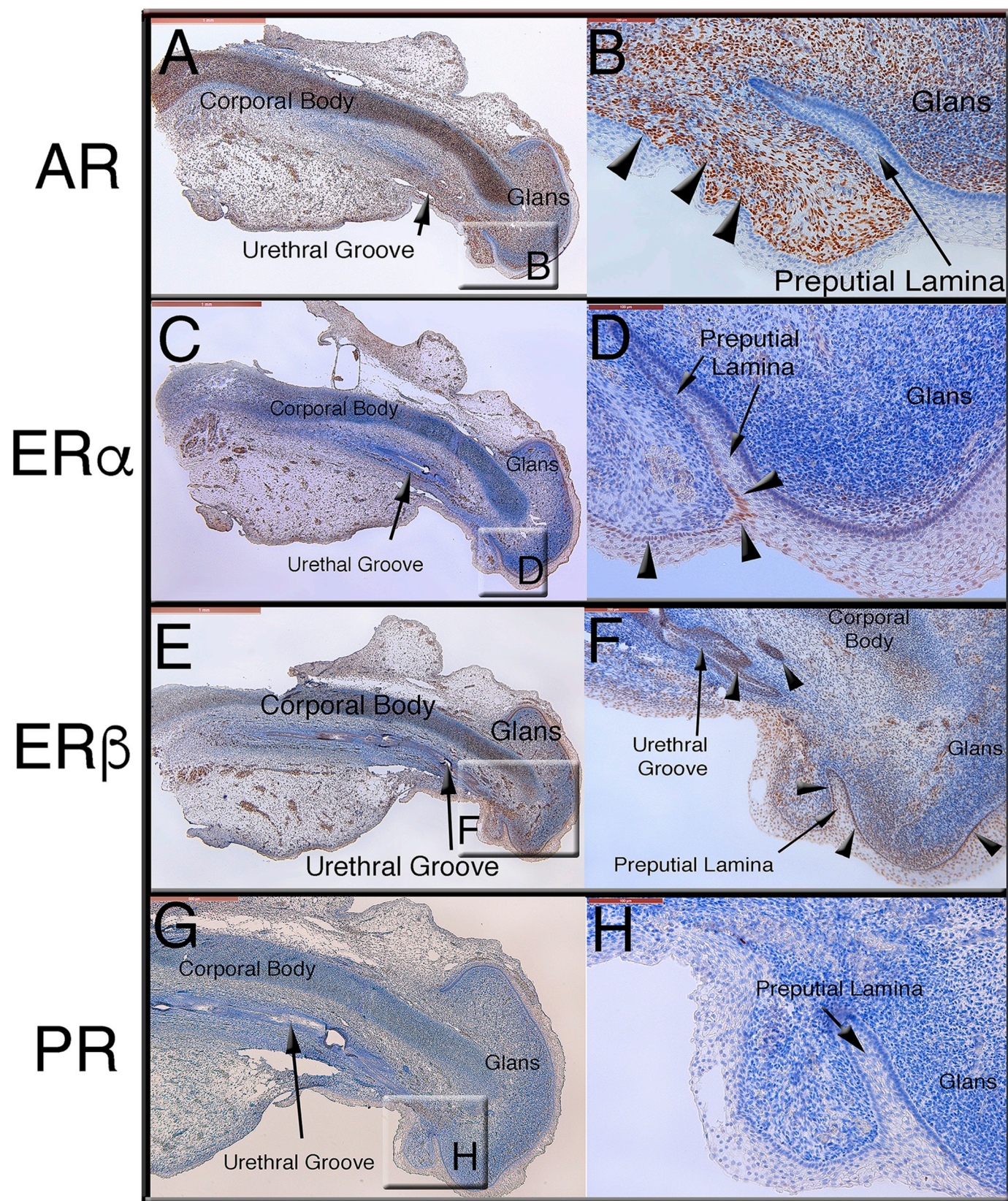


Fig. 6. Sagittal sections of a 11.5 week human penis immunostained for AR (A&B), ER α (C&D), ER β (E&F), and PR (G&H). Note prominent AR expression in the corporal body (A) and in mesenchyme of the glans and mesenchyme associated with the preputial lamina (B, black arrowheads). ER α expression is relatively sparse but can be seen in the basal epithelial cells of the preputial lamina (D, black arrowheads) and in basal cells of the epidermis. In contrast, ER β expression is more prominent localizing to the corporal body, epithelium of the urethral groove, to the epidermis of the glans penis and basal and supra-basal cells of the preputial lamina (E&F, black arrowheads). PR staining was undetectable (H).

Penis:12.5 Weeks Gestation

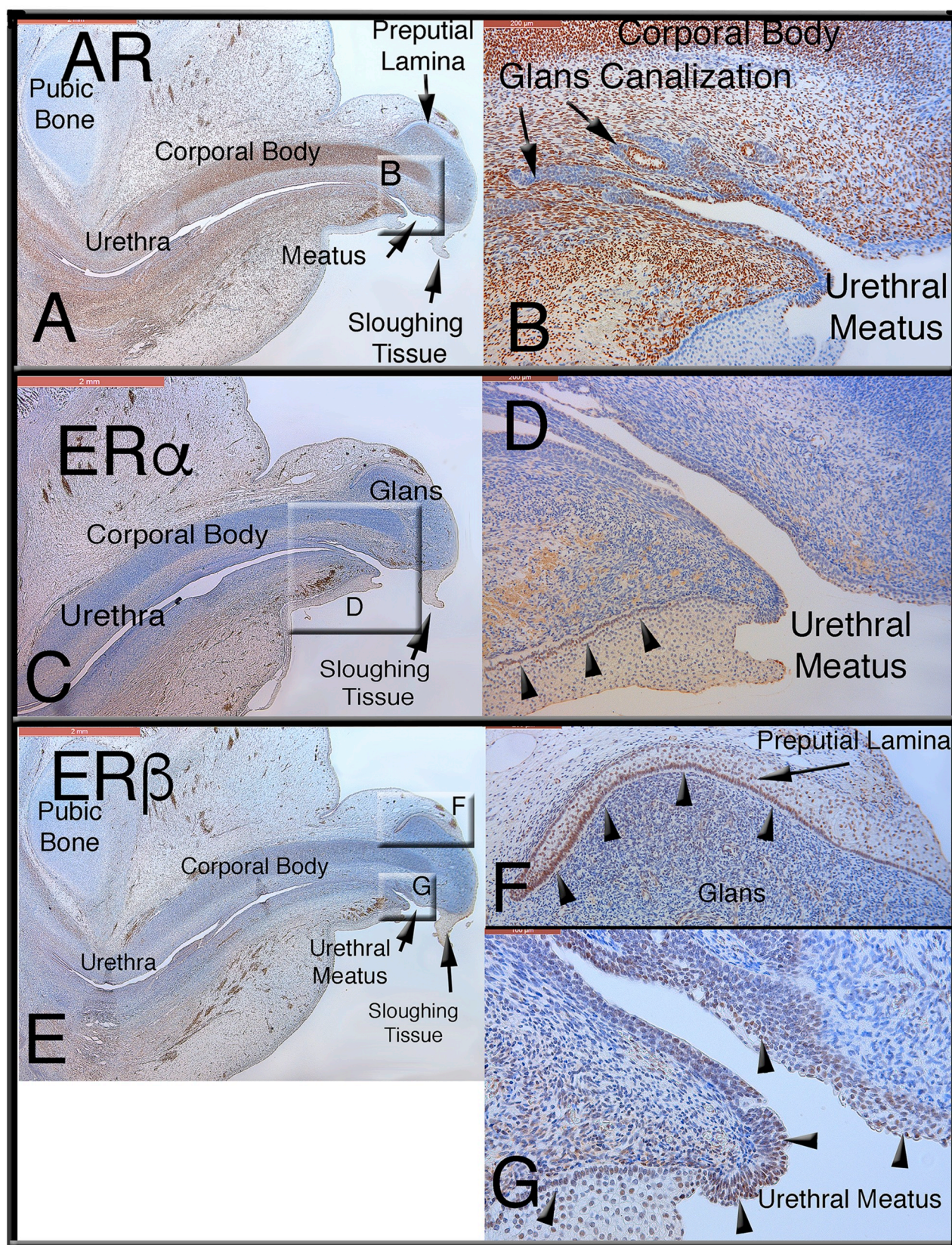


Fig. 7. Sagittal sections of human penis at 12.5 weeks of gestation immunostained for: AR (A&B), ER α (C&D) and ER β (E–G). Note prominent AR expression in the corporal body (A–B) and in epithelium and mesenchyme at the site of canalization and formation of the future urethral meatus (B, black arrows). ER α expression is relatively sparse but is seen in basal cells of the epidermis near the urethral meatus (D, black arrowheads). In contrast, ER β expression is more prominent localizing to the corporal body (E), epithelium and mesenchyme of the urethral meatus (G), and basal and supra-basal cells of the preputial lamina (F, black arrowheads).

