Spring 2024 – Systems Biology of Reproduction
Discussion Outline – Male Reproductive Tract Development & Function
Michael K. Skinner – Biol 475/575
CUE 418, 10:35-11:50 am, Tuesday & Thursday
February 1, 2024
Week 4

Reproduction Tract Development & Function

Primary Papers:

1. Murashima, et al. (2015) Asian J Andrology 17:749-755
2. Zhao, et al. (2017) Science 357:717-720
3. Sakib, et al. (2020) Andrology 8(4):835-841
4. Haider, et al. (2023) Hum Reprod. 38(8):1449-1463.

Discussion

Student 7: Classic Reference #1 above

- What are the developmental steps of the Wolffian/epididymal duct?
- What are the Phenotypes of knockouts that explain the development?
- What technology was used?

Student 8: Reference #2 above

- What is the technology used?
- Where is the expression pattern of the COUP-TF11?
- What does the knockout phenotypes show on regional actions of COUP-TF11?

Student 9: Reference #3 and #4 above

- What is the technology used and how different?
- What organoid cell structures were observed?
- What basic information on male reproductive tract development was obtained?

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INVITED REVIEW

Sperm Biology

Understanding normal and abnormal development of the Wolffian/epididymal duct by using transgenic mice

Aki Murashima[1], Bingfang Xu[2], Barry T Hinton[2]

The development of the Wolffian/epididymal duct is crucial for proper function and, therefore, male fertility. The development of the epididymis is complex; the initial stages form as a transient embryonic kidney; then the mesonephros is formed, which in turn undergoes extensive morphogenesis under the influence of androgens and growth factors. Thus, understanding of its full development requires a wide and multidisciplinary view. This review focuses on mouse models that display abnormalities of the Wolffian duct and mesonephric development, the importance of these mouse models toward understanding male reproductive tract development, and how these models contribute to our understanding of clinical abnormalities in humans such as congenital anomalies of the kidney and urinary tract (CAKUT).

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Keywords: epididymis; mesonephros; transgenic mice; Wolffian duct

INTRODUCTION

Understanding the mechanisms that regulate the development of the Wolffian duct (WD) is important because disruption of epididymal function may arise as a consequence of its abnormal development. Very little is known of either the process of WD development or the nature and causes of congenital defects that lead to male infertility. For example, it is clear that an undeveloped initial segment of the epididymis leads to male infertility[1,2] and considering that the human epididymis has an initial segment-like epithelium,[3] it is important to at least understand the development of this region. There are three developmental processes that are considered to be important during the development of the WD: (1) mesonephros formation, (2) stabilization of the ductal system and further growth, (3) postnatal differentiation (**Figure 1**). Each process is dependent upon developmental factors as shown by WD phenotypic mice carrying mutations of each factor.

This review focuses on mouse models that display abnormalities in WD or mesonephric development, the importance of these mouse models toward understanding male reproductive tract development, and how these models contribute to understanding clinical abnormalities in humans. **Table 1** shows mutations of genes in mice that display Wolffian/epididymal duct phenotypes.

DEVELOPMENT OF WOLFFIAN/EPIDIDYMAL DUCT AND MOUSE MODELS

Mesonephros formation

During development, the nephric duct/Wolffian duct (WD) arises from the anterior, intermediate mesoderm, and extends caudally.[4] In the case of mouse, WD formation begins approximately on embryonic day (E) 8.5 and is completed by reaching the cloaca at E9.5[5] (**Figure 1a** and **1b**). As the WD elongates, it induces the formation of nephric tubules through a mesenchymal-epithelial transition process. The tubules form three kidney primordia: pronephros, mesonephros and metanephros[6] (**Figure 1c**). The pronephros and mesonephros are transient kidneys and degenerate soon after their formation. However, in the mesonephros, the WD and cranial mesonephric tubules (MT) are retained and give rise to the male reproductive tract including the epididymis and efferent ducts, respectively.

Because WD formation is crucial for kidney development in mammals, many mouse models that show abnormal WD or mesonephric development also display urogenital abnormalities. The paired domain transcription factors Pax2 and Pax8 are well-known inducers of the initial formation of the WD.[7,8] The LIM-class homeobox gene *Lim1* is required for the extension of the WD.[9,10] Mice carrying a null mutation of *Emx2*, a mouse homologue of the *Drosophila* head gap gene *empty spiracles* (*ems*), display normal WD development until E10.5, but at later time points the duct degenerates, resulting in lack of a kidney and a failure of the reproductive tract to develop.[11] Mice carrying a null mutation of *Gata3*, which is a transcriptional target of Pax2 and Pax8, also show defects in WD initiation.[12]

Growth factors can differentially regulate gene expression especially through epithelial-mesenchymal interactions. Fibroblast growth factor (FGF) signaling is one of the well analyzed growth factor signaling events during mesonephric formation. *Fgf8* encodes an FGF ligand, which is expressed in the intermediate mesoderm, and lack of its expression results in the absence of the cranial mesonephros and MTs.[13]

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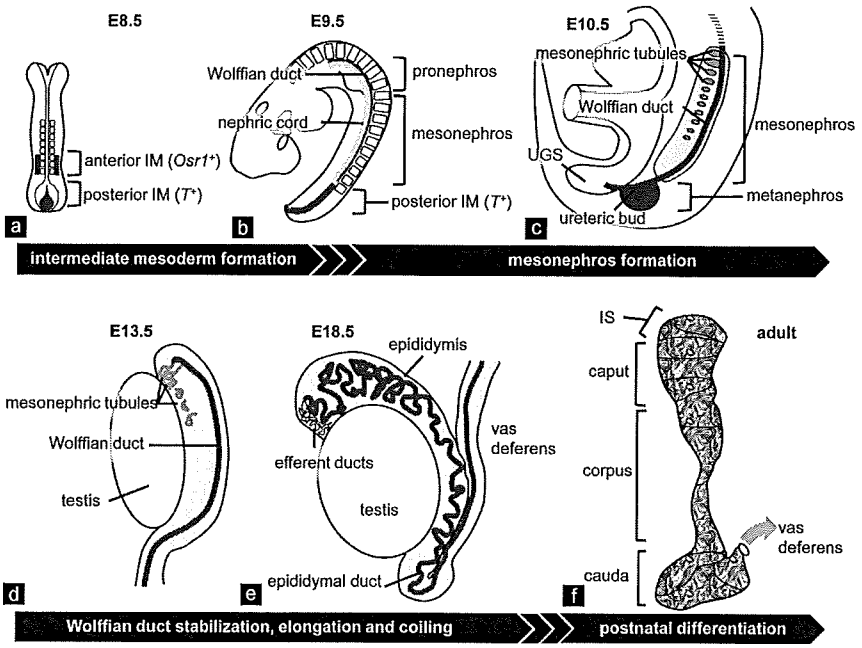

Figure 1: Schematic diagram of mouse Wolffian/epididymal duct development. (**a–c**) The origin of the epididymis is the intermediate mesoderm. Spatiotemporally distinct intermediate mesoderm at E8.5 gives rise to the WD and metanephric mesenchyme.[37] The anterior intermediate mesoderm, which gives rise to the pronephros and the whole WD, is composed of *Osr1*-positive cells at E8.5. The posterior intermediate mesoderm, which gives rise to the metanephric mesenchyme, is positive for *T* at E9.5. The posterior intermediate mesoderm may correspond to axial progenitor cells, which serve as the source of the caudal body trunk.[96,97] The WD begins to form from the anterior intermediate mesoderm at E8.5 and grows posteriorly reaching the urogenital sinus at E9.5.[98] Meanwhile, the pronephros regresses through apoptosis.[99] The WD induces the formation of mesonephric tubules from the mesenchyme (nephric cord) adjacent to the WD in a cranio-caudal manner. At the caudal end of the WD, the metanephros is initiated by ureteric bud formation through the interaction between WD epithelia and the metanephric mesenchyme at E10.5. (**d**) After gonadal sexual differentiation begins, the WD in the female embryo regresses from cranial to caudal while the WD in the male embryo is stabilized. The cranial set of mesonephric tubules connected to the WD is stabilized while the caudal set of mesonephric tubules regresses *via* apoptosis. (**e**) In the male embryo, the stabilized WD begins to coil from the cranial portion at E15.5. The duct continues to elongate and coil throughout development. (**f**) Ductal elongation and coiling continue after birth. The single-layered ductal epithelia undergo differentiation between P15 and P44. At the same time, the regions of the epididymis, initial segment, caput, corpus and cauda, become morphologically distinct. Sperm transport through the duct begins at approximately P35.[59,68] IM: intermediate mesoderm; UGS: urogenital sinus; IS: initial segment.

FGF ligands bind and activate alternatively-spliced forms of four tyrosine kinase FGF receptors (FGFRs 1–4).[14] During mesonephric development, *Fgfr1* is expressed in the mesenchyme while *Fgfr2* is in the epithelium, maintaining the WD and mesonephric mesenchyme.[15] The function of FGFR2 in the WD epithelia is suggested to maintain the caudal part of the WD in the mesonephros by regulating cell proliferation.[16]

Wnt genes encode a family of secreted glycolipoproteins regulating multiple processes during development, including cell proliferation and cell polarity. Among the *Wnt* genes, *Wnt9b* is mainly expressed in the WD epithelium while *Wnt7b* is faintly expressed from E9.5 onward. In animals devoid of *Wnt9b* their MTs are absent, and the epididymis is lacking at birth despite the normal formation of the WD at E10.5.[17] β-catenin-dependent canonical WNT signaling, which mainly regulates cell proliferation and differentiation, is sufficient to rescue MT induction in *Wnt9b* null mice. On the other hand, during metanephric kidney development, attenuation of *Wnt9b* affects the planar cell polarity of the epithelium and lead to tubules with an increased diameter.[18] Further spatiotemporal analyses of epididymal development in this mutant would contribute to our understanding of this molecule in tubulogenesis and its maintenance.

The number of MTs differs between species, and their function as a secretory organ is observed in pigs and humans but not in mice.[19–21] The number of efferent ducts reaching the testis also differs between species.[22,23] It is unclear whether there is a correlation between early MT number and the final number of efferent ducts observed in the adult. MT formation may resemble the formation of the renal nephron; both have the characteristic 'J' or 'S' shape during early development. The nephric tubule is formed through a mesenchymal-to-epithelial transition, and this cellular process is shared between mesonephric and metanephric tubules. *Pax2/8*, *Emx2* and *Lim1* are expressed in the condensed nephric cord and are required for tubulogenesis in addition to WD development.[7–11,24] The Wilms' tumor suppressor gene *Wt-1* and the homeobox gene *Six1* are also expressed in the nephrogenic mesenchymal condensation throughout the nephrogenic cord. Mice lacking *Wt-1* or *Six1* lack caudal MTs while cranial MTs are intact. These observations indicate that the regulation of the cranial and caudal set of MTs is distinct.[25–27] Conversely, lack of the forkhead transcription factors *Foxc1* and *Foxc2*, as well as *Sonic hedgehog* (*Shh*) expressed in the notochord or floor plate, results in supernumerary MT formation, suggesting suppressive effects of these genes on MT formation.[28,29] It is important to uncover how the differential regulation of tubule formation and stabilization along the anterior-posterior axis of the nephrogenic cord is established.

The connection between the rete testis and efferent ducts is observed at E13.5, and testicular fluid transport is detected at the corresponding stage of the rat embryo.[30] The patterning of efferent duct formation is intriguing, but the manner by which they reach the testis is not clear. There are at least two hypotheses on how the efferent ducts could be formed: (1) that a subset of MTs branch and fuse with each other forming the characteristic network of ductules, (2) that branching morphogenesis does not occur and the characteristic

Table 1: Mouse models which show defects in WD/epididymal duct development

Gene	*Type of mutation, Cre driver*	*Phenotype of the mutant*	*References*
Defect in mesonephros formation			
Pax2	KO	Dysgenesis of WD and MD, absence of MT	7
Pax8	KO	Normal	24
Pax2/Pax8	dKO	Dysgenesis of WD and MD, absence of MT	8
Lim1	KO	Dysgenesis of WD	10
	Pax2-Cre	Defect in caudal WD extension	9
Gata3	KO	Dysgenesis of WD and MD, absence of MT	12
Wt-1	KO	Absence of caudal MT	26
Six1	KO	Absence of caudal MT	27
Osr1	KO	Defect in WD extension, absence of MT	100
Emx2	KO	Regression of whole WD	11
Wnt9b	KO	Absence of MT, absence of epididymis	17
Fgf8	*T-Cre*	Regression of cranial mesonephros	13
Fgfr1/2	*T-Cre*	Dysgenesis of WD and MT	13
	Pax3-Cre	Absence of MT	15
Fgfr2	*Hoxb7-Cre*	Regression of caudal WD	16
Shh	KO	Numerous ectopic MT, ectopic UB	29
Foxc1/2	*Foxc1/Mf1^{ch}*, KO	Numerous ectopic MT, ectopic UB	28, 101
c-ret	*ret-k^{-}*	Reduced number of MT	102
Raldh2	KO	Absence of WD	103
Lfng	KO	Blockage of the connection between efferent duct and rete testis	36
Defects in WD stabilization, elongation and coiling			
Ar	*Tfm*, KO	WD regression	40,41
Inhba	KO	Failed to develop ductal coiling in epididymis	53
Sfrp1/2	dKO	Shortened vas deferens	56
Vagl2	*Vangl2$^{lp/lp}$*	Shortened vas deferens	56
Wnt5a	KO	Shortened vas deferens	56
Pkd1	KO, *Pax2-Cre*	Coiling defect, cystic dilation of efferent ducts	54
Defects in postnatal differentiation			
Pten	*Rnase10-Cre*	Dedifferentiation of IS	2
Ros1	KO	Undifferentiated IS	1
Dusp6	KO	Large caput and corpus	67
Frs2	*Hoxb7-Cre*	Morphologically normal	68
	Rnase10-Cre	Abnormal shape of epididymis	68
Ar	*Ap2a-Cre*	Defective epithelial cell differentiation	47
	Rnase10-Cre	Absence of IS, defective epithelial cell differentiation	70
	FoxG-Cre	Absence of IS, defective epithelial cell differentiation	71
	Probasin-Cre	Small epididymis and seminal vesicle	69
Dicer	*Defb4-Cre*	Epithelial cell dedifferentiation	75
miR-29a	*miR-29b1^{UBC}* transgene	Hypoplastic epididymis	77
Lgr4	*Lgr4$^{Gt/Gt}$*	Short, dilated and much less convoluted epididymal ducts	104
	KO	Blockage of efferent duct	105
Shp1	*mev/mev*	Aberrant epididymal region	66
Hoxa11	KO	Transformation of vas deferens to epididymis	79
Hoxa10	KO	Transformation of vas deferens to epididymis	80

WD: wolffian duct; MT: mesonephric tubules; UB: ureteric bud; IS: initial segment; MD: mullerian duct

network of ductules is formed by simple fusion of a subset of MTs. The latter hypothesis would seem more feasible than the first because of the presence of blind-ended tubules. These MTs only fuse to one other MT, leaving one end sealed, hence becoming blind-ended. Obviously, there must be considerable coordination between the fusion events that limit the number of MTs that can fuse[4,5] resulting in the conus (2–3 fused MTs) and the single common ductule.[22] Identification of the genes and processes by which the formation and patterning of the efferent ducts occur is crucial, and the GUDMAP *in situ* hybridization database (http://www.gudmap.org/index.html)[31,32] clearly shows some potential genes that may regulate their formation, e.g., collagen triple helix repeat containing 1*(Cthrc1)*, cortexin 3 *(Ctxn3)* and laminin, alpha1 *(Lama1)*. *Lunatic fringe* (*Lfng*) is one of the mammalian *fringe* genes encoding a modifier of the notch receptor expressed in the developing WD, MTs and testis.[33–35] *Lfng*-null mice show partial bilateral blockage of the connection between the rete testis and the efferent ducts, indicating the involvement of notch signaling in establishing the rete testis-efferent duct boundary.[36]

The origins of nephron progenitor cells are suggested to differ between mesonephros and metanephros.[37] Metanephric mesenchyme is

derived from a posterior immature caudal population, which is positive for *Brachyury* (*T*) expression, and persists in the posterior end of the embryo until body axis extension is complete (**Figure 1a**). On the other hand, the WD and at least part of the mesonephric mesenchyme arise from the anterior intermediate mesoderm, which is defined by *Osr1* expression at E9.5 (**Figure 1b**). These recent studies may indicate that abnormal body axis extension affects the intermediate mesodermal cell fate. It is possible that disruption of the A-P body axis extension affects not only the metanephric mesenchyme but also the mesonephric mesenchymal distribution, and subsequently further male reproductive tract development. Conditionally-induced mutations of the planar cell polarity (PCP) pathway-related genes, *Wnt5a*, *Ror2* and *Vangl2*, which are important for A-P body axis extension, demonstrate that insufficient A-P axis extension of the posterior intermediate mesoderm is correlated with urogenital tract abnormalities.[38] It is clear that more studies are needed to examine the early formation of the intermediate mesoderm and how this translates into development of the WD.

