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Novel culture systems to model testicular function and organization

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**Karolinska
Institutet**

Stockholm 2018

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Published by Karolinska Institutet.

Printed by Universitetservice-AB 2018

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ISBN 978-91-7831-119-4

Novel culture systems to model testicular function and organization

THESIS FOR DOCTORAL DEGREE (Ph.D.)

Friday the 31st of August, 09:00 am

J3:11 Birger & Margareta Blombäck U220032100, Solnavägen 30, Stockholm, Sweden

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To my origins
and to everyone

ABSTRACT

In vitro systems to model the testicular microenvironment are required to study physiology and pathology of the testis, the gonadotoxic effect of environmental and pharmaceutical chemicals, as well as to explore the mechanisms ruling spermatogonial stem cell (SSC) self-renewal and differentiation. The knowledge produced in *in vitro* experiments, using animal, but most importantly utilizing human models, will contribute to the discovery of safe medical treatments and might provide translational tools to treat male infertility problems. The current models are vital to study the testicular microenvironment *in vitro*, but further improvements are required to more closely reconstruct the testicular organization and function found *in vivo*.

The main focus of the research conducted in this thesis was to establish and optimize three-dimensional culture conditions to further improve the current approaches to model testicular architecture and physiology *in vitro*. For that purpose, we explore three distinct approaches: the three-layer gradient system (3-LGS) to culture rat testicular cells suspended in extracellular matrix (ECM) components; the decellularized testicular extracellular matrix (DTM) to culture human testicular cells in their native ECM; and the organ culture system in the air-liquid interface to culture human testicular cells in their native tissue organization.

During our experiments, we found that the 3-LGS promoted the reorganization of rat testicular cells into seminiferous tubule-like organoids with both a functional blood-testis barrier and the capacity to maintain proliferative undifferentiated germ cells. Moreover, the DTM allowed the generation of human testicular organoids that, despite not demonstrating characteristic testicular organization, were able to produce testosterone and inhibin B as well as to maintain spermatogonia proliferating during the entire culture period. Finally, the organ culture system served to maintain human testicular tissue in viable conditions and to demonstrate differences in terms of testicular somatic cell functionality among different patient groups exposed to chemotherapy or treatments against haematological diseases.

In perspective, the rat and human organoid systems demonstrate potential to explore aspects of testicular development and toxicology, but also the possibility to, easier than before, manipulate the culture conditions to study the influence of different niche elements on SSC self-renewal and differentiation. Moreover, the organ culture system showed the aptitude to be used as a quality control tool in the assessment of bio-banked human testicular tissue and to help in determining the best-fit fertility preservation strategy for each group of patients.

LIST OF SCIENTIFIC PAPERS

- I. **João Pedro Alves-Lopes**, Olle Söder & Jan-Bernd Stukenborg
Use of a three-layer gradient system of cells for rat testicular organoid generation
Nature Protocols, 2018, volume 13, pages 248–259
DOI:10.1038/nprot.2017.140
- II. **João Pedro Alves-Lopes**, Olle Söder & Jan-Bernd Stukenborg
Testicular organoid generation by a novel in vitro three-layer gradient system
Biomaterials, 2017, volume 130, pages 76–89
DOI: 10.1016/j.biomaterials.2017.03.025
- III. Yoni Baert, Joery De Kock, **João Pedro Alves-Lopes**, Olle Söder, Jan-Bernd Stukenborg and Ellen Goossens
Primary Human Testicular Cells Self-Organize into Organoids with Testicular Properties
Stem Cell Reports, 2017, volume 8, pages 30–38
DOI: 10.1016/j.stemcr.2016.11.012
- IV. **João Pedro Alves-Lopes**, Magdalena Kurek, Halima Albalushi, Olle Söder, Rod Mitchell, Cecilia Petersen, Kirsi Jahnukainen and Jan-Bernd Stukenborg
In vitro assessment of Sertoli and Leydig cell function in boys subjected to treatment for haematological and oncological diseases
Manuscript

LIST OF SCIENTIFIC PAPERS (not included in the thesis)

- I. **João Pedro Alves-Lopes** and Jan-Bernd Stukenborg
Testicular organoids: a new model to study the testicular microenvironment in vitro?
Human Reproduction Update, 2018, volume 24, pages 176–191
DOI: 10.1093/humupd/dmx036

- II. Jan-Bernd Stukenborg, **João Pedro Alves-Lopes**, Magdalena Kurek, Halima Albalushi, Ahmed Reda, Victoria Keros, Virpi Tohonen, Ragnar Bjarnason, Patrik Romerius, Mikael Sundin, Ulrika Noren Nystrom, Cecilia Langenskiöld, Hartmut Vogt, Lars Henningsohn, Rod Mitchell, Olle Söder, Cecilia Petersen and Kirsi Jahnukainen
Spermatogonial quantity in human prepubertal testicular tissue collected for fertility preservation prior to potentially sterilizing therapy
Human Reproduction, 2018 (in press)
DOI:10.1093/humrep/dey240

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LIST OF ABBREVIATIONS

2D	Two-dimensional
3D	Three-dimensional
3-LGS	Three-layer gradient system
3 β HSD	3 β -hydroxysteroid dehydrogenase
ACTA2	Actin alpha 2 smooth muscle
AMH	Anti-Mullerian hormone
ANOVA	Analysis of variance
BTB	Blood-testis barrier
CK18	Cytokeratin 18
COL1	Collagen type 1
CSF1	Colony stimulating factor 1
DDX4	DEAD-box helicase 4
DMEM	Dulbecco's Modified Eagle's medium
<i>Dpp</i>	<i>Day postpartum</i>
DTM	Decellularized testicular extracellular matrix
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
FGFR3	Fibroblast growth factor receptor 3
FSH	Follicle-stimulating hormone
GDNF	Glial cell derived neurotrophic factor
GnRH	Gonadotropin-releasing hormone
GRO	Growth-regulated alpha/beta/gamma protein
hCG	Human chorionic gonadotrophin
HU	Hydroxyurea
IL1 α	Interleukin 1 alpha

IL6	Interleukin 6
IL8	Interleukin 8
KI67	Marker of proliferation Ki-67
KSR	KnockOut serum replacement
KSR-XF	KnockOut serum replacement – XenoFree
LH	Luteinizing hormone
MCP1	Monocyte chemotactic protein 1
MEM α	Minimum essential medium alpha
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
Pen/strep	Penicillin/streptomycin
PLZF	Promyelocytic leukemia zinc finger protein
RA	Retinoic acid
RAR α	Retinoic acid receptor alpha
rFSH	Recombinant follicle-stimulating hormone
RXR	Retinoid X receptor
SCD	Sickle-cell disease
SOX9	SRY-box 9
SSC	Spermatogonial stem cell
STAR	Steroidogenic acute regulatory protein
STS	Spherical-tubular structure
TNF α	Tumor necrosis factor alpha
UCHL1	Ubiquitin C-terminal hydrolase L1
UTF1	Undifferentiated embryonic cell transcription factor 1
ZO1	Zonula occludens 1

1 INTRODUCTION

The testis is a compartmentalized organ where spermatogenesis and hormone synthesis take place. Both functions are essential for male organisms and regulated by one of the most complex network of cells and molecular signalling in the human body (Fagerberg et al. 2014).

1.1 ORGANIZATION OF THE TESTIS

1.1.1 Interstitial compartment

The interstitial compartment represents 12-15% of the total volume of the adult human testis and provides the structural and nutritional support to seminiferous tubules by means of an organized network of cell-to-cell and cell-to-extracellular matrix (ECM) interactions (Weinbauer et al. 2010). The vascular system in the testis is located in the interstitial compartment and it is not only responsible for delivering of nutrients and endocrine hormones to their target cells, but also to retract metabolic products to be eliminated (Russell et al. 1990). Among the loose organization of the connective tissue, other essential cell populations such as Leydig cells and macrophages perform important roles in hormone production and immunity respectively (Weinbauer et al. 2010; DeFalco et al. 2015). Additionally, macrophages are also known to be involved in the degradation and remodelling of ECM (Valentin et al. 2009) and potentially perform the same role in testicular ECM. The interstitial compartment is also the anatomical space where nerves and fibroblasts are located in the testis (Russell et al. 1990).

1.1.2 Tubular compartment

The tubular compartment is constituted by germ cells and two other supportive somatic cell populations: Sertoli and peritubular cells (Weinbauer et al. 2010). Sertoli cells support spermatogonial stem cell (SSC) self-renewal on the basement membrane and germ cell differentiation towards the lumen of the seminiferous tubules (Clermont 1972). These two processes are physically separated by the blood-testis barrier (BTB). In its turn, the BTB is formed during testicular maturation, when tight-junction complexes are established between adjacent Sertoli cells (Setchell 2008). Some of the proteins composing the tight-junctions, such as occludin and zonula occludens 1 (ZO1), also known as tight junction protein 1, are therefore considered markers for Sertoli cell maturation (Setchell 2008).

The peritubular cells (also known as peritubular myoid cells) are located around the basement membrane, where SSCs and Sertoli cells are settled. Further to their involvement in basement membrane formation (Richardson, Kleinman, and Dym 1995; Schlatt, de Kretser, and Loveland 1996) and seminiferous tubule integrity, peritubular cells spontaneously contract, contributing to the transport of mature sperm towards the distal region of the seminiferous tubules (Weinbauer et al. 2010).

1.1.3 Extracellular matrix

The basement membrane is the ECM found in between the connective tissue and epithelial structures in mammals (Laurie, Leblond, and Martin 1982). In the testis, the basement membrane that separates the seminiferous tubules from the connective tissue is also denominated as tubular wall and is mainly constituted by collagens, laminins and fibronectin (Laurie, Leblond, and Martin 1982; Baert et al. 2015). The interstitial compartment of the testis is also rich in ECM proteins that support the connective tissue and the vasculature (Oguzkurt et al. 2007; Baert et al. 2015). In addition to their contribution to tissue integrity, the components of the ECM are also biological active. More specifically, the ECM components of the tubular wall are involved in the transit regulation of growth factors, such as transforming growth factor beta 3 (TGF- β 3), between the interstitium and the seminiferous tubules. Moreover, collagens and laminins, and also the peptides resulting from their degradation, contribute to the regulation of BTB cellular permeability during spermatogenesis (reviewed in (Siu and Cheng 2004)).

1.1.4 Spermatogonial stem cell niche

Stem cell niches are microenvironments that provide necessary conditions for stem cells to execute their functions on tissue homeostasis and repair. In addition to stem cells, the niche is constituted by cellular and acellular components that mediate proliferation and/or differentiation signalling to stem cells. Although different stem cell niches have their own characteristic composition, somatic cells, ECM, vasculature, soluble factors and adhesion molecules are generally considered to be a part of this microenvironment (Jones and Wagers 2008).

Both rodent and human SSC niches are not restricted to a confined anatomical space, as in other stem cell niches (e.g. intestinal crypts or mammary glands), but rather facultative on the basement membrane of the seminiferous tubules (Ogawa, Ohmura, and Ohbo 2005; Yoshida, Sukeno, and Nabeshima 2007; Ikami et al. 2015). However, some of the characteristic

components of a general stem cell niche have been identified to promote SSC self-renewal and differentiation. For example, Sertoli cells give structural support and produce soluble glial cell derived neurotrophic factor (GDNF) important for SSC proliferation (Meng et al. 2000; Kubota, Avarbock, and Brinster 2004). Moreover, Leydig and peritubular cells are known to release colony stimulating factor 1 (CSF1) that is also thought to be involved in SSC proliferation (Kokkinaki et al. 2009; Oatley et al. 2009). Vasculature and ECM proteins such as laminins have been reported to be associated with SSCs and therefore also considered important constituents of the niche (Yoshida, Sukeno, and Nabeshima 2007; Oatley and Brinster 2012). However, a deeper knowledge in how the SSC niche is constituted and regulated will be of great value to understand mechanisms of disease and to model testicular microenvironment *in vitro*.

1.2 PHYSIOLOGY OF THE TESTIS

1.2.1 Spermatogenesis

The process of germ cell differentiation, known as spermatogenesis, happens in the seminiferous tubules and consists of multiple mitotic divisions of spermatogonia followed by two consecutive meiotic divisions, leading to the formation of haploid cells called round spermatids. In their turn, round spermatids undergo further differentiation by losing the majority of their cytoplasm and forming the acrosome and tail structures, in a process known as spermiogenesis. Finally, sperm is released into the lumen of the seminiferous tubules, concluding the spermatogenesis process (Clermont 1972).

