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:
The application of organoid systems is making its way into 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.

**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 / tests

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### 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 ranging 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 ejaculatory disorders, and they have been associated with chromosomal and gene diseases (e.g. Klinefelter’s syndrome, Y-chromosome deletions, Trisomy 21), undescended tests, infections, torsions, varicoceles, 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 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 gam-
etogenesis 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 resem-
bling 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 pluri-
potent embryonic stem (ES) cells, induced pluripotent stem (iPS) cells
or adult stem cells from adult tissues cultured in a supportive extracellu-
lar matrix (ECM) (usually Matrigel) which, together with morphogenic
and differentiation factors in the culture medium, controls their forma-
tion (Clevers, 2016; Huch et al., 2017). These structures have dimen-
sions 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 func-
tional 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 tes-
ticular 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 regula-
tion, 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 testicu-
lar 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 tube 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 struc-
tures as in vitro models, methodology applied (i.e. in vitro culture, culture
dimensions (2D, 3D), testicular cell suspension or fragments) and out-
come of interest (i.e. organization in vitro), which, together with the exclu-
sion 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 repre-
sented in Tables I and II, along with the main outcomes in terms of cel-
lular 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 inter-
actions 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 interac-
tions 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
decay 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 demon-
strated 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

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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 cocultured 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 cocultures 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; Rondonino et al., 2017) and adult murine (Sato

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**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.
Table 1 2D culture methodologies used to study testicular physiology in vitro.

<table>
<thead>
<tr>
<th>Culture methodology</th>
<th>Cultured cells/tissue</th>
<th>Organization</th>
<th>Differentiation/propagation of germ cells</th>
<th>Species</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-coated surface</td>
<td>7–20 dpp primary Sertoli and peritubular cells</td>
<td>Card-like formation</td>
<td>N/A</td>
<td>Mouse</td>
<td>Hofmann et al. (1992)</td>
</tr>
<tr>
<td></td>
<td>7–20 dpp primary Sertoli and peritubular cells</td>
<td>Sertoli cell aggregates</td>
<td>N/A</td>
<td>Rat</td>
<td>Tung and Fritz (1980), Hadley et al. (1985), Tung and Fritz (1986), Tung and Fritz (1987), Richardson et al. (1993), Schlatt et al. (1996), Hoebe et al. (1999) and El-Ramy et al. (2005)</td>
</tr>
<tr>
<td>Coated Surface</td>
<td>Immortalized Sertoli cells</td>
<td>None</td>
<td>Sg-RS</td>
<td>Rat</td>
<td>Kierszenbaum et al. (1986)</td>
</tr>
<tr>
<td></td>
<td>7–20 dpp primary Sertoli and peritubular cells</td>
<td>Polarized layers of Sertoli cells</td>
<td>PS-RS</td>
<td>Human</td>
<td>Xie et al. (2010)</td>
</tr>
<tr>
<td></td>
<td>7–20 dpp primary Sertoli and peritubular cells</td>
<td>Polarized layers of Sertoli cells</td>
<td>N/A</td>
<td>Rat</td>
<td>Xie et al. (2006)</td>
</tr>
<tr>
<td></td>
<td>7–20 dpp primary Sertoli and peritubular cells</td>
<td>Polarized layers of Sertoli cells</td>
<td>N/A</td>
<td>Human</td>
<td>Tesarki et al. (1998a), Tesarki et al. (1998b), Tesarki et al. (2000) and Sousa et al. (2002)</td>
</tr>
</tbody>
</table>

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

The table provides a summary of various 2D culture methodologies used to study testicular physiology in vitro. Each methodology is characterized by the type of cells/tissue cultured, the organization used, and the species involved. The study details are also provided, including references for further reading.
### Table II 3D culture methodologies to study testicular physiology in vitro.

