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Testicular organoids: a new model to study the testicular microenvironment *in vitro*?

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BACKGROUND: In recent decades, a broad range of strategies have been applied to model the testicular microenvironment *in vitro*. These models have been utilized to study testicular physiology and development. However, a system that allows investigations into testicular organogenesis and its impact in the spermatogonial stem-cell (SSC) niche *in vitro* has not been developed yet. Recently, the creation of tissue-specific organ-like structures called organoids has resurged, helping researchers to answer scientific questions that previous *in vitro* models could not help to elucidate. So far, a small number of publications have concerned the generation of testicular organoids and their application in the field of reproductive medicine and biology.

OBJECTIVE AND RATIONALE: Here, we aim to elucidate whether testicular organoids might be useful in answering current scientific questions about the regulation and function of the SSC niche as well as germ cell proliferation and differentiation, and whether or not the existing *in vitro* models are already sufficient to address them. Moreover, we would like to discuss how an organoid system can be a better solution to address these prominent scientific problems in our field, by the creation of a rationale parallel to those in other areas where organoid systems have been successfully utilized.

SEARCH METHODS: We comprehensively reviewed publications regarding testicular organoids and the methods that most closely led to the formation of these organ-like structures *in vitro* by searching for the following terms in both PubMed and the Web of Science database:

testicular organoid, seminiferous tubule 3D culture, Sertoli cell 3D culture, testicular cord formation *in vitro*, testicular morphogenesis *in vitro*, germ cell 3D culture, *in vitro* spermatogenesis, testicular *de novo* morphogenesis, seminiferous tubule *de novo* morphogenesis, seminiferous tubule-like structures, testicular *in vitro* model and male germ cell niche *in vitro*, with no restrictions to any publishing year. The inclusion criteria were based on the relation with the main topic (i.e. testicular organoids, testicular- and seminiferous-like structures as *in vitro* models), methodology applied (i.e. *in vitro culture*, culture dimensions (2D, 3D), testicular cell suspension or fragments) and outcome of interest (i.e. organization *in vitro*). Publications about grafting of testicular tissue, germ-cell transplantation and female germ-cell culture were excluded.

OUTCOMES: The application of organoid systems is making its first steps in the field of reproductive medicine and biology. A restricted number of publications have reported and characterized testicular organoids and even fewer have denominated such structures by this method. However, we detected that a clear improvement in testicular cell reorganization is recognized when 3D culture conditions are utilized instead of 2D conditions. Depending on the scientific question, testicular organoids might offer a more appropriate *in vitro* model to investigate testicular development and physiology because of the easy manipulation of cell suspensions (inclusion or exclusion of a specific cell population), the fast reorganization of these structures and the controlled *in vitro* conditions, to the same extent as with other organoid strategies reported in other fields.

WIDER IMPLICATIONS: By way of appropriate research questions, we might use testicular organoids to deepen our basic understanding of testicular development and the SSC niche, leading to new methodologies for male infertility treatment.

Key words: testicular organoids / *in vitro* testicular models / 2D and 3D culture / spermatogonial stem-cell niche / Sertoli cells / blood–testis barrier / *in vitro* spermatogenesis / male infertility / testis

Introduction

Why do we need to model the testicular microenvironment *in vitro*?

Male infertility is a multifactorial and complex disease which has been reported to affect ~7% of all males (Krausz, 2011; Nieschlag and Lenzi, 2013). However, a recent study reported a prevalence of male infertility in surveys of general populations range between 9% and 15.8% (Barratt *et al.*, 2017). The reasons for infertility can be grouped into sperm-production problems and blockage of sperm transport as well as ejaculation disorders, and they have been associated with chromosomal and gene diseases (e.g. Klinefelter's syndrome, Y-chromosome deletions, Trisomy 21), undescended testis, infections, torsions, varicocele, medicines, chemicals, radiation damage and or unknown factors that need to be addressed in future studies (Krausz, 2011; Nieschlag and Lenzi, 2013; Song *et al.*, 2016).

Recently, it was stated that although the WHO criterion for normal sperm count is >15 million sperm/mL, 'time to pregnancy' studies reported a decline in fecundity even with sperm concentrations between 30 and 55 million sperm/mL (Virtanen *et al.*, 2017). Another cross-sectional population study performed in the UK found that 1 in 10 men reported unsuccessful attempts to father over a time period of 12 months, which is one of the criteria for infertility (Datta *et al.*, 2016). These studies, together with the reported decline in sperm counts by 52.4% from 1973 until 2011 in men from North America, Europe and Australia (Levine *et al.*, 2017), highlight the need of novel investigation methodologies.

Moreover, cancer and its treatment are often connected to impaired fertility in humans, due to the cancer itself or due to the gonadotoxic effects of chemotherapy (e.g. alkylating agents, radiotherapy) (Jahnukainen *et al.*, 2015). These therapeutic agents directly or indirectly, by acting on somatic testicular cells, affect the spermatogonial stem-cell (SSC) pool and will influence later fertility (Anderson *et al.*, 2015). While storage of sperm is nowadays a clinical routine, patients who are not able to produce sperm (e.g. prepubertal boys) do not have this option yet. Therefore, novel studies on *in vitro* propagation of SSCs and *in vitro* maturation of male germ cells, as well as the development of decontamination protocols to separate cancer from testicular cells *in vitro*, are needed to provide an option to preserve future fertility in these patients (Jahnukainen *et al.*, 2015; de Michele *et al.* 2017b).

In this respect, research focused on sub- or infertility in men has dramatically increased over the last 2 decades (Zhang *et al.*, 2016). It has led to an increasing number of new guidelines for toxicology tests in the pharmaceutical industry focusing on the reproductive organs and it has raised discussion about the effects of environmental pollutants and their effects on fertility in animals and humans (Svechnikov *et al.*, 2014; Brannen *et al.*, 2016). The search for gonadotoxic effects of different compounds is however mostly restricted to animal research due to missing robust *in vitro* systems (Chapin *et al.*, 2016; Brannen *et al.*, 2016). Reproductive toxicology studies, often based on animal experiments, require a relative large number of animals and a long-term experimental research (Brannen *et al.*, 2016), and an *in vitro* system would provide more controllable and faster (e.g. by way of high-throughput analysis methods) evaluation techniques.

The successful production of murine sperm *in vitro* using testicular explant culture conditions, reported for the first time in 2011 (Sato *et al.*, 2011a,b), has subsequently been reported by several research groups (Arkoun *et al.*, 2015; Chapin *et al.*, 2016; Dumont *et al.*, 2016; Reda *et al.*, 2016). However, the system still lacks requirements enabling controlled monitoring of the biological pathways needed to create a robust model to study all aspects crucial to the spermatogenic process (e.g. SSC self-renewal and SSC niche formation and regulation). An *in vitro* methodology which shows robust reproducible results concerning crucial aspects of spermatogenesis in animals would therefore also be beneficial for future studies on human spermatogenesis. Novel cell-culture methodologies established nowadays in other fields of medical research, such as for

example organoids, might provide new tools for research into gametogenesis and its failures, which are missing today.

The organoid concept

Between the 1950s and 1980s, the term organoid had been used to nominate cellular aggregations produced by the reorganization of tissue-specific dissociated cells (Lancaster and Knoblich, 2014; Clevers, 2016). Moscona *et al*. demonstrated that dissociated primary cells from chicken embryos could self-organize into structures resembling the histological architecture of the tissue from where these cells were isolated (Moscona and Moscona, 1952; Weiss and Taylor, 1960). The self-reorganizational properties of dissociated primary cells were fundamental in the creation of *in vitro* models to study the patterns of cellular organization during development.

