

Organoids as tools to investigate the molecular mechanisms of male infertility and its treatments

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Abstract

Organoids are 3D structures characterized by cellular spatial organizations and functions close to the native tissue they mimic. Attempts to create organoids originating from several tissues have now been reported, including the testis. Testicular organoids have the potential to improve our knowledge of the mechanisms that regulate testicular morphogenesis, physiology, and pathophysiology. They could especially prove as useful tools to understand the complex mechanisms involved in the regulation of the germ cell niche in infertility cases as they offer the possibility to control and modify the nature of cell types before self-assembly and thereby opening the perspective for developing innovative methods to restore fertility. To date, there are only few studies targeted at testicular organoids' formation and even less describing the generation of organoids with both testis-specific structure and function. While researchers described interesting applications with regards to testicular tissue morphogenesis and drug toxicity, further research is needed before testicular organoids would eventually lead to the generation of fertilizing spermatozoa. This review will present the conventional systems used to induce *in vitro* maturation of testicular cells, describe the different approaches that have been used for the development of testicular organoids and discuss the potential applications they could have in the field of male reproductive biology.

Reproduction (2021) **161** R103–R112

Introduction

Infertility nowadays occurs in 9 to 15.8% of men and can result from abnormal sperm production/transport or an ejaculation disorder (Barratt *et al.* 2017). Congenital urogenital pathologies such as undescended testes, genetic disorders, sequelae of infections, or medical therapies (e.g. gonadotoxic treatments), all contribute to the delay in or the absence of conception (Nieschlag & Lenzi 2013, Barratt *et al.* 2017). In addition, the significant decrease (52%) of sperm concentrations reported between 1973 and 2011 points to the emergency to identify the reasons and underlying mechanisms of this decline and subsequent male infertility (Levine *et al.* 2017).

Efforts to reproduce spermatogenesis *ex vivo* have been ongoing for more than a hundred years. Several methods of *in vitro* maturation of testicular cells and tissues have been evaluated (reviewed in Reuter *et al.* 2012), with the objective to promote the differentiation of germ cells into fertilization-competent spermatozoa. These can later be used for intracytoplasmic sperm injection to either treat infertility related to spermatogenic failure (Lee *et al.* 2006a, Sun *et al.* 2018) or to restore fertility using prepubertal testicular tissue, cryopreserved before gonadotoxic therapies (Wyns *et al.* 2010, Picton *et al.* 2015, Kanbar *et al.* 2020,

Wyns *et al.* 2020). In the specific context of testicular tissue cryopreservation before cancer treatment, *in vitro* maturation of spermatogonial stem cells (SSCs) present in the prepubertal testis has the advantage of circumventing the risk of disease relapse that exists when tissue or testicular cell suspensions (TCSs) contaminated with cancer cells are transplanted. Currently available methods for *in vitro* maturation of SSCs (within tissue fragments, as isolated seminiferous tubules or TCSs) were recently reviewed (Oliver & Stukenborg 2020) and are summarized in Fig. 1.

Of the different *in vitro* maturation methods described to date, the organotypic culture technique has been the most successful in reproducing spermatogenesis *ex vivo*. Indeed, the birth of healthy fertile progeny has been reported in mice with sperm obtained after culture of 1 to 3 mm³ immature testicular tissue (ITT) fragments using both static and dynamic systems (Sato *et al.* 2011, Yokonishi *et al.* 2014, Komeya *et al.* 2016). However, less favourable outcomes have been obtained with ITT of primates (Heckmann *et al.* 2020) and humans (de Michele *et al.* 2018b, Medrano *et al.* 2018, Portela *et al.* 2019). Only one study on human ITT reported the reproduction of the meiotic process (de Michele *et al.* 2018b) albeit with an extremely poor efficiency that did not allow to fully characterize the generated haploid cells.

