Spring 2022 – 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 3, 2022
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. Richer, et al. (2020) Andrology 8(4):879-891

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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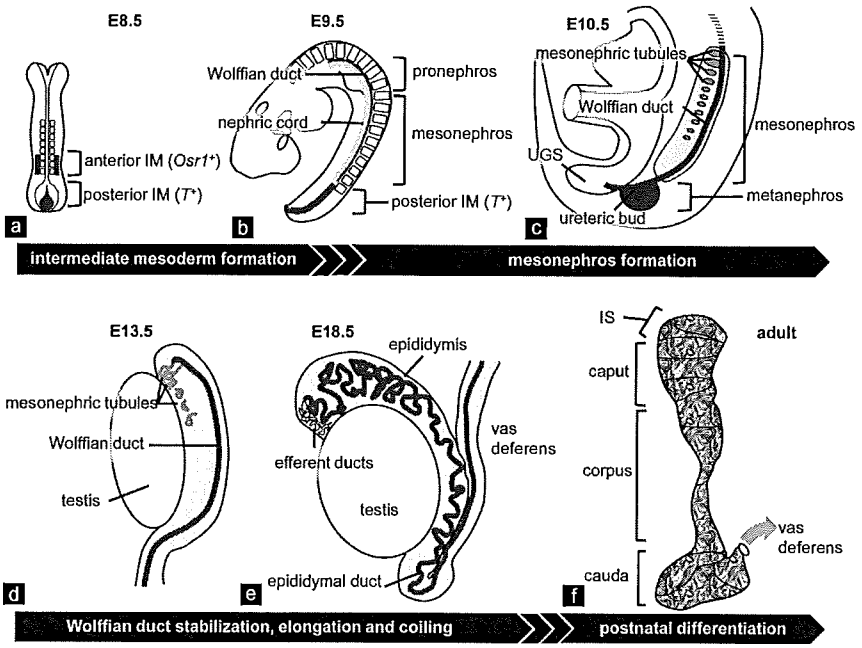

Figure 1: Schematic diagram of mouse Wolffian/epididymal duct development. (**a–c**) The origin of the epididymis is the intermediate mesoderm. Spatiotemporally distinct intermediate mesoderm at E8.5 gives rise to the WD and metanephric mesenchyme.[37] The anterior intermediate mesoderm, which gives 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

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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

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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

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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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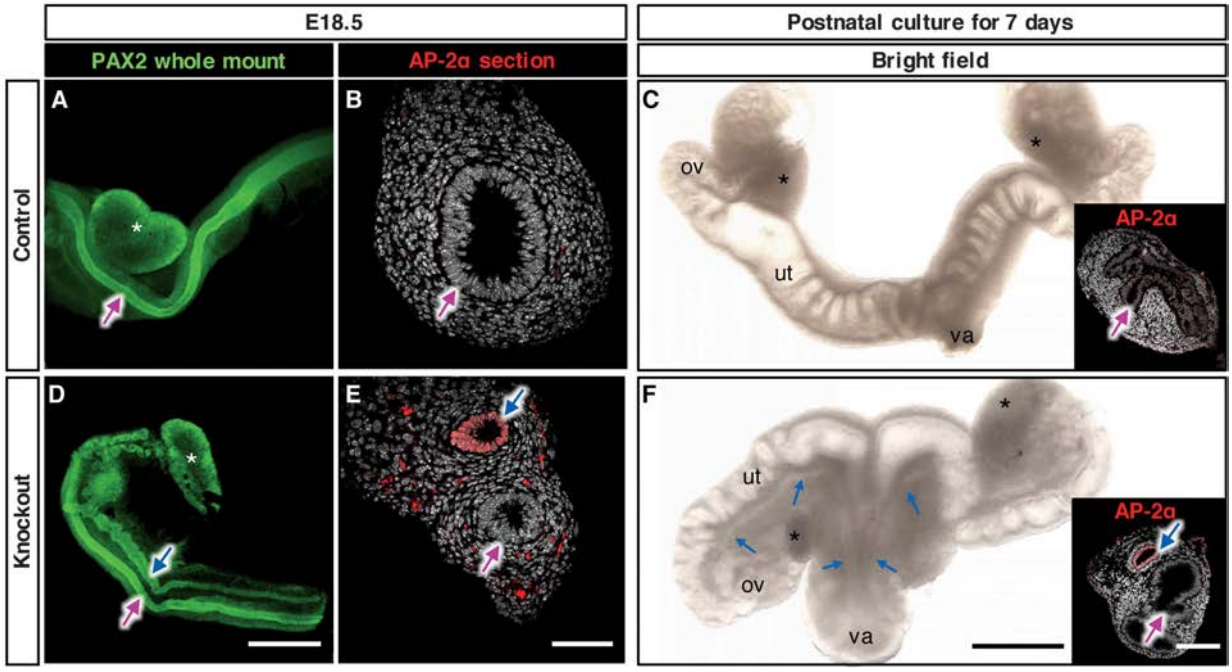

Fig. 1. *Coup-tfII* ablation leads to Wolffian duct retention in the XX embryo. Control and *Coup-tfII* knockout reproductive systems from E18.5 XX embryos were analyzed with (**A** and **D**) whole-mount immunofluorescence for the epithelial marker PAX2 or (**B** and **E**) AP-2α on frozen sections. (**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).

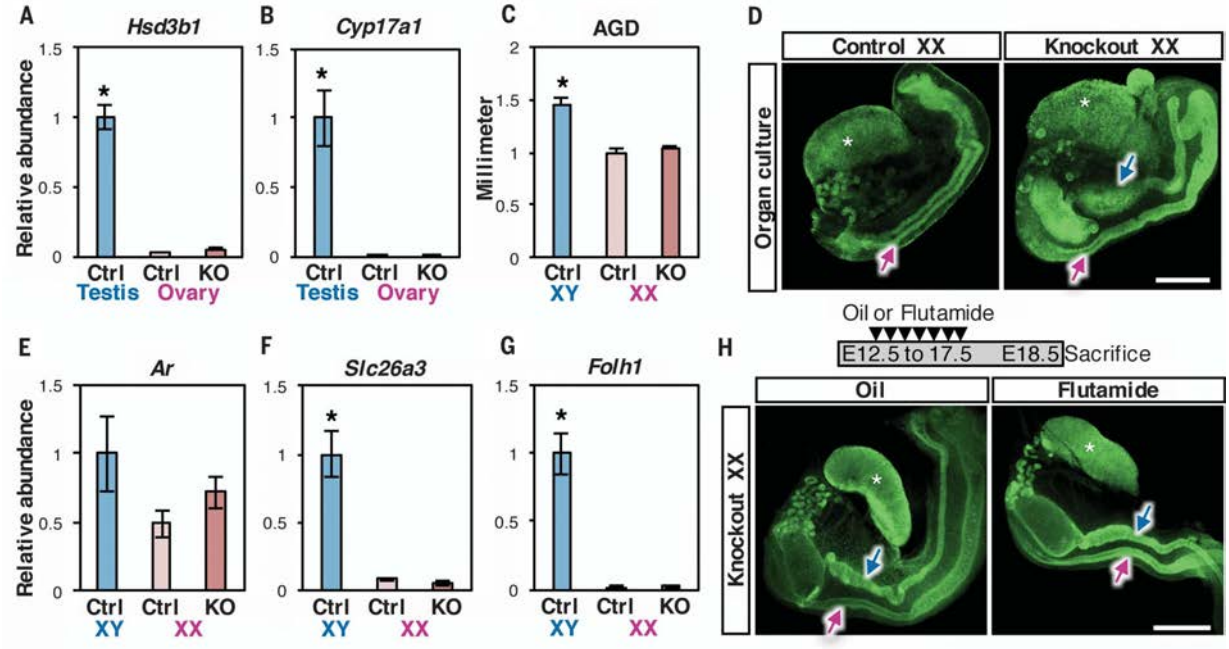

Fig. 2. Wolffian duct retention in *Coup-tfII* knockout XX embryo is independent of androgen production or action. (**A** and **B**) mRNA expression of two rate-limiting steroidogenic enzymes, *Hsd3b1* and *Cyp17a1*, in E14.5 control testis (light blue), control ovary (light pink), and knockout ovary (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.

