ESTABLISHMENT AND CHARACTERIZATION OF A PORCINE TESTIS ORGANOID CULTURE SYSTEM

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By

TAT-CHUAN CHAM

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Professor Ali Honaramooz, DVM, PhD
Department of Veterinary Biomedical Sciences (VBMS)
Western College of Veterinary Medicine
52 Campus Drive
University of Saskatchewan
Saskatoon, Saskatchewan S7N 5B4 Canada

OR

Head of the Department
Department of Veterinary Biomedical Sciences (VBMS)
Western College of Veterinary Medicine
52 Campus Drive
University of Saskatchewan
Saskatoon, Saskatchewan S7N 5B4 Canada

OR

Dean
College of Graduate and Postdoctoral Studies
University of Saskatchewan
116 Thorvaldson Building, 110 Science Place
Saskatoon, Saskatchewan S7N 5C9 Canada

ABSTRACT

The studies presented in this thesis were designed to establish, examine and characterize a porcine testis organoid culture system. The objective of the first study was to establish a porcine testis organoid system and assess different culture conditions for its efficient *in vitro* morphogenesis. We first examined the potential of testis cells from 1-week-old piglets for *de novo* testis organogenesis (*i.e.*, formation of testis organoids) using spheroid-forming low attachment wells in an air-liquid interface culture system. We found that both fresh and cryopreserved neonatal porcine testis cells were able to self-reassemble into testis organoids comprising of testis cord-like structures which could be maintained for at least 4 weeks in culture. Next, we examined several culture conditions including different cell densities, culture durations, media supplementation, and germ cell (gonocyte) ratios to find the optimal conditions for *de novo* tubulogenesis in testis organoids. We found that a testis cell density of 0.8×10^6 cells/organoid and a combined supplementation of 5% knockout serum replacement (KSR) and 10% fetal bovine serum (FBS) in media would improve *de novo* formation of testis cord-like structures. We also observed that the relative number of germ cells in organoids did not decrease over time or differ among supplementation groups. The objective of the second study was to examine the structural and endocrine characteristics of our newly developed porcine testis organoids. We found that testis organoids consisted of tubular and interstitial compartments resembling innate testis tissue. More specifically, germ cells were observed within the testis tubular compartment reconstructed by Sertoli cells, peritubular myoid cells (PTMCs), and a peritubular basement membrane. We also observed the reconstruction of the inter-tubular interstitial compartment containing Leydig cells, collagen fibers, and vascular structures. Furthermore, we showed that testis organoids respond to luteinizing hormone (LH) stimulation and secrete testosterone, suggesting that the organoids also possess the endocrine characteristics of a typical neonatal testis tissue. This study provides a platform for the production of testis organoids, with high structural and functional resemblance to the innate neonatal testis tissue. The new testis organoid system can be used in future studies into *de novo* testis organogenesis or in pharmaco-toxicological testing of various substances on testis formation.

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DEDICATION

This thesis is dedicated to my beloved parents, Daw-Yen Cham and Susan Lin, who always believe in me and support my dreams. Without their love, encouragement, and financial and emotional support, none of this would have happened. Also, to my other family members, Tat-Fatt Cham, Anita Lin, Su-Ching Lin, Jennifer Gerow, Barry Gerow, and Therese Gerow, who always support me and help me get through the hard times in my career and everyday life.

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ORGANIZATION OF THESIS

In **Chapter 1**, a general introduction and the literature review on testis organoid culture systems have been provided. This includes the potential application of testis organoids (**Section 1.1**) and the process of testis development and spermatogenesis especially as related to the criteria for organoid formation (**Section 1.2**). Subsequently, different testis organoid studies have been reviewed and categorized into 'scaffold-based' and 'scaffold-free' culture systems. Next, based on the criteria for organoid formation, the degree of *de novo* organogenesis in different organoid studies has been further discussed and compared (**Section 1.3**). Finally, the objectives and hypotheses of my studies have been listed (**Section 1.4**).

Based on these objectives and hypotheses, I have organized my studies into two data chapters. Each data chapter includes the corresponding objective and hypotheses as well as separate sections for the introduction, materials and methods, results, discussion, and conclusions.

In **Chapter 2**, a porcine testis organoid culture system was established and the effects of different culture conditions on *in vitro* tubulogenesis were assessed. In experiment 1, fresh or cryopreserved testis cells from 1-week-old piglets were seeded in spheroid-forming low attachment culture wells for cell aggregation. The resultant cell spheroids were then cultured using an air-liquid interface system to allow the formation of testis organoids. Samples were collected weekly for 4 weeks for histological analysis. In experiments 2 & 3, to test the effects of various cell densities, supplements, and culture durations on *in vitro* tubulogenesis of organoids, testis tubular structures in different groups were quantified over time. In experiment 4, to test the effects of various supplements and culture durations on germ cell viability in organoids, the relative number of germ cells in groups were quantified over time. In experiment 5, to test the effect of increasing the germ cell ratio on organoid formation, population of testis cells were enriched to contain 60% germ cells and the results were compared with the original germ cell content of ~40%.

In **Chapter 3**, testis cell types, structural components, and endocrine characteristics of the organoids were assessed. In experiment 1, to explore the structural similarity of testis organoids to native testis tissue, testis cell types and structural components in organoids were identified by immunohistochemistry (IHC) and tissue-specific staining. Ultrastructures of testis organoids were further examined by transmission electron microscopy (TEM). In experiment

2, to study the testosterone secretion and luteinizing hormone (LH) responsiveness of testis organoids, testosterone levels were measured over time in culture medium samples from organoids supplemented with or without LH.

In **Chapter 4**, a general discussion is provided, and the potential future directions of testis organoid research are listed and discussed.

LIST OF FIGURES

LIST OF ABBREVIATIONS

NOA	Non-Obstructive Azoospermia
SSC	Spermatogonial Stem Cell(s)
GDNF	Glial cell-Derived Neurotrophic Factor
ECM	Extracellular Matrix (or Matrices)
RS	Round Spermatid(s)
ES	Elongated Spermatid(s)
PTMC	Peritubular Myoid Cell(s)
iPSC	induced-pluripotent stem cell(s)
dpp	days postpartum
DMEM	Dulbecco's Modified Eagle Medium
H&E	Hematoxylin and Eosin
IVF	*In Vitro* Fertilization
AI	Artificial Insemination
KSR	Knockout Serum Replacement
FBS	Fetal Bovine Serum
wk	Week(s)
LH	Luteinizing Hormone
PGC	Primordial Germ Cell(s)
IHC	Immunohistochemistry
PGP9.5	Protein Gene Product 9.5
α-SMA	α-Smooth Muscle Actin
vWF	von Willebrand Factor
CSF1	Colony-Stimulating Factor 1

TEM	Transmission Electron Microscopy
MT	Masson’s Trichrome
PASM	Periodic Schiff-Methenamine
BPA	Bisphenol A

CHAPTER 1

GENERAL INTRODUCTION AND LITERATURE REVIEW[1,2]

1.1 Background and motivation: Why do we need a testis organoid?

Global statistics indicate that approximately 8 to 15% of couples are infertile, where more than half of these cases are caused by male-related factors such as spermatogenic failure (Dohle, et al. 2005, Inhorn and Patrizio 2015, Thoma, et al. 2013). Infertility rates have reached as high as 30% in some populations (Nachtigall 2006, Ombelet, et al. 2008), particularly within areas with high infertility prevalence, such as Central Asia, South Asia, North Africa, sub-Saharan Africa, the Middle East, and Central and Eastern Europe (Mascarenhas, et al. 2012). Currently, many factors are believed to cause or contribute to male infertility. For example, environmental toxicants like cadmium, bisphenol A (BPA), and fungicides have been reported to cause male infertility (Cheng 2014, Cheng, et al. 2011, Sharpe 2010, Siu, et al. 2009, Wong and Cheng 2011). Cancer therapies, such as radiotherapy and chemotherapy, are also believed to be causative, as they introduce numerous gene mutations that negatively impact male germline and testis somatic cells (Ash 1980, Chatterjee, et al. 1994, Ji, et al. 2018, Martinez, et al. 2017, Meistrich, et al. 1992, Rakıcı, et al. 2019, Rivkees and Crawford 1988). Other suggested causes for male infertility include hormonal disorders and genetic defects (Esteves 2015).

Spermatogenic failure, also known as non-obstructive azoospermia (NOA) determined by the complete absence of spermatozoa in the ejaculate, has been recognized as the most severe presentation of male infertility (Dohle, et al. 2005, Esteves 2018, Esteves 2015). Given the legal and ethical implications in human research, it has thus far been impossible to study spermatogenic failure *in situ*. However, the establishment of an artificial testis organoid would permit a much more detailed investigation into the human testis, including stem-cell to somatic-cell interactions, the spermatogonial stem cell (SSC) niche, mechanisms of diseases, and experiments with personalized therapeutic strategies. It was previously discovered that *in vivo* implantation of neonatal testis cells from rats (Gassei, et al. 2006) and pigs (Honaramooz, et al. 2007) under the

[1] Several parts of this literature review have been published as a review paper. Tat-Chuan Cham, Xiongbiao Chen, and Ali Honaramooz (2021). "Current Progress, Challenges, and Future Prospects of Testis Organoids". *Biology of Reproduction* (*in press*). The document has been reformatted from the original version for inclusion in the thesis.

[2] T.C. contributed to conceiving the concept, wrote the first draft of the manuscript, summarized tables, and prepared the figures.

back skin of the immunocompromised mice results in *de novo* testis tissue formation and, in some cases, complete spermatogenesis (Honaramooz, et al. 2007). Although the *de novo* testis tissue reconstructed from the *in vivo* implantation can be further utilized in different studies, unknown factors provided by the host recipient may compromise the outcome of certain experiments that require a more controlled culture environment. In such cases, an *in vitro* testis organoid is preferred, as this provides a more stable and controlled culture environment.

Accordingly, the establishment of an *in vitro* testis organoid culture system from dissociated testis cells has gained increasing attention (Alves-Lopes, et al. 2017, Richer, et al. 2019). Testis organoid has since been considered as a valuable tool in pharmaco-toxicology testing (Jin, et al. 2013) as well as research on viral infection and testicular cancer. Testis organoids were also proposed as a viable alternative model to study the pathogenesis of the Zika virus (Strange, et al. 2018). Testis organoids generated from carcinogenic or transgenic testis cells can also provide an essential tool with which to study the transformed signaling pathways, genetic modification of carcinogenic cells, and the efficiency of cancer therapy, all of which were previously difficult to study. Knocking-out certain genes such as the glial cell line-derived neurotrophic factor (*Gdnf*) is lethal to neonates, as such testis organoids can be utilized in studying the effects of knocking-out a specific gene *in vitro* without sacrificing the experimental subject. Most importantly, testis organoids have tremendous potential to preserve male fertility and even to restore male infertility. This technology could be critical to conservation efforts considering that male infertility and/or death of prepubertal male animals results in the loss of genetic potential in rare and endangered species. It also has important implications for human medicine and health; although approximately 80% of childhood cancers can be cured by chemotherapy or radiation therapy, 30% of patients receiving gonadotoxic cancer treatments are rendered permanently infertile (Council 2003, Thomson, et al. 2002). Based on epidemiological studies, 1 in 1,300 prepubertal male is a childhood cancer survivor in USA (Council 2003); thus, approximately 1 in 5,000 prepubertal males is at the risk of infertility due to the gonadotoxic treatments (Ginsberg 2011). Semen cryopreservation is available for adults before receiving gonadotoxic cancer treatments to preserve the sperm for future artificial insemination (AI) or *in vitro* fertilization (IVF); however, prepubertal boys do not have this option due to the absence of developed spermatozoa (Ibtisham and Honaramooz 2020). Alternatively, testis biopsies obtained from the prepubertal boys before gonadotoxic cancer treatments can be cryopreserved for potential future use such as testis cell isolation (Picton, et al. 2015, Wu, et al. 2012). Considering

this, utilization of the fresh/cryopreserved testis cells in reconstructing a testis organoid culture system that produces lab-generated sperm is an attractive approach to preserving and/or restoring male fertility.

Since the late 20th century, a great variety of scaffold-based and scaffold-free testis cell/organoid culture systems have been established to recapitulate *de novo* testis organogenesis (Alves-Lopes and Stukenborg 2018, Richer, et al. 2019). Despite tremendous efforts, only a few studies successfully reconstructed a compartmentalized testis organoid that is structurally similar to native testis tissue. Also, since not all testis organoid studies performed a complete analysis on testis cell types and structural components, the interpretation of these findings remains unclear. The literature review of various testis organoid culture systems will be further discussed in Section 1.3. Since the goal of a testis organoid culture system is to recapitulate testis organogenesis *in vitro*, understanding testis development and spermatogenesis is crucial in evaluating the organogenic potential of a testis organoid culture system. This will be explained in the next Section.

1.2 Testis development and spermatogenesis

Testis is a structurally complex organ that plays a vital role in spermatogenesis and hormone production in males. Testis organogenesis involves the morphologic and functional development of different testicular spaces, including the tubular and interstitial compartments (Chemes 2001, Rey, et al. 2009, Stukenborg, et al. 2010). The tubular compartment is comprised of immature testis cords in prepubertal males and mature seminiferous tubules in adult males which support the development of germ cells. In either case, the cords/tubules are primarily constructed by two somatic cell types, Sertoli cells and peritubular myoid cells (PTMCs), in addition to male germ cells (Nieschlag 2013). Both Sertoli cells and PTMCs release components of the extracellular matrix (ECM) which itself contributes to the formation of a basement membrane to separate the tubular and interstitial compartments (Tung and Fritz 1987, Tung, et al. 1984). On the other hand, the interstitial compartment is comprised of blood vessels, connective tissue, and interstitial Leydig cells which are androgen producing cells important for steroid synthesis. The native architectural structures and cell orientations in the prepubertal and adult testis are schematically illustrated in **Fig. 1.1A**.

Successful recapitulation of testis organogenesis *in vitro* requires not only the formation of testicular structures but also the production of functional haploid germ cells. Unlike the production

of female germ cells, spermatogenesis is a continuous process of gamete production which takes place throughout the reproductive lifetime/breeding seasons of male animals (Clermont 1972). In humans, the first population of germ cells is primordial germ cells (PGCs), which appear early in embryogenesis in the yolk sac endoderm (Clark 2007). Before birth, PGC mitotically divide to form gonocytes or prospermatogonia, which have large nuclei and prominent nucleoli (Holstein, et al. 1987). In prenatal rodents, gonocytes enter mitotic arrest and reside at the center of the immature testis cords. After birth, gonocytes migrate to the periphery of the testis cords and transform morphologically to form spermatogonial stem cells (SSCs) (Hilscher and Engemann 1992). Testis cords then elongate and develop into lumen-containing seminiferous tubules (Ungewitter and Yao 2013). In rodents, SSCs are suggested to be the earliest undifferentiated spermatogonia (SG), referred to as type A_{single} (A_s) spermatogonia, which reside on the basement membrane of the seminiferous tubule. Type A_s spermatogonium can divide into either two separated A_s spermatogonia (*i.e.* for self-renewal) or a pair of type A_{paired} (A_p) spermatogonia interconnected with an intercellular bridge for further differentiation. Type A_p spermatogonia can then divide mitotically into type $A_{aligned}$ (A_{al}) spermatogonia comprising 4, 8, or 16 interconnected cells which then differentiate into differentiating spermatogonia (type A_1, A_2, A_3, A_4, intermediate, and B spermatogonia) for entering into meiosis. In primates, spermatogonia are also classified as type A and B spermatogonia. Notably, type A spermatogonia in primates are morphologically subcategorized as A_{dark} and A_{pale} spermatogonia, which have similar functions as type A_s, A_p, and A_{al} spermatogonia in rodents. Primate type B spermatogonia are also equivalent to the differentiating spermatogonia in rodents. During meiotic division, type B spermatogonia first differentiate into primary spermatocytes and go through 5 stages including preleptotene, leptotene, zygotene, pachytene, and diplotene to complete meiosis I; each primary spermatocyte then divides into two secondary spermatocytes. Meiosis II follows rapidly after the transition of primary to secondary spermatocytes, and each secondary spermatocyte divides into two haploid round spermatids (RS). The RS then undergo spermiogenesis to form elongated spermatids (ES), which mature before being released as sperm into the lumen of seminiferous tubules (Griswold 2016, Kubota and Brinster 2018). A schematic representation of spermatogenesis is illustrated in **Fig. 1.1B**.