<table>
<thead>
<tr>
<th>Culture methodology</th>
<th>Cultured cells/tissue</th>
<th>Organization</th>
<th>Differentiation/propagation of germ cells</th>
<th>Species</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testicular organ-culture</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Hanging-drop</td>
<td>Foetal testis</td>
<td>N/A</td>
<td>Decreased number of gonocytes</td>
<td>Mouse</td>
<td>Potter and DeFalco (2015)</td>
</tr>
<tr>
<td></td>
<td>Foetal testis</td>
<td>N/A</td>
<td></td>
<td>Human</td>
<td>Jorgensen et al. (2015)</td>
</tr>
<tr>
<td></td>
<td>Adult healthy or cancer testis</td>
<td>N/A</td>
<td>Germ cell proliferation</td>
<td>Human</td>
<td>Jorgensen et al. (2014)</td>
</tr>
<tr>
<td></td>
<td>5 dpp testis</td>
<td>N/A</td>
<td>Sg→RS</td>
<td>Mouse</td>
<td>Suzuki and Sato (2003)</td>
</tr>
<tr>
<td></td>
<td>0.5–5.5 dpp testis</td>
<td>N/A</td>
<td>Sg→Sp. Production of healthy and reproducible offspring</td>
<td>Mouse</td>
<td>Sato et al. (2011a,b), Yokonishi et al. (2014)</td>
</tr>
<tr>
<td></td>
<td>Adult testis</td>
<td>N/A</td>
<td>Sg→RS</td>
<td>Mouse</td>
<td>Sato et al. (2015)</td>
</tr>
<tr>
<td></td>
<td>2.5–7 dpp testis</td>
<td>N/A</td>
<td>Sg→ES</td>
<td>Mouse</td>
<td>Arkoun et al. (2015), Dumont et al. (2015), Reda et al. (2017), and Rondanino et al. (2017)</td>
</tr>
<tr>
<td></td>
<td>6.5 dpp testis</td>
<td>N/A</td>
<td>Sg→Sp</td>
<td>Mouse</td>
<td>Dumont et al. (2016)</td>
</tr>
<tr>
<td></td>
<td>1.4 dpp testis</td>
<td>N/A</td>
<td>None</td>
<td>Rat</td>
<td>Steinberger et al. (1964)</td>
</tr>
<tr>
<td></td>
<td>12 dpp and adult testis</td>
<td>N/A</td>
<td>Sg/PS to PaS</td>
<td>Rat</td>
<td>Steinberger and Steinberger (1965)</td>
</tr>
<tr>
<td></td>
<td>5–7 dpp testis</td>
<td>N/A</td>
<td>Sg→RS</td>
<td>Rat</td>
<td>Reda et al. (2016) and Liu et al. (2016)</td>
</tr>
<tr>
<td></td>
<td>10- to 14-dpp testis</td>
<td>N/A</td>
<td>Sg→meiotic initiation</td>
<td>Calves</td>
<td>Kim et al. (2015)</td>
</tr>
<tr>
<td></td>
<td>Foetal testis</td>
<td>N/A</td>
<td>Decreased number of gonocytes</td>
<td>Human</td>
<td>Lambert et al. (2006)</td>
</tr>
<tr>
<td></td>
<td>Prepubertal testis</td>
<td>N/A</td>
<td>Maintenance of spermatogonia</td>
<td>Human</td>
<td>de Michele et al. (2017a)</td>
</tr>
<tr>
<td></td>
<td>Adult testis</td>
<td>N/A</td>
<td>Decreased number of meiotic and post-meiotic germ cells</td>
<td>Mouse</td>
<td>Roulet et al. (2006)</td>
</tr>
<tr>
<td>Bioreactor and microfluidic devices</td>
<td>Bioreactor</td>
<td>N/A</td>
<td>Generation of morphologically mature spermatozoa</td>
<td>Rat and</td>
<td>Perrard et al. (2016)</td>
</tr>
<tr>
<td></td>
<td>8- or 20 dpp rats and adult human</td>
<td>N/A</td>
<td></td>
<td>Human</td>
<td></td>
</tr>
<tr>
<td>Microfluidic system</td>
<td>0.5–5.5 dpp testis</td>
<td>N/A</td>
<td>Sg→Sp. Production of healthy and reproducible offspring</td>
<td>Mouse</td>
<td>Komeya et al. (2016)</td>
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<tr>
<td>Dissociated testicular cells</td>
<td>Haggling-drop</td>
<td></td>
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<td></td>
<td>Soft Agar Culture System</td>
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<tr>
<td></td>
<td>Adult testis</td>
<td>Cellular aggregates</td>
<td>Progression from diploid to haploid germ cells</td>
<td>Human</td>
<td>Pendergraft et al. (2017)</td>
</tr>
<tr>
<td></td>
<td>7–10 dpp testicular cells</td>
<td>Cellular aggregates</td>
<td></td>
<td>Mouse</td>
<td>(1) Stukenborg et al. (2008), Stukenborg et al. (2009) Review and Abu Elhija et al. (2012)</td>
</tr>
<tr>
<td></td>
<td>5 dpp testicular cells</td>
<td>Cellular aggregates</td>
<td></td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td></td>
<td>13–33 months old testicular cells</td>
<td>Cellular aggregates</td>
<td></td>
<td>N/A</td>
<td></td>
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<tr>
<td></td>
<td>Methylcellulose Culture System</td>
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<td>7–9 dpp testicular cells</td>
<td>Cellular aggregates</td>
<td></td>
<td>Mouse</td>
<td>Stukenborg et al. (2009) Review</td>
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<td>13–33 months old testicular cells</td>
<td>Cellular aggregates</td>
<td></td>
<td>Rhesus monkey</td>
<td>Huleihel et al. (2015) Review</td>
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<tr>
<td>Culture methodology</td>
<td>Cultured cells/tissue</td>
<td>Organization</td>
<td>Differentiation/propagation of germ cells</td>
<td>Species</td>
<td>Study</td>
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<tr>
<td>Matrigel</td>
<td>18 dpp testicular cells</td>
<td>Cord-like formation</td>
<td>Up to RS</td>
<td>Rat</td>
<td>Legendre et al. (2010)</td>
</tr>
<tr>
<td></td>
<td>10 dpp testicular cells</td>
<td>Cord-like formation</td>
<td>Up to PaS</td>
<td>Rat</td>
<td>Hadley et al. (1985)</td>
</tr>
<tr>
<td></td>
<td>7–10 dpp testicular cells</td>
<td>Cord-like formation</td>
<td>N/A</td>
<td>Rat</td>
<td>Hadley et al. (1990) and Gassei et al. (2010)</td>
</tr>
<tr>
<td></td>