Penis: 14 Weeks Gestation

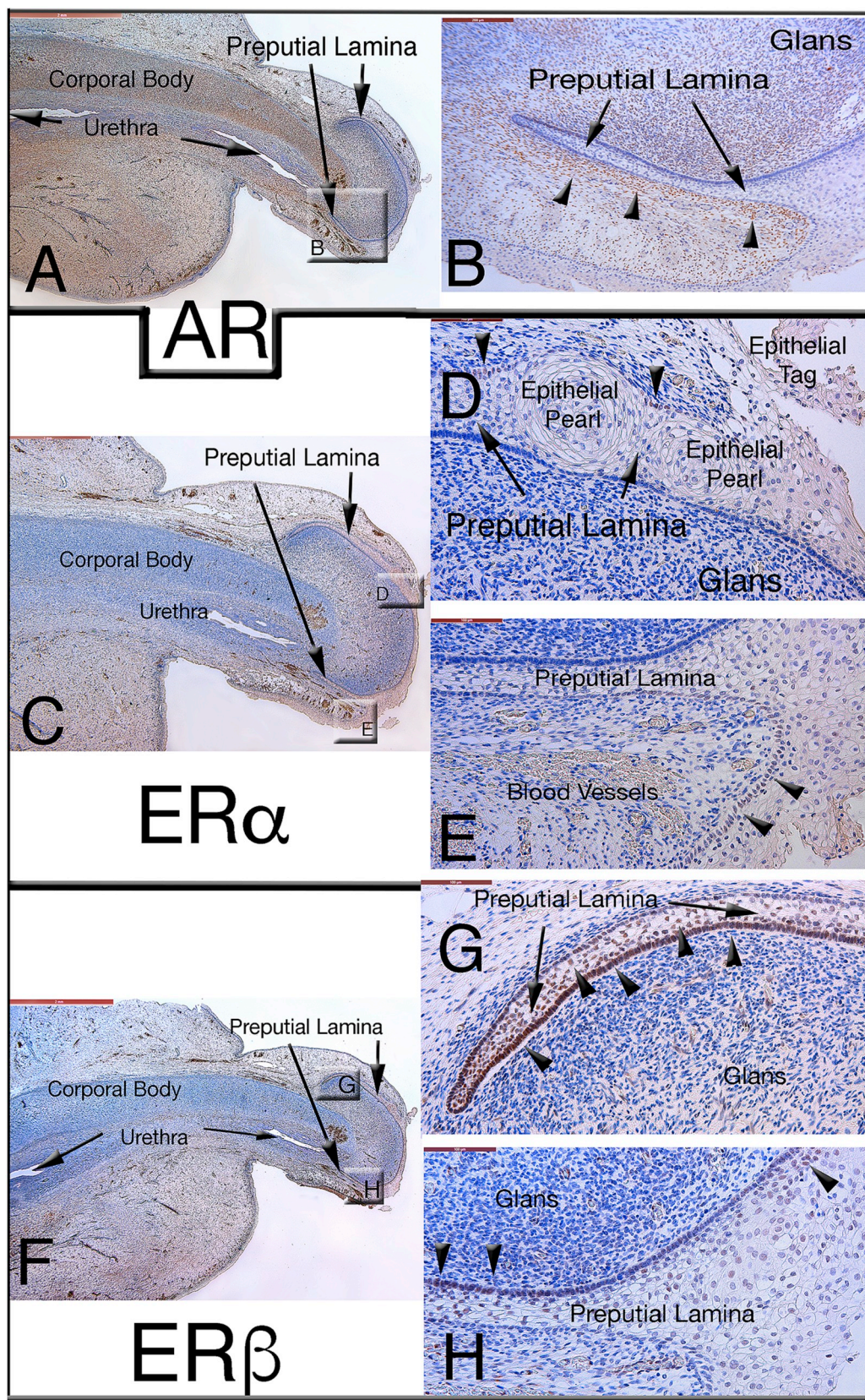


Fig. 8. Sagittal sections of human penis at 14 weeks of gestation immunostained for: AR (A&B), ER α (C–E) and ER β (F–H). Note the prominent AR expression in the corporal body (A) and in mesenchyme surrounding the urethra. Also note AR expression in mesenchyme adjacent to the preputial lamina (black arrowheads) and in the glans (B). ER α expression is relatively sparse but is seen in the basal epithelial cells of the epidermis and preputial lamina (black arrowheads) (D&E). Also note the epithelial pearls within the preputial lamina (black arrows) (D) and blood vessels in the ventral prepuce (E). ER β expression is prominent in the basal and supra-basal epithelial cells of the preputial lamina (black arrowheads) (G&H).

Penis: 15 Weeks Gestation

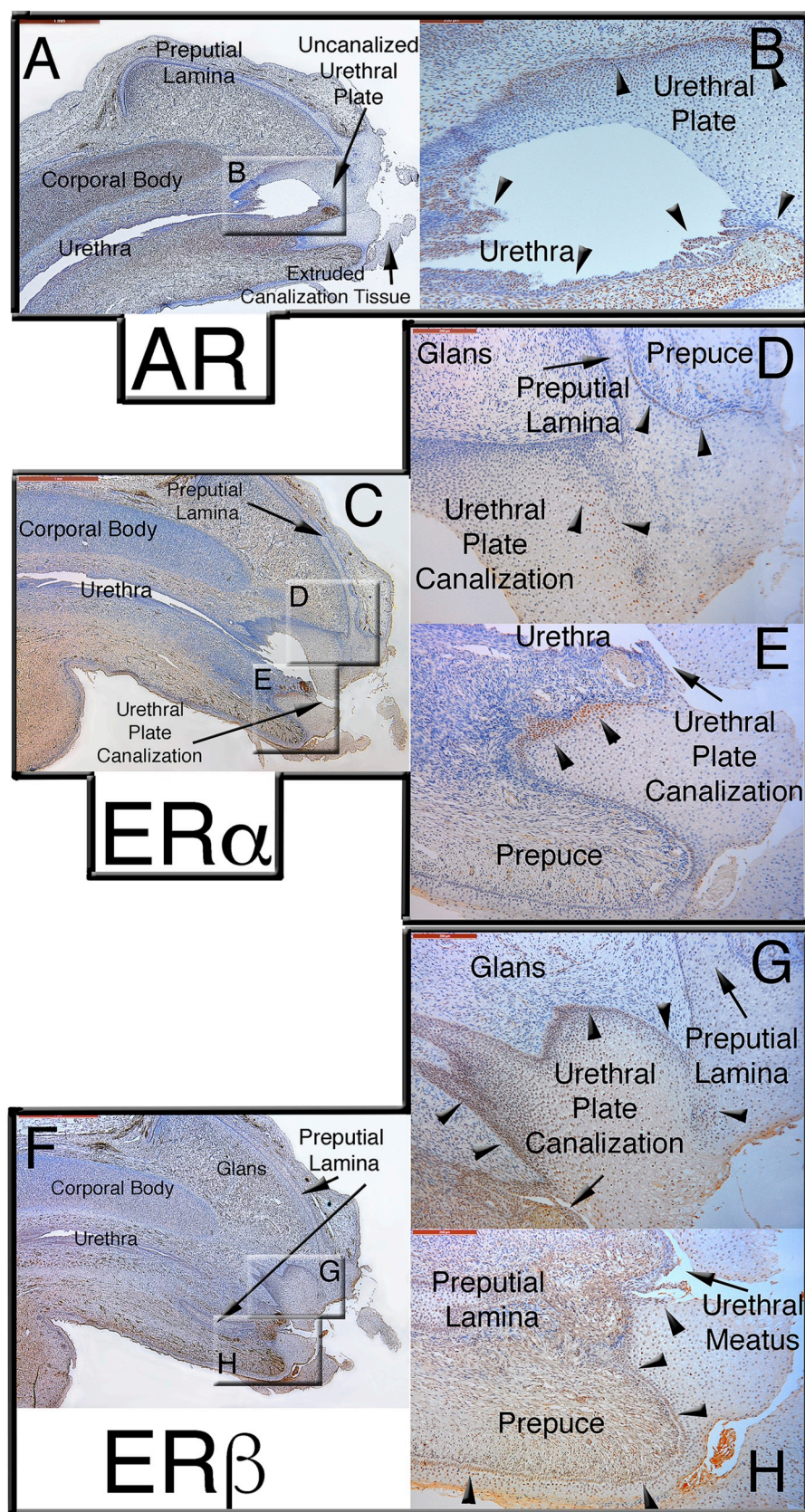


Fig. 9. Sagittal sections of human penis at 15 weeks of gestation immunostained for: AR (A&B), ER α (C–E) and ER β (F,H). Note prominent AR expression in the corporal body (A) and in the epithelium and mesenchyme associated with the canalizing urethral plate (black arrowheads) and mesenchyme of the newly formed urethral meatus (B). ER α expression is relatively sparse but is seen in basal epithelial cells of the epidermis and preputial lamina and in urethral plate (black arrowheads) (D&E). ER β expression is more prominent and seen in the canalizing urethral plate and the forming urethral meatus (black arrowheads) and epidermis (G&H).

