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REVIEW ARTICLE

Three-dimensional testicular organoids as novel *in vitro* models of testicular biology and toxicology

Sadman Sakib[1,2], Anna Voigt[2], Taylor Goldsmith[1,2] and Ina Dobrinski [1,2,*]

[1]Biochemistry & Molecular Biology, Cumming School of Medicine, University of Calgary, Canada and [2]Department of Comparative Biology and Experimental Medicine, Faculty of Veterinary Medicine, University of Calgary, Canada

*Correspondence address. Department of Comparative Biology and Experimental Medicine, Faculty of Veterinary Medicine, University of Calgary, Room 404, Heritage Medical Research Building, 3300 Hospital Drive NW, Calgary, Alberta T2N 4N1, Canada. Tel: 4032106523; Fax: 4032108821; E-mail: idobrins@ucalgary.ca

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Abstract

Organoids are three dimensional structures consisting of multiple cell types that recapitulate the cellular architecture and functionality of native organs. Over the last decade, the advent of organoid research has opened up many avenues for basic and translational studies. Following suit of other disciplines, research groups working in the field of male reproductive biology have started establishing and characterizing testicular organoids. The three-dimensional architectural and functional similarities of organoids to their tissue of origin facilitate study of complex cell interactions, tissue development and establishment of representative, scalable models for drug and toxicity screening. In this review, we discuss the current state of testicular organoid research, their advantages over conventional monolayer culture and their potential applications in the field of reproductive biology and toxicology.

Key words: testis; organoid; morphogenesis; cell–cell interaction; toxicology

Introduction

Originally, the term organoid was used to refer to three-dimensional *in vitro* culture systems for tissue fragments [1, 2]. However, with recent successes in deriving organoids from primary dissociated cells and stem cells [3–13], the term organoid has evolved to include many different systems [14]. Fatehullah *et al.* proposed a definition of the term that currently appears to be the most accurate: 'Here, we define an organoid as an *in vitro* 3D cellular cluster derived exclusively from primary tissue, embryonic stem cells, or induced pluripotent stem cells, capable of self-renewal and self-organization and exhibiting similar organ functionality as the tissue of origin' [4]. Both primary cell and stem cell derived organoids fill different niches of biomedical research. Stem cell derived organoids can be used as an efficient model of organogenesis and development, whereas primary cell derived organoids are useful for drug-toxicity screening and studying the molecular mechanisms of organ specific functions.

Over the last decade, a large number of organoid systems from various organs have been reported, namely intestine [9], liver [15], vasculature [16], pancreas [17] and brain [18]. Because of their architectural and functional resemblance to their respective primary tissues *in vivo*, these organoids were shown to have widespread applications for the study of tissue development, disease modeling, and drug and toxicity screening.

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Although organoids have been successfully derived from different organ systems, testicular organoids only gained attention relatively recently. Here, we discuss the different testicular organoids that have been reported and how such 3D testicular organoid model systems may play an important role in biomedical research, particularly in the field of reproductive toxicity.

Conventional Models of Reproductive Toxicology

Infertility affects 7% of all men [19], 23% of which is due to pathophysiological conditions. Environmental exposure to toxicants such as organic polychlorinated dibenzodioxins, dicarboximide fungicides and heavy metals also contributes to infertility [20–23]. Additionally, gonadotoxic effects from different chemotherapeutic modalities can lead to fertility impairment [24, 25]. Therefore, a robust model that can recapitulate the complex cell–cell communication of the testis *in vivo* is needed for early screening of different drug molecules and to study effects of different toxicants.

Animal Models

Testicular toxicity studies have traditionally been performed in rodents [26–29]. For example, Liu *et al.* characterized the testicular toxicity of 3-methyl-2-(1-hydroxyethyl) quinoxaline-*N*4-monoxide (M4), a metabolite of the synthetic antimicrobial agent Mequindox (MEQ). The authors reported that MEQ triggers oxidative stress, mitochondrial dysfunction and altered junctional protein expressions which lead to disrupted spermatogenesis in mice [26]. Animal models have also been widely used for assessing epigenetic effects of toxins in the testis [30–33]. Environmental exposure to chemicals such as Bisphenol A and phathlates can alter the methylation pattern of the promoter region of a number of different genes such as hippocalcin-like 1 (Hpcal1) genes [34]. It can also cause hypermethylation of estrogen receptor promoter regions in rodents [33]. Maternal exposure to Di-2-(ethylhexyl) phthalate (DEHP) leads to increased DNA methylation and upregulation of DNA methyltransferases in mouse testis [31, 32]. Although experiments such as these have provided important information about the effects of different drugs and toxicants, studies performed in rodents often translate poorly to humans because rodent physiology differs appreciably from humans or large animals [35, 36]. Rodents exhibit genomic responses to inflammatory diseases that are quite different from humans [37]. The activity of certain liver enzymes can also vary between rodents and humans [38] and whole animal models are expensive to maintain [39].

