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Review article

Development of the human female reproductive tract

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ABSTRACT

Development of the human female reproductive tract is reviewed from the ambisexual stage to advanced development of the uterine tube, uterine corpus, uterine cervix and vagina at 22 weeks. Historically this topic has been under-represented in the literature, and for the most part is based upon hematoxylin and eosin stained sections. Recent immunohistochemical studies for PAX2 (reactive with Müllerian epithelium) and FOXA1 (reactive with urogenital sinus epithelium and its known pelvic derivatives) shed light on an age-old debate on the derivation of vaginal epithelium supporting the idea that human vaginal epithelium derives solely from urogenital sinus epithelium. Aside for the vagina, most of the female reproductive tract is derived from the Müllerian ducts, which fuse in the midline to form the uterovaginal canal, the precursor of uterine corpus and uterine cervix an important player in vaginal development as well. Epithelial and mesenchymal differentiation markers are described during human female reproductive tract development (keratins, homeobox proteins (HOXA11 and ISL1), steroid receptors (estrogen receptor alpha and progesterone receptor), transcription factors and signaling molecules (TP63 and RUNX1), which are expressed in a temporally and spatially dynamic fashion. The utility of xenografts and epithelial-mesenchymal tissue recombination studies are reviewed.

1. Introduction

The seminal paper on development of the human female reproductive tract appeared in 1933 (Koff, 1933) and is based upon gray scale histologic images and line drawings. Since then the collective trove on human female reproductive tract development has expanded to include several histologic and immunohistochemical studies (Bulmer, 1957; Cai, 2009; Forsberg, 1996, 1973; Fritsch et al., 2013, 2012; Hunter, 1930; Koff, 1933; Konishi et al., 1984; Mutter and Robboy, 2014; O'Rahilly, 1977, 1983; O'Rahilly and Muller, 1992; Reich and Fritsch, 2014; Sinisi et al., 2003; Sulak et al., 2007; Kurita et al., 2005a). We have recently added four papers to this literature that: (a) provide a modern insight on the derivation of human vaginal epithelium; (b) describe the changing pattern of epithelial differentiation markers and signaling pathways from 8 to 21 weeks; (c) explain how diethylstilbestrol (DES) affects human female reproductive tract development and expression of epithelial differentiation markers (Robboy et al., 2017; Cunha et al., 2017b, 2018) and (d) examined the role of mesenchymal-epithelial interactions in differentiation of human reproductive tract epithelium and the expression of uterine epithelial estrogen receptor alpha and the progesterone receptor (Cunha et al., 2018).

The topic, development of the human female reproductive tract, is important for both academic and clinical reasons. Many congenital malformations of the female reproductive tract result from perturbation of normal morphogenetic mechanisms and their underlying molecular mechanisms. Although rare, many human female reproductive tract malformations result from abnormalities of Müllerian duct (MD) development (Table 1). Many occur spontaneously, while others are elicited by endocrine-disrupting substances, principally those having estrogenic activity. The best example of estrogen-induced malformation of the human female reproductive tract involves the administration of diethylstilbestrol (DES) to pregnant women, which was prescribed from the 1940s to 1971 when the Food and Drug Administration banned its use in pregnant women. Such treatment resulted in a broad spectrum of malformations of the uterine tubes, uterine corpus, cervix and vagina,

Abbreviations: H&E, hematoxylin & eosin; UGS, urogenital sinus, UGE, urogenital sinus epithelium; MD, Müllerian duct; MDE, Müllerian duct epithelium; WD, Wolffian duct; DES, diethylstilbestrol; KRT, keratin

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Table 1
Malformations based upon abnormal Müllerian duct development.

Malformation	Possible cause(s)
Complete absence of the uterus and upper vagina	Failure of MD development or failure of WD development.
Separate hemiuteri	Incomplete fusion of the MDs
Uterus didelphys	Incomplete fusion of the MDs
Uterus duplex	Incomplete fusion of the MDs
Uterus unicornis	Unilateral aplasia of a MD
Bilateral or unilateral doubling of the uterine tubes or uterus	Duplication of MDs
Double vagina	Failure of MD fusion
Double cervix	Failure of MD fusion
Uterus septus	Failure of regression of the septum
Vagina with septum	Failure of regression of the septum
Absence of uterine tubes, uterus, cervix and upper vagina	Effect of Müllerian Inhibiting Substance in males with complete androgen resistance
T-shaped uterotubal junction	Prenatal exposure to DES
Incompetent aplastic cervix	Prenatal exposure to DES
Abnormal uterine lumen	Prenatal exposure to DES
Vaginal adenosis	Prenatal exposure to DES

From (Gray and Skandalakis, 1972; Moore and Persaud, 2003; Sadler, 2004; Reed and Fenton, 2013).

which included T-shaped uterotubal junctions, malformed incompetent cervix, abnormally shaped endometrial cavity, vaginal adenosis (presence of glandular epithelium in the vagina where normally stratified squamous epithelium should reside) as well as clear cell adenocarcinoma of the vagina (Jefferies et al., 1984; Rennell, 1979; Stillman, 1982; Titus-Ernstoff et al., 2010; Herbst et al., 1971, 1975; Robboy et al., 1977, 1984a; Hoover et al., 2011). These observations in human are buttressed with a huge animal literature that preceded/confirmed the effects of exogenous estrogens on reproductive tract development, and have provided a molecular underpinning for the teratogenic effects of natural (17β-estradiol (Forsberg, 1972), 17α-estradiol (Forsberg and Kalland, 1981), estradiol benzoate (Plapinger and Bern, 1979)) and synthetic estrogens (diethylstilbestrol [DES], dienestrol (Forsberg and Kalland, 1981), clomiphene citrate (Gorwill et al., 1982, tamoxifen (Taguchi and Nishizuka, 1985)), nafoxidine (Iguchi et al., 1986), coumestrol (Burroughs et al., 1990) and bisphenol A (BPA) (Newbold et al., 2009)) on urogenital development (Herbst and Bern, 1981; Bern and Talamantes, 1981; Bern et al., 1984; McLachlan et al., 1975, 2001; McLachlan, 1981; Newbold et al., 1983; Newbold and MaLachlan, 1985; Newbold, 1995, 2004, 2008; McLachlan and Newbold, 1996; Kurita et al., 2004; Kurita, 2011; Laronda et al., 2012, 2013). Environmental "endocrine disruptors" appear to adversely affect the health of wildlife and as well as humans (Colborn, 1995, 1994).

Animal studies have been most useful in understanding normal and abnormal female reproductive tract development, in some cases predicting human genital tract malformations even though there are significant differences in anatomy and molecular regulation among species. For example, progesterone receptor regulation in uterine epithelium differs substantially in the mouse versus human (Janne et al., 1975; Horwitz and McGuire, 1979; Kurita et al., 2000, 2005b). In the mouse, uterine epithelial progesterone receptor (PGR) is strongly expressed following ovariectomy and is profoundly down regulated upon estrogen administration, an effect mediated indirectly via stromal estrogen receptor 1 (ESR1) (paracrine mechanism) (Kurita et al., 2000). In contrast, in humans PGR is regulated directly via ESR1 in estrogen-target epithelia (Janne et al., 1975; Horwitz and McGuire, 1979; Kurita et al., 2005b; Cunha et al., 2018). For these reasons we felt it important to study human female reproductive tract development and so developed a xenograft model where observation and analysis of human development were possible ethically in a simulated in-vivo environment. The goals of this paper are (a) to provide detailed information on how to acquire human fetal female reproductive tracts for study, (b) to richly illustrate human female reproductive tract morphogenesis, (c) to review the ontogeny of epithelial and stromal differentiation markers, (d) to illustrate the utility of xenograft experiments designed to directly study the morphogenetic and molecular effects on human female reproductive tract development in vivo, and (e) to validate the role of mesenchymal-epithelial interactions in human female reproductive tract development.

2. Acquisition and isolation of human female reproductive tract

Collection of abortus specimens can only be achieved in locales where legal abortions are possible and where local laws permit investigation of human fetal organs/tissues. Institutional Committees on Human Research should be consulted to acquire authorization to carry out such research. The key proviso is collection of abortus material without patient identifiers. Given that current surgical procedures are disruptive, the initial challenge in human fetal organogenesis is finding the human female reproductive tract in the abortus specimen. Commonly, the bladder and the human female reproductive tract remain attached. Sometimes the bladder and female reproductive tract complex are attached to the proximal end of the free-floating umbilical cord. In other cases an intact pelvis is obtained from which the reproductive tract can be dissected. The key is to understand the developmental anatomy over the time frame of the youngest to the oldest specimens so that the gross morphology can be recognized in the disrupted specimen. Fig. 1 is a montage of human female reproductive tracts from 9 to 22 weeks of gestation.

Gestational age of disrupted surgical specimens was estimated by heel-toe length (Drey et al., 2005). Of import, accurate staging of abortus specimens is not precise, and specimen ages given in peer-reviewed papers are best approximations. As the Carnegie collection website states: "An embryo is assigned a Carnegie stage based on its external features. This staging system is not dependent on the chronological age or the size of the embryo. The stages are in a sense arbitrary levels of maturity based on multiple physical features. Embryos that might have different ages or sizes can be assigned the same Carnegie stage based on their external appearance because of the natural variation which occurs between individuals (Smith, 2016)". In the not-so distant past, abortions involved vaginal delivery of intact embryos/fetuses from which crown-rump measurements were used as a measure of gestational age (Streeter, 1951). Currently, crown-rump measurements are rarely possible. Accordingly, heel-toe length is used to determine fetal maturity, which gives a rough estimate of fetal age.

3. Early Müllerian duct development

Most of the female reproductive tract develops from the MDs whose development is preceded by the Wolffian (mesonephric) duct (WD) growth within the paired urogenital ridges (Fig. 2). Following WD formation, the MDs arise as coelomic epithelial invaginations on the lateral surface of the paired urogenital ridges at 5–6 weeks of gestation (O'Rahilly, 1973). The coelomic invaginations later become the ostia of the uterine tubes. The paired MDs grow caudally within the urogenital ridges using the WDs as "guide wires." Indeed, the WDs are requisite for caudal MD migration to the UGS (Gruenwald, 1941; Kobayashi et al., 2005). During the period of caudal MD migration, urogenital ridge mesenchyme separates the Müllerian and Wolffian ducts cranially (Fig. 3a). More caudally, MD and WD duct epithelia are separated only by their conjoined basement membranes without intervening mesenchyme (Fig. 3b). Even more caudally, the tip of the MD is in direct contact with WD epithelium without an intervening basement membrane (Fig. 3c). In mice Lhx1 is expressed in both the Wolffian and Müllerian ducts, and Wolffian duct-specific Lhx1-knockout elicits Wolffian duct degeneration and loss of the Müllerian ducts (Huang et al., 2014). Müllerian duct-specific knockout of Lhx1 blocks Müllerian duct elongation resulting in loss of the entire endometrium (luminal and glandular epithelium and stroma) (Huang et al., 2014). The track of

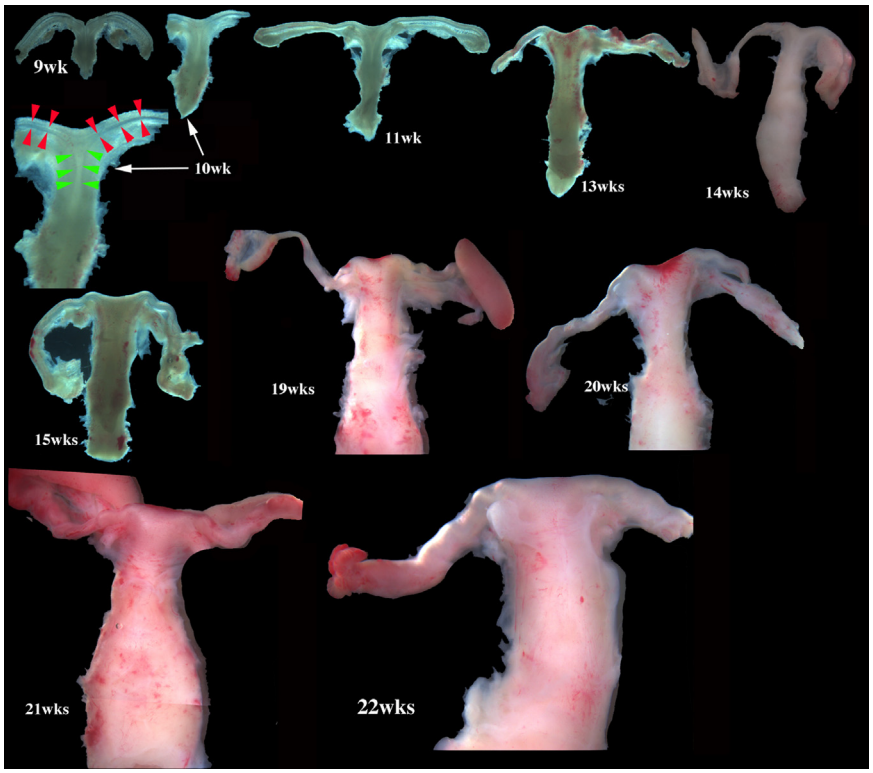

Fig. 1. Wholemount photos of developing human fetal female internal genitalia staged by heel-toe measurements. Note (a) increase in size and morphological complexity with time, and (b) that it is impossible to distinguish the uterine corpus, cervix and vagina. Specimens photographed with transmitted light (9, 10, 11, 13 and 15 weeks) permit visualization of internal (epithelial) organization in regions not too thick. The 10-week specimen is shown at both low and high magnifications. Red arrowheads demarcate the epithelium defining the lumen of the uterine (Fallopian) tube. Green arrowheads define the epithelium lining the uterus. Relative sizes of specimens are not exact, but increase with age. From Robboy et al. (2017) with permission. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the WDs and MDs in route to the UGS exhibits two gentle curves within the paired urogenital ridges that define vertical, horizontal, and vertical portions as seen in frontal view (Fig. 4). The point where the MDs initially contact the urogenital sinus, called the Müllerian tubercle, is a controversial and poorly described entity. The urogenital sinus epithelium at the point of contact with the MDs subsequently proliferates to form the so-called sinovaginal or sinus bulbs (Fig. 5). The point of contact of the Müllerian ducts with the urogenital sinus is a critical step in female reproductive tract development. Failure of merging of the Müllerian ducts with the urogenital sinus can lead to lower