In the last decade, the term organoid has been applied to describe 3D organ-like structures with some organ-specific cell types, structure and functionality. Organoids can be originated by differentiation of pluripotent embryonic stem (ES) cells, induced pluripotent stem (iPS) cells or adult stem cells from adult tissues cultured in a supportive extracellular matrix (ECM) (usually Matrigel) which, together with morphogenic and differentiation factors in the culture medium, controls their formation (Clevers, 2016; Huch *et al*., 2017). These structures have dimensions up to one to two millimetres and their further expansion and maturation is limited by the diffusion range of oxygen and nutrients as they do not have a functional vascular system (Lancaster and Knoblich, 2014). Among the recently generated organ-like structures, researchers have reported the formation of murine lingual (Hisha *et al*., 2013), human brain (Lancaster *et al*., 2013; Quadrato *et al*., 2017), murine and human gut (Sato *et al*., 2009; Drost *et al*., 2015), murine and human prostate (Drost *et al*., 2016b; Chua *et al*., 2014), murine ovary (Laronda *et al*., 2017), murine bladder (Shin *et al*., 2011), human vasculature (Morgan *et al*., 2013; Zheng *et al*., 2012) and human liver (Takebe *et al*., 2013) organoids which exhibit distinct steps of development or functional units of the respective organs. Therefore, organoids have been shown to be suitable systems to model organogenesis and a useful tool in the fields of regenerative medicine, drug discovery and gene therapy.

In this article, we propose to review the methodologies that have most closely generated cellular organizations *in vitro* that model testicular architecture and functionality *in vivo*. Moreover, we will discuss the application of testicular organoids in addressing key questions in the field, such as SSC differentiation, proliferation and niche regulation, by creating a rationale parallel with reported solutions in other fields, where organoid systems have been utilized to answer specific scientific questions that previous models could not help to resolve.

Methods

In order to elaborate a comprehensive review of the application of testicular organoids in basic and translational research in the field of reproductive medicine and biology, we searched for the following terms in both PubMed and the Web of Science database: ((((((((((Testicular organoid) OR (Seminiferous tubules AND three-dimensional culture)) OR (Sertoli cell AND three-dimensional culture)) OR (Testicular cord formation AND *in vitro*)) OR (Testicular morphogenesis AND *in vitro*)) OR (Germ cell AND three-dimensional culture)) OR '*in vitro* spermatogenesis') OR (Testicular AND *de novo* morphogenesis)) OR (Seminiferous tubule AND *de novo* morphogenesis)) OR Seminiferous tubule-like structures) OR Testicular *in vitro* model [Title/Abstract]) OR (Male germ cell niche AND *in vitro*)). The search resulted in the identification of 698 articles in PubMed and 322 articles in Web of Science, with no restrictions to any publishing year. The inclusion criteria was based on the relation with the main topic (i.e. testicular organoids, testicular- and seminiferous-like structures as *in vitro* models), methodology applied (i.e. *in vitro* culture, culture dimensions (2D, 3D), testicular cell suspension or fragments) and outcome of interest (i.e. organization *in vitro*), which, together with the exclusion of publications about grafting of testicular tissue and cells, germ-cell transplantation and female germ-cell culture, resulted in the selection of 71 articles written in English. Moreover, additional relevant publications related with the topics covered in the introduction ($n = 30$) and later in the discussion ($n = 61$) were included in this review (Fig. 1).

Which models have been used to study testicular development and physiology *in vitro*?

Testicular physiology has been investigated for the last century by means of a broad range of 2D and 3D *in vitro* culture models. The 2D and 3D culture methodologies described below are hierarchically represented in Tables I and II, along with the main outcomes in terms of cellular organization and germ-cell proliferation and/or differentiation.

2D models

Using 2D models, testicular cells have been cultured on glass and plastic surfaces of culture dishes in order to explore cell-to-cell interactions between different testicular cell populations *in vitro*. Hofmann *et al*. (1992) produced immortalized cell lines from murine peritubular myoid, Sertoli, Leydig and germ cells, allowing the study of the interactions between different cell types and ECM in 2D conditions *in vitro* (Table I). Moreover, immortalized murine Sertoli, Leydig and germ cells were utilized by Hung *et al*. (2015) to demonstrate that exposure to terbufos (an organophosphate pesticide) leads to increased cell death by apoptosis in all the studied cell populations.

Other researchers have cultured primary rat Sertoli and peritubular myoid cells, either alone or combined in 2D conditions, and demonstrated the importance of cell-to-cell and cell-to-ECM interactions in Sertoli cell organization and the regulation of basement membrane gene expression *in vitro* (Tung and Fritz, 1980, 1986; Hadley *et al*., 1985; Richardson *et al*., 1995; Kierszenbaum *et al*., 1986) (Table I). More specifically, it was demonstrated that important proteins involve in androgen traffic in the testis, such as androgen binding protein, were greatly produced when Sertoli cells were co-cultured with peritubular myoid cells (Tung and Fritz, 1980) or on an ECM produced by co-cultures of these two cell types (Hadley *et al*., 1985). It was also demonstrated that fibronectin, a protein present in the basement membrane of the seminiferous tubules, was expressed in co-cultures of Sertoli and peritubular myoid cells but not in monocultures of Sertoli cells (Tung and Fritz, 1986; Richardson *et al*., 1995), revealing the important interactions of these two cell types in testicular physiology. Additionally, 2D cultures of rat primary testicular cells have also been used to study proliferation responses of co-cultured peritubular myoid and Sertoli cells (Schlatt *et al*., 1996). In this study, it was demonstrated that increased Sertoli cell density resulted in lower rates of proliferation by way of contact inhibition and that this effect could be counteracted

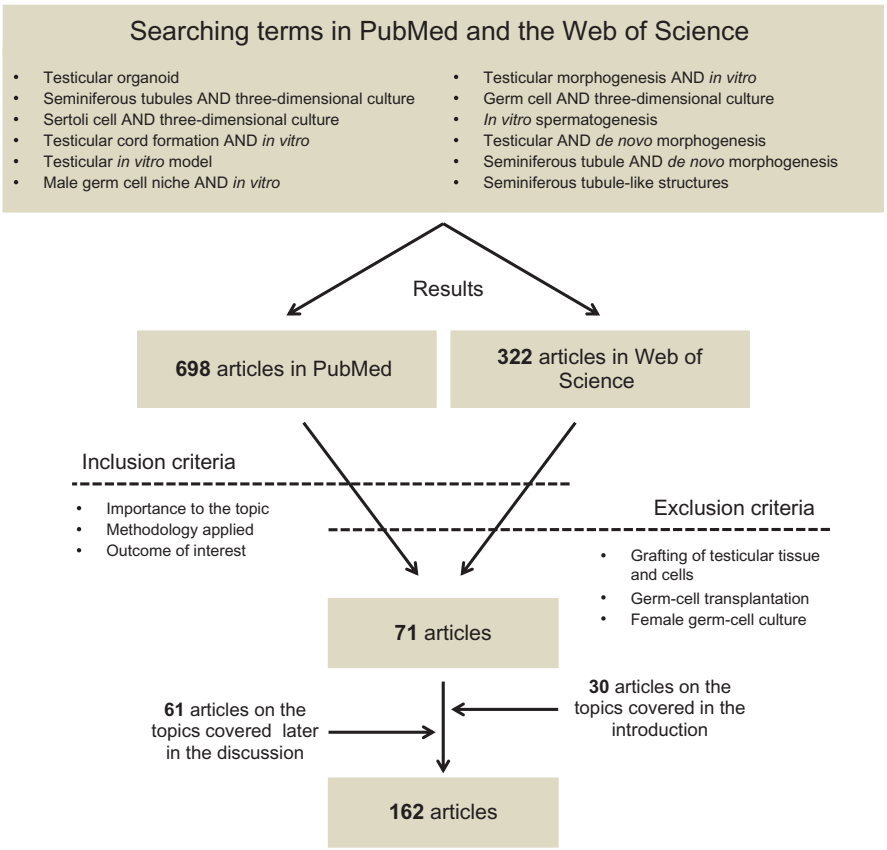

Figure 1 Searching methodology. Selected key words were searched in both PubMed and the Web of Science, resulting in 698 and 322 identified articles, respectively. After the analysis of all publications for the inclusion and exclusion criteria, 71 articles from the initial search were utilized in this review. Relevant articles on the topics covered in the introduction ($n = 30$) and later in the discussion ($n = 61$) were also included in this review.

by FSH supplementation. In additional studies, under 2D culture conditions, researchers have explored the effects of growth factors, cell-signalling molecules and hormones in organization and metabolism of rat (Kierszenbaum *et al.*, 1986; El Ramy *et al.*, 2005; Hoeben *et al.*, 1999; Tung and Fritz, 1987), murine (van der Wee and Hofmann, 1999) and piglet (Saez *et al.*, 1989) testicular cells *in vitro* (Table I).