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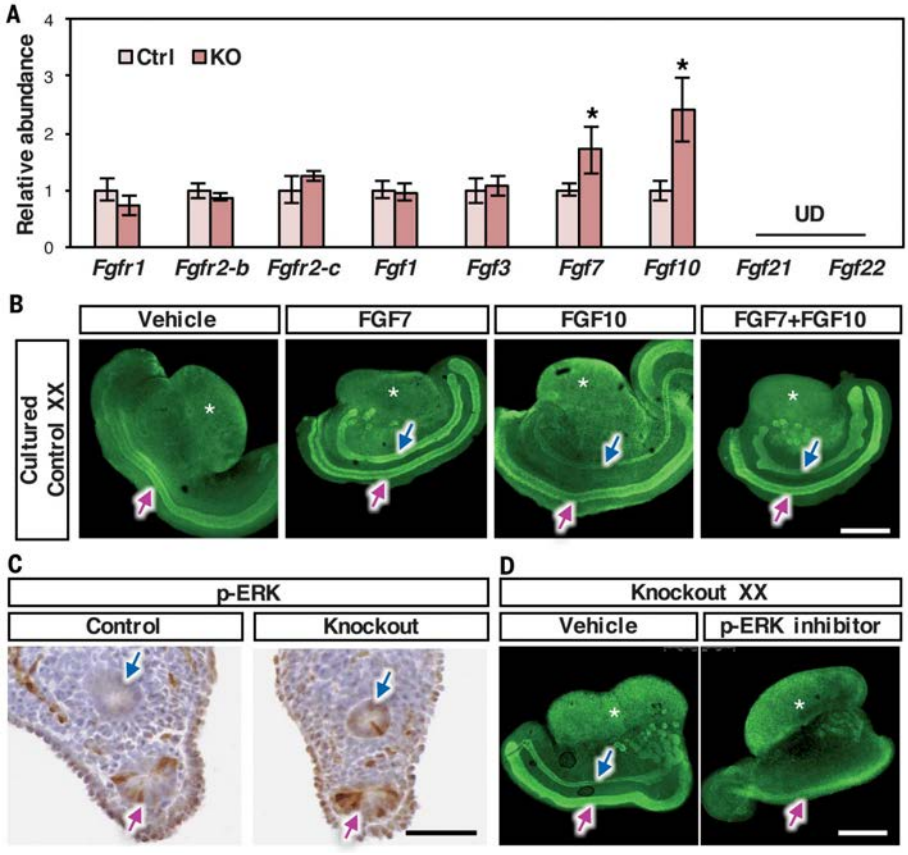

Fig. 3. Enhanced FGF signaling is involved in Wolffian duct retention in the absence of *Coup-tfII*. (**A**) mRNA expression of FGF receptors and ligands in the mesonephroi at E14.5. UD, undetected. Results are shown as mean ± SEM. Asterisks represent statistical significance of $P < 0.05$ compared with 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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Science

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

Science **357** (6352), 717-720.
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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.

Science, this issue p. 717; see also p. 648

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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

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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).

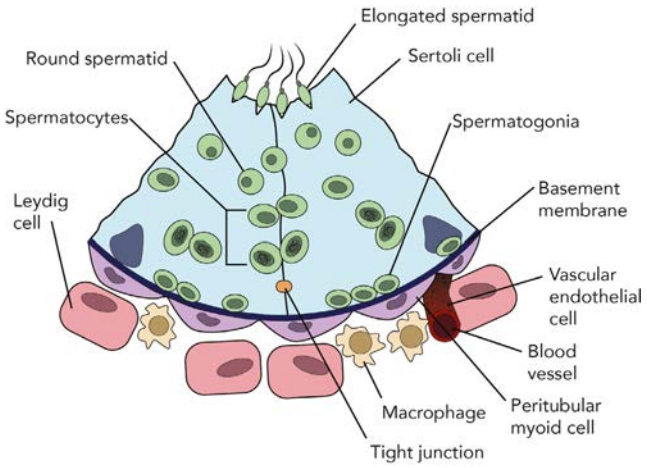

Figure 1 Schematic representation of the germ cell niche. Undifferentiated germ cells including spermatogonial stem cells are localized at the basement membrane and remain in close contact with the Sertoli cells inside the seminiferous epithelium. Peritubular myoid cells line the outer perimeter of the 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).

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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

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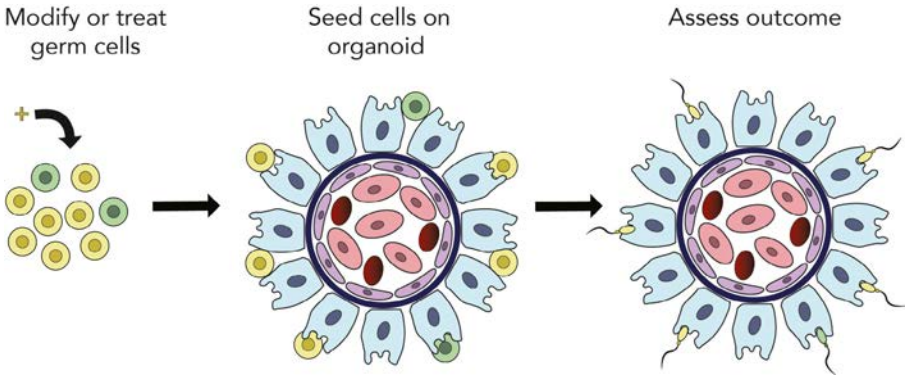

Figure 2 An approach to use testicular organoids in biomedical research. Genetically modified or treated germ cells are seeded on a premade organoid with non-treated testicular cells and assessed for outcomes such as survival, self-renewal, and spermatogenesis.

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).

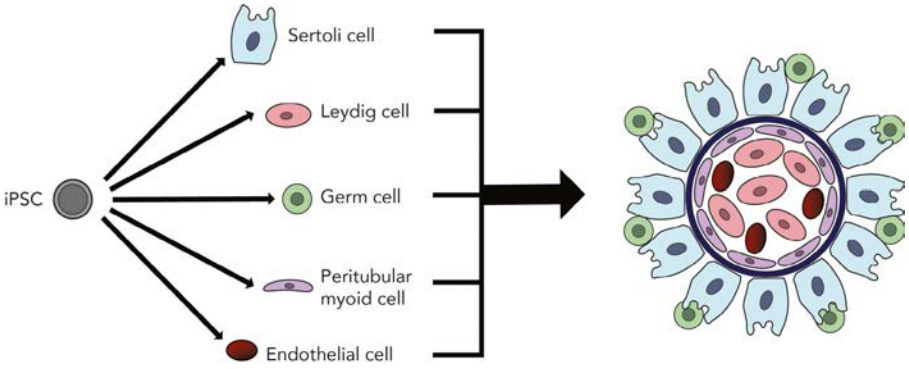

Figure 3 Schematic representation of testicular organoids to study development. Different testicular cells can be derived from iPS cells to generate testicular organoids.

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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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REVIEW ARTICLE

ANDROLOGY WILEY

In-vitro spermatogenesis through testis modelling: Toward the generation of testicular organoids

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Abstract

Background: The testicular organoid concept has recently been introduced in tissue engineering to refer to testicular cell organizations modeling testicular architecture and function. The testicular organoid approach gives control over which and how cells reaggregate, which is not possible in organotypic cultures, thereby extending the applicability of in-vitro spermatogenesis (IVS) systems. However, it remains unclear which culture method and medium allow reassociation of testicular cells into a functional testicular surrogate in-vitro.