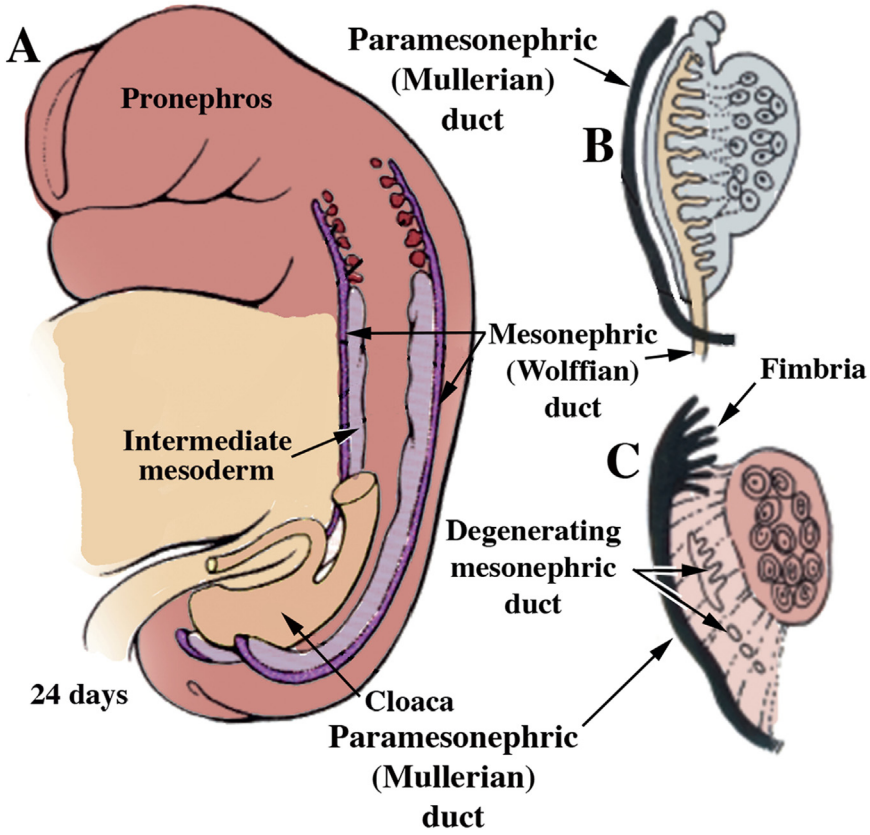

Fig. 2. (A) Formation of the Wolffian (mesonephric) duct, which by 24 days has grown caudally to join the cloaca. At 5–6 weeks the paramesonephric (Müllerian) ducts appear as invaginations of the coelomic epithelium. At 7 weeks (B) the MDs have grown caudally towards the urogenital sinus. Subsequently (C, 8 weeks), the opening of the MDs into the coelomic cavity is fimbriated, and with further growth the MDs reach the UGS, while the Wolffian ducts degenerate. Modified from (Park, 2016) with permission.

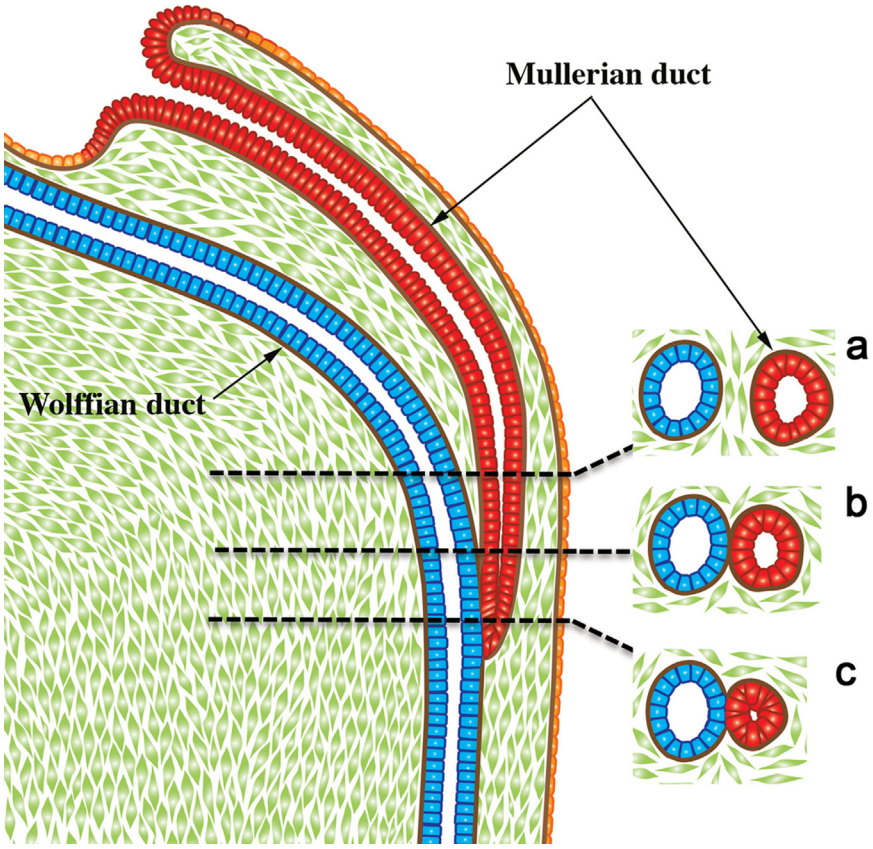

Fig. 3. Diagrammatic representation of the caudal growth of the Müllerian duct using the Wolffian duct as a "guide wire". Note in (a) mesenchyme intervening between the Müllerian and Wolffian ducts, (b) contact of the basement membranes of Müllerian and Wolffian ducts, (c) direct contact of the epithelia of the Müllerian and Wolffian ducts. From Robboy et al. (2017) with permission.

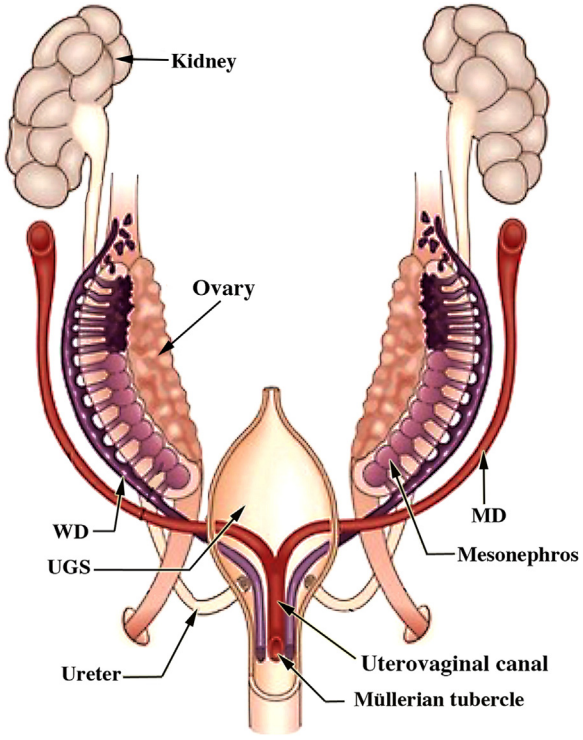

Fig. 4. Diagram of developing human female internal genitalia in the indifferent, bisexual stage (~ 54 days of gestation, Carnegie Stage 22). The Müllerian derivatives are red and Wolffian derivatives are purple. Note the changing anatomical relationships between the Müllerian and Wolffian ducts. From Robboy et al. (2017) with permission. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

vaginal agenesis. Lhfpl2 mutant mice exhibit such a phenotype (Zhao et al., 2016). During the 7th to 8th weeks, the caudal portions of the paired MDs lie between the two Wolffian ducts near the UGS. During the 8th week, the bilateral Müllerian ducts fuse together, and temporarily a midline epithelial septum separates the lumina of the two adjacent Müllerian ducts (Fig. 5). This midline septum largely disappears in the 9th week resulting in formation of the midline uterovaginal canal (Figs. 5 and 6), lined throughout with an undifferentiated simple columnar Müllerian epithelium (Hunter, 1930; Koff, 1933; Robboy et al., 2017).

The degree of midline MD fusion differs in humans versus mice. In humans, midline fusion is extensive, resulting in the midline uterovaginal canal and paired uterine tubes (Fallopian tubes) (Figs. 6 and 7). In mice most of the MDs remain unfused, forming the paired oviducts and the large bilateral uterine horns. Only the caudal portions of the mouse MDs fuse to form the cervical canal and the so-called "Müllerian vagina" (Kurita, 2010). The degree of MD fusion (or non-fusion) as well as disappearance or retention of the midline septum within the uterovaginal canal is the substrate for multiple human congenital malformations (Table 1). Regression of the midline septum within the human uterovaginal canal is not understood, but perhaps bears some similarity to Müllerian duct regression in males, which is triggered by anti-Müllerian hormone (AMH). β-Catenin mediates AMH signaling for Müllerian duct regression during male sexual differentiation (Kobayashi et al., 2011), and perhaps β-catenin mediates regression of the midline septum within the human uterovaginal canal.

As mentioned above, development of the Müllerian ducts in mice and humans appears to be dependent upon the transcription factor, Lhx1 (Huang et al., 2014; Zhang et al., 2017). Müllerian duct-specific knockout of Lhx1 blocks Müllerian duct elongation and results in loss of the entire endometrium and loss of the inner circular but not the outer longitudinal uterine muscle layer (Huang et al., 2014). Congenital absence of the uterus and vagina in humans is associated with a novel

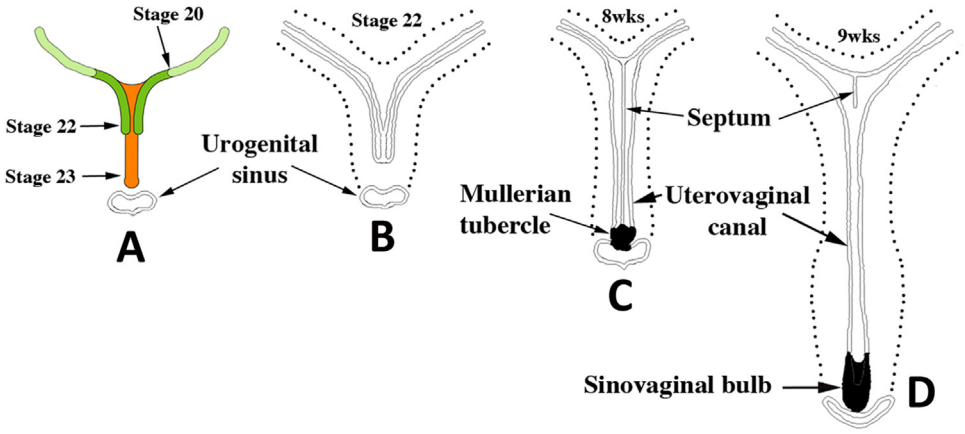

Fig. 5. Early Müllerian duct growth and fusion to form the midline uterovaginal canal. Length of the uterovaginal canal increases with developmental age. In (A) the extent of MD caudal extension is depicted at Carnegie Stages 20–23 (50–56 days). (B-D) depict fusion of the right and left MDs to form the midline uterovaginal canal, formation of the septum and its subsequent disappearance. From Robboy et al. (2017) with permission.

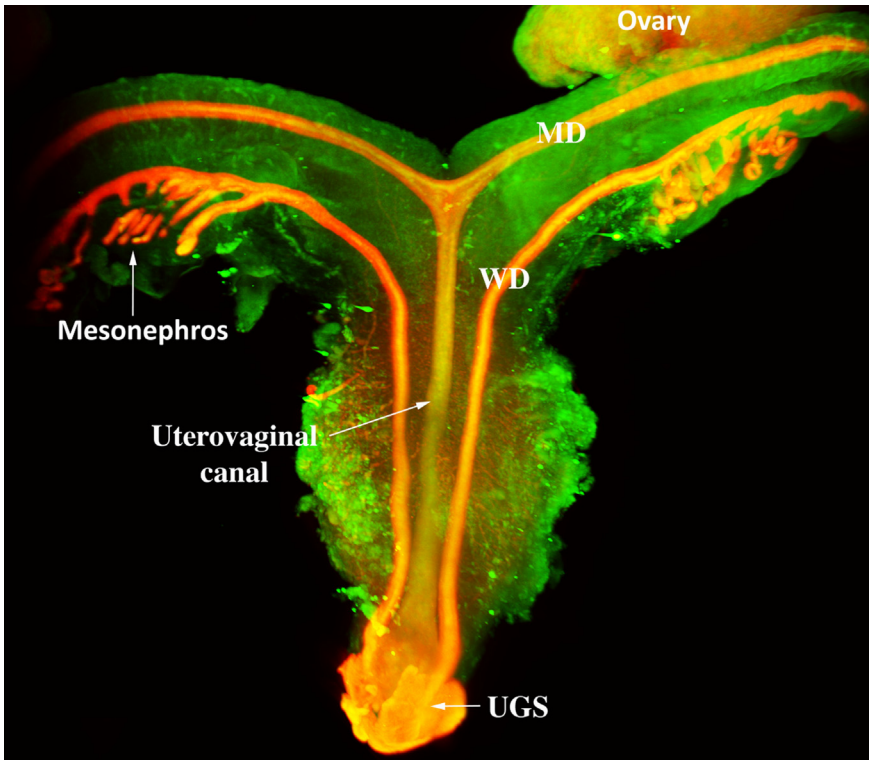

Fig. 6. Light sheet microscopy™ of a human female reproductive tract at 9.5 weeks stained with an antibody to E-cadherin. The mesonephros and Wolffian ducts (WD) are present. Cranially, the unfused MDs are destined to form the uterine tubes. Midline fusion of the MDs has created the uterovaginal canal that terminates caudally by joining the urogenital sinus (UGS).

missense mutation in LHX1 (Zhang et al., 2017).