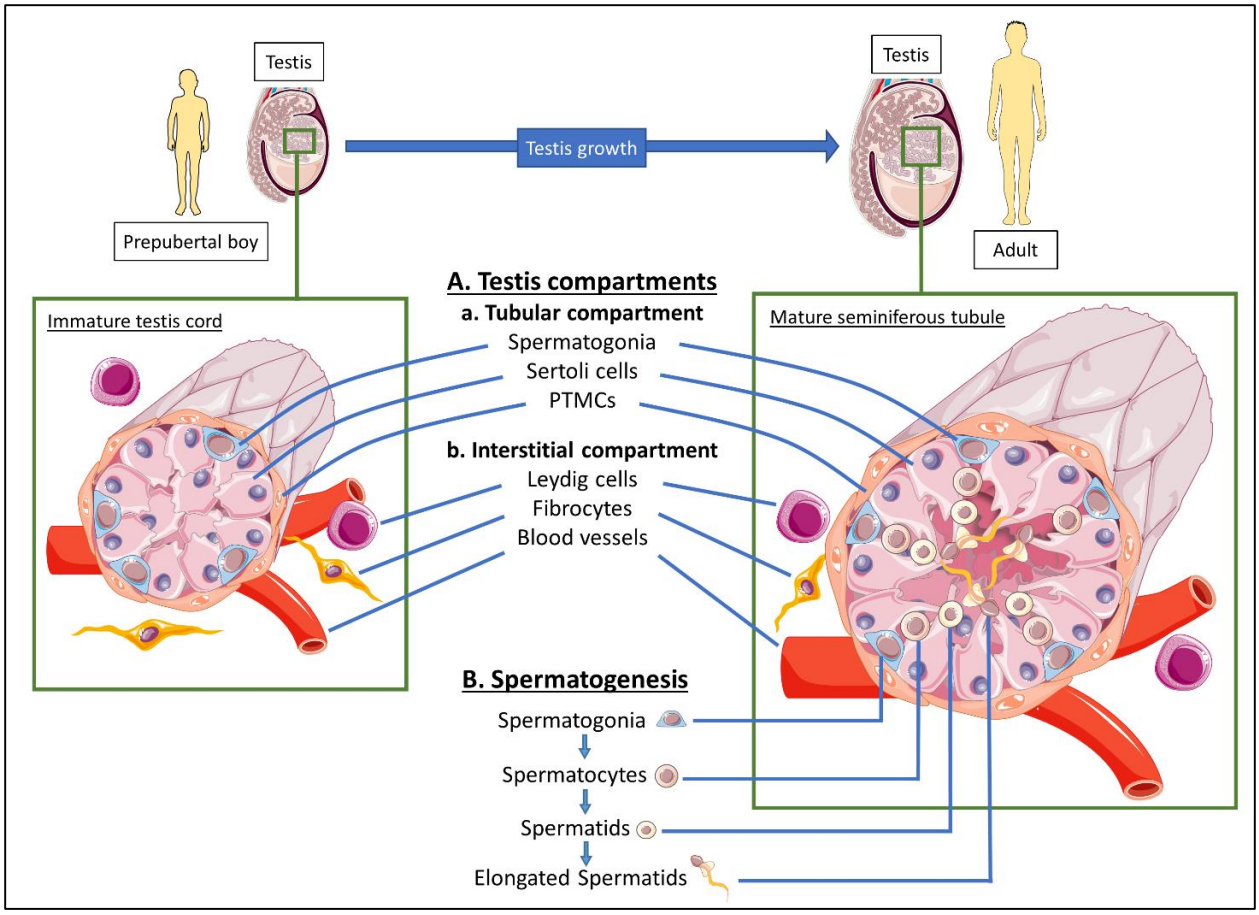

Fig. 1.1 *A schematic representation of the native testis structures and cell orientations in the testis tissues of prepubertal boys and adult males. As the testis develops from an immature to a sexually mature state, the immature testis cords develop into the lumen-containing seminiferous tubules and undergo spermatogenesis to produce functional sperm.* ***(A)*** *Testis tissue consists of* ***(a)*** *tubular, and* ***(b)*** *interstitial compartments.* ***(a)*** *The tubular compartment, including testis cords and seminiferous tubules, is constructed by testis somatic cells such as Sertoli cells and peritubular myoid cells (PTMCs) and incorporates male germ cells (e.g. spermatogonia).* ***(b)*** *The interstitial compartment is comprised of Leydig cells, blood vessels, and connective tissues (fibroblasts, fibrocytes, and collagen fibers).* ***(B)*** *Spermatogenesis starts with the division and differentiation of spermatogonia (spermatogonial stem cells or SSCs) into more advanced germ cells including spermatocytes (primary and secondary spermatocytes), round spermatids which further differentiate into elongated spermatids. Mature sperm are then released into the central lumen of the seminiferous tubule to be carried out of the testis. (Cham, et al. 2021)*

Although the formation of a testis organoid with compartmentalized architectures and completion of the spermatogenic cycle has not yet been achieved, current testis cell/organoid culture systems have shown varying degrees of *de novo* testis organogenesis and spermatogenesis. To summarize the published work to date and better compare the various approaches and outcomes of *de novo* testis organogenesis, we will use a system based on three criteria which have recently been proposed (Edmonds and Woodruff 2020). These criteria include (a) testis cell reassembly, (b) the compartmentalized architectures, and (c) the inclusion of major testis cell types (Sertoli, Leydig, germ, and PTMCs). More specifically, the first step in *de novo* organogenesis is the reassembly of testis cells into newly formed testis structures such as cell aggregates, testis cord-like structures without lumen, or compartmentalized testis tubular structures with lumen. The tubulogenic ability of testis cells is defined as the capacity of the cells to self-assemble into testis cord-like/tubular structures. These *de novo* formed testis structures can contain different testis cell types and cellular orientations, which determine their architectural relevance to native testis tissue. Together, the degree of *de novo* testis organogenesis is considered to be higher if more criteria are achieved by a given testis organoid culture system.

1.3 Current progress in testis organoid culture systems

To date, numerous testis cell culture systems have been established to recapitulate the testis microenvironment and restore *in vitro* spermatogenesis; these are generally classified as either 2D or 3D culture systems (Alves-Lopes and Stukenborg 2018, Komeya, et al. 2018, Richer, et al. 2019). In the scaffold-free 2D culture environment, testis cells initially form a 2D cell monolayer which later proliferate into numerous multilayered cell plaques and cord-like structures over time (Gassei, et al. 2006, Iwanami, et al. 2006, Mäkelä, et al. 2014, Mincheva, et al. 2018, Schlatt, et al. 1996, Sousa, et al. 2002, Tres and Kierszenbaum 1983, Tung and Fritz 1980, 1986, 1987, Tung, et al. 1984, Vigier, et al. 2004, von Kopylow, et al. 2018, Xie, et al. 2010). Although these cord-like structures are morphologically comparable to *in situ* testis cords, their cellular orientation has been reported as disorganized in rodent (Mäkelä, et al. 2014) and human models (von Kopylow, et al. 2018). This lack of physiological architecture and cell topology makes scaffold-free 2D culture systems relatively inefficient in terms of both germ cell maintenance and *in vitro* spermatogenesis (Komeya, et al. 2018, von Kopylow, et al. 2018). Within the body, cells develop in a closely packed 3D multicellular matrix which allows intimate cell-to-cell and cell-to-

extracellular matrix (ECM) communications that are crucial for tissue development. Many research groups have been exploring various scaffold-based and scaffold-free culture methods to recreate this 3D microenvironment and facilitate such interactions. The resultant scaffold-based and scaffold-free testis cell/organoid culture systems from such research are summarized in **Fig. 1.2**. The information in this figure is organized based on the architectural relevance of the *de novo* testis structures to the structure of their respective donor testis (criteria for *de novo* testis organogenesis), and the developmental stages of *in vitro* spermatogenesis. These culture systems are categorized into 'scaffold-based' and 'scaffold-free' systems in the following sections:

1.3.1 Scaffold-based culture systems

Native ECM in the body consists of proteins, polysaccharides, and proteoglycans. Besides cell-to-cell contacts, the 3D ECM architecture also facilitates cell adhesion, cell migration, and the spatial and temporal regulation of growth factors. For instance, laminin was reported to facilitate the reconstruction of testis cord-like structure *in vitro* (Hadley, et al. 1985), but both laminin and collagen are suggested to be necessary for proper regulation of germ cell migration from basal lamina to the seminiferous tubule lumen (Cheng, et al. 2010). Thus, bioengineered scaffolds can be used as a temporary framework within *in vitro* cell culture to simulate the native ECM (Chan and Leong 2008). Hydrogels are typically used as 3D culture scaffolds due to their biocompatibility, cytocompatibility, and good porosity for the diffusion of nutrients, metabolites, and oxygen. Hydrogels are classified as natural or synthetic (Lee and Mooney 2001). Natural hydrogels contain biological ingredients, such as collagen, alginate, agarose, and ECM, which are biocompatible, biodegradable, and have low cytotoxicity (Catoira, et al. 2019, Gyles, et al. 2017). However, there are certain practical limitations in using natural hydrogels, especially since they often have poor mechanical properties and tend to degrade rapidly. Alginate and agarose also lack binding proteins and therefore offer poor cell interactions. Matrigel is made from complex and undefined biological components which limit its clinical application (Hughes, et al. 2010). Synthetic hydrogels (*e.g.* polyethylene), on the other hand, are artificially constructed polymers that possess well-defined components and better mechanical properties. However, they lack cell-adhesion molecules and are thus not ideal for cell encapsulation. Based on hydrogel types (natural

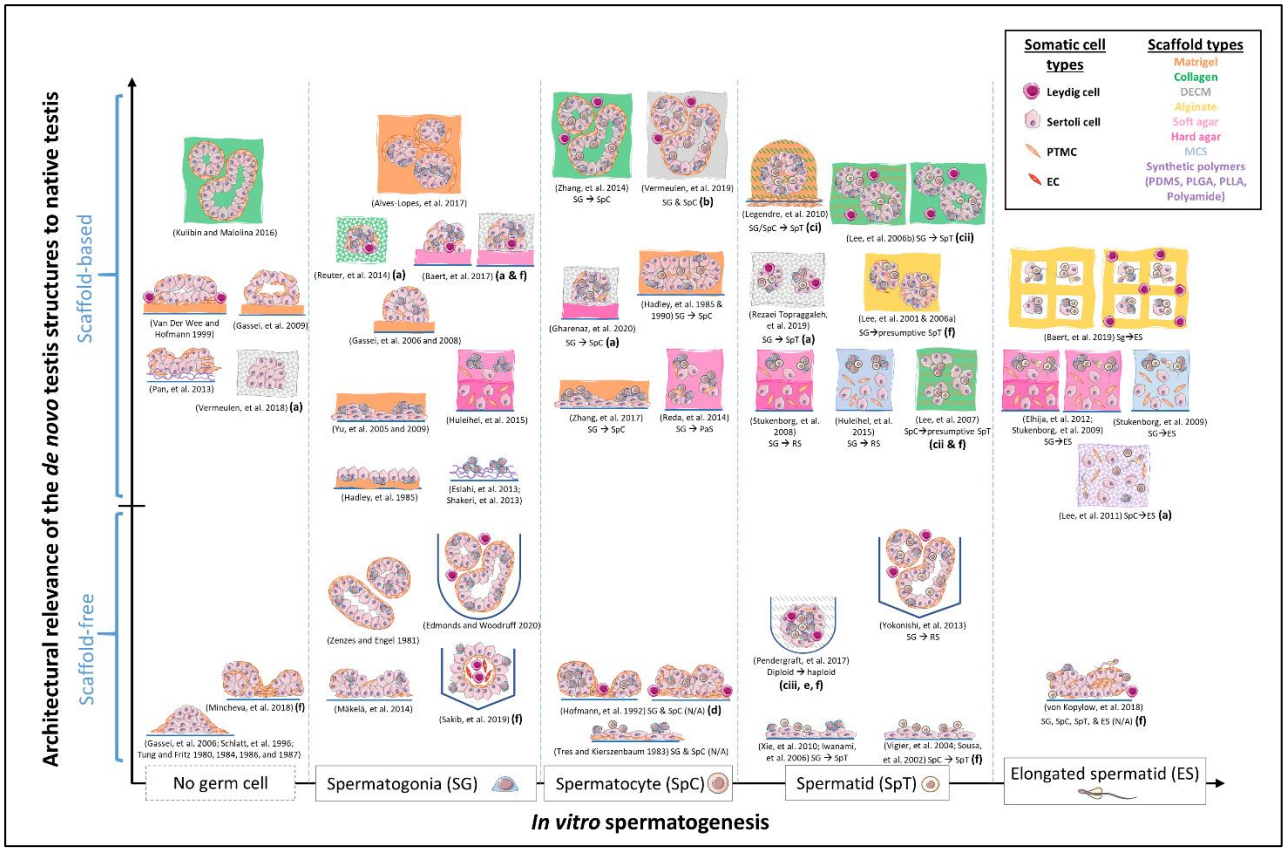

Fig. 1.2 *Schematic summary of scaffold-free and scaffold-based testis cell culture systems illustrating the various cell types, cell orientations, de novo testis structures, and the corresponding scaffolds and/or supplements used. In scaffold-free culture systems, testis cells form a 2D cell monolayer on the culture dish which can further proliferate to form multi-layered cell plagues or cord-like structures over time. The utilization of a rotation culture, a hanging drop culture, or a spheroid-forming low attachment culture well will facilitate in vitro reconstruction of biomimetic testis structures. In scaffold-based culture systems, hydrogel scaffolds act as a temporary frame for the testis cells to form de novo testis structures and promote spermatogenesis. Some studies had spermatocytes (SpCs) instead of spermatogonia (SG) as their cell sources; in these cases, the degree of spermatogenic differentiation is specified beneath the corresponding reference. Different experimental techniques or outcomes are denoted as follows:* ***(a)*** *Use of porous scaffolds seeded with cells.* ***(b)*** *The presence of SCP3 positive cells (marker of meiotic cells) within the testis organoid, with the presence of spermatocytes assumed.* ***(ci-iii)*** *Supplementation with collagen* ***(ci)****, Matrigel* ***(cii)****, or ECM* ***(ciii)*** *molecules. (****d****)* *Use of testis cell lines, including spermatogonia/spermatocyte-like cell lines, PTMC lines, Leydig cell lines, and*

Sertoli cell lines. ***(e)*** *Use of testis cell lines, including Leydig and Sertoli cell lines.* ***(f)*** *Use of human cells. Soft agar: 0.35-0.37% agarose; Hard agar: 0.5-1.5% agarose; PTMC, peritubular myoid cell; EC, endothelial cell; MCS, methylcellulose culture system; N/A, not applicable, spermatogenic cells were present in these studies, but the evaluation of the spermatogenic differentiation was not performed. (Cham, et al. 2021)*

vs. synthetic), different scaffold-based testis cell/organoid culture systems are discussed in the following sections:

1.3.1.1 Natural hydrogel scaffolds

The earliest use of natural hydrogel scaffolds within a testis cell culture was attempted by either overlaying a Matrigel scaffold above the cells (Yu, et al. 2005, Zhang, et al. 2017) or by seeding the cells onto a layer of scaffold that was coated onto the culture dish (Gassei, et al. 2008, Gassei, et al. 2009, Gassei, et al. 2006, Hadley, et al. 1985, Van Der Wee and Hofmann 1999). These studies have offered evidence that natural hydrogel scaffolds can allow cells to grow in both horizontal and vertical directions, guiding the reorganization of 3D cell aggregates or testis cord-like/tubular structures. Since these early attempts, many studies have used scaffolds in their 3D testis cell cultures by embedding dissociated testis cells in various natural hydrogels. For examples, cell-laden hydrogel scaffolds can be fabricated by encapsulating target cells in a designated hydrogel. This is a common and efficient method used for 3D cell culture. The encapsulation of testis cells in natural hydrogels has been established using various components such as alginate (Lee, et al. 2001, Lee, et al. 2006a), agarose (Elhija, et al. 2012, Huleihel, et al. 2015, Reda, et al. 2014, Stukenborg, et al. 2008), collagen (Kulibin and Malolina 2016, Lee, et al. 2007, Lee, et al. 2006b, Zhang, et al. 2014), Matrigel (Alves-Lopes, et al. 2017, Hadley, et al. 1985, Legendre, et al. 2010), decellularized extracellular matrix (DECM) (Vermeulen, et al. 2019), and methylcellulose (MCS) (Huleihel, et al. 2015, Stukenborg, et al. 2009). These studies have shown that different hydrogel scaffolds promote varying degrees of *de novo* testis organogenesis and *in vitro* spermatogenesis from the encapsulated testis cells (**Fig. 1.2**).

Among these studies, several natural hydrogel-based testis organoid studies showed high degrees of *de novo* testis organogenesis. First, Zhang *et al.* (2014) re-aggregated the testis cells from 6-day-old mice into a cell spheroid by rotation culture and encapsulated the cell spheroids in collagen gel. Interestingly, reconstruction of the compartmentalized seminiferous cord-like structures and spermatogenic differentiation of spermatogonia up to SYCP3-positive primary spermatocytes were observed in these collagen scaffolds (Zhang, et al. 2014). Moreover, the DECM hydrogels extracted from decellularized porcine testis tissue were used to encapsulate dissociated testis cells from 4 to 7-day-old piglets to fabricate a DECM-based porcine testis organoid (Vermeulen, et al. 2019). In this case, immunohistochemistry confirmed that their testis organoids consisted of

compartmentalized seminiferous tubule-like structures constructed by Sertoli cells and integrated germ cells, while being surrounded by PTMCs and Leydig cells. The DECM-based testis organoids had a higher number of viable Leydig cells than the collagen-based testis organoids. They were also reported to secrete testosterone and stem cell factor (SCF) and contain synaptonemal complex protein 3 (SCP3)-positive cells (a marker of meiotic cells) which decreased progressively over the time of culture (Vermeulen, et al. 2019). In 2017, Alves-Lopes *et al.* developed a 3D multilayered Matrigel scaffold that facilitated the reorganization of testis cells into spherical-tubular structures (Alves-Lopes, et al. 2017). The spherical-tubular structures were made of Sertoli cells that had become integrated with spermatogonia and were surrounded by PTMCs, in which the latter supports germ cell proliferation and blood-testis barrier (BTB) formation. Although germ cell differentiation was not examined in this 3D multilayered Matrigel scaffold, it provided proof-of-principle that Matrigel scaffolds can facilitate the *in vitro* reconstruction of testis tissue-like structures, including those containing viable germ cells.