Objective: The aim of this paper is to review the different strategies that have been used in an attempt to create testicular organoids and generate spermatozoa. We want to provide an up-to-date list on culture methodologies and media compositions that have been used and determine their role in regulating tubulogenesis and differentiation of testicular cells.

Search method: A literature search was conducted in PubMed, Web of Science, and Scopus to select studies reporting the reorganization of testicular cell suspensions in-vitro, using the keywords: three-dimensional culture, in-vitro spermatogenesis, testicular organoid, testicular scaffold, and tubulogenesis. Papers published before the August 1, 2019, were selected.

Outcome: Only a limited number of studies have concentrated on recreating the testicular architecture in-vitro. While some advances have been made in the testicular organoid research in terms of cellular reorganization, none of the described culture systems is adequate for the reproduction of both the testicular architecture and IVS.

Conclusion: Further improvements in culture methodology and medium composition have to be made before being able to provide both testicular tubulogenesis and spermatogenesis in-vitro.

KEYWORDS

in-vitro spermatogenesis, spermatogonial stem cell niche, testicular organoid, testicular scaffold, tubulogenesis

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1 | INTRODUCTION

Globally, the prevalence of male infertility has been estimated to range between 9% and 16%.[1] Additionally, sperm counts among western populations have more than halved (52.4%) in the past 40 years and keep on decreasing by an average of 1.4% every year.[1] This decrease in sperm count not only relates to fertilization rates but also represents a social and biological crisis.[2] The causes for the catastrophic drop in sperm count are currently unknown. Also, our knowledge on testicular andrology and regulation of spermatogenesis is not complete, hindering research on male infertility, its diagnosis, and treatment.[3] A culture system for the production of haploid spermatozoa starting from testicular cell suspensions would be a powerful tool to study testicular function through gain- or loss-of-function experiments. Additionally, the human culture system for in-vitro spermatogenesis (IVS) could serve as an alternative to animal usage in high-throughput screening assays in the industry. For instance, the European Registration, Evaluation, Authorisation and Restriction of Chemicals regulation (No. 1907/2006) has required toxicity testing for many chemicals on the market. These so-called reprotoxicity tests have been estimated to cost at least €6.7 billion and involve 48.6 million animals.[4] Moreover, prepubertal boys needing to undergo gonadotoxic treatments, and thus being at risk for spermatogonial stem cell (SSC) depletion, will highly benefit from in-vitro derived spermatozoa because they are too young for sperm banking. Cryopreservation of testicular tissue is currently the only experimental procedure to preserve their reproductive function.[5] Autografting of the stored testicular tissue or SSC transplantation is promising fertility preservation strategies. But, the risks related to the transplantation of residual malignant cells limit their application. In-vitro maturation of cryostored SSCs into haploid spermatozoa for later usage in artificial reproduction would bring a promising fertility preservation option for childhood cancer survivors. Similarly, it would benefit the infertility treatment of the wide range of non-obstructive azoospermia patients who are not able to produce spermatozoa but still have SSCs.

Germ cell differentiation relies on the support of Sertoli cells in the tubular compartment of the testis and on factors secreted from the peritubular myoid cells (PTMCs) and other interstitial cells, such as Leydig cells, endothelial cells, fibroblasts, and macrophages. Three types of three-dimensional (3D) IVS culture systems have been successful in rodents: supportive matrix culture systems for testicular cell suspensions,[6] organotypic culture of small testicular fragments[7] and, recently, 3D bioprinting of testicular cell suspensions.[8] Of these, only mouse organotypic culture systems resulted in offspring.[9-11] Up to now, organotypic cultures offer the highest IVS efficiency by preserving the testicular architecture and maintaining complex cellular interactions. The translation of the organotypic culture to humans is still ongoing,[12,13,14] Although promising steps have been made, haploid cells were rarely found and the possible presence of residual differentiating germ cells in the starting material used in these approaches could have biased the evaluation of the results. Also, solid characterization of the in-vitro derived germ cells is still needed.[15] Moreover, this system does not allow researchers to manipulate specific cells before culture, to help understand the many mechanisms controlling testicular physiology and spermatogenesis, and to discover new clinical targets.

Culturing testicular cell suspensions may be a suitable alternative. The testicular organoid concept refers to the reconstitution of a functional in-vitro environment from testicular cell suspensions, compatible with IVS.[16-20] Recapitulation of specific aspects of testicular development and differentiation in-vitro depends on the degree of physical support and the composition of the culture medium, but also on the cell types within the organoid itself. The aim of this review is to identify extrinsic factors driving testicular development from testicular cell suspensions, while supporting SSC differentiation in-vitro.

2 | SEARCH STRATEGY

A literature search was conducted in PubMed, Web of Science, and Scopus with combinations of the following research terms: three-dimensional culture, in-vitro spermatogenesis, testicular organoid, testicular scaffold, and tubulogenesis. To prevent recent articles to be missed, the search strategy was repeated weekly until August 1, 2019. Only studies reporting the reorganization of testicular cell suspensions in-vitro were included with no restrictions to year of publication. Studies using embryonic stem cells, induced pluripotent stem cells, or grafting were excluded. Reference lists of the selected articles were screened for additional relevant studies. Data concerning culture method and culture medium composition were extracted from these articles and divided into three (2D, 3D, and novel 3D strategies) and six groups (basal medium, serum, gonadotrophins, TGFβ-superfamily, other growth factors, and vitamin A), respectively. Because of a limited amount of human studies, rodent studies were also included. Moreover, organotypic culture studies were sometimes discussed to support the findings. Tables S1 and S2 provide detailed data derived from the selected articles.

3 | IMPACT OF THE CULTURE METHOD ON TESTICULAR TUBULOGENESIS IN-VITRO

In this section, the various culture methods that have been used in the past to study tubulogenesis in-vitro starting from testicular cell suspensions are summarized. In recent years, a shift from conventional 2D (Table S1) toward 3D (Table S2) culture models has been observed, because of the latter's ability to replicate the extracellular-matrix (ECM) features (eg, regulation of adhesion, migration, differentiation, and morphogenesis of embedded testicular cells) and

the generation of the complex multi-cellular organization of organs. Accordingly, novel 3D strategies and bio-printers have recently been introduced to facilitate the establishment of the different testicular compartments in-vitro.

3.1 | Two-dimensional cultures have limited potential in testicular tubulogenesis

The initial steps of testicular tubulogenesis have been described in 2D mono-cultures of embryonic mouse Sertoli cells,[21,22] neonatal rat Sertoli cells,[23] or immortalized mouse Sertoli cells,[24] but also in 2D cultures containing all testicular cell types of neonatal rat,[25-30] or adult human.[31,32] Following plating, testicular cells spread to form mono-layered plaques (Figure 1A). The contractile activity of PTMCs reassembles Sertoli cells into mono-layered islands. Subsequently, PTMCs elicit compaction and reaggregation of Sertoli cells into multinodular mounds (Figure 1B) that eventually merge through cytoplasmic projections (protrusions) to form cord-like structures (Figure 1C). Interactions between Sertoli cells and PTMCs result in the deposition of basement membrane components that will separate the tubular (containing undifferentiated Sertoli cells and germ cells) from the surrounding interstitial compartment (containing PTMCs, among others). In response to signals from the basement membrane and interstitium, Sertoli cells within the aggregates differentiate into functional columnar-shaped epithelial cells and establish an apical-basal polarization by becoming perpendicular to the basement membrane upon differentiation (Figure 1D).