4. Organogenesis of the human female reproductive tract

4.1. Uterine tube

The uterine tubes, eponymically called the Fallopian tubes, consist of 4 parts: (a) the funnel-shaped infundibulum with its finger-like projections (fimbria) (Fig. 2C), (b) the ampulla where fertilization typically occurs, (c) the isthmus, immediately lateral to the uterus, and (d) the intramural (or interstitial) portion within the uterine wall terminating in the uterotubal junction (Pauerstein et al., 1974). The uterine tubes develop from the paired cranial portions of the MDs retained after the caudal segments fuse to form the midline uterovaginal canal (Figs. 6–7) (Robboy et al., 2017). The fimbriae of the uterine tubes develop from the irregular ostia of the MDs into the abdominal cavity (Fig. 2C). The epithelium lining the fetal uterine tube is a single layer of columnar cells. As development proceeds two changes occur: (a) Mucosal folds (plicae) form, most prominently in the infundibulum and ampulla, and by 14 weeks a distinct gradient of mucosal plication is evident (Robboy et al., 2017). (b) The mesenchyme surrounding the epithelial tube differentiates into an inner stromal layer in contact with the epithelium and outer circularly oriented smooth muscle layer. In adulthood tubal epithelium contains ciliated cells, secretory cells, and intercalary or "peg" cells (with long slender dark nuclei compressed between adjoining cells).

Epithelial cells of the human fetal uterine tube express multiple differentiation markers (Table 2). Keratins 7, 8 and 19 are expressed from the earliest stages examined (9 weeks) and were maintained thereafter. Androgen receptor and ESR1 were first detected in tubal epithelium at 14 weeks. The progesterone receptor remained undetected in tubal epithelium from 8 to 21 weeks, but was induced by DES in tubal epithelium in xenografts (Cunha et al., 2017a).

4.2. Uterus corpus

The uterine corpus develops from the cranial portion of the midline uterovaginal canal (Koff, 1933; Robboy et al., 2017). The molecular mechanism by which the MDs fuse is poorly understood. However, based upon the human malformation, bicornuate uterus (Campbell,

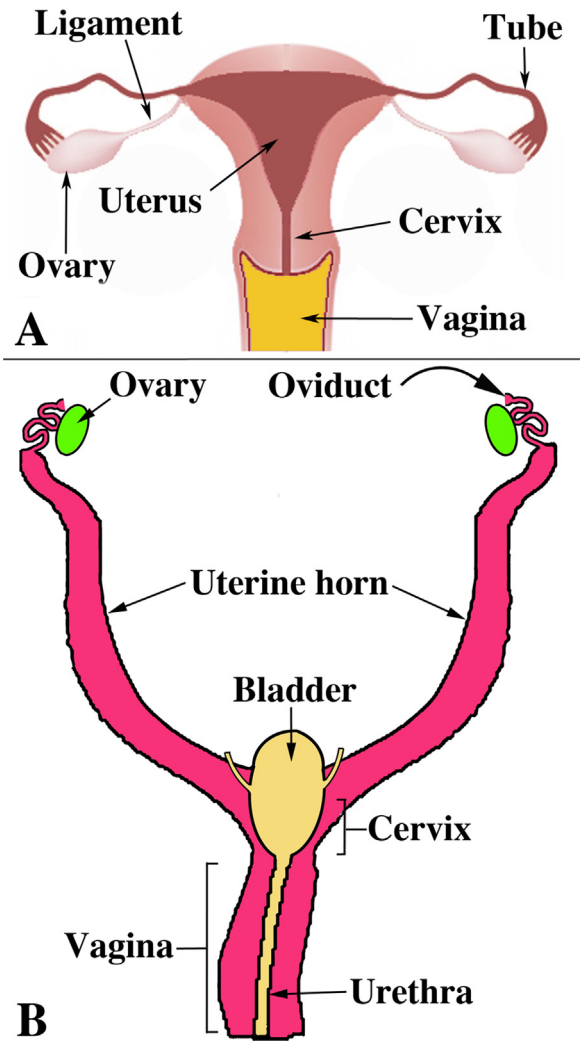

Fig. 7. Diagrams of human (A) and mouse (B) female urogenital tracts emphasizing the marked difference in the degree of MD fusion. From Robboy et al. (2017) with permission.

1952; Davies and Walpole, 1949), it appears that MD fusion begins caudally and progresses cranially. The overall shape of the adult uterotubal junction and the lumen of the uterine corpus are unique. Wholemount specimens photographed with transmitted light reveal epithelial contours within the developing uterovaginal canal (Fig. 8). The basic delta shape of the cranial portion of the uterovaginal canal (uterine corpus anlage) is recognizable by weeks 9–10 (Fig. 6), which subsequently expands and broadens (Robboy et al., 2017) (Fig. 8). Accordingly, transverse sections exhibit marked lateral expansion of the uterovaginal canal cranially and less so caudally (Fig. 8). The overall morphogenetic process surely involves growth in overall size and gradual shape change of the uterine lumen, which will continue to evolve until the final adult morphology is achieved. The uterine corpus remains grossly undeveloped at parturition, undergoing considerable growth postnatally (Cooke et al., 2013). Functional and anatomical maturity is achieved at menarche.

At all stages examined up to 22 weeks the boundary between the uterine corpus and cervix cannot be discerned with certainty in gross specimens (Figs. 1 and 8) or histologically. Glands form within the developing uterine corpus and uterine cervix beginning about 14–15 weeks (Robboy et al., 2017). The rudimentary glands are shallow outpouchings lined by a simple columnar epithelium. Glands are initially found a considerable distance caudal to the uterine fundus, suggesting a regionality in gland formation. Indeed, the first glands to form are probably cervical glands. By 21 weeks uterine and cervical glands are elongated and branched within the stroma (Robboy et al., 2017).

Epithelial cells of the human fetal uterine corpus express various differentiation markers (Table 3). Like the presumptive uterine (Fallopian) tubes, the epithelium of the cranial aspect of the uterovaginal canal (uterine precursor) expresses keratins 7, 8 19 and Runx1 from the earliest stages examined (9 weeks), and these markers are maintained thereafter. ESR1 was first detected in the uterine corpus at 16 weeks in a small subset of epithelial cells, while the surrounding stromal cells are ESR1-positive. At 21 weeks, the epithelium of the uterine corpus contains a mixture of ESR1-reactive and ESR1-unreactive epithelial cells with the former predominant (Cunha et al., 2017b). The progesterone receptor was undetectable in epithelium of the uterine corpus throughout the 9- to 21-week period of study.

Investing and also deep to the glands of the presumptive endometrium is a stromal layer (endometrial stroma) composed of loose mesenchymal cells, best identified by their lack of smooth muscle α-actin reactivity (ACTA2). CD10, a sensitive and diagnostically useful immunohistochemical marker of normal endometrial stroma, was not used in this study.

Myometrial smooth muscle, whose development has been described recently (Robboy et al., 2017; Fritsch et al., 2013), is the most abundant tissue in the uterus. Faint ACTA2 immunoreactivity, indicative of myometrial differentiation, was initially observed in the outer mesenchymal wall of the uterovaginal canal at 8–9 weeks of gestation (Fig. 9A). By the 11–12 weeks ACTA2 was detected most prominently in the middle 2/4^{ths} of the uterovaginal canal corresponding to the region of the presumptive cervix and possibly upper vagina (Fig. 9B-C). At this time the mesenchymal wall of the uterine fundus and inferior vagina showed negligible ACTA2 immunostaining. By the 18th week, all organs of the female reproductive tract displayed strong ACTA2 reactivity within their fibromuscular walls (Fig. 9D), especially in the cervix, which is relatively advanced in development compared to the other female reproductive tract organs, thus confirming a previous study (Fritsch et al., 2013).

4.3. Uterine cervix

The cervix develops from the middle 2/4ths of the uterovaginal canal. Initially, the boundaries between the cervix and the uterine corpus as well as the between the cervix and vagina are indistinct. Koff asserts that the cervix can be “recognized as a spindle-shaped thickening or condensation of the mesenchyme of the genital cord” in 8–10-week fetuses (Koff, 1933), a subtlety which we find impossible to discern (Figs. 1, 8 and 10). Once vaginal fornices become recognizable at roughly 18 weeks (Fig. 10F), the boundary between the exocervix and the vagina becomes obvious. For this reason, in “early stages” of cervical organogenesis, the cervical domain can only be inferred based upon cranial-caudal position within the uterovaginal canal, subtle changes of the endocervical contour in cross-section, and subtle

Table 2
Epithelial differentiation markers in human fetal uterine tube.

KRT6	KRT7	KRT8	KRT10	KRT14	KRT19	TP63	RUNX1	AR	ESR1	PR
–	+	+	–	–	+	–	–	+	+	-*

* Epithelial PR is induced in xenografts by DES, but is otherwise absent from 8 to 21 weeks. From Cunha et al. (2017b).

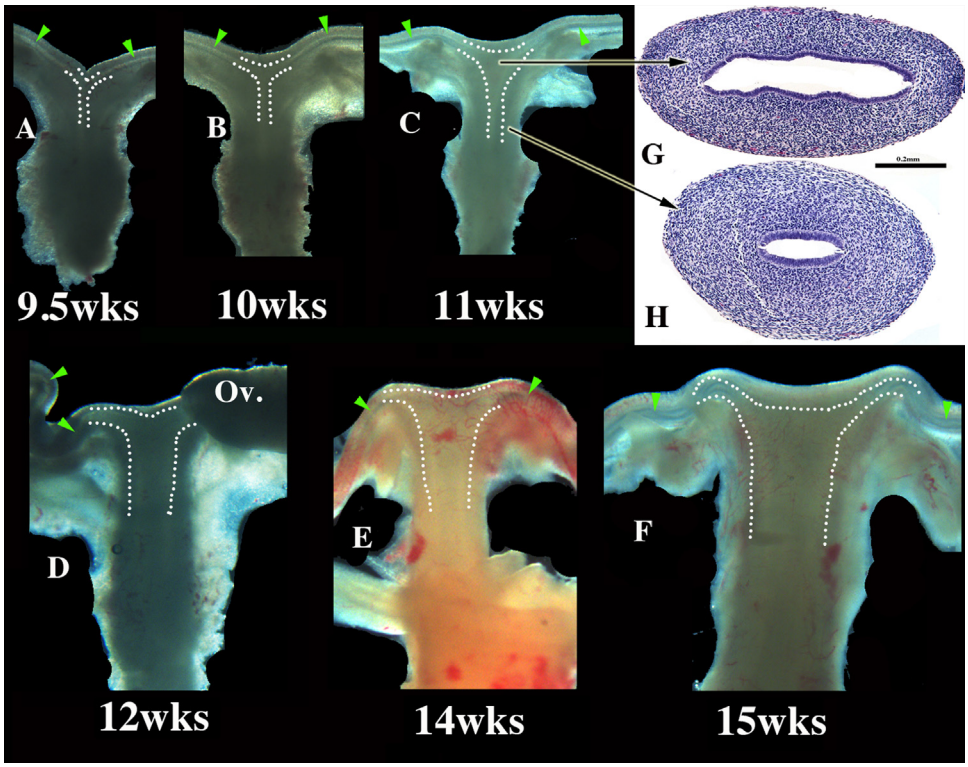

Fig. 8. Wholemount images of human fetal female reproductive tracts (A-F) photographed with transmitted light, and H&E stained transverse sections of the uterovaginal canal (G-H). Dotted lines indicate the contours of the uterotubal junction and the uterine cavity. Green arrowheads indicate the epithelium of the uterine tubes. Note the changing shape of the uterine lumen. The cranial portion is laterally expanded (G) and narrow caudally (H). From Robboy et al. (2017) with permission. (For interpretation of the refenences to color in this figure legend, the reader is referred to the web versin of this article.)

Table 3
Epithelial differentiation markers in human fetal uterine corpus.

KRT6	KRT7	KRT8	KRT10	KRT14	KRT19	TP63	RUNX1	AR	ESR1	PR
–	+	+	–	–	+	–	+	–	-,+*	-#

* Epithelial ESR1 is undetectable at 14 weeks, sparsely expressed at 16 weeks and predominant at 21 weeks. Xenografts of 13-week uterine corpus (before expression of ESR1) when treated with DES exhibit prominent uterine epithelial ESR1 immunoreactivity. # Epithelial PGR is induced in xenografts by DES. From Cunha et al. (2017b).

histodifferentiation of the epithelium.

Initially, simple columnar Müllerian epithelium, indistinguishable from area to area, lines the uterovaginal canal throughout (Fig. 8 G & H). Epithelium of the fetal cervix at 21 weeks, the oldest we examined histologically, was of two types. Caudally, the epithelium was stratified squamous near the external os and on the exocervix. Cranially cervical epithelium is simple columnar with associated glands (Fig. 10E & F). At this time, there is no evidence of mucin expression. During development, the "columnar epithelium lining the caudal portion of the uterovaginal canal becomes stratified" (Koff, 1933) (Fig. 10D). At about 11 weeks "the conversion of columnar to stratified epithelium (within the uterovaginal canal) continues cranially until the transition between the two types becomes abrupt, a point which marks the junction of cervical and vaginal epithelium in the cervical canal" (Koff, 1933). We disagree slightly with Koff's interpretatiom. In our opinion the zone of epithelial stratification within the uterovaginal canal respresents differentiating vaginal epithelium as wells as stratified squamous cervical epithelium (Fig. 10C-F).

The transcription factor, ISL1, may define the boundaries between the endo- and exocervix, uterine corpus and vagina because it is enriched in cervicovaginal mesenchyme, but not uterine mesenchyme (Robboy et al., 2017). ISL1 immunostaining is prominent in vaginal and exocervical mesenchyme (Fig. 10B), but absent in the wall of the endocervix and uterine corpus. The steep reduction in ISL1 staining (Red arrow in Fig. 10B) may denote the future endocervical-exocervical boundary. Immunohistochemical observations show that the domains of ISL1 in cervicovaginal mesenchyme and HOXA11 in uterine mesenchyme have minimal overlap (Cunha et al., 2017b).

HOX genes specify the developmental fate of individual regions of the female reproductive tract. HOXA9 specifies the uterine tube, HOXA10 and HOXA11 specify the uterus and HOXA13 specifies vaginal development (Du and Taylor, 2015). HOXA11 expression is enhanced in human uterine mesenchyme (Cunha et al., 2017b), which in mice is the inducer of uterine epithelial differentiation (Cunha, 1976). Whether inductivity of human uterine mesenchyme is dependent on expression of HOXA11 remains to be determined, likewise for inductivity of human vaginal mesenchyme in relation to expression of ISL1.