Furthermore, 2D co-cultures of human germ cells have been utilized to prove the importance of feeder cells such as Vero (Cremades *et al.*, 1999; Tanaka *et al.*, 2003) or Sertoli cells (Tesarik *et al.*, 1998a; Sousa *et al.*, 2002) and hormonal supplementation (Tesarik *et al.*, 2000, 1998b) in the progress of human spermatogenesis *in vitro* (Table I). Similar studies, where germ cells were co-cultured with Sertoli cells on 2D surfaces, have been carried out using rat (Iwanami *et al.*, 2006; Vigier *et al.*, 2004; Tres and Kierszenbaum, 1983) and buffalo (Xie *et al.*, 2010) cells (Table I). Although progression in the spermatogenic process was observed by means of co-cultures with Vero and Sertoli cells, no cellular arrangements resembling testicular morphology were observed in these 2D cultures.

3D models

Cells and small fragments of tissue can also be cultured in supportive 3D systems in attempts to model the native arrangement and the interactions between cells and ECM. Organ-culture and the combination of dissociated cells with a supportive scaffold have been the two most utilized 3D techniques to culture testicular cells *in vitro* (Table II). As regard organ-culture, small testicular tissue fragments can be cultured integrally, preserving the intrinsic histological organization of the testis. An example of an organ-culture system is the hanging-drop method, where a fragment of testicular tissue is cultured within a small volume of medium placed on the lid of a culture dish. This method has been used to explore the effects of chemical treatments in human testis (Jorgensen *et al.*, 2014) and to study human (Jorgensen *et al.*, 2015) and murine (Potter and DeFalco, 2015) testicular development (Table II). Another organ-culture system is the air–liquid interface system, which consists of the culture of a small testicular tissue piece on a supportive stand and in simultaneous contact with the culture medium and the atmosphere. Steinberger *et al.* adapted the conditions described first by Trowell (1954) to culture immature and adult rat testicular tissue (Steinberger *et al.*, 1964; Steinberger and Steinberger, 1965). The same principal has been recently applied by different groups to promote *in vitro* spermatogenesis using tissue fragments of immature (Suzuki and Sato, 2003; Sato *et al.*, 2011a,b; Yokonishi *et al.*, 2014; Dumont *et al.*, 2015; Arkoun *et al.*, 2015; Dumont *et al.*, 2016; Chapin *et al.*, 2016; Reda *et al.*, 2017; Rondanino *et al.*, 2017) and adult murine (Sato

Table I 2D culture methodologies used to study testicular physiology *in vitro*.

Culture methodology	Cultured cells/tissue	Organization	Differentiation/ propagation of germ cells	Species	Study
Non-coated surface	Immortalized somatic and germ cell lines	Cord-like formation	N/A	Mouse	Hofmann *et al.* (1992)
	7–20 *dpp* primary Sertoli and peritubular cells	Sertoli cell aggregates	N/A	Rat	Tung and Fritz (1980), Hadley *et al.* (1985), Tung and Fritz (1986), Tung and Fritz (1987), Richardson *et al.* (1995), Schlatt *et al.* (1996), Hoeben *et al.* (1999) and El Ramy *et al.* (2005)
	7 *dpp* primary Sertoli and germ cells	None	Sg→RS	Rat	Iwanami *et al.* (2006)
	20–35 *dpp* primary Sertoli and germ cells	None	PS→RS	Rat	Tres and Kierszenbaum (1983) and Vigier *et al.* (2004)
	20–22 *dpp* primary Sertoli and peritubular cells	None	N/A	Rat	Kierszenbaum *et al.* (1986)
	7–8 *dpp* primary germ cells and feeder fibroblasts	None	None	Rat	Marcon *et al.* (2010)
	3–5 months old primary Sertoli and germ cells	None	Sg→RS	Buffalo	Xie *et al.* (2010)
	Adult primary germ cells and Vero cell line	None	RS→ES (1) PS→RS (2)	Human	(1) Cremades *et al.* (1999), (2) Tanaka *et al.* (2003)
	Adult primary germ and Sertoli cells	None	PS→ES PS→RS	Human	Tesarik *et al.* (1998a), Tesarik *et al.* (1998b), (1) Tesarik *et al.* (2000) and Sousa *et al.* (2002)
Coated Surface	Immortalized Sertoli cells	Hollow tubule formation	N/A	Mouse	van der Wee and Hofmann (1999)
	10 *dpp* primary Sertoli and peritubular cells	Polarized layers of Sertoli cells	N/A	Rat	Hadley *et al.* (1985)
	7 *dpp* primary testicular cells	Hollow tubule formation	N/A	Rat	Gassei *et al.* (2006) 7 *dpp*
	7–20 *dpp* primary Sertoli and peritubular cells	Sertoli cell aggregates	N/A	Rat	Tung and Fritz (1986) and Schlatt *et al.* (1996)

Sg, spermatogonia; PS, primary spermatocyte; RS, round spermatids; ES, elongated spermatids; N/A, not applicable; *dpp*, days *post-partum*.

et al., 2015), immature rat (Reda *et al.*, 2016; Liu *et al.*, 2016) and calf (Kim *et al.*, 2015) testes. In these experiments the testis fragments were placed on top of agar stands soaked with medium and cultured in the air–liquid interface (Table II).

Lambrot *et al.* also used the air–liquid interface method, where a membrane was used as a stand for tissue instead of agar blocks, to culture human foetal testis. The group reported a decreased number of germ cells in the cultured human foetal testis after treatment with retinoic acid (Lambrot *et al.*, 2006). In a similar study, where a membrane was also used as a stand to culture human prepubertal testicular tissue, de Michele *et al.* (2017a) maintained spermatogonia proliferating for 139 days and reported the maturation of Sertoli and Leydig cells under the same culture conditions, establishing an important model to study the pubertal transition period *in vitro*. Moreover, using the same methodology, Roulet *et al.* (2006) cultured human adult testicular fragments in order to test germ cell proliferation and differentiation. Although this research group could maintain the somatic microenvironment in the testicular fragments with germ cells proliferating and dividing for 16 days, they also reported that the culture conditions utilized led to a decrease in meiotic and post-meiotic germ cells during the experimental period (Roulet *et al.*, 2006).

Recently, Perrard *et al.* (2016) reported a bioreactor system involving chitosan hydrogel tubes where fragments of rat and human seminiferous tubules were enclosed and immersed in culture medium, allowing germ cells to differentiate up to morphologically mature spermatozoa. However, confirmation of these results by analysis of protein expression profiles of cell-specific markers was missing in this study. Using a different dynamic culture approach to the above, Komeya *et al.* (2016) developed a microfluidic device composed of a continuously flowing medium channel separated from a 160-μm-thick tissue chamber by a nutrient-permeable membrane, which allowed the maintenance of murine testicular tissue and complete spermatogenesis for 6 months. In both systems (bioreactor and microfluidic device) a constant and controlled flow of oxygen and nutrients confers an advantage over previous static organ-culture systems where although the somatic microenvironment could be preserved, germ cell counts decreased over the culture period and differentiation was compromised, probably as a result of poor homoeostasis between the tissue and the medium. This is especially relevant for future long-term cultures of human testicular fragments, where our understanding is still less than that in the mouse, and where a homoeostatic balance between the tissue fragments and the medium might be necessary to mature the somatic microenvironment to the extent of promoting germ cell differentiation.

The culture of testicular cells within a 3D supportive matrix is an alternative approach to study testicular development and spermatogenesis *in vitro*. Suspensions of murine (Abu Elhija *et al.*, 2012; Stukenborg *et al.*, 2008, 2009), rat (Reda *et al.*, 2014) or rhesus monkey (Huleihel *et al.*, 2015) testicular cells within soft-agar resulted in 3D cellular aggregates of somatic and germ cells, demonstrating a beneficial effect in cell-to-cell interaction and ultimately in the progression of spermatogenesis *in vitro* (Table II). Along the same lines, other matrixes such as methylcellulose (mouse (Stukenborg *et al.*, 2009); rhesus monkey (Huleihel *et al.*, 2015)), collagen (mouse (Khajavi *et al.*, 2014; Zhang *et al.*, 2014a); rat (Lee *et al.*, 2006b); human (Lee *et al.*, 2007)), calcium alginate (calf (Lee *et al.*, 2001); human (Lee *et al.*, 2006a)), poly(D,L-lactic-co-glycolic acid) (rat (Lee

Table II 3D culture methodologies to study testicular physiology *in vitro*.