The importance of the basement membrane in testicular cord formation was evidenced by culturing rodent testicular cells in 2D cultures with different ECM coatings, such as laminin,[21,24,26,28,30] fibronectin,[21,24,26] collagen,[23,24,26] seminiferous tubule biomatrix,[23] and Matrigel®.[21,22,24,33] Neonatal rat Sertoli cells cultured on seminiferous tubule biomatrix and Matrigel® reoriented into polarized cells with formation of tight junctions, more closely resembling their in-vivo counterparts than when cultured on plastic surfaces. Moreover, the cells showed better functionality as measured by the secretion of collagen, laminin and fibronectin,[23] and androgen-binding protein.[33] Similarly, enhanced cord-like structures were reported when embryonic mouse Sertoli cells were grown on Matrigel®[21,22] Of the proteins present in these matrices, fibronectin guided the migration of Sertoli cells,[27,34] while laminin strongly stimulated the reorganization of Sertoli cells into cord-like structures.[24,28] Nevertheless, only some of the formed cord-like structures in rodent 2D cultures developed into tubule-like structures with formation of a lumen.[24] Noteworthy, Van der Wee and colleagues did not start from primary testicular cells, but used an immortalized Sertoli cell line.[24] In studies reporting reorganization of adult human testicular cells, cord-like structures could be formed when grown on glass or plastic.[31,32] However, survival of germ cells within the 2D-derived structures was low[32] and the effect of Matrigel® was not evaluated. Altogether, the structures rarely established an organization comparable to that observed in-vivo.[30-32]

3.2 | Three-dimensional cultures improve multilayered testicular cell reorganization

Testicular cells cultured within, rather than onto Matrigel®, reorganized faster and formed improved cord structures displaying better germ cell viability.[33,35] Interestingly, some spermatogonia surrounded by Sertoli cells inside the cord-like structures differentiated up to late pachytene spermatocytes.[33] These results suggest that 3D culture of testicular cells benefits over 2D culture by providing a more physiologically stable system allowing reorganization of embedded testicular cells in-vitro with the potential to improve both tubulogenesis and spermatogenesis. In addition, Hadley et al[35] emphasized the role of laminin as an important factor within Matrigel® driving the reorganization of Sertoli cells into cords. Indeed, laminin is crucial for Sertoli and germ cell survival and differentiation through integrin-dependent signaling.[36] Matrigel® has been used extensively for the embedding of neonatal rat[17,30,37-42] and mouse testicular cells.[19] More post-meiotic germ cells could be observed in neonatal rat testicular cell-derived aggregates within a mixture of Matrigel® and collagen gel, than in collagen gel alone.[40] In accordance with Hadley and colleagues, the authors suggested that laminin was responsible for this effect. Interestingly, culturing neonatal rat testicular cells with an overlay of Matrigel® promoted the multilayered aggregation of testicular cells, while improving survival of spermatogonia.[37] These models have later been used to evaluate the toxic effects of certain chemicals, such as bisphenol A,[42] cadmium[43] and phthalates,[44-47] Likewise, Legendre et al[41] developed an in-vitro model of the blood-testis barrier for toxicology studies. In their model, neonatal rat PTMCs were cultured on the basal side of a transwell bicameral culture chamber, while Sertoli cells and germ cells reorganized into tubule-like structures within Matrigel® at the apical side of the transwell.[41] In their study, round spermatids could be detected. A recent study showed the ability of neonatal rat testicular cells to rebuild tubule-like structures when resuspended in a drop of Matrigel®, placed between two other cell-free Matrigel® layers.[17] In contrast to the conventional single-layer approach, testicular cells organized into tubule-like structures with a blood-testis barrier, maintaining the mitotic activity of undifferentiated spermatogonia. However, because Matrigel® is a mixture of not completely identified ECM proteins (mainly laminin, collagen IV, heparin sulfate proteoglycans, and entactin) and growth factors secreted by a mouse sarcoma, its use in clinical applications is limited.[48]

Collagen has been used to some extent for neonatal mouse[49-51] and adult human testicular cells.[52] Lee and colleagues reported the differentiation of adult human spermatogonia up to presumptive spermatocytes in cellular aggregates within collagen gel.[52] Collagen sponges were later used to provide spatial environment allowing structural reorganization of prepubertal rat testicular cells.[50] However, only the first steps of tubulogenesis could be

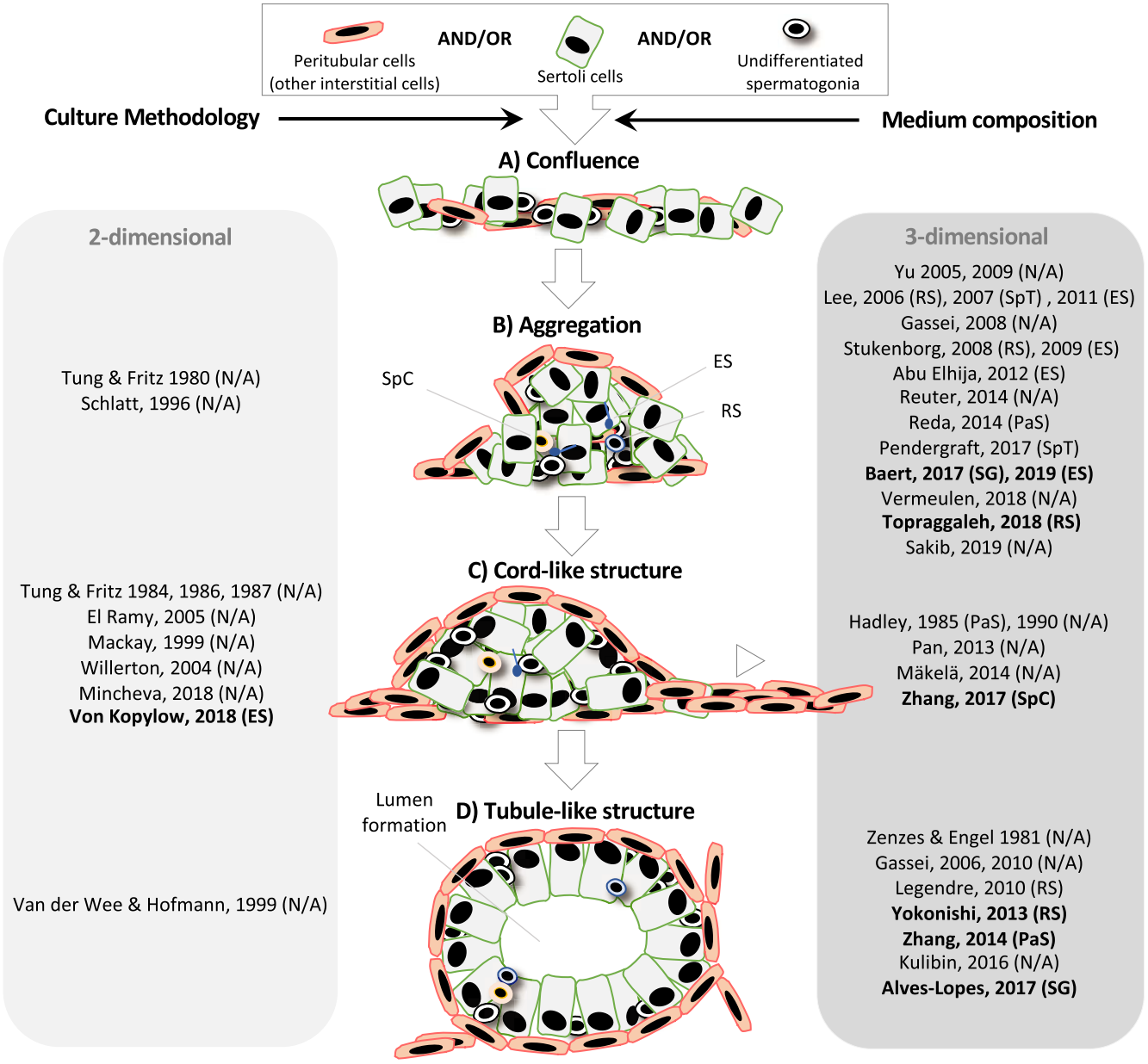

FIGURE 1 Structural reorganization of all or combinations of testicular cell types in-vitro in chronological order. Testicular tubulogenesis in-vitro comprises distinct phases: gaining of cell confluence (A). Aggregation of Sertoli cells into multinodular mounds under influence of contractile peritubular myoid cells (B). Interconnection and merging of multinodular mounds to form cable-like structures (C). Formation of hollow tubules (D). A shift from 2D (light gray table) toward 3D (dark gray table) culture models has been observed because of the latter's ability to improve the cell reorganization. Aside from the culture methodology, medium composition influences the different aspects of testicular tubulogenesis in-vitro. Of the different media-ingredients, KSR has been proven critical (bold). Upon reorganization of the testicular cells, differentiation of spermatogonia could be seen. The most advanced differentiation stage in each study was indicated between parentheses. ES, elongates spermatids; PaS, pachytene spermatocytes; RS, round spermatids; SG, spermatogonia; SpC, spermatocytes; SpT, spermatids