1.3.1.2 Synthetic hydrogel scaffolds

In comparison to natural hydrogels, synthetic hydrogels (*e.g.* polyol, polyester, and polyether) have superior mechanical properties, printability, and consistent qualities. However, they tend to be not biocompatible or biodegradable which can result in poor cell attachment and growth. As a result, testis cells are cultured on the surface of synthetic hydrogel scaffolds rather than being encapsulated within the hydrogel scaffolds. Certain synthetic hydrogels such as poly (L-lactic acid) (PLLA) (Eslahi, et al. 2013) and polyamide nano-filaments (Ultra-Web) (Shakeri, et al. 2013) have been considered for building a nanofibrillar scaffold with which to culture SSCs. These synthetic hydrogel-derived nanofibrillar scaffolds are topologically akin to the native ECM microenvironment, and thus promote SSC proliferation, colony formation, and *in vitro* spermatogenic differentiation (Eslahi, et al. 2013, Shakeri, et al. 2013). Moreover, the reconstruction of testis cord-like structures was reported after somatic testis cells from the 7-day-old rats were cultured on a layer of synthetic poly(dimethylsiloxane) (PDMS) substrate (Pan, et al. 2013). This finding suggests that nanoscale scaffolds exert a strong effect on cell migration and cell-to-cell interactions, as well as cell reorganization of the somatic testis cells into the testis cord-like structures *in vitro*. Unfortunately, the *in vitro* spermatogenic differentiation and *de novo* testis

organogenesis in synthetic hydrogel-based culture systems are not as promising as in natural hydrogel-based cultures due to the poor cytocompatibility of synthetic hydrogels.

Generally, hydrogel scaffolds provide a biological 3D microenvironment for cell attachment, cell growth, cell differentiation, nutrient diffusion, and *de novo* tissue formation. They also provide mechanical support and add tension to the cells which are crucial for cellular functionality (Dado and Levenberg 2009). However, several limitations in scaffold-based culture systems need to be addressed. First, the seeded cells tend to be unevenly distributed and loosely packed within the scaffold, leading to poor cell-to-cell contacts and inconsistent tissue regeneration and organoid formation (Scarritt, et al. 2015). In addition, cell damage may occur during cell encapsulation and/or 3D printing due to mechanical stress (Ning, et al. 2018), compressive forces (Li, et al. 2010, Parkkinen, et al. 1995), or crosslinking-induced cytotoxicity (Fedorovich, et al. 2009, Nguyen and West 2002). To address these issues, several scaffold-free culture systems have been established.

1.3.2. Scaffold-free culture systems

Perhaps the most straightforward and efficient way to make a scaffold-free 3D multicellular culture environment has been to aggregate the cells into a 3D cell spheroid. Zenzes and Engel (1981) first utilized rotation culture by aggregating the dissociated testis cells from neonatal (8 to 10-day-old) or adolescent (18 to 25-day-old) rats into 3D cell spheroids (Zenzes and Engel 1981). Their 3D cell spheroids were able to regenerate testis tubular structures with an organized cell orientation, where Sertoli cells lined the tubule and PTMCs were found around the tubule. Several other scaffold-free culture methods have since been established in which various culture methods were used (*e.g.* rotation culture, hanging drop culture, spheroid-forming low attachment culture well) to successfully build a self-assembling testis organoid (**Fig. 1.2**).

1.3.2.1. Rotation culture

In the rotation culture system, dissociated cells are suspended in a rotating wall vessel that rotates constantly to create microgravity for the formation of cell spheroids (Carpenedo, et al. 2007). Besides the study by Zenzes and Engel (1991), Zhang *et al.* (2014) also re-aggregated the dissociated testicular cells from 6-day-old mice into cell spheroids by rotation culture and encapsulated the spheroids in collagen-based scaffolds. As mentioned in scaffold-based culture system, compartmentalized testis tubular structures with highly accurate cell orientation and the

spermatogenic differentiation of spermatogonia up to SYCP3-positive primary spermatocytes were also observed in these collagen scaffolds. Notably, other studies have shown that the encapsulation of testis cells in collagen gel alone (without forming cell spheroids) only facilitates the reconstruction of Sertoli cell-cysts or cell aggregates instead of testis tubular structures (Lee, et al. 2007, Lee, et al. 2006b). This indicates that both spheroid formation and the use of collagen-based scaffold are crucial in *de novo* testis tubulogenesis.

1.3.2.2. Hanging drop culture

In the hanging drop culture system, dissociated cells are suspended in a droplet of media that allows cells to aggregate into a cell spheroid using the gravitational force and the surface tension of the droplet (Achilli, et al. 2012, Timmins and Nielsen 2007). The size of cell aggregates can be controlled by changing the cell density or volume of the droplet. Pendergraft *et al.* (2017) utilized the hanging drop culture in their trials. In their methodology, *in vitro* propagated primary testis cells from 56 to 61-year-old men were aggregated into a compact cell spheroid which was then transferred to a U-bottom low attachment well for further culture (Pendergraft, et al. 2017). The reconstruction of testis tubular structure was not observed in this study, perhaps due to the age of the testis cell donors. Still, the production of testosterone and upregulation of post-meiotic genes (*PRM1* and *Acrosin*) were detected, indicating that these testis organoids possessed endocrine function and could support *in vitro* spermatogenic differentiation (Pendergraft, et al. 2017).

1.3.2.3. Spheroid-forming low attachment culture wells

The spheroid-forming low attachment culture wells allow cells to be freely suspended in the media instead of attaching to the wall of the well. With the gravitational force, dissociated cells are condensed at the center of the well, which maximizes cell-to-cell contacts and facilitates the formation of a 3D cell spheroid. Yokonishi *et al.* (2013) formed compact cell spheroids by culturing dissociated testis cells from 0.5–5.5 days postpartum (*dpp*) neonatal mice in V-bottom low attachment wells. These cell spheroids were then cultured on top of an agarose gel soaked in the media, which is reminiscent of the air-liquid interface culture method. Interestingly, the reconstruction of testis tubular structures with the incorporation of germ cells was observed, and the spermatogenic differentiation from SSCs to the round spermatid stage was detected by expression of meiotic markers (SYCP1 and *Acr*-GFP) and the presence of PAS-positive acrosomal cap (Yokonishi, et al. 2013). Conversely, Sakib *et al.* (2019) also generated testis organoids from

pig, mouse, macaque, and human donors using similar cell spheroids of a smaller size and a microwell centrifugal aggregation system (Sakib, et al. 2019b). In this study, the testis organoids featured tubular structures that were completely reversed compared with the native testis architecture. Yet, they provided proof of principle that testis cells of various mammalian species maintain the morphogenic capacity to autonomously reconstruct compartmentalized and polarized testis tissue-like structures *in vitro*. Recently, Edmonds and Woodruff (2020) compared the reconstruction of *de novo* testis tissue from immature murine testis cells in scaffold-free *vs.* Matrigel scaffold-based culture conditions using non-adherent microwells and Matrigel coated plates, respectively (Edmonds and Woodruff 2020). They found that both groups were able to assemble into multicellular testis organoids possessing distinct tubular and interstitial compartments that had physiologically relevant cell orientation and endocrine function. This study indicated that the reconstruction of the *de novo* murine testis organoids from immature testis cells can be achieved with or without the use of Matrigel scaffold.

It is worth mentioning that scaffold-free cell spheroid culture methods have several limitations. First, a high number of donor cells are required to form a sufficient number of highly compact cell spheroids. A specific germ cell-to-somatic cell ratio is also necessary for the reconstruction of *de novo* testis structures (Sakib, et al. 2019b) which requires additional tissue samples, as well as extra cost and time. In addition, the most critical issue with scaffold-free cultures is the cell necrosis that occurs within the core of large cell spheroids due to insufficient oxygen and nutrient perfusion (Hirschhaeuser, et al. 2010). Nevertheless, scaffold-free cell spheroid culture favors cell growth over scaffold-based cultures, and they can be easily performed without the requirement of scaffold fabrication. Therefore, the scaffold-free spheroid-forming low attachment wells combined with the air-liquid interface culture system have been chosen in this study to establish a neonatal porcine testis organoid culture system. Using this methodology, testis organoids can be generated from both fresh and cryopreserved testis cells. Next, the effects of cell density, supplementation types, culture duration, and germ cell ratio on *de novo* testis tubulogenesis of organoids were assessed. Furthermore, testis cell types, structural components, and endocrine characteristics of the reconstructed organoids were evaluated.

1.4 Objectives and Hypotheses

The overall hypothesis of this thesis was that testis organoid culture systems can be established using neonatal porcine testis cells in spheroid-forming low attachment wells combined with an air-liquid interface culture system. The general objectives were to establish, examine, and characterize the resultant porcine testis organoid systems. Experiments were designed to test the following specific objectives and hypotheses:

1.4.1 Establishment of a porcine testis organoid system and assessment of culture conditions for its efficient in vitro morphogenesis

1.4.1.1 Objective

- To establish a porcine testis organoid system and assess different culture conditions for its efficient *in vitro* morphogenesis.

1.4.1.2 Hypotheses

- Using neonatal piglet testis cells in a reported mouse testis organoid system will result in the formation of a porcine testis organoid system.
- Using frozen-thawed testis cells will result in the formation of testis organoids containing compartmentalized testis cord-like structures similar to organoids derived from fresh testis cells.
- Different culture conditions (by changing cell density, culture duration, media supplementation, and gonocyte ratios) will affect the efficiency of *in vitro* tubulogenesis in organoid formation.
- Different supplementations and culture durations will affect germ cell viability in the testis organoids.

1.4.2 Cell types, structural components, and endocrine characteristics of porcine testis organoids

1.4.2.1 Objective

- To examine the structural and endocrine characteristics of the porcine testis organoid system.

1.4.2.2 Hypotheses

- Porcine testis organoids will have various testis cell types and structural components that resemble intact porcine testis tissue *in situ*.

- Porcine testis organoids will secrete testosterone and this secretion will be induced by luteinizing hormone (LH).

CHAPTER 2

ESTABLISHMENT OF A PORCINE TESTIS ORGANOID SYSTEM AND ASSESSMENT OF CULTURE CONDITIONS FOR ITS EFFICIENT *IN VITRO* MORPHOGENESIS[3]

2.1. Abstract

The objective of this study was to examine the capability of fresh and frozen-thawed neonatal porcine testis cells to reconstruct a testis organoid. We cultured dissociated fresh or cryopreserved testis cells from 1-week-old piglets in ultra-low attachment U-bottom wells to allow the formation of cell spheroids. The cell spheroids were then cultured on top of agarose gels immersed in media in the form of an air-liquid interface culture. This led to formation of testis organoids with tubular structures that resembled those in the age-matched testis. We then examined the effect of a number of culture conditions on tubular reconstruction in the organoids which included changing the cell density, time of culture, and media supplementation (serum or its replacement). We observed that a testis cell density of 0.8×10^6 cells/organoid and a combined supplementation of 5% knock-out serum replacement (KSR) + 10% fetal bovine serum (FBS) improved *de novo* tubulogenesis in the organoids. The number of germ cells in the organoids did not significantly change as a result of media supplementation or time in culture. Increasing the ratio of germ cells (gonocytes - from 40% to 60%) in the initial population of cells also did not improve the results and led to multifocal necrosis and poor morphogenesis in the resultant organoids. Therefore, we established a novel and efficient culture system for the formation of porcine testis organoids.

2.2. Introduction

The term 'organoid' has been defined most accurately as "an *in vitro* 3D cellular cluster derived exclusively from primary tissues, embryonic stem cells, or induced pluripotent stem cells, capable of self-renewal and self-organization and exhibiting similar organ functionality as the tissue of origin" (Fatehullah, et al. 2016). Various organoid culture systems have been developed, including for intestine (Sato, et al. 2009), vasculature (Morgan, et al. 2013), liver (Takebe, et al. 2013), pancreas (Boj, et al. 2015), and brain (Quadrato, et al. 2017). However, testis organoid culture systems have only more recently become a topic of intense interest. The establishment of an *in*

[3] T.C. contributed to the conceiving and designing of the study, performed the experiments, and wrote the first draft of the thesis.

vitro testis organoid culture system provides a new platform for experimentations on testis function with widespread applications. This includes applications in basic science such as physiology, genetic manipulation, and pharmaco-toxicological testing, as well as in clinical applications such as potential fertility/genetic preservation.

Although a few different testis organoid culture systems have been established using rodent models (Alves-Lopes and Stukenborg 2018, Edmonds and Woodruff 2020), rodents are not the ideal animal models for human diseases and disorders due to their pathophysiological differences (Perlman 2016, Seok, et al. 2013). To date, only a few studies have utilized testis cells derived from adult men to build testis organoids (Baert, et al. 2017, Lee, et al. 2006a, Lee, et al. 2007). It is worth noting that the reconstruction of a compartmentalized testis tubular structure has not been reported in these latter studies. Many studies have also shown that the *de novo* tubulogenic ability of primary testis cells is age-dependent; primary testis cells derived from neonatal/prepubertal animals retain their tubulogenic ability, whereas pubertal/adult-derived primary testis display a delayed or absent tubulogenic potential (Alves-Lopes and Stukenborg 2018, Edmonds and Woodruff 2020). Although the use of neonatal/prepubertal testis cells may produce a more desirable result in establishing a human testis organoid, the scarcity of available donor tissue and the ethical concerns related to obtaining such tissues hinder their use in practice. Thus far, only a single study has demonstrated the ability of primary testis cells from 6-month-old and 5-year-old prepubertal children to reconstruct a compartmentalized testis organoid (Sakib, et al. 2019b). However, these testis organoids were observed to have a reversed architectural structure with its seminiferous tubules described as being 'inside-out'. This is possibly due to the low cell density (1,000 cells/organoid) and small size of organoids (100-150 μm in diameter) which might only support the reconstruction of small cell spheroids rather than testis tubular structures. More studies are required to investigate the potential *de novo* testis organogenesis of neonatal/immature primary testis cells from human or alternative animal models. Since pigs share physiological and anatomical similarities with humans, they are increasingly viewed as an alternative non-rodent model species for biomedical, pharmaceutical, and xenotransplantation studies (Ibtisham, et al. 2020, Ibtisham and Honaramooz 2020). Due to these reasons, neonatal porcine testis cells have been selected as the cell sources in this study for the establishment of testis organoid culture systems.

Although the use of porcine testis cells in formation of testis organoids has been reported, several limitations were reported in those studies. As indicated earlier, Sakib *et al.* (2019) generated testis organoids using prepubertal porcine testicular cells in a microwell centrifugal aggregation system (Sakib, et al. 2019b). However, these organoids display an inside-out and completely reversed architectural structures compared with testis tubular structures *in situ*. Also in 2019, Vermeulen, *et al.* created a porcine testis organoid system using DECM and collagen scaffolds which consisted of testis tubular structures constructed by Sertoli cells, along with the integration of germ cells, and surrounded by PTMCs and Leydig cells (Vermeulen, et al. 2019). However, a progressive decrease in the number of germ cells in cross-sections of testis tubular structures was observed up to a point of reaching zero germ cells on day 45. Also, the reconstructed tubular structures were mostly incomplete and did not resemble native testis tubular structures. Therefore, there is a race in the field to introduce a suitable testis organoid system that can replicate the *in situ* form and function of the testis using pig models.

A first research question in this study was what culture system should we examine for formation of porcine testis organoids? As mentioned in Section 1.3.2.3., the scaffold-free cell spheroid culture method has been proven effective in promoting the reconstruction of a compartmentalized testis organoid in rodent models (Edmonds and Woodruff 2020, Yokonishi, et al. 2013). We therefore attempted to use a similar culture methodology in establishing a pig testis organoid system. Next, we tested different culture conditions in order to find the optimal culture condition for efficient organoid formation and maintenance.

2.3. Materials and methods

2.3.1. Testis collection and preparation

Testes from 1-week-old Yorkshire-cross piglets (Camborough-22 × Line 65; PIC Canada, Winnipeg, MB, Canada) were collected regularly (30-40 testis pairs per wk) through aseptic castration at the Prairie Swine Center (a USask–affiliated facility). All experimental procedures involving animals were reviewed and approved by the University of Saskatchewan's Institutional Animal Care and Use Committee (Animal Use Protocol# 20080042). The testes were kept in Dulbecco's phosphate-buffered saline (DPBS; Cat. no. 20-031-CV; Mediatech, USA), containing 1% antibiotics solution (penicillin and streptomycin; Cat. No. 30-002-CI, Mediatech), and were transported on ice. In the laboratory, the testes were rinsed three times with DPBS prior to

processing. The tunica albuginea and excess connective tissues were removed to isolate testicular parenchyma for further tissue digestion and cell isolation.