Culture methodology			Cultured cells/tissue	Organization	Differentiation/ propagation of germ cells	Species	Study
Testicular organ-culture	Hanging-drop		Foetal testis	N/A		Mouse	Potter and DeFalco (2015)
			Foetal testis	N/A	Decreased number of gonocytes	Human	Jorgensen *et al.* (2015)
			Adult healthy or cancer testis	N/A	Germ cell proliferation	Human	Jorgensen *et al.* (2014)
	Air–liquid interface		5 d*pp* testis	N/A	Sg→RS	Mouse	Suzuki and Sato (2003)
			0.5–5.5 d*pp* testis	N/A	Sg→Sp. Production of healthy and reproducible offspring	Mouse	Sato *et al.* (2011a,b), Yokonishi *et al.* (2014)
			Adult testis	N/A	Sg→RS	Mouse	Sato *et al.* (2015)
			2.5–7 d*pp* testis	N/A	Sg→ES	Mouse	Arkoun *et al.* (2015), Dumont *et al.* (2015), Reda *et al.* (2017), and Rondanino *et al.* (2017)
			6.5 d*pp* testis	N/A	Sg→Sp	Mouse	Dumont *et al.* (2016)
			14 d*pp* testis	N/A	None	Rat	Steinberger *et al.* (1964)
			12 d*pp* and adult testis	N/A	Sg/PS to PaS	Rat	Steinberger and Steinberger (1965)
			5–7 d*pp* testis	N/A	Sg→RS	Rat	Reda *et al.* (2016) and Liu *et al.* (2016)
			10- to 14-d*pp* testis	N/A	Sg→meiotic initiation	Calves	Kim *et al.* (2015)
			Foetal testis	N/A	Decreased number of gonocytes	Human	Lambrot *et al.* (2006)
			Prepubertal testis	N/A	Maintenance of spermatogonia	Human	de Michele *et al.* (2017a)
			Adult testis	N/A	Decreased number of meiotic and post-meiotic germ cells	Human	Roulet *et al.* (2006)
	Bioreactor and microfluidic devices	Bioreactor	8- or 20 d*pp* rats and adult human	N/A	Generation of morphologically mature spermatozoa	Rat and Human	Perrard *et al.* (2016)
		Microfluidic system	0.5–5.5 d*pp* testis	N/A	Sg→Sp. Production of healthy and reproducible offspring	Mouse	Komeya *et al.* (2016)
Dissociated testicular cells	Hanging-drop		Adult testis	Cellular aggregates (Testicular Organoid)	Progression from diploid to haploid germ cells	Human	Pendergraft *et al.* (2017)
	Soft matrixes	Soft Agar Culture System	7–10 d*pp* testicular cells	Cellular aggregates	Sg→ES Sg→RS (!)	Mouse	(!) Stukenborg *et al.* (2008), Stukenborg *et al.* (2009) Review and Abu Elhija *et al.* (2012)
			5 d*pp* testicular cells	Cellular aggregates	Sg→PaS	Rat	Reda *et al.* (2014)
			13–33 months old testicular cells	Cellular aggregates	N/A	Rhesus monkey	Huleihel *et al.* (2015) Review
		Methylcellulose Culture System	7–9 d*pp* testicular cells	Cellular aggregates	Sg→ES	Mouse	Stukenborg *et al.* (2009) Review
			13–33 months old testicular cells	Cellular aggregates	Sg→RS	Rhesus monkey	Huleihel *et al.* (2015) Review

Continued

Table II *Continued*

Culture methodology		Cultured cells/tissue	Organization	Differentiation/ propagation of germ cells	Species	Study
	Matrigel	18 *dpp* testicular cells	Cord-like formation	Up to RS	Rat	Legendre *et al.* (2010)
		10 *dpp* testicular cells	Cord-like formation	Up to PaS	Rat	Hadley *et al.* (1985)
		7–10 *dpp* testicular cells	Cord-like formation	N/A	Rat	Hadley *et al.* (1990) and Gassei *et al.* (2010)
		5–7 *dpp* testicular cells	Cellular aggregates	N/A	Rat	Yu *et al.* (2009), Wegner *et al.* (2013) Protocol, Harris *et al.* (2015), and Harris *et al.* (2016)
		7 *dpp* testicular cells	Sertoli cell aggregates	N/A	Rat	Gassei *et al.* (2008)
		6 *dpp* testicular cells	Cord-like formation	Sg→meiotic initiation	Rat	Zhang *et al.* (2017)
		20 *dpp* testicular cells	Seminiferous tubule-like structures (Testicular Organoid)	Maintenance of proliferative undifferentiated germ cells	Rat	Alves-Lopes *et al.* (2017)
	Collagen	6 *dpp* testicular cells	Seminiferous tubule-like structures	Sg→PS	Mouse	Zhang *et al.* (2014a)
		18 *dpp* testicular cells	cyst-like structures	Sg→RS	Rat	Lee *et al.* (2006b)
		Adult testicular cells	Cellular aggregates	Spermatocytes up to presumptive spermatids	Human	Lee *et al.* (2007)
	Calcium alginate	3 *dpp* testicular cells	Cellular aggregates	Gonocytes to presumptive spermatids	Calves	Lee *et al.* (2001)
		Adult testicular cells	Cellular aggregates	Up to presumptive spermatids	Human	Lee *et al.* (2006a)
	PGAL	18 *dpp* testicular cells	Cellular aggregates	Spermatocytes up to presumptive spermatids	Rat	Lee *et al.* (2011)
Hard matrixes	Decellularized matrix	17–19 *dpp* (mouse) or 18 *dpp* (rat) testicular cells	Cellular aggregates	N/A	Mouse and Rat	Enders *et al.* (1986)
		Adult and 15-year-old (active spermatogenesis up to meiosis)	Cellular aggregates (Testicular Organoid)	Maintenance of proliferative spermatogonia	Human	Baert *et al.* (2017a,b), Baert and Goossens (2017) Protocol and Baert, Rombaut, and Goossens (2017) Protocol
	Sponges	7 *dpp* testicular cells	Cellular aggregates	None	Rat	Reuter *et al.* (2014)
	Nanotubes	7 *dpp* testicular cells	Cord-like formation	N/A	Rat	Pan *et al.* (2013)
Self-support	Cellular pellets on air–liquid interface	0.5–5.5 *dpp* testicular cells	Seminiferous tubule-like structures	Sg→RS	Mouse	Yokonishi *et al.* (2013)
	Rotation Cultures	New-born to adult testicular cells	Seminiferous tubule-like structures	N/A	Rat	Zenzes and Engel (1981)

Sg, spermatogonia; PS, primary spermatocyte; PaS, pachytene spermatocyte; RS, round spermatids; ES, elongated spermatids; Sp, sperm; PGAL, poly(D,L-lactic-co-glycolic acid; N/A, not applicable; *dpp*, days *post-partum*.

et al., 2011)) and Matrigel (rat (Hadley et al., 1985, 1990; Gassei et al., 2008, 2010; Legendre et al., 2010; Wegner et al., 2013; Zhang et al., 2017; Alves-Lopes et al., 2017)) have been combined with testicular cells from the stated species to explore the potential in cellular reorganization and germ cell differentiation offered by these 3D scaffolds (Table II). Instead of utilizing the previously mentioned matrixes, other researchers developed decellularized testicular matrixes to culture newly seeded rat and human cells (Enders et al., 1986; Baert et al., 2015, 2017a,b; Baert and Goossens, 2017). In these studies, the presence of native components of testicular ECM such as collagen, laminin and fibronectin, and close to *in vivo* structural organization, was thought to better guide testicular cells to reorganize *in vitro*. Furthermore, the utilization of collagen sponges (Reuter et al., 2014) and carbon nanotubes (Pan et al., 2013) to explore the effect of structural and topographic clues in rat testicular organogenesis *in vitro* was also reported, resulting in the formation of tubule-like structures (Table II). However, no germ cell differentiation was reported in these studies.