The simple columnar epithelium of the uterovaginal canal remains as such during development of the uterine tube, uterine corpus and endocervix as discussed above, and glands of simple columnar epithelium are well developed within the cervix at 21 weeks of gestation (Fig. 10E). More caudally, the simple columnar epithelium of the uterovaginal canal differentiates into a stratified squamous epithelium in the vagina and exocervix, a process preceded by conversion of the uterovaginal canal into the solid vaginal plate. To follow this process of epithelial differentiation in the vagina and exocervix, we employed immunohistochemistry using a panel of relevant keratin antibodies known to be reactive to either simple columnar, stratified squamous (or both) epithelia (Moll et al., 1982, 2008).

KRT19 expression is particularly dynamic, changing dramatically in certain areas. KRT19 immunostaining was seen at 9 weeks in simple columnar epithelium of the Müllerian duct and uterovaginal canal and in the solid vaginal plate at 12 weeks of gestation (Cunha et al., 2017b). At this stage, KRT19 and the PAX2 immunostaining (indicative of

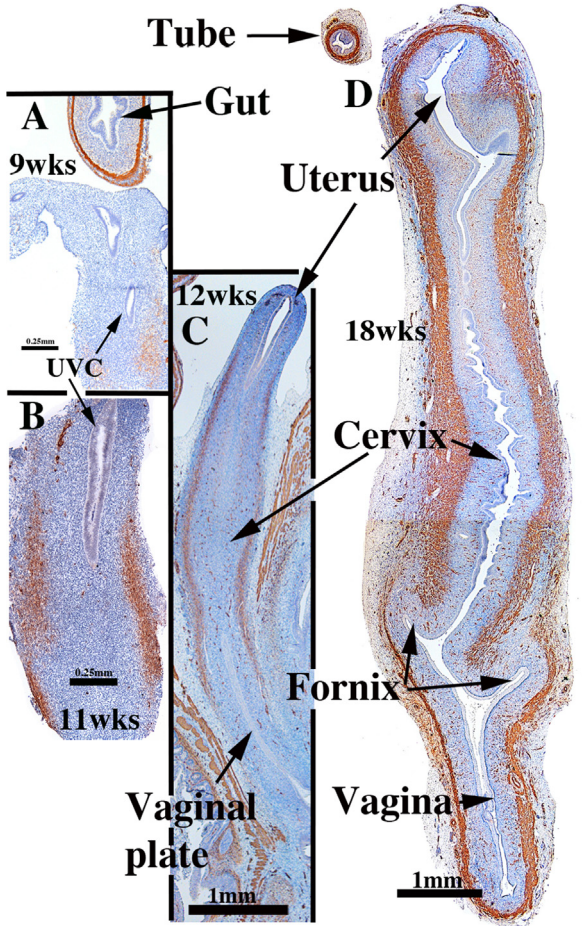

Fig. 9. α-Actin immunoreactivity in human female reproductive tracts at (A) 9, (B) 11, (C) 12, and (D) 18 weeks. Faint α-actin immunoreactivity first appears focally in mesenchyme of the 9-week uterovaginal canal, increasing in intensity by the 11 weeks. By 12 weeks α-actin immunoreactivity is strong in the middle 2/4ths of the developing reproductive tract, and by 18 weeks, is strongly expressed throughout the developing female reproductive tract. From Robboy et al. (2017) with permission.

Müllerian epithelium) are congruent (Cunha et al., 2017b; Robboy et al., 2017). At 14 weeks and 16 weeks, the solid vaginal plate was KRT19-negative (Fig. 10C-D and insets) (Cunha et al., 2017b), and a sharp boundary was revealed with KRT19-reactive stratified epithelium associated with the lumen, as well as KRT19-positive simple columnar epithelium that extends cranially to the epithelium of the uterine corpus (Fig. 10C-D and insets) (Cunha et al., 2017b). There are several possible interpretations. The absence of KRT19 staining in the solid vaginal plate versus the intense KRT19 staining in the stratified epithelium lining the lumen immediately cranial to the solid vaginal plate may reflect the UGE/MDE boundary. The KRT19-negative solid vaginal plate is FOXa1-positive indicative of endodermal urogenital sinus epithelium, whereas the KRT19-positive epithelium extending uninterrupted to the uterine corpus is PAX2-positive indicative of Müllerian epithelium (Cunha et al., 2017b). This zone of positive/negative KRT19 reactivity is likely to be one of constant flux until the final distribution of Müllerian and urogenital sinus epithelium is achieved as discussed below.

The epithelium lining the uterine cervix eventually differentiates into stratified squamous cells in the exocervix, but remains simple columnar with associated glands in the endocervix (Fig. 10E & F). Accordingly, epithelial differentiation in the uterine cervix exhibits patterns of differentiation markers shared by both the uterine corpus and vagina depending on cranial-caudal position (Table 4). Keratins 7, 8 and 19 were detected in the cervical region of the uterovaginal canal from as early as 9 weeks, which is consistent with the presence of simple columnar epithelium. Expression of these keratins was maintained in the endocervical epithelium to 21 weeks. Keratin 14 is a protein normally expressed in basal cells of stratified epithelia, including in mature vaginal epithelium (Moll et al., 1982, 2008). Mature exocervical and vaginal epithelium seen in our 21 weeks specimen, exhibited KRT14 reactivity in basal cells as expected (Cunha et al., 2017b). In the developing human female reproductive tract KRT14 expression is clearly dynamic, temporally regulated and not strictly related to epithelial stratification. This is illustrated in the 18-week specimen in which the vaginal fornices, exocervix and endocervix are readily identified. The stratified squamous epithelia of the vagina and exocervix were KRT14-negative with the exception of a patch of KRT14-positivity in epithelium of the caudal-most segment of the vagina, yet earlier the solid vaginal plate is KRT14-positive (Cunha et al., 2017b) (Table 4). In cervicovaginal epithelia of mice keratin14 is expressed early in postnatal development, and keratin 14 has been shown to be down stream of p63 and Runx1 (Laronda et al., 2013). The organotypic expression pattern of TP63 and RUNX1 in the developing human female reproductive tract (Cunha et al., 2017b) departs substantially from that seen in the mouse (Kurita and Cunha, 2001; Laronda et al., 2013).

At 16 and 18 weeks the cervical epithelia (exocervix and endocervix) were uniformly ESR1-negative, whereas at 21 weeks ESR1 was strongly reactive in the presumptive epithelium of the exocervix, but patchy in epithelium of the endocervix. Thus, while ESR1 expression exhibits regionality of expression, its relevance to exocervical and endocervical boundaries is uncertain. In xenografts, DES strongly induced ESR1 reactivity in the “cervical region” (Cunha et al., 2017a). The progesterone receptor was not detected in the epithelium and stroma of the uterine cervix from 8 to 21 weeks.

4.4. Vagina

The origin of vaginal epithelium has been debated ever since Johannes Müller described the duct that bears his name (Müllerian duct) (Müller, 1830). Vaginal epithelium has been proposed to receive contributions from epithelia of the urogenital sinus, Müllerian and Wolffian ducts alone or in combination (Koff, 1933; O'Rahilly, 1977; Forsberg, 1978; Bulmer, 1957). Historically, Koff's publication in 1933 and Bulmer's in 1957 were the two most seminal works examining the origin of the human vaginal epithelium (Bulmer, 1957; Koff, 1933). Both investigators understood that Müllerian and urogenital sinus epithelia play major roles. Koff asserted that Müllerian epithelium formed most of the vaginal epithelium, assigning a minor contribution from urogenital sinus epithelium (UGE) to vaginal epithelium. Bulmer stated that UGE upgrowth “forms the whole of (the vaginal) epithelial lining” (Bulmer, 1957). For both studies the interpretations were based solely upon hematoxylin and eosin (H&E) stained sections without further verification.

To explore this issue, we carried out immunohistochemical studies of PAX2 and FOXA1 expression (Robboy et al., 2017). PAX2 is a protein expressed in Müllerian epithelium (Kurita, 2010), while FOXA1 is a protein expressed in endodermal urogenital sinus epithelium and its derivatives (Diez-Roux et al., 2011; Robboy et al., 2017; Besnard et al., 2004). The anatomical distribution of these two differentiation markers is dynamic during development of the female reproductive tract (See Fig. 15 for summary). At 11–12-weeks the cranial aspect of the uterovaginal canal has a lumen lined with a simple columnar epithelium (Fig. 8G-H), while the caudal aspect of the uterovaginal canal becomes occluded forming the solid vaginal plate abutting the introital epithelium. At 12 weeks the epithelia of the uterovaginal canal and almost all of the vaginal plate was PAX2-reactive (Figs. 11A-B and 15). The most caudal portion of the vaginal plate was PAX2-negative and FOXA1-positive (Figs. 11B & C and 15). Epithelia of the bladder and urethra

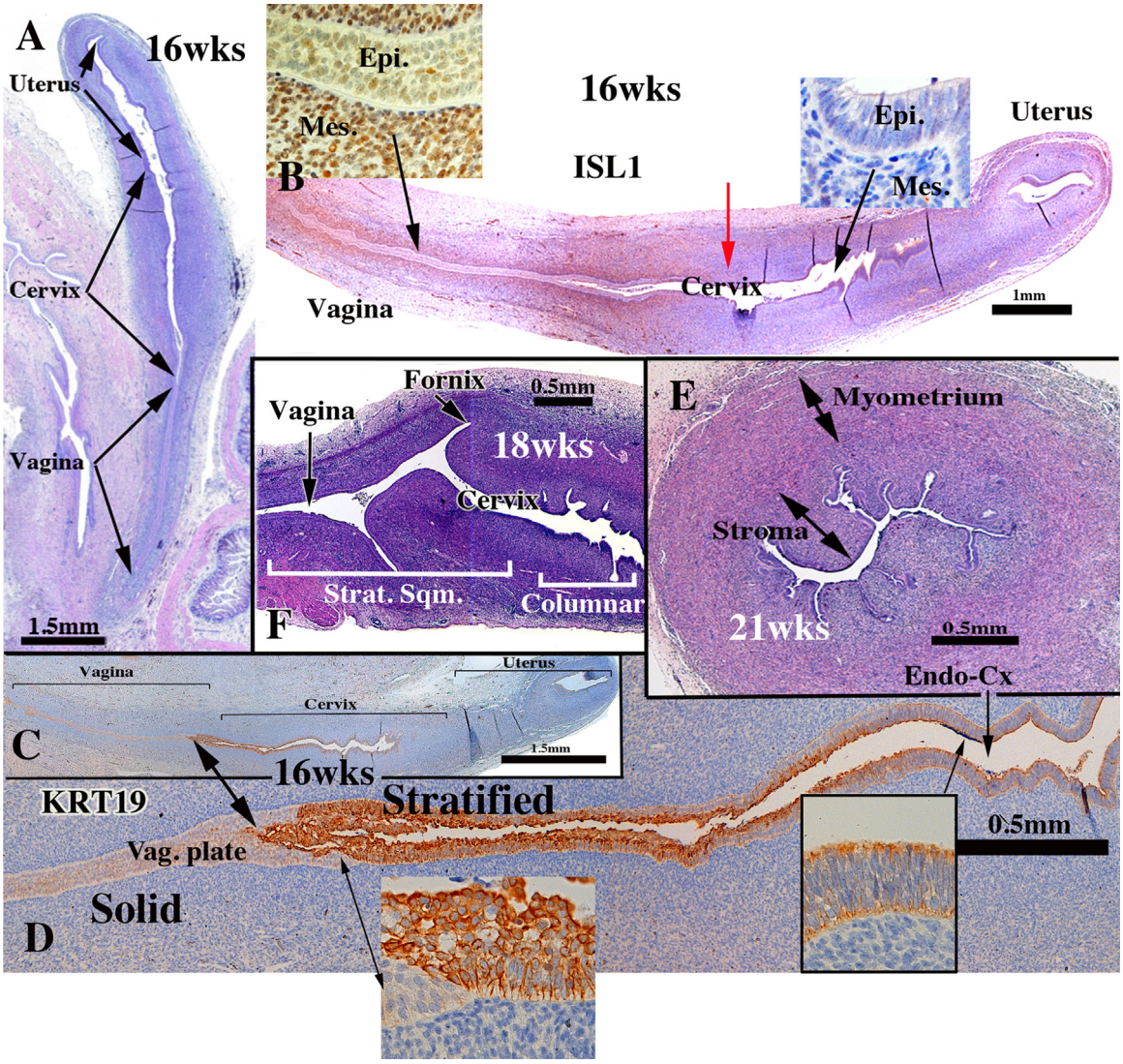

Fig. 10. Cervical development. (A-D) are sagittal sections of the female reproductive tract of a 16 week fetus (A = H&E, B = ISL1 immunostain, C-D = keratin 19 immunostain). Boundaries between the vagina, cervix and uterus are nebulous up to 18 weeks, when vaginal fornices become apparent (F). (B) ISL1 immunostaining is strong in vaginal stroma, absent in uterine stroma, with a sharp fall off in staining intensity at the mid-point of the uterovaginal canal (red arrow in B). Keratin 19 immunostaining (C-D) may also be indicative of vaginal-exocervical boundary. Cervical glands are prominent at 21 weeks of gestation (E). Epi. = Epithelium, Mes. = mesenchyme, Strat. Sqm. = Stratified squamous, Vag. Plate = vaginal plate, Endo-Cx = endocervix. From Robboy et al. (2017) with permission.

were FOXA1-positive as expected (Fig. 11D). During weeks 14–16the solid vaginal plate increased in extent, and at 16 weeks constituted about 1/3rd of the developing female reproductive tract's total length (Figs. 12 A & D and 15). At 16 weeks the solid vaginal plate consisted of FOXA1-reactive epithelial cells (of UGS origin) (Figs. 12C & D and 15), while more cranially the stratified epithelium associated with a lumen was PAX2-reactive (Fig. 12A & B). Clearly, from 12 to 16 weeks the domain of UGS-derived FOXA1-positive epithelial cells had expanded cranially (see Fig. 15). When the vaginal fornices became defined at 18 weeks of gestation (Figs. 10F & 13A & D-E, and 15), epithelia of the uterine tube, uterine corpus, uterine cervix, and the upper (cranial) portion of the vagina remained PAX2- and KRT19-reactive (and thus Müllerian in origin), while the lower 2/3rds of the vagina was lined with FOXA1-reactive epithelial cells (Figs. 13C-D and 15), thus suggesting a substantial contribution of UGE to developing vaginal epithelium as proposed previously (Bulmer, 1957). At 18 weeks of gestation the vaginal vault and the exocervix were lined by a stratified squamous epithelium (Fig. 10F), which was PAX2-reactive (Fig. 15).