2.3.2. Testis cell isolation

Testis cell isolation was performed using methods previously established in our laboratory (three-step enzymatic digestion) (Awang-Junaidi and Honaramooz 2018, Yang and Honaramooz 2011). Briefly, testis parenchyma was thoroughly minced with fine scissors for 5 min, suspended in 5 mL of DPBS, vortexed for 30 sec in a test tube shaker (Reax Top; Cat. No. 541-10000; Heidolph Instrument, UK) and digested with 1 mL of 0.2% collagenase IV (Cat. No. C-153; SigmaAldrich, ON, Canada), 0.1% hyaluronidase (Cat. No. H-3884; Sigma-Aldrich), and 0.01% DNase (Cat. No. DN25; Sigma-Aldrich) in Dulbecco's Modified Eagle Medium (DMEM; Cat. No. 10-013-CM; Mediatech) supplemented with 1% w/v antibiotics at 37 °C for 15 min. FBS (Cat. No. A15-701; PAA Laboratories, ON, Canada) was added to stop the digestion. To remove cell clumps and undigested tissue segments, the suspension was vortexed for another 30 sec and filtered through a 40 µm filter (Cat. No. 3522340; BD Biosciences, CA, USA). The filtrate cell suspension was then centrifuged at 500×*g* at 16 °C for 5 min to form a cell pellet and the supernatant was removed. The cell pellet was then re-suspended with 20 mL of the lysis buffer and placed at room temperature (RT) for 30 min for erythrolysis. The lysis buffer contains 156 mmol/L ammonium chloride (NH_4Cl; Cat. No. A9434; Sigma-Aldrich), 10 mmol/L potassium bicarbonate ($KHCO_3$; Cat. No. 237205; Sigma-Aldrich), and 0.1 mmol/L disodium ethylenediaminetetraacetate (Na_2EDTA; Cat. No. E6635; Sigma-Aldrich) in sterile distilled water. The cell-lysis buffer mixture was then centrifuged at 500×*g* at 16 °C for 5 min and the supernatant was removed. Lastly, the cell pellet was washed with DPBS and re-suspended in 5 mL of DMEM supplemented with 10% KSR. The cell number and viability of the resultant cell suspension was measured using the trypan blue exclusion assay.

2.3.3. Testis cell cryopreservation

The fresh testis cells were either used directly for organoid formation or cryopreserved using our protocols with minor modifications (Honaramooz, et al. 2002). Briefly, the cryoprotectant was first made with FBS, DMEM, and dimethyl sulfoxide (DMSO) at a 1:3:1 ratio, and stored at 4 °C. The cell suspension was then mixed with ice-cold cryoprotectant at 1: 1 ratio to make a cell-DMSO mixture. For example, 1 mL of FBS, 3 mL of DMEM, and 1 mL of DMSO were mixed with 5 mL

of cell suspension to make a 10 mL cell-DMSO mixture. Next, cell-DMSO mixture was quickly dispensed in cryovials (1 mL/vial) and the cryovials were placed in a freezing container and stored at -20 °C for 30 min. The freezing container was then stored in -80 °C freezer overnight and transferred to liquid nitrogen storage.

2.3.4. Germ cell enrichment

To increase the germ cell ratio among isolated testis cells, a germ cell enrichment procedure was performed using a Nycodenz centrifugation protocol which has been developed in our laboratory (Yang and Honaramooz 2011). Briefly, 3 mL of 17% Nycodenz in DPBS (Histodenz; Cat. No. D2158; Sigma-Aldrich) was first added and settled at the bottom of a 15 mL-graduated conical tube. 2 mL of testis cell suspension at a concentration of ~2×10^7cells /mL was then added gently on top of the Nycodenz solution, and the mixture was centrifuged at 500×*g* at 4 °C for 15 min. The supernatant was discarded, and the cell pellet was resuspended in DMEM culture media to form ~80% germ cell-enriched testis cell suspension. The ~80% germ cell-enriched testis cells were then mixed with the same volume of freshly isolated unenriched testis cells (containing 40% gonocytes) to obtain a germ cell population of ~60% for testis organoid formation.

2.3.5. Testis organoid culture system

Fresh or cryopreserved testis cells (1.0×10^6cells/well) were first cultured in U-bottom 96-well plates for 24 hr to form cell spheroids. The cell spheroids were then cultured on small pieces (1×1×0.5 cm) of 1.5% agarose gel bedding immersed in DMEM supplemented with 10% KSR (**Fig. 2.1**). This culture method is referred to as an air-liquid interface culture system, which allows the cell spheroids to receive sufficient gaseous exchange from the air above and nutrients from the media below. The cell spheroids were further cultured for 4 wk in the same air-liquid interface system to form the testis organoids and the media were changed every other day. The size of testis organoids was examined under light microscopy and samples collected weekly for histological analysis.

Next, different testis cell densities (1.0×10^6, 0.8×10^6, or 0.6×10^6/well) were tested to determine the optimal cell density for tubulogenesis in organoid formation. Furthermore, different media supplementations including 10% KSR, 10% FBS, 10% KSR+5% FBS, or 5% KSR+10% FBS were tested to determine the optimal supplement for efficient tubulogenesis in the organoids.

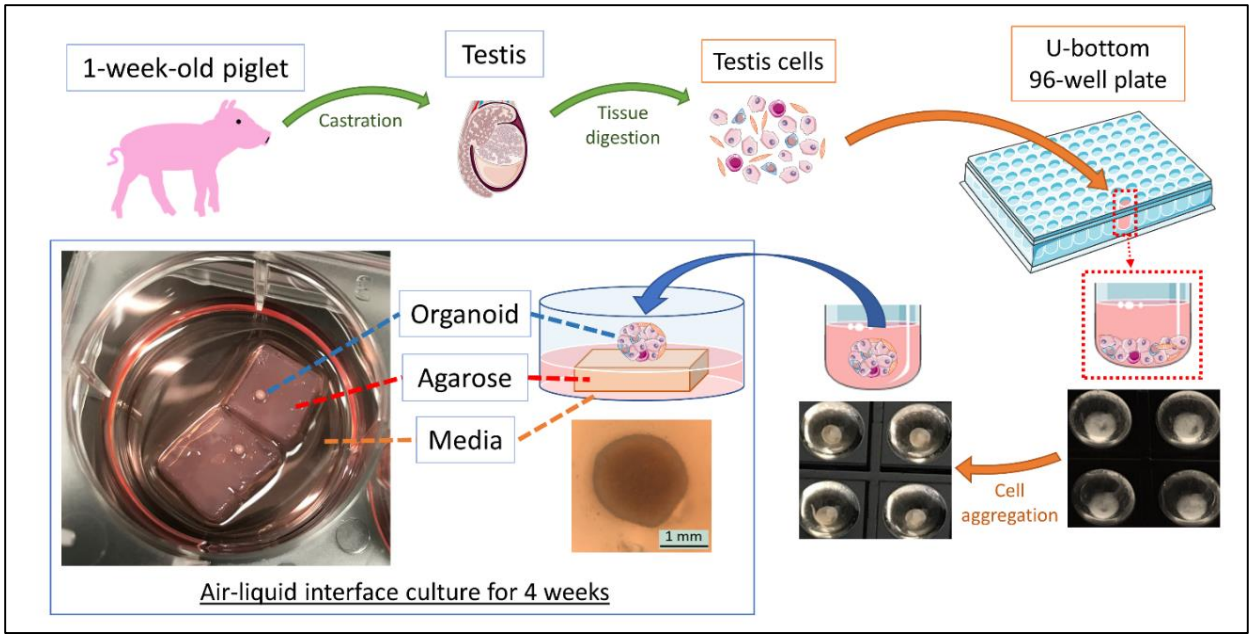

Figure 2.1. *Schematic summary of the procedures for formation of testis organoids. Testis tissues were collected from 1-week-old piglets and digested to isolate fresh testis cells. The testis cells (1.0×10^6cells/well) were then cultured in U-bottom 96-well plates for 24 hr to form cell spheroids. The cell spheroids were then cultured on small pieces (1×1×0.5 cm) of 1.5% agarose gel bedding immersed in media. This culture method is referred to as an air-liquid interface culture system. The cell spheroids were further cultured for 4 wk in this culture system to form the testis organoids, and the media were changed every other day.*

2.3.6. Histological evaluation and immunohistochemistry (IHC) of germ cells

The collected samples were fixed overnight in Bouin's solution for 4 hr, then rinsed with and stored in 70% ethanol. Samples were processed using standard histological preparations, embedded in paraffin, and sectioned at 5 μm thickness. The sections were deparaffinized and stained with hematoxylin and eosin (H&E) or used for immunohistochemistry (IHC). The H&E staining was performed using previously described protocols (Fayaz, et al. 2020). Immunohistochemical staining for α-smooth muscle actin (α-SMA) was performed using an automated staining platform. Heat-induced epitope retrieval was performed, and the primary antibody for α-SMA (Mouse anti- α-SMA clone ASM-1, Leica Biosystems Inc, Buffalo Grove, IL, Cat No. PA0943) was applied for 30 min at 1:100 dilutions. This was followed by detection using an HRP-labelled polymer reagent (EnVision+ System - HRP Labelled Polymer, Agilent Technologies Canada Inc., Mississauga, ON, Cat No. K4003), and the staining was visualized using 3,3'-diaminobenzidine tetrahydrochloride (DAB)[h] as the chromogen (Dako Liquid DAB+ Substrate Chromogen System, Agilent Technologies Canada Inc., Mississauga, ON, Cat No. GV823). On the other hand, the immunohistochemical staining for UCHL 1 (a marker for gonocytes) was performed to allow quantification of germ cells in the organoids over time. Briefly, paraffin-embedded tissue sections were deparaffinized with xylene and rehydrated with graded ethanol. Heat induced antigen retrieval was performed at 98°C in citrate buffer (pH 6.3, Vector Laboratories, Cat No. H-3300) and Tris-EDTA buffer (pH 9.2-9.4, 1.21g/L of Tris and 0.37g/L of EDTA in distilled water) for 30 min, respectively; the tissue sections were then washed in DPBS for three times. Next, endogenous peroxidase was inactivated by 0.3% hydrogen peroxide in distilled water for 15 min at 37°C and the tissue sections were washed three times with DPBS. The tissue sections were then incubated overnight at 4°C with anti-UCHL 1 antibody (1:900, Abcam, Cat No. ab8189) in 2.5% horse serum (blocking agent, Vector, Cat No. MP-7500). After primary antibody binding, the tissue sections were rinsed three times with DPBS and incubated with HRP-labeled anti-mouse/rabbit secondary antibody (universal anti-mouse/rabbit Ig, Vector, Cat No. MP-7500) for 1 hr at RT. The tissue sections were then incubated with DAB chromogen (Vector laboratories, Cat No. SK-4105) for 3 min and counter-stained with hematoxylin for 5 min. Sections were dehydrated with graded ethanol, xylene, and sealed with mounting media and cover slips. Histological analysis of slides was performed using a light microscope equipped with digital

photomicrography (Northern Eclipse Image Analysis software version 7.0, Empix Imaging, Mississauga, ON, Canada).

2.3.7. Statistical analyses

Statistical analyses included data from quantification of density of tubular cross sections (number of tubules in cross-sections/mm^2), tubular relative area (% of tubular area compared with the total cross-section area), and the relative germ cell number (% of germ cells compared with total cells on a cross-section). In the first analysis, the effects of cell density (1.0×10^6 *vs.* 0.8×10^6 *vs.* 0.6×10^6 testis cells) and culture duration (1 to 4 wk) on density of tubular cross sections and tubular relative area in organoids were examined. ImageJ software was used for quantification and two-way ANOVA with Turkey's post-hoc test in SPSS (IBM SPSS Statistics for Windows, Version 25.0) were used for statistical analysis. Percentages were transformed (using Arcsin function) prior to ANOVA. Data were expressed as means ± SEM. $P<0.05$ was considered significant. In the second part of analysis, the effects of media supplementation (10% KSR *vs.* 10% FBS *vs.* 10% KSR+5% FBS < 5% KSR+10% FBS) and culture duration (1 to 4 wk) on density of tubular cross sections and tubular relative area in organoids were examined. We again used a two-way ANOVA with Turkey's post-hoc test for these two factors. In the third part of analysis, the effects of media supplementation (10% KSR *vs.* 10% FBS *vs.* 10% KSR+5% FBS *vs.* 5% KSR+10% FBS) and culture duration (1 to 4 wk) on relative germ cell number in organoids were examined. We again used a two-way ANOVA with Turkey's post-hoc test for these two factors.

2.4. Results

2.4.1. Experiment 1: Organoid formation using fresh or cryopreserved testis cells in spheroid-forming low attachment wells combined with an air-liquid interface culture system

To test the tubulogenic potential of neonatal porcine testis cells, testis cell spheroids were made by low-attachment U-bottom well and further cultured in an air-liquid interface system. At day 0, cell spheroids were collected prior to the air-liquid interface culture for histological examination. The testis cells were closely packed in the cell spheroids at day 0; however, no tubular reconstruction was observed (**Fig. 2.2A**). The cell spheroids were further cultured in either air-liquid interface system (on an agarose base, **Fig. 2.2C-F**) or directly in media (without the support of an agarose base, **Fig. 2.2B**) for 4 wk, and the samples were collected weekly for histological

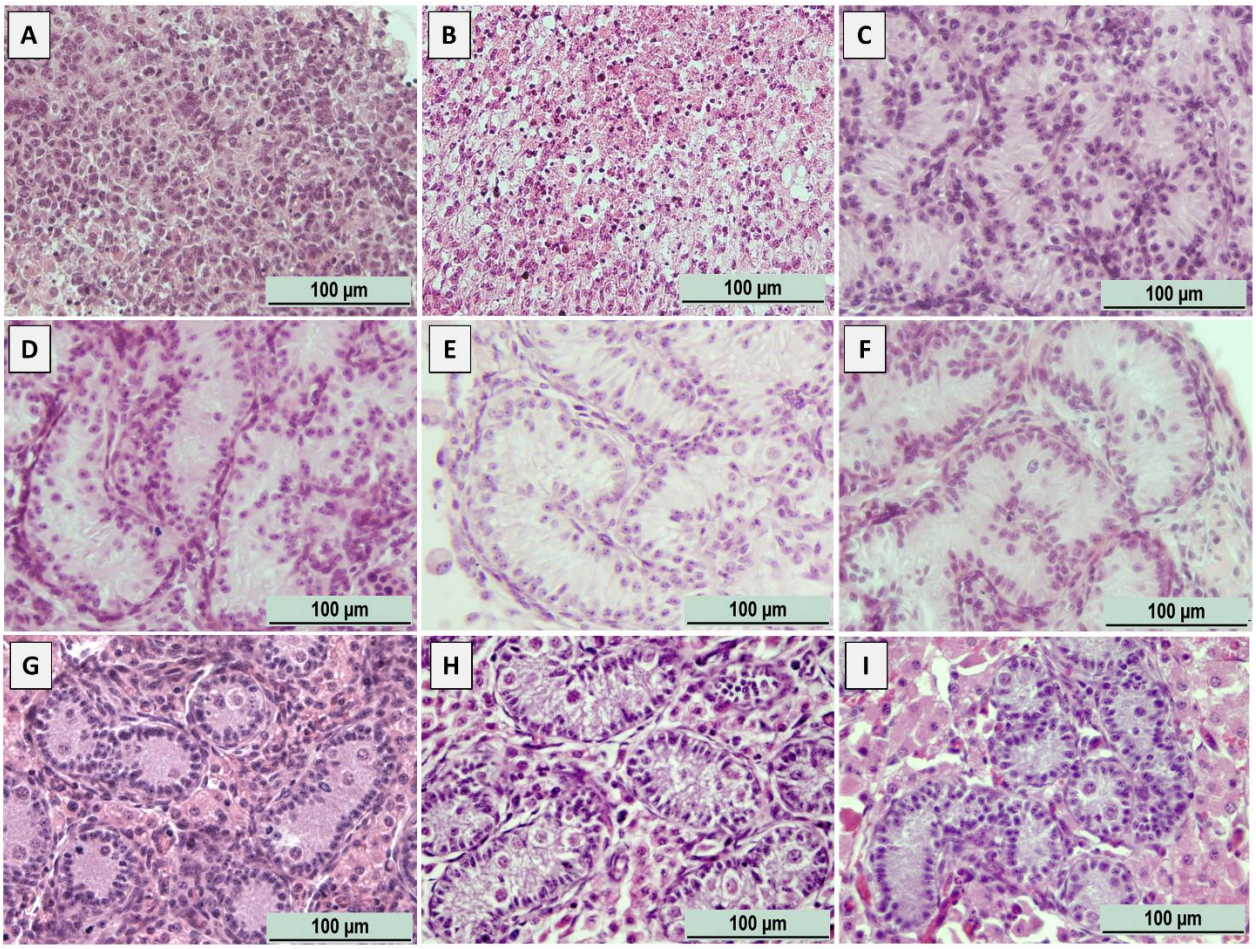

Figure 2.2. *Representative histological micrographs of testis spheroids and organoids formed after culturing of fresh piglet testis cells as well as control intact testes.* ***(A)*** *Cell spheroids from day 0 comprised randomly distributed testis cells without any tubular reconstruction.* ***(B)*** *Cell spheroids cultured directly in the media (without the support of an agarose base) did not form any testis tubular structures after 1 week of culture, and most cells underwent apoptosis or necrosis.* ***(C-F)*** *Cell spheroids cultured in air-liquid interface system showed initial formation of testis tubules at Wk 1* ***(C)*** *which were further developed at Wk 2* ***(D)****, and were maintained to Wk 3* ***(E)****, and Wk 4* ***(F)****.* ***(G-I)*** *Controls are intact testis tissues from* ***(G)*** *1-wk-old,* ***(H)*** *2-wk-old, and* ***(I)*** *4-wk-old piglets.*

examination. Also, control groups of intact testis tissues from 1-, 2-, and 4-wk-old piglets were collected for comparison (**Fig. 2.2G-I**). Cell spheroids cultured directly in media (without the support of agarose base) do not form any tubular structure after 1 week of culture, and most cells undergo apoptosis or necrosis (**Fig. 2.2B**). On the other hands, the reconstruction of testis tubular structures resembling the testis cords in control group was observed in cell spheroids cultured in air-liquid interface system (on an agarose base) as early as one week (**Fig. 2.2C&G**). Moreover, these testis tubular structures were maintained for 4 wk (the end of experiments) in the air-liquid interface system (**Fig. 2.2C-F**).

