Research article

Morphogenesis of penis and spongy urethra during human gestation

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Abstract

Background. Every year, approximately 500,000 children in the world are born with congenital abnormalities of the urinary system and kidneys. Therefore, pediatricians and urologists must understand the normal processes that lead to male sexual differentiation. **Objective.** The aim of this study was to describe in detail the process that occurs during masculinization of the fetus, which leads to the formation of male structures under normal conditions. **Methods.** Fifty-four fetuses with gestation periods between four and 18 weeks were collected, which were considered normal, did not have any signs of external anatomic abnormalities or any alteration in their development, and were a product of spontaneous abortions and tubal pregnancies. The urogenital sinus region was collected and prepared for scanning electron microscopy and high-resolution optical microscopy to observe the cellular characteristics of the urogenital fold during external development in male embryos. **Results.** This work shows the formation of the glans and spongy urethra in a detailed manner from the eighth week of embryonic development, carefully describing the role of the labioscrotal folds and the fusion of the walls of the urogenital fold during the subsequent stages of development to form the proximal part of the urinary tract. **Conclusion.** The formation of the penile urethra from the urethral fold and its posterior fusion have a probable role of ectodermal cells, in addition to the endodermal origin established previously.

Keywords: urogenital system, sry gene, sex differentiation, sex determination processes, gestational age.

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Introduction

The urogenital system in mammals, the bladder, urethra, and external genitals develop from epithelium and cloacal mesenchyme, which is a transient embryonic cavity at the tail end of the posterior intestine. Urogenital malformations are frequent in humans and are often seen with malformations of the genital tubercle, penis in men and clitoris in women, and defects in the separation of the urethral and rectal compartments (1). Each year, it is estimated that 6% of births worldwide (approximately 8 million infants) have serious genetic defects. Out of this 6%, approximately 1% of human fetuses show congenital anomalies in the urinary tract and kidneys (2, 3). Therefore, it is crucial for pediatricians and urologists to understand the normal processes that lead to sexual differentiation.

Even though the male external genital differentiation is a very detailed process, there are still migration and cellular differentiation processes that need to be studied further, since in many cases, most of the information has been obtained from studies in mice models (4-6). Despite the fact that, by studying murines, relevant information has been found and access to experimental manipulation has been given, it is important to note that they have some anatomical and morphological differences when compared to humans, which presents a difficulty when trying to use direct analogies (7) (8).

To elucidate this process, in this study were used specimens considered normal human males, from eight to fourteenth week of gestation, and a detailed description of the development of the external genitalia was performed using optical and scanning microscopy, showing the different processes of migration and cellular proliferation that result in the differentiation of male structures.

Materials and methods

Samples

For this research, the samples were collected over a period of a year and six months; and were obtained from spontaneous or frustrated abortions and ectopic pregnancies and were acquired from the Hospital Universitario del Valle de la ciudad de Cali, with the collaboration of the Department of Pathology and Obstetrics Gynecology from Universidad del Valle.

From the samples obtained, 54 embryos and fetuses that were between the eighth and fourteenth week of gestation and did not have any external morphological defects, such as low implantation of ears or some kind of mutilation in limbs, were selected (Table 1). The samples were placed in 10% buffered formalin. The entire body of the small specimens and pelvic blocks of the larger specimens were included.

Determination of embryonic age

Classification of the gestational age was performed with the help of a stereoscope, based on age tables (9), parameters of normal fetal growth, and information from the Department of Morphology of Universidad del Valle, Cali – Colombia (10).

From the embryos and fetuses obtained, the gonads were processed to differentiate the testicles from the ovaries to be associated with the external genitalia. The morphological analyses and age determinations were conducted by two experts from the Pathology and Obstetric Gynecology Department of Universidad del Valle.