Finally, cellular aggregates can themselves work as 3D scaffolds and support cellular reorganization into testicular-like structures (Table II). One example was shown in the experimental work carried out by Zenzes *et al.* where dissociated rat testicular cells were placed in rotation cultures to explore the effects of specific cell populations and testicular maturational stages in *de novo* tissue formation (Zenzes and Engel, 1981). In another study, immature murine testicular cells were allowed to form aggregates and were later cultured on top of agar stands in an air–liquid interface which could maintain and promote the initial steps of germ-cell differentiation (Yokonishi et al., 2013).

Nevertheless, the arrival of more challenging scientific questions will impose a need to improve the existent *in vitro* models and create room for the implementation of innovative culture techniques. The establishment of novel approaches in the field of reproductive medicine and biology might simply occur via the application of *in vitro* culture technologies already being used in other areas such as bioprinting (Murphy and Atala, 2014; Vermeulen et al., 2017) or organoid cultures (Lancaster and Knoblich, 2014), the latter of which is the focus of this review.

Testicular organoids

Up to now, a restricted number of research groups have reported and characterized testicular organoids, as testis organ-like structures that partially model testicular histology and physiology by way of reorganization of dissociated testicular cells *in vitro*. Pendergraft et al. (2017) reported the generation of a functional testicular organoid system by co-culture of adult human SSCs, and immortalized human Leydig and Sertoli cells in a hanging drop of medium supplemented with solubilized human testis ECM. Although characteristic histological organization of the testis was not recognized, the group reported the maintenance and viability of the compact testicular organoids for 3 weeks and production of testosterone with or without hCG stimulation, for the same period of time. Moreover, a small fraction of diploid germ cells were reported to transit to the haploid stage. The model was also utilized to create dose–toxicity curves of chemotherapeutic drugs on testicular organoids, leading the authors to suggest their system for preliminary toxicology studies of new drugs (Pendergraft et al., 2017).

Recently, Baert et al. (2017a), in collaboration with our lab, described the generation of human testicular organoids by seeding adult and 15-year-old (with active spermatogenesis up to meiosis) testicular cells on decellularized adult testicular matrixes as scaffolds. Despite the fact that histological similarities with human testis were not detected over the time in culture, the inoculated cells demonstrated the capacity to remodel the scaffold and become reorganized in compact structures capable of testosterone and inhibin B production as well as cytokine secretion. Moreover, germ cells were proliferative for up to 4 weeks and undifferentiated germ cells could be maintained for the same culture period, suggesting this as a model to study undifferentiated germ cell propagation and testicular toxicology *in vitro* (Baert et al., 2017a).

Lately, we also described a 3D model, the three-layer gradient system that allows the reorganization of 20-day-old rat testicular cells into testicular organoids after 7 days in culture (Alves-Lopes et al., 2017). These testicular organoids were mainly constituted by Sertoli and germ cells organized in spherical-tubular structures. Moreover, a functional blood–testis barrier was reported among neighbour Sertoli cells and proliferative undifferentiated germ cells could be observed on these structures up to 21 days. Furthermore, the similarity of the results obtained with our model, in terms of germ cell maintenance and blood–testis barrier integrity, to those obtained previously in *in vivo* studies on the effect of retinoic acid and pro-inflammatory cytokines in testicular physiology, led us to propose this as testicular organoid model to search for unknown factors involved in SSC proliferation and differentiation (Alves-Lopes et al., 2017).

Methodologies that most closely generate testicular organoids

A clear improvement in testicular cell reorganization is recognized in the transition from 2D to 3D culture conditions (Fig. 2). Although the majority of 2D testicular cell co-cultures have resulted in cord-like structures where aggregates of Sertoli cells are connected by 'cables' of peritubular myoid cells (Tung and Fritz, 1986; Richardson et al., 1995; Schlatt et al., 1996; Gassei et al., 2006), there are reports of the formation of seminiferous tubule-like structures, designated 'nodules' and 'protrusions' when cells were cultured for 21 (Tung and Fritz, 1987) and 49 days (Tung and Fritz, 1980), respectively (Fig. 2). These experiments led to seminiferous tubule-like structures as result of overlapping and folded cell layers and the long period of culture, but the organization of the Sertoli cells was not similar to that observed in the epithelium of seminiferous tubules. However, Sertoli cells were more organized and formed epithelial layers when co-cultured on a layer of reconstituted ECM (Hadley et al., 1985; van der Wee and Hofmann, 1999; Gassei et al., 2006) (Fig. 2). The effect of ECM in testicular cell reorganization is even more pronounced when cells are co-cultured within the matrix (e.g. Matrigel (Hadley et al., 1985; Legendre et al., 2010; Alves-Lopes et al., 2017), or collagen (Zhang et al., 2014a)). In these experiments, Sertoli cells rearranged themselves into tubule-like structures surrounded by newly produced basal lamina and/or peritubular cells, faster than in 2D conditions (Fig. 2). Moreover, tight junctions and tight junction protein components (e.g. claudin-11 and zonula occludens-1) were detected between the reorganized Sertoli cells, which could also support germ cells at different stages of differentiation (Fig. 2). Finally, the 3D support offered by the initial cell aggregate was found to be effective in the generation of murine seminiferous tubule-like structures (Yokonishi et al., 2013). The 3D support given by the cellular aggregate itself was also observed when new-born (8-10-day-old *post-partum*) and juvenile (18-25-day-old *post-partum*) rat testicular cells were cultured in rotation and allowed to form seminiferous tubule-like structures (Zenzes and Engel,