Table 4
Epithelial differentiation markers in human fetal uterine cervix.

KRT6	KRT7	KRT8	KRT10	KRT14	KRT19	TP63	RUNX1	AR	ESR1	PR
-/+#	+	+	–	-/+*	+	+	+	–	-**	-##

Stratified epithelium was KRT6-reactive, while simple columnar epithelium in the (presumed) endocervix) was KRT6-negative.
* KRT14 reactivity was absent from the putative endocervix at 18 and 21 weeks, but was observed in areas of stratified squamous epithelium considered to be exocervix.
** Epithelial ESR1 was generally negative until reactivity appeared ≥ 21 weeks.
DES induced epithelial PGR- and ESR1-reactivity in xenografts. From (Cunha et al., 2017b).

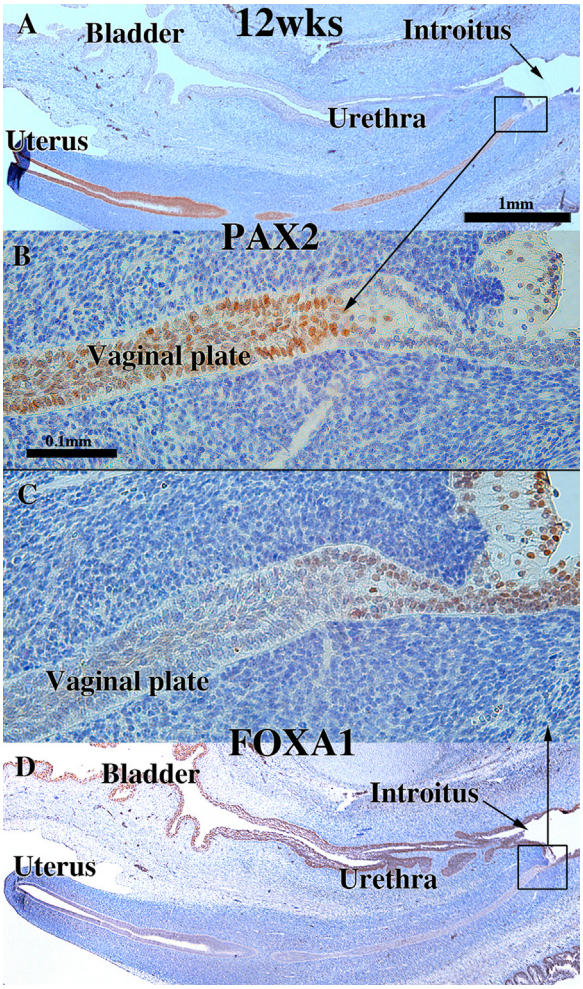

Fig. 11. Sagittal sections of a 12-week human female fetal reproductive tract immunostained with PAX2 (A-B) and FOXA1 (C-D). PAX2-reactive epithelial cells extend to near the junction with urethral epithelium (A-B). FOXA1-reactive epithelial cells extend only a short distance into the solid vaginal plate (C-D). Scale bar in A also refers to D. Scale bar in D also refers to C. From Robboy et al. (2017) with permission.

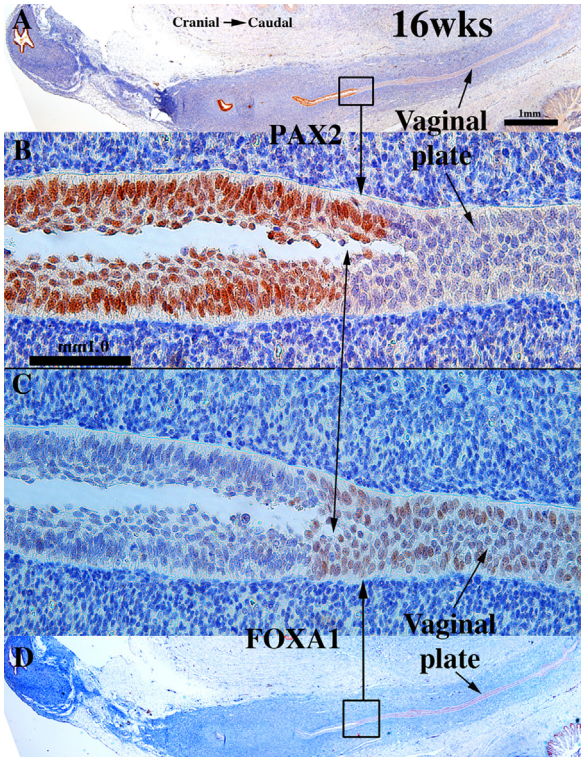

Fig. 12. Sagittal sections of a 16-week human female fetal reproductive tract immunostained with PAX2 (A-B) and FOXA1 (C-D). FOXA1-reactive epithelial cells form all of the solid vaginal plate (C-D). PAX2-reactive epithelial cells constitute a epithelium lining the lumen of the uterovaginal canal (A-B). Scale bar in A also refers to D. Scale bar in D also refers to C. From Robboy et al. (2017) with permission.

This is consistent with the idea that simple columnar Müllerian epithelium of the caudal portion of the uterovaginal canal can transform directly into stratified squamous epithelium independent of UGE as is the case during development of mouse vaginal epithelium (Kurita and Cunha, 2001).

At 21 weeks vaginal epithelium exhibited estrogenic stimulation throughout and was many cell layers thick, presumably due to elevated endogenous estrogen (Oakey, 1970). The vaginal fornices, which were evident at 18 weeks (Fig. 10F), were “filled in” (Fig. 14B) by epithelial proliferation. Epithelial FOXA1 staining was now observed from the introitus to the exocervix, while epithelial PAX2 immunostaining remained confined to the endocervix (Figs. 14C-D and 15) and the uterine body, suggesting the replacement of Müllerian epithelium by urogenital sinus epithelium in the vagina. The abrupt change in FOXA1/PAX2 staining occurred at the same point in adjacent sections (Fig. 14C, E, F). The dynamic changes in the distribution of PAX2-reactive Müllerian epithelium and FOXA1-reactive UGE is summarized in Fig. 15. These data support Bulmer's proposal that the entire vaginal epithelium derives from urogenital sinus epithelium (Bulmer, 1957). While our observations support displacement of Müllerian epithelium by UGE upgrowth, the molecular mechanistic scenario in human vaginal development requires further research. Also, since the oldest specimen analyzed in our study came from a 21-week fetus, further changes in the relative contribution MDE versus UGE to human vaginal epithelium may occur at later stages. How these changes in the expression pattern of these markers relate to DES-induced adenosis is discussed below.

Epithelial cells of the human fetal vagina express a variety of differentiation markers whose expression changes during development (Table 5). Keratin 6 was not detected in the simple columnar epithelial cells of the uterovaginal canal. However, KRT6 was prominently expressed in epithelium of the solid vaginal plate epithelium at 12 weeks of gestation and thereafter, a pattern consistent with its known expression in basal and suprabasal cells of stratified epithelia (Moll et al., 1982, 2008). Keratin 19 was expressed in simple columnar epithelium of the uterovaginal canal at 9 weeks, and at 12–16 weeks was expressed the simple columnar epithelia of the uterine corpus, the cervix and stratified epithelium immediately cranial to the solid vaginal plate, which itself was KRT19-negative (Fig. 10C-D) (Cunha et al., 2017b). At 18 and 21 weeks, KRT19 was detected in epithelium of the upper vagina, but not in epithelium of the lower vagina (Fig. 13E, arrowheads). Keratin 14 was first detected in patches within the vaginal plate at 14 weeks and more broadly in the vaginal plate at 16 weeks. At 21 weeks KRT14 was expressed in basal and supra-basal cells of stratified vaginal epithelium (Cunha et al., 2017b) (Table 5). ESR1 was first detected in isolated patches in epithelium of the vaginal plate at 14 weeks. At 16 weeks ESR1 was uniformly expressed within the solid vaginal plate, and at 21 weeks the thick estrogen-stimulated vaginal epithelium expressed ESR1 strongly in basal and suprabasal epithelial cells (Cunha et al., 2017b) (Table 5). Runx1 was detected the uterovaginal canal at 9 weeks, in the solid vaginal plate at 12 weeks and was maintained thereafter during differentiation of the vaginal epithelium (Cunha et al., 2017b). TP63 was initially detected in the solid vaginal plate at 12 weeks and was maintained thereafter during differentiation of the vaginal epithelium. At 21 weeks TP63 was uniformly expressed in basal

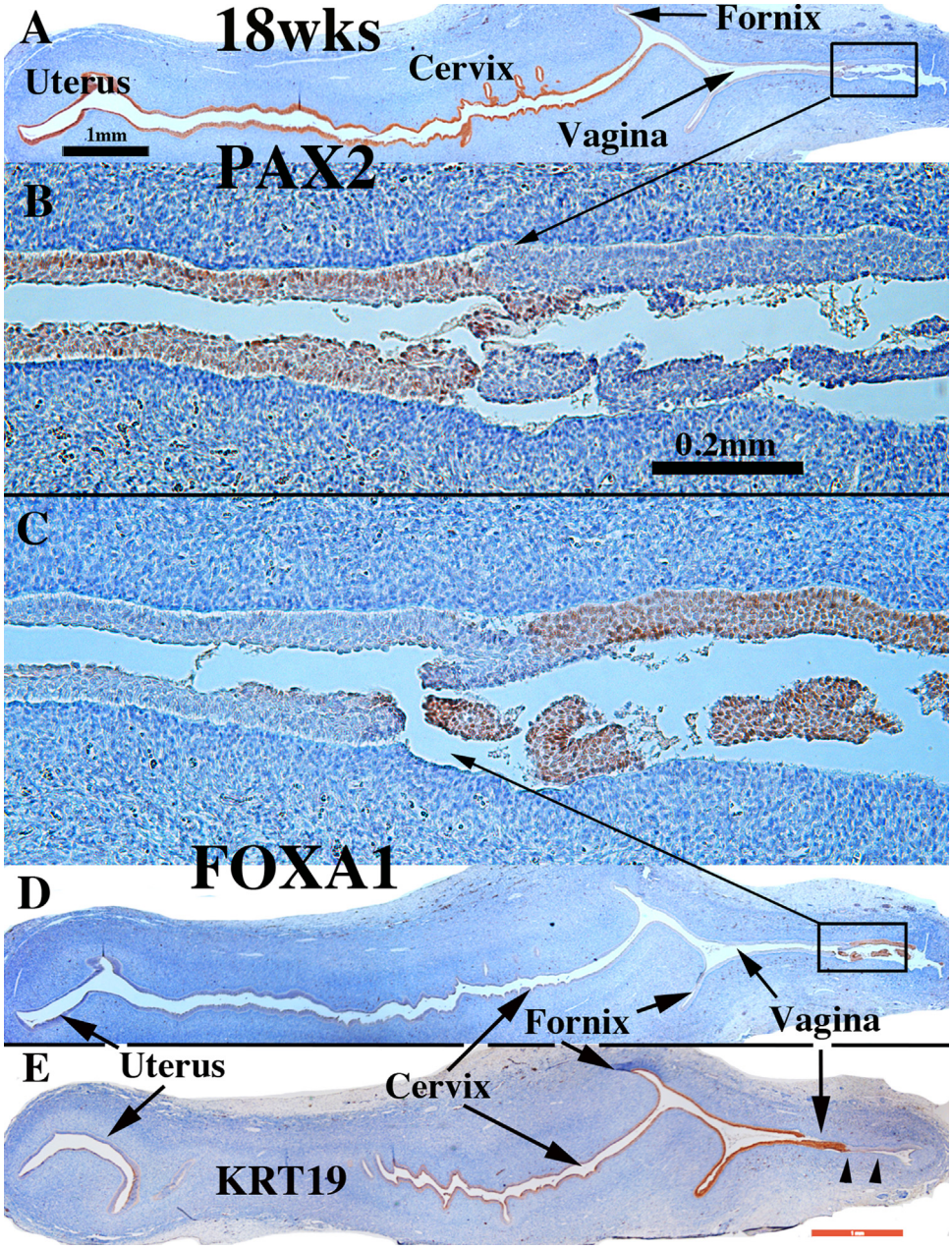

Fig. 13. Sagittal sections of an 18-week human female fetal reproductive tract immunostained with PAX2 (A-B) and FOXA1 (C-D). PAX2-reactive epithelial cells line the lumen of the female reproductive tract from the uterine tube to the cranial aspect of the vagina (A-B). PAX2 staining is present, but weak in vaginal epithelium (A-B). FOXA1-reactive epithelial cells line the lower (caudal) vagina (C-D). Note abrupt fall off in KRT19 staining in the lower vagina (arrowheads). Scale bar in A also refers to D. Scale bar in D also refers to C. (E) KRT19 mirrors PAX2 immunostaining. From Robboy et al. (2017) with permission.

cells of vaginal epithelium, and KRT10 was detected in the thick estrogen-stimulated vaginal epithelium for the first time at 21 weeks (Cunha et al., 2017b). The progesterone receptor was not detected in vaginal epithelium from 8 to 21 weeks, but was induced by DES in vaginal epithelium in xenografts (Cunha et al., 2017a) (Table 5). Thus, the pattern of epithelial marker expression is dynamic during human vaginal development, presumably reflecting changes in the MD/UGE distribution.

4.5. Xenograft studies

4.5.1. Xenografts of human fetal female reproductive tracts

Xenograft studies provide the unique opportunity to study human female reproductive tract development, estrogen-induced malformations and their molecular mechanisms. The underpinning of this approach to organogenesis/pathogenesis within the human derives from our studies published in 1982 in which human fetal female reproductive tracts were grown in athymic mouse hosts treated with DES and other hormonally active agents (Cunha et al., 1987b, 1987a; Robboy et al., 1982a; Taguchi et al., 1983). Recently, we have (a) revisited our xenograft model of human female fetal reproductive tract development, (b) provided new data to validate normal development in xenografts grown in untreated hosts and (c) explored the effects of DES exposure on morphogenesis, differentiation and molecular expression within human female fetal reproductive tracts (Cunha et al., 2017a, 2018).