1.2.2 Hormone production

Testicular hormone production is under the influence of the hypothalamic-pituitary system located in the brain. Hypothalamus releases gonadotropin-releasing hormone (GnRH) that activates the production of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) by the pituitary. Both gonadotropins LH and FSH reach the testis via the blood stream and stimulate Leydig and Sertoli cells, respectively. Leydig cells produce testosterone that not only stimulates spermatogenesis and the development of secondary sexual characteristics, but also inhibits the production of GnRH and both gonadotropins, in a negative feed-back regulatory way. In its turn, FSH indirectly supports spermatogenesis via its effect on Sertoli cell metabolism. Among other hormones, Sertoli cells produce inhibin B that acts on the pituitary decreasing the production of FSH (Weinbauer et al. 2010).

1.3 FERTILITY PRESERVATION

In the last decade, concerns about how to preserve male fertility have increased not only because the lack of solutions but also due to the increased prevalence of infertility problems among men at all ages (Krausz 2011; Barratt et al. 2017). There are several causes of male infertility including sperm-production problems, infections, chromosomal diseases, torsions, medicines, chemicals and factors that are not known yet (Krausz 2011; Nieschlag and Lenzi 2013). Chemotherapy and radiation used to treat malignant and haematological diseases also lead to infertility. Although adult individuals have the option to cryopreserve their semen before being exposed to these treatments, patients who do not produce sperm (e.g. pre-pubertal boys) have restricted and ineffective fertility preservation options (Jahnukainen, Mitchell, and Stukenborg 2015; Stukenborg, Jahnukainen, et al. 2018). For this group of patients with restricted alternatives to preserve their fertility, experimental approaches such as SSC transplantation, testicular tissue transplantation or *in vitro* germ cell maturation are under intense research development, with the final aim of translating them to safe clinical practices in the near future (Anderson et al. 2015).

The current experimental work to find treatments for infertility is mainly focused on the impact of chemotherapy and radiation on germ cells, marginalizing the side effects of these therapies on testicular somatic cells. However, alkylating agents (e.g. cyclophosphamide and busulphan) as well as irradiation are known to damage important components of the SSC niche such as Sertoli and Leydig cells, with an indirect effect on germ cells viability (review in (Stukenborg, Jahnukainen, et al. 2018)). These facts suggest that an integrated understanding of the damage induced in both germ and somatic compartments is necessary to refine our *in vitro* experimental strategies for germ cell differentiation and consequent treatment of male infertility.

1.4 IN VITRO TESTICULAR MODELS

A broad variety of two-dimensional (2D) and three-dimensional (3D) models have been used to investigate testicular physiology and disease *in vitro* (Figure 1). While the 2D models have been invaluable in the understanding of the testicular microenvironment, testicular cells perform generally better, in terms of organization and function, when cultured in 3D systems (Alves-Lopes and Stukenborg 2018).

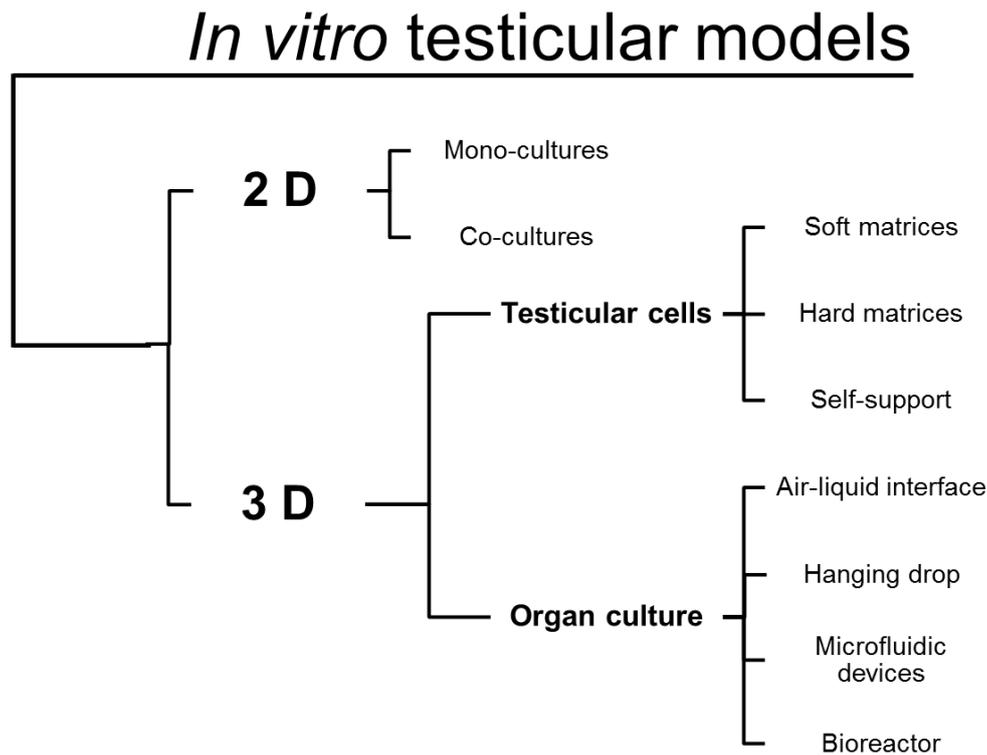


Figure 1. Hierarchical distribution of the models used to study testicular microenvironment *in vitro*.

1.4.1 Two-dimensional models

Testicular cells have been utilized in 2D mono- or co-cultures in order to investigate the role of ECM and growth factors on somatic and germ cell biology (Figure 1). In a study by van der Wee and Hofmann, a mouse immortalized Sertoli line was applied to demonstrate the importance of laminins and collagen type IV in cord-like structure formation *in vitro* (van der Wee and Hofmann 1999). Other groups used 2D testicular mono- and co-cultures to show the required interaction between Sertoli and peritubular cells in the regulation of ECM expression as well as the testicular cell reorganization capabilities *in vitro* (Tung and Fritz 1980; Hadley et al. 1985; Kierszenbaum et al. 1986; Richardson, Kleinman, and Dym 1995; Mincheva et al. 2018). Using the same type of cultures, Schlatt and colleagues demonstrated that rat Sertoli cells proliferation decreases by cell-contact inhibition and that this effect could be reverted by FSH (Schlatt, de Kretser, and Loveland 1996). Moreover, germ cells have also been co-cultured with Sertoli and Vero cells in 2D conditions. In these studies, the significance of Vero (Cremades et al. 1999; Tanaka et al. 2003) and Sertoli cells in supporting the progression of germ cell differentiation was demonstrated in both human (Tesarik et al. 1998; Sousa et al. 2002) and animal models (Tres and Kierszenbaum 1983; Vigier et al. 2004; Xie et al. 2010) (reviewed in (Alves-Lopes and Stukenborg 2018)).

1.4.2 Three-dimensional models

Testicular cells can also be cultured in 3D conditions in an attempt to model the original cellular organization and interactions *in vivo*. Cells have been either combined with supportive scaffolds or cultured in small fragments of the native testicular tissue (Figure 1) (reviewed in (Alves-Lopes and Stukenborg 2018)).

1.4.2.1 Testicular cells cultured in three-dimensional scaffolds

When considering 3D cultures of cells, soft-agar is an example of a soft matrix used to support testicular cells from mouse (Stukenborg et al. 2008; Stukenborg et al. 2009; Abu Elhija et al. 2012), rat (Reda et al. 2014) and rhesus monkey (Huleihel, Nourashrafeddin, and Plant 2015). Although, cells formed aggregates without characteristic testicular organization, the interactions between the different cells types were enough to promote germ cell differentiation (Stukenborg et al. 2009; Abu Elhija et al. 2012). Following the same experimental line, other soft matrices, such as collagen (Khajavi et al. 2014; Zhang et al. 2014), methylcellulose (Stukenborg et al. 2009; Huleihel, Nourashrafeddin, and Plant 2015), calcium alginate (Lee, Kaproth, and Parks 2001; Lee et al. 2006) and matrigel (a mixture of ECM proteins and growth factors produce from Engelbreth-Holm-Swarm mouse sarcoma) (Hadley et al. 1985; Gassei, Ehmcke, and Schlatt 2008; Legendre et al. 2010; Zhang et al. 2017) have been used to investigate reorganization and differentiation of testicular cells *in vitro* (reviewed in (Alves-Lopes and Stukenborg 2018)).

An alternative to soft matrices are hard matrices obtain either from synthetic or natural sources. Synthetic carbon nanotubes (Pan, Chi, and Schlatt 2013) and collagen sponges (Reuter et al. 2014) were utilized to manipulate rat testicular cell organization into tubular aggregates *in vitro*. Moreover, tubular ECM scaffolds produced by decellularization of dissociated seminiferous tubules were used to support 3D cultures of rat testicular cells (Enders, Henson, and Millette 1986).

Last but not least, testicular cells can themselves support the formation of complex 3D structures that demonstrate similarities with the organization and function of the testis. In a study by Zenzes and colleagues, it was shown that immature rat testicular cells cultured in a rotational system could reorganize into seminiferous tubule-like structures (Zenzes and Engel 1981). Moreover, testicular cell aggregates from immature mouse displayed the capacity to reorganized into seminiferous tubule-like structures that could support initial steps of spermatogenesis, when simply cultured on top of agarose stands (Yokonishi et al. 2013).

1.4.2.2 Testicular cells cultured in the native tissue (organ cultures)

The model most successfully used to culture testicular tissue is the air-liquid interface system. During 1960's, Steinberger and collaborators adapted the methodology developed by Trowell and colleagues (Trowell 1954) in order to culture small fragments of rat testicular tissue on a supportive stand ensuring simultaneous contact with the medium and the incubation atmosphere (Steinberger, Steinberger, and Perloff 1964; Steinberger and Steinberger 1965). The air-liquid interface culture conditions have been recently modified to differentiate mouse (Suzuki and Sato 2003; Sato et al. 2011; Sato et al. 2015; Dumont et al. 2016; Reda et al. 2017), rat (Reda et al. 2016) and calf (Kim et al. 2015) germ cells by means of an optimized culture medium supplementation and the use of agarose stands to support the tissue. Instead of using agarose stands, other groups used membranes as support for human fetal (Lambrot et al. 2006), pre-pubertal (de Michele et al. 2017) and adult testicular tissue (Roulet et al. 2006). In the study using pre-pubertal tissue, de Michele and colleagues demonstrated evidences of Sertoli and Leydig cell maturation that, paradoxically, was accompanied by decreasing numbers of germ cells along the culture period (de Michele et al. 2017).

Another strategy to culture testicular tissue is the hanging-drop technology. In this model, the tissue is fragmented in small parts and cultured in a drop of medium hanging on plastic or glass. This system has been utilizing to investigate the effect of different molecules on testicular microenvironment (Jorgensen et al. 2014) and development (Jorgensen et al. 2015; Potter and DeFalco 2015).

More recently, a small number of publications have reported the development of dynamic culture systems where testicular tissue is exposed to a continuous and controlled flux of fresh medium. One of these studies described a bioreactor where seminiferous tubules from rat and human were cultured enclosed in permeable chitosan hydrogel tubes (Perrard et al. 2016). Although specific-cell characterization was not presented, the authors of this study reported the differentiation of both rat and human germ cells into spermatozoa (Perrard et al. 2016). In another publication, Komeya and colleagues conceived a microfluidic device composed of two adjacent channels (tissue and medium channels) separated by a permeable membrane (Komeya et al. 2016). This culture design permitted continuous spermatogenesis using mouse testicular tissue during 6 months (Komeya et al. 2016).

1.5 ORGANOID

The term organoid was mainly employed from the 50's until the 80's of the last century to nominate the structures formed by the reorganization capacity of primary dissociated cells *in*

in vitro (Moscona and Moscona 1952; Weiss and Taylor 1960; Clevers 2016). These studies represented the first steps in our understanding of cellular organization during development. More recently, the term organoid has become widely used, but this time to describe 3D cellular structures that partially model the organization and function of a certain organ. These organ-like structures can originate from pluripotent or adult stem cells under the support of a 3D ECM scaffold (generally matrigel) and influence of a tissue-specific cocktail of morphogens and growth factors (Lancaster and Knoblich 2014; Huch et al. 2017). Typically, organoids grow few millimetres in size but their further expansion, into more complex and matured organ-like structures, is conditioned by the lack of a functional vascular system (Lancaster and Knoblich 2014). Organoids have been generated to model brain (Lancaster et al. 2013; Quadrato et al. 2017), gut (Sato et al. 2009; Drost et al. 2015), liver (Takebe et al. 2013) and prostate (Chua et al. 2014; Drost et al. 2016), among other organs. These organ-like structures are therefore promising tools to investigate several steps of development, function and disease in an organ-specific way *in vitro*.