Scanning Electron Microscope

A scanning electron microscope was used to examine detailed cellular development during the maturation of the external genitalia. We chose scanning electron microscopy (SEM) over transmission electron microscopy (TEM) to give detailed surface imaging of specimens. Because SEM generates a three-dimensional topographic image and can analyze a greater area than TEM of the specimen at once. For SEM, the tissue samples were fixed in 2% paraformaldehyde, 2% glutaraldehyde and 0.1M phosphate buffer (PH 7.4) for 48h at 4 °C. The tissues were then set on 2% tannic acid for 2h, washed with distilled water for 1h and treated with 1% osmium tetroxide for 2 h at room temperature, dehydrated with increasing concentrations of ethanol, the samples were taken to the dry critical point, and finally covered with gold and observed using a Jeol 5800 *scanning electron microscope* (JEOL Ltd.). Musashino, Akishima, Tokyo 196-8558, Japan.

Optical Microscopy

For optical microscopy, the samples were rehydrated with different concentrations of isoamyl acetate and mixtures of pure alcohol and isoamyl until decreasing alcohol concentrations were achieved, followed by washing with 0.1% phosphate buffer. Afterwards, they were passed through alcohols at increasing concentrations and then with propylene oxide. They were then infiltrated with histological resin and polymerized at 60 °C to be cut with a glass blade in an ultra-microtome, stained with toluidine blue, and H&E mounted (11) (12), and observed (13).

Ethical approval

This study was carried out in accordance with the ethical standards of the Universidad Surcolombiana institutional committee on human experimentation. (MEMORANDO No. 052, October 26, 2020).

Results

Morphological characteristics of the external genitalia development

Eighth week of development

In the eighth week of gestation, the main events included growth of the genital tubercle and formation of the epithelial appendix. The overlapping ectodermal cells at the distal end level, where the glans originate, and from that point forward, this tubercle is known as the phallus.

The urogenital fold is now longer and has less depth as a result of the growth of the phallus (Fig. 1, A y B); (Table 1)

Table 1. Discrimination of a sample of fifty-four embryos and fetuses between the eighth and fourteenth week of gestation used in this study. These samples had no visible external defects and were considered normal.

Number of Embryos and Fetuses	Age (weeks)	Cephalo-Caudal Length (mm)	Morphological Characteristics
5	8	23 - 31	* Flat nose * Separated eyes
6	9	32 - 44	* Eyes closing * Round head
6	10	45 - 56	* Intestines in the umbilical cord * No physiological hernia * Erect head * Molded extremities
9	11	57 - 69	* Head half the length CC. * External genitalia beginning to differentiate
11	12	70 - 81	* Head still dominant * Sex determined. * Length of definitive members
11	13	82- 97	* There are signs of hair
6	14	98 - 110	* Head upright * Nail development

Ninth week of development

Further development can be observed in ectodermal cells of the urogenital fold. The phallus continues to grow, and at this point is the structure with the most genital development. The glans is now more defined, and it lies at the end of the urogenital fold and conservation of the epithelial appendix. The labioscrotal swellings increase in volume and grow towards the anal region, in the direction of the middle line where they will fuse posteriorly; as a result of this growth, the anal fold disappears (Fig. 1C).

Changes in the phallus fold and mesoderm; the shape of the linear genitalia changes to rhomboid, showing proliferation of the cells on the outer part of the urogenital fold. This defined space allows for the organization of the penile urethra. The superficial ectoderm, which serves as a lining for the urogenital fold from the beginning, serves as an origin point for the epithelium of the urethra (Fig. 1D)

The phallus' mesoderm shows cellular proliferation that is related to the deepest region of the fold; these cellular clusters will give origin to cavernous bodies (Asterisks, Fig. 1D)