Stabilization of the ductal system and further growth: elongation and coiling

During embryogenesis, the mesonephros gives rise to a stable male reproductive tract whereas the mesonephros in the female regresses (**Figure 1d** and **1e**). Androgens produced in the testis are a major factor regulating this stabilization.[39–42] Following gonadal sex differentiation, the testis begins to produce the androgen, testosterone, at approximately E12.5.[43,44] Unlike for other androgen-dependent organs, such as the prostate and seminal vesicle, it has been suggested that locally-produced, and not systemic androgen, from the testis is necessary for WD stabilization.[45] Indeed, fluorescence labeling of an androgen ligand shows that androgen is transported within the luminal fluid.[30] However, there are studies showing that testicular androgen delivered via the systemic circulation is sufficient to prevent WD regression. Subcutaneous testicular grafts stabilize the WD in female marsupial embryos.[46] Androgens act through the androgen receptor (AR), a member of the nuclear receptor superfamily. The expression of AR is mainly detected in the mesenchyme surrounding WD epithelia at E13.5 in the mouse. Tissue-specific *Ar* knockout (KO) analyses demonstrate that WD stabilization and coiling is induced in the absence of epithelial-expressed *Ar*, demonstrating the importance of *Ar* in the mesenchyme.[47] This finding is consistent with the observation from tissue recombination experiments on androgen-insensitive *Testicular feminized* (*Tfm*) mice.[48,49] Several growth factors, including FGF and Epidermal growth factor (EGF), are suggested to mediate androgen functions in the prostate and WD.[50–52] However, the molecular mechanisms by which androgens regulate these genes *in vivo* are not known.

To create a long, highly-convoluted epididymal duct, the WD begins to elongate and coil from E15.5, following stabilization (**Figure 1e**). This process is also androgen-dependent, but growth factor signaling has been reported to regulate this elongation event. Tomaszewski *et al.* reported that *Inhba*, a subunit of both inhibins and activins, is a regional paracrine factor in mouse mesonephroi that controls coiling of the epithelium in the anterior WD.[53] *Pkd1*, whose mutation accounts for 85% of autosomal dominant polycystic kidney disease, and is a membrane-spanning glycoprotein involved in growth factor signaling transduction and cytoskeleton dynamics. Epithelial coiling is absent from the *Pkd1* mutant.[54] In both mutations, epithelial cell proliferation is attenuated. Recently, mathematical modeling has suggested that epididymal tubule morphogenesis is dependent upon the cell proliferation area in the tubule and mechanical resistance from the tissues surrounding the tubule.[55]

The secreted frizzled-related proteins (SFRPs) antagonize WNT ligand protein binding to its receptor FZD. The double KO (dKO) of *Sfrp1* and *Sfrp2* genes results in a shortened WD and vas deferens.[56] Androgen administration to these animals never rescues this phenotype, indicating that the abnormalities in *Sfrp1/2* dKO mutant male embryos are not caused by insufficient production of testosterone from the testes, but may reflect insensitivity of some target tissues to androgens.[56] It is also possible to consider that these phenotypes are, at least partially, a secondary consequence of the A-P extension defect of intermediate mesoderm formation described above. Although recent analyses have partially revealed the molecular mechanisms of ductal morphogenesis, further analyses should be performed including how androgen signaling regulates these molecules.

Postnatal differentiation: regional differentiation and epithelial cell differentiation

The epididymis consists of distinct anatomical regions that vary between species. However, in the mouse four regions can be defined: initial segment and caput, corpus and cauda epididymidis (**Figure 1f**). Each region is further divided into many segments characterized by expression of specific mRNAs, proteins and a repertoire of cell types.[57,58] The segments, divided by septa, are observed after birth and are distinct during puberty, postnatal (P) days 14–35. Impaired epididymal regionalization or epithelial cell differentiation results in male infertility. For example, if the initial segment does not develop, then male infertility results. Data from efferent duct ligation (EDL) experiments suggested that luminal fluid coming from testis is responsible for the maintenance of initial segment cell survival, proliferation and differentiation.[59,60]

Several growth factors, including FGFs 2,4 and 8, are detected in testicular fluid, and *Fgfrs* are expressed in the epithelium of the initial segment.[61,62] During normal development, high activity of the MAPK pathway, especially p-MAPK1/3 (p-ERK1/2), is detected in the initial segment.[60] EDL abolishes their activities, emphasizing the importance of lumicrine factors regulating their activity.[60] *Ros1* encodes an orphan receptor tyrosine kinase that is expressed in few epithelia, among them the WD and its derivatives.[63–65] Loss of *Ros1* expression or a naturally-occurring mutation of *Shp1* (*me*^{v}), a negative regulator of ROS1, results in abnormal differentiation of the initial segment.[1,66] *RNase10-Cre* drives gene recombination in the initial segment epithelia from P17 onward. *RNase-Cre*-mediated mutation in *Pten*, a negative regulator of PIP3/AKT signaling, induces dedifferentiation of the initial segment.[2] In these animals, abnormal differentiation results in an abnormally shaped initial segment. MAPK signaling regulators such as DUSP6 and FRS2 play important roles in epididymal cell proliferation and survival during postnatal development.[67,68]

Androgens are important regulators of epididymal development from embryonic to adult stages. From later stages of development to the adult stage, *Ar* expression in the epithelia is greater than that in the mesenchyme. Several *Ar* KO mice have been reported, and the majority show a hypoplastic epididymis and defective epithelial cell differentiation.[47,69–71] A differentiated epididymal epithelium is pseudostratified and comprises principal, clear, narrow, basal and recently-identified dendritic cells throughout the duct.[72,73] Similar to other pseudostratified epithelia, for example the trachea, the epididymal luminal environment regulates secretion and absorption of ions, water, organic solutes and proteins.[74] The molecular mechanisms of epididymal epithelial differentiation are not clear. Chimeric mutation of the *Ar* indicates that defective epithelial cell differentiation is cell-autonomous.[47] Dicer and small RNAs also regulate epididymal

development and epithelial cell differentiation partially through androgen action.[75–77]

Hox genes are evolutionarily-conserved transcriptional regulators that determine body patterning.[78] As found for body plan formation, vertebrae and the gut, *Hox* genes, *Hoxa10* and *Hoxa11* are suggested to determine the boundary between the epididymis and vas deferens.[79–81] Later studies by Snyder *et al.*[82] showed that there were additional region-specific (efferent ducts, epididymis and vas deferens) *Hox* transcripts that may define boundaries along the reproductive tract during development.

POSSIBLE CONTRIBUTION OF MOUSE MODELS TO UNDERSTAND HUMAN CINICAL ABNORMALITIES

One of the most well-known congenital anomalies of the epididymis or vas deferens is congenital bilateral absence of the vas deferens (CBAVD). It occurs in 1%–2% of men with infertility.[83] 60%–90% of the CBAVD men harbor at least one associated *cystic fibrosis transmembrane conductance regulator* (*CFTR*) gene mutation.[84] 10%–40% of CBAVD men do not have recognizable *CFTR* gene abnormalities accompanied by unilateral renal agenesis (URA).[85] Presumably, CBAVD patients have disrupted morphogenesis of the early mesonephros owing to the mutation of genes.[86] Those genes involved in mesonephros formation, e.g., *Pax2*, *Wt-1* and *Fgfs*, may be viable candidate genes responsible for CBAVD with renal malformation.

Conversely, congenital anomalies of kidney and urinary tract (CAKUT) often carry mutations in genes, such as *PAX2* and *WT-1*, and male mice carrying mutations of these genes also exhibit reproductive tract malformations.[87] Syndromes with renal tract abnormalities also carry mutations in the genes described above. Branchio-Oto-Renal (BOR) syndrome is a genetic condition that typically disrupts the development of tissues in the neck and causes malformations of the ears and kidneys. *EYA1*, the human homolog of the *Drosophila eyes absent* gene, is the most common gene responsible for BOR.[88] Further, *Foxc1* regulates *Eya1* expression.[28] Mutations in the *SIX1* gene can be detected in 2% of individuals with the clinical diagnosis of BOR.[89] Mutations in both *ROR2*[90] and *WNT5A*[91] have been implicated in a rare genetic disease, Robinow syndrome, which exhibits several defects such as dwarfism, hydronephrosis and genital abnormalities. Because these syndromes often exhibit lethal abnormalities, it is still unclear if these mutations affect male fertility in humans.

Epididymal disjunction is the failure of the efferent ducts to reach the testis, which may reflect the failure of the efferent ducts to elongate, and presumably coil, during their development.[92–95] Interestingly, one study[95] has shown that 30%–79% of boys with an undescended testis also have Wolffian duct abnormalities, of which 25% display epididymal disjunction. Therefore, it is important that epididymal abnormalities be detected at orchidopexy, or other male infertility, which may be classified as idiopathic, will result. As mentioned above, it is not clear how the efferent ducts form, elongate, are directed toward the testis and then fuse with the rete testis. Obviously, mouse models that display epididymal disjunction will greatly aid our understanding of this abnormality.

SUMMARY

One of the striking characteristics of the epididymis is its complex developmental process. The primordium of the epididymis, the mesonephros, arises as a part of the transient kidney, and its stability and differentiation are regulated by hormonal signaling including by androgens and growth factors. In human, it transforms its morphology to form a 6 m duct that is coiled and packed into a three-dimensional organ of approximately 10 cm in length. Recent studies utilizing a variety of transgenic mice have revealed the molecular contribution of numerous factors at each stage of epididymal development. The molecular dissection of the developmental mechanisms of the epididymis has just begun. Integrative understanding of the hierarchy and interaction of each factor will provide new directions in this field. Considering that the epididymis shares its origin with the urinary tract, it is noteworthy that the molecular mechanisms which lead to kidney mal-development, such as CAKUT, may provide significant insight for the mesonephros derivative mal-development, such as CBAVD and *vice versa*.

COMPETING FINANCIAL INTERESTS

Neither author declares a competing interest.

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DEVELOPMENTAL BIOLOGY

Elimination of the male reproductive tract in the female embryo is promoted by COUP-TFII in mice

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The sexual differentiation paradigm contends that the female pattern of the reproductive system is established by default because the male reproductive tracts (Wolffian ducts) in the female degenerate owing to a lack of androgen. Here, we discovered that female mouse embryos lacking *Coup-tfII* (chicken ovalbumin upstream promoter transcription factor II) in the Wolffian duct mesenchyme became intersex—possessing both female and male reproductive tracts. Retention of Wolffian ducts was not caused by ectopic androgen production or action. Instead, enhanced phosphorylated extracellular signal-regulated kinase signaling in Wolffian duct epithelium was responsible for the retention of male structures in an androgen-independent manner. We thus suggest that elimination of Wolffian ducts in female embryos is actively promoted by COUP-TFII, which suppresses a mesenchyme-epithelium cross-talk responsible for Wolffian duct maintenance.

Sexually dimorphic establishment of reproductive tracts epitomizes the anatomical difference between males and females. This dimorphic establishment depends on two concurrent events during embryogenesis: regression of one of the two primitive ducts (Müllerian and Wolffian ducts) and maintenance of the other. These two events ensure that the embryo retains only one reproductive tract that corresponds to its sex: Müllerian duct for the XX individual and Wolffian duct for the XY individual (*1*). In the 1950s, Alfred Jost provided the first evidence for what became the foundation of the sexual differentiation paradigm: XY embryos retain Wolffian ducts through the action of testis-derived androgen, whereas XX embryos lose Wolffian ducts as a result of a lack of androgens (*2*–*5*).

The action of androgen on the Wolffian duct is mediated through androgen receptors in the mesenchyme surrounding Wolffian ducts (*6*, *7*). It is well established that mesenchyme-derived factors govern the fate and differentiation of ductal epithelium (*8*). The orphan nuclear receptor COUP-TFII (chicken ovalbumin upstream promoter transcription factor II, or NR2F2) is a mesenchyme-specific regulator in many developing organs, including the mesonephros, where Wolffian ducts develop (*9*). COUP-TFII expression in Wolffian duct mesenchyme overlapped with Wilms' Tumor 1 (WT1) (fig. S1A), another mesenchyme-specific transcriptional factor (*10*). To investigate the role of COUP-TFII in Wolffian duct regression, we used the tamoxifen-inducible $Wt1^{CreERT2}$ mouse model that targeted *Coup-tfII* deletion in $Wt1^{+}$ mesenchymal cells (fig. S1B). In the control ($Wt1^{CreERT2-}$;$Coup\text{-}tfII^{f/f}$) female, COUP-TFII remained in the mesenchymal cells of mesonephroi from embryonic day 12.5 (E12.5) to E16.5 (fig. S1, C to E), the developmental window that encompassed initiation (E14.5) and completion (E16.5) of Wolffian duct regression in XX embryos (fig. 2SA). In the knockout ($Wt1^{CreERT2+}$;$Coup\text{-}tfII^{f/f}$) female, COUP-TFII ablation began 24 hours after the first tamoxifen injection (fig. S1F) and was completed by E14.5 (fig. S1, G and H). Ablation of *Coup-tfII* was further confirmed with reverse transcription polymerase chain reaction (RT-PCR) (fig. S2B). These results demonstrated an efficient ablation of *Coup-tfII* in WT1-positive Wolffian duct mesenchyme in XX embryos.

The impact of *Coup-tfII* ablation on XX mesonephroi was first examined at E18.5, when dimorphic development of reproductive tracts is completed. The control XX embryos contained only Müllerian ducts that were visualized by immunostaining of the epithelial marker PAX2 (Fig. 1A). Other control genotypes that include $Wt1^{CreERT2+}$;$Coup\text{-}tfII^{f/+}$ and $Wt1^{CreERT2+}$;$Coup\text{-}tfII^{+/+}$ female embryos also developed normally, with only Müllerian ducts (fig. S3A). Knockout XX littermates, however, had both Müllerian and Wolffian ducts in the mesonephros (Fig. 1D). The identity of the Wolffian duct was confirmed by the presence of Wolffian duct epithelium marker transcription factor AP-2α (AP-2α) (Fig. 1, B and E) (*7*). Embryos in which *Coup-tfII* was knocked out died soon after birth. We therefore developed an organ culture system that allowed us to maintain E18.5 XX mesonephros with ovaries for 7 days to investigate whether Wolffian ducts remained present postnatally. At the end of culture, Wolffian ducts were still present in knockout tissues, along with the components of female reproductive tracts (Fig. 1, C and F), indicating that Wolffian duct maintenance in knockout XX persisted after birth and was not a transient event.

Retention of Wolffian ducts in the *Coup-tfII* knockout XX embryo points to a possible action of androgens based on the Jost paradigm (*11*). $Wt1^{CreERT2}$ targets *Coup-tfII* deletion not only in mesonephroi but also somatic cells of the ovary (*12*), raising the possibility that an ovary in which *Coup-tfII* has been knocked out could synthesize androgens ectopically. We compared the transcriptomes between control and knockout ovaries at E14.5 and E16.5, during which Wolffian duct regression occurs. The transcriptome of the knockout ovary was not different from the control ovary, with the exception of 10 differentially expressed genes (including *Coup-tfII*) (table S1). None of these genes were associated with androgen production. Furthermore, mRNA expression of two rate-limiting enzymes—hydroxy-δ-5-steroid dehydrogenase, 3β- and steroid δ-isomerase 1(*Hsd3b1*) and cytochrome P450 17A1 (*Cyp17a1*)—for steroidogenesis was not different between control and knockout ovaries and nearly undetectable compared with the wild-type fetal testis (a positive control) (Fig. 2, A and B). A lack of androgen-producing capacity in the ovary was corroborated with unchanged anogenital distance (AGD), an androgen-sensitive parameter, between control and knockout XX at E18.5 (Fig. 2C). To exclude the possibility that androgens came from other resources in the knockout embryo, we removed the mesonephroi from XX embryos and cultured them for 4 days. After culture, Wolffian ducts regressed in control XX mesonephros, as expected, whereas in knockout XX, either in the presence or absence of ovaries, Wolffian ducts were maintained (Fig. 2D and fig. S3B). These results indicated a lack of androgen production in the XX knockout ovaries and led us to speculate that Wolffian duct retention in XX embryos could be the result of ectopic activation of the androgen pathway in the absence of *Coup-tfII*. This possibility was excluded based on of the finding that expression of androgen receptor (*Ar*) and two androgen-induced genes—folate hydrolase 1 (*Folh1*) and solute carrier family 26 member 3 (*Slc26a3*) (*13*, *14*)—was not different between control and knockout XX mesonephroi (Fig. 2, E to G). To rule out the involvement of androgens, we exposed the dam that carried control and knockout embryos to the androgen antagonist flutamide (Fig. 2H) (*15*). This regimen was sufficient to prevent Wolffian duct maintenance resulting from ectopic androgen action in XX embryos (*16*). Despite the verified action of flutamide (fig. S4), Wolffian ducts were still retained in knockout XX embryos (Fig. 2H). Thus, the maintenance of Wolffian ducts in the *Coup-tfII* knockout XX embryo is not due to ectopic production or action of androgens.