1.5.1 Testicular organoids

Although several of the 3D methodologies mentioned before produced cellular aggregates demonstrating similarities to testicular organization and function (Zenzes and Engel 1981; Hadley et al. 1985; Legendre et al. 2010; Yokonishi et al. 2013; Zhang et al. 2014), those structures were not termed organoids. Indeed very few studies have been published reporting the formation and characterization of testicular organoids. Excluding the methods described in this thesis to generate human and rat testicular organoids (Baert et al. 2017; Alves-Lopes, Söder, and Stukenborg 2018), only one other method has been published on the same topic. This report, by Pendergraft and colleagues, described the co-culture of human primary SSCs and immortalized Leydig and Sertoli cells in a hanging drop of medium containing solubilized human testicular ECM (Pendergraft et al. 2017). Even though the resulting organoids were compact cellular aggregates without characteristic testicular organization, it was reported that these testis-like structures could produce testosterone and promote the passage of a small fraction of diploid germ cells into haploid cells (Pendergraft et al. 2017). Moreover, these human organoids have been also proposed to test the infection mechanisms of Zika virus in the testis (Strange et al. 2018). Therefore, this and the methods described in this thesis to produce testicular organoids are innovative technologies with potential to back up the previous models and promote the study of the testicular microenvironment *in vitro* (reviewed in (Alves-Lopes and Stukenborg 2018)).

2 AIMS

The main aim of this study was to explore different strategies to generate and optimize culture conditions to model testicular organization and functionality *in vitro* (Figure 2). Ultimately, the developed culture systems were intended to maintain and mature male germ cells from human pre- and pubertal testicular tissue.

More specifically,

- We proposed to investigate the effect of various 3D techniques on testicular somatic cell maturation *in vitro*.
- We intended to explore the suitability of these previously developed systems to maintain immature germ cells *in vitro*.
- We intended to study the effect of signalling agents, such as retinoic acid (RA), IL1 α , TNF α and gonadotropins on germ cell maintenance as well as somatic cell function and organization using the previous testicular models.
- We also proposed to use the culture systems as tools to evaluate the functionality of pre- and pubertal human testicular tissue, in an attempt to refine future fertility preservation strategies for different patient groups.

Strategies to model testicular organization and function <i>in vitro</i>			
Model	Three-layer gradient system (3-LGS)	Decellularized testicular extracellular matrix (DTM)	Organ culture
To investigate	Organization and function	Organization and function	Function
Species	Rat	Human	Human
Tissue/cells	Cells	Cells	Tissue
Age	5-8-, 20- and 60-dpp	Pubertal and adult	Pre- and pubertal
Main outcome	Testicular organoid modelling organization and function	Testicular organoid modelling function	Tissue explant modelling function

Figure 2. Specifications of the 3D models investigated to accomplish the aims of this thesis.

3 MATERIALS AND METHODS

3.1 ETHICS

3.1.1 Rodent

Sprague Dawley male rats with an age range of 5–60-day *postpartum* (*dpp*) were delivered by Charles River (Sulzfeld, Germany) at our animal house. Animal experiments were conducted in agreement with ethics permit number 280/14 accorded by Karolinska Institutet ethical committee.

3.1.2 Human

Adult testicular tissue was collected from six patients submitted to bilateral orchiectomy at the Urology Department, Universitair Ziekenhuis Brussel in agreement with ethics permit number 2014/243. Cadaveric testes were collected from the autopsy service of the Universitair Ziekenhuis Brussel in accord with ethics permit number 2012/336. Pubertal testicular tissue from a 15-year-old was obtained within the fertility preservation program at UZ Brussel's Center for Reproductive Medicine and according the ethics permit number 2015/V9.

Prepubertal testicular tissue was obtained from patients in Sweden, Finland and Iceland who were facing treatments associated with a very high risk of infertility (Skinner et al. 2017) and because of that were offered to enrol in the experimental fertility preservation program "NORDFERTIL". Ethical approval was obtained from the Karolinska Institutet ethical committee and the Regional Ethics Board in Stockholm (Dnr 2013-2129-31-3), the National Ethics Board of Iceland in Reykjavik (VSN 15-002) and the Ethics Board of the University of Helsinki (426/13/03/03/2015).

3.2 TESTICULAR SAMPLES

3.2.1 Rodent

Testicular tissue from 5–8-, 20- and 60-*dpp* rats was harvested and used to obtain single cell suspensions by enzymatic digestion.

3.2.2 Human

3.2.2.1 Decellularized testicular extracellular matrix

Pubertal and adult testicular tissues from patients with average age of 15- and 75-years-old, respectively, were enzymatically digested into single cell suspensions. Cadaveric testes were utilized to prepare decellularized testicular extracellular matrix (DTM) scaffolds.

3.2.2.2 Organ culture

Pre- and pubertal testicular samples from 18 patients enrolled in the NORDFERTIL cohort were mechanically split into small fragments. The 18 biological replicates were divided into four groups according their previous exposure to chemotherapeutic drugs and haematological conditions: (1) biopsy performed before being exposed to chemotherapy (4.6 ± 3.3 [mean \pm standard deviation] years of age), (2) biopsy performed on patients with sickle-cell disease (SCD) (5.8 ± 1.6 years of age) after being exposed to hydroxyurea (HU), (3) biopsy performed after being exposed to non-alkylating agents (9.6 ± 3.9 years of age) and (4) biopsy performed after being exposed to alkylating agents (8.8 ± 3.1 years of age).

3.3 TISSUE DIGESTION

Decapsulated fresh rodent and cryopreserved human testicular tissue were enzymatically dissociated using a two-step digestion protocol. Briefly, samples were mechanically dissected into small fragments and firstly digested in a culture medium solution supplemented with collagenase 1A (1 mg/mL) in order to dissociate the interstitial compartment (for human tissue, supplementation with DNase type 1 [0.5 mg/mL] was also used in the first step of digestion to avoid excessive cellular aggregation). After, the remaining tissues (mostly seminiferous tubules fragments) were processed in a second medium solution supplemented with collagenase 1A (1 mg/mL), hyaluronidase (0.5 mg/mL) and DNase type 1 (0.5 mg/mL). Testicular cells isolated from both digestion steps were combined and checked for their viability and concentration.

3.4 CULTURE SYSTEMS

Three different culture systems were utilized for the experiments described in this thesis. Two of them, the three-layer gradient system (3-LGS) and the DTM scaffold, were used to generate testicular organoids from primary testicular cells. The third culture system, the organ culture in the air-liquid interface, was applied to culture small fragments of testicular tissue (Figure 3).

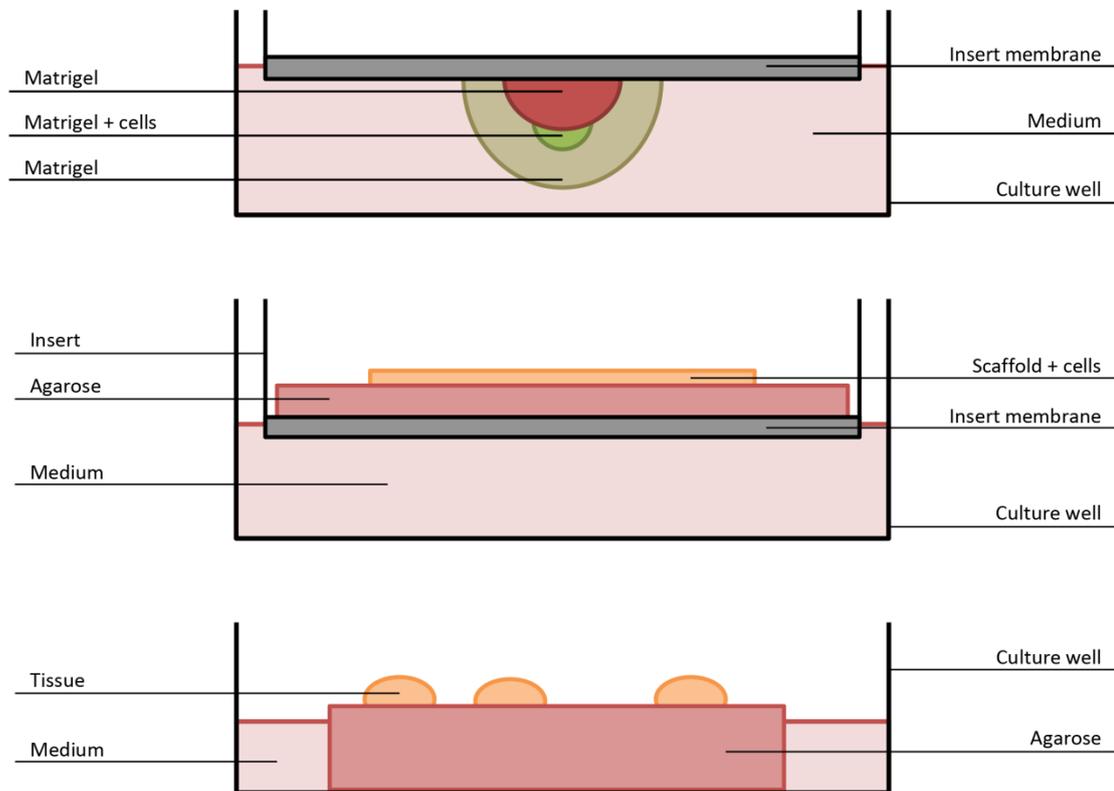


Figure 3. Schematic representation of the 3D models used in the experiments for this thesis: the three-layer gradient system (3-LGS) to generate rat testicular organoids (top); the decellularized testicular extracellular matrix (DTM) to produce human testicular organoids (middle); the organ culture in the air-liquid interface (bottom).

3.4.1 Three-layer gradient system

In order to set up the 3-LGS, three concentric drops of Corning® Matrigel® diluted 1:1 with culture medium (matrigel) were applied on the bottom membrane side of hanging cell inserts (Figure 3). A first 5- μ L drop of matrigel was applied and permitted to gel. Secondly, a 3- μ L drop of matrigel combined with rat testicular cells was placed on top of the first drop and allowed to gel. After, a third 8- μ L drop of matrigel was pipetted on top of the previous drops and permitted to gel. For controls conditions, a 16- μ L drop of matrigel combined with rat testicular cells was applied on the bottom membrane side of independent hanging cell inserts. The hanging cell inserts were finally placed in 24-well plates with 0.6 mL of culture medium.

3.4.2 Decellularized testicular extracellular matrix

DTM scaffolds were prepared from small fragments (approx. 1 cm³) of cadaveric testes as previously described by Baert and colleagues (Baert et al. 2015). Cells were removed using a saline 1% (w/v) sodium dodecyl sulphate solution and by mechanical agitation. After being washed and sterilized, the decellularized fragments were sectioned into 90- μ m scaffolds.

Subsequently, scaffolds were placed on top of 0.35% (w/v) agarose stand, which were, in their turn, positioned on the apical compartment of hanging cell inserts (Figure 3). The culture system was after placed in 24-well plates and soaked with culture medium. Finally, testicular cells suspended in culture medium were pipetted on top of the arrangement with or without (controls) DTM scaffolds.

3.4.3 Organ culture in the air-liquid interface

Testicular tissues were dissected into small fragments and placed on top of 0.35% (w/v) agarose stands in the air-liquid interface as described previously (Reda et al. 2016; Reda et al. 2017) (Figure 3). Culture medium was added to the 6-well plates until the edge of the stand in order to soak the agarose but without covering the testicular samples.

3.5 CULTURE CONDITIONS

3.5.1 Rat testicular organoids

Organoids were generated and cultured in minimum essential medium alpha (MEM α), 1% (v/v) penicillin/streptomycin (pen/strep), 10 % KnockOut serum replacement (KSR). Supplementation with 10 nM–100 μ M of RA, 10 nM–10 μ M of a RA receptor alpha (RAR α) antagonist (ER 50891), 1 nM–1 μ M of retinoid X receptor (RXR) antagonist (HX 531), IL1 α at 1–10 ng/mL and TNF α at 1–10 ng/mL were utilized depending on the experimental conditions. Organoid cultures were incubated at 35 °C, in a 5% CO₂ atmosphere and half of the culture medium (0.3 mL) was refreshed every second day.

3.5.2 Human testicular organoids

Organoids were produced and cultured in KnockOut Dulbecco's modified Eagle's medium (DMEM), 1% (v/v) pen/strep, 10% (v/v) KSR XenoFree (KSR-XF), 1x GlutaMax supplemented with 5 IU/L of both human chorionic gonadotrophin (hCG) and/or recombinant FSH (rFSH) according the experimental conditions. Organoids were cultured in the gas-liquid interface in an atmosphere containing 5% CO₂, at 35 °C, for four weeks. Culture medium (0.6 mL) was changed at each collection time point or otherwise weekly.

3.5.3 Human organ culture

Testicular fragments, from all patients groups, were cultured in NutriStem® medium (2 mL) supplemented with 1% (v/v) pen/strep, 10⁻⁷ M melatonin and 10% (v/v) KSR-XF that was

changed every week. The tissue was incubated in the air-liquid interface, in an atmosphere containing 5% CO₂, at 35 °C, for 21 days.