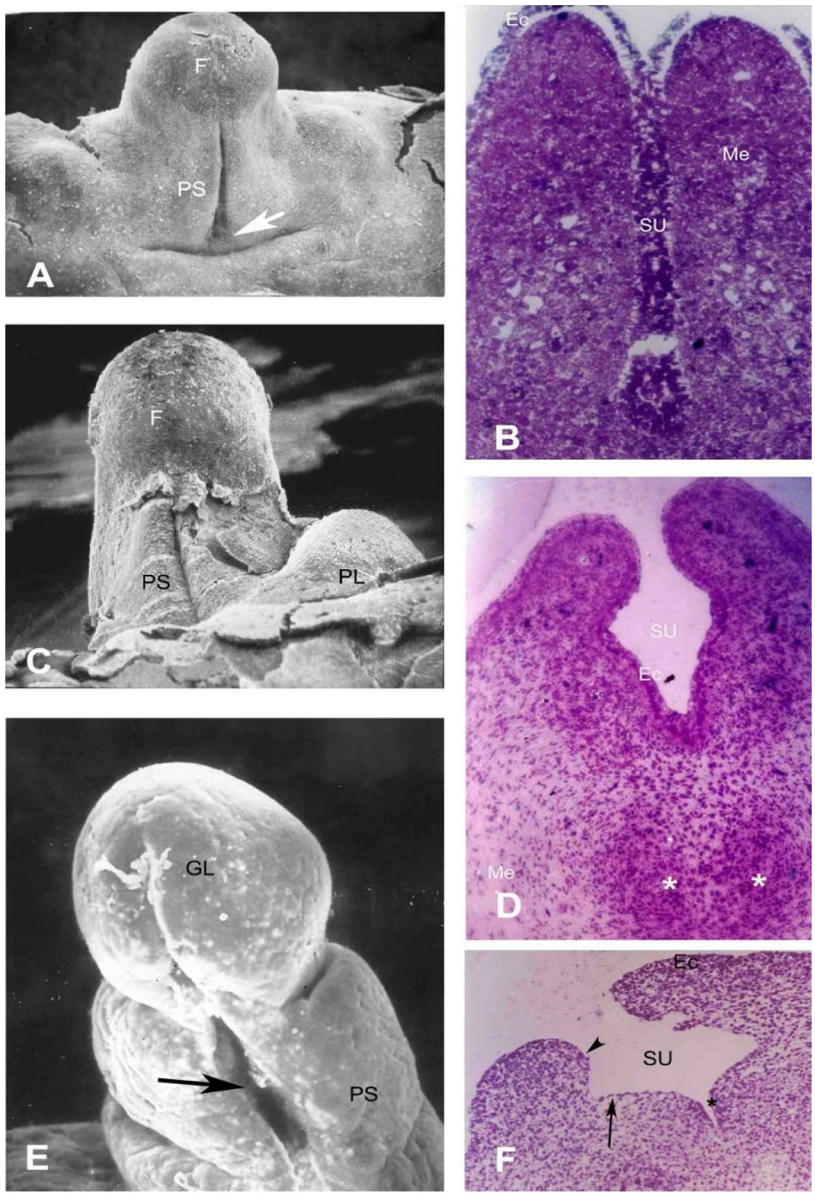

Figure 1. Microphotographs of the genital area of masculine embryos between eight and ten weeks of gestation (1A) Scanning electron microphotography of the genital area of an embryo at eight weeks of development, showing the phallus (f), fold of the urogenital sulcus (PS), and anal fold (arrow) (40X). (1B) During this phase, the superficial epithelium (Ec) can be observed covering the urogenital sulcus (SU) and mesoderm (Me) (400x). (1C) Detailed picture of the genital area of a nine-week-old embryo, labioscrotal folds (PL), and folds of the urogenital sulcus (PS) and phallus (f). Magnification 80X; (1D) also, the urogenital sulcus (PS) can be seen covered with epithelial cells (Ec) on its internal and external parts, and in the internal mesoderm (Me), internal conglomerates organized in a circular shape (asterisk) can be seen (150X). 1E Details of the glans (GL) of a ten-week-old fetus, where the urogenital sulcus (arrow) and folds of the urogenital sulcus (PS) can be observed. (1F) Likewise, the transformation of the urogenital sulcus (SU) can be witnessed because of the presence of external elevation (top of the arrow) and internal elevation (asterisk), which is surrounded by the superficial ectoderm (arrow) (80X).