To test whether organoids can be generated from cryopreserved testis cells, frozen-thawed testis cells from 1-wk-old piglet were used to form organoids using our culture system. Similar to the findings in organoids derived from fresh cells, cryopreserved testis cells retained the capability to reconstruct testis cord-like structures as early as one week in our culture system (**Fig. 2.3B**). Also, these tubular structures could be maintained for at least 4 wk (the end of experiments) in our culture system (**Fig. 2.3B-E**).

2.4.2. Experiment 2: Effects of cell density and culture duration on in vitro tubulogenesis of organoids

To examine the effects of cell density and time of culture on *in vitro* tubulogenesis of organoids, the density of tubular cross sections and tubular relative area of organoids in different cell density groups (1.0×10^6 *vs.* 0.8×10^6 *vs.* 0.6×10^6 testis cells/organoid) were quantified (**Fig. 2.5&2.6**). Density of tubular cross sections was defined as the number of tubules in cross-sections/mm^2; while, tubular relative area was defined as the % of tubular area compared with the total cross-section area (**Fig. 2.4**). The density of tubular cross sections in the 0.8×10^6 cell density group was higher than other groups in the first 2 wk of culture ($P<0.05$), but density of tubular cross sections did not differ among different cell density groups at 3 and 4 wk ($P>0.05$). While density of tubular cross sections in the 0.8×10^6 cell density group did not change over time, density of tubular cross sections increased in the 1.0×10^6 cell density group at wk 3, and in the 0.6×10^6 cell density group at 4 wk of culture ($P<0.05$).

The tubular relative area in the 0.8×10^6 cell density group was higher than other groups at 1, 3, and 4 wk ($P<0.05$). A significant increase in tubular relative area was observed at least at one time point in all groups over the length of culture. Such an increase was observed at wk 2 for both

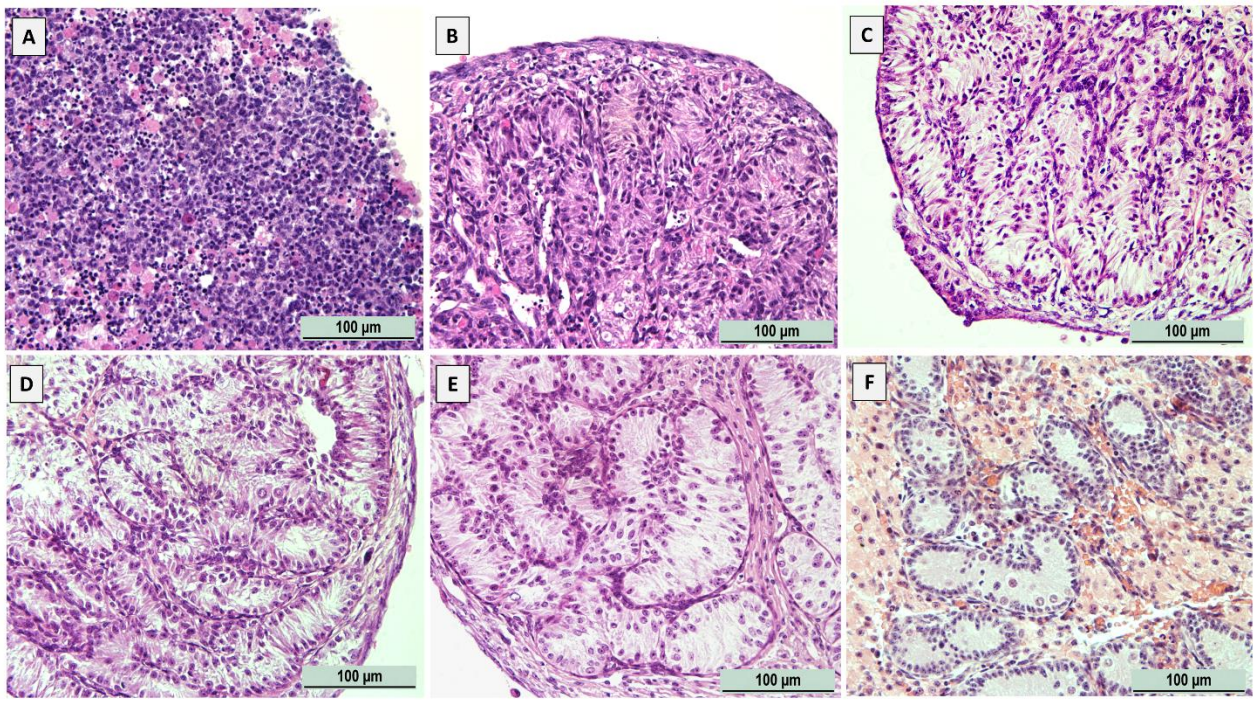

Figure 2.3. *Representative histological micrographs of testis spheroids and organoids formed after culturing of cryopreserved piglet testis cells as well as control intact testis.* ***(A)*** *Cell spheroids from day 0 comprised randomly distributed testis cells without any tubular reconstruction.* ***(B-E)*** *Cell spheroids generated from cryopreserved testis cells showed initial formation of testis tubules at wk 1* ***(B)*** *which were further developed at wk 2* ***(C)****, and were maintained to wk 3* ***(D)****, and wk 4* ***(E)****.* ***(F)*** *Controls are intact testis tissues from 1-wk-old piglets.*

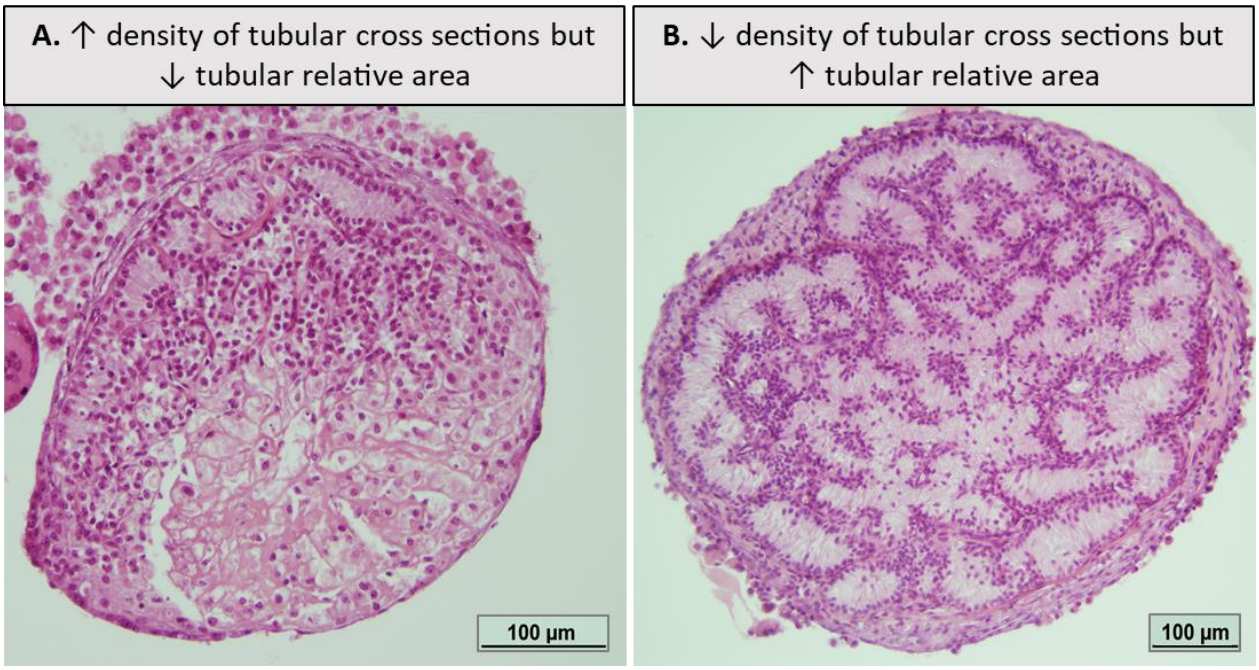

Figure 2.4*. Representative histological micrographs of testis organoids with varying degrees of tubulogenesis.* ***(A)*** *Organoids with high density of tubular cross sections but low tubular relative area, as compared with* ***(B)*** *a testis organoid with low density of tubular cross sections but high tubular relative area. Notably, large areas of necrosis were observed in* ***(A)****.*

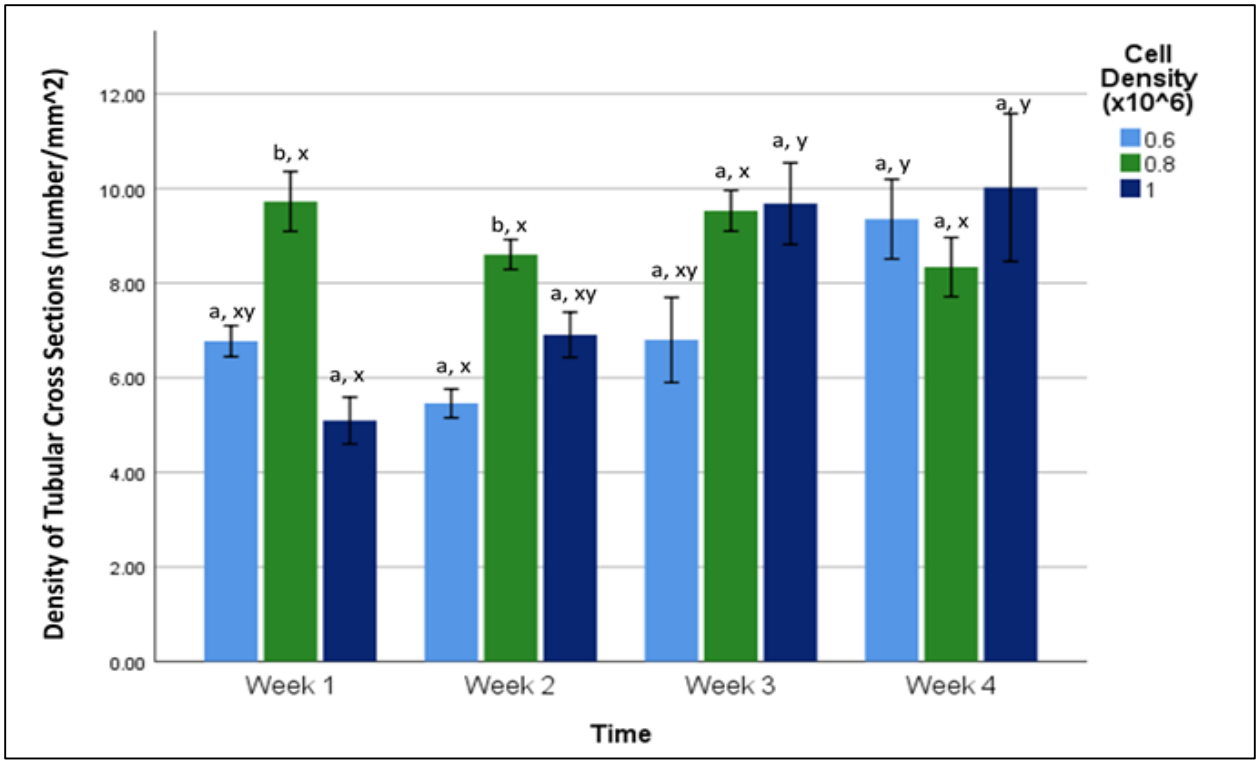

Figure 2.5. *Density of tubular cross sections (tubular sections/mm^2) of testis organoids resulting from different cell densities (1.0×10^6 vs. 0.8×10^6 vs. 0.6×10^6 testis cells) over time (1 to 4 wk). Data are presented as mean ± SEM.* ^{abc} *Data with different letters differ significantly among cell densities (P<0.05).* ^{xyz} *Data with different letters differ significantly over time (P<0.05). n=3 replications*

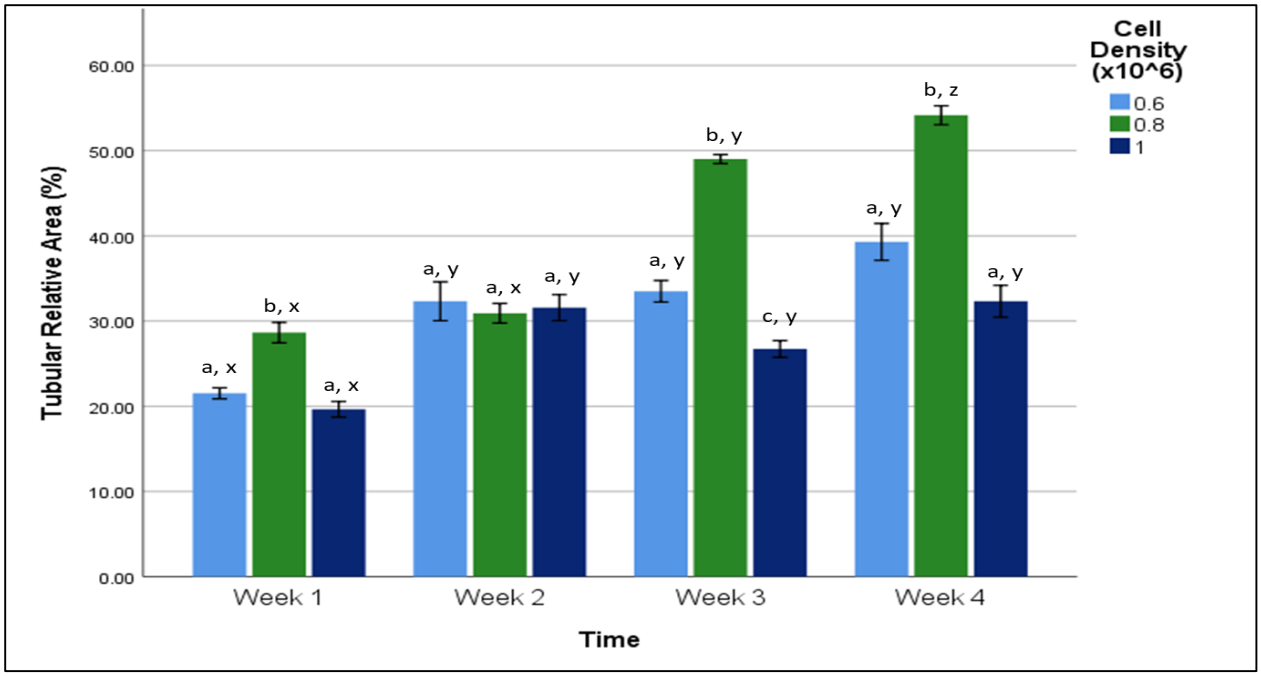

Figure 2.6. *Tubular relative area (% of tubular area compared with the total cross-section area) of testis organoids with different cell densities (1.0×10^6 vs. 0.8×10^6 vs. 0.6×10^6 testis cells) over time (1 to 4 wk). Data are presented as mean ± SEM.* ^{abc} *Data with different letters differ significantly among cell density groups ($P<0.05$).* ^{xyz} *Data with different letters differ significantly over time ($P<0.05$). n=3 replications*

1.0×10^6 and 0.6×10^6 cell density groups, whereas it was observed at wk 3 and 4 for the 0.8×10^6 cell density group ($P<0.05$).

2.4.3. Experiment 3: Effects of media supplementation and culture duration on organoid size and in vitro tubulogenesis in organoids

In this experiment, we aimed to examine the effects of media supplementation and culture duration on the size, density of tubular cross sections, and tubular relative area of the testis organoids (**Fig. 2.7, 2.10, 2.11**). A decrease in the size of organoids in all groups was observed at wk 1 of culture (**Fig. 2.7**). It is noteworthy that the size of organoids in the 10% FBS group decreased considerably compared with other groups, especially at 1 and 4 wk. The size of organoids in the 10% KSR, 10% KSR+5% FBS, and 5% KSR+10% FBS groups was maintained from wk 2 to 4 of culture. In our supplementary experiments of two-dimensional (2D) testis cell culture, rapid proliferation of somatic cells was observed in FBS-supplemented media compared with those in KSR-supplemented media (**Fig. 2.8**). In another supplementary experiments, we detected extent growth of smooth muscle cells in the capsule surrounding the organoid (**Fig. 2.9**).