observed. Because the mitotic activity of Sertoli cells is crucial for the elongation of the cords,[53] mostly prepubertal rodent testicular cells have been used to establish tubulogenesis in-vitro. But, for ethical reasons, immature testicular cells are difficult to obtain from human. However, recent findings in rodent studies using collagen matrices suggest that adult testicular cells could be an alternative source of proliferating Sertoli cells.[49,51] It was hypothesized that due to their undifferentiated status, neonatal testicular cells adapted easier to culture conditions. However, adult mouse Sertoli cells continued to proliferate within collagen.[49] The authors further reported that laminin around Sertoli cell aggregates regulated the alignment of the cells. Interestingly, Kulibin & Malolina[51] identified two distinct populations of adult mouse Sertoli cells, with different location in-vivo. Collagen-embedded Sertoli cells originating from the rete testis maintained their mitotic activity and ability to form cord-like structures in-vitro. Likewise, Mäkelä et al[54]

demonstrated that adult mouse Sertoli cells in seminiferous tubule cultures maintain the capacity to proliferate through a process of dedifferentiation.

Artificial 3D scaffolds, such as soft agarose,[6,55-57] methylcellulose,[6] poly(D,L-lactic-co-glycolic acid),[58] and polydimethylsiloxane nanotubes,[59] have also been used to culture rodent testicular cells. Promising results have been obtained by Stukenborg et al,[6] who were the first to report the successful generation of elongated spermatids in mouse testicular cell aggregates in soft agarose and methylcellulose. The former continued to be used to generate elongated spermatozoa in mouse,[55] but also pachytene spermatocytes in rat testicular cell aggregates.[56] Notably, agarose is not cell-interactive and might be responsible for the low efficiency of tubulogenesis and spermatogenesis.[6,55,56] Interestingly, germ cells outside of de-novo formed SC aggregates died, presumably because of lack of support.[56]

The lack of reorganization of testicular cells within scaffold-based culture systems questions the necessity of a scaffold to recreate tubulogenesis in-vitro. For instance, de-novo formation of seminiferous tubule-like structures supporting germ cell differentiation has been reported without scaffold.[60] Indeed, in their study, tubulogenesis relied solely on the self-reorganization potential of cultured neonatal mouse testicular cell aggregates during two weeks on agarose gel at the gas-liquid interphase. Although spermatogonia progressed up to meiosis, the lack of a circulatory system might have hampered germ cells to differentiate completely.[60] In rotation culture, neonatal rat testicular cells were able to reorganize into tubule-like structures, whereas adult testicular cells regained this capacity only when deprived of germ cells.[61] Pendergraft et al[18] cultured adult SSCs and immortalized Sertoli and Leydig cells in a hanging drop of medium supplemented with human testicular ECM. The 23-day-long culture period did not result in the characteristic reorganization of the testicular cells into tubule-like structures either. However, elongated spermatids, high cell viability, and steroidogenic activity were reported in the testicular organoids. Phenotypical differences between immortalized and primary cells may have influenced the ability of the cultured cells to reorganize and promote IVS.[18,62] Interestingly, the authors used the developed human testicular organoids to study the reprotoxicity of the Zika virus.[63] Similarly, neonatal mouse testicular organoids formed in microwell plates supplemented with Matrigel® were used to test phthalates exposition to testicular function.[19] Although the testicular organoids had a testis-specific structure and expressed tight junctions, the tubular and interstitial compartments were reversed. Moreover, prepubertal human testicular cells could reorganize into organoids without the supplementation of Matrigel®.

Altogether, testicular cells could reorganize faster and show better organization in 3D matrices, while prepubertal testicular cells could also reorganize in a scaffold-free environment. In-vivo, differentiation of Sertoli cells occurs until puberty, leading to functional and structural changes that confer the cells the ability to support spermatogenesis. While the mitotic activity of Sertoli cells decreases during maturation, the expression of differentiation markers, such as transferrin, androgen-binding protein, androgen-receptors, and follicle stimulating hormone-receptors, increases. Interestingly, recent findings suggest that adult Sertoli cells can reacquire mitotic activity under appropriate culture conditions.[49,51,54] In-vivo, adult Sertoli cells in cords reorient themselves into elongated polarized cells at the basement membrane and develop a blood-testis barrier. Finally, they secrete fluid to create a lumen and become seminiferous tubules (Figure 1D). The blood-testis barrier consists of a network of cellular junctions that restricts the flow of factors to the differentiating germ cells, thereby dividing the tubules into two distinct microenvironments. In-vitro, some aspects of Sertoli cell differentiation could be observed in 3D culture. However, in most studies, Sertoli cells did not mature further than cord-like structures.[33,35,42,54,59] In general, Sertoli cells remained partially differentiated, which may have hindered meiotic progression of germ cells.[30,56,59] Only when in-vitro derived testicular cell aggregates were grafted under the skin of immunodeficient mice, more advanced morphological changes could take place.[30] The presence of microvessels and other still unknown factors that are lacking in-vitro is hypothesized to account for the better reorganizing properties of the grafts.[64,65]

3.3 | Novel 3D strategies for testicular tubulogenesis

The nanostructure and composition of the matrix influence the physiological and morphological behavior of the cultured cells. Yet, 2D and 3D cultures only allow initial steps of tubulogenesis. Reorganization of testicular cells into hollow tubules of mature Sertoli cells requires novel culture methodologies. For therapeutic uses, artificial scaffolds and/or defined recombinant human ECM proteins should be used. However, artificial scaffolds often lack or have limited biocompatibility, while the usage of synthetic biological scaffolds still results in highly unorganized testicular cells. Because the composition of ECM is tissue-specific, decellularized testicular matrix (DTM) was considered as an optimal scaffold for recellularization with testicular cells. We were the first to derive and develop natural cytocompatible scaffolds from human testis.[66] Agitating testicular fragments of 1 cm^3 for 24 h in 1% sodium dodecyl sulfate detergent was more effective in decellularizing and removing DNA from the tissue while maintaining important ECM proteins compared to treatment in 1% Triton X-100.[66] Following recellularization of these scaffolds with adult or pubertal human testicular cells at the gas-liquid interphase, testicular somatic and germ cells attached to the scaffold and created 3D mini-testicular tissues referred to as testicular organoids.[16] The testicular organoids were composed of proliferative spermatogonia and functional niche cells secreting testosterone and inhibin B for at least four weeks. However, complete differentiation of germ cells was not expected because the testicular organoids did not show the critical testicular compartmentalization. Remarkably, the morphology and functionality of testicular cells grown on DTM

were similar to DTM-free conditions. Recently, Vermeulen et al[67] compared several decellularization protocols for prepubertal porcine testicular fragments. Following recellularization of the different scaffolds with adult human Sertoli cells, 0.01% sodium dodecyl sulfate + 1% Triton X-100 and 0.05% trypsin + 0.02% ethylenediaminetetraacetic + 3% Triton X-100 showed the most promising results in terms of attachment, proliferation, and functionality of Sertoli cells within newly formed cellular aggregates. Another study fabricated testis-derived scaffolds by incubating ram testicular tissue in sodium dodecyl sulfate + Triton X-100 for 48h, followed by acidification in acid peptin solution and freeze-drying cycles.[20] Although tubular-like structures were not observed following inoculation of the scaffolds with neonatal mouse testicular cells, post-meiotic cells could be generated.