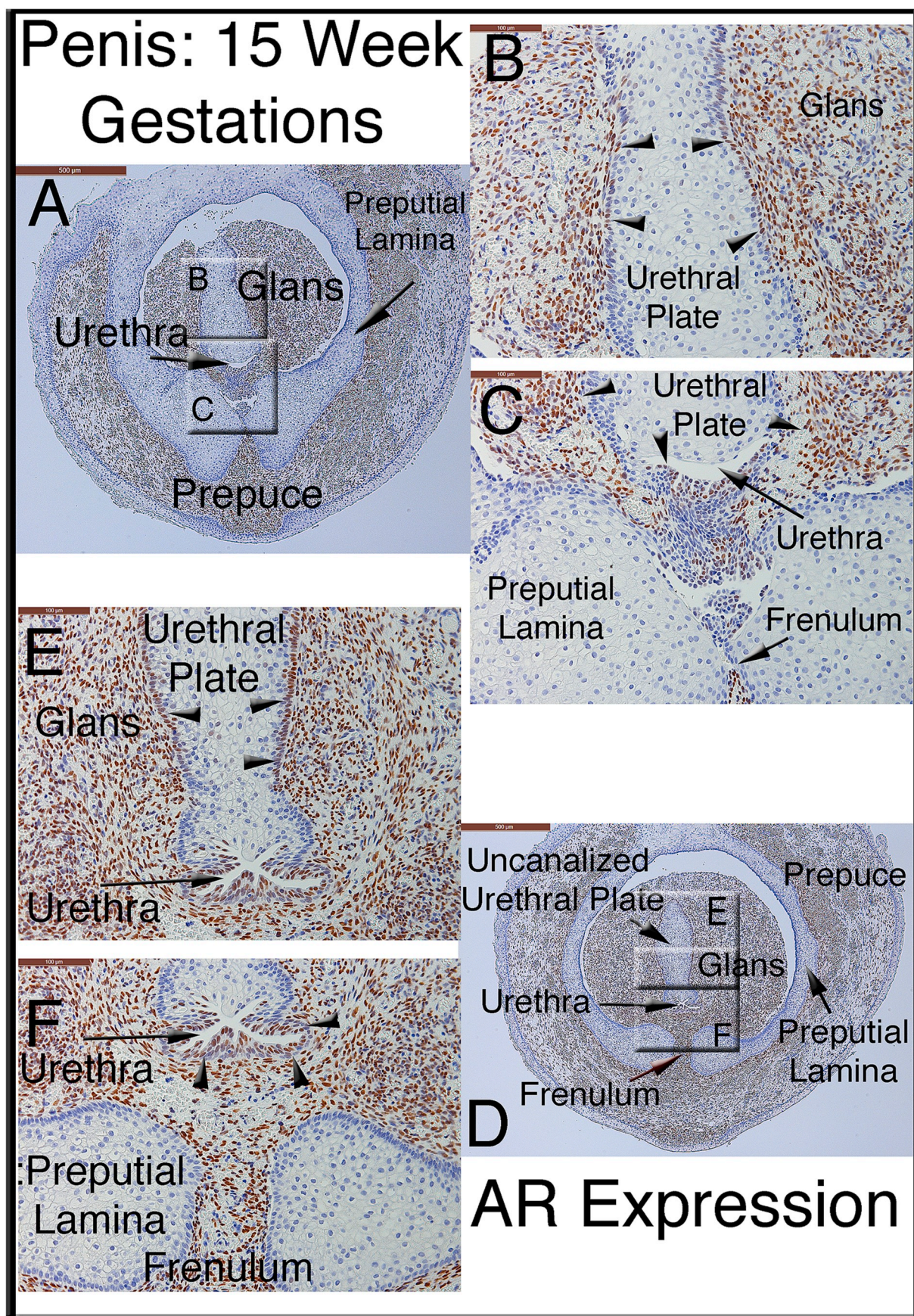


Fig. 10. Transverse sections of human penis at 15 weeks of gestation immunostained for AR. Note prominent AR expression in glans mesenchyme and basal epithelial cells of the urethral plate (black arrowheads) (B&E) and in the canalizing and remodeling urethral plate (C). Also note the preferential AR expression in epithelial cells of the ventral aspect of the newly formed urethra (black arrowheads) (E, E-F) and within the frenulum (D-F).