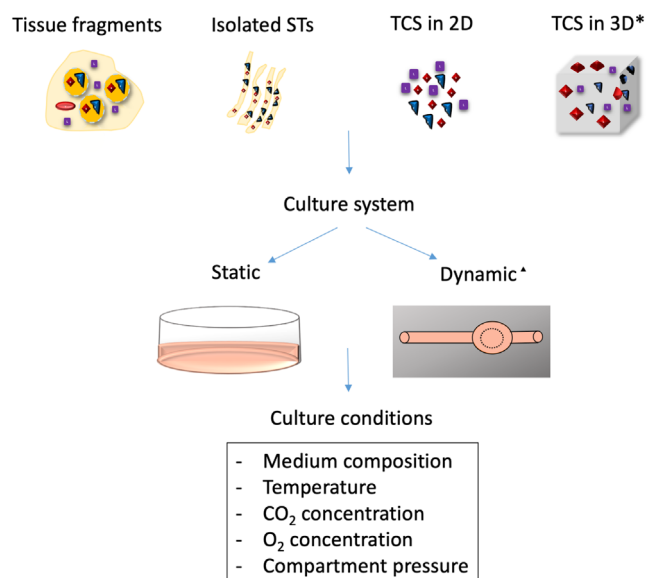


Figure 1 Different available options for germ cell *in vitro* maturation from intact or disaggregated testicular tissue. *: with or without scaffold – including testicular organoids; ▲: Microfluidic, shaking/rotating culture or shaken bioreactors; ST: seminiferous tubule; TCS: testicular cell suspension.

While 2D-cell culture was the first technique used for *in vitro* maturation of TCSs, the completion of spermatogenesis starting from prepubertal TCSs was only achieved in cattle and rodents, but the generated haploid cells were either abnormal or incompletely characterized (Izadyar *et al.* 2003, Iwanami *et al.* 2006, Xie *et al.* 2010). Attempts to culture adult human TCSs were previously reported but were either not reproducible (Tesarik *et al.* 1999), not suitable for clinical application due to the use of animal components (Cremades *et al.* 1999, Sousa *et al.* 2002), or did not demonstrate any germ cell differentiation despite some cellular reorganization (von Kopylow *et al.* 2018). The absence of a proper

spatial arrangement of the testicular cells was assumed to be a major factor to explain failed *in vitro* maturation in 2D culture (Reuter *et al.* 2014, Stukenborg *et al.* 2018).

Hence, researchers turned their efforts to 3D-cell culture systems which were able to better mimic the *in vivo* cell configuration required for more physiologic cellular responses (Petersen *et al.* 1992, Bissell *et al.* 2002) and normal germ cell differentiation (Stukenborg *et al.* 2009). Scaffold-free (e.g. hanging drops, cellular aggregates) or scaffold-based (e.g. hydrogel, decellularized ECM) approaches were both considered (Edmondson *et al.* 2014).

Limited meiotic progression was noted with the scaffold-free approach (Yokonishi *et al.* 2013), leading to the use of scaffold-based approaches in most of the available studies.

While many of these studies did not investigate germ cell development or failed to obtain post-meiotic cells (Hadley *et al.* 1985, Gassei *et al.* 2006, 2010, Reda *et al.* 2014, Reuter *et al.* 2014, Zhang *et al.* 2014, 2017, Vermeulen *et al.* 2018), haploid germ cells were eventually generated in systems using various natural and synthetic scaffolds (Table 1).

Overall, compared to the 2D system, improved cell organization and viability were observed (reviewed in Richer *et al.* 2020). Most importantly, human round spermatids able to fertilize mice oocytes were obtained in a Matrigel™-based culture system (Sun *et al.* 2018). Unfortunately, despite some encouraging results, the efficiency of spermatogenesis in 3D culture systems remained low with a limited recapitulation of the testis-specific architecture.

Paralleling these developments, the concept of organoids was added to the field of 3D culture (reviewed in Velasco *et al.* 2020). Although definitions varied since the 1950s, an organoid was eventually defined as ‘a unit of function of a given organ that can reproduce, in culture, a biological structure similar in

Table 1 Most commonly used scaffolds in 3D culture of testicular cells reporting germ cell differentiation up to the haploid stage.

Scaffolds	Species	Reference
Natural		
ECM-derived scaffolds		
Collagen	Rat	Lee <i>et al.</i> (2006b)
Matrigel™*	Rat	Lee <i>et al.</i> (2006b), Legendre <i>et al.</i> (2010)
	Humans [^]	Sun <i>et al.</i> (2018)
Decellularized ECM [†]	Humans [^]	Ashouri Movassagh <i>et al.</i> (2020)
Seaweed-derived scaffolds		
Alginate	Mouse	Baert <i>et al.</i> (2019)
Soft-Agarose	Mouse	Stukenborg <i>et al.</i> (2008), Stukenborg <i>et al.</i> (2009), Abu Elhija <i>et al.</i> (2012)
Synthetic		
PLGA	Rat	Lee <i>et al.</i> (2011)
Methylcellulose	Mouse	Stukenborg <i>et al.</i> (2009)
	Rhesus monkey	Huleihel <i>et al.</i> (2015)
	Humans [^]	Abofoul-Azab <i>et al.</i> (2019)

*Extracted from mice Engelbreth–Holm–Swarm tumour cells and mainly composed of laminin, collagen IV, and entactin (Hughes *et al.* 2010);

[†]solid or solubilized form; All reports used prepubertal testicular tissue except studies marked with a [^] that used adult tissue.