3.6 MIGRATION ASSAY

The generation of rat organoids was followed daily using a bright-field TE 2000 microscope and recorded with an Infinity 1-2C digital camera for 7 days. In order to compare the migration profile among 5–8-, 20- and 60-dpp organoids, the area occupied by cells was measured during the 7-day culture period utilizing the image analysis program ImageJ. Relative migration area was calculated using the following equation, where x stands for day of analysis (0–7).

$$\text{Relative migration area} = \frac{\text{colony area day 0} - \text{colony area day x}}{\text{colony area day 0}} \times 100$$

3.7 HISTOCHEMISTRY

Testicular organoids and organ cultured fragments were fixed in 4% paraformaldehyde or in Bouin's solution. After dehydration, samples were paraffin embedded and cut in 5-µm thick sections. Finally, organoid and tissue sections were stained with periodic Acid-Schiff solution and mounted.

3.8 WHOLE-MOUNT STAINING

Rat and human testicular organoids were fixed in 4% paraformaldehyde and, after washing, antigen retrieved in sodium citrate buffer (pH 6) at 95 °C. After permeabilization, unspecific binding sites were serum blocked and endogenous peroxidase activity prevented with methanol, 3% (v/v) hydrogen peroxide (the last step just for human organoids). Incubation with specific primary antibodies was followed by washing steps and incubation with fluorescent-dye conjugated secondary antibodies, for rat organoids. For human organoids, incubation with horseradish peroxidase-conjugated secondary antibodies was followed by development with Tyramide Signal amplification Plus Cy3 or Fluorescein Systems. DAPI was used as counter staining and organoids were 3D-mounted (rat organoids) or mounted with anti-fade mounting medium between the slide and coverslip (human organoids).

3.9 IMMUNOFLUORESCENCE

Paraffin embedded sections were de-paraffinized and rehydrated using xylene and a descending ethanol series, respectively. Subsequently, samples were antigen retrieved in 10 mM sodium citrate buffer (pH 6) at 95 °C and after blocked with serum from the same host

where the secondary antibody was produced. Incubation with the primary antibodies of interest was performed overnight, at 4 °C, and was followed by washing steps. Finally, tissue sections were incubated with fluorescent-dye conjugated secondary antibodies and mounted with anti-fade mounting medium containing DAPI.

3.10 COUNTING

3.10.1 Rat testicular organoids

Superficial 3D projections of whole-mounted organoids were generated by confocal microscopy and using a Z-stack command. Testicular cells stained for specific markers as well as total number of cells were counted using the image analysis program ImageJ. Finally, the ratio specific marked cells/total cells was used to compare the effect of different medium supplementations.

3.10.2 Human organ culture

The number of germ cells [DEAD-box helicase 4 (DDX4)-positive cells] per total seminiferous cord cross-sections and the percentage of seminiferous cord cross-sections positive for Sertoli cell markers of interest [ZO1, Anti-Mullerian hormone (AMH) and Cytokeratin 18 (CK18)] were determined using a fluorescence microscope.

3.11 BLOOD-TESTIS BARRIER FUNCTIONALITY

Rat organoids cultured for seven days were harvested, washed in phosphate buffered saline (PBS) and incubated with PBS, 0.4% (w/v) Evans Blue for 0.5 h, at 35 °C. Organoid impermeability to Evans Blue was verified by confocal microscopy using the acquisition settings for Cy3. Moreover, rat organoids were whole-mount stained against ZO1 and occludin in order to verify the distribution pattern of tight-junction proteins among Sertoli cells.

3.12 HORMONE ANALYSIS

Conditioned medium was collected from human testicular organoid and organ cultures in order to evaluate the production of testosterone, inhibin B and AMH during the culture period. Hormone concentrations were determined by specific enzyme-linked immunosorbent assays (ELISAs) according to the manufacturer's instructions. Two technical replicates were performed for each individual sample. Resulting average absorbance values were matched to

testosterone (ng/mL), inhibin B (pg/ml) and AMH (ng/mL) concentrations using a 4 Parameter Logistics, linear regression and cubic regression curves-fit, respectively.

3.13 CYTOKINE ANALYSIS

In order to quantify factors produced by human testicular organoids, an antibody array targeting 80 human cytokines was utilized to test conditioned medium from one- and four-week cultures following the manufacturer's instructions. After subtracting the background, sample reads were normalized to the positive controls and relative cytokine production levels were compared between one- and four-week conditioned medium arrays.

3.14 STATISTICAL ANALYSIS

3.14.1 Rat testicular organoids

Statistical significance among different groups was calculated using SigmaPlot 12.0 software by means of one-way analysis of variance (ANOVA) and one-way repeated measures ANOVA with correction for multiple comparisons. Means and standard deviations were used for analysis and significance was considered when p -value was < 0.05 .

3.14.2 Human testicular organoids

Mean levels of hormones and cytokines (\pm standard deviations) were compared by two-way ANOVA with correction for multiple comparisons using GraphPad Prism 6 or IBM Statistics 20 software. Statistical significance was considered when p -value was < 0.05 .

3.14.3 Human organ culture

Statistical analyses were carried out in SigmaPlot 13.0 software and the results presented as mean \pm standard deviation. The t-test was applied when the normality and equal variance tests were passed (Shapiro-Wilk and Brown-Forsythe, respectively). If normality and equal variance tests failed, the Mann–Whitney U test was used to evaluate differences.

4 RESULTS AND DISCUSSION

4.1 DEVELOPMENT OF THE THREE-LAYER GRADIENT SYSTEM AND GENERATION OF RAT TESTICULAR ORGANOID (PAPER I AND II)

Primary testicular cells, obtained by enzymatic digestion of 20-dpp rat testis, formed spherical-tubular structures (STSs) like seminiferous tubules, when suspended in a 3- μ L layer of matrigel and placed between two other layers of matrigel without cells (5- and 8- μ L layers) (Figure 3). This 3D culture arrangement, the 3-LGS, was compared to control conditions where testicular cells, at the same concentration as in the 3- μ L layer of the 3-LGS, were suspended in one only layer of matrigel with the same volume as the sum of the three layers (16 μ L) in the 3-LGS. Although cellular migration and reorganization were observed in control conditions, no STSs were formed as in the 3-LGS setting, demonstrating the beneficial effect of cell culture compartmentalization and the creation of a gradient of cells in *de novo* organization of rat testicular cells *in vitro*.

The STSs created after 5–7 days in culture by the 3-LGS protocol demonstrated similarities to seminiferous tubules in terms of organization and function. Because of that, we termed these cellular arrangements testicular organoids. Testicular organoids were mainly constituted of Sertoli and germ cells [(SRY-box 9 (SOX9)- and DDX4-positive cells, respectively)]. Moreover, undifferentiated germ cells maintained proliferation [Promyelocytic leukemia zinc finger (PLZF) and proliferating cell nuclear antigen (PCNA) double positive cells] up to 21 days in these testicular organoids. Further functionality was demonstrated by the presence of an *in vitro* formed BTB in the organoids, confirmed by expression of occludin and ZO1 between neighbouring Sertoli cells and by the organoid impermeability to Evans Blue (a small molecule known not to cross healthy BTB *in vivo*). In addition, testicular organoids could be generated with an efficiency of around 96%. Along with the higher reproducibility, the relative small number of cells and volume of matrigel used in the 3-LGS makes this culture method both appropriate for high-throughput analysis, as well as efficient regarding the reduced number of laboratory animals needed per technical replicate when compared with other methods (Alves-Lopes, Söder, and Stukenborg 2018).

The majority of 3D cell culture strategies generally use a supportive matrix where cells are homogeneously suspended as we utilized for our control conditions but without success in

terms of reorganization. On the other hand, the compartmentalized approach applied in the 3-LGS favoured the generation of organized structures as described in a previous report where the same principle was employed (Hagiwara, Peng, and Ho 2015). In this study, Hagiwara and colleagues described the formation of branch-like structures when highly concentrated human epithelial lung cell suspensions were cultured in a limited volume (1 μ L) enclosed by only matrigel (Hagiwara, Peng, and Ho 2015). The 3-LGS, in the current study, uses a layered structure that both increases the surface area where factors can be exchanged between compartments with and without cells, and allows the application of a bigger cell suspension volume (3 μ L) leading to the development of larger organoids.

The generation of testicular organoids under the 3-LGS culture conditions could be explained by Hagiwara's and colleagues' hypothesis, which describes the formation of branch-like structures by the action of activating factors on the adjacent cells and diffusion of inhibiting factors to the surrounding matrix and medium (Hagiwara, Peng, and Ho 2015). This theory was backed up by our own hypothetical model where we took into account the concepts of molecular diffusion and concentration gradient to explain the formation of testicular organoids (Alves-Lopes, Soder, and Stukenborg 2017). More specifically, we hypothesize that two main concentration gradients are created in the 3-LGS: cellular metabolites were highly concentrated in the layer with cells and diffused into the layers without cells and into the medium; on the other hand, factors present in the matrigel and in the culture medium, which are consumed by cells, diffused from the layers without cells and medium into the layer with cells (Figure 4). In the boundaries of adjacent layers, the concentration of cellular products was lower and the concentration of matrigel and medium factors was higher than in the centre of the layer with cells. Consequently, in the regions between layers, cells had the most optimal concentration of cell-culture factors together with free space to reorganize into testicular organoids (Figure 4).

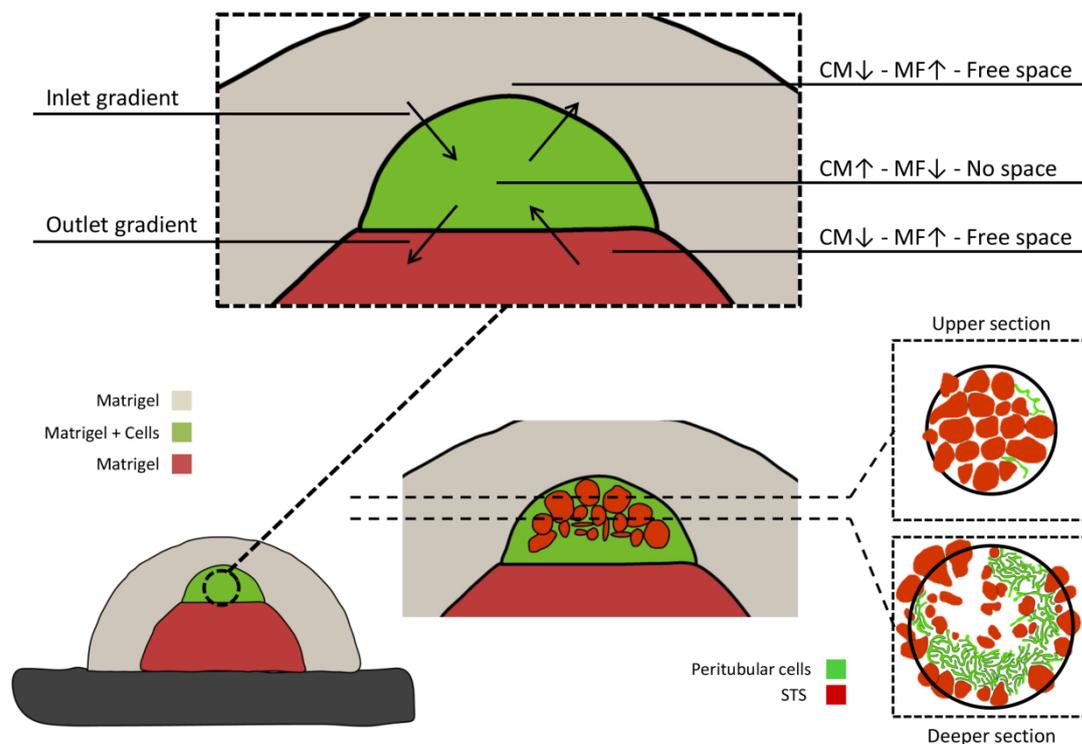


Figure 4. Hypothetical model to explain the reorganization of testicular cells from 20-day *postpartum* rat under the influence of the 3-LGS culture conditions. (CM) cellular metabolites; (MF) matrigel and medium factors. Figure adapted from (Alves-Lopes, Soder, and Stukenborg 2017).

4.2 RAT TESTICULAR ORGANOID CHARACTERIZATION AND VALIDATION (PAPER II)

After the development of the 3-LGS and generation of rat testicular organoids, we further characterized and validated this culture system to study germ-to-somatic cell associations *in vitro*.

4.2.1 Cellular concentration and developmental stage influenced testicular cell reorganization

In order to explore the most optimal conditions for testicular organoid formation, different cellular concentrations (ranging 5.5–88 million 20-*dpp* rat cells/mL) were tested. The concentration of 44 million cells/mL was selected because most of the initially seeded cells formed organoids constituted by connected STSs, which facilitated the handling in downstream analytic steps.