The methodology begins with the abortus specimen and isolation of the fetal reproductive tract. For "young" specimens ($\leq$ 14 weeks), the entire human reproductive tract can be grafted under the renal capsule, which is the preferred graft site (Cunha and Baskin, 2016). Older

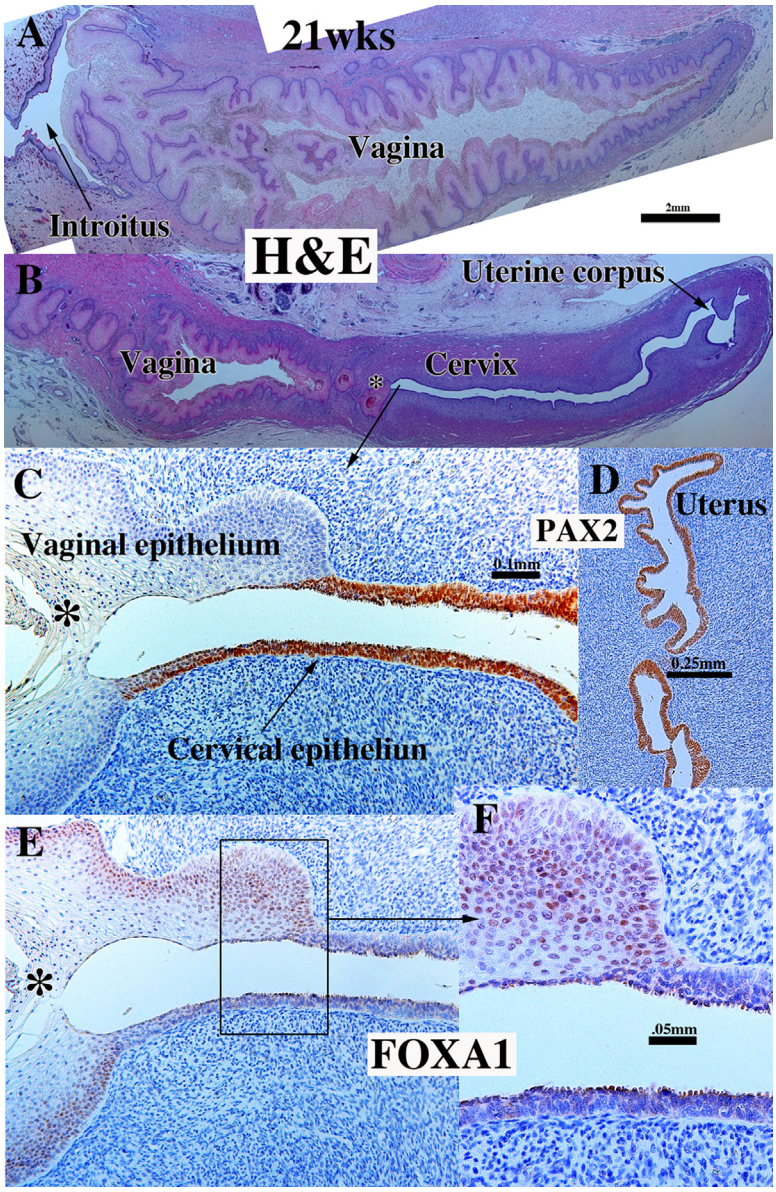

Fig. 14. Sagittal sections of a 21-week female reproductive tract. Section (A) depicts the lower portion of the vagina (introitus to upper vagina). Section (B) depicts the upper portion of the specimen (vagina, cervix and uterine corpus) (both (A & B) are H&E stain. The vaginal epithelium is many layers thick due to estrogenic stimulation (A-B). Sections (C, E & F) are adjacent sections. In (B, C & E) note the abrupt transition in epithelial differentiation at the vaginal/cervical border and the change in PAX2/FOXA1 staining (indicative of a MD/UGE boundary). The vaginal epithelium is PAX2-negative (C), while epithelium of the uterine corpus (D) is strongly PAX2-reactive. FOXA1 immunostaining was seen uniformly throughout the entire vagina, and FOXA1 immunostaining abruptly stopped at the vaginal/cervical border (E-F). Asterisks in (B, C and E) are at the vaginal exocervical boundary. From Robboy et al. (2017) with permission.

specimens can be split into right and left halves that in turn can be cut into cranial-caudal segments. This is particularly useful when contralateral control and Rx-treated groups are necessary. The endocrine status of the host mice can be precisely controlled via ovariectomy and exogenous hormone treatment. Xenografts can be grown for various periods, which in all cases should be > 1 week to avoid effects that may be due to the "healing in" process. We typically grow grafts for 2–4 weeks.

Based upon several published studies (Cunha et al., 1987b, 1987a, 2017a; Robboy et al., 1982a; Taguchi et al., 1983), human fetal female reproductive tracts grown in untreated hosts develop normally and express the normal complement of epithelial and mesenchymal differentiation markers (Cunha et al., 1987b, 1987a, 2017a; Robboy et al., 1982a; Taguchi et al., 1983). Our most recent study (Cunha et al., 2017a) has extended our previous work by exploring the effect that DES exerts on epithelial differentiation marker expression. Accordingly, xenografts were grown in ovariectomized mice that were either untreated or treated with a 20 mg subcutaneous DES pellet. DES stimulated endometrial and cervical gland formation (Fig. 16E) and increased plication (folding) of tubal epithelium (Fig. 16C).

DES also affected ESR1 expression in tubal and uterine epithelium. Control specimens (non-grafted human uterine tubes (Cunha et al., 2017b) and tubal grafts grown in untreated ovariectomized hosts) disclosed uniform epithelial ESR1-reactivity (Fig. 16A & B). Grafts of uterine tube treated with DES exhibited weak ESR1-reactivity at best with many epithelial cells being ESR1-negative (Fig. 16C). Uterine epithelium at 16 weeks and younger is ESR1-negative and such ESR1-negativity is maintained in xenografts to untreated ovariectomized hosts (Fig. 16B & D). DES treatment induced ESR1 in the uterine epithelium (Fig. 16E).

Use of xenografts confirmed our previous report (Robboy et al., 1982a) of DES-induced vaginal adenosis. Vaginal adenosis is the presence of simple columnar epithelium on the surface or as submucosal glands in the vagina (Robboy et al., 1982b). Adenosis was never observed in vaginal grafts grown in untreated hosts (Cunha et al., 1987b, 1987a, 2017a; Robboy et al., 1982a; Taguchi et al., 1983). In contrast, xenografts in one DES-treated specimen from a previous study (Robboy et al., 1982a) and in two specimens in our recent study (Cunha et al., 2017a) disclosed the presence of adenosis (Fig. 17). The histology of the adenosis was of the embryonic (immature) form, and not that seen as the more differentiated tuboendometrial or mucinous forms. While DES elicited formation of a thickened and glycogenated squamous

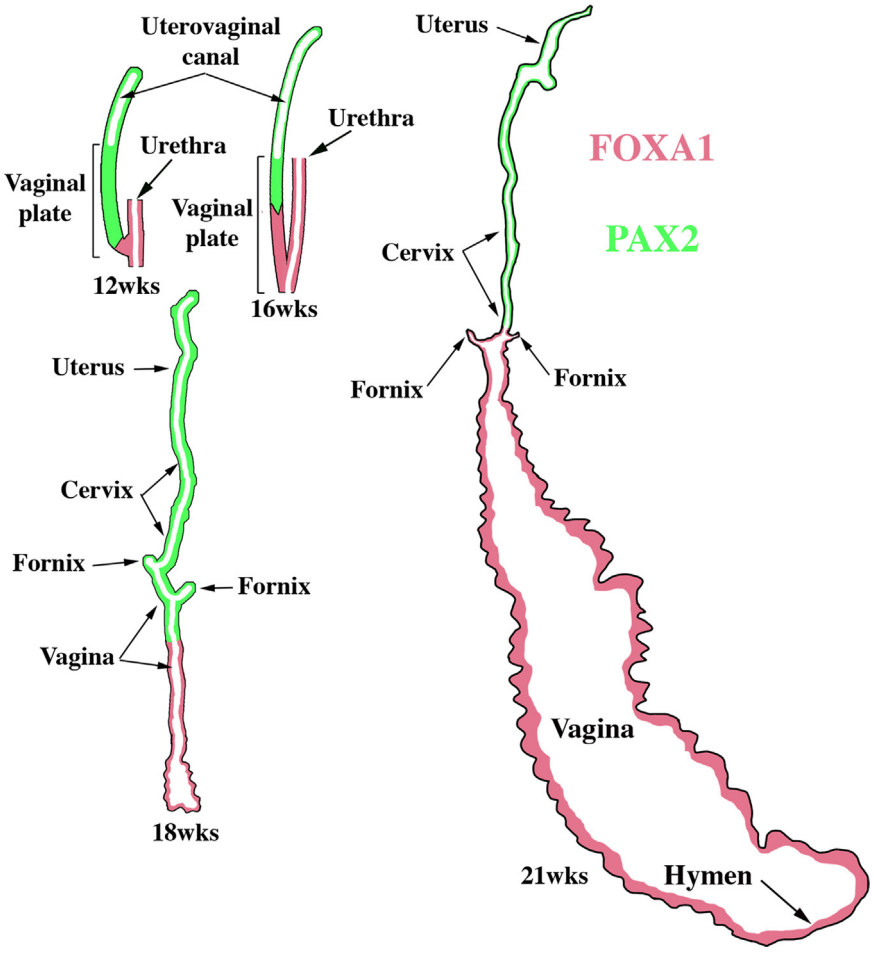

Fig. 15. Summary of the changing patterns of PAX2 epithelial expression (green) indicative of Müllerian epithelium and FOXA1 epithelial expression (red) indicative of endodermal urogenital sinus epithelium over the time frame of 12–21 weeks. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

epithelium as anticipated, simple columnar epithelium (adenosis) was also present, and both epithelial types strongly expressed ESR1 (Fig. 17A). PGR, not present at the time of grafting, was also expressed in both the adenotic and the mature vaginal epithelium in response to DES (Fig. 17B). The DES-stimulated hyperplastic vaginal epithelium expressed keratins 6 (not illustrated) and 14 (Fig. 17C), TP63 (Fig. 17D), and RUNX1 (Fig. 17F), while the adenotic glandular vaginal epithelium expressed keratins 7 (Fig. 17E) and 8 (not illustrated). DES-induced adenosis was PAX2-reactive and FOXA1-negative, whereas the mature stratified vaginal epithelium was PAX2-negative and FOXA1-reactive (Fig. 17G-H), confirming that DES-induced adenosis is occurring in residual Müllerian epithelium. Table 6 summarizes the differences in marker expression in the mature stratified squamous epithelium versus adenosis.

4.5.2. Xenografts of tissue recombinants composed of mouse vaginal mesenchyme and human fetal uterine tubal epithelium

Studies in mice have shown that differentiation of Müllerian epithelium is induced and specified by cues from the mesenchyme (Cunha, 1976; Kurita et al., 2001; Kurita, 2011). Accordingly, vaginal mesenchyme instructively induces uterine epithelium to undergo vaginal epithelial differentiation (VgM + UtE⇒vaginal differentiation), and uterine mesenchyme instructively induces vaginal epithelium to undergo uterine epithelial differentiation (UtM + VgE⇒uterine differentiation). To determine whether mesenchymal induction plays a role in human Müllerian epithelial differentiation, tissue recombinants were prepared with 3-day postnatal mouse vaginal mesenchyme and human uterine tubal epithelium (mVgM + hTubE) from 12- to 13-week fetuses and grown under the renal capsule of ovariectomized female athymic mice. The mouse hosts were treated with DES (via subcutaneous pellet) to promote stratified squamous vaginal differentiation. In response to DES grafts of human fetal vagina differentiate a thick glycogenated stratified epithelium (Fig. 17), while grafts of human fetal uterine tube remain simple columnar (Fig. 16C) (Cunha et al., 2017a).

Epithelial differentiation markers (androgen receptor [AR], KRT6, TP63 and RUNX1) differ considerably in the human tubal versus human vaginal epithelium (Table 7) (Cunha et al., 2017a). In mVgM + hTubE recombinants grown in DES-treated female athymic mouse hosts, the

Table 5
Epithelial differentiation markers in human fetal vagina.

KRT6	KRT7	KRT8	KRT10	KRT14	KRT19	TP63	RUNX1	AR	ESR1	PGR
+	–	–	+*	+	+	+	+	–	+	-,+#

*KRT10 was only detected in the 21-week specimen showing intense estrogenic stimulation.
Epithelial PGR is induced in xenografts by DES. From Cunha et al. (2017b).