Penis: 16 Weeks Gestation

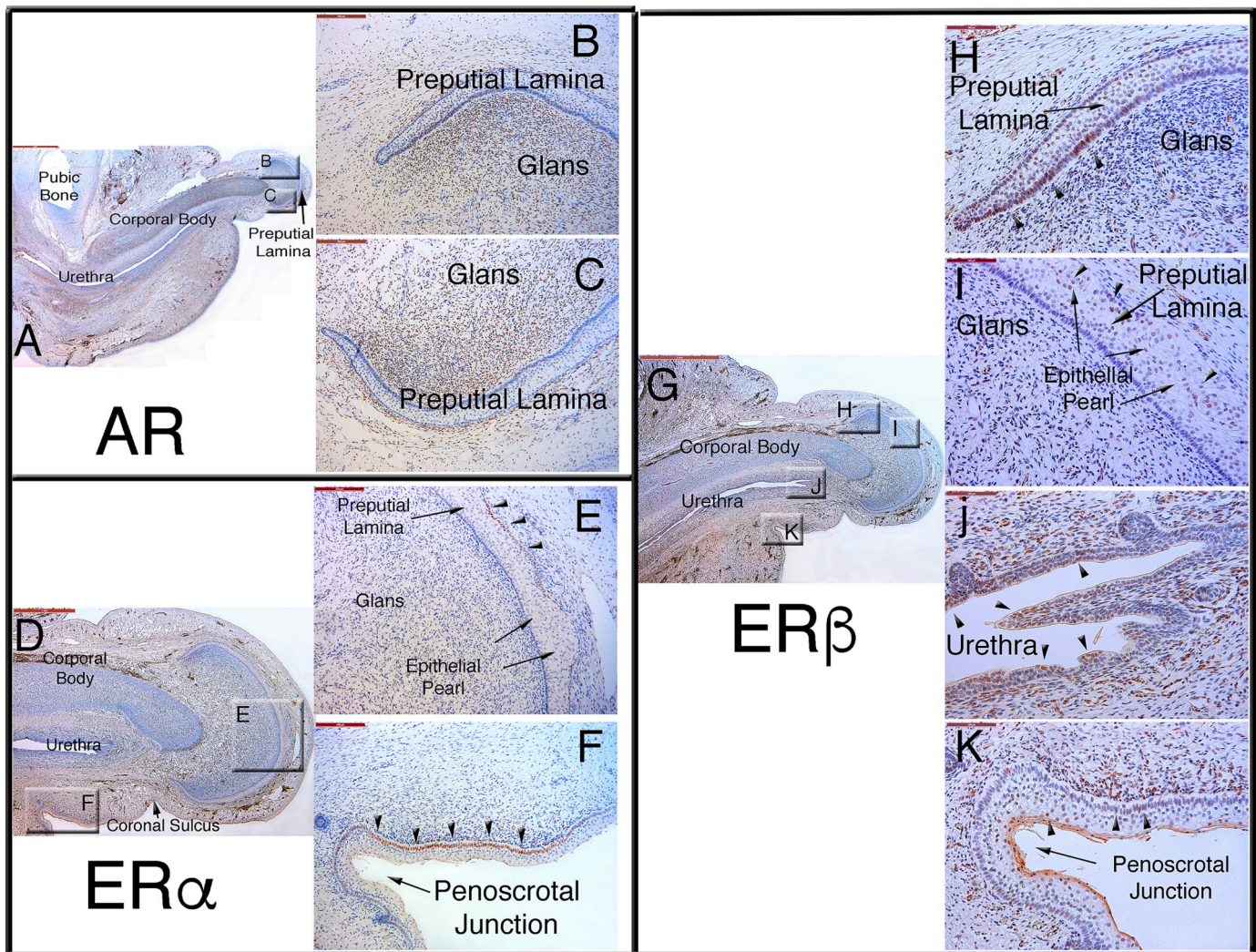


Fig. 11. Sagittal sections of human penis at 16 weeks of gestation. Human penis immunostained for: AR (A–C), ER α (D–F) and ER β (G–K). Note AR expression in the corporal body (A) and in mesenchyme of the glans and the basal epithelial layer of the preputial lamina (B&C). ER α is seen in the outer basal epithelial cells of the preputial lamina (E) and in basal epidermal cells of the penoscrotal junction (black arrowheads) (E&F). ER β is seen in the preputial lamina and in the glans (black arrowheads) (H&I), in the urethral epithelium and surrounding mesenchyme (black arrowheads) (J) and in sporadic epithelial and mesenchymal cells near the penoscrotal junction (black arrowheads) (K).

epithelial of the preputial lamina facing the preputial mesenchyme, in the mesenchyme associated with preputial lamina and in the basal epithelial layer of the epithelium at the penoscrotal junction (Fig. 11 E&F). ER β was expressed in the preputial lamina predominantly in the basal layer of the epithelium and sparsely in mesenchyme of the glans (Fig. 11 H&I). ER β was also expressed in urethral epithelium and the mesenchyme surrounding the urethra (Fig. 11J). ER β was sporadically expressed in epithelial and mesenchymal cells near the future penoscrotal junction (Fig. 11K, arrowhead).

3.2. AR, ER β and ER α expression in the developing clitoris

In the human clitoris AR was expressed at 9.5 weeks of gestation in the corporal body and the mesenchyme ventral to the corporal body where the vestibular groove will form (Fig. 12A & B (black arrowheads)). ER α expression was relatively sparse at 9.5 weeks in the clitoris but was seen in mesenchyme ventral to the corporal body

(Fig. 12C and D (black arrowheads)). In contrast, ER β expression was particularly prominent in of the epidermis of the glans clitoris and the urethral epithelium (Fig. 12F and G (black arrowheads)). PR staining was undetectable (Fig. 6H).

At 11 weeks of gestation AR was expressed in mesenchyme of the ventral-lateral region of the distal aspect of the human clitoris (Fig. 13 A & C, arrowheads). In the vestibular groove AR expression was prominently expressed in mesenchyme associated with the vestibular groove (Fig. 13D (black arrowheads)). Note the paucity of AR expression in the human clitoris compared to homologous regions of the human fetal penis (Figs. 4 and 5).

At 12.5 weeks of gestation the human clitoris expressed the AR, ER α and ER β (Fig. 14). AR was prominently expressed in (a) the corporal body and glans (Fig. 14A and B), (b) sparsely but well-defined in epithelium of the preputial lamina and associated mesenchyme (Fig. 14B) and (c) in epidermal aggregates apparently destined to be sloughed/jettisoned (Fig. 14A). ER α expression was relatively sparse but was

Clitoris: 9.5 Weeks Gestation

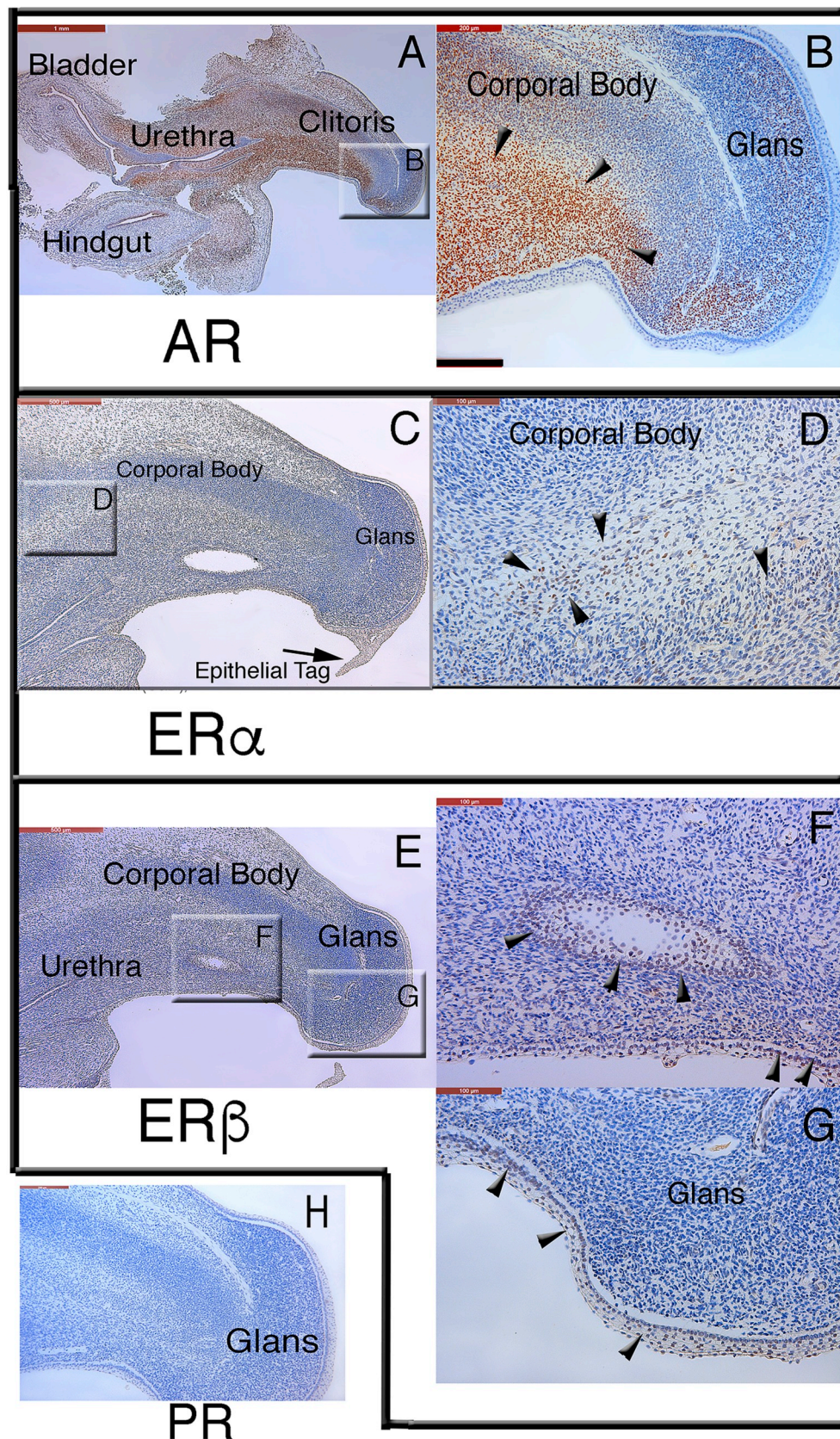


Fig. 12. Sagittal sections of human clitoris at 9.5 weeks of gestation immunostained for AR (A&B), ER α (C&D), ER β (E–G), and PR (H). Note prominent AR expression just ventral to the future vestibular groove as well as in the corporal body (A&B, black arrowheads). ER α expression is relatively sparse but can be seen in mesenchyme ventral to the corporal body (D, black arrowheads). In contrast, ER β is more prominent localized to epidermis of the glans clitoris and urethral epithelium (F&G, black arrowheads). PR staining was undetectable (H).