ECM, extracellular matrix; PLGA, Poly (lactic-co-glycolic acid).

architecture and function to its counterpart *in vivo*' (Simian & Bissell 2017).

The potential of organoid technology to accurately mimic biological structures *in vitro*, as already demonstrated for numerous animal and human organs (e.g. lung, liver, pancreas fallopian tube) (reviewed in Kim *et al.* 2020), was recently extended to the testis.

This review, therefore, aims at first to present the different approaches that have so far been applied to generate testicular organoids (TOs), secondly to analyse how far research has progressed with achieving *in vitro* spermatogenesis by using TO culture, and thirdly to highlight the scientific advances made to understand testicular morphogenesis and (patho-)physiology. Eventually, the numerous applications that TOs could offer in the field of male reproduction in the future will be discussed.

Testicular organoid generation

Several groups recently attempted to generate TOs in both animals and humans (see Tables 2 and 3 respectively). The first group to introduce the term 'testicular organoid' studied the culture of human pubertal TCSs on adult decellularized testicular tissue slices. The study showed that adult human TCSs were able to self-organize whether a decellularized tissue scaffold was present or not. Despite the lack of the typical native tissue architecture (expected in a proper organoid), spermatogonia and their important niche cells were maintained for 1 month *in vitro*. Leydig and Sertoli cell functionalities were demonstrated by the respective detection of testosterone and inhibin B in culture supernatants (Baert *et al.* 2017). This inability of adult Sertoli cells to reorganize into cords was also reported when cultured onto prepubertal porcine decellularized ITT scaffolds (Vermeulen *et al.* 2018).

The first time testicular organoids were shown to harbour seminiferous-tubule-like structures with an accurate cell configuration was when TCSs from prepubertal rats (5–8 and 20 days old) were spread on several layers of Matrigel™ (Alves-Lopes *et al.* 2017). Consistent with the previous studies, the use of pubertal (60-days-old rats) TCSs did not lead to the formation of seminiferous-tubule-like structures, suggesting that only immature TCSs can.

Using a hanging drop co-culture system followed by the culture of cell clusters on low attachment plates, somatic (immortalized Leydig cells and Sertoli cells) and germ cells, from adult men, were also unable to rearrange properly although a medium enriched with a solubilized form of testicular ECM was used (Pendergraft *et al.* 2017).

In contrast to all the studies using immature tissue, an abnormal cell reorganization was observed when TCSs isolated from prepubertal pigs were cultured in microwell centrifugal aggregation plates to generate the

TOs. Germ cells and Sertoli cells were both located at the outer side, while Leydig and endothelial cells were found on the inner side (Sakib *et al.* 2019). Although the exact architecture of the native organ was not obtained, two distinct compartments separated by a basement membrane were established. This led to an attenuated response to retinoic acid (RA) compared to 2D culture, suggesting that the cellular interactions modulated the response to RA similarly to that *in vivo* (Endo *et al.* 2017). Interestingly, application of the protocol to mice, rhesus macaques, and human prepubertal TCSs resulted in the formation of TO-like structures with a similar organization, suggesting that the system can be applied to all mammalian TCSs (Sakib *et al.* 2019).

Eventually, in large mammals, the first TOs to have an accurate tissue-specific structure were obtained in 2019 by Vermeulen *et al.* (2019). TCSs from prepubertal pigs were cultured in collagen and in a solubilized form of decellularized testicular tissue. Germ cells and Sertoli cells were located inside the tubule-like structures and surrounded by a basement membrane while both peritubular and Leydig cells were located on the outside. However, regardless of the matrix, the number of germ cells decreased, and their differentiation was limited to the stage of spermatocytes during the 45 days culture period. This suggests that either cell–cell interactions/assembly (required for germ cell differentiation) were not identical to the native organ, or that the culture environment (media composition and conditions) was unsuitable.

Several factors have been shown to influence TO formation. Self-assembly of testicular cells appeared to be age-dependent as observed in rats (Alves-Lopes *et al.* 2017). This observation was further confirmed in a recent study in mice, where organoid formation was either absent or delayed when peripubertal vs prepubertal TCSs were used. Also, when the same protocol was applied to adult murine and human TCSs, no cellular self-assembly was observed (Edmonds & Woodruff 2020). Interestingly, immature cells (and not their secreted factors) were able to rescue the assembly of adult cells when co-cultured together, suggesting that one or several cell types (e.g. immature Sertoli cells) in prepubertal TCSs can drive TO assembly (Edmonds & Woodruff 2020).