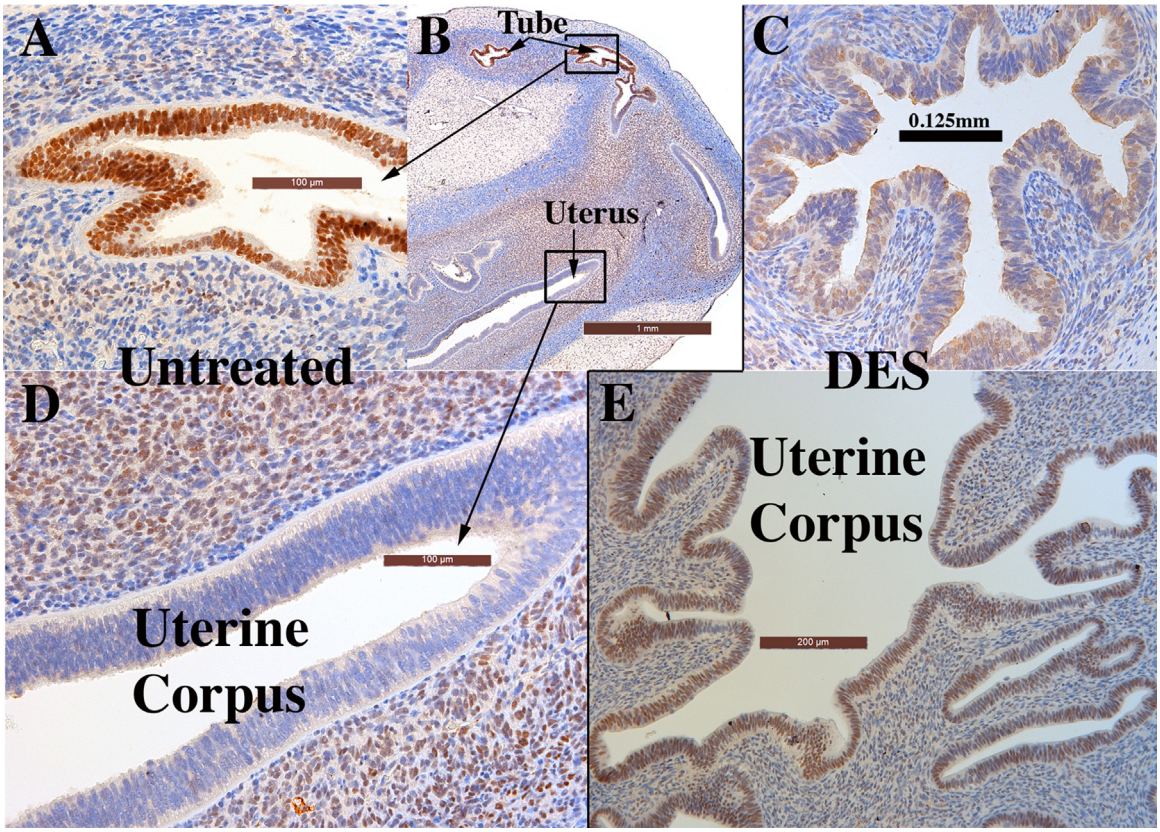

Fig. 16. Xenografts of twin 13-week human female reproductive tracts grown for 4 weeks in untreated ovariectomized (A, B & D) and ovariectomized DES-treated athymic mice (C & E). Note in (A-B) uniform strong ESR1 immunostaining of the tubal epithelium in the untreated ovariectomized host, and (C) the mixture of weakly ESR1-positive and ESR1-negative tubal epithelial cells in the DES-treated specimen. Sections (B & D) depict the uterine corpus of a xenograft grown in an untreated ovariectomized host. The epithelium of the uterine corpus is ESR1-negative, while the uterine mesenchyme is ESR1-reactive. Section (E) depicts the uterine corpus of the twin 13-week specimen grown in a DES-treated ovariectomized host. Note the strong uniform expression of ESR1 in the uterine epithelium. Note also the substantial DES-induced plication of the uterine tube (compare B & C), and the profound DES-induced uterine gland formation (compare (B & E).

mouse vaginal mesenchyme elicited a shift in epithelial histodifferentiation and differentiation marker expression from uterine tubal epithelial differentiation to vaginal epithelial differentiation. The shift in epithelial differentiation elicited by vaginal mesenchyme was incomplete as some tubal epithelial markers (KRT14 and KRT19) remained unchanged in mVgM + hTubE recombinants (Table 7), suggesting that mouse vaginal mesenchyme was able to elicit only a subset of vaginal differentiation markers in the tubal epithelium (Cunha et al., 2018). This partial shift in epithelial differentiation may be due to the age of the epithelium Fig. 18.

4.5.3. Use of tissue recombinants to explore direct versus paracrine regulation of ESR1 and PGR in human fetal uterine epithelium

ESR1 is first expressed in mesenchymal cells of the human uterine corpus at about 14 weeks, but rarely in uterine epithelium before the 21st gestational week, when the endogenous serum estrogen levels are elevated (Oakey, 1970). This suggests that uterine epithelial ESR1 may be estrogen induced, an idea confirmed in human fetal uterine xenografts in which epithelial ESR1 was broadly induced by DES (Fig. 16) (Cunha et al., 2018). PGR is undetectable in human fetal reproductive tracts from 9 to 21 weeks, but is also induced throughout the human fetal reproductive tracts in DES-treated xenografts (Cunha et al., 2018). Thus, there are two potential mechanisms of DES induction of uterine epithelial ESR1 and PGR: (a) DES may induce uterine epithelial ESR1 and PGR directly via epithelial ESR1 whose expression is below the sensitivity of immunohistochemistry. (b) Alternatively, DES may induce uterine epithelial ESR1 and PGR indirectly via mesenchymal ESR1 (paracrine mechanism). To distinguish between these two scenarios, we prepared tissue recombinants composed of epithelium of the human corpus (hUtE) and uterine mesenchyme from either *Esr1*-positive wild-type mice or *Esr1*-negative *Esr1*KO mice (wt UtM + hUtE and *Esr1*KO UtM + hUtE) (Cunha et al., 2018). All tissue recombinants were grown for 4 weeks in ovariectomized hosts treated with a subcutaneous 20 mg DES pellet. As expected, wt UtM + hUtE tissue recombinants consistently (6/6) contained an ESR1- and PGR-reactive human uterine epithelium (Fig. 19A & B). *Esr1*KO UtM + hUtE tissue recombinants also (5/5) contained an ESR1- and PGR-reactive human uterine epithelium even though the surrounding stromal cells were ESR1-negative (Fig. 19C & D) (Cunha et al., 2018), thus confirming that DES acts directly on human uterine epithelium.

5. Discussion

The tacit, but usually unproven, assumption inherent in animal models is that they reflect the underlying morphogenetic and molecular mechanisms of human biology. This approach is generally useful, even though substantial differences exist between human and animal anatomy, development and pathology. Accordingly, it is critical to understand both the similarities and differences between human and laboratory animal development. This, of course, necessitates a modern approach to human development in which the full technical palate is utilized. Sadly, most of our knowledge of human female reproductive tract development rests upon ancient studies based primarily upon H&E stained sections. This paper on human female reproductive tract development chronicles recent studies that shed new light on this topic and provide practical information facilitating future exploration.

The early papers on development of the human female reproductive tract by Koff in 1933 and Bulmer in 1957 (Koff, 1933; Bulmer, 1957)

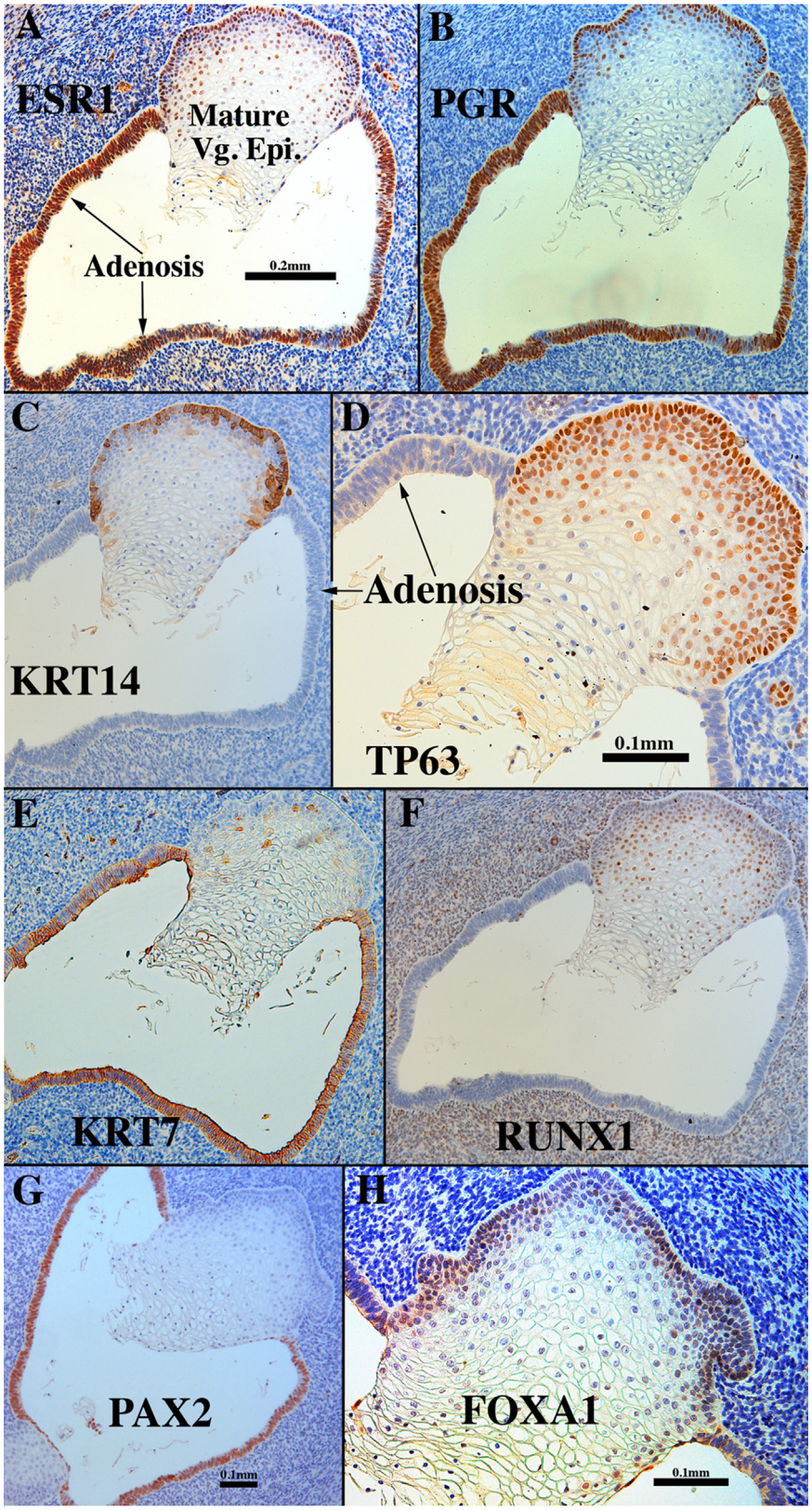

Fig. 17. Sections of a 13-week vaginal specimen grown for 1 month in a DES-treated host via a 20 mg subcutaneous pellet and immunostained as indicated. Mature stratified squamous vaginal epithelium and simple columnar adenotic epithelium exhibit remarkably different marker profiles (see Table 6).

have been augmented by immunohistochemical studies of Fritsch et al. covering the period of 8–34 weeks of gestation (Fritsch et al., 2012, 2013) and our studies covering the period of 9–21 weeks (Robboy et al., 2017; Cunha et al., 2017b). Taken together, these immunohistochemical studies show that several differentiation markers are present throughout this entire period, while others have a unique temporal expression profile. Note the substantial concurrence between our and Fritsch's studies (Table 8) (Fritsch et al., 2012, 2013; Robboy

Table 6
Differences in differentiation marker expression in mature stratified squamous vaginal epithelium versus adenosis in xenografts grown in ovariectomized DES-treated hosts.

Differentiation markers	Mature stratified squamous epithelium	Adenosis
ESR1	Present	Present
PGR	Present	Present
KRT14	Present	Absent
TP63	Present	Absent
KRT7	Absent	Present
RUNX1	Present	Absent
PAX1	Absent	Present
FOXA1	Present	Absent

From (Cunha et al., 2017a).

Table 7
Epithelial differentiation of human uterine tube, human vagina and mVgM + hTubE tissue recombinants.

Feature	Uterine tube	Vagina	mVgM + hTubE
Simple columnar epithelium	Yes	No	No
Stratified squamous epithelium	No	Yes	Yes
KRT6	No	Yes	Yes
TP63	No	Yes	Yes
Androgen receptor	Yes	No	No
RUNX1	No	Yes	Yes
KRT14	No	Yes	No
KRT19	Yes	No	Yes

From Cunha et al. (2018).

et al., 2017; Cunha et al., 2017b).

The derivation of human vaginal epithelium has been debated for decades with Koff (1933) asserting that vaginal epithelium is mostly derived from Müllerian epithelium with a minor contribution ascribed to UGE, while Bulmer (1957) concluded the opposite, namely that virtually all of the human vaginal epithelial lining derives from UGS. Reich and Fritsch (2014) assert that squamous epithelia of the exocervix and the upper vagina are of Müllerian origin, thus supporting Koff's interpretation. Unfortunately, these previous studies lacked markers indicative of both Müllerian and urogenital sinus epithelia. Our work, using PAX2 (a Müllerian epithelial marker) and FOXA1 (an endodermal UGE marker), supports Bulmer's interpretation by showing that UGE of the vaginal plate grows cranially to completely replace the Müllerian epithelium to the level of the cervical os. The replacement of Müllerian epithelium by UGE appears to be perturbed by exposure to DES, which generates PAX2-positive adenotic epithelium in the vagina, an epithelium that appears to be retained columnar Müllerian epithelium. Our studies, showing that the lining epithelium of the human vagina is of urogenital sinus origin, nonetheless emphasize the need to further decipher this complicated process.

The human vaginal plate is a fascinating dynamic structure not having a mouse counterpart. The human vaginal plate first appears at about 11 weeks of gestation through occlusion of the Müllerian-derived uterovaginal canal, originally lined with a simple columnar epithelium. At this age, the vaginal is plate is almost entirely PAX2 reactive, indicative of its Müllerian origin. As gestation progresses during the next 4 weeks, the vaginal plate lengthens, and an increasing percentage of the vaginal plate shows reactivity with FOXA2, indicative of UGS origin. Ultimately, the vaginal plate “disappears” due to its canalization. Concurrently, the cranial PAX2 (Müllerian) reactive area shrinks in extent. What triggers this change remains unknown. One potential explanation is differential growth of Müllerian versus urogenital sinus epithelia, with UGE out-competing Müllerian epithelium. Another possibility may involve differential adhesion of basal epithelial cells of Müllerian versus urogenital sinus origin to the epithelial basement membrane mediated via hemidesmosomes (Cunha et al., 1978).

The vaginal plate was initially considered to be uniform throughout, due to its homogeneous microscopic appearance in H&E histology. Clearly the squamous epithelial cells of the vaginal plate (and its derivative, vaginal epithelium) are not uniform, based upon immunohistochemical observations of keratins 7, 14 and 19, RUNX1 and ESR1 (Cunha et al., 2017b). Also, the vaginal plate is described as being a solid structure throughout. Yet it in some areas it appears to have a central lumen and with solid lateral “wings” (Koff, 1933; Robboy et al., 2017). Given the difficulty of obtaining human specimens, all of our specimens were sectioned sagittally or transversely. A series of coronal sections would be most helpful. Three-dimensional graphics allowing for specimen rotation would also be useful. Indeed, modern imaging techniques such as optical projection tomography, thick section confocal microscopy or light-sheet microscopy™ can be used to more fully understand development of the human vaginal plate.