AR Expression Human Clitoris: 11 Weeks Gestation

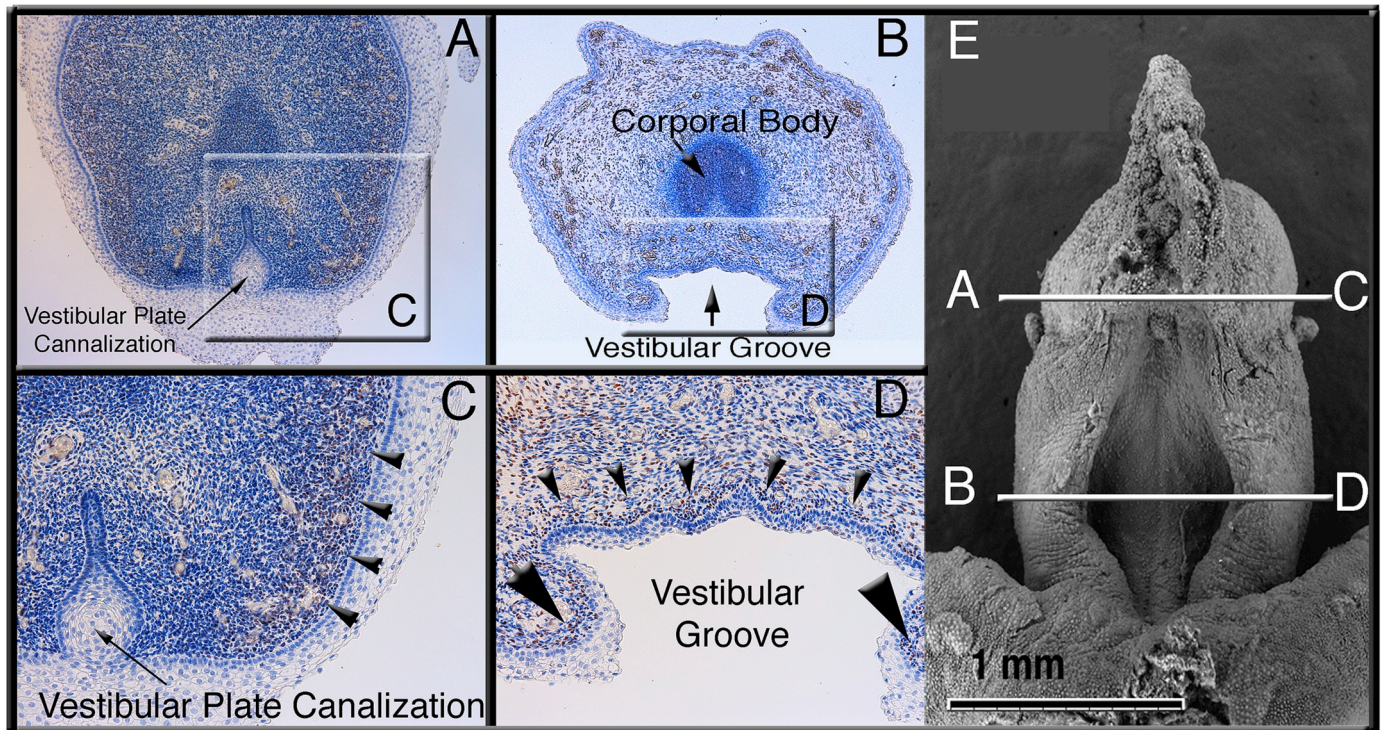


Fig. 13. Transverse sections of the human clitoris at 11 week gestation immunostained for AR (A–D). Representative scanning electron microscopic image (E) showing relative location of each histologic section. Note clear localization of the AR in the ventral-lateral mesenchyme (B, black arrowheads) and the mesenchyme just under the urethral groove (D, black arrowheads). Note the paucity of AR expression compared to the male specimens (see Figs. 5 and 8).

clearly seen in the basal epithelial cells of the preputial lamina (Fig. 14D). ER β expression was prominent in the corporal body, basal and supra-basal epithelial cells of the preputial lamina, epidermal surface aggregates apparently destined to be sloughed/jettisoned and in the epithelial tag (Fig. 14 E&F).

At 13 weeks of gestation the human clitoris expressed AR (Fig. 15) in the corporal body, in the peri-urethral mesenchymal tissue extending up to the bladder neck, in the mesenchyme of the glans clitoris (Fig. 15B), in mesenchyme adjacent to the preputial lamina (Fig. 15B arrow) and in the mesenchyme adjacent to the vestibular groove (Fig. 15B arrowheads).

At 15 weeks gestation AR was expressed in the human fetal clitoris (a) within the corporal body, (b) in the crura of the clitoris, (c) in mesenchyme of the glans clitoris, (d) in the mesenchyme associated with the preputial lamina, (e) in mesenchyme and the superficial epithelial layer of the vestibular groove and (f) in the superficial epithelial layer of the fully formed urethra (Fig. 16).

AR, ER α and ER β were detected in the human fetal clitoris at 16 weeks (Fig. 17). The corporal body was intensely AR-positive (Fig. 17A). AR was also detected in the glans and in mesenchyme surrounding the prepuce (Fig. 17B), while the preputial lamina was AR-negative. Sporadic ER α expression was seen in mesenchyme adjacent to the urethra (Fig. 17D). Extensive ER β expression was seen in corporal body, glans and in the preputial lamina (Fig. 17 E&F).

4. Discussion

The human penis and clitoris develop from the ambisexual genital tubercle starting at 8–9 weeks of gestation (Fig. 1). In males the urethral plate and in females the analogous vestibular plate undergo canalization to form the urethral groove and vestibular groove, respectively (Li et al., 2015). In males the urethral folds fuse to form the penile urethra,

while urethral development within the penile glans results from direct canalization of the urethral plate and extensive epithelial remodeling (Li et al., 2015; Shen et al., 2016). In females vestibular fold fusion does not take place resulting in an open vestibular groove (Overland et al., 2016).

In the course of both penile and clitoral development aggregates of epidermal cells are seen on the surface of the skin of these organs (Figs. 1–3). The most highly developed of these epidermal aggregates is the epithelial tag seen near the tip of the developing penis and clitoris, but smaller aggregates are also seen transiently on the ventral penile and clitoral surface usually on the glans or near the glans/shaft interface. With development, all of these epidermal aggregates including the epithelial tag disappear, presumably being sloughed/jettisoned. The significance of the sloughing epidermal tissue and the transient epithelial tag is unknown.

The developing human penis and clitoris expresses not only AR but also ER α and ER β (Figs. 4–17) (Tables 2 and 3). These observations are compatible with the hypothesis that androgens play a role in normal development, and estrogens play a role in abnormal development of the penis and/or clitoris (Zheng et al., 2015). Clearly, in humans, androgens have been shown to be necessary for development of the penile urethra by mediating fusion of the urethral folds to form the urethra within the penile shaft (Baskin et al., 2018; Shen et al., 2016) (Figs. 1–3). The strategic localization of the AR in epithelium and mesenchyme of the fusing urethral folds and in the epithelium and associated mesenchyme of the urethral plate provide the underpinning for androgen action in penile urethral development (Figs. 4–11).

Five processes act in synchrony for successful formation of the urethra within the glans penis: (a) Extension of the urethral plate to near the tip of the glans to meet surface ectodermal epithelium (epidermis) (Liu et al., 2018), (b) Canalization of the glanular urethral plate to form the urethral lumen (Baskin et al., 2018; Liu et al., 2018), (c)