To identify the androgen-independent mechanism underlying Wolffian duct retention in the female in which *Coup-tfII* had been knocked out, we turned our attention to epidermal growth factor (EGF) and fibroblast growth factor (FGF) signaling pathways for their putative ability to promote Wolffian duct maintenance (*17*, *18*). We first examined by means of RT-PCR the expression of *Egf* and its receptor *Egfr*. Their expression

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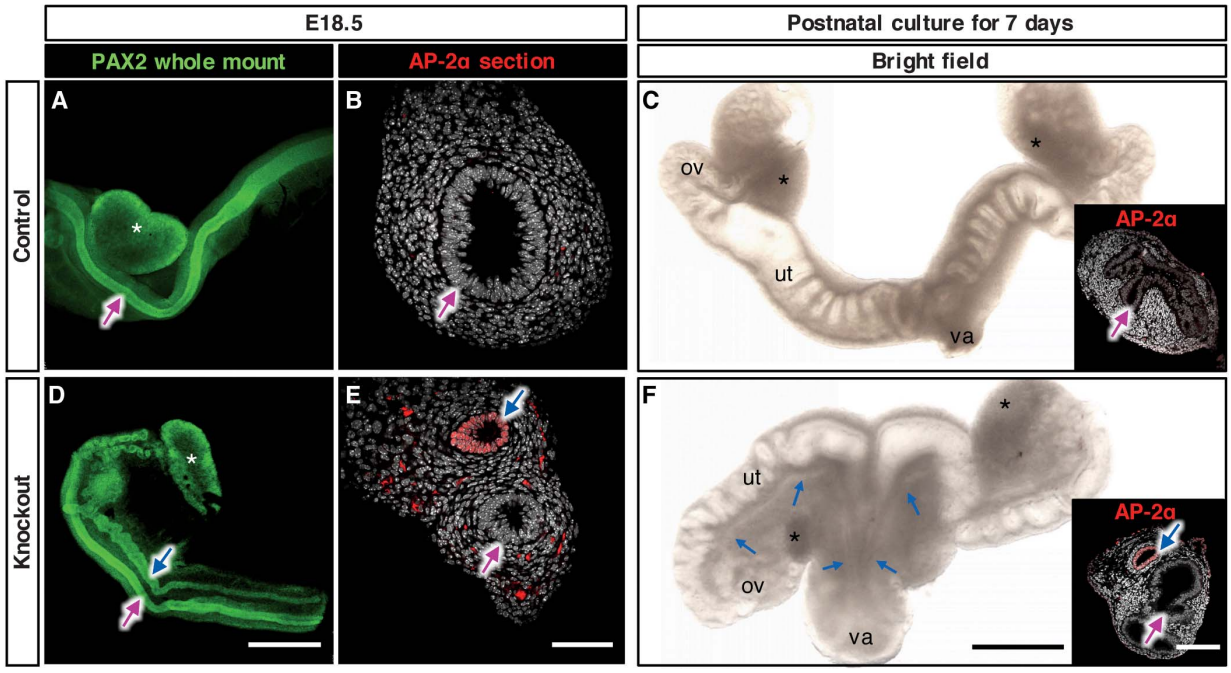

Fig. 1. *Coup-tfII* ablation leads to Wolffian duct retention in the XX embryo. Control and *Coup-tfII* knockout reproductive systems from E18.5 XX embryos were analyzed with (**A** and **D**) whole-mount immunofluorescence for the epithelial marker PAX2 or (**B** and **E**) AP-2α on frozen sections. (**C** and **F**) Control and knockout mesonephroi from E18.5 XX embryos were cultured for 7 days and analyzed with bright field microscopy or (insets) AP-2α immunofluorescence on frozen sections. Blue arrows indicate Wolffian ducts, magenta arrows indicate Müllerian ducts, and asterisks indicate ovary. ov, oviduct; ut, uterus; va, vagina. Scales bars, (A), (C), (D), (F), 0.5 mm; (B), (C) inset, (E), and (F) inset, 50 μm. *n* = 14 embryos in (A); *n* = 3 embryos per each genotype in (B), (C), (E), (F), and *n* = 23 embryos in (D).

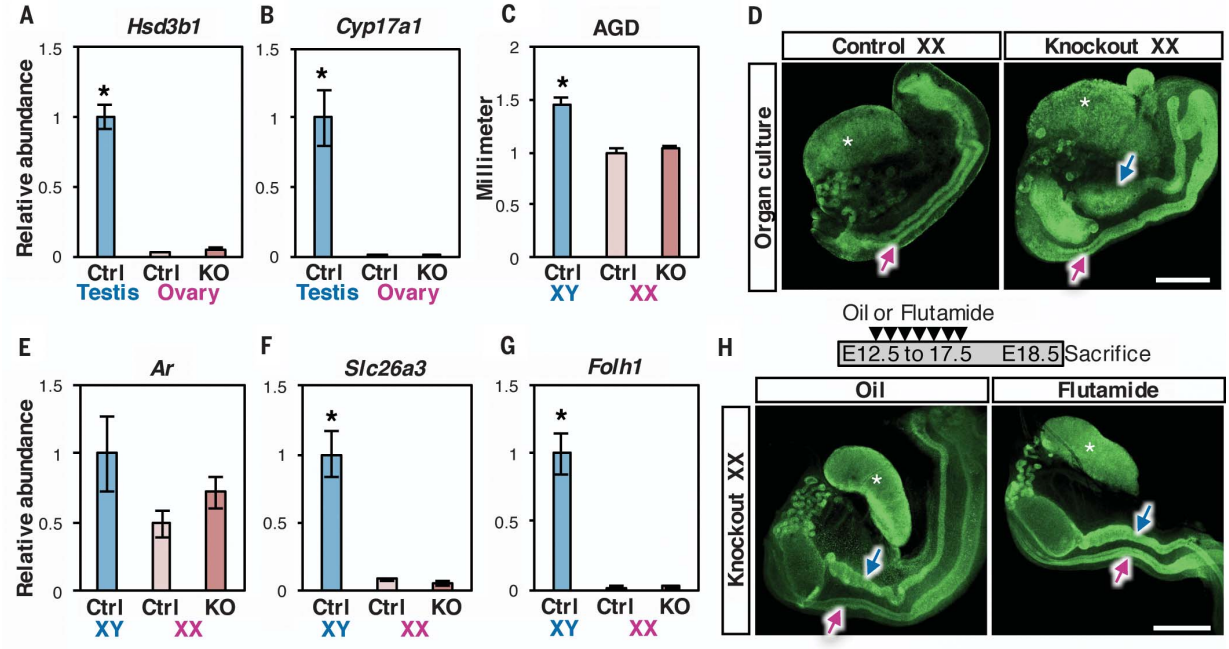

Fig. 2. Wolffian duct retention in *Coup-tfII* knockout XX embryo is independent of androgen production or action. (**A** and **B**) mRNA expression of two rate-limiting steroidogenic enzymes, *Hsd3b1* and *Cyp17a1*, in E14.5 control testis (light blue), control ovary (light pink), and knockout ovary (dark pink). (**C**) AGD of the control and knockout embryos at E18.5. (**D**) PAX2 whole-mount immunofluorescence of ovaries and mesonephroi after 4-day culture. *n* = 7 embryos per genotype. (**E** to **G**) mRNA expression of androgen receptor *Ar* and androgen-responsive genes (*Slc26a3* and *Folh1*) in control XY, control XX, and knockout XX mesonephroi. Results are shown as mean ± SEM. Asterisks in (A), (B), (C), (F), and (G) represent statistical significance of $P < 0.05$ compared with either control or knockout XX samples by means of one-way analysis of variance followed by Tukey's test [*n* = 8 embryos per each group in (A) and (B), *n* = 8 to 11 embryos in (C); and *n* = 8 embryos in (E) to (G)]. (**H**) Knockout XX embryos were exposed to either vehicle (oil) or androgen receptor antagonist flutamide in utero once daily from E12.5 to E17.5. Samples were collected at E18.5 and analyzed with PAX2 whole-mount immunofluorescence. *n* = 3 embryos per genotype. Blue arrows indicate Wolffian ducts, magenta arrows indicate Müllerian ducts, and white asterisk indicates ovary. Scale bar, (D) and (H), 0.5 mm.

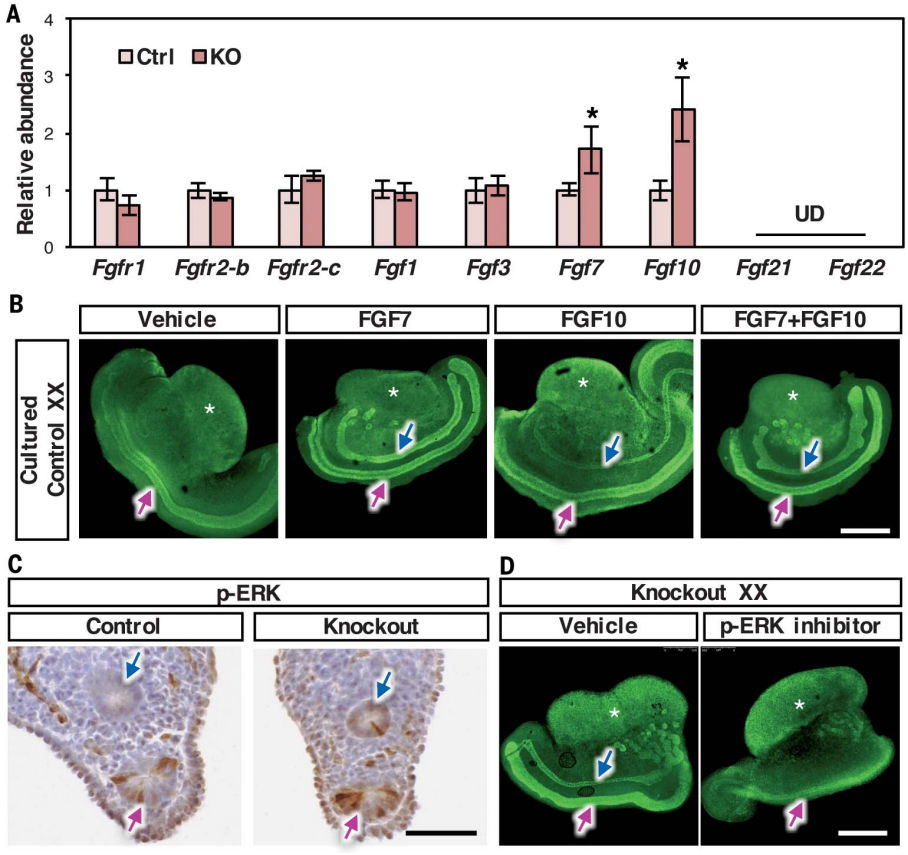

Fig. 3. Enhanced FGF signaling is involved in Wolffian duct retention in the absence of *Coup-tfII*. (**A**) mRNA expression of FGF receptors and ligands in the mesonephroi at E14.5. UD, undetected. Results are shown as mean ± SEM. Asterisks represent statistical significance of $P < 0.05$ compared with control females by using Student's *t* test ($n = 8$ embryos for each genotype). (**B**) Whole-mount immunofluorescence of PAX2 of 2-day cultured E14.5 wild-type XX mesonephroi in the presence of vehicle, or FGF7, FGF10, or FGF7+FGF10. (**C**) p-ERK immunohistochemistry of control and knockout XX mesonephroi at E14.5. (**D**) PAX2 whole-mount immunofluorescence in vehicle- or p-ERK inhibitor–treated knockout XX genital ridges. $n = 3$ embryos per group in (B) to (D). Scale bars, (B) and (D), 0.5 mm; (C) 50 μm. Blue arrows indicate Wolffian ducts, magenta arrows indicate Müllerian ducts, and white asterisk indicates ovary.

was not different between control and knockout XX mesonephroi (fig. S5A). Components of FGF signaling, in contrast, exhibited distinct changes in the absence of *Coup-tfII*. FGFR2 is the major FGF receptor in the Wolffian duct epithelium (*19*), and its binding ligands include FGF1, -3, -7, -10, -21, and -22 (*20*). mRNA expression of FGF receptors (*Fgfr1*, *Fgfr2-b*, and *Fgfr2-c*) and most ligands were unaltered (*Fgf1* and *Fgf3*) or undetectable (*Fgf21* and *Fgf22*) in knockout XX mesonephroi compared with the control (Fig. 3A). However, expression of *Fgf7* and *Fgf10* was increased significantly in knockout XX mesonephroi at E14.5 (Fig. 3A) and E16.5 (fig. S5B). To investigate whether FGF7 and FGF10 were capable of reproducing the Wolffian duct maintenance phenotype in the wild-type female, we cultured E14.5 wild-type XX mesonephroi for 2 days in the presence of vehicle, FGF7, FGF10, or FGF7+FGF10. In the vehicle-treated group, Wolffian ducts regressed after 2-day culture, similar to the in vivo situation. In contrast, presence of FGF7, FGF10, or FGF7+FGF10 maintained the Wolffian duct in the wild-type XX mesonephroi (Fig. 3B).

FGF7 and FGF10 are expressed in the mesonephric mesenchyme, the same cellular compartment as COUP-TFII (*21*, *22*). These FGFs bind FGFR2 in Wolffian duct epithelium and activate two intracellular signaling components, phosphorylated protein kinase B (p-AKT) and phosphorylated extracellular signal-regulated kinase (p-ERK) (*20*). Loss of *Coup-tfII* did not change p-AKT activation in Wolffian ducts (fig. S5C). The presence of p-ERK, conversely, became detected in Wolffian duct epithelium of knockout XX compared with the control XX at E14.5 (Fig. 3C) and E16.5 (fig. S5D). These results indicate that loss of mesenchymal *Coup-tfII* led to an enhanced activity of p-ERK signaling in Wolffian duct epithelium. We then tested whether elevated p-ERK signaling was the cause of Wolffian duct maintenance by culturing the *Coup-tfII* knockout mesonephroi with a p-ERK specific inhibitor PD0325901 (*23*). In the vehicle-treated group, Wolffian ducts in the knockout XX were maintained in culture. Conversely, the p-ERK inhibitor eliminated Wolffian ducts in the knockout XX mesonephroi (Fig. 3D), indicating that enhanced p-ERK signaling was involved in Wolffian duct retention in the *Coup-tfII* knockout XX embryo.

We have shown that instead of a passive process occurring as a result of the absence of androgens, elimination of the male reproductive tract in the female embryo is actively promoted by COUP-TFII through its action in the Wolffian duct mesenchyme. COUP-TFII in the mesenchyme inhibits expression of FGFs, which otherwise activate the p-ERK pathway in the Wolffian duct epithelium for its maintenance. The function of COUP-TFII in facilitating Wolffian duct elimination is not restricted to XX embryos; when the testis was removed from the *Coup-tfII* knockout XY mesonephros, Wolffian ducts remained present despite a lack of androgens (fig. S6). These findings reveal unexpected mechanisms underlying the dimorphic development of the Wolffian ducts via COUP-TFII. In addition, maintenance of male reproductive tracts without androgens prompts a reassessment of the role of androgens in this process, which presumably is to antagonize the action of COUP-TFII.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/357/6352/717/suppl/DC1
Materials and Methods
Figs. S1 to S6
Tables S1 and S2
References

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Elimination of the male reproductive tract in the female embryo is promoted by COUP-TFII in mice

Fei Zhao, Heather L. Franco, Karina F. Rodriguez, Paula R. Brown, Ming-Jer Tsai, Sophia Y. Tsai and Humphrey H.-C. Yao

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The makings of the reproductive tract

Every embryo, regardless of its sex, contains both male and female primitive reproductive tracts before sexual differentiation. To establish a sex-specific reproductive system, female embryos need to remove the components of male tracts. The general consensus contends that removal of the male tracts occurs by default, a passive outcome owing to a lack of testis-derived androgens. Working in mice, Zhao *et al.* discovered that this process instead was actively promoted by the transcription factor COUP-TFII (see the Perspective by Swain). Without the action of this factor, embryos retained male reproductive tracts, independently of androgen action. These findings unveil unexpected mechanisms underlying the sexually dimorphic establishment of reproductive tracts.

