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Development of the external genitalia

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Development of the external genitalia is a complicated processes involving a constellation of individual disparate morphogenetic events. External genitalia consist of the penis, clitoris, labia majora and minora and scrotum in humans, while mice lack labia. The penis and clitoris develop from the ambisexual genital tubercle (GT), an embryonic appendage within the perineum, which has some molecular mechanistic features in common with limb appendages. For example, the distal urethral epithelium of the mouse GT, when transplanted to the chick limb bud, elicits digit duplication in the limb, an effect ascribed to sonic hedgehog in embryonic mouse urethral epithelium (Perriton et al., 2002). Development of body appendages such as the penis, clitoris and limb are immensely complex involving distal outgrowth of primordia, mesenchymal condensation, differentiation of skeletal elements (limb bones and os penis and os clitoris [in mice]), formation of erectile bodies, polarity [dorsal-ventral, proximal-distal and medial-lateral], muscle differentiation (skeletal muscle in limbs and smooth muscle in the penis and clitoris), epithelial differentiation and unique morphogenetic processes. Penile development involves all 3 germ layers, ectoderm forming penile and preputial epidermis, endoderm forming most of the urethra, and mesoderm forming erectile bodies, dermis and connective tissue stroma. The urethral meatus of both males and females constitutes an interface between ectoderm and endoderm. Of course, all of these disparate developmental/differentiation events are regulated via molecular pathways unique to each type of developmental process. One feature particularly important is androgenic regulation of development of external genitalia that defines whether the embryonic GT develops into a penis or a clitoris.

From an experimental perspective, molecular pathways have been pursued mostly in mice utilizing the power of genetic manipulation. One of the main justifications of studying development of the external genitalia is the extremely prevalent occurrence of hypospadias in humans, a congenital abnormality of the penile urethra with an incidence of approximately 1:200–1:300 male births (Baskin, 2000; Cunha et al., 2015). Recently, use of the mouse as a model of human hypospadias has been questioned based upon significant mouse-human differences in adult penile anatomy and the developmental processes dictating the adult penile form. Accordingly, many of the reported forms of mouse hypospadias are substantially different from human hypospadias (Cunha et al., 2015). However, careful, judicious focus upon common features of development of mouse and human external genitalia has certainly advanced the field.

In this special issue on development of external genitalia, we have gathered experts on this topic for both mouse and human studies. The diversity and richness of our field is in part due to its position at crossroads of developmental biology as well as endocrinology. An extremely useful feature of development of the external genitalia derives from the fact that the ultimate developmental fate of the ambisexual GT is determined by the presence or absence of androgens. Accordingly, masculine development of the GT can be elicited in genetically male or female primordia by androgens, and conversely feminization of the GT can be elicited by various methods of abrogating androgen action. This feature gives the investigator unprecedented control of experimental systems to explore normal molecular pathways as well as malformations such as hypospadias.

The paper by Cunha et al. sets the stage by describing differences in the adult anatomy and developmental processes in human and mouse external genitalia (Cunha et al., 2019d). Full appreciation of these human-mouse differences requires expertise in anatomy, developmental biology and endocrinology. Given the importance of sex steroids in normal and abnormal development of external genitalia, one must be aware of the ontogeny of androgen and estrogen levels throughout gestation, the ontogeny of androgen and estrogen receptors, as well as the substantial differences in mouse versus human serum hormone-binding proteins during pre- and early postnatal development. Mouse alpha fetoprotein (AFP) binds estrogens at high affinity and neutralizes estrogen action during the perinatal period, whereas this is not a factor in perinatal humans. This singular fact has profound implications regarding experimental design and interpretation of data as discussed (Cunha et al., 2019d). Nonetheless, mice have been used extensively as a model for development and pathogenesis of human external genitalia, and certain similarities in developmental processes and hormone action provide ample justification for the judicious use of mouse models for

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human reproductive tract development. Indeed, certain human genetic disorders affecting external genitalia are congruent with mouse studies. However, anatomic, developmental and endocrinologic differences exist between mice and humans that (a) must be appreciated and (b) considered with caution when extrapolating information between animal models and humans.