The lack of a circulatory system might have hampered germ cells to differentiate completely in tubule-like structures as mentioned before.[60] Recently, organotypic culture of mouse testes in microfluidic devices containing a circulatory system has shown increased efficiency and duration of spermatogenesis.[11,68] Hosting testicular organoids in microfluidic devices might thus improve their functionality. Alternatively, reducing the size of the sample might also increase the availability of shortcoming nutrients and oxygen.[69] In a study from our group, a mix of juvenile mouse interstitial cells and alginate was printed in a macroporous scaffold and neonatal tubular cells were seeded into the scaffold pores. In parallel, all testicular cell populations were cultured on cell-free scaffolds. Although tubules were not formed in-vitro, both approaches resulted in the formation of small-sized cellular aggregates and completion of spermatogenesis.[8]

4 | IMPACT OF MEDIUM COMPOSITION ON TUBULOGENESIS IN-VITRO

In this section, the various medium compositions that have been used in the past to study testicular tubulogenesis in-vitro starting from testicular cell suspensions will be summarized. A shift from undefined serum-containing media toward defined serum-free media has been observed. Several factors have been reported to stimulate tubulogenesis, SSC propagation, and spermatogenesis in-vitro.

4.1 | Basal medium

Since the first studies on testicular tubulogenesis in-vitro, culture conditions have improved considerably in order to ensure the growth and maintenance of testicular cells. To date, studies of testicular tubulogenesis have mostly employed traditional basal media, such as Eagle's minimal essential medium (MEM)[23,25-27,37,44,56,61] and its variations α-MEM,[8,17,20,60] α-MEM/F12,[51] Dulbecco's modified MEM (DMEM),[21,22,24,28,30-33,35,38,39,50,56,59] KnockOut DMEM,[8] and DMEM/F12.[6,19,29,40-42,52,54,56-58,67] In contrast, the use of Roswell Park Memorial Institute (RPMI) 1640[49,55] and StemPro-34-based media[18] has rarely been reported. Because of their scarcity and tendency for apoptosis in culture, SSCs should ideally be propagated in-vitro prior to IVS. StemPro-34-based media have extensively been used for this purpose in mice.[70] Recently, Kojima et al[69] identified insulin as the factor in StemPro-34-based media responsible for testicular somatic cell growth in neonatal mouse organotypic culture. The use of DMEM/F12-based medium resulted in a reduction of human testicular somatic cell overgrowth and may thus improve germ cell survival.[71]

4.2 | Serum

Early 2D cultures of primary testicular cells typically used medium supplemented with serum.[21,22,25-27,29] However, none of these studies reported the successful reorganization of primary testicular cells into tubule-like structures. The morphology of Sertoli cells did not change in culture with the addition of 2.5% fetal calf serum (FCS).[23] Nevertheless, the addition of 10% FCS to immortalized Sertoli cells resulted in the transition from flat cords to tubular-like structures.[24] The authors suggested that hepatocyte growth factor, found in FCS, was responsible for the observed morphogenic effects. Because SV40 large T antigen was used to immortalize the Sertoli cells, the possibility exist that the oncogene also targeted pathways for cellular differentiation. Interestingly, in rat cord-like structures cultured in Matrigel®, lumen formation could be observed in the absence of FCS.[30] Others observed enrichment of Sertoli cells in serum-free conditions.[28,30,41] The authors explained this by the overgrowth of PTMCs when serum was added to the medium.[28,30,41] Indeed, Legendre et al[41] demonstrated that 10% FCS overstimulated PTMC proliferation. On the other hand, 10% FCS has successfully been used by Lee et al[40,58] who reported the generation of round rat spermatids in cellular aggregates in a mixture of Matrigel® and collagen gel and in collagen gel alone, and presumptive elongated rat spermatids in aggregates in macroporous poly(D,L-lactic-co-glycolic acid)-based scaffolds. Using human adult testicular cells, the same authors reported the generation of elongated spermatids in collagen gel following 12 days of culture.[52] Noteworthy, 30% FCS was used to generate testicular organoids from immortalized Sertoli cells and Leydig cells supporting progression of diploid to haploid cells.[18] Aside from the immortalized cell line, starting material collected from adult men should be processed adequately because it can be contaminated with residual differentiating germ cells.

The age-related changes in the concentration of serum proteins in addition to the batch variability make serum inappropriate to speculate about the mechanisms and factors driving testicular tubulogenesis and spermatogenesis in-vitro. Undefined factors within serum may also have hampered these processes. As such, for further improvements of culture media, serum was replaced by the more reliable KSR supplement, commonly used to culture undifferentiated embryonic stem cells.[9,72] KSR consists of lipid-rich

albumin (AlbuMAX), amino acids, vitamins, transferrin, antioxidants, insulin, and trace elements.[72] α-MEM supplemented with KSR was previously found to allow IVS in neonatal mouse testicular organotypic cultures.[9] The authors ascribed the effects of KSR to AlbuMAX,[9,69] which are believed to be exerted through the binding of albumin to other molecules.[73] In selected studies starting from testicular cell suspensions, KSR has successfully been used for the generation of meiotic[49] and post-meiotic germ cells in mice,[20,60] meiotic initiation of rat spermatogonia,[42] and maintenance of the mitotic activity of spermatogonia in rat[17] and humans.[16] However, in a study by von Kopylow et al,[32] human spermatogonia were found only in low quantities in cord-like structures following three months of culture in 2D uncoated dishes in DMEM supplemented with 15% KSR and growth factors (10 ng/mL insulin growth factor, 40 ng/mL embryonic growth factor, and 20 ng/mL fibroblast growth factor 1, 2, and 9). Noteworthy, the absence of a physiological 3D microenvironment may have accounted for this observation. The addition of 10% KSR to RPMI-1640 promoted the formation of tubule-like structures with formation of a blood-testis barrier from dissociated neonatal rat testicular cells within collagen matrix.[49] Within the structures, mature Sertoli cells reoriented to engulf germ cells and promoted germ cell proliferation and differentiation. Absence of KSR resulted in differences in behavior and morphology of Sertoli cells and PTMCs, and decreased aggregation of Sertoli cells.[49] Recently, culture systems with a Matrigel® overlay got improved by incorporating KSR, which promoted testicular cell survival and proliferation and meiotic induction of neonatal rat spermatogonia.[42] In addition, most studies reporting the creation of testicular organoids incorporated 10% KSR in the culture medium.[8,16,17,20,60] However, control conditions without KSR were not performed. The optimal concentration of KSR is believed to be around 10% because higher concentrations did not improve germ cell differentiation and testosterone production of mouse testicular cells in organotypic culture.[74]