Tenth week of development

The external genitalia continue to develop; the phallus is the predominant element with very defined glans and conserving the epithelial appendix (Fig. 1, E y F). At the end of the week, the balanopreputial sulcus appears as well as an ectoderm invagination at the middle line level of the glans, forming an ectothermic fold that carries on with the urogenital fold, which aids in the formation of the spongy urethra's glans. The labioscrotoral folds show an important development approaching the midline, causing the anal fold to disappear, resulting in a better appreciation of the anal opening. The folds of the urogenital fold show prominent to the sides of the fold, which has now acquired more depth (Fig. 1E y 1F)

The urogenital fold continues to change in shape and size, and its superficial borders continue to become closer as a result of the growth of the internal mesoderm. In the middle area, mesodermic growth that produces a couple of bulges that slightly change the shape of the groove light can be observed (Fig. 1F).

Eleventh week of development

In this week, the phallus has a defined glans and balanopreputial groove; however, the epithelial appendix has now disappeared, furthermore, an invagination of ectodermal cells at the glans level is observed causing internal cell proliferation, at the end of this invagination the area where the external urethral orifice is be located will be determined (Fig. 2, A y B)

The labioescrotoral folds bind at middle line level giving place to the scrotal bags and scrotal raphe; As for the urogenital sinus' folds they start to partially close during this week, showing as areas with a lot of closeness but without a real fusion.

The urethra is now completely closed, covered with epithelial cells, and has started to have a well-defined star shape (dotted line, Fig. 2B).

Twelfth week of development

The external urethral orifice is defined at the glans; the glans on the ventral side shows partial closure of the ectodermal invagination, specifically at the distal portion near the urethral orifice (Fig. 2C).

The medium raphe is not consolidated in the mesoderm and its proliferation forms a bulge in this area; this event is a result of the increase in cellular concentration at the midline level where the urogenital sinus folds have closed. The superficial ectoderm proliferated, and formation of the foreskin as an ectoderm fold was observed (Fig. 2D).

Thirteenth week of development

The internal area of the urethral orifice that forms the glandular portion of the spongy urethra is now covered by epithelium that

was previously invaginated around the tenth week of gestation (Fig. 2E) The urethra exhibited a characteristic star shape covered by the epithelium in the process of cellular differentiation (Fig. 2F)

The superficial ectoderm completely covers the closing, and it proliferates into a stratified epithelium with basal cells other than superficial cells. The raphe is now formed with a more ectodermal mesodermal bulge, and it can be seen that the mesoderm surrounding the urethra is filled with small blood vessels, whereas the vascularization of the peripheral mesoderm has larger blood vessels.

Fourteenth week of development

During this week of development, the external urethral orifice is observed, this orifice is very wide during its initial stages, but will eventually close (ou, Fig. 2G)