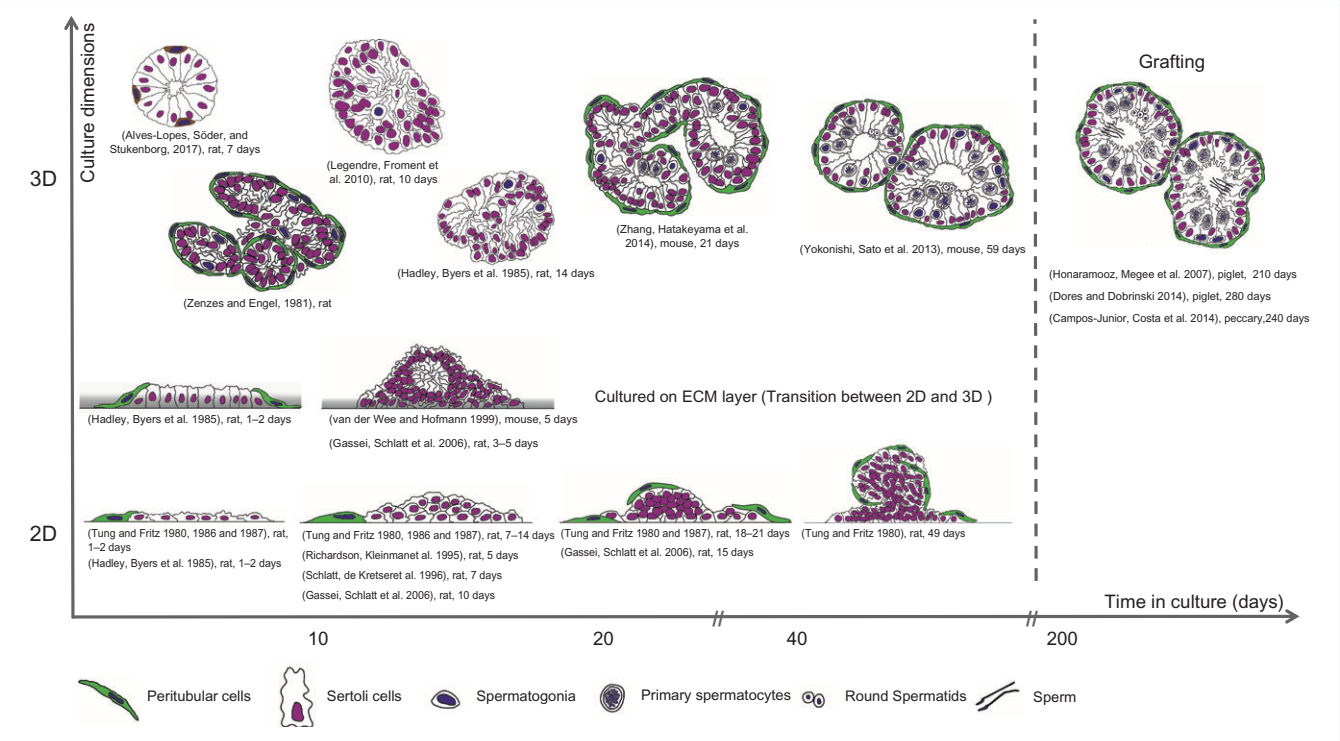

Figure 2 Testicular cell organization *in vitro* varies with culture dimensions and time in culture. More complex cell associations are observed over time in 2D conditions, but a clear improvement in organization is observed when going from 2D to 3D culture conditions *in vitro*. The latter conditions facilitate cellular organization, and over time in culture they enable the appearance of characteristic testicular features such as formation of the blood–testis barrier and spermatogenesis progression, in contrast to the cellular associations obtained in 2D conditions over the same period of time. Some improvement in testicular cell organization is already observed when cells are cultured on a layer of reconstituted ECM (transition from 2D to 3D). Additionally, grafting of pellets or suspensions of cells embedded in extracellular matrix (ECM) under the skin of immunodeficient mice has demonstrated a beneficial effect in cellular reorganization. Cultured in 3D conditions and over a long period of time, these testicular cells become reorganized into testis-like tissue, leading to initiation and progression of spermatogenesis. Although the grafting techniques do not permit the generation of testicular organoids under controlled conditions, important evidence, such as the use of high cell concentrations, the development of vasculature and the roles of still unknown host morphogenic factors, might be applied to the development of testicular organoids exclusively *in vitro*. The species utilized and the culture period of each experiment is specifically mentioned in the figure after the corresponding reference.

1981) (Fig. 2). Together, these observations suggest that cells can better and faster reorganize themselves in testicular-like structures when a 3D support is applied rather than a 2D system where more disorganized cellular constructs can be observed over the time of culture, with overlapping and folding of cellular layers (Fig. 2).

Although 3D culture conditions favour the generation of testicular organ-like structures, not all 3D models have allowed such reorganization. The differences seem to be related to the nature of the scaffold, the cell concentration and the maturational stage of the donor. As regards the scaffold, the soft-agar culture system is an example of a 3D culture condition where organized testicular-like structures are not observed (Stukenborg *et al.*, 2008; Abu Elhija *et al.*, 2012; Reda *et al.*, 2014). This could be due to the fact that soft-agar does not contain some of the basal lamina components such as collagen and laminin which are present in reconstituted ECM such as Matrigel. These proteins provide testicular cells with an environment close to those *in vivo* and spatial clues for cellular reorganization *in vitro* (Legendre *et al.*, 2010; Hadley *et al.*, 1985; Alves-Lopes *et al.*, 2017). The application of collagen sponges is another example of a 3D scaffold which does not allow testicular cells to form seminiferous tubule-like structures, most probably because the cells cannot recycle the ECM and create an appropriate histological organization; instead they just occupy the cavities of the scaffold (Reuter *et al.*, 2014).

Concerning cell concentration, Zhang *et al.* (2014a) demonstrated that pellets of dissociated murine testicular cells embedded in a collagen matrix could form seminiferous tubule-like structures. However, this histological pattern was not observed when rat testicular cells were combined with collagen at a concentration of ~2.5 million cells/mL (Lee *et al.*, 2006b). We also observed that higher cell concentrations benefits the formation of better testicular organoids from 20-day-old rat cells (Supplementary data in Alves-Lopes *et al.* (2017)). These findings suggest that an increase in cell concentration might favour the formation of bigger and more complex organoid structures *in vitro*, probably due to the reduced distance between cells and consequently easier cell-to-cell and paracrine communications. This might finally avoid the formation of more disconnected and disperse cell aggregates as we observed in our *in vitro* experiments (effect of cell concentration on testicular organoid formation in Alves-Lopes *et al.*, 2017).

Moreover, as mentioned before, Zenzes and Engel (1981) showed that new-born and juvenile rat testicular cells can reorganize themselves in seminiferous tubule-like structures in rotation culture. However, in the same study, it was demonstrated that a mixture of all testicular cell types from adult rats cannot regenerate in the same way showing that the maturational stage of the donor has a role in testicular organoid formation. We also observed this phenomenon in our studies, where we reported that

5-8- and 20-day-old, but not 60-day-old, but not 60 days old, rat testicular cells could reorganize in *in vitro* 3D culture conditions (Alves-Lopes *et al.*, 2017).

A more advanced status of cellular reorganization and testicular functionality was achieved when pellets or suspensions of cells were embedded in ECM and grafted under the skin or kidney capsule of immunodeficient mice (Fig. 2). This methodology was applied to generate testicular-like structures from immature piglet (Dufour *et al.*, 2002; Honaramooz *et al.*, 2007; Kita *et al.*, 2007; Dores and Dobrinski, 2014), marmoset monkey (Aeckerle *et al.*, 2013), lamb (Arregui *et al.*, 2008), peccary (Campos-Junior *et al.*, 2014), rat (Kita *et al.*, 2007; Gassei *et al.*, 2006, 2008, 2010) and murine (Kita *et al.*, 2007; Zhang *et al.*, 2014b) testicular cells. In some of these studies, testicular functionality was restored in these *de-novo* created tubules, leading to initiation and progression of spermatogenesis up to haploid-cell stages (Honaramooz *et al.*, 2007; Arregui *et al.*, 2008; Dores and Dobrinski, 2014) (Fig. 2), which in some cases were shown to fertilize donor oocytes, generate embryos (Campos-Junior *et al.*, 2014) (Fig. 2) and produce offspring (in mice Kita *et al.*, 2007; Zhang *et al.*, 2014b). The generation of testicular organ-like structures by grafting of cell suspensions offers an important platform to study testicular development and functionality, with the possibility to include, exclude or genetically modify a specific a cell population before grafting. However, if the study design needs a more controlled environment, the unknown factors that the host provides to the grafted cells can compromise the outcome of the experiment. In such cases, an exclusively *in vitro* system that generates similar structures would be preferable. However, translation of the results obtained by grafting to a completely *in vitro* system has not been achieved so far. The use of high cell concentrations, the development of vasculature and the role of the still unknown host morphogenic factors seem to be key aspects in testicular cell reorganization under grafting conditions *in vivo* that are still missing in the majority of *in vitro* approaches applied.

Why do we need testicular organoids?

Are the previous models not sufficient to address the scientific questions in the field? Although the *in vitro* methodologies used up to now have provided important information about the production of ECM and its influence on testicular reorganization, testicular toxicology (Steinberger and Klinefelter, 1993; Rodriguez and Bustos-Obregon, 2000; Yu *et al.*, 2009; Marcon *et al.*, 2010; Jorgensen *et al.*, 2014; Harris *et al.*, 2015, 2016; Goldstein *et al.*, 2016) and germ cell differentiation *in vitro*, novel techniques such as bioprinting (Murphy and Atala, 2014; Vermeulen *et al.*, 2017) and organoid cultures (Lancaster and Knoblich, 2014) are arising and will back up the previous methods. Testicular organoids might provide a new and promising variation on already existing methods, helping researchers to answer scientific questions in a simple and efficient way because of the easy manipulation of cell suspensions, the relatively fast reorganization of these structures and the controlled *in vitro* conditions (Alves-Lopes *et al.*, 2017; Baert *et al.*, 2017a; Pendergraft *et al.*, 2017).

One of the possible applications of testicular organoids is manipulation of a gene of interest in a chosen cell population, which would lead to less costly and laborious knockout strategies. In addition, use of testicular organoids could be a solution in studies focusing on genes that are lethal if knocked-out early in life, thereby making them difficult if not impossible to study. One example in this regard is glial-cell-line-derived neurotrophic factor (GDNF) and its receptor Gfrα-1, both of which are important in the SSC niche in the testis (Moore *et al.*, 1996; Enomoto *et al.*, 1998; Pichel *et al.*, 1996). To overcome this issue, testicular cell suspensions could be transfected by electroporation or viral infection, as already demonstrated *in vivo* (Yomogida *et al.*, 2002; Ikawa *et al.*, 2002; Kanatsu-Shinohara *et al.*, 2002) and *in vitro* (Miura *et al.*, 2007; Kanatsu-Shinohara *et al.*, 2012; Li *et al.*, 2013), or the site-specific genome modified by Cas9 RNA-guided endonuclease (Cho *et al.*, 2013; Cong *et al.*, 2013), after being allowed to form testicular organoids in culture. This strategy might also be used to overcome the problems regarding low efficiency in gene delivery *in vivo* and in organ-culture systems by simply transfecting single cell suspensions before testicular organoid formation.