The cell density of the TCS also seemed to play a role in TO formation. Notably, it was shown that immature TCSs used at high concentration or after centrifugation as a cell pellet formed seminiferous-tubule-like structures, regardless of the addition of an ECM-based hydrogel when cultured at the air–liquid interphase (Yokonishi *et al.* 2013, Zhang *et al.* 2014, Vermeulen *et al.* 2019). The benefit of high cell density was further highlighted by an improved organization of rat TOs generated in Matrigel™ when higher cell concentrations were used (Alves-Lopes *et al.* 2017).

The need for a supportive scaffold or ECM components for TO formation is unclear as most studies were not

Table 2 List of animal studies reporting generation of testicular organoids.

Reference	Species	Cell maturity		Culture method	Testis-specific architecture	Major outcomes	GC differentiation stage*
		Stage	Age				
Alves-Lopes et al. (2017)	Rat	Prepubertal Peripubertal Adult	5–8 days-old 20 days-old 60 days-old	Culture of one drop containing TCSs into Matrigel™ placed between two cell-free layers of Matrigel™.	Yes	- Only immature TCSs reform TO. - Identification of SC for 21 days but reduction of their relative numbers from day 7 (14%) to day 14 (4%). - Establishment of some BTB components. - Responsiveness of TOs to IL1a, TNFa and RA. - Functionality of LCs and SCs confirmed by quantification of testosterone and inhibin B. - Detection of post-meiotic GCs inside TOs.	NI
Rezaei Topraggaleh et al. (2019)	Mouse	Prepubertal	3–5 days-old	Culture of TCSs onto a chemically crosslinked hydrogel derived from ram decellularized testicular tissue.	No	Mouse and macaque: Inverted organization between intra- and extra-tubular compartments.	Haploid (TNP1).
Sakib et al. (2019)	Mouse Rhesus macaque Pig	Prepubertal	8–10 days-old 2 years-old 1-week-old	Centrifugation of TCSs in aggregation centrifugal plates before culture. Matrigel™ only added for mice TCSs.	No	- Expression of BTB proteins. - Attenuated responsiveness to RA compared to 2D culture.	–
Vermeulen et al. (2019)	Pig	Prepubertal	<7 days old	Culture of TCSs into drops of collagen or a solubilized form of decellularized testicular tissue at the air-liquid interphase.	Yes	- Significant decrease of GC numbers. - LC and SC functionality maintained for 45 days as demonstrated by testosterone and SCF quantification.	Identification of meiotic cells (SCP3) but no post-meiotic cells (CREM).
Goldsmith et al. (2020)	Pig	Prepubertal	1 week old	Centrifugation of normal SCs or SCs knockdown for <i>ODF2</i> and <i>IFT88</i> in aggregation centrifugal plates before culture.	No	- 5/150 <i>IFT88</i> -KO organoids had a distinct basement membrane vs 128/150 in the control group. - No outcome for <i>ODF2</i> KO organoids	NI
Edmonds & Woodruff (2020)	Mouse	–	–	Culture of TCSs inside microwells moulded in agarose blocks allowing diffusion of the media.	Yes	- Self-assembly only with immature TCSs. - Long-term endocrine function (80 days) with preserved gonadotropin responsiveness. - Immature TCSs rescue the self-assembly when co-cultured with mature cells.	Identification of GCs (DDX4) and SSCs (SALL4) but no meiotic cells (SCP3).

* Identified through IHC staining.

BTB, blood-testis barrier; CREM, cAMP responsive element modulator; DDX4, DEAD-box helicase 4; GC, germ cell; *IFT88*, intraflagellar transport protein 88; IHC, immunohistochemistry; IL1a, interleukin 1 alpha; KO, knock-out; LC, Leydig cell; NI, Not investigated; *ODF2*, outer dense fibre protein 2; RA, retinoic acid; SALL4, Sal-like protein 4; SC, Sertoli cell; SCF, stem cell factor; SCP3, synaptonemal complex protein 3; SG, spermatogonia; SSC, spermatogonial stem cell; TCS, testicular cell suspension; TNFa, tumor necrosis factor alpha; TNP1, transition protein 1; TO, testicular organoid.