Density of tubular cross sections in the 10% FBS group was higher than other groups, regardless of the time of culture ($P<0.05$; **Fig. 2.10**). In comparison, density of tubular cross sections of the 10% KSR group was lower than other groups in the first 3 wk of culture ($P<0.05$). A significant increase in the density of tubular cross sections was observed at least at one time point in all groups over the course of culture. Such an increase was observed in the 10% FBS, 10% KSR+5% FBS, 5% KSR+10% FBS groups at wk 2 of culture, whereas an increase was observed for the 10% KSR group at wk 4 ($P<0.05$).

Tubular relative area of the 5% KSR+10% FBS group was higher than other groups at wk 1, 3, and 4 ($P<0.05$; **Fig. 2.11**). In comparison, the tubular relative area of the 10% FBS group was lower than other groups regardless of the time of culture ($P<0.05$). A significant increase in tubular relative area was observed at least at one time point in all groups over time of culture. Such an increase was observed at wk 2 of culture in all groups, also at wk 3 for the 5% KSR+10% FBS groups ($P<0.05$).

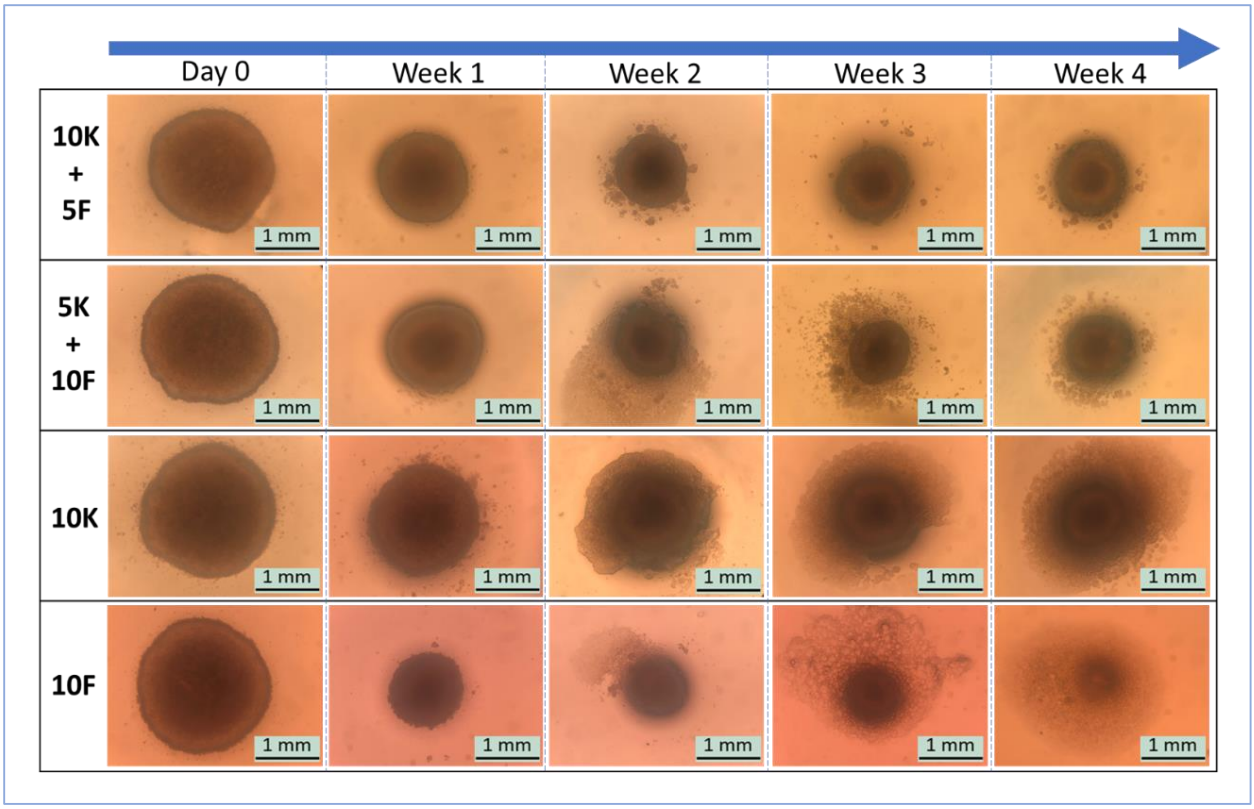

Figure 2.7. *Sizes of testis organoids in different media supplementation groups as observed under light microscopy over time. A decrease in size was noticed for testis organoids supplemented with 10% KSR+5% FBS (10K+5F), 5% KSR+10% FBS (5K+10F), 10% KSR (10K), or 10% FBS (10F) over time.*

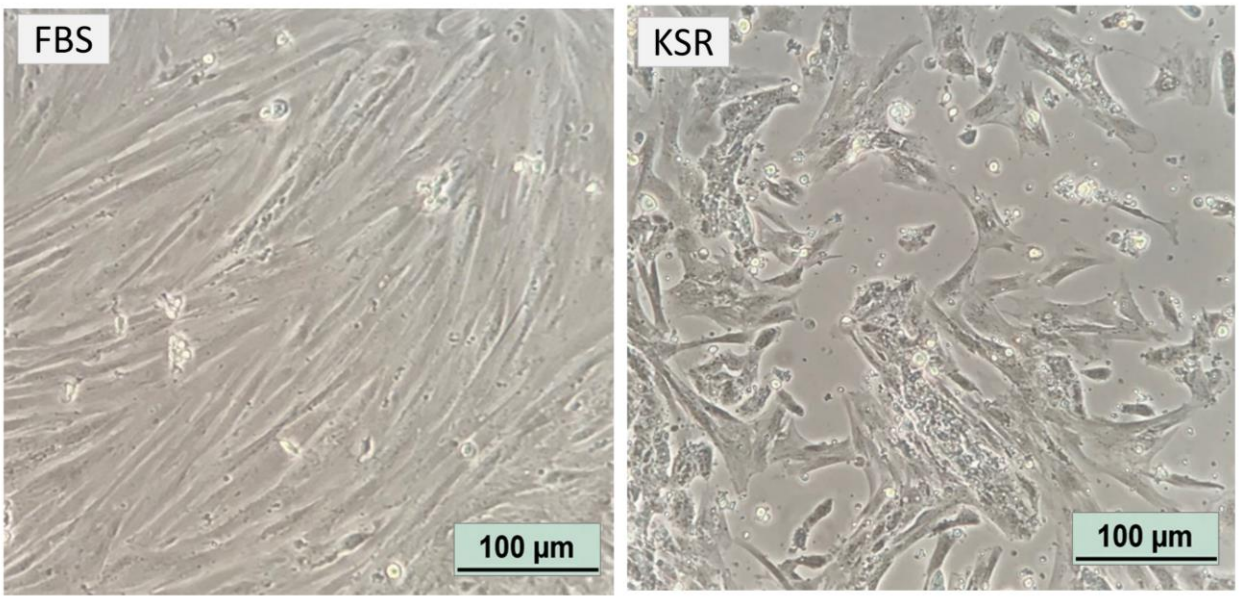

Figure 2.8. *Two-dimensional (2D) culture of testis cells in media supplemented with either knock-out serum replacement (KSR) or fetal bovine serum (FBS). Rapid proliferation of somatic cells cultured in FBS-supplemented media (left) compared with those in KSR-supplemented media (right).*

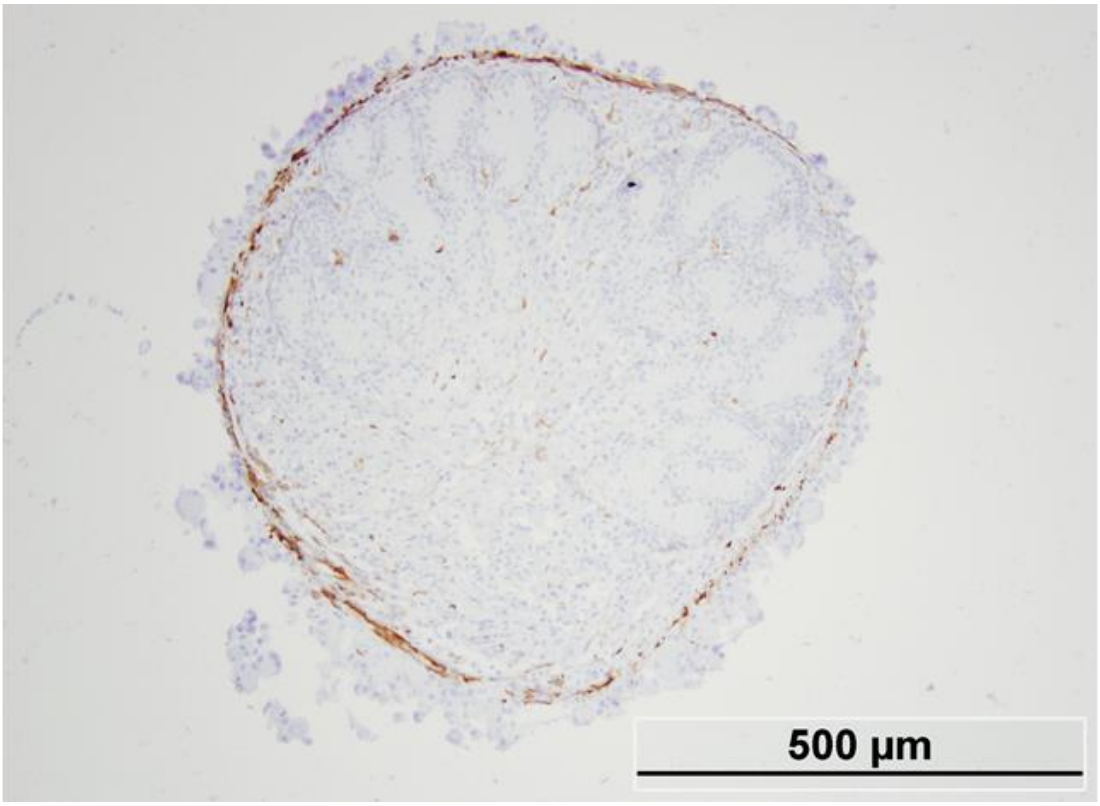

Figure 2.9. *Immunohistochemistry to detect α-smooth muscle actin (α-SMA)* within *smooth muscle cells in the testis organoid. Note the extent of smooth muscle cells in the capsule surrounding the organoid.*

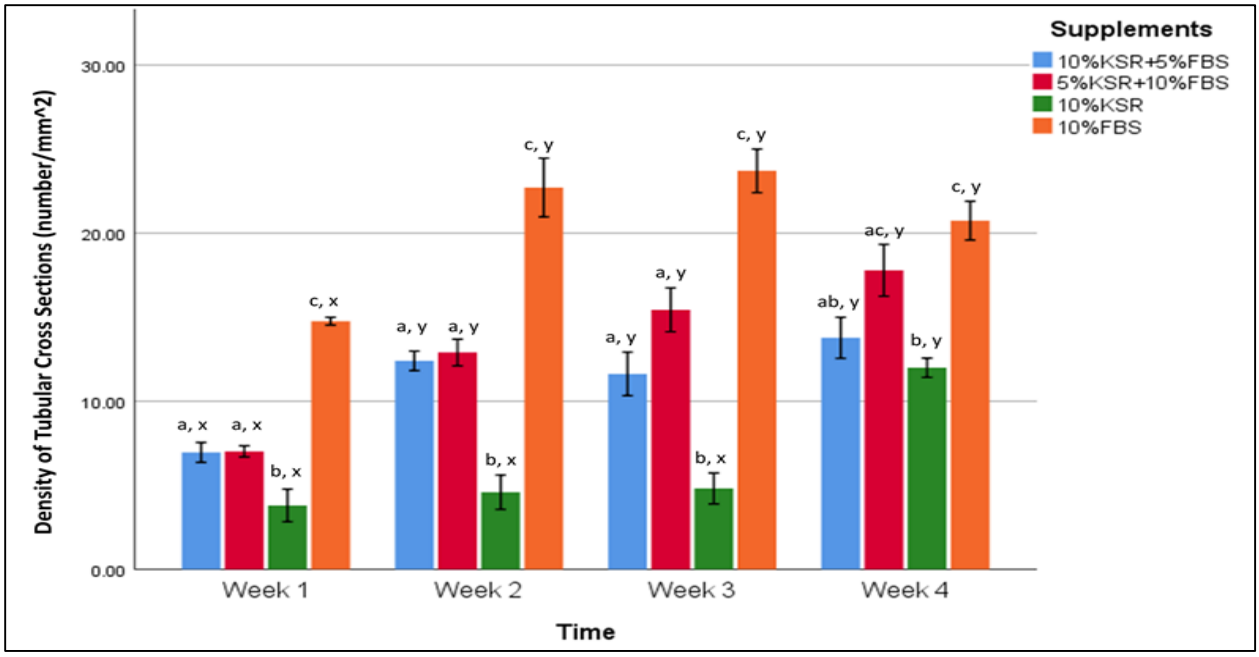

Figure 2.10. *Density of tubular cross sections (tubular sections/mm^2) of testis organoids from different media supplementation groups (10% KSR vs. 10% FBS vs. 10% KSR+5% FBS vs. 5% KSR+10% FBS) over time (1 to 4 wk). Data are presented as mean ± SEM.* ^{abc} *Data with different letters differ significantly among media supplementation groups (P<0.05).* ^{xyz} *Data with different letters differ significantly over time (P<0.05). n=3 replications*

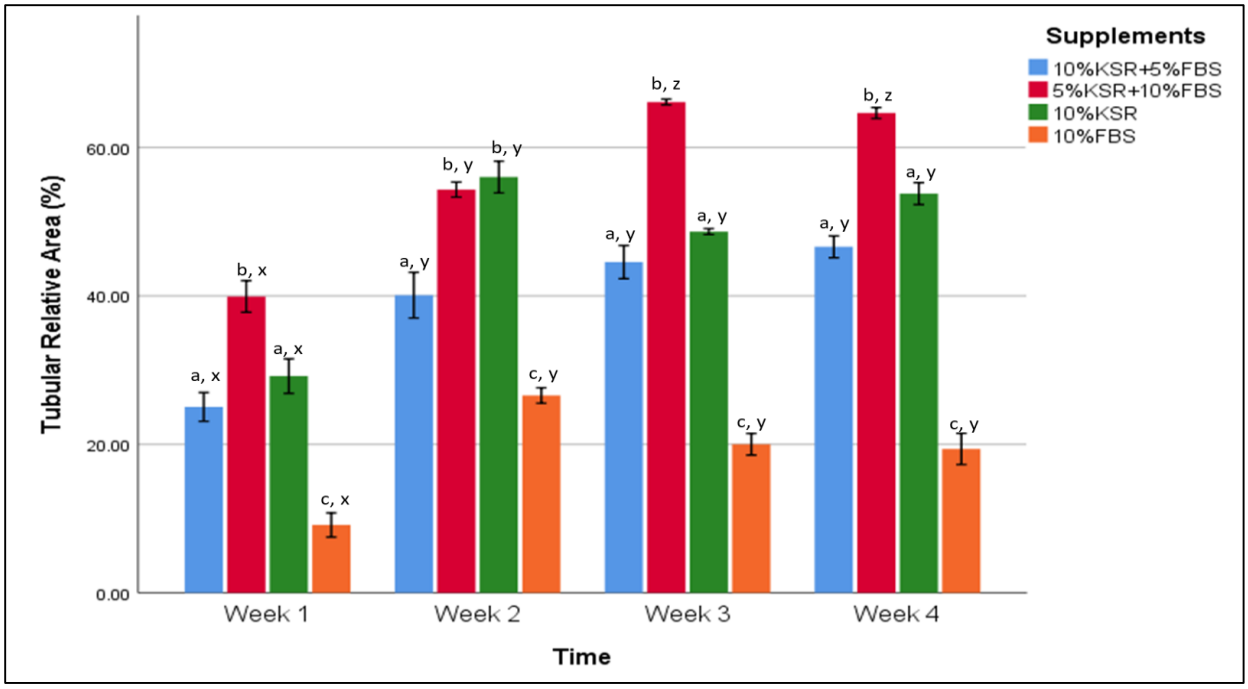

Figure 2.11. *Tubular relative area (% of tubular area compared with the total cross-section area) of testis organoids from different media supplementation groups (10% KSR vs. 10% FBS vs. 10% KSR+5% FBS vs. 5% KSR+10% FBS) over time (1 to 4 wk). Data are presented as mean ± SEM.* ^{abc} *Data with different letters differ significantly among supplement groups (P<0.05).* ^{xyz} *Data with different letters differ significantly over time (P<0.05). n=3 replications*

2.4.4. *Experiment 4: Effects of media supplementation and culture duration on germ cell ratios in organoids*

To examine the effects of media supplementation and culture duration on germ cell ratios in organoids, the relative germ cell number in organoids were quantified. Surprisingly, the relative number of germ cells did not differ significantly among supplementation groups ($P>0.05$; **Fig. 2.12**). It is worth mentioning that since the total number of cells in the FBS group was relatively lower than other groups, the relative germ cell number in the 10 % FBS group was slightly higher than other groups at wk 1, 2, and 4. Moreover, although a slight decrease in the number of germ cells was observed in all groups over time of culture, no statistical differences were detected ($P>0.05$). In our supplementary results (**Fig. 2.13**), testis organoids cultured in FBS only groups have smaller size and lower total cell number compared with testis organoids cultured in KSR only or KSR+FBS combined supplementation groups.