In addition to experiments with 20-*dpp* cells, 5–8- and 60-*dpp* rat testicular cells at concentrations ranging 5.5–88 million cells/ mL were employed to explore the effect of cellular developmental stage in organoid formation. Under the bright-field microscope, STS formation was observed for all concentrations of 20-*dpp* cells and for low concentrations

(5.5–22 million cells/mL) of 5–8-dpp cells. Although formation of compact cellular aggregates was observed for high concentrations of 5–8-dpp cells (44–88 million cells/mL), no STSs were observed in these colonies. Regarding experiments with 60-dpp rat cells, STSs generation was not detected for all concentrations investigated.

The expression of SOX9 and actin alpha 2 smooth muscle (ACTA2) (Sertoli and peritubular cell markers, respectively) was also evaluated for organoids generated from 5–8- and 20-dpp cells at both low- and high-concentrations (11 and 44 million cells/mL, respectively). At both low- and high-concentrations of 20-dpp cells, peritubular cells were observed mainly at the periphery of the STSs colonies formed by Sertoli cells. On the other hand, when 5–8-dpp cells were utilized, peritubular cells were observed mostly throughout the colonies and Sertoli cells formed few STSs at low- and even less at high-concentration. This evaluation was not feasible for 60-dpp cells because STSs were not produced.

To further assess the differences among 5–8-, 20- and 60-dpp rat testicular cells in terms of reorganization *in vitro*, their migration profile was monitored under the 3-LGS culture conditions. Overall, it was observed that 5–8-dpp cells migrated quicker but ended in less compact colonies than 20-dpp testicular cells. Regarding 60-dpp cells, migration was not observed using the same culture conditions.

Altogether, our experiments detected differences in terms of cellular reorganization *in vitro* among the distinct ages analysed. This might be related to the variances in the relative number of Sertoli cells present in the rat testis at the distinct developmental stages, as reported for human (Rey 2003). Sertoli cell numbers are relatively low in 60-dpp when compared to 20-dpp rat testis because of the relative high number of germ cells in the adult testis. This dilution effect might result in reduced interactions between Sertoli cells in culture and, consequently, absence of STSs formation. As regards 5–8-dpp, Sertoli cell numbers are also expected to be relatively low in comparison to 20-dpp rat testis but in this case, because of the relative higher volume occupied for the interstitial compartment in the pre-pubertal testis. This, together with the fact that 5–8-dpp peritubular cells are more immature and proliferative might justify the decreased numbers of STSs observed in organoids generated with testicular cells from 5–8-dpp cells. Moreover, the formation of a static peritubular cell network in the organoids generated from 5–8-dpp cells might have further blocked Sertoli cell reorganization into STSs.

As previously mentioned, further characterization and validation of the model was performed using organoids generated from 20-*dpp* rat testicular cells at the concentration of 44 million cells/mL.

4.2.2 Undifferentiated germ cells proliferated and organized as *in vivo*

Undifferentiated germ cells maintained proliferation (PLZF and PCNA double positive cells) up to 21 days in culture. Moreover, undifferentiated germ cells formed cellular chains connected by cytoplasmic bridges as observed on the basement membrane of 20-*dpp* rat seminiferous tubules. These findings, together with the observation of BTB formation in the STSs, indicated that testicular organoids generated by the 3-LGS partially mimicked the structure and function of rat testis *in vitro*. Therefore, the model might be used in the future to improve culture conditions for undifferentiated germ cell proliferation and to test BTB formation and integrity *in vitro*.

4.2.3 Retinoic acid improved germ cell maintenance in organoid cultures

The percentage of germ cells was higher than in controls, when testicular organoid cultures were supplemented with RA (1 μ M) during 10 days. This finding is in line with *in vivo* observations of decreased germ cell numbers in the seminiferous tubules of vitamin A-deficient rats and their subsequent recovery to normal cell counts after RA administration (Pelt and Rooij 1991).

To further confirm the role of RA in germ cell maintenance, medium supplementation with ER 50891 (RAR α antagonist) or HX 531 (RXRs antagonist) were used in 10-day cultures. At molarities that inhibit RAR α , supplementation with ER 50891 showed no effect on germ cell maintenance. Instead, higher molarities of ER 50891, known to inhibit RAR γ and β subunits, decreased germ cell percentage. Regarding HX 531 experiments, no differences in terms of germ cell maintenance were observed for all molarities tested. These observations are in accordance with *in vivo* studies that demonstrate γ as the RAR subtype involved (Gely-Pernot et al. 2012; Ikami et al. 2015) and RXRs as being non-essential (Krezel et al. 1996; Hogarth 2015) in germ cell response to RA.

4.2.4 IL1 α and TNF α disturbed organoid formation and decreased germ cell maintenance

Testicular organoids generated in presence of IL1 α (1 or 10 ng/mL) displayed STSs with a smaller average area than controls. The same effect was confirmed for culture conditions where TNF α was added at the concentration of 10 ng/mL. The negative effect of both pro-inflammatory cytokines in organoid structure was further confirmed by the observation that IL1 α (1 ng/mL) led to absence of occludin and a decreased expression of ZO1 on the STSs, while TNF α (1 ng/mL) also disturbed occludin and ZO1 expression patterns but less extensively than IL1 α conditions. Moreover, both IL1 α and TNF α decreased the number of germ cells maintained in organoid cultures. More specifically, IL1 α (1 or 10 ng/mL) reduced the percentage of germ cells from 6.3% (controls) to close to zero (0.2%), while increasing concentrations of TNF α (1–10 ng/mL) led to decreasing germ cell counts (4.8–2.4%, respectively), but to lesser extent than IL1 α . The similarities of these results to those reported *in vivo* studies, where the administration of the same cytokines to adult rats led to BTB impairment and reduction in germ cell counts (Sarkar et al. 2008), additionally validated the testicular organoids generated by the 3-LGS to investigate the role of bioactive molecules on BTB formation and integrity as well as germ cell maintenance *in vitro*.

4.2.5 Limitations and future applications

Although organoids generated by the 3-LGS partially model the function and organization of rat testis, several limitations were still detected such as decreasing germ cell maintenance and absence of post-meiotic differentiation during the period of culture. Moreover, the 3-LGS setting was not enough to produce organoids from 5–8- and 60-*dpp* rat testicular cells and the matrigel (derived from mouse tissue) used in its configuration of layers does not have a completely characterised composition. Nevertheless, sorting and/or genetic engineering of a specific cell population from the initial testicular cell suspensions might improve the reorganization of the remaining/modified cells in culture. Moreover, the application of other matrices with a fully characterized composition might contribute for more defined 3-LGS culture conditions.

Additionally, upgraded versions of the 3-LGS might be utilized in future experiments to generate human testicular organoids from primary and/or pluripotent stem cells. Furthermore, the system might be employed to generate organoids for other specific tissues and to explore

the concept of compartmentalized cell culture by the application of distinct cell populations and growth factors, at desired concentrations, in its different layers or compartments.

4.3 GENERATION OF HUMAN TESTICULAR ORGANOIDS (PAPER III)

In a further approach to model testicular function and organization, in collaboration with Baert and colleagues, the potential of testicular DTM to promote cellular reorganization into organoids was tested on human adult or pubertal testicular cells.

4.3.1 Organoid formation was independent of decellularized testicular extracellular matrix support

Adult or pubertal cells were seeded on culture systems with 90 µm-thick DTM (Figure 3) or without this scaffold (controls). Although testicular cells occupied both tubular and interstitial compartments of the DTM during the first day in culture, over the time, cells deformed the scaffold and organized into condensed spheroids that remained until the end of the culture. This spatial and temporal pattern of organization, also observed for cells seeded on soft-agar without DTM, might be to a certain extent explained by the contractile capabilities of peritubular cells (smooth-muscle cells) present in the initial cell suspensions.

4.3.2 Testicular somatic elements were present and functional in organoids

The presence of peritubular cells was confirmed by immunofluorescence staining against ACTA2. Round-shaped peritubular cells co-expressing collagen type 1 (COL1) were observed in the first day of culture, while after 4 weeks, ACTA2 positive cells were elongated and distributed on a reorganized network of COL1. These findings demonstrated that peritubular cells present in culture were partly functional and further suggested their key contribution for spherical organoid generation and remodelling of the ECM scaffold over the culture period.

Sertoli cell aggregates expressing ZO1 were also present in organoids from the first day until the end of the culture time. Additionally, inhibin B was detected in the collected media during the entire culture period. Although Sertoli cell functionality could be in part modelled in these culture conditions, their organization was not comparable with the situation found *in vivo*. This lack of reorganization might be related to the advanced stage of maturation and/or to the interference of other testicular cell populations present in the organoids.

Leydig cells, identified by co-expression of steroidogenic acute regulatory protein (STAR) and 3 β -hydroxysteroid dehydrogenase (3 β HSD), were other testicular somatic cell population present throughout the whole culture period. Furthermore, Leydig cell activity was demonstrated by the detection of testosterone in the collected media. The results regarding the different somatic cell types distribution in the organoids and hormone measurements were similar between adult and pubertal cultures in either DTM or control conditions.

Altogether, these results showed that testicular functionality could be partially achieved, but similarities to the native testicular organization could not yet be demonstrated in organoids generated with or without the support of DTM. This histological disorganization might be in part due to the negative effect of peritubular cells in the process of reorganization. These cells created a cellular and ECM network during the culture period that might condition migration and epithelia formation by Sertoli cells. Moreover, testicular organoids produced cytokines involved with the inflammatory response, such as IL6, IL8, growth-regulated alpha/beta/gamma protein (GRO) and monocyte chemotactic protein 1 (MCP1), which might have a negative effect on testicular cell reorganization *in vitro*, as demonstrated in the previous experiments with IL1 α and TNF α in rat testicular organoids.

4.3.3 Organoids supported spermatogonia maintenance and proliferation

Spermatogonia were identified in the organoid cultures for 4 weeks by the expression of ubiquitin C-terminal hydrolase L1 (UCHL1), undifferentiated embryonic cell transcription factor 1 (UTF1) or fibroblast growth factor receptor 3 (FGFR3) in combination with the germ cell marker DDX4. Spermatogonial cells were observed as single cells or in cellular clusters and to be mitotically active [DDX4 and marker of proliferation Ki-67 (KI67) co-expression]. Although the numbers of proliferative spermatogonia appeared to decrease, the mitotic activity of these cells could be maintained over the time in culture, demonstrating that other key functional features of the human testis could be modelled using organoid cultures. These results were similar between adult and pubertal cultures in either DTM or control conditions.

4.3.4 Limitations and future applications

The effort to promote reorganization of primary testicular cells *in vitro* by means of a supporting DTM scaffold did not show advantages over the control conditions, where cells reorganized in the same fashion without DTM scaffold sustenance. In order to overcome this

problem, harder matrices might be a possible alternative in future experiments to avoid the fast degradation and remodelling, as well as to allow seeded cells to occupy the appropriate compartments of the scaffold. Moreover, cells and factors thought to have a negative effect on the reorganization process might be sorted out and/or blocked, respectively, from organoid cultures.

Another limitation of this method is related to the decreasing numbers and the absence of spermatogonial cell differentiation over the culture period. However, because spermatogonia were mitotically active until the end of cultures, testicular organoids might be used to search for novel factors involved in spermatogonial cell proliferation and to support initial steps of differentiation. Moreover, testicular organoids might be used to provide the supportive somatic environment for further differentiation of spermatogonial cell like-cells derived from pluripotent stem cells as already described for rodents (Zhou et al. 2016). Finally, the system is also suggested as a tool to test the toxic effects of drugs and chemicals on the integrity of the somatic environment and spermatogonia maintenance.

4.4 ORGAN CULTURE SYSTEM TO CULTURE HUMAN IMMATURE TESTICULAR TISSUE (PAPER IV)

In the last approach to model the testicular microenvironment *in vitro*, we used organ culture conditions to test the functionality of immature testicular tissue from pre- and pubertal boys subjected to treatments for malignant and haematological diseases. The presence of germ cells and the functionality of the testicular somatic cells were evaluated for four different patient groups used in this study during the 21-day culture period.

4.4.1 Organ culture conditions did not support long-term germ cell maintenance

The germ cell numbers per seminiferous cord cross-sections were close to zero in the group of SCD patients treated with HU and in the group treated with alkylating agents, at the time of biopsy, suggesting that SCD and/or its treatment with HU as well as the treatment with alkylating agents have a negative impact on germ cell maintenance, as demonstrated in previous reports (Poganitsch-Korhonen et al. 2017; Stukenborg, Alves-Lopes, et al. 2018). Although the remaining two groups (non-exposed to chemotherapy and treated with non-alkylating agents) had germ cell numbers per seminiferous cord cross-sections comparable to reference values (Masliukaite et al. 2016) at the time of biopsy, a decrease in these cell numbers, to values close to zero, was verified after 21 days in culture.