designed to answer this question or it led to controversial results. Indeed, ECM-derived hydrogels were used in four studies to generate human (Pendergraft *et al.* 2017), porcine (Vermeulen *et al.* 2019), rat (Alves-Lopes *et al.* 2017), and murine (Rezaei Topraggaleh *et al.* 2019) TOs, but the absence of a control group without hydrogel prevented drawing any conclusion regarding the benefit of the ECM-hydrogel for TO development. Only two studies compared the impact of the presence/absence of ECM compounds (Matrigel™) on prepubertal mice TO formation. The first demonstrated that TCSs failed to form organoids when Matrigel™ was absent (Sakib *et al.* 2019), by contrast with the second where organized and functional TOs were obtained without any Matrigel™ supplementation (Edmonds & Woodruff 2020). It is thus still not clear in which conditions the endogenous production of ECM components by the TCSs (Maekawa *et al.* 1996) would be sufficient to promote TO formation and in which conditions the addition of a testis-specific-ECM-component would be required.

The ratio of different testicular cell types could be another factor to influence TO assembly, similarly to what happens during gonadogenesis where the number of Sertoli cells predetermines the size of the germ and Leydig cell populations of the adult testis (Rebourcet *et al.* 2017). Using different germ and somatic stem cell ratios to generate, TOs could therefore prove useful in determining the optimal conditions for cell assembly.

TOs as tools to study testicular morphogenesis and pathophysiology

TOs could serve as a tool to evaluate the role of different factors or cell types in testicular morphogenesis and physiology.

The utility of the TO model to understand the molecular mechanisms involved in testicular development was first highlighted in the study of Alves-Lopes *et al.* where an impaired formation of TOs was observed after exposure to interleukin 1 alpha (IL-1a) and TNF 1 alpha (TNF1a) (Alves-Lopes *et al.* 2017). These observations were in line with those made *in vivo* where the injection of IL-1a and TNF alpha (TNFa) led to the disruption or destabilization of the blood-testis barrier (BTB), respectively (Sarkar *et al.* 2008, Cheng & Mruk 2012).

The main advantage of TOs is that the testicular cells can be sorted and selected enabling the modification of a cell of interest or its replacement by another cell before cell assembly (Fig. 2A).

For instance, TO-like structures were recently used to study the role of primary cilia (present on testicular somatic cells) in testicular morphogenesis (Goldsmith *et al.* 2020). Indeed, following inhibition of primary cilia assembly, through CRISPR Cas9-mediated gene editing of a structural component of primary cilia and siRNA-mediated gene silencing of a functional component of the intraflagellar transport system in isolated porcine

Sertoli cells, the authors observed an abnormal cellular assembly and organization compared to controls.

The assembly of testicular cells either originating from different individuals (e.g. genetically normal and abnormal), modified using gene-editing techniques, or exposed to external factors (e.g. drugs, toxins) may help in understanding the molecular mechanisms involved in testicular dysfunction. While immunofluorescent labelling of testicular cells before TO formation proved useful to study the cellular organization in TOs (Pendergraft *et al.* 2017), genetic transgene integration of fluorescent proteins (e.g. GFP or mCherry) in cell types of interest would be required for longer-term studies of TO formation (Fig. 2A).

Moreover, TOs could help improve our knowledge of the roles of different cell types within the testicular microenvironment. For example, endothelial cells (Bhang *et al.* 2018) and testicular macrophages (DeFalco *et al.* 2015) were both recently identified as important players in the SSC niche regulation. Developing TOs enriched with or lacking one or more cell types (Fig. 2B) could therefore prove useful in understanding their exact roles in testicular pathophysiology.

Disease modelling could represent another application of TOs allowing both the study of the disease itself and the development of patient-tailored treatments, as described in organoids from various tissue types (Fatehullah *et al.* 2016). Notably, TOs can be potentially generated with TCSs isolated from patients suffering from infertility conditions such as Klinefelter syndrome, early maturation arrest, cryptorchidism, or testicular cancer. The generated disease-modelling TOs could then be compared under different conditions with TOs developed with cells from fertile/healthy men and used as an assay for subsequent evaluation of a candidate therapeutic agent. In this regard, TOs were already recently used as models to study drug toxicity (chemotherapeutic agents: busulfan, doxorubicin, cisplatin, and etoposide) (Pendergraft *et al.* 2017) and also to assess the impact of environmental toxins (phthalate derivatives) (Sakib *et al.* 2019) and microbiological agents (Zika virus) (Strange *et al.* 2018) on the testis *in vitro*. In the future, human TOs could be a promising and improved alternative to animal models for pharmaceutical drug development and testing.