Two-Dimensional Monolayer Culture

Testicular cells (primary or immortalized) cultured on plastic tissue culture plates, due to their ease and low cost of maintenance, have been the standard platform for understanding male reproductive biology and for drug and toxicity screens *in vitro*. Co-cultures of primary and immortalized somatic (Sertoli, peritubular myoid and Leydig cells) cells and germ cells facilitated study of cell–cell and cell–ECM interactions [40–43]. In 1985, Hadley *et al.* using Sertoli cell 2D culture and Sertoli-myoid cell co-culture described the important role of the basement membrane in the testicular microenvironment [43]. Sertoli cells grown on reconstituted basement membrane could form polarized monolayers similar to *in vivo*. They also maintained tight junctions and undifferentiated germ cells [43]. Co-cultures of testicular cells have also been used to investigate the effects of hormones such as FSH; growth factors such as HGF, FGF2 and FGF9; signalling molecules, drugs and environmental toxicants such as Bisphenol A, and reactive oxygen species on testicular somatic and germ cells [42, 44–50]. Although, these 2D culture modalities have provided us with much information on testicular biology and toxicology, they often fail to mimic organ specific toxicity [51, 52].These 2D cultures, often grown on rigid and planar surfaces modify cellular architecture and can lead to inappropriate and biologically irrelevant cell–cell interactions [53–55].

Organ Culture

Organ culture methods were applied to address the lack of 3D cell–cell interactions of 2D culture. In organ culture, small testicular tissue fragments rather than single cells are placed in cultures [56]. In 2011, Sato *et al.* reported the birth of healthy mice after intra-cytoplasmic sperm injection of sperm produced by *in vitro* organ culture [57]. Since then a number of groups have reported using organ cultures to study spermatogenesis in rodents [58–61] and bovids [62]. These testis organ culture systems can also be used for assessing reproductive toxicity. A proof of principle was recently reported by Nakamura *et al.*, where testis fragments were treated with increasing dosages of ethinylestradiol (EE), a well-known testicular toxicant. EE treatment led to a reduction in viable germ cells and a reduction of estrogen receptor 1, cytochrome P450, family 11, subfamily a, and polypeptide 1 in a dose-dependent manner [63]. It was also shown that organ culture of rat fetal testes can recapitulate the epigenetic reprogramming in gonocytes [64] indicating that these models could be used for assessing testicular epigenetics.

Testicular Tissue and Cell Grafting

Although organ culture models have been used successfully to study testicular biology and toxicity in rodents, the system falls short when it comes to studying large animals or humans. Testicular organ culture also does not allow study of testicular morphogenesis. Autologous and xenogeneic transplantation of testicular tissue and cells were developed to address these shortcomings [65–69]. Autologous transplantation of cryopreserved prepubertal primate tissue supports production of fertilization competent sperm [69]. Xenotransplantation of testicular tissue from different animal species into immunodeficient mice also results in spermatogenesis [65–68]. Since the metabolism of toxicants such as phthalates is qualitatively similar between human and mouse [70], testis tissue xenografting is a unique model for toxicological assays. It also allows for reproductive toxicological studies on testicular tissue from non-human primates or humans where *in vivo* experiments cannot be performed due to ethical or regulatory issues [71, 72]. For example, chronic exposure of mice carrying testicular tissue fragments as grafts from pre-pubertal rhesus macaques to phthalate esters revealed that long-term, low-dose [0, 10, 500 mg/kg Di-n-Butyl and Di-(2-EthylHexyl)] exposures led to impaired steroidogenesis and spermatogenesis in a dose-dependent manner [72]. Reconstitution of functional testis tissue from xenografted testicular cells is a complementary bioassay where cells from a pre-pubertal donor are grafted ectopically to immunocompromised mice [73]. Xenografted cells are capable of re-establishing the germ cell niche environment and can support full spermatogenesis. The system can be utilized to study the effects of

different environmental and experimental factors on reproductive function [74, 75].