Given the complexity of developing external genitalia, it is imperative that the investigator know exactly where observations are made in serial section sets particularly along the proximal-distal axis of the developing GT as developmental events differ dramatically on a proximal-distal basis. For this reason, some of the most informative data comes from three-dimensional (3D) immunohistochemistry. The paper by Isaacson et al. reviews the 3D reconstruction techniques that have previously enabled paradigm shifts in our understanding of human embryonic and fetal development (Isaacson et al., 2019). Light sheet fluorescence microscopy (LSFM), a recently-developed technique, uses thin planes of light to optically section whole-mount immunolabeled biologic specimens that facilitates research into gene expression and tissue dynamics at extremely high resolution. Our group has applied LSFM to study developing human fetal external genitalia, internal genitalia and kidneys. This work describes the technique for preparing, clearing, immunostaining and imaging human fetal urogenital specimens. Our data represents the first description of utilization of LSFM on the developing human urogenital system during embryonic and fetal periods.

Examination of the literature on development of mouse external genitalia reveals a preponderant emphasis on penile development, with considerably less emphasis on mouse clitoral development. Perhaps the reason for this disparity is the use of mouse penile development as a model for human hypospadias. The goal of the report on mouse clitoral development by Cunha et al. is (a) to provide the first detailed description of mouse clitoral development, and (b) to compare mouse and human clitoral development (Cunha et al., 2019a). For this purpose, external genitalia of female mice were examined by wholemount microscopy, histology and immunohistochemistry from 14 days of gestation to 10 days postnatal. Human clitoral development was examined by these techniques as well as by scanning electron microscopy and optical projection tomography from 8 to 19 weeks of gestation. This study has revealed dramatic differences in adult mouse and human clitoral anatomy and accordingly dramatic differences in developmental events in mouse versus human clitoris. The prominent perineal appendage in adult female mice is prepuce, formed via fusion of the embryonic preputial swellings, and is not the clitoris. The adult mouse clitoris is an internal organ defined by a U-shaped clitoral lamina that develops from the female preputial lamina. In contrast, the human clitoris develops from the GT and is in many respects a smaller anatomic version of the human penis having all of the external and internal elements except the urethra. The human clitoris (like the human penis) has a glans projecting into the vaginal vestibule. Adult morphology and developmental processes are virtually non-comparable in the mouse versus human clitoris.

Literature on development of the human prepuce begins in 1866 (Schweigger-Seidel, 1866). Since then several theories of human preputial development have emerged. In the course of examining development of the human glans penis, novel data emerged suggesting an entirely different developmental mechanism (Cunha et al., 2019c). For this purpose 30 human fetal specimens were studied from 9 to 17 weeks of gestation. Transverse and sagittal sections revealed that the epidermis of the glans is considerably thicker than that of the penile shaft. Our studies revealed a morphogenetic mechanism of human preputial development that involves splitting of the thick epidermis of the glans into the preputial lamina and the epidermis via the intrusion of mesenchyme containing an abundance of red blood cells and blood vessels. This process begins at 10–11 weeks of gestation in the proximal aspect of the glans and extends distally. The process appears to be androgen-dependent and mediated via androgen receptors strategically localized to the morphogenetic process. 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. Substantial differences in preputial development in mouse versus human are discussed.

While penile development is androgen-dependent is a global sense, the paper by Cunha et al. points out the counter-intuitive idea that many of the individual steps in penile development are in fact androgen-independent, and thus are in common with clitoral development (Cunha et al., 2019b). Developmental events in the embryonic GT common to human males and females include formation of the (a) the GT, (b) the urethral plate, (c) the urethral groove, (d) the glans, (e) the prepuce and (f) the corporal body. For humans 6 of 13 individual developmental steps in penile development are accordingly interpreted as androgen-independent. For certain developmental events, this conclusion is bolstered by observations in androgen-insensitive patients and androgen receptor mutant mice. Moreover, during human penile development certain events occur before production of androgens by the testes. For mice 5 of 11 individual developmental steps were interpreted as androgen-independent, based in part upon analysis of androgen-insensitive mutants.

Recent evidence suggests that estrogens along with androgens are involved in development of male external genitalia. To explore the mechanism of action of androgens and estrogens in development of human external genitalia, immunohistochemistry was used to define the precise location of the androgen receptor (AR), and estrogen receptors alpha (ERα, ESR1) and beta (ERβ, ESR2) in the developing human penis and clitoris (Baskin et al., 2019). AR was expressed in (a) the developing corporal body, (b) in mesenchyme associated with the urethral plate and canalizing urethral groove, (c) in the forming penile urethra (the fusing urethral folds), (d) in mesenchyme associated with the preputial lamina, (e) in mesenchyme and epithelium of the urethral meatus, and (f) in mesenchyme of the glans penis. ERα was expressed sparingly in ventral epidermis, while ERβ was expressed more broadly 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, (e) in epithelium and mesenchyme of the urethral meatus and (f) in penile epidermis. In the developing human clitoris AR was generally expressed in the same areas as that of the penis, but overall staining intensity was reduced, except for the corporal body of the clitoris, which was intensely AR-positive. ERα and ERβ expression in the clitoris was similar to that of the penis. Patterns of AR, ERα and ERβ expression are consistent with the known normal actions of androgens in males and the abnormal masculinizing effects of androgen on developing human female external genitalia, as well as the presumed teratogenic effects of exogenous estrogens.