TOs as a tool to restore fertility

TOs could be generated with TCSs isolated from ITT of prepubertal patients affected by haematological cancers (Fig. 2C) with the objective of creating an artificial testis free of cancer cells that can be transplanted back to the cured patient. Indeed, cryopreservation of ITT before potential gonadotoxic treatment is now ethically accepted in several centres (Wyns *et al.* 2011, Picton *et al.* 2015, Valli-Pulaski *et al.* 2019, Kanbar *et al.* 2020). Autotransplantation of the tissue back to patients after treatment completion is considered a promising method

Table 3 List of human studies reporting generation of testicular organoids.

Reference	Cell maturity		Culture method	Testis-specific architecture	Major outcomes	GC differentiation stage*
	Stage	Age				
Baert <i>et al.</i> (2017)	Peripubertal	15 years old	Culture of TCSs onto agar blocks or decellularized adult testicular tissue slices.	No	Maintenance of SG and somatic cells for 1 month. LC and SC functionality confirmed by quantification of testosterone and inhibin B.	NI
Pendergraft <i>et al.</i> (2017)	Adult	56–61 years old	Culture of GCs together with immortalized LCs and SCs within a media enriched with a solubilized form of decellularized testicular tissue into hanging drop plates for 48 h and transferred to ultra-low attachment plates.	No	LC functionality confirmed by testosterone quantification. Transition of a small percentage of GC from the diploid to the haploid stage.	Haploid†
Strange <i>et al.</i> (2018)	Adult	N/A	Culture of SG together with primary LCs and SCs within a media enriched with a solubilized form of decellularized testicular tissue into hanging drop plates for 48 h and transferred to ultra-low attachment plates.	No	Reduced viability in TOs infected by the Zika virus as shown by a decrease of the ATP production/TO. Reduced expression of markers of SSCs (<i>ZBTB16</i>), SCs and LCs (<i>CYP19A1</i> , <i>STAR</i>) in infected TOs.	NI
Sakib <i>et al.</i> (2019)	Prepubertal	6 month old and 5 years old	Centrifugation of TCSs in aggregation centrifugal plates before culture.	No	Inverted organization between intra- and extra-tubular compartments.	NI

*identified through IHC staining; † protamine-1 and acrosin.

CYP19A1, cytochrome P450 family 19 subfamily A member 1; GC, germ cell; IHC, immunohistochemistry; LC, Leydig cell; N/A, no information about the age of the patients; NI, Not investigated; SC, Sertoli cell; SG, spermatogonia; SSC, spermatogonial stem cell; *STAR*, steroidogenic acute regulatory protein; TCS, testicular cell suspension; TO, testicular organoid; *ZBTB16*, zinc finger and BTB domain containing protein 16.

to restore their fertility. The birth of a healthy baby monkey after ICSI using autograft-derived spermatozoa was the proof of the concept (Fayomi *et al.* 2019). However, infiltration of the testis by malignant cells can occur in up to 21% of young boys affected by leukaemia (Kim *et al.* 1986). Hence, elimination of cancer cells before organoid generation with either cell-sorting techniques (Dovey *et al.* 2013) or with specific culture protocols (Sadri-Ardekani *et al.* 2014) could be applied to eliminate the risk of cancer cell reintroduction. Once the safety and efficacy of such protocols are proven, TOs created with the selected decontaminated cells could then be considered for transplantation. This has the advantage of exposing TOs to the *in vivo* macroenvironment, including blood-circulating factors, required for spermatogenesis (de Michele *et al.* 2017b).

No studies have evaluated the grafting of TOs so far although human cerebral organoids were shown to survive and re-vascularize after xenotransplantation to the mouse (Mansour *et al.* 2018). Knowledge of the real potential of a TO environment to support germ cell differentiation is thus still lacking.

Remaining challenges with TOs

As only immature TCSs were shown to possess the ability to reorganize with a testis-specific architecture

(Alves-Lopes *et al.* 2017, Edmonds & Woodruff 2020), a major challenge will be to create TOs harbouring this specific organization with cells from adults presenting with testicular failure. This issue can be circumvented by creating TO chimeras by co-culture of immature and adult TCSs as recently demonstrated in mice (Edmonds & Woodruff 2020), although access to immature human Sertoli cells may be difficult for evident ethical reasons. However, Sertoli cells generated from patient-derived-induced pluripotent stem cells (iPSCs) (Rodriguez Gutierrez *et al.* 2018) could represent an interesting alternative to drive the assembly of adult TOs offering a new way to study infertility in adult males (Fig. 2D).