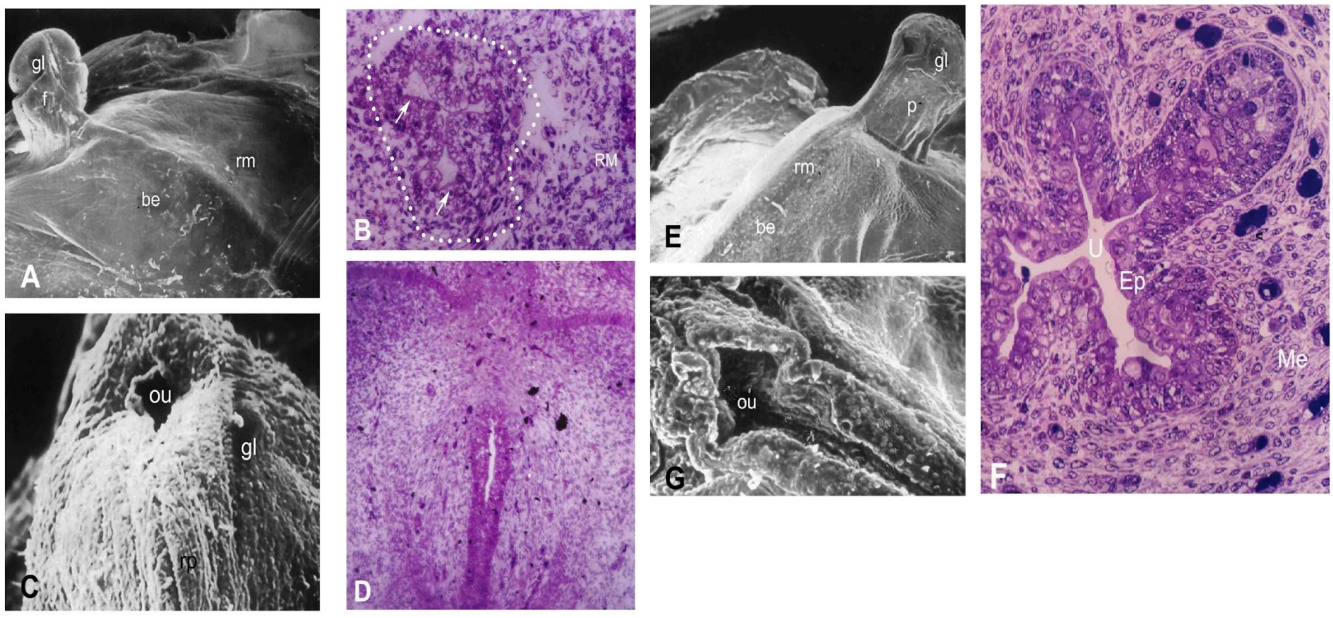

Figure 2. Microphotographs of the genital area of masculine embryos between 11 and 14 weeks of gestation (A-G). 2A At the eleventh week of development, the phallus (f) can be seen at the end of which the glans (gl) can be observed, and the closed medium raphe (rm) also shows laterally from the scrotal bags (be) (30X). 2B The cross-section of the phallus showing the medium raphe (RM) without being completely consolidated; the urethra (dotted lines) is covered by the ectoderm (arrows) (400X). 2C On the twelfth week of development, the glans and the external urethral orifice were clearly developed (150X). 2D The foreskin (pr), which is made up of the ectoderm (Ec), medium raphe (RM), mesoderm (Me), and urethral orifice (U), can be seen (400X). 2E After 13 weeks of development, the penis (p) is evident, and at the extreme of it, the glans (gl); at the inferior portion of the penis, the scrotal bags (be) and the medium raphe are located (30X). 2F The penile urethra (U) can be seen to have a stared shape during the 13 weeks; it is lined with an epithelium (Ep) that is being differentiated with prominent nucleus; the mesoderm (Me) can be seen surrounding the urethra (400X). 2G In the fourteenth week of development, a detailed external urethral orifice (ou) can be seen (200X).

Discussion

The male external genitals originate from structures formed during the ambisexual stage, which are differentiated, giving origin to their defined structure (14),(15, 16). Sexual differentiation is determined genetically following hormonal action: once the SRY gene is expressed, testosterone production increases, influencing the central nervous system as well as the internal and external genitals that have not yet been differentiated, which initiates the maturation process that induces masculinization of the fetus (17) (18),(19), (20), (21).

Earlier, at the level of the cloaca on its cephalic portion and at the middle line, an elevation known at the genital tubercle where the penis will originate appears (22), (23).

Sexual differentiation of the external genitalia occurs after differentiation of the gonads and expression of sexual steroid receptors on the genital tubercle. The three germinal layers play a role in the formation of the external genitalia. The genital mesoderm conforms to the stromal tissue of the phallus, the endodermally derived urethral plate forms the entire epithelium of the urethral tube, and an ectodermal epithelium envelope forms the skin that surrounds it (19), (24) (25).

The study of human embryos that are a result of spontaneous abortions can provide a lot of information to understand normal and altered embryological development (26). During the ambisexual phase, between the sixth and twelfth weeks of gestation, the genital tubercle is very similar in males and females, and they are almost the same size (23), making it difficult to identify any changes during this period (2)

In the sixth week of intrauterine life, the urorectal fold grows, dividing the cloaca into two parts: the anterior part that gives origin to the urogenital sinus and a posterior part that gives origin to the anal region. Simultaneously, the urogenital sinus originates from urogenital folds that form scrotal bags (15), (27). Two weeks later, the testicles differentiate and secrete testosterone, which acts over the genital tubercle and the urogenital folds, inducing masculinization of the external genitalia (6). For the development of the folds, the genital tubercle grows to form the penis, whereas the urogenital fold enlarges and closes, forming the spongy portion of the masculine urethra on the medium raphe, which starts in the eighth week of intrauterine life following a fusion that starts from the base to the tip of the phallus (28).