4.3 | Gonadotrophins/androgens

Follicle stimulating hormone (FSH) and luteinizing hormone (LH) are gonadotrophins secreted by the pituitary in response to gonadotrophin releasing hormone from the hypothalamus. Gonadotrophins have a central role in testicular development and function. While LH is responsible for testosterone production by the Leydig cells,[75] FSH is a known driver of Sertoli cell proliferation after birth until they are fully mature at the onset of spermatogenesis during puberty. Inhibin B secretion by Sertoli cells will in turn modulate pituitary FSH secretion through negative feedback.[76,77] In response to FSH, mature Sertoli cells also support spermatogenesis through direct communication with germ cells. Moreover, the testicular somatic compartment provides germ cells with a paracrine milieu of Sertoli cell-secreted growth factors such as stem cell factor (SCF), transforming growth factor (TGF)-β, fibroblast growth factor (FGF), glial-cell line-derived neurotrophic factor (GDNF), androgen-binding protein, and transferrin. The high affinity of androgen-binding proteins for androgens contributes to the high intra-testicular concentrations of testosterone, necessary for meiotic entry.[78,79] Elevated secretion of androgen-binding protein was previously shown in co-cultures of neonatal rat Sertoli cells and PTMCs in MEM supplemented with 100 ng/mL FSH.[25] Moreover, 10 ng/mL FSH was shown to increase mRNA levels of GDNF, an import factor for SSC self-renewal.[54] Testosterone was incorporated in culture of rat[33,35,40,41,58] and human[52] testicular cells, while human chorionic gonadotrophin (hCG) was used as a surrogate for LH in culture of rat,[50,56] mouse,[6,20] and human[16] testicular cells. However, the effects of testosterone or hCG have not been further characterized. Interestingly, Reda et al[56] highlighted the role of glutamine in steroidogenesis of neonatal rat testicular cells in soft agarose. Glutamine,[18,24,32,33,35,67] and its more stable alternative formulation Glutamax[8,16,50] have also been included in the culture medium by other researchers, but the effects have not been further characterized. Cord-like structures could be obtained from neonatal rat testicular cells in Matrigel® in serum-free defined DMEM supplemented with 100 ng/mL FSH and 10^{-8} M testosterone.[33,35] However, the authors did not further characterize the effects of the gonadotrophins on testicular tubulogenesis. In another study, 200 ng/mL FSH was shown to stimulate Sertoli cell reaggregation on laminin.[28] In addition, mound formation could only be observed in the presence of FSH.[28] On the contrary, Gassei et al[38] suggested that Sertoli cell aggregation was FSH-independent. Neither 10-100 ng/mL FSH, nor anti-FSH receptor antibodies (1:100-1:1000) disturbed Sertoli cell aggregation. Despite these observations, it should be noted that Sertoli cells did not proliferate in this culture setup. Therefore, these results should be interpreted carefully. Schlatt et al[28] further showed that contact-inhibition decreased the mitotic activity of densely packed Sertoli cells at high cell densities, an event also observed by Pan et al[59] and originally described by Steinberger & Steinberger[80] in cultured rat Sertoli cells. Proliferation could partially be reinitiated through the addition of 200 ng/mL FSH in the culture medium.[28] Accordingly, testicular cell densities and ratios should be monitored to prevent somatic cell outgrowth, a major obstacle for SSC culture.

The crucial role of gonadotrophins in germ cell differentiation was highlighted by Stukenborg et al.[6] Only when DMEM/F12 was supplemented with physiological concentrations of 5 IU/L FSH and hCG, neonatal mouse testicular cells in soft agarose and methylcellulose could differentiate elongated spermatids.[6] Although the differentiation efficiency to haploid cells was low, the number of tetraploid cells (representing meiotic primary spermatocytes) increased in cultures with gonadotrophin supplementation. Interestingly, high serum levels of FSH (>12 IU/L) correlated with poor differentiation of human germ cells in-vitro, while levels of 1-6 IU/L have been associated with a good differentiation potential.[52] In agreement with previous work on the steroidogenic and anti-apoptotic effects of hCG supplementation,[81,82] 5 IU/L hCG drove testosterone production in Leydig cells and enhanced germ cell survival.[6] Comparable results were obtained by Reda et al[56] by culturing neonatal rat testicular cells in soft agarose in a similar medium composition. Nonetheless,

when neonatal rat testicular cells were cultured in collagen sponges using the same medium composition, the steroidogenic and anti-apoptotic effects of gonadotrophin supplementation could not be observed.[50] A possible explanation for this observation is the inefficient biodegradability of the collagen sponges, or their lack of bioactivity. Stimulation of adult human and neonatal mouse testicular cells on DTM with gonadotrophins (5 IU/L FSH and hCG) did not further increase inhibin B and testosterone secretion by Sertoli and Leydig cells, respectively.[16,20] Baert et al[16] reasoned that the age of the adult human donors could be the cause for the lack of response of the testicular cells. Alternatively, since control cultures (also showing inhibin B and testosterone secretion) included KSR or Glutamax, and since these factors have known gonadotrophin-like effects,[56] the addition of gonadotrophins might have been superfluous. Noteworthy, spermatids have been derived from in-vitro systems of unorganized testicular cells in the absence of gonadotrophins, but supplemented with FCS[55] or KSR.[8,32] It therefore remains difficult to evaluate the need of gonadotrophins in cultures studying testicular development, especially in cultures supplemented with undefined or lipid-rich serum.[16,18,20,40,41,52,54,58]

4.4 | TGFβ-superfamily

The TGFß-superfamily contains a large group of proteins controlling testicular development such as activin A and GDNF. Activin A is a key regulator of Sertoli cell proliferation during embryogenesis and early postnatal life.[83,84] Its production decreases during puberty (when Sertoli cells differentiate) through the antagonizing effects of follistatin and FSH-induced inhibin B. Activin A directly contributes to the establishment of Sertoli cell numbers, thereby determining the testis size and daily spermatozoa production.[85,86] Interestingly, it was previously reported that non-proliferative adult Sertoli cells treated with 50 ng/mL activin A could dedifferentiate to a prepubertal phenotype exhibiting the ability to proliferate.[87] The regulatory network of activin A, follistatin, and FSH may also regulate spermatogenesis. Indeed, activin A treatment reduced the expression of the germ cell differentiation marker KIT proto-oncogene receptor tyrosine kinase (*KIT*) transcript levels in organotypic hanging drop cultures of human testis cancer samples.[88] With the roles of activin and FSH on Sertoli cell proliferation and tubulogenesis in mind, rat Sertoli cells in 3D Matrigel® surprisingly did not lose the ability to aggregate when treated with the activin antagonists follistatin (50-500 ng/mL) or FSH (10-100 ng/mL).[38] The authors deduced that Sertoli cell aggregation was not an activin-specific event, but could be the result of combined effects of different factors.

The role of GDNF as the most important regulator of SSC survival and self-renewal is well established.[89,90] GDNF is an important medium component for murine and human SSC propagation culture systems.[70,90,91] The secretion of GDNF in Sertoli cells is mostly FSH-dependent.[92] Additionally, retinoic acid (RA) treatment was shown to downregulate GDNF, further supporting the role of GDNF in inhibiting differentiation and maintaining the undifferentiated state of SSCs.[93] The culture medium used to generate organoids from immortalized human testicular cells was similar to the one used for mouse SSC propagation, comprising 10 ng/mL GDNF.[18,63] Ten ng/mL GDNF was also added to 10% KSR to stimulate SSC self-renewal in tubule-like structures, a culture setup able to support the initial steps of meiosis in mouse.[60] In another study, 100 ng/mL GDNF was combined with a cocktail of growth factors (20 ng/mL FGF1, FGF2 and FGF9, 10 ng/mL insulin growth factor and 40 ng/mL epidermal growth factor) to generate cord-like structures from human testicular cells in 2D culture.[32] In their 2D culture setup, germ cells could not be maintained and some somatic cells dedifferentiated. Nevertheless, the tubulogenesis-inducing effects of GDNF were not evaluated in these studies. Moreover, recent studies have shown tubulogenesis without GNDF supplementation.[17,30,39,41,49,51,61]