The paper by Cripps et al. focusses upon the role of estrogen in development of mouse penile development through use of mice in which in which the aromatase (Cyp19a1) gene has been knockout, designated as ArKO mice (Cripps et al., 2019). Estrogen production is completely ablated in these mutant mice, which had a mild hypospadias phenotype. The hypospadias seen in these ARKO mice was almost identical to that reported for ERα knockout mice suggesting that ERα is the predominant receptor for mediating estrogen action during development of the mouse penis. Preputial development was also accelerated in ARKO mice suggesting that estrogen is required for normal preputial development and placement of the mature urethral opening of the penis. Using qPCR, expression of keratin genes and key urethral patterning genes were altered as a result of impaired estrogen signaling. These data allow a better understanding of how anti-estrogenic as well as estrogenic endocrine disruptors affect urethral and preputial development.

The paper by Mattiske et al. reveals the importance of a long non-coding RNA molecule known as Leat1 (long non-coding RNA, EphrinB2

associated transcript 1), which may play a role in defective anorectal and urogenital malformations (Mattiske et al., 2019). lncRNA is syntenic with EfnB2 (which encodes EphrinB2) and is expressed during embryonic development of the genital tubercle. While lncRNAs have varied functions, many are known to regulate their neighbouring genes. Eph/Ephrin bidirectional signaling molecules mediate many patterning pathways in early embryonic development, including cloacal septation and penile urethral development. This paper investigates the role of Leat1 and its possible regulation of EphrinB2 during development of the female reproductive tract. Mattiske et al. show that loss of Leat1leads to reduced EfnB2 expression in the developing female genital tubercle, reduced anogenital distance and decreased fertility.

Internal fertilization in terrestrial mammals necessitates the evolution of external genitalia sufficient to

fertilize eggs housed deep inside the female. Accordingly, sexual dimorphism of mammalian external genitalia has become highly pronounced, and the molecular regulation of highly complex morphogenetic processes of penile development has resulted in novel spatial and temporal patterns of gene expression. Recent studies delineating the genetic regulation of external genitalia development, largely derived from development of the murine GT, have vastly enlightened the field of reproductive developmental biology. Analysis of murine homologs of human genes, deleted in the mouse, have revealed defects in GT outgrowth and urethral development that can be ascribed to deletion of specific genes in the developing murine external genitalia. The review by Haller and Ma details how these murine genetic models have advanced a rapidly growing body of knowledge detailing the spatial-temporal genetic regulation of external genitalia development.

The review by Hyuga et al. focusses on early and late stages of GT development in male and female mouse embryos (Hyuga et al., 2019). The authors review their earlier studies on the role of various growth factors (Fibroblast growth factor) and Wnt genes that function during GT formation. In addition, the authors present their novel ideas concerning epithelial-mesenchymal interaction between developing urethral epithelia and its associated mesenchyme during androgen-dependent penile urethral development in mouse embryos. Mafb, belonging to AP-1 gene family, was identified as a key androgen-responsive mesenchymal gene. During masculinization of the urethra, bilateral mesenchymal cell condensation and migration was shown to be essential for normal penile urethral development. The dynamic mode of epithelial-mesenchymal interaction for both early and late phases of ExG development is discussed.

This special issue on development of external genitalia provides for the first time in-depth morphogenetic, immunohistochemical, electron microscopic and Light Sheet immunofluorescent analysis of human penile, clitoral and preputial development in conjunction with morphogenetic and molecular studies of the interacting gene pathways of mouse GT development. The complexity and richness of developmental and endocrinologic advances provide a wealth of research opportunities for the developmental biologist as well as the endocrinologist that encompasses epithelial-mesenchymal interactions, epithelial and mesenchymal remodeling, directed outgrowth, establishment of multiple axes of polarity, bone and cartilage biology, hormone action and steroid receptor biology. This timely compendium of the current state of our field will hopefully serve as a launching pad for new investigators to explore this fascinating and evolutionarily important topic.

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