Organoids could increase the reproductive potential of ITT fragments as a fertility restoration strategy (Table 4). Most notably, their production can be non-time limited and in high numbers, if coupled to efficacious cell expansion protocols. However, while the culture of entire tissue pieces already allowed the completion of spermatogenesis and generation of offspring in mice (Sato *et al.* 2011), research groups who reported on the formation of TOs from prepubertal tissue paid more attention to their architecture and somatic cell functionalities rather than germ cell differentiation. A major problem resides in the difficulty to maintain germ cells *in vitro* and in achieving their differentiation as was reported by several groups working with TOs (Vermeulen *et al.* 2019, Edmonds

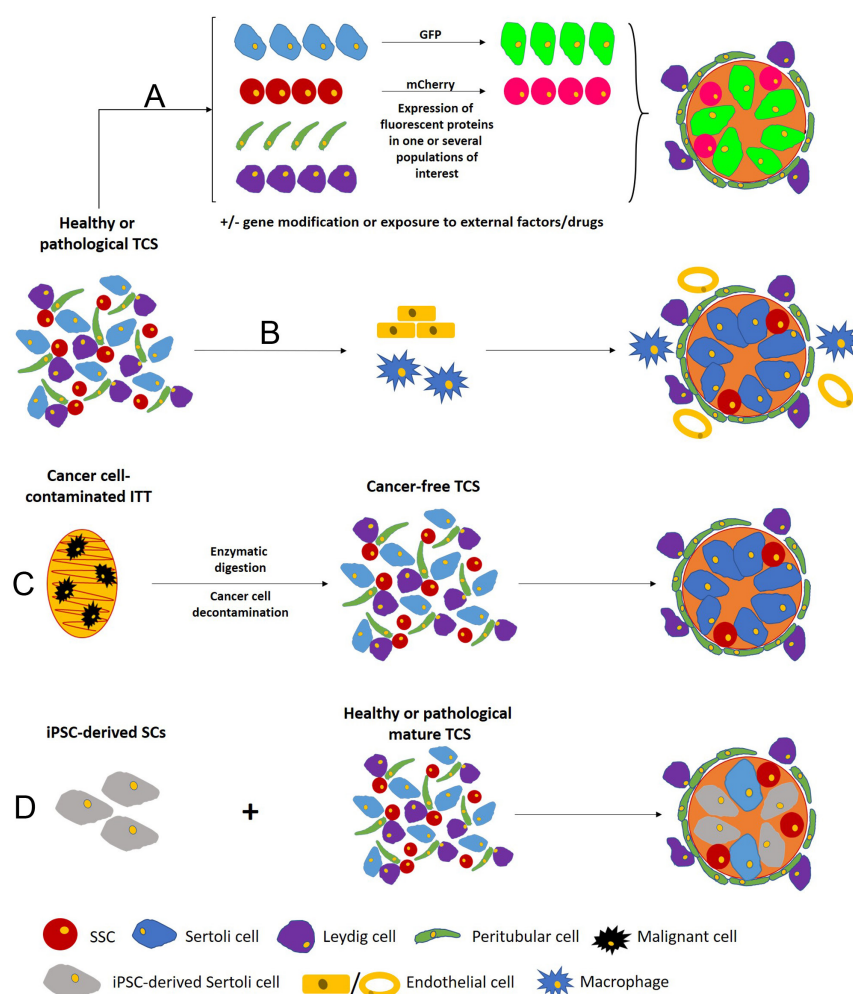


Figure 2 Potential application of TOs. (A) TCSs isolated from pathological testicular tissue (e.g. testicular cancer, Klinefelter syndrome, maturation arrest, etc.) used to generate TOs and compare their cellular organization and functionalities to healthy TOs. Genetic modifications to induce expression of fluorescent proteins in one or several populations of interest in healthy TCSs or to explore gene defects (e.g. through CRISPR-Cas9 technology) and monitor the role of each cell type in testicular morphogenesis. (B) TOs used to study the role of cell types (e.g. testicular endothelial cells and testicular macrophages) involved in the regulation of the GC niche. (C) In the case of contamination of ITT by malignant cells (e.g. in the case of leukaemia, lymphoma, metastasizing cancers), TOs are generated following specific decontamination and used for *in vitro* maturation or *in vivo* transplantation. (D) Considering the limited access to ITT for research purposes and the inability of adult SCs to drive TO self-assembly, immature SC-like cells could be generated from iPSCs and mixed with adult TCSs, either healthy or pathological, to promote the generation of chimeric TOs. Cas9, CRISPR associated protein 9; CRISPR, clustered regularly interspaced short palindromic repeats; GC, germ cell; GFP, green fluorescent protein; iPSC, induced pluripotent stem cell; ITT, immature testicular tissue; IVM, *in vitro* maturation; SC, Sertoli cell; SSC, spermatogonial stem cell; TCS, testicular cell suspension; TO, testicular organoid.