2.4.5. *Experiment 5: Organoid formation using germ cell-enriched testis cells*

Since the number of germ cells in the organoids up to this point was relatively low in comparison with the native testis tissue, populations of testis cells enriched to 60% gonocytes were examined for their organoid formation capacity. However, multifocal necrosis and poor tubular morphogenesis were observed in the testis organoids generated from 60% germ cell-enriched testis cells at the first week of culture (**Fig. 2.14**).

2.5. Discussion

The general objective of this chapter was to establish a porcine testis organoid system and assess different culture conditions for its efficient *in vitro* morphogenesis. The first hypothesis was that using neonatal piglet testis cells in a reported mouse testis organoid system would also result in the formation of a porcine testis organoid system. Our findings showed that neonatal porcine testis cells from 1-week-old piglet indeed retain the ability to reconstruct compartmentalized testis cord-like structures *in vitro*, similar to the reported mouse testis organoids (Edmonds and Woodruff 2020, Yokonishi, et al. 2013). One important condition in this culture system is the air-liquid interface culture that improves nutrient and oxygen perfusion in the organoids. Our preliminary experiments have shown that cell aggregates cultured directly in media (without the support of an agarose base) do not form any tubular structures and most cells undergo apoptosis or necrosis (**Fig.**

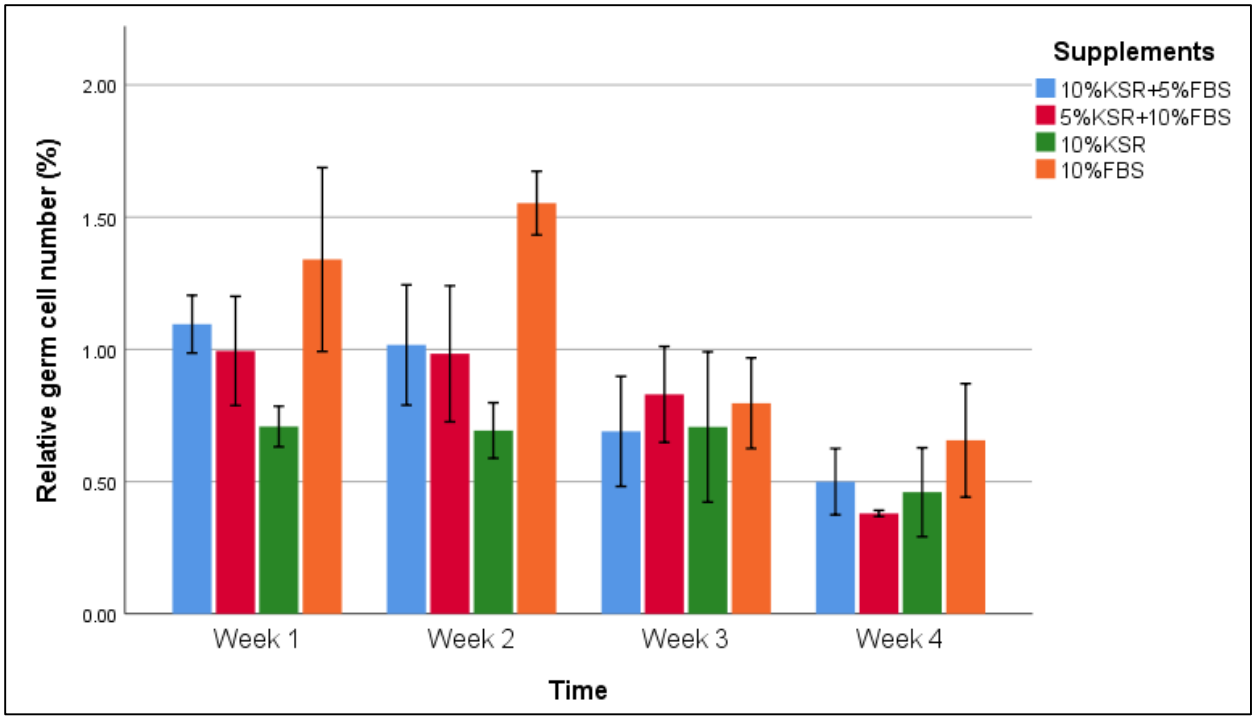

Figure 2.12. *Relative germ cell number (% of germ cells to total cells in a cross-section) in testis organoids from different media supplementation groups (10% KSR vs. 10% FBS vs. 10% KSR+5% FBS vs. 5% KSR+10% FBS) over time (1 to 4 wk). Data are presented as mean ± SEM. Although a slight decrease in the number of germ cells was observed over time of culture in all groups, no statistical differences were found (P>0.05). Also, no statistical differences were observed among different supplement groups (P>0.05). n=3 replications*

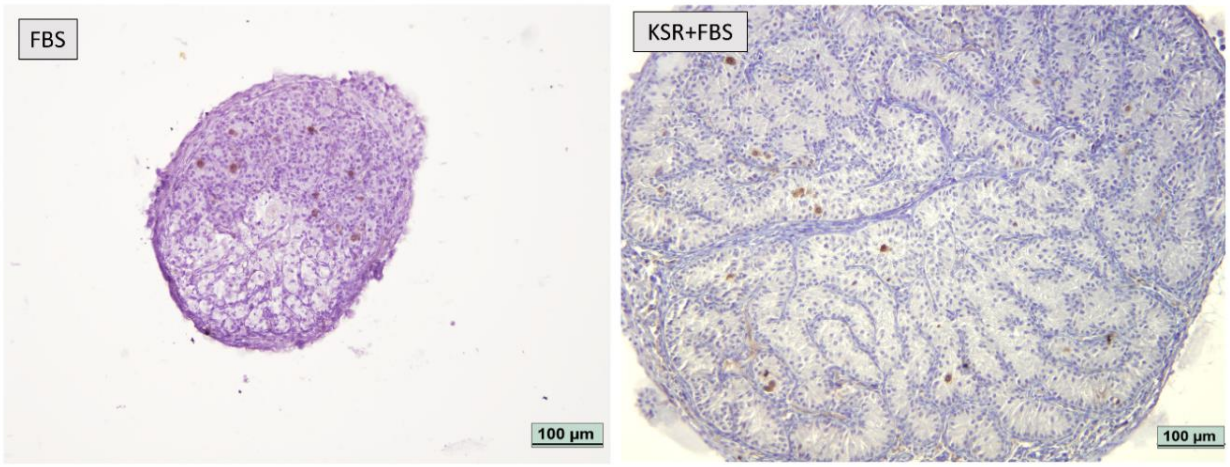

Fig. 2.13. *Representative histological micrographs of testis organoids in different media supplementation groups. Testis organoids in the FBS groups had a smaller size and lower total cell numbers, which inversely increased the relative number of germ cells (left), whereas organoids in KSR+FBS groups had a larger size and higher total cell numbers (right). Gonocytes (immature germ cells) were detected using UCHL-1 immunostaining (brown).*

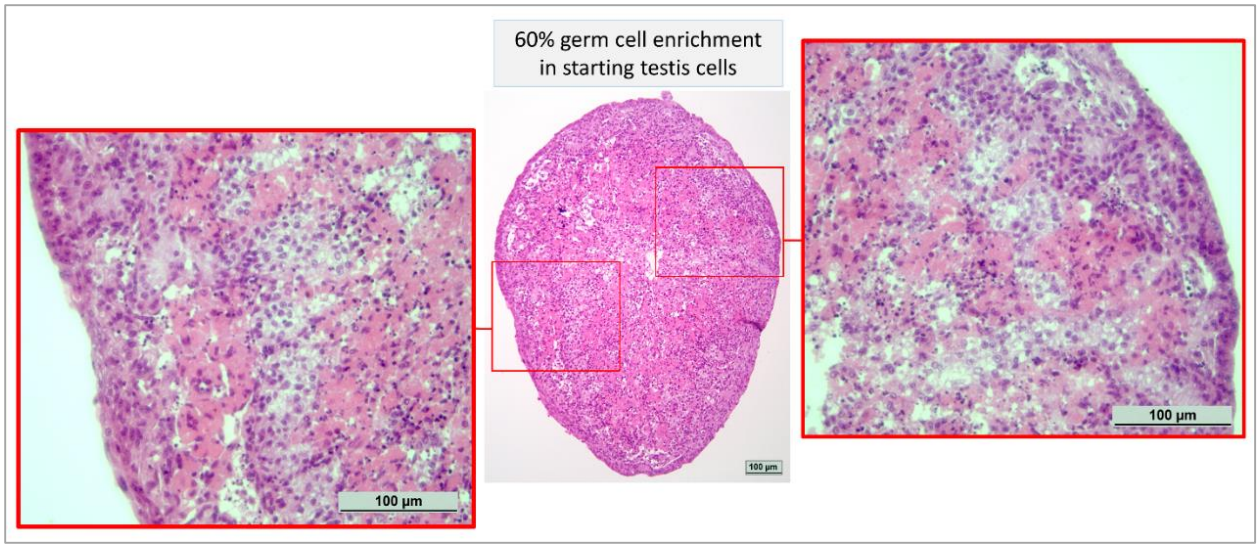

Figure 2.14. *Effect of increasing the germ cell ratio from 40% to 60% on testis organoid formation. Multifocal necrosis and poor tubular morphogenesis were observed in week 1 of testis organoid reconstructed from germ cell-enriched testis cells (60% germ cells).*

2.2B). The same observations have been reported in murine testis organoids using similar culture methodologies (Yokonishi, et al. 2013). The air-liquid interface culture system has been widely used in testis tissue culture to minimize hypoxia-induced cell death (Gholami, et al. 2020). This culture system can be easily set up; however, it requires a stable culture environment to prevent tissue from falling into the culture media. Modifications such as making the agarose base in a microwell inset might potentially solve this problem. Besides the air-liquid interface system, microfluidic and rotation culture systems have also been developed to continuously provide fresh media to the tissue and increase the surface area for nutrient/oxygen diffusion (Komeya, et al. 2016, Nakamura, et al. 2017). It will be interesting to compare the testis tubulogenic efficiency and organoid formation between the microfluidic device and air-liquid interface systems in future research.

The next hypothesis was that using frozen-thawed testis cells would result in the formation of testis organoids containing compartmentalized testis cord-like structures similar to organoids derived from fresh testis cells. Our findings showed that frozen-thawed testis cells from 1-week-old piglets were also capable of generating testis organoids using our culture system. Similar results were also reported by Sakib, *et al.* (2019b) where frozen-thawed neonatal porcine testis cells were able to reconstruct a compartmentalized testis organoid using a low-attachment microwell culture system (Sakib, et al. 2019b). This is an important observation since it suggests the feasibility of using cryopreserved human testis cells for building a human testis organoid in basic science research or potential clinical applications (Richer, et al. 2019, Sakib, et al. 2019a, Sakib, et al. 2019c).

Another hypothesis was that different culture conditions (by changing cell density, culture duration, media supplementation, and gonocyte ratios) would affect the efficiency of *in vitro* tubulogenesis in organoid formation. First, the number of cells used to form a testis organoid, also known as cell density, has varied in different studies. In general, a higher cell density is expected to promote intimate cell-to-cell interactions, which should improve *de novo* testis organogenesis. However, high cell densities also increase the size of organoids hence causing poor perfusion of oxygen and nutrients to the center of the organoid, which can eventually lead to central necrosis of the organoids. On the other hand, low cell densities decrease the size of organoids and thereby improve nutrient/oxygen perfusion in the organoids (Hirschhaeuser, et al. 2010), but organoid formation may be hindered due to insufficient number of initial testis cells (Sakib, et al. 2019b). In scaffold-

free cell spheroid culture systems, a cell density per organoid ranging from 10^3 to 2×10^6 cells has been reported (Edmonds and Woodruff 2020, Sakib, et al. 2019b, Yokonishi, et al. 2013). In our preliminary experiments, 2×10^6 cells/organoid caused central necrosis in the resultant organoids, while 0.5×10^6 cells/organoid resulted in delayed or impaired tubulogenesis, and the organoids were too small to be histologically processed. Therefore, mid-range cell densities including 0.6×10^6, 0.8×10^6, and 1×10^6 cells/organoid were investigated further for their organoid formation and tubulogenic efficiency. In this study, we defined tubulogenic efficiency by quantifying the density of tubular cross sections and tubular relative area. The density of tubular cross sections represents the number of tubular sections per mm^2 of cross-sections. Notably, high density of tubular cross sections does not necessarily indicate superior tubular reconstruction. High density of tubular cross sections can be interpreted as: 1) The reconstructed tubular structures are highly convoluted since even a single tubule can create multiple tubular cross-sections. 2) Necrosis may occur and split a tubule into multiple segments, creating a false impression of multiple tubular sections (**Fig. 2.4**). On the other hand, the tubular relative area indicates the relative area occupied by viable tubular structures within a testis organoid. Therefore, a high tubular relative area indicates superior tubular reconstruction (**Fig. 2.4**). Statistical analyses show that the 0.8×10^6 testis cells group reconstructed testis organoids with the highest tubular relative area. Thus, 0.8×10^6 testis cells per well is an optimal cell density to generate a porcine testis organoid.

Furthermore, there has been no consensus on the most suitable composition of the culture media supplements for formation of testis organoids (Richer, et al. 2019). For serum-based culture media, fetal bovine serum (FBS), knock-out serum replacement (KSR), or both combined have been used in organoid culture systems. FBS is abstracted from serum of fetal calves and hence contains various growth factors and biological substances with various effects on cells especially on their growth and development. This makes FBS a common serum supplement for *in vitro* cell culture. The addition of 10% FBS in a Sertoli cell line 2D culture was reported to induce the formation of testis tubular structures (Van Der Wee and Hofmann 1999). FBS was also reported to stimulate PTMC proliferation and support *de novo* testis tubulogenesis (Gassei, et al. 2006, Schlatt, et al. 1996). However, the inconsistent quality due to the supplier and batch variability and unidentified components in FBS make it challenging for researchers to study the key mechanisms and components that induce *de novo* testis tubulogenesis. Also, since the reconstruction of testis tubular structures has also been reported in a serum-free Matrigel-based culture system (Gassei, et al.

2006), the key inducers in both FBS and Matrigel that trigger *de novo* testis tubulogenesis remain unknown. In comparison, KSR has defined components and consistent quality between batches, which is commonly used in embryonic stem cell culture to avoid unwanted cell differentiation (Sato, et al. 2011). Sato, *et al.* (2011) were first to show that KSR supplementation is a crucial supplement in producing murine haploid spermatozoa *in vitro*; the fertilization ability of the lab-generated spermatozoa to produce viable euploid offspring was also confirmed in this study (Sato, et al. 2011). Later, Yokonishi, *et al.* (2013) used KSR supplementation in murine testis organoid system, which resulted in the formation of a compartmentalized testis organoid with tubular structures and the spermatogenic differentiation up to round spermatids was suggested (Yokonishi, et al. 2013). Most scaffold-based or scaffold-free testis organoid systems have also used KSR as a media supplement to maintain spermatogonia proliferation in organoids (Alves-Lopes, et al. 2017, Baert, et al. 2017), or to support spermatogenic differentiation from spermatogonia up to spermatocytes (Gharenaz, et al. 2020, Zhang, et al. 2014, Zhang, et al. 2017), spermatids (Rezaei Topraggaleh, et al. 2019), or even elongated spermatids (Baert, et al. 2019). Zhang *et al.* (2014) also showed that *de novo* testis tubulogenesis and the formation of compartmentalized seminiferous tubule-like structures only occurred in KSR supplemented groups (Zhang, et al. 2014). To combine the beneficial effects of FBS on *de novo* tubulogenesis and KSR on germ cell maintenance, we adopted a strategy of using a combination of KSR and FBS for media supplementation in this study.

We first observed the change in the size of organoids in different supplementation groups over time of culture. Our results showed that the size of organoids decreases abruptly in all groups, and they condensed into spheroids after 1 wk of culture, likely due to the contractile action of PTMCs and cell reorganization. In fact, based on IHC, smooth muscle cells were also observed within the capsule surrounding the testis organoids, which might play a role in size contraction of organoids (**Fig. 2.9**). Notably, the sizes of organoids in the 10% FBS (only) group were considerably smaller than other media supplementation groups. FBS has been reported to promote the proliferation of somatic testis cells which would theoretically better support the formation of testis cord-like structures *in vitro* (Richer, et al. 2019, Van Der Wee and Hofmann 1999). Our supplementary results also showed that somatic cells such as PTMCs proliferate more rapidly in FBS- than in KSR-supplemented media (**Fig. 2.8**), which may explain the intensive condensation of organoids in the 10% FBS only group.