<td>5–7 dpp testicular cells</td>
<td>Cellular aggregates</td>
<td>N/A</td>
<td>Rat</td>
<td>Yu et al. (2009), Wegner et al. (2013) Protocol, Harris et al. (2015), and Harris et al. (2016)</td>
</tr>
<tr>
<td></td>
<td>7 dpp testicular cells</td>
<td>Sertoli cell aggregates</td>
<td>N/A</td>
<td>Rat</td>
<td>Gassei et al. (2008)</td>
</tr>
<tr>
<td></td>
<td>6 dpp testicular cells</td>
<td>Cord-like formation</td>
<td>Sg→meiotic initiation</td>
<td>Rat</td>
<td>Zhang et al. (2017)</td>
</tr>
<tr>
<td></td>
<td>20 dpp testicular cells</td>
<td>Seminiferous tubule-like structures (Testicular Organoid)</td>
<td>Maintenance of proliferative undifferentiated germ cells</td>
<td>Rat</td>
<td>Alves-Lopes et al. (2017)</td>
</tr>
<tr>
<td>Collagen</td>
<td>6 dpp testicular cells</td>
<td>Seminiferous tubule-like structures</td>
<td>Sg→PS</td>
<td>Mouse</td>
<td>Zhang et al. (2014a)</td>
</tr>
<tr>
<td></td>
<td>18 dpp testicular cells</td>
<td>cyst-like structures</td>
<td>Sg→RS</td>
<td>Rat</td>
<td>Lee et al. (2006b)</td>
</tr>
<tr>
<td></td>
<td>Adult testicular cells</td>
<td>Cellular aggregates</td>
<td>Spermatocytes up to presumptive spermatids</td>
<td>Human</td>
<td>Lee et al. (2007)</td>
</tr>
<tr>
<td>Calcium alginate</td>
<td>3 dpp testicular cells</td>
<td>Cellular aggregates</td>
<td>Gonocytes to presumptive spermatids</td>
<td>Calves</td>
<td>Lee et al. (2001)</td>
</tr>
<tr>
<td></td>
<td>Adult testicular cells</td>
<td>Cellular aggregates</td>
<td>Up to presumptive spermatids</td>
<td>Human</td>
<td>Lee et al. (2006a)</td>
</tr>
<tr>
<td>PGAL</td>
<td>18 dpp testicular cells</td>
<td>Cellular aggregates</td>
<td>Spermatocytes up to presumptive spermatids</td>
<td>Rat</td>
<td>Lee et al. (2011)</td>
</tr>
<tr>
<td>Hard matrixes</td>
<td>Decellularized matrix</td>
<td>Cellular aggregates</td>
<td>N/A</td>
<td>Mouse and Rat</td>
<td>Enders et al. (1986)</td>
</tr>
<tr>
<td></td>
<td>Adult and 15-year-old (active spermatogenesis up to meiosis)</td>
<td>Cellular aggregates (Testicular Organoid)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sponges</td>
<td>7 dpp testicular cells</td>
<td>Cellular aggregates</td>
<td>None</td>
<td>Rat</td>
<td>Reuter et al. (2014)</td>
</tr>
<tr>
<td>Nanotubes</td>
<td>7 dpp testicular cells</td>
<td>Cord-like formation</td>
<td>N/A</td>
<td>Rat</td>
<td>Pan et al. (2013)</td>
</tr>
<tr>
<td>Cellular pellets on air–liquid interface</td>
<td>0.5–5.5 dpp testicular cells</td>
<td>Seminiferous tubule-like structures</td>
<td>Sg→RS</td>
<td>Mouse</td>
<td>Yokonishi et al. (2013)</td>
</tr>
<tr>
<td>Rotation Cultures</td>
<td>New-born to adult testicular cells</td>
<td>Seminiferous tubule-like structures</td>
<td>N/A</td>
<td>Rat</td>
<td>Zenzes and Engel (1981)</td>
</tr>
</tbody>
</table>

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 matrices, other researchers developed decellularized testicular matrices 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 maturation 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 matrices 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 inhibit 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, 2018).
Testicular organoids

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 benefit 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 favor 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, 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; Dones 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; Gassier 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; Dones 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 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 morphogenetic 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 toxicity (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 Gfrα1, 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 transflecting 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 iPSC cells (Spence et al., 2011). These organoids can be genetically manipulated by electroporation (Fuji et al., 2015) or viral (Drost et al., 2016a) delivery of transgenics or by Cas9 RNA-guided endonuclease (Drost et al., 2016a; Fuji et al., 2015) to study cell signalling and stem-cell niche homeostasis 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 mucoسا 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 an 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 is 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...
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 (Bucay et al., 2009; Yang et al., 2015; Kjartansdottir et al., 2015) 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.

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.
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 convenient methodology, 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.

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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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The authors declare that they have no competing financial interests.

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