In Vitro Tubule Reconstitution

Although grafting of testicular tissue or cells provides a powerful platform to study testis function in different species, it is not without its shortcomings. The grafted tissue can experience hypoxic damage due to delayed vascularization from the host. It is also less accessible for manipulation and/or observation than an *in vitro* system. This led to the establishment of *in vitro* models of testicular reconstitution or morphogenesis where tubule like structures are generated from dissociated testicular single cells. Testicular cells from rodents and pigs cultured on supportive biomaterials such as ECM proteins or agar formed seminiferous tubule-like structures [76–79]. Dores *et al.* showed that *in vitro* tubule formation can be used as an assay to study the effect of an experimental agent namely Ciliobrevin D (an inhibitor of primary cilia) on testicular morphogenesis [78]. Since *in vitro* tubule formation depends entirely on cellular morphogenetic capacity with no external forces promoting a desired geometry, the tissue architecture of the *de novo* formed tubule may vary widely. It also requires a large number of cells, which can limit the utility of the system when dealing with limited samples such as those obtained from biopsies. Thus, model systems that allow reproducible recapitulation of architecture and function are needed. To address this need, testicular organoid models were investigated.

Testicular Organoid Models

3D testicular organoids can serve as an intermediate platform between 2D culture systems and animal models. Organoids can be used as a physiologically more relevant model system to study cell–cell interactions, development and tissue morphogenesis [80]. They also pave the way for high-throughput drug and toxicity screening with more reliable and biologically relevant readouts [3, 5, 7, 81].

So far, only a few groups have reported generating testicular organoids from testicular single cells [11–13, 82].

Baert *et al.* reported generation of human testicular organoids. Both adult and pre-pubertal (15-year-old) testicular cells were placed on decellularized adult testicular extra cellular matrix (ECM). Adult and prepubertal cells colonized, remodeled and compacted the ECM scaffold to generate spheroidal organoids. ECM scaffold-free cells also formed similar organoids. These organoids had no morphological similarity with human testis and did not produce a well-defined germ cell niche. However, they produced testosterone, inhibin B and several different cytokines such as interleukin 6. The Sertoli cells also expressed tight junction proteins similar to *in vivo*. The organoid model maintained undifferentiated germ cells for up to 4 weeks. This indicated the potential application of the system for studying effects of different drugs and toxicants on testicular paracrine signaling [82].

Alves-Lopes *et al.* described generation of testicular organoid from 20d old rat testicular cells using a novel three-layer Matrigel gradient system. The Sertoli and germ cells in this organoid system formed spherical tubular structures. Although, peritubular myoid cells were present, they did not appear to actively participate in the self organization process. Sertoli cells in the organoid gave rise to a functional blood testes barrier. The spherical tubules could maintain undifferentiated germ cells for up to 21 days. The authors also reported that their organoids were responsive to retinoic acid treatment similar to previous reports [83–85] and were sensitive to the pro-inflammatory cytokines tumor necrosis factor alpha (TNFα) and interleukin 1 alpha (IL1α) leading to impaired organoid formation, reduction of germ cell maintenance and loss of blood-testes barrier integrity as previously reported for testis *in vivo* [86–88]. This proof of principle points to the utility of the model for assaying the effect of experimental factors and drugs on testicular function [11].

Pendergraft *et al.* described generation of a human testicular organoid system using adult germ cells and immortalized Leydig and Sertoli cells using the hanging drop culture system. The culture media was supplemented with solubilized human testis ECM. These organoids could be maintained in culture for up to 21 days and produced testosterone. Although the organoid lacked testicular tissue architecture, it appeared to support haploid germ cell transition. The authors assessed four different cytotoxic compounds: busulfan, cisplatin, doxorubicin and etoposide to evaluate the model's utility for toxicity screening [12]. Organoids were exposed to increasing concentrations of the compounds for 48 h, which lead to a dose-dependent decrease in viability and increase in apoptotic cells. Organoids also displayed IC_{50} values significantly higher than corresponding 2D cultures. Another report by the same group described using organoids to model Zika virus infection. Testicular organoids were generated and then infected with Zika virus, effectively showing a reduction in testicular cell viability and decline in testosterone production. This suggests that testicular organoids can serve as a tool for infectious disease modeling [13].