4.4.2 Sertoli cell protein expression was similar among the different patient groups

We also analysed the expression of ZO1, AMH and CK18 in Sertoli cells present at the time of biopsy and in the testicular fragments cultured for 7, 14 and 21 days, for all patient groups. These proteins are used as markers for Sertoli cell maturation. More specifically, immature Sertoli cells demonstrate a high expression profile of CK18 and AMH, which is inverted when cells become mature (Sharpe et al. 2003). Additionally, ZO1 is considered to be a marker of Sertoli cell maturation, once its expression increases in parallel with BTB formation in maturing testis (Setchell 2008).

Concerning ZO1, the percentage of seminiferous cord cross-sections expressing this protein was consistent during the entire period of culture (81-91% at day 0 and 33-97% at day 21) among the four different patient groups at each time point (0, 7, 14 and 21 days).

Regarding AMH expression, a reduced percentage of positive seminiferous cord cross-sections was detected in the group of patients exposed to non-alkylating agents compared to the group not treated with chemotherapy, at day 0. This might be explained by the fact that the group of patients exposed to non-alkylating agents is on average older than the remaining patient groups and consequently having a more mature testicular microenvironment. Despite the differences at day 0, a pronounced decline in AMH expression was observed for all groups from day 0 to day 7, culminating with almost no expression of this protein in the seminiferous cord cross-sections at day 21 of culture, demonstrating some evidences of Sertoli cell maturation in these organ culture conditions.

In contrast, an increased percentage of CK18-positive seminiferous cord cross-sections was observed in the group of patients exposed to alkylating agents in comparison to the group not treated with chemotherapy, at day 0. Moreover, an increasing trend in the percentage of seminiferous cords expressing CK18 was observed for all groups throughout the 21 days of culture. These results are in discordance to the indications of Sertoli cell maturation demonstrated by the expression of AMH and ZO1 in the same samples and are suggestive of Sertoli cell dysfunctional or de-differentiation (Sharpe et al. 2003).

Altogether, these results indicate that despite the different treatments among the four patient groups, the organ culture system used in this study might be not enough to support Sertoli cell maturation, a consequence of which might be germ cell lost during the culture period.

4.4.3 Testicular somatic functionality from sickle-cell disease patients treated with hydroxyurea was diminished *in vitro*

In order to evaluate the testicular somatic functionality, we determined the hormone profile in the culture medium for all patient groups at each experimental time point (day 7, 14 and 21).

The concentration of testosterone in the conditioned medium from testicular cultures of SCD patients treated with HU was lower, at all time points, than in the cultures of testicular tissue from patients not exposed to chemotherapy. Moreover, the concentrations of inhibin B and AMH in the collected medium were also lower in the group of SCD patients treated with HU compared to the group not exposed to chemotherapy at day 7, 14 and 21.

Our results suggested that the SCD itself and/or the treatment with HU impair the normal hormone production of Sertoli and Leydig cells, which most likely occurred *in vivo* (before the testicular biopsy) and was reflected *in vitro*. More studies will be necessary in order to explain whether the cause of this reduced somatic functionality is due to the SCD itself or to the treatment with HU. However, the existence of this compromised somatic environment, together with the fact that few germ cells were present at the time of biopsy, highlights the need of alternative fertility preservation strategies for this group of patients that might not rely on the utilization of their biopsied testicular tissue.

4.4.4 Limitations and future applications

Although the organ culture model was successfully applied to mature mouse and rat testicular tissue (Sato et al. 2011; Reda et al. 2016), our results demonstrated that this system is not enough to maintain human germ cells in long-term cultures, corroborating the results from other groups (de Michele et al. 2017). A possible explanation for these distinct outcomes might be related to differences in tempo of testicular maturation between rodents and humans (Stukenborg, Colon, and Soder 2010). *In vivo*, the maturation of the somatic testicular environment takes weeks for rodents but years for humans. This means that in culture, maintenance and differentiation of rodent germ cells is supported by simultaneous somatic cell maturation. However, in the human context, the culture period and/or

conditions appeared unable to mature the somatic environment fast enough to even maintain germ cells.

Despite its limitations, the organ culture system served to elucidate differences in terms of testicular somatic functionality among distinct groups of patients subjected to different treatments against malignant and haematological diseases. For that reason, we suggest the methodology to test the functionality of pre- and pubertal testicular tissue in short-term cultures. The resulting information will help in directing patients, exposed to different treatment protocols, to the most adequate fertility preservation approach for each individual.

5 CONCLUSIONS

Throughout these studies, we utilized three distinct models in an attempt to model testicular organization and function *in vitro*. We demonstrated that using the 3-LGS, it was possible to explore both reorganization and functionality of the generated rat organoids. However, while using the DTM to generate human testicular organoids and the organ culture system, only functional aspects could be modelled.

More specifically, we verified that:

- The layout of the 3-LGS benefited the reorganization of 20-dpp rat testicular cells into structures similar to *in vivo* seminiferous tubules in a way never achieved exclusively *in vitro*.
- These seminiferous tubule-like structures, which we designated testicular organoids, displayed some features of the testicular function, namely, the presence of a *de novo* formed BTB and proliferative undifferentiated germ cells.
- Testicular organoids could be used to model the known *in vivo* effect of RA, IL1 α and TNF α on testicular microenvironment, validating the system to study the role of other factors of interest on germ cell maintenance as well as somatic cells function and organization *in vitro*.
- Although DTM did not support the reorganization of human testicular cells into a similar arrangement as found *in vivo*, the generated organoids displayed some characteristics of testicular functionality, namely, the presence of proliferative spermatogonia and production of testosterone and inhibin B.
- The organ culture conditions utilized in this study are yet not enough to maintain and differentiate human germ cells, as already demonstrated for other species.
- The organ culture system could be used to identify differences in parameters related to somatic functionality among testicular tissue samples from pre- and pubertal boys subjected to malignant and haematological treatments.
- Testicular tissue from SCD patients treated with HU had a compromised testicular somatic environment. This, together with the fact that few germ cells were present in the testicular sample of these patients at the time of biopsy, highlights the need for new fertility preservation strategies for this group of patients.

6 ACKNOWLEDGEMENTS

To everyone who has been involved in this journey, I would like to express my gratitude.

To **Jan-Bernd Stukenborg**, my main supervisor, I would like to thank for the opportunity to join his research group and the Initial Training Network “GrowSperm”. Moreover, I would like to acknowledge the freedom to experiment new ideas and the constant availability to discuss results as well as the next steps of my study plan.

For receiving me in their laboratories and research groups, as well as for all the support given during my studies, I would like to thank my co-supervisors **Olle Söder** and **Ellen Goossens**.

I am grateful to **Maria João Gama**, my mentor, for all reasonable words and unconditional support since I started in the paths of research.

Moreover, I would like to thank all friends and colleagues from the old Astrid Lindgren Barnsjukhus, from the Initial Training Network “GrwoSperm”, from the Vrije Universiteit Brussel, from KI housing in Vårberg and at the BioClinicum for whole the friendship and support.

To all my friends in Portugal, I would like to thank for all the remote support and constant availability to be themselves.

To my family, I am thankful not only for the encouragement and trust, but also for the upbringing given during all these years.

I also would like to express my gratitude and appreciation to Stockholm for being my harbour during my studies in Sweden.

Finally, I would like to thank all the foundations and funding institutions for the support given to the research projects related with the results presented in this book. Namely the Kronprinsessan Lovisas Förening För Barnsjukvård/ Stiftelsen Axel Tielmans, the Frimurare Barnhuset in Stockholm Foundation, the Swedish Childhood Cancer Foundation, the Samariten Foundation, the Sällskapet Barnavård Foundation, the Sigurd och Elsa Goljes Minne Foundation and the EU-FP7-PEOPLE-2013-ITN (603568) “Growsperm”.

Thank you!

Zelong Dou
Sara Albuquerque
Jacopo Fontana
Dong Liang
Ola Nilsson
Valeria Messina
Mari Wilhelmsson
Débora Michelatto
Danyang Li
Shambhu Prasad
Daniel Salamon
Susanne Hallberg
Mariana Paulson
Wei Han
Yoni Baert
Swati Sharma
Andre Van Steirteghem
Carolina von Grothusen
Iga Dobkowska
Magdalena Kurek
Jennifer Frithiof
Débora Michelatto
Svetlana Lajic
Daniel Salamon
Susanne Hallberg
Mariana Paulson
Wei Han
Yoni Baert
Swati Sharma
Andre Van Steirteghem
Olle Söder
Emelie Benji
Mi Hou
Suzanna Queckborner
Svetlana Lajic
Daniel Salamon
Susanne Hallberg
Mariana Paulson
Wei Han
Yoni Baert
Swati Sharma
Andre Van Steirteghem
Christine Carlsson-Skwirut
Sandra Brogård
Elisavet Ntemou
Andrea Merker
Dorina Ujvari
Ans van Pelt
Marsida Hutka
Ellie Landman
Konstantin Svechnikov
Nikolaos Tsesmetzis
Elizabeth Oliver
Lalit Kumar
Yunhan Zhao
Martin Ritzen
Valentina Pampanini
Bettina Celvin
Stefan Schlatt
Elena Vicini
Marsida Hutka
Ellie Landman
Konstantin Svechnikov
Kristiina Tammimies
Kai Zhou
Martin Becker
Alexandra Gkourogianni
Kai Zhou
Lena Sahlin
Anna Sandberg
Liang Zhang
Valentina Pampanini
Bettina Celvin
Stefan Schlatt
Elena Vicini
Marsida Hutka
Ellie Landman
Konstantin Svechnikov
Ellen Goossens
Kirsi Jahnukainen
Rod Mitchell
Ahmed Reda
Lena Sahlin
Anna Sandberg
Liang Zhang
Valentina Pampanini
Bettina Celvin
Stefan Schlatt
Elena Vicini
Marsida Hutka
Ellie Landman
Konstantin Svechnikov
Nageswara Boggavarapu
Isabel Wagner
Ieva Masliukaite
Lisa Rabkina
Lilly Velentza
Klas Blomgren
Elena di Martino
Luise Landreh
Maria João Gama
Eugenie Quartier
Prashant Kadam
Julen Goicolea
Luís Lopes
Ahmed Osman
Niels Geijsen
Monique Havermans
Cecilia Dominguez
Yvonne Pierre
Maryam Iravani
Halima Albalushi
Camilla Adle
Prashant Kadam
Julen Goicolea
Luís Lopes
Ahmed Osman
Niels Geijsen
Monique Havermans
Domingyka Batkovskyyte
Zelia Sahin
Leif Karlsson
Joaquin Custodio
Therese Cedervall
Nikolas Herold
Annelies Vant Westeinde
Dirk de Rooij
Sowmya Ramesh
Marie Artaki
Ahmed Osman
Niels Geijsen
Monique Havermans
Jan-Bernd Stukenborg
Emilia Schwertner
Mina Mincheva
Bas Jansen
Xin Xuan Lin
Sarita Panula
Rodrigo Marcondes
Joana Portela
Jan-Bernd Stukenborg
Emilia Schwertner
Mina Mincheva
Bas Jansen
Xin Xuan Lin
Sarita Panula
Rodrigo Marcondes
Joana Portela