The undifferentiated simple columnar Müllerian epithelium lining the embryonic uterovaginal canal differentiates and matures in the adult as one of three basic forms: a tuboendometrial form with various degrees of ciliation in the uterine (Fallopian) tube, endometrium, and as adenosis in the vagina. The second basic form, depicted by mucinous columnar epithelial cells, typifies the endocervical epithelium, which in the adult when found on the exocervical is called “ectropion” and when extending into the vagina is called adenosis. Finally, in the vagina and exocervix a stratified squamous epithelium forms. These various forms of epithelial differentiation may be induced and specified by mesenchymal-epithelial interactions in both normal and abnormal development of the human female reproductive tract.

Mesenchymal-epithelial interactions are known to play a central role in differentiation of the murine female reproductive tract. Mouse vaginal mesenchyme is capable of inducing certain aspects of vaginal differentiation in human fetal uterine tube epithelium. The cellular target of mesenchymal induction within the epithelium may be relatively rare epithelial stem cells, or may be a large subset of the fetal epithelium. It is perhaps worth noting that organs of the female reproductive tract (especially the uterus) undergo dramatic morphogenetic and growth changes during reproductive cycles due in part to the presence of stem cells (Maruyama, 2015).

Mesenchymal-epithelial interactions between rodent and human tissues are so highly conserved that rodent mesenchymal inducers can reprogram differentiation of human fetal and adult epithelia (Aboseif et al., 1999; Cunha et al., 1983). This approach can be extended by combining mutant mouse mesenchyme plus human fetal epithelium or human mesenchyme plus mutant mouse epithelium in experiments designed to explore molecular mechanisms of human organogenesis as illustrated in our *Esr1*KO UtM + hUtE studies. This approach was used to verify that estrogenic up-regulation of both ESR1 and PGR in the human fetal uterine epithelium is mediated directly via epithelial ESR1 (Cunha et al., 2018).

An important finding from our prior work is that differentiation of myometrial smooth muscle from uterine mesenchyme in mice depends upon a signal(s) from uterine epithelium (Cunha et al., 1992). Likewise, urinary bladder epithelium induces detrusor smooth muscle differentiation (Baskin et al., 1996, 2001). In the bladder, this effect of epithelium (smooth muscle induction) appears to be non-specific as a range of epithelia can induce smooth muscle differentiation in bladder mesenchyme (DiSandro et al., 1998), and in the bladder this effect appears to be mediated by sonic hedgehog (Cao et al., 2010). Whether similar developmental mechanisms of myometrial differentiation are at play in differentiation of human myometrium could be examined in tissue recombination studies.

Another fascinating area to explore is why and where does the clear cell adenocarcinoma develop in DES daughters. One thought proffered is that DES acts as a carcinogen, directly affecting the epithelium to transform into a cancer (Saeed et al., 2009). Alternatively, DES may act more like a teratogen via the developing vaginal stroma (Cunha et al., 1977; Kurita, 2011; Robboy, 1983). The process may involve inhibition

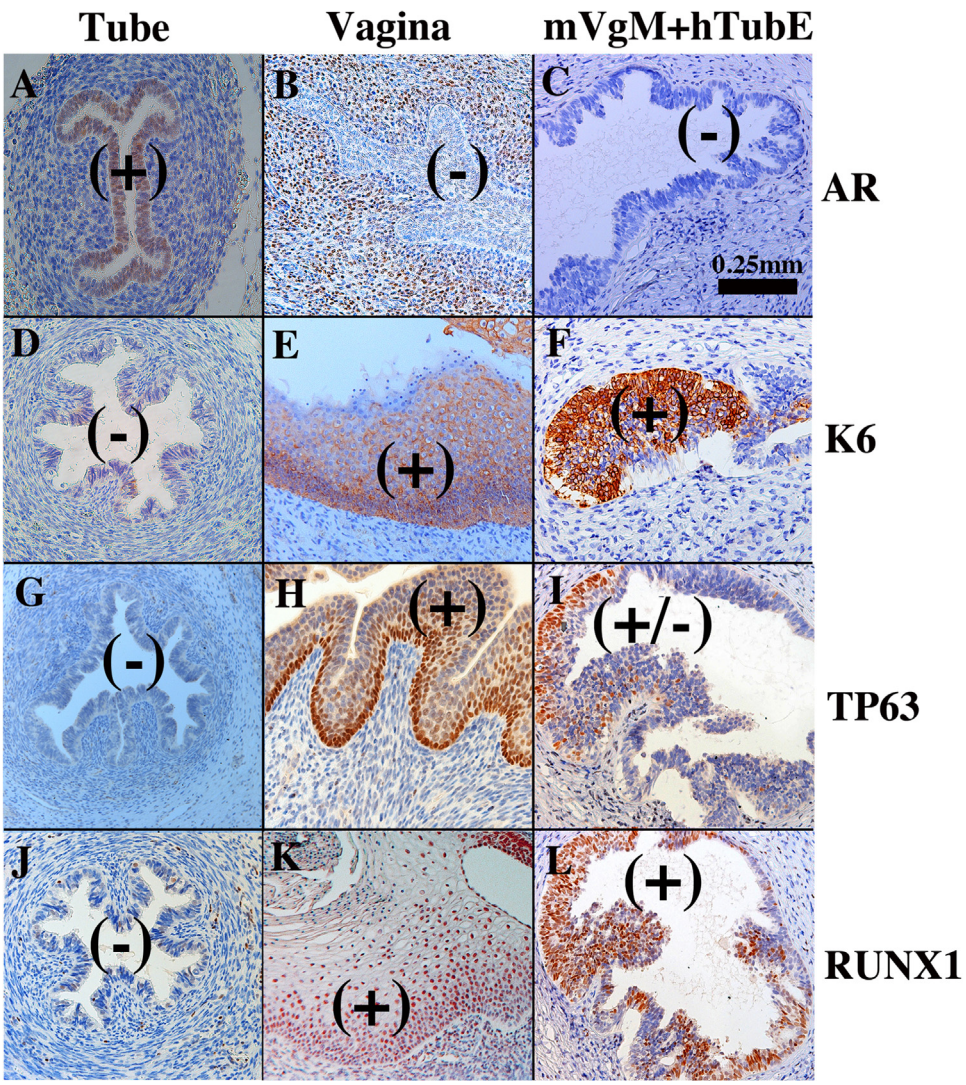

Fig. 18. Tissue recombinants composed of neonatal mouse vaginal mesenchyme plus 13-week human fetal uterine tube epithelium (mVgM + hTubE) grown for 4 weeks in DES-treated hosts and immunostained for various vaginal epithelial markers as indicated. Human uterine tube (A, D, G, J) and vagina (B, E, H, K) at 16–18 weeks of gestation serve as controls. Note induction of KRT6, TP63 and RUNX1 and down regulation of AR in epithelium of the mVgM + hTubE recombinants, indicative of an effect of mouse vaginal mesenchyme on expression of differentiation markers in human tubal epithelium. (+) and (-) indicate epithelial marker expression. From Cunha et al. (2018) with permission.

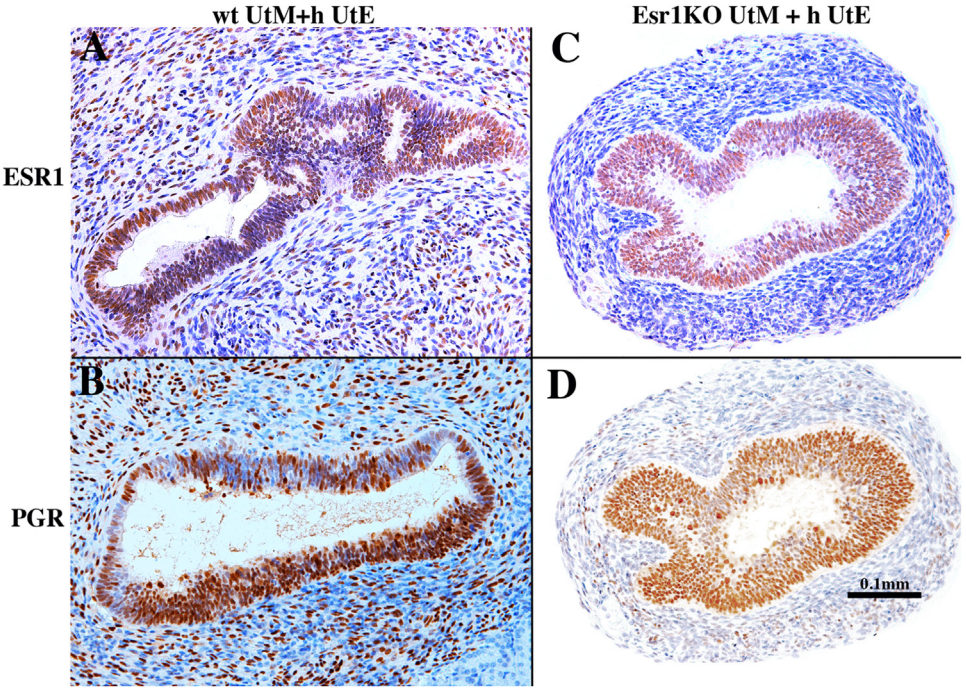

Fig. 19. Tissue recombinants composed of neonatal mouse wild-type uterine mesenchyme plus human fetal uterine tube epithelium (wt UtM + hUtE) (A &B) and αERKO uterine mesenchyme plus human uterine tube epithelium *Esr1*KO UtM + h UtE) (C & D) grown in DES-treated hosts and immunostained for ESR1 (A & C) and PGR (B & D). DES induced ESR1 and PGR even when the mesenchyme was genetically devoid of ESR1. Sections (C) and (D) are adjacent sections stained for ESR1 (C) and PGR (D). From Cunha et al. (2018) with permission.

Table 8
Proteins detected by immunohistochemistry in epithelia*** of the human fetal female reproductive tract.

Protein	Fritsch*	Cunha group**	Other groups
E-cadherin	YES	ND	
Vimentin	YES	ND	
TP63	YES	YES	
Laminin	YES	ND	
Ki67	YES	YES	
KRT19	YES	YES	
KRT17	YES	ND	
KRT14	YES	YES	
KRT13	YES	ND	
KRT10	ND	YES	
KRT8	YES	YES	
KRT7	ND	YES	
KRT6	ND	YES	
PAX2	YES	YES	
BCL-2	YES	ND	
FOXA1	ND	YES	
HOXA13	YES	ND	
BMP4	YES	ND	
RUNX1	ND	YES	
ESR1	ND	YES	YES#
PGR	ND	NO	
AR	ND	YES	
ACTA2	YES	YES	
HOXA11	ND	YES	
ISL1	ND	YES	
Uroplakin	ND	YES	

***See text and references for detail, tissue expression and temporal changes. ND = not done.
* (Fritsch et al., 2012, 2013).
** (Robboy et al., 2017; Cunha et al., 2017b).
# (Glatstein and Yeh, 1995).

of upgrowth of the UGE to replace the native columnar Müllerian epithelium. The resultant retention of simple columnar Müllerian epithelium in the vagina is called adenosis. Of import, cancers in patients consistently occur at a specific location, namely the junction where metaplastic squamous epithelium (the “healing” manifestation of mucinous adenotic epithelium) abuts glycogenated squamous epithelium (Robboy et al., 1982b), presumably representing UGE. This hypothesis can be assessed with PAX2 and FOXA2 immunohistochemistry, if tumor specimens are available that were serially blocked when removed and locations noted. Our earlier work showed that all clear cell adenocarcinomas arise in beds of tuboendometrial epithelium, and where at least a single focus of atypical tuboendometrial epithelium was present, suggesting a transition from a teratogenic origin of tuboendometrial adenosis, through atypicality, to eventual cancer (Robboy et al., 1984b).

The main findings of our studies are listed below:

1. PAX2 and FOXa1 immunostaining support Bulmer's proposal that human vaginal epithelium derives solely from urogenital sinus epithelium.
2. That portion of Müllerian ducts and uterovaginal canal lined by simple columnar epithelium (endocervix, uterine corpus and uterine tube) expresses keratins 7, 8 and 19.
3. That portion of the female reproductive tract undergoing stratified squamous differentiation (exocervix and vagina) expresses keratins 6, 14 and 10 in an age-dependent fashion.
4. TP63 and RUNX1 are expressed in vaginal epithelium prior to keratin 14, as these two transcription factors are known to be upstream from keratin 14.
5. HOXA11 is expressed in uterine mesenchyme and ISL1 is expressed in vaginal mesenchyme.
6. The ontogeny of estrogen receptor alpha (ESR1), progesterone receptor and the androgen receptor provides the mechanistic underpinning for the teratogenicity of estrogens, progestins and androgens on female reproductive tract development.
7. Normal morphogenesis and normal patterns of differentiation marker expression occur in xenografts of human female reproductive tracts grown in untreated hosts.
8. DES treatment of human female reproductive tract xenografts: (a) stimulated endometrial/cervical gland formation, (b) increased plication (folding) of tubal epithelium, (c) elicited stratified squamous maturation of vaginal epithelium and (d) elicited vaginal adenosis.
9. DES treatment of xenografts also induced ESR1 in epithelia of the uterine corpus, cervix and globally induced PGR in most cells of the developing human female reproductive tract.
10. DES treatment of xenografts induced vaginal adenosis and was associated with altered expression of epithelial marker proteins.
11. DES perturbed smooth muscle patterning in xenografts of the uterine tube.
12. Mouse vaginal mesenchyme can induce human tubal epithelium to undergo partial conversion to a vaginal phenotype, thus demonstrating the importance of mesenchymal-epithelial interactions in development of the human female reproductive tract.
13. DES-induction of uterine epithelial progesterone receptor (PGR) and estrogen receptor 1 (ESR1) is mediated directly via epithelial ESR1 and not via a paracrine mechanism.

Investigation of human female reproductive tract development can be extended through application of the full palate of modern biological techniques including modern imaging techniques, xenografting, single cell RNA-seq, tissue recombinant and crispr-cas9 technologies. Many carcinogenetic processes recapitulate to some extent developmental processes, and thus modern studies of human organogenesis and epithelial differentiation may provide important insights into human pathogenesis.

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