Clitoris: 12.5 weeks Gestation

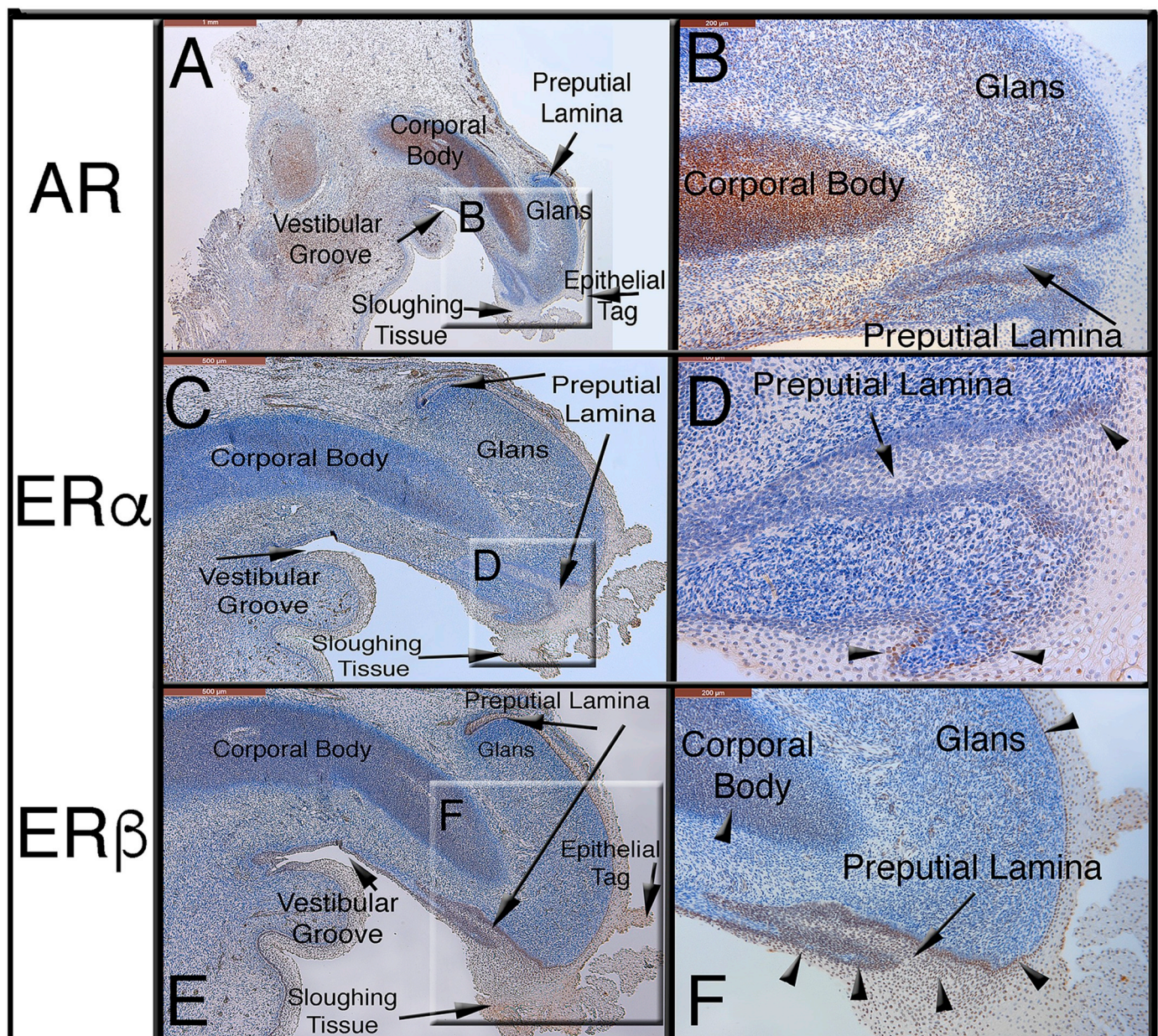


Fig. 14. Sagittal sections of human clitoris at 12.5 weeks of gestation immunostained for: AR (A&B), ER α (C&D) and ER β (E&F). Note the prominent AR expression in the corporal body (A) and sparse but well-defined expression in epithelium and associated mesenchyme of the preputial lamina (B). ER α expression is relatively sparse but is seen in basal cells of the epidermis and preputial lamina (D, black arrowheads). In contrast, ER β expression is more prominent localizing to the corporal body, basal and supra-basal epithelial cells of the preputial lamina, sloughing epidermal tissue and epithelial tag. (E&F, black arrowheads).

midline mesenchymal confluence to separate the glanular urethra from the epidermis (Baskin et al., 2018; Liu et al., 2018), (d) Reabsorption or remodeling of endodermal epithelial cells ventral to the mesenchymal confluence (Liu et al., 2018; Shen et al., 2016) and (e) Remodeling of epithelial channels in the distal glans and formation of the distal glanular urethra (Liu et al., 2018). AR is expressed in epithelial and mesenchymal cells precisely in these regions of profound epithelial and mesenchymal remodeling.

The presence of AR in comparable sites within the developing human clitoris (Figs. 12–17) (albeit less intense staining compared to males) provide the molecular mechanism for masculinization of female external genitalia under abnormal conditions of high levels of in utero androgen exposure. Androgens are necessary for penile growth and

elongation (Cunha et al., 2019a) as well as for development of the prostate, seminal vesicle, vas deferens and epididymis (Cunha et al., 2018). As noted, penile development is an exceptionally complex many-step process in which approximately half of the individual steps are androgen-dependent, while almost half of the individual steps in penile development are androgen-independent (Cunha et al., 2019a). These androgen-independent events in human penile and clitoral development include: formation of the (a) genital tubercle, (b) urethral/vestibular plate, (c) the corporal body, (d) glans, (e) prepuce, (f) urethral/vestibular groove and (g) neuronal development (Cunha et al., 2019a; Baskin et al., 1998, 1999).

In humans, genotypic XX fetuses exposed prenatally to high levels of androgens undergo varying degrees of virilization of the clitoris

AR Expression Human Clitoris: 13 Week Gestation

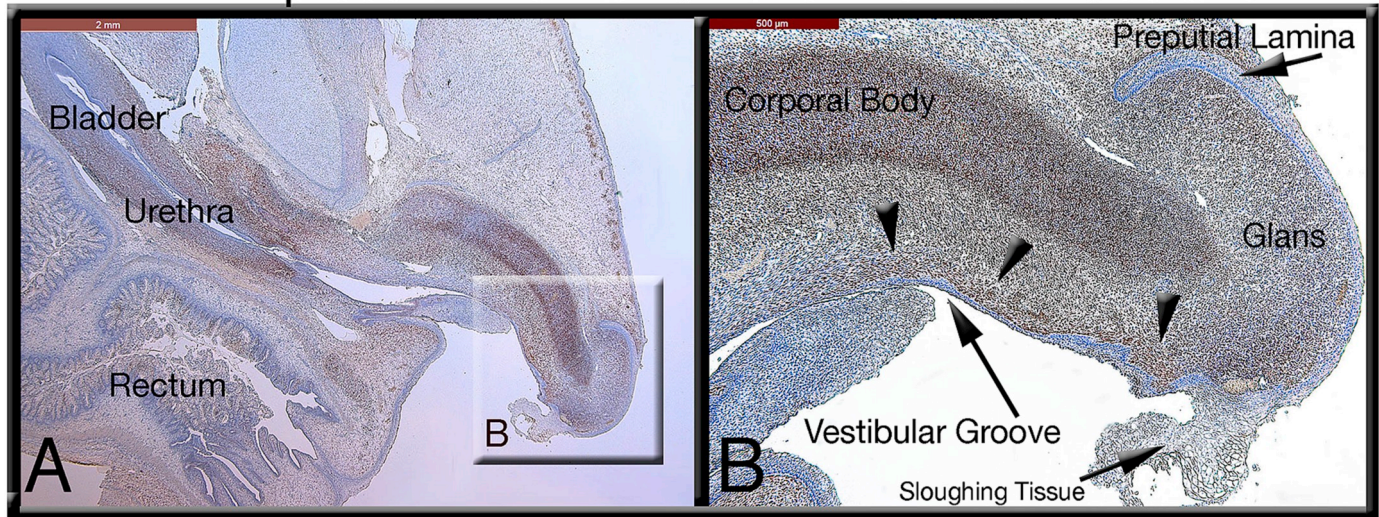


Fig. 15. Sagittal sections of human clitoris at 13 weeks of gestation immunostained for AR. Note the prominent AR expression in the corporal body (A) and in the peri-urethral mesenchyme extending up to the bladder neck, in mesenchyme of the glans clitoris, in mesenchyme associated with the preputial lamina and in mesenchyme ventral to the corporal body (black arrowheads) in the region of the vestibular groove (B).