Recently, our group generated and characterized a testicular organoid model from pre-pubertal porcine testicular cells by using a microwell centrifugal aggregation system (see Fig. 1). The resulting organoids have a tissue architecture that is similar to testis *in vivo* [89]. These organoids have a clearly delineated exterior (seminiferous epithelium) and interior compartment (interstitial) separated by the basement membrane. Germ cells and Sertoli cells are in the exterior compartment. The peritubular myoid cells are localized along the interior of the basement membrane and the Leydig and endothelial cells are at the core of the interior compartment (see Fig. 1). We demonstrated that our organoid generation methodology is widely applicable across species, including mice, primates and humans. The Sertoli cells in these organoids express tight junction proteins. Germ cells in the organoids displayed an attenuated response to retinoic acid stimulation compared with conventional 2D culture indicating that the tissue architecture in the organoid modulates response to retinoic acid similar to testis *in vivo* [90]. Germ cells in organoids had fewer autophagosomes than those in 2D culture. Autophagy is a self-degradation and recycling mechanism that occurs at a basal level in every tissue [91, 92] and is an important process for normal protein turnover and maintenance of homeostasis [92, 93]. As a stress response mechanism autophagy serves to clear accumulating proteins and organelles crucial for the continuous renovation of the cell [94, 95]. Lower numbers of autophagosome in germ cells in organoids indicate reduced cellular stress when physiological cell interactions are maintained compared with cells in monolayer culture. Environmental toxicants can also trigger autophagy [93, 96] and autophagy as a biomarker for toxicity within the male reproductive tract has been described [97–99]. Exposure to increasing doses of di-(2-ethylhexyl) phthalate, a commonly used plasticizer, induced an increase in the number of autophagosomes in germ cells in a dose-dependent manner in 2D culture [99] and this observation could be replicated in organoids [89].