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REVIEW ARTICLE

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Testicular organoids to study cell–cell interactions in the mammalian testis

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ABSTRACT

Background: Over the last ten years, three-dimensional organoid culture has garnered renewed interest, as organoids generated from primary cells or stem cells with cell associations and functions similar to organs in vivo can be a powerful tool to study tissue-specific cell–cell interactions in vitro. Very recently, a few interesting approaches have been put forth for generating testicular organoids for studying the germ cell niche microenvironment.

Aim: To review different model systems that have been employed to study germ cell biology and testicular cell–cell interactions and discuss how the organoid approach can address some of the shortcomings of those systems.

Results and Conclusion: Testicular organoids that bear architectural and functional similarities to their in vivo counterparts are a powerful model system to study cell–cell interactions in the germ cell niche. Organoids enable studying samples in humans and other large animals where in vivo experiments are not possible, allow modeling of testicular disease and malignancies and may provide a platform to design more precise therapeutic interventions.

INTRODUCTION

Historically, the term 'organoid' has been used to describe different three-dimensional (3D) cell culture systems such as organ cultures (Barcellos-Hoff *et al.*, 1989); 3D cell clusters derived from tissue-specific primary cells or from adult and/or pluripotent stem cells (Simian *et al.*, 2001; Sato *et al.*, 2009; Shamir & Ewald, 2014; Huch & Koo, 2015; Matano *et al.*, 2015; Schepers *et al.*, 2016; Czerniecki *et al.*, 2018; Forsythe *et al.*, 2018). However, for this review, we will be using the definition of the term 'organoid' put forth by Fatehullah and colleagues in 2016 as we find it to be most appropriate: 'Here we define an organoid as an in vitro 3D cellular cluster derived exclusively from primary tissue, embryonic stem cells, or induced pluripotent stem cells, capable of self-renewal and self-organization, and exhibiting similar organ functionality as the tissue of origin' (Fatehullah *et al.*, 2016). Methodologies for the derivation of organoids from primary cells and stem cells have been well established for different organ systems. While stem cell-derived organoids can be a useful model to study development, organoids produced by self-organization of primary cells can be utilized to study tissue-specific morphogenesis, cellular function, and effects of different experimental factors.

A number of different organs such as intestine (Sato *et al.*, 2009), stomach (Barker *et al.*, 2010), pancreas (Huch *et al.*, 2013), liver (Takebe *et al.*, 2013), vasculature (Morgan *et al.*, 2013), colon (Sato *et al.*, 2011), pancreas (Boj *et al.*, 2015), mammary gland (Simian *et al.*, 2001), and brain (Quadrato *et al.*, 2017) have been modeled in 3D organoid systems. These organoids displayed cell associations and functions similar to their in vivo counterparts. As a result, these organoids can serve as an intermediary between conventional two-dimensional (2D) cultures and animal models. Compared to 2D cultures on plastic plates, 3D organoids composed of different cell types with a tissue-specific architecture and function are more biologically relevant for studying tissue microenvironments. They can also be used to study the effects of different experimental factors on the niche environment and cell–cell interactions (Jabs *et al.*, 2017; McCauley & Wells, 2017; Czerniecki *et al.*, 2018; Forsythe *et al.*, 2018). Organoids derived from both primary and stem cells allow easier and greater access for modulating signal transduction pathways compared to animal models (Lancaster & Knoblich, 2014; Shamir & Ewald, 2014; Fatehullah *et al.*, 2016).

Despite increased interest in organoid systems over the last decade, it is only very recently that testicular organoids have

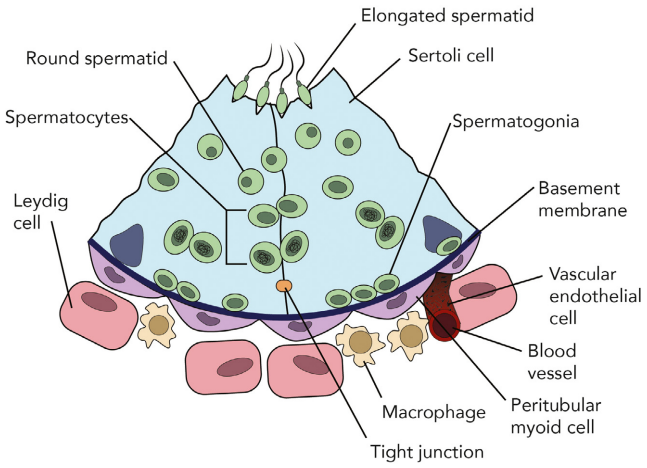
started to garner interest (Alves-Lopes *et al.*, 2017; Baert *et al.*, 2017; Pendergraft *et al.*, 2017; Strange *et al.*, 2018; Sakib *et al.*, 2019). In this review, we discuss the different model systems that have been used to study the germ cell niche to illustrate the utility of organoids in the context of existing approaches. Then, we will discuss how the recently reported testicular organoid systems may be employed to understand the niche maintenance, cell interactions, and important testicular functions such as spermatogenesis and testosterone production.

IMPORTANCE OF UNDERSTANDING THE GERM CELL NICHE

The mammalian testis is a complex multicellular system that carries out two primary functions, namely production of spermatozoa and androgens (Tesarik *et al.*, 1998; Oatley & Brinster, 2012). The testis is broadly divided into two distinct compartments—the seminiferous tubules and interstitial tissue (Fig. 1). Sertoli and germ cells are the primary cell types of the seminiferous epithelium. Undifferentiated germ cells are localized along the basement membrane in the basal compartment of the epithelium and remain in close contact with Sertoli cells (Oatley & Brinster, 2012). Sertoli cells produce factors like glial cell line-derived neurotrophic factor (GDNF) (Tadokoro *et al.*, 2002), fibroblast growth factor 2 (FGF2) (Mullaney & Skinner, 1992), and Wnt ligands (Tanwar *et al.*, 2010; Takase & Nusse, 2016) to maintain germ cell homeostasis. Sertoli cells also produce extracellular matrix (ECM) proteins collagen IV and laminin to contribute to the basement membrane. Adjacent Sertoli cells form tight junctions which constitute the blood–testis barrier that divides the seminiferous epithelium into the basal and adluminal compartments (Richardson *et al.*, 1995; Oatley & Brinster, 2012). During spermatogenesis, the undifferentiated germ cells migrate from the basal compartment to the adluminal compartment and differentiate into spermatocytes, spermatids, and spermatozoa. The interstitium is mainly composed of peritubular myoid cells, Leydig cells, endothelial cells, and testicular macrophages (Oatley & Brinster, 2012; DeFalco *et al.*, 2015; Bhang *et al.*, 2018). A primary function of Leydig cells is to produce testosterone for maintenance of spermatogenesis (Lejeune *et al.*, 1998). The peritubular myoid cells produce growth factors such as GDNF and CSF1 (Chen *et al.*, 2016; de Rooij 2017, Oatley *et al.*, 2009) and ECM proteins such as collagen IV, laminin, and fibronectin which contribute to the basement membrane (Richardson *et al.*, 1995). Spermatogenesis relies on the cell–cell interactions in the germ cell niche. The relative cell associations of different testicular somatic cells and germ cells in the testicular microenvironment allow for spatial and temporal regulation of cell–cell signaling to control germ cell fate (de Rooij, 2001; Oatley & Brinster, 2012; Spradling *et al.* 2001) (Fig. 1).

Around 7% of men suffer from infertility (Krausz, 2011). Pathophysiological conditions, exposure to environmental chemicals, radiation, cancer, and chemotherapeutic agents can lead to infertility (Schrader *et al.*, 2001; Siu *et al.*, 2009; Kashir *et al.*, 2010; Sharpe, 2010; Krausz, 2011; Lee & Shin, 2013; Cheng, 2014). A thorough understanding of the germ cell niche would allow for better intervention strategies for infertility. Storage of spermatozoa is a common fertility preservation technique for people undergoing chemotherapy. However, for pre-pubertal boys that is not possible. From pre-pubertal individuals, a small testicular biopsy containing germ cells may be cryopreserved that could later be transplanted back to the patient (Fayomi *et al.*, 2019), germ cells could be transplanted into the testes to rebuild the depleted germ cell pool or propagated by in vitro spermatogenesis. Thus, study of the germ cell niche and factors that allow for efficient germ cell colonization and spermatogenesis can lead to better fertility preservation (in vitro maturation and spermatogenesis) and/or restoration strategies (germ cell transplantation) (Alves-Lopes & Stukenborg, 2017).

Figure 1 Schematic representation of the germ cell niche. Undifferentiated germ cells including spermatogonial stem cells are localized at the basement membrane and remain in close contact with the Sertoli cells inside the seminiferous epithelium. Peritubular myoid cells line the outer perimeter of the basement membrane, and Leydig cells, vascular cells, and testicular macrophages are located in the interstitium. As spermatogonia differentiate, they traverse the tight junctions and move from the basal to the adluminal compartment of the seminiferous epithelium.



MODEL SYSTEMS TO STUDY THE GERM CELL NICHE

Animal models such as rodents have traditionally been used to study testicular biology and germ cell function (Akingbemi *et al.*, 2001; Park *et al.*, 2002; Gaido *et al.*, 2007; Yoshida *et al.*, 2007; Oatley *et al.*, 2011; Takase & Nusse, 2016; Liu *et al.*, 2017). However, experiments performed with rodent models may not translate well to humans due to physiological differences between rodents and humans or other larger animals (Seok *et al.*, 2013; Zanger & Schwab, 2013; González & Dobrinski, 2015; Gutierrez *et al.*, 2015).

Two-dimensional culture of testicular cells (primary or immortalized) with conventional glass or plastic tissue culture plates has played an important role for understanding niche biology. Co-culture systems of different testicular somatic cells and germ cells were some of the earliest attempts to study niche biology in vitro. Such systems allowed insights into how germ cells interact with the surrounding cells and ECM proteins in the testis microenvironment (Hadley *et al.*, 1985; Kierszenbaum *et al.*, 1986; Hofmann *et al.*, 1992; Richardson *et al.*, 1995). These 2D culture systems have also allowed early investigations on the effects of growth factors, hormones, and experimental factors on niche biology (Kierszenbaum *et al.*, 1986; Tung & Fritz, 1987; Saez *et al.*, 1989; Tesarik *et al.*, 1998; van der Wee & Hofmann, 1999; El Ramy *et al.*, 2005; Hung *et al.*, 2016; Gong *et al.*, 2017).

Although 2D culture systems provide much insight into testicular biology, these monolayer systems grown on hard plastic or glass surfaces can lead to physiologically irrelevant tissue architecture and inappropriate cell communications (Pampaloni *et al*., 2007; Yamada & Cukierman, 2007; Horvath *et al*., 2016). As a result, they often fail to mimic tissue-specific biological responses (Abbott, 2003; Mazzoleni *et al*., 2009).

To address this lack of biologically relevant cell association and intercellular communication, testis organ culture systems were developed where small pieces of the testes were cultured in appropriate media conditions (Steinberger *et al*. 1964). In organ culture systems, the germ cell niche is kept intact. As a result, experimental readouts from such a platform are more translatable to the situation in vivo. Culture of mouse testes fragments on agar at the gas–liquid interphase was first to achieve in vitro spermatogenesis (Sato *et al*., 2011). Similar experiments have also been performed for bovids (Kim *et al*., 2015) and rodents (Reda *et al*., 2016; Nakamura *et al*., 2017; Gholami *et al*., 2018; Sanjo *et al*., 2018).

Due to their relatively large size accompanied by a lack of functional vasculature, organ culture systems may experience reduced gas and nutrient diffusion and as a result are challenging to maintain for longer durations. This limits the utility of the system for experiments involving long-term culture of samples from human and other large animals. An alternative approach to address these shortcomings is testis tissue or cell xenotransplantation, where testis tissue fragments from larger animals are grafted into the back of immunodeficient mice (Honaramooz *et al*., 2002; Zeng *et al*., 2009; Pukazhenthi *et al*., 2015; Liu *et al*., 2016). This allows the study of samples from larger animals for a longer duration in a biologically competent environment. Both autologous and xenogenic testis grafts can generate spermatozoa (Fayomi *et al*., 2019; Pukazhenthi *et al*., 2015; Zeng *et al*., 2009), which makes these systems a good platform to study spermatogenesis. Testis tissue grafts can also be used to study reproductive toxicology, particularly for larger mammals and non-human primates where in vivo studies can be cost prohibitive (Jahnukainen *et al*., 2006; Rodriguez-Sosa *et al*. 2014).

In addition to small testis tissue fragments, testicular cells can also be ectopically grafted into immunodeficient mice where they undergo self-organization and rebuild the testes microenvironment, and can support spermatogenesis (Honaramooz *et al*., 2007; Kita *et al*., 2007). This system can be used to obtain developmental and morphogenic readouts, where different cells of the testes can be sorted and genetically modified or treated with experimental factors and then grafted and observed for generation of tubules capable of supporting spermatogenesis (Dores & Dobrinski, 2014; Dores *et al*., 2015).

Although testis tissue or cell grafting can be a robust model to study niche function in vivo, an initial delay in angiogenesis by the recipient species can lead to hypoxic damage and impairment of tissue functions, particularly for cells of the seminiferous epithelium. The approach still requires experimental animals and a long observation time frame. To address these limitations, in vitro tubule morphogenesis systems were developed, where testicular cells with the aid of a supportive biomaterial can form tubular structures with testis-specific morphology and functions (Dores *et al*. 2017; Gassei *et al*., 2006; Reda *et al*., 2014; Yokonishi *et al*., 2013). These systems can be used as a readout for morphogenic outcomes arising from specific manipulations; for example, Dores *et al*. showed testicular cells treated with a small molecule to prevent formation of primary cilia lead to impaired tubular morphogenesis in vitro (Dores *et al*. 2017).

In vitro tubular morphogenesis assays are prone to variability and require relatively large cell numbers. This makes it challenging to obtain quantitative, reproducible data across experiments, and it becomes difficult to quantify. Thus, in vitro model systems with reproducible testis-specific architecture and functions are needed. Testicular organoid systems address that need.

ORGANOIDS TO STUDY THE GERM CELL NICHE

One of the first reports on testicular organoids was by Baert *et al*. (2017), where testicular organoids were generated from adult and pre-pubertal (15-year-old) human testicular cells. The cells were cultured on ECM obtained by decellularization of adult human testes. No difference in morphology was observed between organoids that were generated with ECM or without. These organoids were able to secrete inhibin B, testosterone, and cytokines. Sertoli cells in the organoids also gave rise to tight junction proteins and supported germ cells for up to 4 weeks (Baert *et al*., 2017).

Alves-Lopes *et al*. (2017) reported using a three-layer Matrigel gradient system to generate rat testicular organoids from 5-, 8- and 20-day-old animals. In this system, Sertoli cells and germ cells formed tubule-like structures, which were surrounded by peritubular myoid cells. These tubule-like structures also had a functional blood testes barrier and could support undifferentiated germ cells for up to 21 days. Exposure to inflammatory cytokines tumor necrosis factor alpha (TNFα) and interleukin 1 alpha (IL1α) perturbed morphogenesis led to germ cell loss and blood testes barrier permeability. These effects are similar to what has been shown for testes in vivo (Sarkar *et al*., 2008; Yan *et al*., 2008; Cheng & Mruk, 2012). The organoid system developed by Alves-Lopes *et al*. showed retinoic acid response similar to testes (van Pelt & de Rooij, 1991; Li *et al*., 2011; Endo *et al*., 2017).

In 2017, another testicular organoid system was reported by Pendergraft and colleagues (Pendergraft *et al*., 2017). A simple hanging drop culture method was used to generate human organoids from adult germ cells and immortalized Leydig and Sertoli cells. Although these organoids did not have a testis-specific morphology, they could produce testosterone and appeared to support haploid germ cells. The same group also reported modeling Zika virus infection using their testicular organoids. The organoids were infected with Zika virus and showed loss of cell viability and reduced testosterone secretion (Strange *et al*., 2018).