7 REFERENCES

- Abu Elhija M, Lunenfeld E, Schlatt S, and Huleihel M. Differentiation of murine male germ cells to spermatozoa in a soft agar culture system. *Asian Journal of Andrology* 2012; **14**; 285-293.
- Alves-Lopes JP, Soder O, and Stukenborg JB. Testicular organoid generation by a novel in vitro three-layer gradient system. *Biomaterials* 2017; **130**; 76-89.
- Alves-Lopes JP and Stukenborg J-B. Testicular organoids: a new model to study the testicular microenvironment in vitro? *Human Reproduction Update* 2018; **24**; 176-191.
- Alves-Lopes JP, Söder O, and Stukenborg J-B. Use of a three-layer gradient system of cells for rat testicular organoid generation. *Nature Protocols* 2018; **13**; 248-259.
- Anderson RA, Mitchell RT, Kelsey TW, Spears N, Telfer EE, and Wallace WH. Cancer treatment and gonadal function: experimental and established strategies for fertility preservation in children and young adults. *Lancet Diabetes Endocrinol* 2015; **3**; 556-567.
- Baert Y, De Kock J, Alves-Lopes JP, Söder O, Stukenborg J-B, and Goossens E. Primary Human Testicular Cells Self-Organize into Organoids with Testicular Properties. *Stem Cell Reports* 2017; **1**; 30-38.
- Baert Y, Stukenborg J-B, Landreh M, De Kock J, Jörnvall H, Söder O, and Goossens E. Derivation and characterization of a cytocompatible scaffold from human testis. *Human Reproduction* 2015; **30**; 256-267.
- Barratt CLR, Bjorndahl L, De Jonge CJ, Lamb DJ, Osorio Martini F, McLachlan R, Oates RD, van der Poel S, St John B, Sigman M, *et al.* The diagnosis of male infertility: an analysis of the evidence to support the development of global WHO guidance-challenges and future research opportunities. *Hum Reprod Update* 2017; **23**; 1-21.
- Chua CW, Shibata M, Lei M, Toivanen R, Barlow LJ, Bergren Sarah K, Badani KK, McKiernan JM, Benson MC, Hibshoosh H, *et al.* Single luminal epithelial progenitors can generate prostate organoids in culture. *Nat Cell Biol* 2014; **16**; 951-961.
- Clermont Y. Kinetics of spermatogenesis in mammals: seminiferous epithelium cycle and spermatogonial renewal. *Physiol Rev* 1972; **52**; 198-236.
- Clevers H. Modeling Development and Disease with Organoids. *Cell* 2016; **165**; 1586-1597.
- Cremades N, Bernabeu R, Barros A, and Sousa M. In-vitro maturation of round spermatids using co-culture on Vero cells. *Human Reproduction* 1999; **14**; 1287-1293.
- de Michele F, Poels J, Weerens L, Petit C, Evrard Z, Ambroise J, Gruson D, and Wyns C. Preserved seminiferous tubule integrity with spermatogonial survival and induction of Sertoli and Leydig cell maturation after long-term organotypic culture of prepubertal human testicular tissue. *Hum Reprod* 2017; **32**; 32-45.
- DeFalco T, Potter SJ, Williams AV, Waller B, Kan MJ, and Capel B. Macrophages Contribute to the Spermatogonial Niche in the Adult Testis. *Cell Rep* 2015; **12**; 1107-1119.

- Drost J, Karthaus WR, Gao D, Driehuis E, Sawyers CL, Chen Y, and Clevers H. Organoid culture systems for prostate epithelial and cancer tissue. *Nat. Protocols* 2016: **11**; 347-358.
- Drost J, van Jaarsveld RH, Ponsioen B, Zimmerlin C, van Boxtel R, Buijs A, Sachs N, Overmeer RM, Offerhaus GJ, Begthel H, *et al.* Sequential cancer mutations in cultured human intestinal stem cells. *Nature* 2015: **521**; 43-47.
- Dumont L, Oblette A, Rondanino C, Jumeau F, Bironneau A, Liot D, Duchesne V, Wils J, and Rives N. Vitamin A prevents round spermatid nuclear damage and promotes the production of motile sperm during in vitro maturation of vitrified pre-pubertal mouse testicular tissue. *Mol Hum Reprod* 2016: **22**; 819-832.
- Enders GC, Henson JH, and Millette CF. Sertoli cell binding to isolated testicular basement membrane. *J Cell Biol* 1986: **103**; 1109-1119.
- Fagerberg L, Hallstrom BM, Oksvold P, Kampf C, Djureinovic D, Odeberg J, Habuka M, Tahmasebpoor S, Danielsson A, Edlund K, *et al.* Analysis of the human tissue-specific expression by genome-wide integration of transcriptomics and antibody-based proteomics. *Mol Cell Proteomics* 2014: **13**; 397-406.
- Gassei K, Ehmcke J, and Schlatt S. Initiation of testicular tubulogenesis is controlled by neurotrophic tyrosine receptor kinases in a three-dimensional Sertoli cell aggregation assay. *Reproduction* 2008: **136**; 459-469.
- Gely-Pernot A, Raverdeau M, Célébi C, Dennefeld C, Feret B, Klopfenstein M, Yoshida S, Ghyselinck NB, and Mark M. Spermatogonia Differentiation Requires Retinoic Acid Receptor γ . *Endocrinology* 2012: **153**; 438-449.
- Hadley MA, Byers SW, Suárez-Quian CA, Kleinman HK, and Dym M. Extracellular matrix regulates Sertoli cell differentiation, testicular cord formation, and germ cell development in vitro. *The Journal of Cell Biology* 1985: **101**; 1511-1522.
- Hagiwara M, Peng F, and Ho C-M. In vitro reconstruction of branched tubular structures from lung epithelial cells in high cell concentration gradient environment. *Sci. Rep.* 2015: **5**; 8454.
- Hogarth C. 9 - Retinoic acid metabolism, signaling, and function in the adult testis A2 - Griswold, Michael D. *Sertoli Cell Biology (Second Edition)*. 2015. Academic Press, Oxford, pp 247-272.
- Huch M, Knoblich JA, Lutolf MP, and Martinez-Arias A. The hope and the hype of organoid research. *Development* 2017: **144**; 938-941.
- Huleihel M, Nourashrafeddin S, and Plant TM. Application of three-dimensional culture systems to study mammalian spermatogenesis, with an emphasis on the rhesus monkey (*Macaca mulatta*). *Asian Journal of Andrology* 2015: **17**; 972-980.
- Ikami K, Tokue M, Sugimoto R, Noda C, Kobayashi S, Hara K, and Yoshida S. Hierarchical differentiation competence in response to retinoic acid ensures stem cell maintenance during mouse spermatogenesis. *Development* 2015: **142**; 1582-1592.
- Jahnukainen K, Mitchell RT, and Stukenborg JB. Testicular function and fertility preservation after treatment for haematological cancer. *Curr Opin Endocrinol Diabetes Obes* 2015: **22**; 217-223.
- Jones DL and Wagers AJ. No place like home: anatomy and function of the stem cell niche. *Nat Rev Mol Cell Biol* 2008: **9**; 11-21.

- Jorgensen A, Nielsen JE, Perlman S, Lundvall L, Mitchell RT, Juul A, and Rajpert-De Meyts E. Ex vivo culture of human fetal gonads: manipulation of meiosis signalling by retinoic acid treatment disrupts testis development. *Hum Reprod* 2015; **30**; 2351-2363.
- Jorgensen A, Young J, Nielsen JE, Joensen UN, Toft BG, Rajpert-De Meyts E, and Loveland KL. Hanging drop cultures of human testis and testis cancer samples: a model used to investigate activin treatment effects in a preserved niche. *Br J Cancer* 2014; **110**; 2604-2614.
- Khajavi N, Akbari M, Abdolsamadi HR, Abolhassani F, Dehpour AR, Koruji M, and Habibi Roudkenar M. Role of Somatic Testicular Cells during Mouse Spermatogenesis in Three-Dimensional Collagen Gel Culture System. *Cell J* 2014; **16**; 79-90.
- Kierszenbaum AL, Crowell JA, Shabanowitz RB, DePhilip RM, and Tres LL. Protein secretory patterns of rat Sertoli and peritubular cells are influenced by culture conditions. *Biol Reprod* 1986; **35**; 239-251.
- Kim KJ, Kim BG, Kim YH, Lee YA, Kim BJ, Jung SE, Cho YJ, Lee SH, and Ryu BY. In Vitro Spermatogenesis Using Bovine Testis Tissue Culture Techniques. *Tissue Engineering and Regenerative Medicine* 2015; **12**; 314-323.
- Kokkinaki M, Lee TL, He Z, Jiang J, Golestaneh N, Hofmann MC, Chan WY, and Dym M. The molecular signature of spermatogonial stem/progenitor cells in the 6-day-old mouse testis. *Biol Reprod* 2009; **80**; 707-717.
- Komeya M, Kimura H, Nakamura H, Yokonishi T, Sato T, Kojima K, Hayashi K, Katagiri K, Yamanaka H, Sanjo H, *et al.* Long-term ex vivo maintenance of testis tissues producing fertile sperm in a microfluidic device. *Scientific Reports* 2016; **6**; 21472.
- Krausz C. Male infertility: pathogenesis and clinical diagnosis. *Best Pract Res Clin Endocrinol Metab* 2011; **25**; 271-285.
- Krezel W, Dupé V, Mark M, Dierich A, Kastner P, and Chambon P. RXR gamma null mice are apparently normal and compound RXR alpha +/-RXR beta -/-RXR gamma -/- mutant mice are viable. *Proc Natl Acad Sci U S A* 1996; **93**; 9010-9014.
- Kubota H, Avarbock MR, and Brinster RL. Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. *Proc Natl Acad Sci U S A* 2004; **101**; 16489-16494.
- Lambrot R, Coffigny H, Pairault C, Donnadiou AC, Frydman R, Habert R, and Rouiller-Fabre V. Use of organ culture to study the human fetal testis development: effect of retinoic acid. *J Clin Endocrinol Metab* 2006; **91**; 2696-2703.
- Lancaster MA and Knoblich JA. Organogenesis in a dish: Modeling development and disease using organoid technologies. *Science* 2014; **345**; 1247125.
- Lancaster MA, Renner M, Martin C-A, Wenzel D, Bicknell LS, Hurles ME, Homfray T, Penninger JM, Jackson AP, and Knoblich JA. Cerebral organoids model human brain development and microcephaly. *Nature* 2013; **501**; 373-379.
- Laurie GW, Leblond CP, and Martin GR. Localization of type IV collagen, laminin, heparan sulfate proteoglycan, and fibronectin to the basal lamina of basement membranes. *J Cell Biol* 1982; **95**; 340-344.

- Lee DR, Kaproth MT, and Parks JE. In Vitro Production of Haploid Germ Cells from Fresh or Frozen-Thawed Testicular Cells of Neonatal Bulls. *Biology of Reproduction* 2001: **65**; 873-878.
- Lee DR, Kim K-S, Yang YH, Oh HS, Lee SH, Chung TG, Cho JH, Kim HJ, Yoon TK, and Cha KY. Isolation of male germ stem cell-like cells from testicular tissue of non-obstructive azoospermic patients and differentiation into haploid male germ cells in vitro. *Human Reproduction* 2006: **21**; 471-476.
- Legendre A, Froment P, Desmots S, Lecomte A, Habert R, and Lemazurier E. An engineered 3D blood-testis barrier model for the assessment of reproductive toxicity potential. *Biomaterials* 2010: **31**; 4492-4505.
- Masliukaite I, Hagen JM, Jahnukainen K, Stukenborg JB, Repping S, van der Veen F, van Wely M, and van Pelt AM. Establishing reference values for age-related spermatogonial quantity in prepubertal human testes: a systematic review and meta-analysis. *Fertil Steril* 2016: **106**; 1652-1657.
- Meng X, Lindahl M, Hyvonen ME, Parvinen M, de Rooij DG, Hess MW, Raatikainen-Ahokas A, Sainio K, Rauvala H, Lakso M, *et al.* Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. *Science* 2000: **287**; 1489-1493.
- Mincheva M, Sandhowe-Klaverkamp R, Wistuba J, Redmann K, Stukenborg JB, Kliesch S, and Schlatt S. Reassembly of adult human testicular cells: can testis cord-like structures be created in vitro? *Mol Hum Reprod* 2018: **24**; 55-63.
- Moscona A and Moscona H. The dissociation and aggregation of cells from organ rudiments of the early chick embryo. *J Anat* 1952: **86**; 287-301.
- Nieschlag E and Lenzi A. The conventional management of male infertility. *Int J Gynaecol Obstet* 2013: **123** (2); S31-35.
- Oatley JM and Brinster RL. The germline stem cell niche unit in mammalian testes. *Physiol Rev* 2012: **92**; 577-595.
- Oatley JM, Oatley MJ, Avarbock MR, Tobias JW, and Brinster RL. Colony stimulating factor 1 is an extrinsic stimulator of mouse spermatogonial stem cell self-renewal. *Development* 2009: **136**; 1191-1199.
- Ogawa T, Ohmura M, and Ohbo K. The niche for spermatogonial stem cells in the mammalian testis. *Int J Hematol* 2005: **82**; 381-388.
- Oguzkurt P, Kayaselcuk F, Tuncer I, Alkan M, and Hicsonmez A. Evaluation of extracellular matrix protein composition in sacs associated with undescended testis, hydrocele, inguinal hernia, and peritoneum. *Urology* 2007: **70**; 346-350.
- Pan F, Chi L, and Schlatt S. Effects of nanostructures and mouse embryonic stem cells on in vitro morphogenesis of rat testicular cords. *PLoS One* 2013: **8**; e60054.
- Pelt AMMV and Rooij DGD. Retinoic Acid Is Able to Reinitiate Spermatogenesis in Vitamin A-Deficient Rats and High Replicate Doses Support the Full Development of Spermatogenic Cells. *Endocrinology* 1991: **128**; 697-704.
- Pendergraft SS, Sadri-Ardekani H, Atala A, and Bishop CE. Three-dimensional testicular organoid: a novel tool for the study of human spermatogenesis and gonadotoxicity in vitro. *Biol Reprod* 2017: **96**; 720-732.
- Perrard MH, Sereni N, Schluth-Bolard C, Blondet A, SG DE, Plotton I, Morel-Journel N, Lejeune H, David L, and Durand P. Complete Human and Rat Ex Vivo