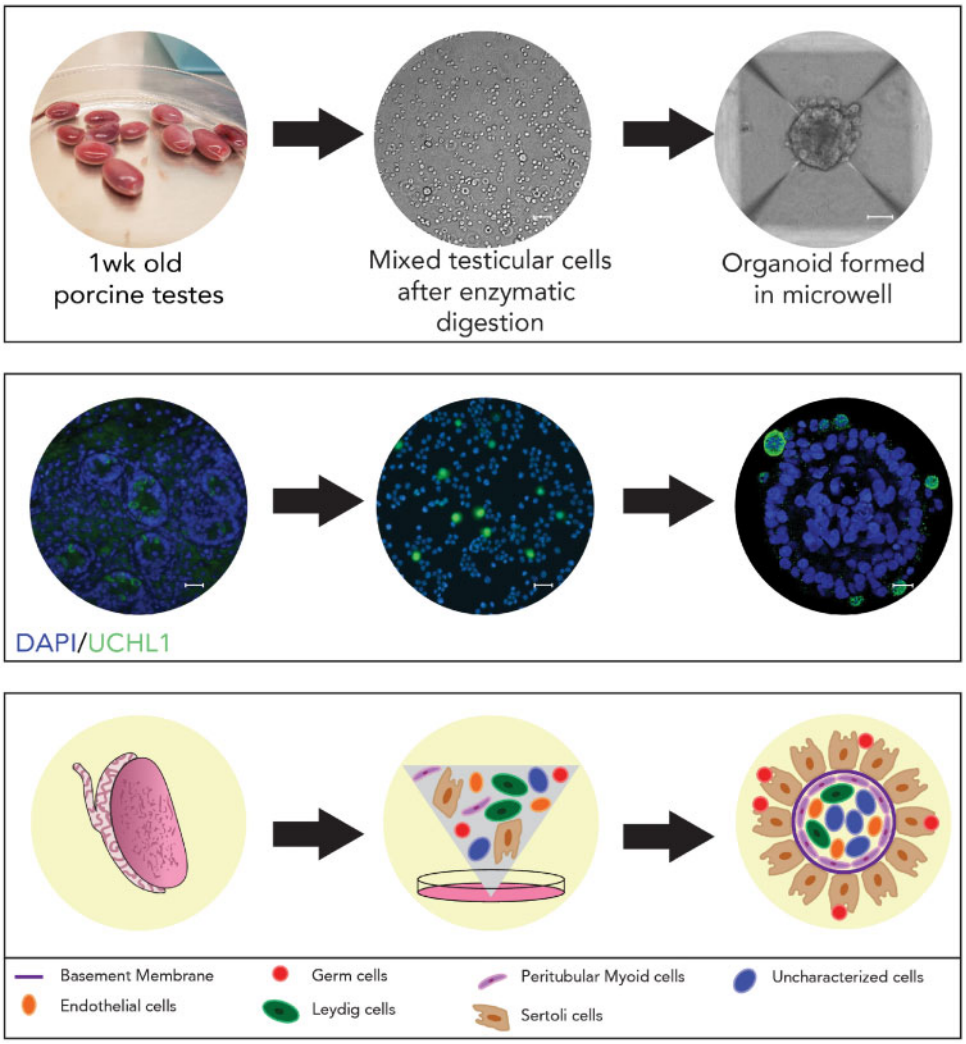

Figure 1: testicular organoid formation in microwell culture. Top panel (left to right): 1-Week old porcine testes are enzymatically digested into single cells (Scale bar = 20 μm), which undergo self organization into an organoid (Scale bar = 50 μm) after 5 days of culture in microwell. Middle panel (left to right): Immunofluorescence characterization of testis cells and organoid. DAPI-nuclear stain, UCHL1-germ cell marker. Scale bars = 20 μm. Bottom panel (left to right): A schematic representation of testicular organoid formation from testis tissue-derived single cells undergoing self-organization in microwells. This figure has been modified from Sakib *et al.* [89].

Similarly, exposure of cells to a small molecule inhibitor of primary cilia led to a loss of morphogenic capacity. These initial observations point towards the utility of testicular organoid systems for screening the effects of drugs and toxins on morphogenesis and cell function [89].

Potential Applications in Male Reproductive Biology and Toxicology

An effective model of *in vitro* spermatogenesis, particularly for non-rodent mammalian species remains elusive. As spermatogenesis is a multifactorial complex process which requires the coordination of germ cells and testicular somatic cells [100], an *in vitro* model that maintains testis specific cell associations is essential. Studies have shown that testis specific architecture is required for germ cell homeostasis [90] and a number of paracrine factors, such as glial cell line-derived neurotropic factor, colony-stimulating factor 1 [101–105] and signalling molecules such as wnt6 and wnt3a released by somatic cells are required for germ cell maintenance [106, 107]. Thus, a testicular organoid model with testis specific architecture and function can serve as a bridge between 2D culture and animal models. A 3D organoid composed of all different testicular cell types provides an accessible *in vitro* model to inform a more thorough understanding of how germ cells interact with their niche. Drug and toxicity screening in such models would provide more physiologically relevant readouts than 2D culture modalities [51, 108].

Primordial germ cells can undergo a series of epigenetic modifications such as the erasure of parental imprinting and demethylation during development. Environmental toxicants like bisphenol A and phthalates can cause epigenetic changes such as DNA methylation, histone modifications, and expression of different non-coding RNAs which can impact testicular functions [31–33]. Such epigenetic changes can happen to not only in germ cells but also in different testicular somatic cells in a transgenerational manner [109]. Studying such epigenetic mechanisms using a 3D organoid system may more accurately recapitulate the situation *in vivo*.

Testicular organoids generated from primary cells can also be an important tool for disease modeling. As the starting cell populations used are single cells [11–13, 82], different cell types can be isolated and genetically modified or exposed to environmental factors and then recombined to generate organoids with specific disease phenotypes. Testicular cancer tissue could be used to generate organoid models of testicular malignancy. Such models would be invaluable for early testing of pharmaceutical and chemotherapeutic interventions.

Organoids can also be a great boon for the field of development and regenerative medicine. Existing protocols for derivation of germ cells from induced pluripotent cells (iPSCs) remain inefficient [110, 111]. Combining these existing protocols with testicular organoids could enhance germ cell derivation efficiency. These protocols may also be combined with iPSC derived somatic cell derivation protocols [112–114] to generate entire testicular organoids from iPSCs. This could provide a powerful model for understanding testicular development as shown in other organoid systems [80, 115], allowing us to study and investigate therapeutic interventions to congenital male infertility syndromes.

Concluding Remarks

Testicular organoids that recapitulate testicular cytoarchitecture and function allow for a more thorough investigation of the germ cell niche. This in turn can lead to development of better interventions to regulate and modify germ cell proliferation and differentiation *in vitro*. It would also pave way for a more reliable model of testicular development and disease, and a platform to test experimental and environmental factors with readouts expected to mimic *in vivo* conditions more closely allowing for quicker translation to clinical applications.

Conflict of interest statement. None declared.

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