Recently, our group reported generation of testicular organoids by using the microwell culture system (Sakib *et al*., 2019). Testicular cells were isolated from pre-pubertal pig, mice, primate, and humans, seeded into microwells, and cultured for 5 days to generate organoids from all four species. The organoids had testis-specific morphology, and the Sertoli cells expressed tight junction proteins. The organoid microenvironment modulated the effects of retinoic acid on germ cells similar to testis in vivo as the germ cells on the organoids exhibited an attenuated response to retinoic acid stimulation (Lord *et al*., 2018; Sakib *et al*., 2019). Germ cells in the organoids also experienced reduced levels of autophagy compared

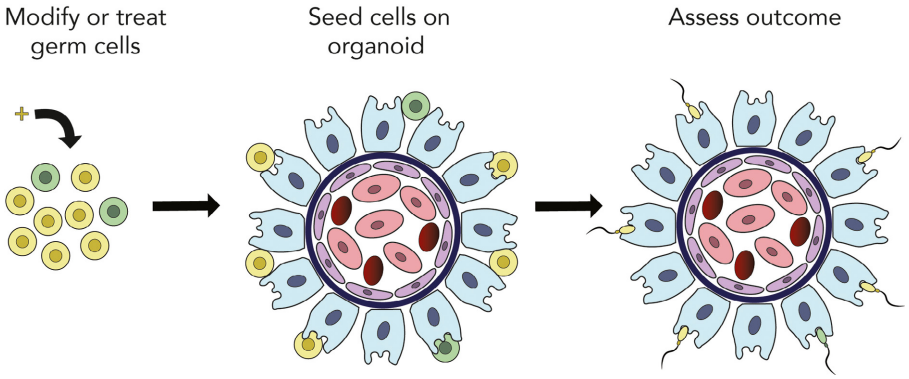


Figure 2 An approach to use testicular organoids in biomedical research. Genetically modified or treated germ cells are seeded on a premade organoid with non-treated testicular cells and assessed for outcomes such as survival, self-renewal, and spermatogenesis.

to cells cultured in 2D monolayers indicating that germ cells located in an engineered niche experience reduced cellular stress. Reproducing the findings previously obtained in tubules formed in vitro (Dores *et al.* 2017) inhibition of primary cilia on testicular somatic cells led to impaired organoid formation, highlighting the utility of organoids to study aspects of morphogenesis.

POTENTIAL APPLICATIONS OF TESTICULAR ORGANOIDS

The mammalian germ cell niche is complex. The different cells in the interstitium and seminiferous epithelium all contribute to control germ cell fate (Meng *et al.*, 2000; Kubota *et al.*, 2004; Yoshida *et al.*, 2006; Oatley *et al.*, 2009, 2011; Oatley & Brinster, 2012; Takase & Nusse, 2016; Chassot *et al.*, 2017). Less studied components of the niche such as cells of the vascular system (Yoshida *et al.*, 2007; Bhang *et al.*, 2018), peritubular myoid cells (Chen *et al.*, 2016), and testicular macrophages (DeFalco *et al.*, 2015) also contribute to niche maintenance and control of germ cell homeostasis. Organoids provide a reproducible in vitro model system to help understand the mechanisms involved in governing this niche. As organoids are generated from single cell suspensions, the system allows manipulation of key signaling pathways in specific cell types. For example, a gene of interest can be deleted or overexpressed or treatments can be performed in a particular cell population, the niche can be engineered by combining the modified cells with other testicular cell populations in organoids to enable analysis of the specific effects of the manipulation on morphogenesis and cell–cell interactions (Fig. 2). While cell type- and stage-specific genetic manipulations are possible in genetically modified mouse models, organoids can provide an alternative approach in target species where generating animal models would be logistically impossible, or to study human testis.

In vitro spermatogenesis, especially in non-rodent species, remains a challenge. The testicular microenvironment and specific cell associations are essential for germ cell homeostasis (Oatley & Brinster, 2012; Lord *et al.*, 2018). Thus, studying factors and signaling pathways that influence spermatogenesis in an organoid system will be more informative and translatable to the in vivo situation than studies in 2D culture (Fig. 2).

Testicular organoids also provide an exciting avenue for disease modeling. Different testicular cell types can be sorted, modified, and recombined to generate testicular organoids displaying specific disease phenotypes. Testicular cells from patients suffering from infertility conditions such as Klinefelter syndrome, Down syndrome, or testicular cancer can be used to generate disease-specific organoids to better understand the underlying mechanisms. Such model systems will also provide a platform for high-throughput drug screening.

Methods for derivation of germ cells from induced pluripotent cells (iPSCs) have been reported (Easley *et al.*, 2012; Zhao *et al.*, 2018). Although most of these reports have shown generation of germ like cells and haploid cells in vitro, the yield remains inefficient. Combining these protocols with testicular organoids may improve efficiency. As most of the iPSC-derived germ cells remain immature, such cells can be placed in an organoid system for further maturation. Derivation of Sertoli-like and Leydig-like cells from pluripotent cells has also been reported (Bucay *et al.*, 2009; Yang *et al.*, 2015; Rodríguez Gutiérrez *et al.*, 2018). An approach combining all these different testicular cell derivation techniques can be utilized to generate organoids entirely from pluripotent cells allowing the study of testicular development and organogenesis in vitro (Fig. 3).

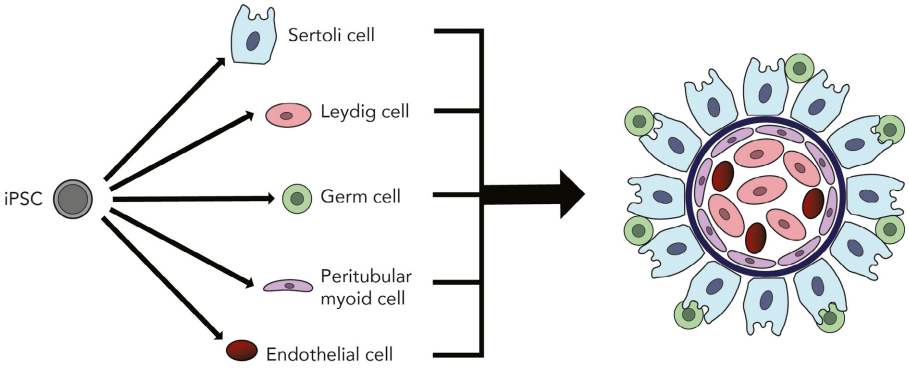


Figure 3 Schematic representation of testicular organoids to study development. Different testicular cells can be derived from iPS cells to generate testicular organoids.

CONCLUSION

Testicular organoids that bear architectural and functional similarities to their in vivo counterparts are a powerful model system to study the germ cell niche and its intricate cell interactions. This is particularly useful for studying samples in humans and other large animals where in vivo experiments are not possible. Organoids will also allow modeling of different testicular diseases and malignancies which would pave the way for more precise therapeutic interventions.

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Mini-Review

Developments in reproductive biology and medicine

Human organoid systems in modeling reproductive tissue development, function, and disease

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ABSTRACT

Research focused on human reproductive biology has primarily relied upon clinical samples affording mainly descriptive studies with limited implementation of functional or mechanistic understanding. More importantly, restricted access to human embryonic material has necessitated the use of animals, primarily rats and mice, and short-term primary cell cultures derived from human patient material. While reproductive developmental processes are generally conserved across mammals, specific features unique to human reproduction have resulted in the development of human-based *in vitro* systems designed to retain or recapitulate key molecular and cellular processes important in humans. Of note, major advances in 3D epithelial stem cell-based systems modeling human reproductive organ development have been made. These cultures, broadly referred to as organoids, enable research aimed at understanding cellular hierarchies and processes controlling cellular differentiation and function. Moreover, organoids allow the preclinical testing of pharmacological substances, both from safety and efficacy standpoints, and hold large potential in driving aspects of personalized medicine that were previously not possible with traditional models. In this mini-review, we focus on summarizing the current state of regenerative organoid culture systems of the female and male reproductive tracts that model organ development, maintenance, and function. Specifically, we will introduce stem cell-based organoid models of the ovary/fallopian tube, endometrium, cervix, prostate gland, and testes. We will also describe organoid systems of the pre-implanting blastocyst and trophoblast, as the blastocyst and its extraembryonic trophectoderm are central to fetal, maternal, and overall pregnancy health. We describe the foundational studies leading to their development and outline the utility as well as specific limitations that are unique and common to many of these *in vitro* platforms.

Keywords: ovary / fallopian tube / endometrium / cervix / prostate / testis / blastocyst / placenta / stem and progenitor cells / organoids

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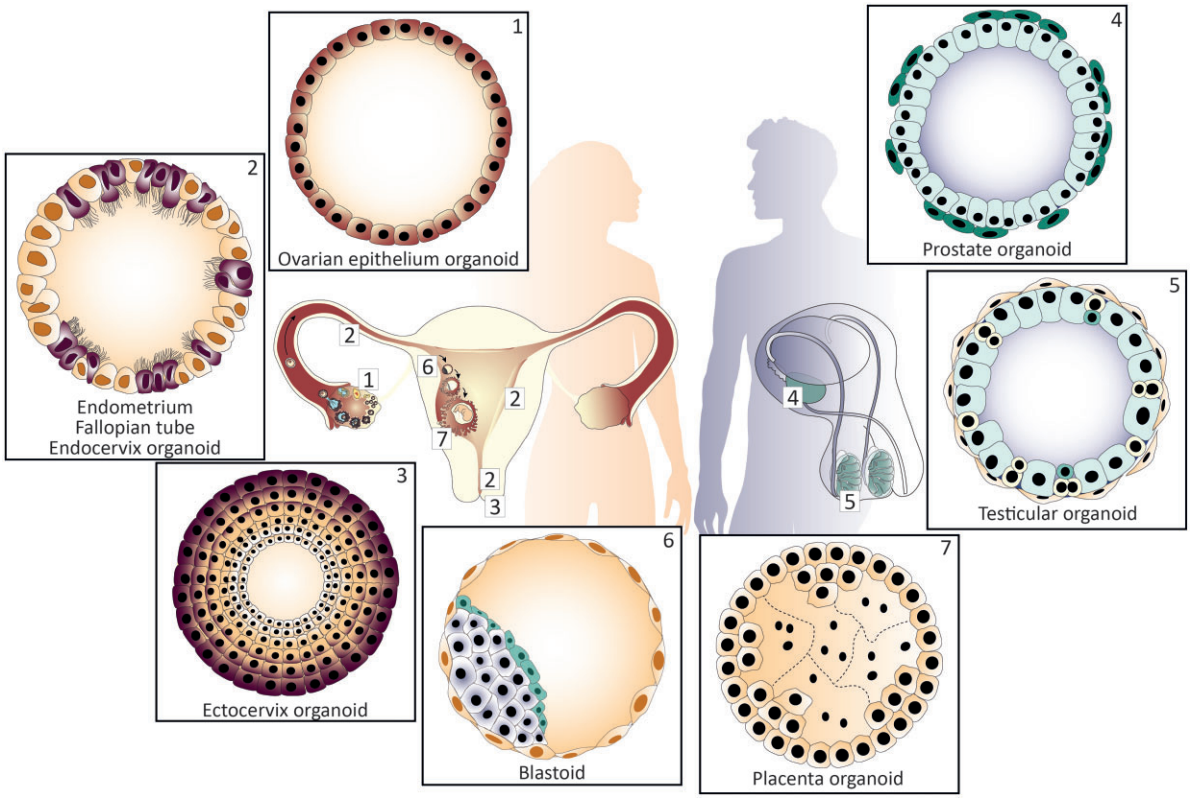
GRAPHICAL ABSTRACT



3D culture systems for reproductive tissue organogenesis

Introduction

The female and male reproductive systems are formed by the ovaries, fallopian tubes, uterus, cervix and vagina, and the testis, epididymis, vas deferens, prostate gland, and seminal vesicles, respectively. As arising and developing within the female reproductive tract, the blastocyst and placenta are related in a broader sense. Most female reproductive tract structures, such as the fallopian tube, the endometrium as the uterine inner surface, the cervix, the ovary, and the placenta, are formed by an epithelial lining and an underlying supporting mesenchyme. Indeed, well-coordinated cellular interactions play central roles in organ function and, most likely, dysfunction. Approaches used to model male and female reproductive tract biology have traditionally relied upon short-term primary cell cultures and tissue/organ explants that are challenging to establish and manipulate and are often difficult to access. Alternatively, researchers have also utilized immortalized or transformed cell lines that are karyotypically abnormal, lack genetic diversity, do not establish a basal-apical polarized orientation, and model poorly defined states of cell development (Adey *et al.*, 2013; Lee *et al.*, 2016; Abbas *et al.*, 2020). Cell lines have contributed significantly to our understanding of reproductive tract biology. However, their simplistic 2D and unicellular designs that fail to recapitulate cell spatial complexity and heterogeneity, render such model systems sub-optimal for research focused on understanding organ development and function. Likewise, while animal models that retain an intact physiology have been indispensable for the generation of knowledge in reproductive biology, species-specific differences in developmental timing, tissue cell type organization, and molecular programs controlling cell fate and function limit the utility of animal models and necessitate the complementary use of human cell-based platforms (Carter, 2007; Okae *et al.*, 2018; Ruane *et al.*, 2022).

In the last decade, 3D organoid models largely recapitulating the cellular and physiological characteristics of reproductive tract mucosal surface structures have become important tools for studying development, function, and disease in reproductive biology (Alzamil *et al.*, 2021; Chumduri and Turco, 2021; Francés-Herrero *et al.*, 2022; Yang *et al.*, 2022). Common key components for establishing such 3D cultures include a defined set of signaling molecules as well as an extracellular matrix (ECM) (such as Matrigel) that serves to foster progenitor cell-basal lamina engagement (Clevers, 2016; O'Connell and Winter, 2020). Once embedded in the ECM, cells or tissue fragments initiate spontaneous self-organization into tissue-specific organoids that recapitulate cell sub-lineages and cell patterning, and provide organ-like systems suitable for addressing processes central to regenerative maintenance, cell differentiation, and cell/tissue function (Sato *et al.*, 2009; Clevers, 2016). At the heart of self-assembling organoids are tissue-specific adult stem cells, namely pluripotent embryonic stem cells (ESC) (Reubinoff *et al.*, 2000), or induced pluripotent stem cells (iPSC) (Maherali *et al.*, 2007; Clevers, 2016). Of note, the conditions and resources designed to isolate, expand, and establish stem cell lines from human reproductive tissues are surprisingly unknown or not yet developed, with the exception of unipotent trophoblast stem cell lines (Okae *et al.*, 2018). Consequently, most reproductive organoids (RO) depend upon isolating complex stem/progenitor cell populations enriched from primary tissue sources (Karthaus *et al.*, 2014; Kessler *et al.*, 2015; Turco *et al.*, 2017; Haider *et al.*, 2018). These cell preparations facilitate long-term regenerative culture and sub-lineage differentiation potential (Kessler *et al.*, 2015; Turco *et al.*, 2017, 2018; Lõhmussaar *et al.*, 2021). Additionally, ESC- and iPSC-based RO are increasingly being used to

refine current organotypic models and have already been established for trophoblast and fallopian tube organoids, as well as blastocyst-like structures (called blastoids) (Yucer *et al.*, 2017; Castel *et al.*, 2020; Kagawa *et al.*, 2022). The blastoid has been demonstrated to self-organize into spatially conserved epiblast-, hypoblast-, and trophectoderm-like lineages (Liu *et al.*, 2021, 2022; Sozen *et al.*, 2021; Kagawa *et al.*, 2022; Pham *et al.*, 2022).

Together, these quickly evolving *in vitro* 3D systems have significantly improved the tools at hand to model most human developmental processes. Given the field's enthusiastic uptake of established organoid systems to address developmental and disease-focused questions in reproductive biology, this mini-review sets out to concisely and accurately summarize the current state of female and male RO modeling both major and well-developed organ and tissue structures, including their key advantages and limitations. Specifically, we describe organoids modeling the cellular architecture and development of the ovary, fallopian tube, endometrial lining, and cervix of the female, organoids modeling the prostate gland and testes of the male, and organoids modeling the blastocyst and placenta of the conceptus. As well, we aim to offer perspectives of where further improvements and future applications can or are being made to RO platforms.