AR Expression Human Fetal Clitoris: 15 Weeks Gestation

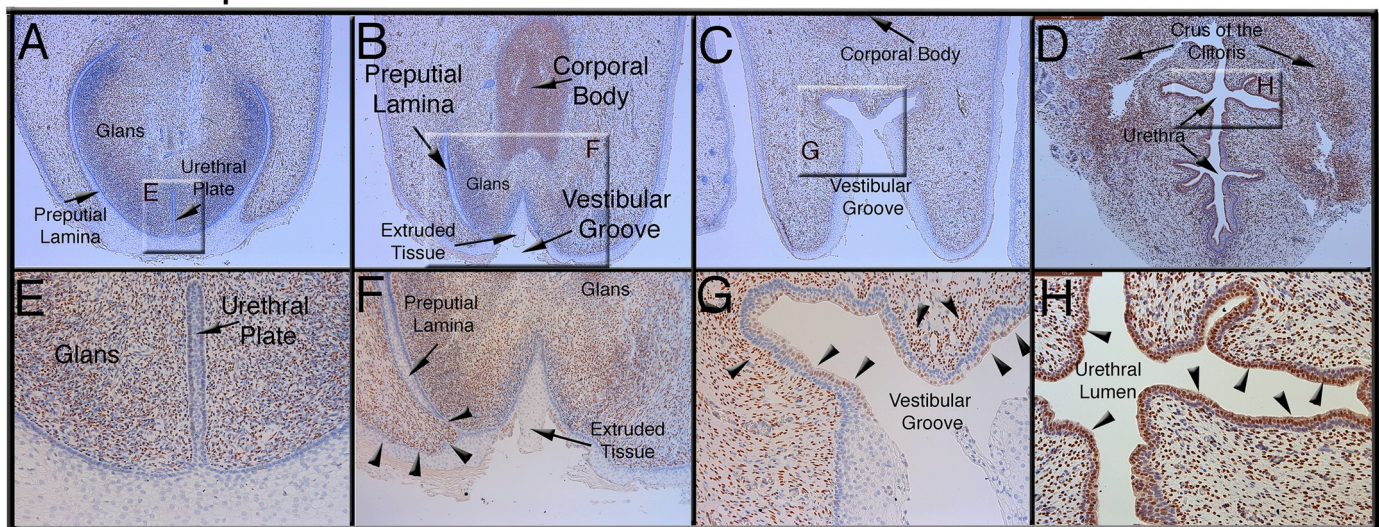


Fig. 16. Transverse sections of human clitoris at 15 weeks of gestation immunostained for AR. Note the prominent AR expression in the corporal body (B), crus of the clitoris (D), in mesenchyme of the glans clitoris (E), in mesenchyme adjacent to the urethral plate (E) and in the preputial lamina (black arrowheads) (F), in superficial layers of epithelium of the vestibular groove and associated mesenchyme (black arrowheads) (G) and in superficial layers of urethral epithelium and associated mesenchyme (black arrowheads) (H).

(Baskin, 1999). The most common cause of virilization of the clitoris is congenital adrenal hyperplasia (Speiser et al., 2018). The most extreme cases of congenital adrenal hyperplasia results in normal penile development in XX patients (Speiser et al., 2018). Maternal exposure to androgenic drugs may also cause prenatal virilization (Clark et al., 1988). The degree of masculinization of such prenatally androgenized human females is thought to be determined by the timing and level of androgens (Clark et al., 1988). The expression of AR in the developing clitoris is surely the mechanistic basis for such masculinization.

In humans, disruption of androgen metabolism can lead to impaired virilization and specifically disruption in fusion of the urethral folds resulting in hypospadias. For example, a deficiency in the enzyme 5 α -reductase-type-2 in human males inhibits the conversion of testosterone to the more active androgen, dihydrotestosterone, and results in

atypical external genitalia with severe hypospadias (Wilson, 2001). Another form of impaired androgen action involves the genetics of testicular development. SF-1 and NR5A1 regulate testicular development, and mutations in these genes are associated with under virilization of the genitalia from decreased production of androgens resulting in hypospadias and micropenis (Peycelon et al., 2017; Kohler and Achermann, 2010). Finally, defects in the AR can lead to partial or complete feminization (complete androgen insensitivity syndrome) of the external genitalia in humans with a XY karyotype (Batista et al., 2018). As noted, the phenotype of an XY genotype with non-functional AR has subtle, but detectable anatomic differences compared to normal XX females (Yucel et al., 2004; Wilson et al., 2011). Such anatomic differences may imply a possible role of estrogens in abnormal development of the external genitalia as proposed (Jost, 1953; Zheng et al., 2015).

Clitoris: 16 Weeks Gestation

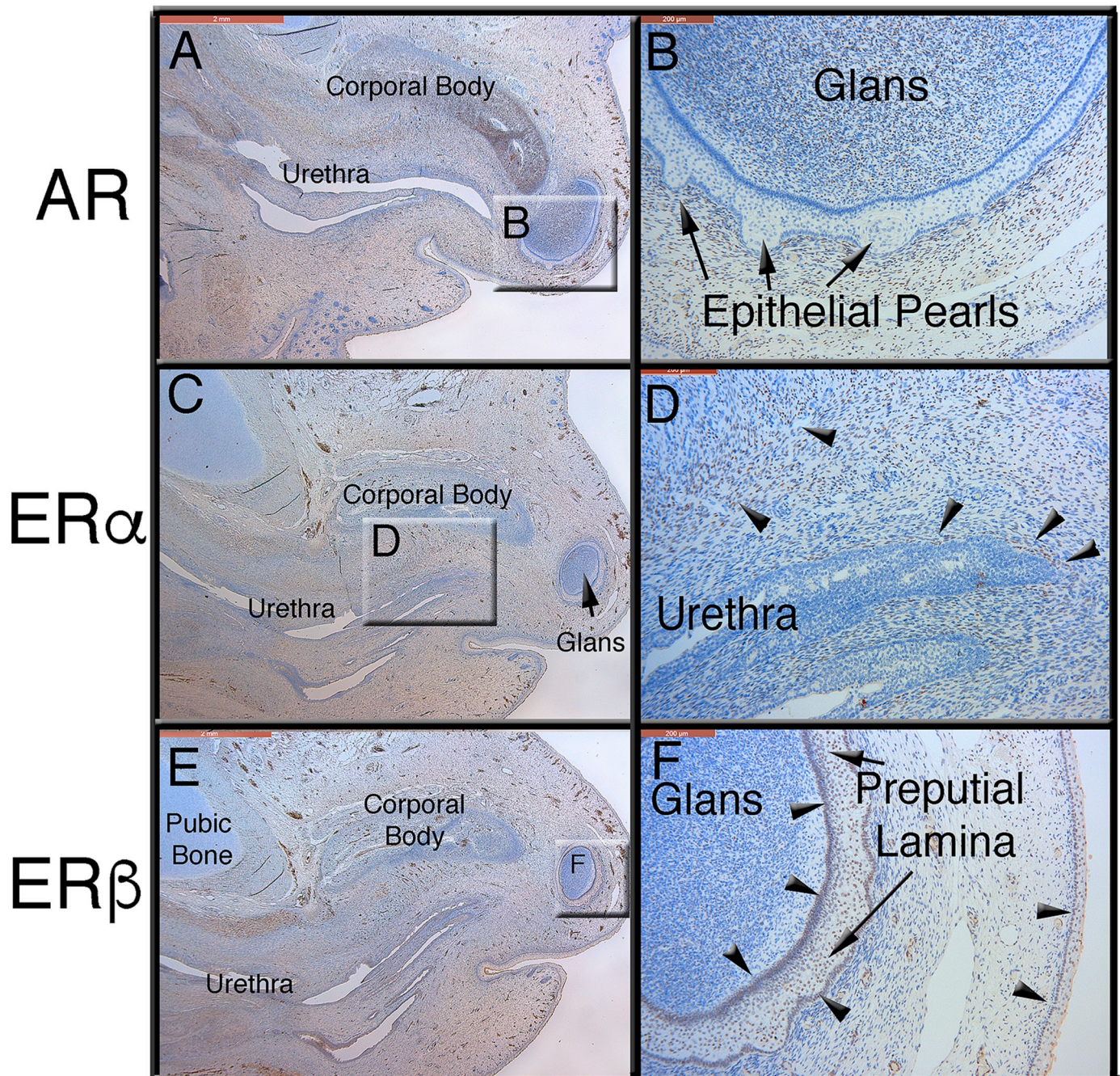


Fig. 17. Sagittal sections of human clitoris at 16 week gestation human immunostained for: AR (A&B), ERα (C&D) and ERβ (E&F). Note, AR expression in the corporal body (A), in mesenchyme of the glans and in mesenchyme surrounding prepuce (B). The preputial lamina was AR-negative. Sporadic ERα expression in the (black arrowheads) mesenchyme (D). Extensive ERβ expression in the corporal body, glans and preputial lamina (black arrowheads) (E&F).

AR is expressed during the indifferent stage as early as 8 weeks of gestation (perhaps earlier) specifically in the mesenchyme surrounding the urethral plate and in penile and clitoral corporal bodies (Fig. 4) (Cunha et al., 2019a). Testosterone formation by the human fetal testes is initiated at 8–10 of gestation (Siiteri and Wilson, 1974a), and the first evidence of androgen action in human male fetuses is the formation of prostatic buds at 10 weeks of gestation (Cunha et al., 2018). Prior to 10 weeks the genital tubercle of human males and females is identical in size and morphology (Fig. 1). A corporal body rudiment (condensed mesenchyme) is present in the developing penis and clitoris during the

ambisexual stage (8–9 weeks) (Fig. 4) before morphological evidence of androgen action and before testosterone production by the testes (Tapanainen et al., 1981; Siiteri and Wilson, 1974b), even though AR is expressed in the corporal body rudiment in both male and female genital tubercles. AR is also expressed in the mesenchyme surrounding the urethral plate, the urethral groove and the tubular urethra at 9 weeks of gestation (Fig. 5). Thus, it appears that AR either precedes the formation of testosterone by the testes or is present at the earliest stage of androgen production, perhaps before levels of testosterone are sufficient to initiate masculine development. Notably, this timing of AR

Table 2
Androgen Receptor, Estrogen Receptor α & β Expression in the Developing Penis.