Between the eighth and ninth weeks of gestation, Leydig cells differentiate in the testicles and secrete testosterone (15), (29). Testosterone and/or dihydrotestosterone (a more potent androgen) masculinize the genital tubercle, Wolffian duct, and the urogenital sinus. As a result, the anogenital

distance increases, followed by elongation of the genital tubercle and eventually the formation of urethral plaque. The urethral plaque originates in the endoderm (confirmed by positive immunohistochemistry for Foxa1) (30),(31),(32); and extends from the base of the urethra to the proximal region of the future penis' glans (33), (34).

The genital tubercle that becomes the penis contains tissues derived from the three germ layers: the ectoderm gives rise to the skin of the phallus and foreskin (35), the cavernous bodies and connective tissue of the penis stroma are of mesodermal origin, and the endoderm originates from the penis urethra (4). The urethral plaque canalices distal to the proximal region (Fig. 1A, arrow) to form an open structure that simulates a zipper, the epithelial proliferation (identified for the expression of Ki67) (23), is abundant in the canalizing urethra and on the floor of the urethral sulcus, this event could be responsible for its lateral expansion.

The initial canalization of the urethral plaque extends distally to the coronal sulcus, indicating that the urethral sulcus does not extend inside the glans. The coronal sulcus now defined on the glans is prominent from the penis shaft at approximately ten weeks of gestation (Fig. 1E), becoming an important mechanism in the differentiation between the penis' body and the glans (35). As previously mentioned, the urethra of the penis' body forms as a result of the canalization of the urethral plaque, whose folds are initially open and eventually fuse to form the tubular structure. In contrast, inside the glans, the urethral lumen is formed by limited canalization that does not include the formation of urethral plaques (34) (36).

Interestingly, two morphogenetic mechanisms occur during the development of the urethra in humans. Direct canalization of the urethra in the glans region and formation of the urethra from the urethral sulcus open from its base. Merging events are related to the formation of the urethra inside the body of the penis (arrow, vertical space) (34), (35), (37).

The fusion of urethral folds during penile urethra formation is a complex process. As the medial edges of the urethral folds approach each other, longitudinally intertwined ridges are formed that initially approach each other without merging, leaving clean channels between the lumen of the developing urethra and the exterior, which are initially evident (Fig. 1E, black arrow) (37). With the progression of penile urethra development, the medial edges of the urethral folds fuse in the midline to form the median raphe of the penis. (closed zipper).

From a broad perspective, the process during the formation of the penile urethra (in the body) consists of three separate events: (a) the fusion of the whole epidermis com-

pletes the ventral skin of the penis. (b) Fusion of the endoderm forms the penile urethra. (c) Finally, after removal of the midline epithelial seam, a ventral confluence is established between the right and left mesenchyme of the urethra (23).

Anatomic and immunohistochemical studies reinforce the idea that in humans, the urethra's epithelium originates in the endoderm of the urogenital sinus (4) and that the urethral meatus represents an interphase between the ectoderm epithelium and the epithelium of the urethra's endoderm. The entire human urethra is formed as a result of the growth of the urethral plaque in the genital tubercle and the fusion of the urethral folds along the body of the penis.

Further evidence of the endodermal origin of the urethral epithelium is the expression of FOXA1 during urethral development (23),(35). In contrast, Glenister theory states that the glandular urethra forms from the skin (ectodermal intrusion) growing inside the gland and meets the endoderm-derived urethra at the junction of the shaft of the penis with the glans (37).

Conclusion

Taken together the results of this study, we identified the participation of cells with a possible ectodermic origin in different canalization processes and in the formation of the urethra; however, more specific data are needed to confirm the origin of these cells. Furthermore, information related to the origin and derivation of urethral epithelium based on data collected from developing penises requires validation using adult specimens.

Author contribution statements

A.R.V and M.G.F contributed to the design and implementation of the research, analysis of the results, and writing of the manuscript.

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Conflict of interest The authors declare that they have no conflict of interest.

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