Female reproductive tract 3D organotypic systems

Ovary

The ovary is formed by an ovarian surface epithelial layer that encapsulates the outer cortex hosting primordial follicles, and an inner medulla containing blood vessels, lymphatics, and nerves (Fig. 1). In early human development, at around 6-week post-conception, pro-ovary signals initiate the sex determination of primordial germ cells (PGCs) into oogonia (Burgoyne *et al.*, 1988; Taketo-Hosotani *et al.*, 1989; Habara *et al.*, 2021; Luo *et al.*, 2021; Ford *et al.*, 2022). Subsequently finely coordinated signals inducing mitosis, first steps of meiosis, and apoptosis result in a pool of immature oocytes surrounded by pre-granulosa cells, forming primordial follicles (Block, 1953; Mehlmann, 2005; Tiwari *et al.*, 2015). At puberty, oogenesis is continued and each month of the female reproductive life, the precise interaction between oocytes, female gonadal somatic cells (fGSCs; granulosa, theca, and stromal cells), and hormonal signals orchestrate oogenesis, ovulation, and the formation of the corpus luteum (Gougeon, 1996; Mehlmann, 2005). Notably, failures in this network are considered risk factors for infertility (due to PCOS), ovulation defects, poor oocyte quality, and cancer (Richards and Pangas, 2010).

Organoids modeling oogenesis

Rising infertility rates of 12–24% in developed countries in the last decade (Zhang *et al.*, 2022) have increased efforts to establish *in vitro* conditions for self-renewal and differentiation of PGCs. An important step forward was the discovery of persisting oocyte stem cells (OSCs) in adult ovaries thereby shifting a long-standing paradigm in reproductive biology (Johnson *et al.*, 2004). The identification of DEAD-box helicase 4 (DDX4) as a cell surface marker suitable for retrieving the rare pool of OSCs (~0.014 ± 0.002%; White *et al.*, 2012) from mouse ovaries in 2009 (Zou *et al.*, 2009) was the foundation for successfully purifying OSCs from adult human ovarian cortical tissues. Besides *DDX4*, PR domain zinc finger protein 1 (*PRDM1*), developmental pluripotency-associated protein 3 (*DPPA3*), interferon-induced transmembrane protein 3 (*IFITM3*), telomerase reverse transcriptase (*TERT*), and fibroblast growth factor receptor 3 (*FGFR3*) are widely accepted germline genes verifying OSC populations (White *et al.*, 2012; Chitiashvili *et al.*, 2022). Since then, multiple corroborating studies supported not only the existence of OSCs in adulthood across species but also demonstrated IVM of isolated OSCs into oocytes (Zou *et al.*, 2009; White *et al.*, 2012; Woods and Tilly, 2013; Alberico *et al.*, 2022). As an alternative to isolating the rare OSC pool, various studies developed PGC-like cells (PGCLC) from iPSCs or ESCs (Hayashi *et al.*, 2012; Sasaki *et al.*, 2015; Yamashiro *et al.*, 2018; Murase *et al.*, 2020), all with the same goal: to develop mature follicles releasing fertilizable oocytes. Apparently, stage-specific growth factors, hormones, and fGSCs from various prenatal time points constitute important key steps forward (Hikabe *et al.*, 2016; Herta *et al.*, 2018; Li *et al.*, 2021). Two groups established and further developed protocols using PGCLCs or isolated OSCs in 2D-like organoid conditions. Oocyte growth and maturation were achieved by timely coordinated supplementation with estrogen receptor antagonists, FSH, bone morphogenetic protein 15 (BMP15), growth differentiation factor 9 (GDF9), leukemia inhibitory factor (LIF), fibroblast growth factor (FGF), epidermal growth factor (EGF), glial cell line-derived neutrotrophic factor (GDNF), and hCG, yielding a robust number of follicles each possessing a single oocyte surrounded by cumulus and granulosa cells (Hikabe *et al.*, 2016; Li *et al.*, 2021). However, shortcomings, such as flattening of follicles as well as deviations of mechanical forces in 2D-like cultures, might provoke *in vitro* side effects (Choi *et al.*, 2014). Consequently, alternative biomimetic approaches are developed to provide appropriate ovarian scaffolds including bio-printing natural or synthetic polymers or de-cellularizing ovaries, the latter creating cell-free ECM-containing structures (Dolmans and Amorim, 2019; Xiang *et al.*, 2021). In this way, Pors *et al.* (2019) optimized de-cellularization of human ovaries and reported beneficial effects on follicle viability after re-cellularization with human ovarian stromal cells. However, a routinely applicable 3D *in vitro* protocol allowing for reproducible oocyte differentiation and maturation has not been established yet. Beside 3D oogenesis, organoid conditions for the normal human ovarian surface epithelium (Fig. 2) were established providing an *in vitro* model for studying early events of ovarian cancer (OC) (Kwong *et al.*, 2009). Along this line, OC organoids were developed by cultivating with supplements such as B27, N2, nicotinamide, n-acetylcysteine, p38 inhibitor (i), Noggin, r-spondin-1 (RSPO1), epidermal growth factor (EGF), FGF10, insulin-like growth factor 1 (IGF1), hepatocyte growth factor (HGF), neuregulin 1 (NRG1), Y27632, A8301, Forskolin, and β-estradiol (exact compositions are summarized and compared by Yang et al., 2021), and allowing for long-term cultivation and drug screening. At time of writing, several groups have established patient-derived OC organoid platforms mimicking primary tumor- and patient-specific characteristics, which represents an important step towards personalized medicine (Kopper *et al.*, 2019; Maenhoudt *et al.*, 2020; Nanki *et al.*, 2020; Yang *et al.*, 2021; Chen *et al.*, 2022). One major obstacle might be the strong dependency of oocyte development and maturation on supporting stromal cells. Accordingly, the integration of the original microenvironment, represented by stromal and immune cells, and intrinsic vascular systems will be one of the main future objectives in order to mimic the complex structures and physiology of human ovaries.

Fallopian tubes

Fallopian tubes (FTs) form the bridge between the ovaries and the uterine horns, and represent channels for oocyte and sperm

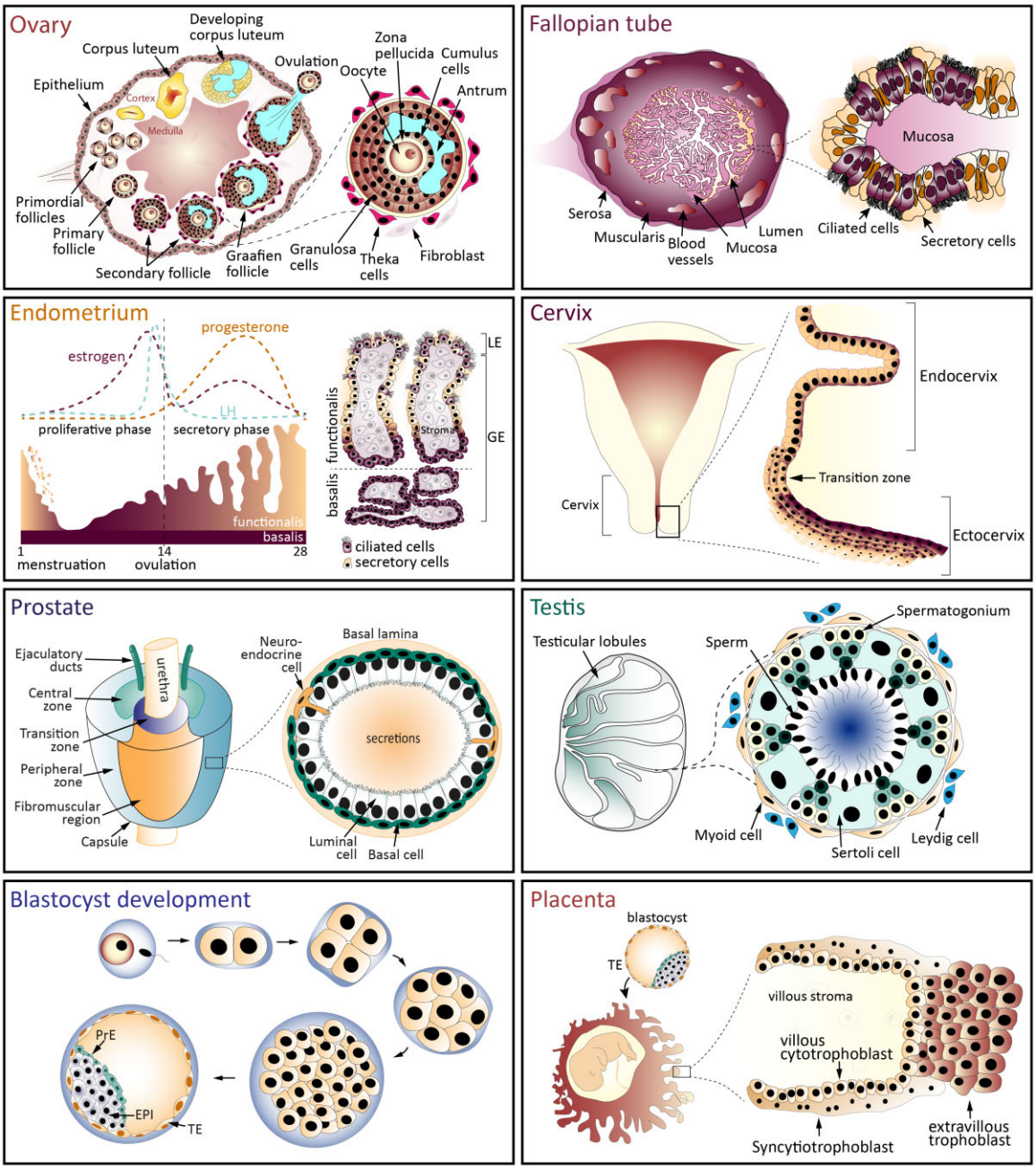


Figure 1. Morphology and cellular composition of human female and male reproductive tissues. Ovary: Each month granulosa and theca cells govern follicle development. After ovulation, corpus luteum-derived progesterone maintains pregnancy until placenta-derived hormones take control. Fallopian tube: Secretory and ciliated cells cover the mucosal surface supporting germ cell movement and survival. Endometrium: Female hormones regulate the monthly endometrial morphogenesis. Until ovulation, follicular estrogen induces endometrial proliferation while progesterone orchestrates differentiation and secretory maturation into ciliated and secretory cells of luminal epithelium (LE) and glandular epithelium (GE) segments. Cervix: The glandular epithelium of the endocervix meets the stratified squamous epithelium of the ectocervix at the transition zone. Prostate: The adult prostate is divided into one fibromuscular region and three glandular zones (central, transition, and peripheral zone). Prostate glands are composed of basal (pool of prostate stem cells), luminal (secrete proteins such as prostate-specific antigen), and neuroendocrine cells (regulatory function) (McNeal, 1981; Di Sant'Agnese, 1998; Henry *et al.*, 2018). Testis: The human testis consist of lobules containing seminiferous tubules where sperms are produced. Supported by Sertoli, Leydig, and Myoid cells, spermatogonia differentiate into mature sperm cells that are released into the duct system (Fietz and Bergmann, 2017). Blastocyst development: At 5- to 7-day post-fertilization, three founding lineages are formed: epiblast (EPI, embryonic), trophectoderm (TE, extraembryonic), primitive endoderm (PrE, extraembryonic) (Meistermann *et al.*, 2021). Placenta: The trophectoderm (TE) gives rise to placental epithelial cells, the trophoblasts. During first trimester pregnancy, villous cytotrophoblasts differentiate into hormone-producing syncytiotrophoblasts or decidua-invading extravillous trophoblasts.

transport, and eventually fertilization. Human FTs are divided in four regions (from medial to lateral: isthmus, ampulla, infundibulum, and fimbriae) and are lined by a columnar epithelium (FTE) comprising ciliated and secretory cells (Eddy and Pauerstein, 1980) (Fig. 1). Ciliated cells are equipped with rhythmically waving slender cilia whose movements are precisely orchestrated by hormone levels during the menstrual cycle (Lyons *et al.*, 2002). The number of ciliated cells is continuously increasing towards the distal end of FTs possibly supporting oocyte capture and conveyance (Crow *et al.*, 1994). Secretory cells

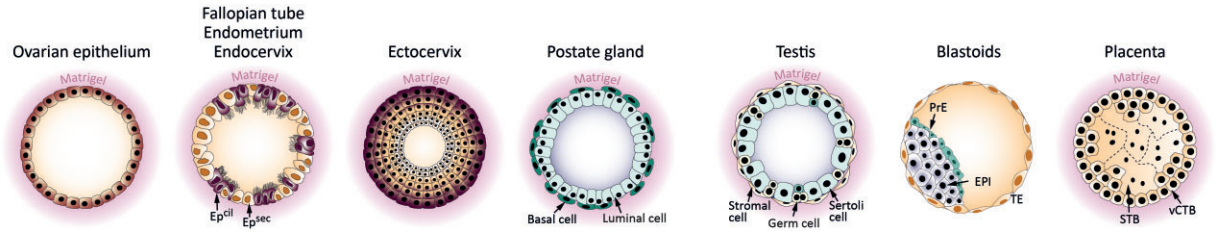


Figure 2. Organoid structures modeling female and male physiological reproductive tissues. Ovarian surface epithelial cells form a single cell-layered organoid. Under differentiating conditions, organoids of the fallopian tube, the endometrial epithelium, and the endocervix comprise ciliated (Ep^{cil}) and secretory cells (Ep^{sec}). Ectocervical organoids are formed by a stratified epithelium. Prostate gland organoids consist of luminal cells and few basal cells at the outer layers. Testis organoids are formed by germ cells and a supporting stroma, including Sertoli cells. Currently, the testis organoid illustration represents an idealized, hypothetical model. Embryonic stem cells/induced pluripotent stem cells self-organize into blastoids comprising a trophectoderm (TE) surrounding the epiblast (EPI) and the primitive endoderm (PrE). Trophoblast organoids develop outer layers of villous cytotrophoblasts (vCTB) and an inner structure of syncytiotrophoblasts (SCT). Please note that independent from the cell source, a basal-apical polarity is established towards the center of reproductive organoids.

produce a nutrient-rich fluid providing a supportive environment for survival, transport, and fertilization of the gametes (Leese *et al.*, 2001). Secretory cells are more prominent at the site of fertilization, the ampulla, which is also the most frequent region for ectopic pregnancies (Wong and Clark, 1968). Beside their role in reproduction, various studies identified FT secretory cells in precursor lesions of high-grade serous epithelial OC, which is the most common and lethal OC subtype (Levanon *et al.*, 2010; Kurman and Shih, 2011; Zheng *et al.*, 2018).

Organoids modeling fallopian tubes

First attempts of *in vitro* cultivation in 1990s struggled with difficulties of maintaining FTEs, and reported a rapid loss of differentiation characteristics under conventional 2D culture conditions (Henriksen *et al.*, 1990). Twenty years later, the first 3D cultures were performed. Paik *et al.* (2012) demonstrated that a small subset of FTE cells ($CD44^+$, $ITGA6^+$) embedded in equal amounts of Matrigel and prostate epithelial cell growth medium, can give rise to self-renewing spheres in 3D cultures. But only in 2015, Kessler and colleagues successfully established hormone-responsive FTE organoids that were maintained long-term and recapitulated the FT tissue architecture (Fig. 2). Basically, the authors embedded isolated FTE cells into undiluted Matrigel and incubated the cells in the presence of Wnt activators (RSPO1, wingless-type MMTV integration site family, member 3A (WNT3A)), transforming growth factor beta (TGFβ) inhibitors (noggin, SB431542), mitogens (EGF, FGF10), and Rock inhibitor (Y27632). Similar to the *in vivo* FTE, FT organoids comprised secretory and ciliated cells. FT organoid cultures were shown to be preserved by WNT that induces hTERT, axis inhibition protein 2 (AXIN2), cMYC, and neurogenic locus notch homolog protein 1 (NOTCH1). Interestingly, inhibition of NOTCH activity provoked a strong induction of ciliogenesis revealing a WNT-NOTCH-dependent axis ensuring stemness and self-renewal of FT organoids (Kessler *et al.*, 2015). In 2017, Yucer et al. established protocols to mimic *in vivo* FT development by using iPSCs that were first differentiated into intermediate mesoderm cells and subsequently into FTE precursors. Embedded into Matrigel, these cells also developed into hormone-responsive FT organoids comprising ciliated and secretory cells (Yucer *et al.*, 2017). In 2020, researchers achieved spheroid and organoid structures comprising ciliated and secretory cells when cultivating primary FTE on top of gelatin- or Matrigel-coated wells (Chang *et al.*, 2020). Notably, another group revealed that FTEs from the infundibulum gave rise to larger organoids and identified aldehyde dehydrogenase as a stem/progenitor FTE cell marker associated with increased organoid formation (Rose *et al.*, 2020). In summary, FT organoids derived from primary cells and iPSCs represent powerful tools to faithfully recapitulate cellular compositions in FTE subjected to cyclic hormonal fluctuations.