Hormone Receptor	Urethral Plate		Epidermis		Corporal Body		Glans		Preputia Lamina		Urethral Groove Epithelium		Urethra Epithelium		Glans Canalization		Prepuce		Peno-Scrotal Junction		
	AR	ER β	AR	ER α	AR	ER β	AR	ER α	AR	ER β	AR	ER α	AR	ER β	AR	ER α	AR	ER β	AR	ER α	ER β
8 weeks	-	+	-	-	+	+	-	-	+	+	+	+	+	+	-	-	-	-	-	-	+
9 "	+	+	-	-	+	+	-	-	+	+	+	+	+	+	+	-	+	+	+	-	+
11 "	+	+	-	-	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	-	+
12 "	-	-	-	-	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	-	+
14 "	-	-	-	-	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	-	+
15 "	-	-	-	-	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	-	+
16 "	-	+	-	-	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	-	+

Table 3
Androgen Receptor, Estrogen Receptor α & β Expression in the Developing Clitoris.

Hormone Receptor	Vestibular Plate		Epidermis		Corporal Body		Glans		Preputia Lamina		Vestibular Groove Epithelium		Vestibular Groove Mesenchyme		Urethra Epithelium		Prepuce	
	AR	ER α	ER β	AR	ER α	ER β	AR	ER α	ER β	AR	ER α	ER β	AR	ER α	ER β	AR	ER α	ER β
9 "	-	-	+	-	-	+	+	-	-	-	-	+	+	-	+	-	-	-
11 "	-	-	+	-	-	+	+	-	-	-	-	+	+	-	+	+	+	+
12 "	-	-	+	-	-	+	+	+	+	+	+	+	+	-	+	+	+	+
13 "	+	-	+	-	-	+	+	-	-	-	+	+	+	+	+	+	+	+
15 "	+	-	+	-	-	+	+	-	-	-	+	+	+	+	+	+	+	+
16 "	-	-	-	-	-	+	+	-	-	+	-	+	+	+	+	+	+	+

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

versus testosterone production is seen in developmental events shown to be androgen-independent (Cunha et al., 2019a) such as formation of the corporal body, genital tubercle and urethral plate.

Later in human penile development (≥ 10 weeks) when androgen-dependent events are under way, AR was detected in strategic anatomic sites, namely: in mesenchyme and epithelium of the fusing urethral folds (Figs. 6–8). AR expression was continuously maintained in the penile corporal body to at least 16 weeks (and most likely thereafter) (Fig. 11). An important developmental event from 10 to 16 weeks is penile and corporal body growth, which is surely androgen-dependent. AR is also seen during this time frame in mesenchyme associated with the penile urethra, glans and prepuce suggesting that androgen action continues to be important in these regions/structures (Figs. 6–11).

Urethral formation within the glans involves direct canalization of the urethral plate and considerable epithelial remodeling without formation of a urethral groove (Liu et al., 2018). This process is clearly androgen-dependent and only occurs in males. Accordingly, AR expression is prominent within the glans both in the mesenchyme and in the canalizing and remodeling glanular urethra (Figs. 6–10). This finding is consistent with our previous work on formation of the glans and prepuce in the developing penis (Liu et al., 2018). Taking together, AR is expressed in the developing penis in strategic sites such as: (a) in the mesenchyme associated with the urethral groove, (b) the mesenchyme associated with the fusing urethral folds, (c) the mesenchyme associated with the newly formed tubular urethra, (d) the mesenchyme associated with canalizing urethral plate, and (e) remodeling and formation of the glanular urethra (Tables 2 and 3). These observations support the role of androgens as necessary for several aspects of penile development and especially penile urethral development. Finally, we note the presence of AR in mesenchyme associated with a variety of epithelia within the developing penis (urethral plate, urethral epithelium, preputial lamina) (Figs. 4–11). This juxtaposition of AR-positive mesenchyme with epithelia that may or may not express AR is compatible with the idea that certain developmental steps in penile development may be mediated via paracrine influences. In this regard, mesenchyme may be the critical and fundamental androgen target subsequently regulating epithelial development as has been shown to be the case in prostatic development (Cunha et al., 1987).

One aspect of clitoral development is that AR expression is consistently less prominent when compared to homologous structures in the developing penis, specifically in the areas of the vestibular groove and the corporal body. An impressive example of the difference in AR expression between male and female is seen in the intense AR staining of the mesenchyme around the urethral groove of the developing penis in comparison to the sparse AR staining vestibular groove of the clitoris (Compare Figs. 5 and 13). Such differences in the level of AR expression is most apparent between 11 and 15 weeks of gestation when production of testosterone by the fetal testes is particularly high (Siiteri and Wilson, 1974a), suggesting the possibility that AR within the developing human penis is up-regulated by androgens as has been reported previously (Grino et al., 1990) (Block et al., 1991). This is not the case for the corporal body of females in which AR expression is prominent and comparable to that in the developing penis at 8–9 weeks, prior to androgen production by the testes (Siiteri and Wilson, 1974a). It is notable that genital tubercle size and penile/clitoral size is similar/identical during the time period studied (Fig. 2) (11–16 weeks) (Cunha et al., 2019a), while later in development growth of the penis outstrips that of the clitoris. For example, compare size of the newborn penis to clitoris (Feldman and Smith, 1975).

Both ER α and ER β are expressed in the developing penis and clitoris. ER α expression was consistently less prominent than ER β expression in both the male and female specimens. ER α expression was present, albeit sparse in the corporal body of the penis and clitoris at all development stages studied (best seen in Fig. 11D). Interestingly, ER α was strategically localized to the epithelial cells of the preputial lamina and in areas of canalization and remodeling of the glanular urethra

plate (Fig. 14D). These strategic sites of ER α expression are consistent with the idea that estrogenic endocrine disruptors may play a role in human hypospadias, an idea that has gained traction recently (Baskin et al., 2001; Yee and Baskin, 2010). In the 16-week male specimen, ER α expression localized to the basal cells of the now distinguishable future penoscrotal junction (Fig. 11F). The expression of ER α at the penoscrotal junction may play a role in the etiology of penile webbing (Bonitz and Hanna, 2016).

The progesterone receptor (PR) was not detected at any stages in the developing human penis or clitoris. This is perhaps not surprising as human PR is induced by estrogen via ER α , a concept verified in both cell lines and in many tissues (Janne et al., 1975; Horwitz and McGuire, 1979). The absence of PR immunostaining could be due to two factors: (a) the paucity of ER α expression and (b) insufficient estrogen levels. It is notable that PR is not expressed in the developing human female reproductive tract from 8 to 15 weeks apparently due to insufficient serum estrogen (Cunha GR et al., 2017a). Estrogen treatment of athymic mice bearing grafts of human fetal female reproductive tracts elicits PR expression throughout all developing organs of the human female reproductive tract (Cunha GR et al., 2017b).

ER β , in contrast to ER α , was consistently more prominent in the developing penis and clitoris. ER β expression was first seen at the indifferent stage of 8 weeks of gestation in the urethral plate and epidermis as well as sparsely in the genital tubercle mesenchyme (Fig. 4). ER β expression was clearly seen in the corporal body and glans at 11 and 12 weeks of gestation of both the developing penis and clitoris (Figs. 6, 7, 13 and 14). Also, ER β was prominently expressed in the urethra/vestibular epithelium, the preputial lamina and the area of urethral/vestibular plate canalization as well as in remodeling in the penile glanular urethra. The functional role of ER β in human penile and clitoral development needs further examination as penile and clitoral morphology is normal in ER β knockout mice (Antal et al., 2008). It is reasonable to hypothesize that ER β (in contrast to ER α) plays the more prominent role during normal development of male and female external genitalia based upon its more extensive expression compared to ER α .

In summary, AR, ER α and ER β are all expressed to varying degrees and in strategic locations in the developing penis and clitoris. AR is expressed in close proximity to the urethral groove, in the fusing urethral folds and in the forming penile and glanular urethras which supports a critical requisite role of androgens in normal human penile urethral development. The expression of ER α and ER β in epithelial cells of the preputial lamina and in areas of canalization and remodeling of the glanular urethra plate is consistent with the possible role of estrogens in normal penile urethral and preputial development and in the etiology of hypospadias.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.diff.2019.08.005>.

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