The 3D organization of organoids confers advantage over the conventional 2D conditions because cell-to-cell and cell-to-ECM relationships are better modulated. Following this approach, testicular organoids could also be applied to explore testicular development by tracking the reorganization process and the interactions between different cell populations in a 3D environment mimicking the *in vivo* situation better than 2D culture conditions (Fig. 2). Moreover, the influence of distinct components of the SSC niche can be investigated by means of testicular organoids because these systems allow the modification, inclusion or exclusion of parts of this microenvironment, helping researchers to understand their complex interactions. This strategy will give to researchers a simpler and more efficient tool to identify unknown factors responsible for SSC propagation and its complex mechanism of differentiation, in comparison with current models.

Future perspectives

Organoids as tools to answer scientific questions

Organoids for different organs have been employed to study development, stem-cell to stromal–cell interactions and mechanisms of disease, or to experiment with personalized therapy strategies. Among these models are the intestinal organoids, consisting of small-intestine-crypt-villus-like structures generated from murine primary adult stem cells (Sato *et al.*, 2009) and more recently from human ES and iPS cells (Spence *et al.*, 2011). These organoids can be genetically manipulated by electroporation (Fujii *et al.*, 2015) or viral (Drost *et al.*, 2016a) delivery of transgenes or by Cas9 RNA-guided endonuclease (Drost *et al.*, 2016a; Fujii *et al.*, 2015) to study cell signalling and stem-cell niche homoeostasis mechanisms of the intestinal crypt.

Another important improvement in the field of regenerative medicine was the establishment of protocols to create artificial vasculature *in vitro* (Morgan *et al.*, 2013; Zheng *et al.*, 2012). This is an important aspect because lack of a vascular network limits the size of the organoids, since nutrients can only reach the cells by diffusion. The presence of microvasculature in organoids is also important as regards possible transplantation of an *in vitro* generated organ. To address this aspect, researchers thought to combine human umbilical vein endothelial cells (HUVECs) (Takebe *et al.*, 2013) or human dermal microvascular endothelial cells (Heller *et al.*, 2016) in the initial cell suspensions that later generated vascularized liver-buds and buccal mucosa organoids.

In vitro models of diseases representing a situation closer to that *in vivo* are another application of organoid technologies. The generation of prostate organoids from healthy primary cells and cancer cells has

been reported (Drost *et al.*, 2016b; Chua *et al.*, 2014). Moreover, genetic modifications in commonly affected genes of colorectal cancer have been induced in primary cells, by Cas9 RNA-guided endonuclease (Drost *et al.*, 2015) or viral transfection (Li *et al.*, 2014), which were subsequently cultured in a 3D system to form intestinal organoids. Such approaches are promising in modelling cancer and its microenvironment, along with other *in vitro* techniques and *in vivo* models.

In addition to the above, organoids formed from immature primary cells or early-stage differentiated pluripotent stem cells give the opportunity to study the initial steps of development of various organs (Takebe *et al.*, 2013; Lancaster *et al.*, 2013; Takasato *et al.*, 2015). Co-culture of hepatic endoderm cells differentiated from human iPS cells with HUVECs and human mesenchymal stem cells in Matrigel resulted in liver-bud organoids modelling early human liver development *in vitro* (Takebe *et al.*, 2013). Moreover, cerebral organoids displaying distinct brain regions have also been generated by the differentiation of human ES cells (Lancaster *et al.*, 2013). Although not completely as observed *in vivo*, these organoids demonstrated distinct characteristics of human brain organogenesis, making them valuable in the study of cerebral development *in vitro*. Another example of a developmental study *in vitro* is the formation of kidney organoids from human iPS cells in 3D culture conditions (Takasato *et al.*, 2015). The genetic transcriptional similarities between the organoids generated *in vitro* and the human foetal kidney in the first trimester make this system a promising tool to study cellular interactions during development and to model human kidney diseases.

The described strategies have already been demonstrated to be important in exploring physiology, pathology and the development of various organs *in vitro*. In the next section, we outline potential experiments by applying the concepts and methodologies described for the generation of other organoids to study, among other things, SSC niche, testicular disease and development. In view of this, a testicular organoid simply constituted of Sertoli and germ cells will be used as the platform to design and explain our proposed testicular organoid applications (Fig. 3).

Testicular organoids: exploring niche, disease and developmental events

There is an urgent need to understand the SSC niche and the basic mechanisms governing this microenvironment. This information would provide valuable clues about the processes of SSC self-renewal and differentiation *in vivo* that can afterwards be logically translated to *in vitro* applications. The niche of SSCs is simpler and more localized in small organisms such as *Caenorhabditis elegans* and *Drosophila melanogaster* and, because of this, much more studied and understood. In these organisms, SSCs are closely located to specialized somatic cells in the apical compartment of the male gonads that promotes SSCs self-renewal. SSCs differentiation starts when they move away from these locations (Kimble and White, 1981; Tulina and Matunis, 2001; Kiger *et al.*, 2001). However, in mammals the SSC niche is not restricted to one individual location, but rather distributed throughout the seminiferous tubules in the testis (Ogawa *et al.*, 2005; Yoshida *et al.*, 2007; Ikami *et al.*, 2015). Although a lot remains unknown, studies using mice suggested that components of the vascular system (Yoshida *et al.*, 2007) and paracrine factors secreted by stromal cells, such as GDNF (Meng *et al.*, 2000; Kubota *et al.*, 2004) and colony-stimulating factor 1 (CSF-1) (Kokkinaki *et al.*, 2009; Oatley *et al.*, 2009), might have an essential role in the SSC niche *in vivo* by promoting SSC self-renewal.

An *in vitro* system that supports the SSC niche would be appreciated in the study of SSC self-renewal and differentiation. For this purpose, testicular organoids might offer a suitable model and a simple approach to test candidate factors related to SSC self-renewal, such as paracrine factors secreted in the SSC niche (e.g. GDNF and CSF-1), because distinct niche components could be reassembled and manipulated *in vitro*. One possibility for testing these paracrine factors might be the generation of a testicular organoid system composed of Sertoli and SSCs cultured in medium supplemented with growth factors of interest. The potential of the tested growth factor in SSC self-renewal or differentiation could be verified by the increased capacity of a organoid cultured in testing conditions to support SSCs when compared with a organoid cultured in control conditions (Fig. 3A).

As discussed above, the mammalian SSC niche is restricted to facultative regions of the seminiferous tubules and just a few cells from the whole Sertoli cell population in the testis are associated with this niche (Ogawa *et al.*, 2005; Yoshida *et al.*, 2007). To model this situation *in vitro*, an organoid system composed of wild-type Sertoli cells, SSCs and a minimal fraction of green fluorescent protein (GFP)-marked Sertoli cells over-expressing a candidate factor for SSC self-renewal might be applied (Fig. 3B). Primary Sertoli cells could be genetically modified by electroporation or viral delivery of transgenes, or by Cas9 RNA-guided endonuclease and then co-cultured with SSCs and wild-type Sertoli cells. In this hypothetical system, it would be interesting to investigate first if there would be increased proliferation or self-renewal of SSCs particularly associated with the GFP-positive Sertoli cells, and secondly if there would be decreased self-renewal and/or initiation of differentiation of those germ cells that would be progressively further from the GFP-positive cells and harboured by the wild-type Sertoli cells (Fig. 3B).

Although Sertoli cells are necessary components in the SSC niche, other players such as microvasculature are thought to have an important role in this microenvironment (Yoshida *et al.*, 2007). In order to investigate the role of microvasculature in the SSC niche, a testicular organoid generated from wild-type Sertoli cells, SSCs and an endothelial cell line, such as HUVECs, might be used to generate a capillary network in a manner similar to that achieved for liver-bud and buccal mucosa organoids (Takebe *et al.*, 2013; Heller *et al.*, 2016). In this theoretical system, one might explore the effect of endothelial cells in SSC self-renewal by comparison of SSC proliferation rates between organoids with and without capillary network (Fig. 3C). Moreover, the indirect effect of endothelial cells on SSC self-renewal via expression of a particular factor by Sertoli cells might also be investigated. To explore this, a gene of interest would be associated with the expression of GFP in Sertoli cells. Comparison of vascularized and non-vascularized organoids would allow identification of the effect of endothelial cells on expression of the investigated factor via GFP expression in Sertoli cells and ultimately the effect on SSC self-renewal by way of the proliferation rate of these cells (Fig. 3D).