4.5 | Other growth factors

Male sex differentiation starts with the Y-chromosome-specific expression of sex-determining region Y (*Sry*) in the fetal indifferent bipotential gonads through its main downstream effector, the transcription factor SOX9.[94] FGF9 is an established downstream effector of SRY/SOX9 in pre-Sertoli cells of the developing fetal testis where it is responsible for differentiation of pre-Sertoli cells to Sertoli cells. Factors secreted by Sertoli cells will subsequently drive the commitment of precursor somatic testicular niche cells toward male sex differentiation.[95] FGF9 induces proliferation of Sertoli cells and also suppresses meiotic entry of germ cells by antagonizing retinoic acid 8 (*Stra8*) expression.[94] The meiotic block of germ cells during fetal life occurs through the upregulation of NANOS2, referred to as the meiotic gatekeeper.[96] It is an RNA-binding protein that silences the RA/SCF/KIT axis that is essential for meiotic entry of germ cells. Nonetheless, experiments focusing on the role of FGFs in testicular tubulogenesis are scarce.[18,22,24,29,32] Consistent with the previous work from Colvin et al[94] in-vivo, 10 ng/mL FGF9 enhanced proliferation and aggregation of fetal mouse Sertoli cells into cord-like structures on Matrigel®.[22] Moreover, in the presence of 20 ng/mL FGF9, adult human testicular cells reorganized into cord-like structures in 2D culture.[32] Few studies have focused on FGF2, a key regulator of in-vitro SSC propagation. In co-culture of fetal and prepubertal rat Sertoli cells and SSCs, it was shown that 4-10 ng/mL FGF2 acted as a testicular morphogen through survival and mitogenic actions.[97] However, it was recently reported that 10 µg FGF2/adult mouse testis induces differentiation of spermatogonia.[98] Although the number of spermatogonia increased under influence of FGF2, these cells developed into a differentiation-prone subset expressing the RA receptor RARγ. Moreover, FGF2 signaling was shown to suppress both GDNF production and degradation of RA in-vivo.[98] Fifty ng/mL FGF9 and FGF2 were found to promote reorganization of prepubertal rat Sertoli cells into aggregates. The FGF2-treated aggregates were bigger than the FGF9-treated ones. This difference could be attributed to the survival and mitogenic actions of FGF2 on PTMCs. The authors further suggested that FGFs take part into the

remodeling of the basement membrane of the tubules by regulating the expression of proteinases.[29]

Originally identified for their nerve growth-stimulating activity, several neurotrophins have been identified in the developing testis of rodents[99-102] and humans.[103] These neurotrophins comprise brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and neurotrophin (NTF) 3. The actions of NGF, BDNF, and NTF3 are mediated through the neurotrophin-specific tyrosine kinase receptors (NTRK) 1, NTRK2, and NTRK3, respectively. It was suggested that neurotrophins could have a role in testicular development.[101] When treated with high doses of the NTRK inhibitor K252a (5-10 nM), Sertoli cells did not aggregate.[38] A similar dose-dependent effect on Sertoli cells was shown with the NTRK1-specific antagonist AG879 (10-20 μM).[38] However, treatment of the testicular cells with NTF3 (10-100 ng/mL) did not stimulate aggregation, nor rescued the inhibitory effects of the NTRK antagonists.[38] The disturbance of K252a on Sertoli cell aggregation was also observed in humans, where relatively high doses (500 nM and higher) prevented cluster formation by inhibiting the protrusion of PTMCs.[32]

4.6 | Vitamin A

In order to improve the meiotic process that has long been proven difficult in IVS,[104] the supplementation of culture media with retinoids (retinol and RA) has been considered. RA is the biological active metabolite produced from retinol. RA plays a central role in meiotic entry of germ cells in both rodents and humans.[105,106] The actions of RA in the testis are mediated through the RA receptors, of which RARα, RARß, and RARγ are expressed on Sertoli cells, round spermatids, and undifferentiated spermatogonia, respectively.[107] RA directly induces the transition from undifferentiated KIT^- to differentiating KIT^+ spermatogonia by upregulating STRA8. Indirectly, RA also does so by stimulating the secretion of bone morphogenetic protein 4 and SCF by Sertoli cells and by downregulating GDNF.[93] This will, in turn, induce the synthesis of meiotic markers in germ cells, such as synaptonemal complex protein 3 and meiosis-specific recombinase.[93]

In initial culture conditions, serum acted as a source of RA.[108] Serum-free culture media were supplemented with 50 ng/mL vitamin A for the culturing of immature rat testicular cells in Matrigel®[33,35]. Later, its in-vitro role shifted to promoting differentiation of germ cells.[17-20,40,41,52,58] Indeed, Lee et al generated round spermatids in-vitro in rat and presumptive elongated spermatids in human using a medium supplemented with a combination of RA and retinol.[40,52] Legendre et al[41] observed round spermatids in their in-vitro model with little adjustments to the medium composition used by Lee et al.[40,52] RA has later been used at different concentrations to generate post-meiotic germ cells in testicular organoids made from adult human and neonatal mouse testicular cells.[18,20] Although Topraggaleh et al observed a significantly higher expression of post-meiotic genes following administration of 1 μM RA, expression of *Stra8* did not change significantly in culture, nor did synaptonemal complex protein 3.[20] Using a three-layer gradient system of Matrigel®, Alves-Lopes et al[17] investigated the role of RA in IVS. Through treatment of the testicular organoids with 10 nM-10 μM RA and the RA antagonist ER 50 981, they concluded that RA improved germ cell counts (12%) in 21 days culture compared with controls (7%). However, when a higher concentration of RA (10 μM) was used, this effect was countered. Noteworthy, it was recently demonstrated in neonatal mouse organotypic cultures that 10 μM retinol was more effective than RA in inducing seminiferous tubule growth and meiosis.[109] Similarly, the effects of RA on germ cells in human testicular organoids were weaker compared to the effects on germ cells in 2D culture.[19] These studies support the idea that reorganized PTMCs around the seminiferous tubules may act as RA-degrading barrier that inhibits RA actions in the tubules through cytochrome P450 hydroxylase enzymes.[107]

5 | CONCLUSION

Most IVS studies using testicular cell suspensions have focused on obtaining post-meiotic germ cells without paying attention to also improve the reestablishment of the testicular architecture. However, the testicular cell organization is pivotal in achieving spermatogenesis in-vitro. With this review, we summarized and compared studies aiming to recreate an adequate in-vitro environment for testicular cells in order to mimic testicular tubule formation and germ cell differentiation in-vitro. The testicular organoid concept is emerging in tissue engineering and might allow the creation of a functional human testicular surrogate from isolated testicular cells, especially with the emergence of 3D bioprinting. The regulation of testicular tubulogenesis in-vitro remains poorly understood as tubular-like structures were rarely able to support IVS. Moreover, most of the selected studies have been conducted in rodents. Although rodent IVS systems can provide much insight into human spermatogenesis, it is crucial to develop systems that recapitulate the actual human spermatogenesis as this process shows differences with rodents. Given the long cycle of human spermatogenesis, it will be necessary to maintain long-term testicular cell cultures, while providing signals important for germ cell differentiation. Taking into account the different steps in testis development and germ cell differentiation (mitosis, meiosis, and spermiogenesis), sequential culture media might need to be developed in order to promote tubulogenesis and germ cell differentiation. The results suggest prepubertal testicular cells possess a self-assembly potential that has to be taken full advantage of by improving the medium composition. Nonetheless, if adult testicular cells cannot be induced to dedifferentiate into morphogenic cells, 3D bioprinting technology might be required because it gives control over cell deposition and scaffold design. This concern is particularly relevant for humans as prepubertal material is scarce. From the medium ingredients, KSR has been proven critical for the reorganization and in-vitro maturation of rodent testicular cells. However, the exact factor within KSR responsible for this

has yet to be defined. Although KSR was also successful in maintaining human germ cells in testicular organoids, it remains to be tested whether this is sufficient to induce complete differentiation of human SSCs. Possibly, other combinations of factors are needed with respect to tubulogenesis. However, because of the rich medium compositions used in selected studies, it is difficult to make definite conclusions. Recent findings suggest that FGFs and neurotrophins require more research focus. Furthermore, vitamin A derivates may be used to improve the efficiency of spermatogenesis. Other cell types and factors which have not been studied in included studies, for example, endothelial cells, BMP's, and SCF, deserve more attention.

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CONFLICT OF INTEREST

The authors declare there are no conflicts of interest.

AUTHORS' CONTRIBUTIONS

GR contributed to conception and design, literature search, and manuscript writing; GR, Y.B, and EG contributed to manuscript revision and final approval of the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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