- Spermatogenesis from Fresh or Frozen Testicular Tissue. *Biol Reprod* 2016: **95**; 89, 81-10.
- Poganitsch-Korhonen M, Masliukaite I, Nurmio M, Lähteenmäki P, van Wely M, van Pelt AMM, Jahnukainen K, and Stukenborg JB. Decreased spermatogonial quantity in prepubertal boys with leukaemia treated with alkylating agents. *Leukemia* 2017: **31**; 1460-1463.
- Potter SJ and DeFalco T. Using Ex Vivo Upright Droplet Cultures of Whole Fetal Organs to Study Developmental Processes during Mouse Organogenesis. *Jove-Journal of Visualized Experiments* 2015.
- Quadrato G, Nguyen T, Macosko EZ, Sherwood JL, Min Yang S, Berger DR, Maria N, Scholvin J, Goldman M, Kinney JP, *et al.* Cell diversity and network dynamics in photosensitive human brain organoids. *Nature* 2017: **545**; 48-53.
- Reda A, Albalushi H, Montalvo SC, Nurmio M, Sahin Z, Hou M, Geijsen N, Toppari J, Soder O, and Stukenborg JB. Knock-Out Serum Replacement and Melatonin Effects on Germ Cell Differentiation in Murine Testicular Explant Cultures. *Ann Biomed Eng* 2017: **45**; 1783-1794.
- Reda A, Hou M, Landreh L, Kjartansdóttir KR, Svechnikov K, Söder O, and Stukenborg J-B. In vitro Spermatogenesis – Optimal Culture Conditions for Testicular Cell Survival, Germ Cell Differentiation, and Steroidogenesis in Rats. *Frontiers in Endocrinology* 2014: **5**; 21.
- Reda A, Hou M, Winton TR, Chapin RE, Söder O, and Stukenborg J-B. In vitro differentiation of rat spermatogonia into round spermatids in tissue culture. *Molecular Human Reproduction* 2016: **22**; 601-612.
- Reuter K, Ehmcke J, Stukenborg J-B, Simoni M, Damm OS, Redmann K, Schlatt S, and Wistuba J. Reassembly of somatic cells and testicular organogenesis in vitro. *Tissue and Cell* 2014: **46**; 86-96.
- Rey R. Regulation of Spermatogenesis. In Söder, O (ed) *The Developing Testis. Physiology and Pathophysiology*. 2003. Endocrine Development, Basel, pp 38-55.
- Richardson LL, Kleinman HK, and Dym M. Basement membrane gene expression by Sertoli and peritubular myoid cells in vitro in the rat. *Biology of Reproduction* 1995: **52**; 320-330.
- Roulet V, Denis H, Staub C, Le Tortorec A, Delaleu B, Satie AP, Patard JJ, Jegou B, and Dejuqc-Rainsford N. Human testis in organotypic culture: application for basic or clinical research. *Hum Reprod* 2006: **21**; 1564-1575.
- Russell LD, Ettl RA, Hikim APS, and Clegg ED. *Histological and Histopathological Evaluation of the Testis* 1990. Cache River Press, Claerwater, FL.
- Sarkar O, Mathur PP, Cheng CY, and Mruk DD. Interleukin 1 Alpha (IL1A) Is a Novel Regulator of the Blood-Testis Barrier in the Rat. *Biology of Reproduction* 2008: **78**; 445-454.
- Sato T, Katagiri K, Gohbara A, Inoue K, Ogonuki N, Ogura A, Kubota Y, and Ogawa T. In vitro production of functional sperm in cultured neonatal mouse testes. *Nature* 2011: **471**; 504-507.
- Sato T, Katagiri K, Kojima K, Komeya M, Yao M, and Ogawa T. In Vitro Spermatogenesis in Explanted Adult Mouse Testis Tissues. *PLoS One* 2015: **10**; e0130171.

- Sato T, Vries RG, Snippert HJ, van de Wetering M, Barker N, Stange DE, van Es JH, Abo A, Kujala P, Peters PJ, *et al.* Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature* 2009: **459**; 262-265.
- Schlatt S, de Kretser DM, and Loveland KL. Discriminative analysis of rat Sertoli and peritubular cells and their proliferation in vitro: evidence for follicle-stimulating hormone-mediated contact inhibition of Sertoli cell mitosis. *Biology of Reproduction* 1996: **55**; 227-235.
- Setchell BP. Blood-Testis Barrier, Junctional and Transport Proteins and Spermatogenesis. In Cheng, CY (ed) *Molecular Mechanisms in Spermatogenesis*. 2008. Springer New York, New York, NY, pp 212-233.
- Setchell BP. Blood-testis barrier, junctional and transport proteins and spermatogenesis. *Adv Exp Med Biol* 2008: **636**; 212-233.
- Sharpe RM, McKinnell C, Kivlin C, and Fisher JS. Proliferation and functional maturation of Sertoli cells, and their relevance to disorders of testis function in adulthood. *Reproduction* 2003: **125**; 769-784.
- Siu MK and Cheng CY. Dynamic cross-talk between cells and the extracellular matrix in the testis. *Bioessays* 2004: **26**; 978-992.
- Skinner R, Mulder RL, Kremer LC, Hudson MM, Constone LS, Bardi E, Boekhout A, Borgmann-Staudt A, Brown MC, Cohn R, *et al.* Recommendations for gonadotoxicity surveillance in male childhood, adolescent, and young adult cancer survivors: a report from the International Late Effects of Childhood Cancer Guideline Harmonization Group in collaboration with the PanCareSurFup Consortium. *Lancet Oncol* 2017: **18**; 75-90.
- Sousa M, Cremades N, Alves C, Silva J, and Barros A. Developmental potential of human spermatogenic cells co-cultured with Sertoli cells. *Human Reproduction* 2002: **17**; 161-172.
- Steinberger A and Steinberger E. Differentiation of rat seminiferous epithelium in organ culture. *Journal of Reproduction and Fertility* 1965: **9**; 243-248.
- Steinberger A, Steinberger E, and Perloff WH. Mammalian testes in organ culture. *Experimental Cell Research* 1964: **36**; 19-27.
- Strange DP, Zarandi NP, Trivedi G, Atala A, Bishop CE, Sadri-Ardekani H, and Verma S. Human testicular organoid system as a novel tool to study Zika virus pathogenesis. *Emerg Microbes Infect* 2018: **7**; 82.
- Stukenborg J-B, Schlatt S, Simoni M, Yeung C-H, Elhija MA, Luetjens CM, Huleihel M, and Wistuba J. New horizons for in vitro spermatogenesis? An update on novel three-dimensional culture systems as tools for meiotic and post-meiotic differentiation of testicular germ cells. *Molecular Human Reproduction* 2009: **15**; 521-529.
- Stukenborg J-B, Wistuba J, Luetjens CM, Elhija MA, Huleihel M, Lunenfeld E, Gromoll J, Nieschlag E, and Schlatt S. Coculture of Spermatogonia With Somatic Cells in a Novel Three-Dimensional Soft-Agar-Culture-System. *Journal of Andrology* 2008: **29**; 312-329.
- Stukenborg JB, Alves-Lopes JP, Kurek M, Albalushi H, Reda A, Keros V, Tohonon V, Bjarnason R, Romerius P, Sundin M, *et al.* Spermatogonial quantity in human prepubertal testicular tissue collected for fertility preservation prior to potentially sterilizing therapy. *Human Reproduction* 2018; (in press).

- Stukenborg JB, Colon E, and Soder O. Ontogenesis of testis development and function in humans. *Sex Dev* 2010; **4**; 199-212.
- Stukenborg JB, Jahnukainen K, Hutka M, and Mitchell RT. Cancer treatment in childhood and testicular function: the importance of the somatic environment. *Endocr Connect* 2018; **7**; 69-87.
- Suzuki S and Sato K. The fertilising ability of spermatogenic cells derived from cultured mouse immature testicular tissue. *Zygote* 2003; **11**; 307-316.
- Takebe T, Sekine K, Enomura M, Koike H, Kimura M, Ogaeri T, Zhang R-R, Ueno Y, Zheng Y-W, Koike N, *et al.* Vascularized and functional human liver from an iPSC-derived organ bud transplant. *Nature* 2013; **499**; 481-484.
- Tanaka A, Nagayoshi M, Awata S, Mawatari Y, Tanaka I, and Kusunoki H. Completion of meiosis in human primary spermatocytes through in vitro coculture with Vero cells. *Fertility and Sterility* 2003; **79** (1); 795-801.
- Tesarik J, Greco E, Rienzi L, Ubaldi F, Guido M, Cohen-Bacrie P, and Mendoza C. Differentiation of spermatogenic cells during in-vitro culture of testicular biopsy samples from patients with obstructive azoospermia: effect of recombinant follicle stimulating hormone. *Human Reproduction* 1998; **13**; 2772-2781.
- Tres LL and Kierszenbaum AL. Viability of rat spermatogenic cells in vitro is facilitated by their coculture with Sertoli cells in serum-free hormone-supplemented medium. *Proc Natl Acad Sci U S A* 1983; **80**; 3377-3381.
- Trowell OA. A modified technique for organ culture in vitro. *Experimental Cell Research* 1954; **6**; 246-248.
- Tung PS and Fritz IB. Interactions of Sertoli Cells with Myoid Cells in vitro. *Biology of Reproduction* 1980; **23**; 207-217.
- Valentin JE, Stewart-Akers AM, Gilbert TW, and Badylak SF. Macrophage participation in the degradation and remodeling of extracellular matrix scaffolds. *Tissue Eng Part A* 2009; **15**; 1687-1694.
- van der Wee K and Hofmann MC. An in vitro tubule assay identifies HGF as a morphogen for the formation of seminiferous tubules in the postnatal mouse testis. *Experimental Cell Research* 1999; **252**; 175-185.
- Weinbauer GF, Luetjens CM, Simoni M, and Nieschlag E. Physiology of Testicular Function. In Nieschlag, E, Behre, HM, and Nieschlag, S (eds) *Andrology: Male Reproductive Health and Dysfunction*. 2010. Springer Berlin Heidelberg, Berlin, Heidelberg, pp 11-59.
- Weiss P and Taylor AC. Reconstitution of complete organs from single-cell suspensions of chick embryos in advanced stages of differentiation. *Proc Natl Acad Sci U S A* 1960; **46**; 1177-1185.
- Vigier M, Weiss M, Perrard MH, Godet M, and Durand P. The effects of FSH and of testosterone on the completion of meiosis and the very early steps of spermiogenesis of the rat: an in vitro study. *Journal of Molecular Endocrinology* 2004; **33**; 729-742.
- Xie B, Qin Z, Huang B, Xie T, Yao H, Wei Y, Yang X, Shi D, and Jiang H. In Vitro Culture and Differentiation of Buffalo (*Bubalus bubalis*) Spermatogonia. *Reproduction in Domestic Animals* 2010; **45**; 275-282.

- Yokonishi T, Sato T, Katagiri K, Komeya M, Kubota Y, and Ogawa T. In Vitro Reconstruction of Mouse Seminiferous Tubules Supporting Germ Cell Differentiation. *Biology of Reproduction* 2013: **89**; 15.
- Yoshida S, Sukeno M, and Nabeshima Y. A vasculature-associated niche for undifferentiated spermatogonia in the mouse testis. *Science* 2007: **317**; 1722-1726.
- Zenzes MT and Engel W. The capacity of testicular cells of the postnatal rat to reorganize into histotypic structures. *Differentiation* 1981: **20**; 157-161.
- Zhang J, Hatakeyama J, Eto K, and Abe S. Reconstruction of a seminiferous tubule-like structure in a 3 dimensional culture system of re-aggregated mouse neonatal testicular cells within a collagen matrix. *Gen Comp Endocrinol* 2014: **205**; 121-132.
- Zhang X, Wang L, Zhang X, Ren L, Shi W, Tian Y, Zhu J, and Zhang T. The use of KnockOut serum replacement (KSR) in three dimensional rat testicular cells co-culture model: An improved male reproductive toxicity testing system. *Food Chem Toxicol* 2017: **106**; 487-495.
- Zhou Q, Wang M, Yuan Y, Wang X, Fu R, Wan H, Xie M, Liu M, Guo X, Zheng Y, *et al.* Complete Meiosis from Embryonic Stem Cell-Derived Germ Cells In Vitro. *Cell Stem Cell* 2016: **18**; 330-340.