Testicular organoids might also be applied to study testicular cancer *in vitro*. Organoids generated from Sertoli cells of carcinogenic testicular tissues could be used to study the influence of cancer microenvironment on germ cell proliferation *in vitro*. Moreover, the generation of testicular organoids from carcinogenic testicular tissue could help to identify transformed signalling pathways and genetic modifications that lead to

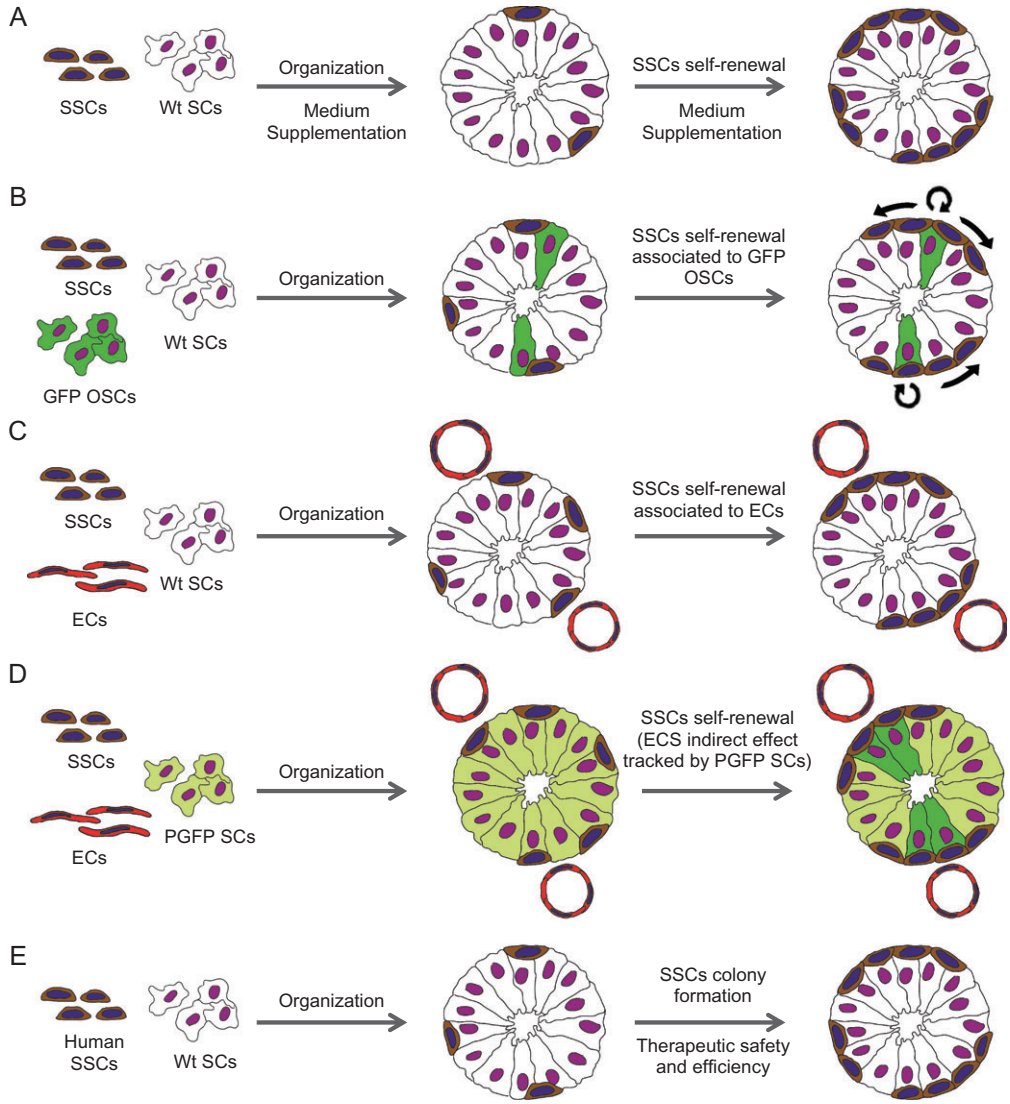

Figure 3 Hypothetical testicular organoid applications. (**A**) Generation of testicular organoids composed of wild-type Sertoli cells (Wt SCs) and spermatogonial stem cells (SSC) in culture conditions supplemented with candidate factors for SSC self-renewal or differentiation. (**B**) Testicular organoid composed of Wt SCs, SSCs and a minimal fraction of green fluorescent protein (GFP)-marked Sertoli cells over-expressing (GFP OSCs) a candidate factor for SSC self-renewal; (round arrow) proliferation or self-renewal of SSCs special associated with GFP-positive Sertoli cells; (bended arrow) decreased self-renewal and/or initiation of differentiation. (**C**) Testicular organoid generated from Wt SCs, SSCs and endothelial cells (ECs). (**D**) Tracking the indirect effect of endothelial cells in SSC self-renewal by the application of testicular organoids composed of Sertoli cells expressing GFP associated with the expression of a gene of a factor of interest (PGFP SCs), SSCs and ECs. (**E**) Testicular organoids formed by human SSCs and Wt SCs to assess SSC proliferation and therapeutic safety and efficiency.

unbalanced tissue homoeostasis in both carcinogenic and non-carcinogenic cells of the testicular cancer microenvironment.

Understanding of the mechanisms regulating development is fundamental in the field of regenerative medicine and ultimately our knowledge of testicular development might be applied to the generation and differentiation of testicular cells *in vitro*. As demonstrated in regard to other organs (Takebe *et al.*, 2013; Lancaster *et al.*, 2013; Takasato *et al.*, 2015), the application of human ES and iPS cells to model initial stages of testicular development will potentiate studies in this area, especially if access to human foetal material is restricted. Several protocols to differentiate ES and iPS cells or transdifferentiate somatic cells into testicular somatic (ES (Bucay *et al.*, 2009; Yang *et al.*, 2015; Kjartansdottir *et al.*, 2015); transdifferentiation (Buganim *et al.*, 2012)) or germ-cell lines (ES (Bucay *et al.*, 2009; Lim *et al.*, 2014; Kjartansdottir *et al.*, 2015); iPS (Panula *et al.*, 2011; Yang *et al.*, 2012; Cai *et al.*, 2013); transdifferentiation (Medrano *et al.*, 2016; Ge *et al.*, 2015)) have already been reported and sooner or later 3D co-cultures of these early differentiated cells might produce testicular organoids for the study of testicular development. However, more standardized and reproducible protocols to differentiate pluripotent stem cells into testicular cells are needed to generate consistent results in terms of organoid formation and experimental outcomes.

In addition to the above, human testicular organoids produced from primary cells or derived from the differentiation of pluripotent stem cells might also represent a platform to test the safety and efficiency of future *in vivo* genetic therapies (Fig. 3E), which have already been employed to rescue spermatogenesis *in vivo* in a murine model (Yomogida *et al.*, 2002; Ikawa *et al.*, 2002; Kanatsu-Shinohara *et al.*, 2002), representing one possible solution to the problem of the lack of an *in vivo* model as regards the human testis.

Conclusions

The development of testicular organoids will bring the opportunity to explore testicular physiology *in vitro* by means of simpler and more convenient methodologies, as already demonstrated in other scientific areas, allowing researchers to address more challenging questions. More complete comprehension of how the germ cell niche is regulated will be essential to manipulate SSC self-renewal and differentiation *in vitro* and extend these methodologies to clinical applications in reproductive medicine. To achieve this goal, the experimental strategies outlined in this review might represent the first steps in the application of testicular organoids in the search for unknown factors ruling this microenvironment. Overall, testicular organoids do not represent a revolutionary technology but instead an innovative platform to reassemble testis-like structures on a small scale and in a controlled *in vitro* environment that in the short term can be applied to back up previous models in answering current and future scientific interrogations in the field of reproductive medicine and biology.

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Authors' roles

J.P.A.L. and J.-B.S. designed the review. J.P.A.L. performed the literature review, analyses of the data and conceived the article. J.P.A.L. and J.-B.S wrote the article.

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Conflicts of interest

The authors declare that they have no competing financial interests.

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