Endometrium

The human endometrium forms the inner lining of the uterus and comprises a single-layered columnar epithelium supported by an underlying stromal compartment. During reproductive life, it undergoes monthly remodeling of shedding, regeneration, and differentiation, a process that depends on stem cells residing in the basal layers, and hormonal regulation (Fig. 1). Until ovulation, follicular estrogen (E_2) induces endometrial proliferation while corpus luteum-derived progesterone governs differentiation and secretory maturation into ciliated and secretory cells of luminal and glandular segments. Beside hormonal regulation, cross-talk between epithelial and stromal components is assumed to ensure optimal endometrial refinement for blastocyst attachment and implantation, and maintenance of pregnancy in mammals. It is remarkable that the menstrual cycle occurs only in a limited number of mammals including primates, some species of bat, the spiny mouse, and the elephant shrew (Catalini and Fedder, 2020). The murine endometrium, for example, is not shed but controlled by an estrous cycle divided into four stages (proestrus, estrus, metestrus, and diestrus) that repeats every 4–5 days until pregnancy occurs (Byers *et al.*, 2012). Hence it might be reasonably assumed that species-specific differences would exist, hindering comparability between mouse and human. Consequently, the development of reliable *in vitro* systems modeling human endometrial glands was urgently needed.

Organoids modeling the epithelial endometrium

The first endometrial *in vitro* studies aimed to reconstruct the entire endometrium by including stromal and epithelial components. Stacks of endometrial epithelium, Matrigel, and matrix-embedded stromal cells or tissue explants preserving the initial *in vivo* tissue integrity were used to investigate hormone-driven proliferation (Arnold *et al.*, 2001; Pierro *et al.*, 2001; Illouz *et al.*, 2003). However, limited maintenance of intact histological structures and complicated assessment of proliferative responses emerged as general shortcomings in these models. In 2005, Bläuer *et al.* (2005) applied conditions for short-term culturing of hormone-sensitive epithelial organoids in 3D. Epithelial fragments were embedded in undiluted Matrigel and cultured with stromal cells from the same donors, growing on the plastic surface of the transwell, allowing for indirect cross-talk studies (Bläuer *et al.*, 2005). However, it took another 12 years until conditions were established to generate long-term cultures of

genetically stable and hormone-responsive 3D endometrial gland organoids (EG-ORG) that reproduce the molecular and histological phenotypes of the uterine cycling epithelium (Fig. 2) (Boretto *et al.*, 2017; Turco *et al.*, 2017). Highest yields of organoids were achieved by supplementing typical organoid components (N2/ITS, B27, EGF, FGF10) with the Wnt inducer R-Spondin, and the TGFβ/BMP inhibitors A83-01/Noggin. Hormonal-induced epithelial alterations were simulated by sequentially adding E_2 and progesterone. To better mimic signals from decidualized stromal cells and, eventually from a conceptus, progesterone, cAMP, prolactin, hCG, and human placental lactogen were added to E_2-primed organoid cultures, resulting in an increase of markers associated with ciliogenesis and secretory differentiation. In 2019, the mutual dependency of E_2 and NOTCH signaling during the proliferative phase was demonstrated in human EG-ORG (Haider *et al.*, 2019). Interestingly, while E_2 triggers the multiciliogenesis transcriptional program, E_2-induced attenuation of NOTCH1 signaling is required to provide the permissive environment. Notably, progesterone treatment was shown to attenuate estrogen-regulated genes (Hewitt *et al.*, 2022). These findings are in line with recent sequencing data demonstrating the tight control of secretory and ciliated lineage determination by WNT and NOTCH signaling in EG-ORG and endometrial tissue (Garcia-Alonso *et al.*, 2021). Additionally, EG-ORG were successfully developed from first trimester decidua, decidual parts attached to term placentae, and from menstrual flow, the latter two approaches highlighting non-invasive alternatives for patient-derived EG-ORG platforms (Turco *et al.*, 2017; Haider *et al.*, 2019; Marinić *et al.*, 2020; Cindrova-Davies *et al.*, 2021).

Important limitations of EG-ORGs exist including the lack of a stromal compartment, as well as the inverted orientation of the apical surface that is positioned towards the center challenging the investigation of interactions with blastocysts or pathogens. Inspired by these shortcomings, researchers developed a Matrigel-free co-culture model where endometrial stromal and epithelial cells self-organized into spheroid-like structures with a single layer of epithelial cells surrounding the stromal cells (Murphy *et al.*, 2019; Wiwatpanit *et al.*, 2020). However, these structures were only stable for 14–28 days likely because essential factors supporting long-term maintenance were not added. To address these issues, self-aggregating hormone-responsive co-cultures of human decidua-derived EG-ORG (according to published protocols) and endometrial stromal fibroblasts developed from pluripotent stem cells were tested (Cheung *et al.*, 2021). When exposed to two rounds of 16-day decidualization induced by sequential treatment of E_2 and cAMP/medroxyprogesterone acetate and separated by a 10-day period of hormone withdrawal, this co-culture system induced specific endometrial markers in the appropriate compartments and a cyclic response to alternating hormone levels. In 2021, so called endometrial assembloids were developed containing EG-ORG and single-cell suspensions of patient-matched stromal cells in hydrogel scaffolds (Rawlings *et al.*, 2021). An implantation model was further constructed by embedding human embryos into these endometrial assembloids. Interestingly, the blastocysts expanded and provoked proliferation of polar trophectoderm, however disintegration of the structures over time limited their long-term culture and experimental utility (Rawlings *et al.*, 2021). More recently, 2D re-constituted endometrial epithelial cells exhibiting apical/basal polarity obtained from hormonally stimulated EG-ORGs have been used to model blastocyst implantation using a synthetic pluripotent stem cell (PSC)-based blastoid model (Heidari Khoei *et al.*, 2023). EG-ORG have also been established from a broad spectrum of endometrial pathologies, including endometriosis, endometrial hyperplasia, and endometrial cancer, thereby widening the medical applicability (Boretto *et al.*, 2019). In summary, well-advanced EG-ORG are constantly evolving for studying physiological as well as pathological processes involved in endometrial development and function.

Cervix

The cervix connects the uterine cavity to the vagina. In doing so, one of the primary functions of the cervix is to serve as a physical barrier between the external environment and the uterus, providing protection from infection and pathogen entry. The cervix also functions to facilitate the passage of spermatozoa into the uterine cavity and plays central roles in fertility and pregnancy. In terms of positioning within the female reproductive tract and epithelial architecture, there are two major regions: the endocervix and the ectocervix (Fig. 1). The endocervix, contiguous with the uterus, is a mucosal structure characterized by columnar epithelia, whereas the ectocervix is a stratified squamous epithelial tissue; these distinct regions are separated by the squamocolumnar junctional zone sharing tissue features of each epithelial subtype (Chumduri *et al.*, 2021). Endo- and ecto-cervical regions can be distinguished by combinations of cytokeratins, including KRT7, KRT8, and KRT18, that define the endocervical region and KRT5, KRT14, and KRT17 that define the ectocervix (Chumduri *et al.*, 2021; Lõhmussaar *et al.*, 2021). Columnar endocervical epithelial cells form a glandular monolayer important for the production of mucins that line and lubricate the cervical canal. In contrast, the stratified and cornified nature of the squamous ectocervical epithelium establishes a protective barrier against many pathogen types (e.g. viral, bacterial, fungal), where expression of the transcription factor TP63 in basal progenitors of the ectocervix is essential for tissue organization and maintenance (Kurita *et al.*, 2004). While the cervix plays a central role in female health by mitigating ascending infections, it also is susceptible to viral-induced cell transformation (primarily human papilloma virus-mediated) leading to the development of cervical cancer, the second leading cause of death in females worldwide (Sung *et al.*, 2021). Critically, research focused on studying cervical homeostasis/establishment, routes of infection, and cancer development have been significantly hampered by inadequate *in vitro* human systems that have traditionally relied on genetically unstable viral-transformed and cervical cancer cell lines such as HeLa and HT-3. Below we describe the recent development of human cervical organoid systems that recapitulate ecto- and endocervical epithelial structure, as well as provide new platforms for modeling female reproductive tract infection and cervical cancer development.

Organoids modeling the cervix

In 2021, two independent studies described the establishment of adult cervical organoids (Chumduri *et al.*, 2021; Lõhmussaar *et al.*, 2021). In both instances, organoids were established from patient-derived endo- and ectocervical tissue specimens obtained from hysterectomy procedures. As with many epithelial-based organoid systems, single cell suspensions generated by combinations of mechanical and enzymatic digestion are embedded into a matrix modeling a basement membrane niche (Cultrex's Basement Membrane Extract (Lõhmussaar *et al.*, 2021) or Matrigel (Chumduri *et al.*, 2021)). Survival, progenitor expansion, and inhibition of cell differentiation are achieved by adding inhibitors targeting BMP, p38 mitogen activated protein kinase (MAPK), rho-associated protein kinase (ROCK), and TGFβ pathways, as well as

of growth (i.e. FGF and EGF) and other factors including nicotinamide, β-estradiol, and the protein kinase A (PKA) agonist forskolin (Chumduri *et al.*, 2021; Lõhmussaar *et al.*, 2021). In these culture conditions, ectocervical organoids can be maintained and passaged over 6 months and spontaneously generate correctly orientated striated squamous epithelia-like structures that closely resemble *in vivo* tissue architecture (Chumduri *et al.*, 2021; Lõhmussaar *et al.*, 2021) (Fig. 2). This media formulation is also used in endocervical organoids but requires the addition of Wnt signaling potentiators (i.e. Wnt3a, R-spondin1, or CHIR99021) (Chumduri *et al.*, 2021; Lõhmussaar *et al.*, 2021). These culture conditions are consistent with the paradigm that underlying stromal cells of the endo- and ectocervix produce factors that either promote or inhibit Wnt signaling, respectively (Chumduri *et al.*, 2021). These requirements also highlight the limitation of current cervical organoid models in that the underlying stromal compartment, including immune cells, is not fully recapitulated.

Even with limitations related to the lack of cellular complexity, human cervical organoids open new opportunities for studying not only cervical biology, but also cervical-related diseases. To date, cervical organoids have been used in modeling HIV infection (Lõhmussaar *et al.*, 2021), and cervical tumor pap-brush sampling has led to the generation of both squamous and adenocarcinoma-like organoids that could be expanded for long periods of time displaying varying degrees of chromosomal instability (Lõhmussaar *et al.*, 2021). Together, both hysterectomy and pap-brush sampling approaches used to generate normal and malignant cervical organoids establish important *in vitro* tools for modeling viral-induced infection and transformation, and create opportunities for precision patient-focused medicine.

Male reproductive tract 3D organotypic systems

Prostate

The human prostate is a male reproductive gland, which lies directly beneath the bladder and wraps around the urethra and the ejaculatory ducts. Histologically it can be divided into five different zones: the central zone, the peripheral zone, the transition zone, a fibromuscular region, and a surrounding capsule (Fig. 1) (Singh and Bolla, 2022). The glands, comprising basal cells (BC), luminal cells (LC) and neuroendocrine cells, produce alkaline seminal fluid for balancing the vaginal acidity thereby supporting the sperm lifespan (Henry *et al.*, 2018; Singh and Bolla, 2022).

Organoids modeling prostate gland development and disease

Various research papers, for example Lang *et al.* (2001), have demonstrated the cultivation and characteristics of spheroids as well as ECM-based 3D cultures derived from primary prostate cells and cell lines, as summarized by Patrício *et al.* (2022). However, in 2014, the group of Hans Clevers were the first to establish defined conditions for long-term cultivation of genetically stable human prostate organoids that resembled the *in vivo* composition of prostate glands. Isolated prostate cells cultivated in Matrigel and culture medium supplemented with EGF, Noggin, RSPO1, A8301, dihydrotestosterone, FGF10, FGF2, prostaglandin E2 (PGE2), nicotinamide, and p38i gave rise to single- and double-layered organoids after 2–3 weeks (Karthaus *et al.*, 2014). These structures mainly contained LC with some BC at the outer layers; neuroendocrine cells were not detectable (Fig. 2). The organoids exhibited key features such as the expression of prostate-specific antigen and intact androgen receptor signaling. In this work as well as other corroborating studies, individual organoids were formed from either BC or LC. Notably, significant differences between BC- and LC-derived organoids were observed. BC were highly efficient at establishing organoids, but these organoids predominantly consisted of BC and only sporadically formed luminal structures. LC-derived organoids were formed at a much lower efficiency, but they immediately formed lumen. Interestingly, BC were detectable indicating that luminal cells could generate BC (Karthaus *et al.*, 2014; Park *et al.*, 2016). Following these studies, prostate organoids were also developed from ESCs (Calderon-Gierszal and Prins, 2015) and iPSCs (Hepburn *et al.*, 2020) representing prostate organoid formation independent of primary tissue. In addition to benign/non-cancerous prostate organoid modeling, cancer organoids from various cancer states have been also been developed (Gao *et al.*, 2014; Drost *et al.*, 2016; Puca *et al.*, 2018). However, shortcomings highlighting that initially detectable prostate cancer hallmarks are lost in organoid culture, and metastasis-derived organoids form at a low efficiency and stability, suggest that the current culture conditions are optimized for physiologically normal tissue organoids (Servant *et al.*, 2021). In summary, prostate organoids represent a powerful tool to investigate prostate gland development and function, however, there is a need to optimize the culture conditions supporting cancer cell phenotypes.

Testis

Starting at early puberty, spermatogenesis is a continuous process occurring in the seminiferous epithelium of the testis, and is supported by various stromal cells (Li *et al.*, 2014) (Fig. 1). Specifically, Sertoli cells orchestrate the maturation of spermatogonia into sperm, Myoid cells contribute to the structure of the testis and promote the export of mature sperms to the seminal vesicles, and androgen-producing Leydig cells provoke masculinization before birth and support testosterone production in the adult (Wilhelm *et al.*, 2007; Zirkin and Papadopoulos, 2018). Furthermore, the testicular structure comprises another characteristic, the blood–testis barrier (BTB), which separates the blood from the seminiferous tubules. Unlike other blood–tissue barriers, including the blood–brain barrier and the blood–retina barrier that are formed by tight junctions between endothelial cells surrounded by pericytes and other supporting cells, the mammalian BTB comprises specialized junctions between adjacent Sertoli cells close to the basement membrane (Cheng and Mruk, 2012). It separates a basal (spermatogonial renewal and differentiation) from an apical (spermiogenesis) compartment, however, the BTB is not a static structure but undergoes restructuring for passing differentiating spermatocytes towards the lumina (Russell and Peterson, 1985). Testicular tumors account for only 1% of all tumors in males where more than 95% are of germ cell origin with an overall good prognosis for even widely metastasized tumors (Feldman, 2008; Moch *et al.*, 2016). This could explain why organoids modeling testicular cancers have not been developed yet.

Organoids modeling spermatogenesis

Male infertility and the lack of reliable male-dependent birth control inspired numerous researchers to develop *in vitro* 3D artificial testis (AT) mimicking testicular cyto-architectures for studying and manipulating human spermatogenesis. Various ECM proteins, such as collagen gels (Lee *et al.*, 2007), Matrigel (Sun *et al.*, 2018; Oliver *et al.*, 2021), soft agar (Gholami *et al.*, 2018), or decellularized testicular scaffolds (Baert *et al.*, 2017) are utilized to form scaffolds for dissociated testicular cells. Indeed, cell

type-specific marker expression identified Leydig cells [STAR (steroidogenic acute regulatory protein)], 3 beta-hydroxysteroid dehydrogenase (3βHSD), telomerase associated protein 1 (TEP1), Myoid cells [actine alpha 2, smooth muscle (ACTA2), CD34], Sertoli cells [sry-box transcription factor 9 (SOX9), clusterin (CLU), gata binding protein 4 (GATA4)], and germ cells [thy-1 cell surface antigen (THY1), promyelocytic leukemia zinc finger protein (PLZF), ubiquitin C-terminal hydrolase L1 (UCHL1), DEAD-box helicase 4 (DDX4)] in AT structures (Baert *et al.*, 2017; Pendergraft *et al.*, 2017; Oliver *et al.*, 2021). However, the limited germ cell maintenance represents one of the current obstacles (Baert *et al.*, 2017; Oliver *et al.*, 2021). A recent case report colonizing bio-printed hydrogel structures with adult human testicular cells reported enhanced gene expression involved in spermatogonial stem cell maintenance, even after 12 days (Robinson *et al.*, 2022). Another study mixing testicular cells, specific culture media, and extracted human testis ECM into hanging drops achieved extended maintenance and differentiation of germ cells for more than 30 days (Pendergraft *et al.*, 2017). To the best of our knowledge, a functional Sertoli cell-formed BTB, expressing tight junction proteins and being impermeable for Evans blue dye, was only demonstrated in a specialized three-layer model developed from dissociated testicular rat cells where cell-containing Matrigel was placed between two layers of cell-free Matrigel (Alves-Lopes *et al.*, 2017). Taken together, AT represent a seminal *in vitro* tool for studying spermatogenesis, however there is an explicit need for improving maintenance and differentiation of male germ cells in order to advance developmental medicine.