& Woodruff 2020). Indeed, in rodent TOs, germ cells differentiated until the spermatid stage in one report (Rezaei Topraggaleh *et al.* 2019), while other groups reported no differentiation (Edmonds & Woodruff 2020) or did not include this outcome in their studies (Alves-Lopes *et al.* 2017, Sakib *et al.* 2019). In non-rodent mammalian TOs, germ cell differentiation was reported but either with very low efficiency (Pendergraft *et al.* 2017) or restricted to the spermatocyte stage (Vermeulen *et al.* 2019).

These limited results in terms of spermatogenesis efficiency, especially with regard to larger mammals and primates, are mainly due to the general lack of knowledge on media components needed to reproduce spermatogenesis *in vitro* (de Michele *et al.* 2017a, de Michele *et al.* 2018a,b, Heckmann *et al.* 2020). However, as studies at the genome level during testis development, puberty, and adulthood (Hermann *et al.* 2018, Guo *et al.* 2020) start to unravel the cues and signals required for tubule morphogenesis up to the differentiation of germ cells, it is expected that optimal culture media components will be identified.

Sequential culture systems that provide the required growth factors specific to each stage of TO development (Vermeulen *et al.* 2019) coupled to microfluidic systems

providing specific concentration gradients (e.g. required to maintain a balance between stem cell self-renewal and differentiation) (Fatehullah *et al.* 2016) might contribute to the achievement of a faithful reproduction of the testis *ex vivo*.

Conclusion and perspectives

We are still in the early stages of TO formation with substantial room for improvement when addressing the numerous challenges and perspectives. TOs with structure and function closely mimicking those of the native tissue will highly likely serve as a powerful tool to study testis development, regulating mechanisms of the germ cell niche, and testicular disorders, including those leading to infertility. These are the key to developing successful methods for SSC-based therapy. Moreover, the capacity to produce self-renewing TOs in high numbers, with the possibility of modifying cell types before testicular cells' self-assembly, will greatly contribute to the understanding of the respective roles of each testicular cell-subpopulation.

As vast heterogeneity was observed in both the protocols for TO generation and TOs morphological/

Table 4 Advantages and limitations of human testicular organoids compared to immature testicular tissue (ITT) fragments.

	Organoid	ITT fragment
Physiological cells communication and adhesion/cohesion	No [†]	Yes
Possibility of large-scale production	Yes	No
Possibility to be vascularized <i>in vivo</i> and <i>in vitro</i>	Yes*	Yes
Long-term maintenance <i>in vitro</i>	Yes*	Yes*
Possibility of genetic modification	Yes	No
Possibility to be cryopreserved and stored	Yes	Yes
Potential to restore spermatogenesis when niche is damaged	Yes	No
Potential for safe use for fertility restoration in cases of haematological or metastasizing diseases	Yes	No [‡]

[†]partially, to be further explored; *Both *in vivo* (Mansour *et al.* 2018) and *in vitro* (through tissue engineering) (Grebenyuk & Ranga 2019) are possible but have so far not been applied to the testis; [‡]12 weeks in mice; ^{*}6 months in mice; [‡] only in the absence of malignant cell contamination.

functional features among studies, it is of prime importance to develop standardized protocols to ensure the reproducibility and comparability of future research outcomes on TOs.

Finally, the prospect of combining organoid technology with new bioengineering techniques (e.g. bioprinting, microfluidics), as was already attempted with double-cell constructs in alginate (Baert *et al.* 2019) or multi-organ-on-a-chip (Baert *et al.* 2020) (also referred to as assembloids) (Marton & Pasca 2020), holds promise in developing integral study models of spermatogenesis.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

Some of the studies cited in this review were supported by grants from the Fonds National de la Recherche Scientifique de Belgique (grant Télévie No. 7451016F and 7650818), the foundation Salus Sanguinis and Belgian foundation against cancer (FDC 2016-141).

Authors contribution statement

MK and MV performed the literature search and wrote the manuscript, CW performed critical revision of the manuscript and approved the final version.

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Received 5 September 2020

First decision 25 September 2020

Revised Manuscript received 22 February 2021

Accepted 2 March 2021