Pregnancy-related 3D organotypic systems

Pre- and post-implantation embryos

The development of the human embryo takes place within the female reproductive tract and, as such, cellular events during pre- and post-implantation are difficult to study. Traditionally, aspects of embryo development have been modeled using pluripotent stem cells derived from the epiblast lineage or by using iPSC that, when cultured in 2D, have generated important insight into epiblast lineage maintenance and early stages of embryo development (D'Amour *et al.*, 2005; Linneberg-Agerholm *et al.*, 2019; Mackinlay *et al.*, 2021). Likewise, surplus human blastocysts donated to research have contributed enormously to our understanding of cellular events in early human development (Kang *et al.*, 2014; Shahbazi *et al.*, 2016, 2017). However, the use of human embryonic tissues and blastocysts is rare and their use in research is subject to major ethical restrictions, limiting the overall utility of this material.

3D systems modeling blastocyst development

Recently, major advances in the development of *in vitro* stem cell-based blastocyst models have been made. Specifically, stepwise iterations of expanded PSCs (Sozen *et al.*, 2021), embryonic PSC lines (Yanagida *et al.*, 2021; Yu *et al.*, 2021; Kagawa *et al.*, 2022), commercial iPSC lines (Yanagida *et al.*, 2021; Yu *et al.*, 2021; Kagawa *et al.*, 2022), and iPSCs derived from reprogrammed patient fibroblasts (Liu *et al.*, 2021) have been used under varying conditions to generate blastocyst-like structures that morphologically resemble pre-implantation embryonic Day 6 human blastocysts. Collectively termed blastoids and established with varying degrees of efficiency (2–80%), PSCs first must be cultured in media that promote a naïve pluripotent state [using inhibitors targeting mitogen-activated protein kinase (MEK), WNT, B-raf proto-oncogene (BRAF), ROCK, and SRC proto-oncogene, non-receptor tyrosine kinase (SRC), and the growth factors LIF and Activin A, abbreviated as 5i/L/A (Theunissen *et al.*, 2014) or using inhibitors targeting MEK, WNT, and atypical protein kinase C, and recombinant LIF (abbreviated as PXGL) (Rostovskaya *et al.*, 2019)]. Importantly, this pluripotent naïve state enables cell differentiation into all three founding cell lineages and, when placed in non-adherent culture vessels that promote 3D self-assembly, PSC-derived cells spatially organize into epiblast- ($OCT4^+$, $NANOG^+$, $SOX2^+$), hypoblast- ($GATA4^+$, $GATA6^+$, $SOX17^+$), and trophectoderm-like ($CDX2^+$, $GATA3^+$, $TFAP2C^+$) cells with a blastocoel-like cavity (Fan *et al.*, 2021; Liu *et al.*, 2021; Sozen *et al.*, 2021; Yanagida *et al.*, 2021; Yu *et al.*, 2021; Kagawa *et al.*, 2022) (Fig. 2). Variations in cell plating number and media pulses that independently prioritize the expansion of trophectoderm, epiblast, and hypoblast lineages have been described; the approach using PXGL-derived naïve PSCs initially exposed to trophoblast promoting HIPPO (lysophosphatidic acid), TGFβ (A38-01), ERK (PD0325901), and ROCK (Y-27632) chemical inhibitors in conjunction with LIF (Kagawa *et al.*, 2022) produce self-organizing blastoids that most closely recapitulate blastocyst morphology, lineage-specific molecular programs, and the step-wise processes of implantation (Kagawa *et al.*, 2022).

While still in their infancy, blastoid models provide exciting *in vitro* platforms to study pre-and post-implantation embryogenesis akin to human E5–E8 of development, as well as cellular and molecular processes governing blastocyst implantation. Relevant to the latter, using a blastoid-endometrial epithelial cell co-culture system, Kagawa *et al.* (2022) showed the importance of epiblast-polar trophectoderm cross-talk in instructing blastocyst attachment. Moving forward, blastoids can serve as tools central to understanding human developmental biology (particularly pre/post-implantation processes), early pregnancy loss, chemical screening and safety for embryos, and even patient-focused fertility assessments.

Placenta

Specialized placental cells called trophoblasts, derived from the trophectoderm of the implanting blastocyst, perform many of the placenta's transport, endocrine, and immunogenic functions (Fig. 1) (Knöfler *et al.*, 2019; Turco and Moffett, 2019). In rodents and primates that have haemochorial placentas, nutrient and oxygen transfer between mother and fetus is controlled in part by the actions of syncytium-forming and invasive trophoblast subtypes (Abbas *et al.*, 2020; Aplin and Jones, 2021). While rodent and human trophoblasts perform similar functions, trophoblast developmental trajectories in rodents and humans are different. These differences are underlined by distinct species-specific molecular programs controlling trophoblast stem cell maintenance and differentiation (Kunath *et al.*, 2014; Blakeley *et al.*, 2015; Petropoulos *et al.*, 2016; Okae *et al.*, 2018). Indeed, conditions for mouse trophoblast stem cell (mTSC) establishment were first reported in 1998 (Tanaka *et al.*, 1998), 20 years prior to the derivation of the human equivalent (Okae *et al.*, 2018). While mTSCs require exogenous FGF4 and TGFβ1, human (h)TSC selection necessitates EGF, canonical Wingless/Integrated (Wnt) activity, and suppression of TGFβ signaling (Okae *et al.*, 2018). Because of these distinctions, significant limitations exist when using rodent models to study human trophoblast stem cell biology. Concurrently, major ethical constraints, difficulties in accessing first trimester placental tissue for research, and sub-optimal *in vitro* systems have also limited progress in human placental research.

Organoids modeling placental development

In 2018, three separate reports described the establishment of 2D (Okae *et al.*, 2018) and 3D (Fig. 2) (Haider *et al.*, 2018; Turco *et al.*, 2018) long-term regenerative trophoblast cultures. Derived from progenitor cytotrophoblasts (CTB) of first trimester chorionic villi (<8 weeks gestation), 2D hTSC lines and 3D trophoblast organoids exhibit key trophoblast identity criteria. For example, hTSCs and trophoblast organoids express trophoblast lineage genes [*GATA3*, keratin 7 (*KRT7*), transcription factor AP-2 gamma (*TFAP2C*), lack expression of class I HLA molecules, though this is contentious (Sheridan *et al.*, 2021)], exhibit hypomethylation of the ETS transcription factor 5 (*ELF5*) promoter, and express chromosome 19 miRNA cluster micro RNAs (Lee *et al.*, 2016). Stem-like states in 2D and 3D can be perpetuated long-term in the presence of WNT-activating and EGF signals concurrent with TGFβ and ROCK inhibition (Haider *et al.*, 2018; Okae *et al.*, 2018; Turco *et al.*, 2018). Whereas both hTSC line- and primary CTB-derived organoids spontaneously fuse to generate hormone-producing multinucleated syncytiotrophoblast (SCT) scattered within the organoid's core, hTSC lines cultured in 2D require forskolin to form SCT (Okae *et al.*, 2018; Sheridan *et al.*, 2021). In both 2D and 3D conditions, the removal of Wnt-activating factors promotes CTB differentiation into invasive matrix-degrading extravillous trophoblasts (EVT).

Trophoblast organoids provide a substantial improvement over past *in vitro* trophoblastic systems where trophoblast cell lineage complexity largely mirrors cell composition *in vivo* (Haider *et al.*, 2018; Turco *et al.*, 2018). Furthermore, cell differentiation trajectories along the extravillous and villous pathways assessed by single cell transcriptomics show overall good correlation (Shannon *et al.*, 2022a,b). However, limitations in the organoid model remain, especially with respect to the inverted orientation placing the syncytial-like structures within the organoid's core and the simplistic nature of the model that excludes extraembryonic mesoderm and decidual cell types that interact with and likely shape trophoblast biology *in vivo*; regarding the former limitation, a recent preprint from the Coyne laboratory describes an approach that corrects the inverted nature of the organoid (Yang *et al.*, 2023). By subjecting organoids initially established in Matrigel (i.e. inverted orientation) to suspension culture combined with gentle agitation, Yang *et al.* (2023) describe a system that produces large syncytial structures (>60 nuclei) correctly situated on the outside of the organoid that secrete high levels of SCT-associated factors, such as hCG.

An additional consideration when designing a trophoblast organoid system relates to the choice of trophoblast progenitor cell source to use. In addition to freshly isolated primary CTB preparations, TSC lines can generate organoids that recapitulate an inverted CTB/SCT orientation. However, TSC line-derived organoids may resemble more closely EVT progenitor-like cells, both transcriptionally and in their surface expression of ITGA2 and HLA-G (Sheridan *et al.*, 2021). Moreover, TSC lines cultured in 2D or 3D show detectable levels of Class I HLA-A/B, where 3D culture appears to dampen, but not eliminate, their expression (Sheridan *et al.*, 2021). A recent preprint supports these findings, showing that progenitors in hTSC line-derived organoids resemble a developmentally downstream state that is transcriptionally akin to column CTB (Shannon *et al.*, 2022b). Given that hTSC lines are commercially available, more accessible than freshly isolated CTBs, and provide major improvements over pre-existing choriocarcinoma and immortalized cell lines for studying trophoblast biology, a careful assessment of both the strengths and weaknesses of hTSC lines is warranted. To complicate this further, new reports describing the derivation of hTSC lines from PSC sources (Wei *et al.*, 2021; Karvas *et al.*, 2022; Soncin *et al.*, 2022) and from the direct reprogramming of adult somatic cells (called induced TSCs) (Castel *et al.*, 2020; Tan *et al.*, 2022), that in theory can or have (Karvas *et al.*, 2022) been applied to organoid conditions, will also need to be carefully evaluated.

To date, trophoblast organoids have been used to examine molecular processes governing stem cell maintenance (Meinhardt *et al.*, 2020; Shannon *et al.*, 2022a) as well as cell differentiation along the villous (Meinhardt *et al.*, 2020) and extravillous (Haider *et al.*, 2022) pathways. For example, chemical inhibition as well as CRISP-Cas9-mediated knockout of the Hippo signaling-dependent transcriptional co-activator YAP1 in CTB- and JEG-3-derived trophoblast organoids, respectively, led to a profound decrease in progenitor cell proliferation and results in unrestrained differentiation into SCT-like structures, demonstrating that YAP1 signaling is critical for trophoblast progenitor maintenance (Meinhardt *et al.*, 2020). In trophoblast organoids instructed to differentiate into EVT, a time-delayed exogenous TGFβ impulse is required for the expression of many markers characteristic of mature, decidua-embedded EVTs (e.g. FN1, DAO), whereas TGFβ inhibition, essential for EVT progenitor formation, results in the development of pro-migratory/pro-invasive features, highlighting that decidual cell-derived TGFβ plays a central role in controlling EVT development (Haider *et al.*, 2022). Trophoblast organoids have also been used to examine routes of vertical viral infection and subsequent trophoblast-mediated inflammatory responses in decidual cells. For example, in response to human cytomegalovirus infection, trophoblast organoids induce a Type III interferon response that leads to protection of decidual cells (Yang *et al.*, 2022). iPSC-derived TSC organoids have also been used to examine ZIKA and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus interactions with trophoblasts (Karvas *et al.*, 2022). While serving as useful tools to model infection, both the inverted nature of the trophoblast organoid and expression of MHC class I ligands normally not expressed by progenitor CTB or SCT does highlight important limitations of this system that necessitate the careful interpretation and/or tempering of findings related to pathogen-host modeling.

Current challenges, future directions, and conclusions

While the RO platforms described here provide important *in vitro* advancements over previous cell culture systems, major limitations exist and opportunities for model-improvement are still needed. Two common limitations shared across all ROs are the lack of cellular complexity and the absence of an intact physiology (i.e. vascular system, immune system). The uni-lineage nature of ROs that are primarily epithelial cell based, while advantageous from both reductionist and reproducibility standpoints, does ignore critical cross-talk between mesenchymal and immune cell populations that most likely shape organ development and function. Attempts to increase cellular complexity have made use of ROs co-cultured with stromal (i.e. trophoblast and endometrial organoid co-cultures) (Yang *et al.*, 2022) or immune cells (i.e. endometrial organoids with peripheral blood leukocytes) (Dolat and Valdivia, 2021). While conceptually appealing, particularly with respect to the incorporation of reproductive mucosal immune cell populations, such as tissue resident macrophages and natural killer cells into the RO culture design, important challenges remain. For instance, the

phenotype and function(s) of mucosal immune cells residing within both the female and male reproductive tract are thought to be fined-tuned by their tissue environments, and when transplanted into *in vitro* culture rapidly change their phenotype and are no longer representative of the *in vivo* biology. Along this line, we should keep in mind that RO cultivation based on the manipulation of essential signaling pathways, such as Wnt and TGFβ, was specifically optimized for supporting epithelial growth and differentiation. Therefore, it is likely that in co-culture scenarios mesenchymal cells will respond differently to pathway manipulations thereby compromising interpretation of experimental outcomes.

The application of microfluidic organ-on-chip systems that recapitulate endothelial-epithelial cell cross-talk, tissue permeability, and the effects of fluid shear stress on cellular processes, to our knowledge, has not been applied to female RO systems. However, organ-on-chip systems using cell lines and primary cells cultured in 2D have been used to model the placenta barrier (Blundell *et al.*, 2016; Nishiguchi *et al.*, 2019), endometrial physiology (Ahn *et al.*, 2021), and endo- and ecto-cervical epithelial cell interactions (Tantengco *et al.*, 2022). Additionally, multi-organ-on-chip testes and liver organoid co-cultures have been established to model hepatic cell metabolism of androgens (Baert *et al.*, 2020). Taken together, these advances suggest the broader use of RO platforms incorporating microfluidic systems to better model organ physiology will be the major next steps in RO culture.

Lastly, another refinement that will need to be considered for RO relates to the basement membrane ECM used. Currently, Matrigel or similar mouse tumor-derived basement membrane products (e.g. Cultrex BME, Geltrex) are most often used in organoid culture. These products are enriched for the basement membrane proteins collagen IV and laminin that help to establish cell-matrix anchoring and polarity of progenitor cells required for organoid establishment. However, the composition and concentration of specific factors within these products are poorly defined and vary according to lot production. Moreover, control of matrix stiffness is difficult to achieve using Matrigel-like products, and therefore the ability to replicate and maintain substratum stiffness of specific reproductive tissue progenitor niches is not possible. To address this, the use of stable hydrogel scaffolds incorporating basement membrane matrix fragments designed to engage specific cell surface receptors are now beginning to be used in spheroids and organoids (Cruz-Acuña *et al.*, 2017; Gjorevski *et al.*, 2022). At the same time, decellularized tissue-specific matrix extracts are also being tested as improvements over Matrigel (Giobbe *et al.*, 2019). Decellularization requires a combination of enzymatic, chemical, and physical removal of cells resulting in a scaffold comprising the complex mixture of structural and functional proteins (Gilbert *et al.*, 2006; Francés-Herrero *et al.*, 2022). So far, this approach has been successfully applied for reconstructing a human ovary (Pors *et al.*, 2019; Pennarossa *et al.*, 2021), growth and differentiation of human spermatogonial stem cells (Bashiri *et al.*, 2021; Salem *et al.*, 2023), and for constructing a 3D endometrium (Olalekan *et al.*, 2017).

The establishment and refinement of reproductive tissue organoid platforms have led to major improvements over traditional human primary and cell line models. Already, human RO have generated novel insights into the molecular drivers controlling reproductive tissue development. Their use in modeling human disease development and pharmacological screening, especially when complemented with existing animal models, will likely lead to improvements in our understanding of reproductive biology and disease.

Data availability

No new data were generated or analysed in support of this research.

Authors' roles

Conceptualization: S.H. and A.G.B.; Writing—original draft: S.H. and A.G.B.; Writing—review and editing: S.H. and A.G.B.; Funding acquisition: S.H. and A.G.B.; Illustration concept: S.H. and A.G.B; Illustration design and performance: S.H.

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Conflict of interest

The authors declare that no competing interests exist.

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