Next, to determine the effects of media supplementation on tubulogenic efficiency in organoids, we compared the density of tubular cross sections and tubular relative area in organoids resulting from 10% KSR, 10% FBS, or combined supplementation of 10% KSR+5% FBS or 5% KSR+10% FBS. Our results have shown that the combined supplementation of 5% KSR+10% FBS improves *de novo* tubulogenesis of organoids in comparison to other supplement groups. It is worth noting that the organoids in the 10% FBS only group had a significantly higher density of tubular cross sections than other supplementation groups. This might be due to the rapidly proliferating somatic cells in FBS-supplemented media that formed a more convoluted tubular structure in organoids. However, excessive cell proliferation could have also contributed to cell death and necrosis in the 10% FBS only group. Indeed, the tubular relative area of the 10% FBS only group was significantly lower than other groups, perhaps due to the tissue necrosis and cell death. It is noteworthy that massive cell death and necrosis may also play a role in the decreases in size of organoids (**Fig. 2.4**). On the other hand, the 10% KSR+5% FBS and 5% KSR+10% FBS groups had a higher density of tubular cross sections than the 10% KSR at the first 3 wk of culture ($P<0.05$). Interestingly, the tubular relative area of the 5% KSR+10% FBS group was higher than both 10% KSR+5% FBS and 5% KSR groups at wk 1, 3, and 4 ($P<0.05$). This observation indicates that the combination of KSR and FBS improved tubular reconstruction and viability of testis organoids; therefore, 5% KSR+10% FBS is considered to be the optimal media supplementation for the porcine testis organoid system.

Next, to determine the effects of media supplementation on germ cell ratios within organoids, the relative germ cell numbers in different supplement groups were quantified. Our findings indicated that changing supplements did not affect the number of germ cells in testis organoids. Also, no significant decrease in the number of germ cells was observed in the organoids over 4 wk of culture. It is worth noting that the germ cell ratio was slightly higher in the FBS groups due to the fact that organoids in FBS groups had a smaller size and lower total cell numbers (**Fig. 2.7 & 2.13**), which inversely increased the relative germ cell number. Furthermore, it was reported that the germ cell number in organoids was increased by enriching the initial population of germ cells from 5% to 25% (Sakib, et al. 2019b). Our laboratory has developed a three-step digestion protocol to isolate primary testis cells containing ~40% of germ cells (gonocytes) from neonatal porcine testis (Awang-Junaidi and Honaramooz 2018, Yang, et al. 2010). Therefore, our initial testis cells already had a fairly high germ cell ration (~40%) but we wanted to investigate whether further

increasing the germ cell ratio would affect organoid formation in our system. Our laboratory also has introduced a novel method using Nycodenz centrifugation which can efficiently enrich germ cells (gonocytes) to ~80% in the resultant population of neonatal porcine testis cells (Yang and Honaramooz 2011). We set out to examine the effect of using germ cell-enriched testis cells resulted in ~60% germ cell-enriched testis cells, for which we combined equal volumes of cell mixtures of ~40% and ~80% germ cells. Since excessive germ cells hamper organoid formation (Sakib, et al. 2019b), only testis cells containing ~60% germ cells (rather than ~80%) were used for this experiment. However, our findings showed that multifocal necrosis and poor morphogenesis were observed in the reconstructed organoid at the first week of culture. A possible reason for the cell death and poor tubular reconstruction can be the lack of sufficient somatic cells to support *de novo* testis organogenesis (Sakib, et al. 2019b). More studies are required to explore the possibility of increasing the number of germ cells in organoids.

2.6. Conclusions

A porcine testis organoid culture system was established for neonatal testis cells from 1-week-old piglet using spheroid-forming low attachment wells combined with an air-liquid interface culture system. Both fresh and cryopreserved testis cells were capable of forming testis organoids comprising testis cord-like structures. The organoids could be maintained for at least 4 wk in an air-liquid interface culture system. Furthermore, a testis cell density of 0.8×10^6 cells/organoid and a combined supplementation of 5% KSR+10% FBS in media was deemed suitable for improving *de novo* tubulogenesis of testis cord-like structures in organoids. Although slight decreases in the number of germ cells (gonocytes) was observed over 4 wk of culture, this decrease was not statistically significant and media supplements did not affect the number of germ cells. Further studies are required to explore the possibility of increasing the number of germ cells in organoids.

2.7. Transition

In chapter 2, a porcine testis organoid culture system was established, and different culture conditions were tested for efficient *de novo* tubulogenesis in organoids. In chapter 3, the structural components and endocrine characteristics of the reconstructed testis organoids will be discussed.

CHAPTER 3

CELL TYPES, STRUCTURAL COMPONENTS, AND ENDOCRINE CHARACTERISTICS OF PORCINE TESTIS ORGANOIDS[4]

3.1. Abstract

The objective of this study was to characterize the testis organoids that result from our novel culture system for neonatal testis cells. Briefly, freshly isolated testis cells from 1-week-old piglets were cultured in ultra-low attachment U-bottom wells to allow the formation of cell spheroids. The cell spheroids were then cultured on top of agarose gels soaked in media in an air-liquid interface culture. The resultant testis organoids were then examined for their cell type, structural components, and endocrine function using routine histology, tissue-specific staining, immunohistochemistry (IHC), transmission electron microscopy, and hormone measurements of the culture media. The results indicated that testis organoids consisted of a variety of cell types and components commonly found in the neonatal testis including immature germ cells (gonocytes), Sertoli cells, peritubular myoid cells (PTMCs), peritubular basement membrane, and inter-tubular interstitium containing Leydig cells, collagen fibers and vascular structures. At the ultrastructural level, testis epithelium-like structure and Leydig cell maturation were observed in the organoids. Furthermore, testis organoids were capable of releasing testosterone especially in response to luteinizing hormone (LH) stimulation. These results show that our testis organoids have cellular, structural, and functional resemblance to the native testis tissue.

3.2. Introduction

Spermatogonial stem cell (SSC) niches are the microenvironment that provide molecular factors and acellular components to regulate the proliferation and/or differentiation signaling to SSC. Although SSC niches are complex and some aspects of their function and regulation remain elusive, a number of factors that promote SSC self-renewal and differentiation have been identified. For examples, growth factors secreted from testis somatic and interstitial cells are reported to have beneficial effects on SSC growth and development. Glial cell-derived neurotrophic factor (GDNF), which is produced by Sertoli cells, promotes SSC proliferation (Kubota, et al. 2004). Colony-stimulating factor 1 (CSF1), which is produced by Leydig and PTMCs, also promotes SSC

[4] T.C. contributed to the conceiving and designing of the study, performed the experiments, and wrote the first draft of the thesis.

proliferation (Kokkinaki, et al. 2009, Oatley, et al. 2009). *In vitro* studies have also shown that the co-culture of testicular cell lines, such as Sertoli cells and Leydig cells, with germ cells triggers meiotic division and germ cell differentiation in 2D culture methods (Le Magueresse-Battistoni, et al. 1991, Nagao 1989, Rassoulzadegan, et al. 1993). That said, the presence of main testis cell types, including germ cells, Sertoli cells, PTMCs, and Leydig cells, is crucial in a successful organoid system (Edmonds and Woodruff 2020).

The cell-to-cell and cell-to-ECM interactions within the interstitial compartment also provides molecular signaling for the regulation of spermatogenesis (Nieschlag 2013). As mentioned in Section 1.2, testis consists of interstitial and tubular compartments, which are constituted by different cell populations with unique spatial orientations. Sertoli cells regulate SSC self-renewal and differentiation, and support the migration and development of spermatocytes toward the lumen. PTMCs, located around seminiferous tubules, support the tubular integrity, spontaneous contraction of seminiferous tubules and are involved in the formation of basement membrane (Schlatt, et al. 1996). Basement membrane provides the site of attachment for Sertoli cells and separates seminiferous tubules from the interstitial compartment. The interstitial compartment is the anatomical space between tubular compartments which consists of loose connective tissue with cell populations such as Leydig cells, macrophages, and fibroblasts (Nieschlag 2013). Leydig cells produce testosterone which stimulates spermatogenesis and is responsible for the development and maintenance of male secondary sex organs. Blood vessels are also located within the interstitial space. Based on the criteria of organoid formation in Section 1.2, it is necessary to confirm the presence of the main testis cell types (*e.g.* germ cells, Sertoli cells, PTMCs, and Leydig cells) and compartmentalized structures in the organoids (Edmonds and Woodruff 2020).

The regulation of hormonal activity related to spermatogenesis can be simply described as being related to the hypothalamic-pituitary-gonadal (HPG) axis. Hypothalamus releases gonadotropin-releasing hormone (GnRH) which acts on the anterior pituitary gland to stimulate the release of luteinizing hormone (LH) and follicular stimulating hormone (FSH). FSH and LH reach the testis via blood circulation and bind with the receptors on Sertoli cells and Leydig cells, respectively, to trigger spermatogenesis and steroidogenesis. FSH affects the metabolism of Sertoli cells and indirectly stimulates spermatogenesis. Activated Sertoli cells produce inhibin B to inhibit further secretion of FSH, through a negative feedback mechanism. Activated Leydig cells release

testosterone that stimulates the development of secondary sexual characteristics and inhibits further production of GnRH and LH (Nieschlag 2013). To test whether testis organoids possess endocrine capabilities, some studies have measured the testosterone levels in the organoid culture media (Edmonds and Woodruff 2020, Vermeulen, et al. 2019).

Together, the objective of second study was to examine the cell types, structural components, and endocrine functionality of our organoid culture system.

3.3. Materials and Methods

3.3.1. *Testis collection and preparation*

Testes were collected from 1-week-old Yorkshire-cross piglets as described in Chapter 2.3.1.

3.3.2. *Testis cell isolation*

Testis cells were isolated using the protocols established in our lab as described in Section 2.3.2.

3.3.3. *Testis organoid culture system*

Testis organoids were generated using low attachment U-bottom well and the organoids were further cultured in air-liquid interface culture system as described in Section 2.3.5.

3.3.4. *Immunohistochemistry (IHC) and tissue-specific staining*

To observe the cell orientation and structural components in the organoids, immunostaining of testis cell types, and tissue-specific staining were performed. IHC for α-smooth muscle actin (α-SMA) and von Willebrand Factor (vWF) were performed using an automated staining platform as described in Section 2.3.6. The primary antibodies for vWF (Rabbit anti-human vWF, Agilent Technologies Canada Inc., Mississauga, ON, Cat No. IR527) were applied for 30 min at 1:1000 dilution. Masson's trichrome (MT) and periodic Schiff-methenamine (PASM) staining were also performed using an automated staining platform. The IHC for UCHL 1, GATA-1, and CYP17A1 was performed using protocols previously mentioned in Section 2.3.6. The primary antibodies used in this experiment include anti-UCHL 1 antibody (1:900, Abcam, Cat No. ab8189), anti-GATA-1 antibody (1:200, Santa Cruz Biotechnology, Cat No. sc-1237), and anti-CYP17A1 antibody (1:50, Santa Cruz Biotechnology, Cat No. sc-374244). The secondary antibodies included the HRP-labeled anti-mouse/rabbit secondary antibody (universal anti-mouse/rabbit Ig, Vector,

Cat No. MP-7500) and anti-goat secondary antibody (Abcam, Cat No. ab97100). Histological analysis of slides was performed using routine light microscopy as described in Section 2.3.6.

3.3.5. Transmission electron microscopy (TEM)

Transmission electron microscopy (TEM) was performed to observe the ultrastructure of the organoids. The fixation, embedding, and sectioning of samples for TEM were performed using previously reported protocols in our laboratory (Awang-Junaidi, et al. 2020). Briefly, the tissue samples were first fixed in ice-cold 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (4 °C, pH 7.2) for 4 hr. The fixed tissue samples were then washed by immersion in 0.1 M sodium cacodylate buffer for 10 min and stored in fresh 0.1 M sodium cacodylate buffer at 4 °C. Post-fixation was performed by immersion in 1% osmium tetroxide for 1 h at room temperature. The tissue samples were then washed by distilled water and dehydrated with graded ethanol. Next, tissue samples were immersed in LR White resin mix diluted with pure ethanol in 1:1 and 2:1 ratio for 1 h, respectively, and finally in pure LR White resin mix for 2 h. The samples were then polymerized at 65 °C overnight and sectioned at 100 nm using a Leica Ultracut UCT. The sectioned samples were observed under a transmission electron microscope (HT7700, Hitachi) at an accelerating voltage of 80 kV.

3.3.6. Luteinizing hormone (LH) induction and testosterone measurements

Testosterone secretion from Leydig cells is induced by luteinizing hormone (LH) in the intact testis. Therefore, organoids were cultured with LH (100 ng/ml, Lutropin-V; Bio-niche Animal Health) starting at day 6 and the culture media were collected every other day for testosterone measurements. Also, culture media from control organoid groups without LH supplementation were collected for comparison. The testosterone measurements were performed using testosterone ELISA kits (Cayman, Ann Arbor, MI, Cat. No. 582701). For statistical analysis, the effect of LH supplement on testosterone levels in media was examined. Welch's ANOVA with Games Howell post-hoc test was used for statistical analysis. Data were log transformed prior to ANOVA for normalization. Data are expressed as means ± SEM. $P<0.05$ was considered significant.

3.4 Results

3.4.1 *Experiment 1: Cell types and structural components of organoids*

To identify the cell types and structural components in the organoids, IHC and tissue-specific staining were performed. Gonocytes (immature germ cells; **Fig. 3.1A**) were observed within the testis tubular structures reconstructed by Sertoli cells (**Fig. 3.1B**) and PTMCs (**Fig. 3.1C**). In addition, reconstruction of the peritubular basement membrane and inter-tubular interstitium containing Leydig cells (**Fig. 3.1D**), collagen fibers (**Fig. 3.2**) and vascular structures (**Fig. 3.3**) were also observed in the testis organoids.

Furthermore, TEM imaging was performed to study the ultrastructure of the organoids. Testis epithelium-like structures comprising Sertoli cells, basement membrane, and PTMCs resembling native testis epithelium were observed in the organoids (**Fig. 3.4A&B**). Moreover, lipid droplets were observed within the Leydig cells in week 1 organoids (**Fig. 3.4D**). Immature Leydig cells in the control testis tissue from 1-week-old piglet do not contain any lipid droplets (**Fig. 3.4C**), suggesting the initial androgen synthesis and *in vitro* maturation of Leydig cells in the organoids (**Fig. 3.4D**).

3.4.2. *Experiment 2: Testosterone secretion and LH responsiveness of organoids*

To determine the endocrine functionality and LH-responsiveness of testis organoids, testosterone levels (ng/mL) in the organoid culture media were measured over time of culture. During days 9 to 30 of culture, testosterone levels in the LH supplemented groups were significantly higher than control groups due to LH stimulation (**Fig. 3.5**), suggesting that Leydig cells in the organoids can be induced by LH to release more testosterone.

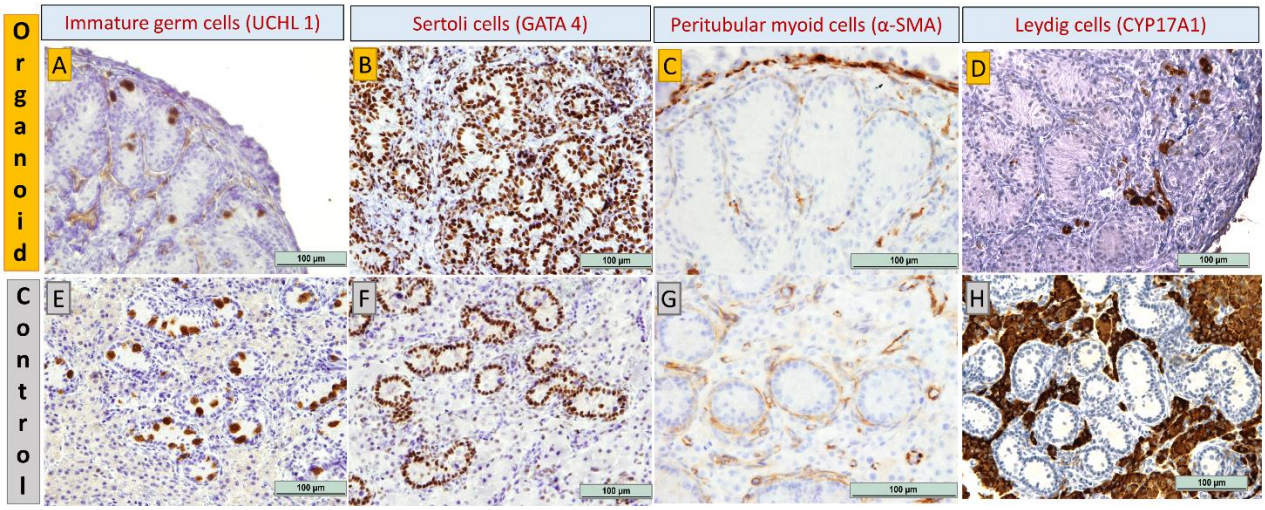

Figure 3.1. *Immunohistochemistry to detect immature germ cells, Sertoli cells, peritubular myoid cells, and Leydig cells in testis organoids and intact testes. Upper row (organoid): Immature germ cells* ***(A)*** *were detected using UCHL-1 and were observed within the tubular structures constructed by Sertoli cells* ***(B)*** *detected by GATA4, and peritubular myoid cells* ***(C)*** *detected by α-SMA. Also, Leydig cells* ***(D)*** *were detected using CYP17A1 and were observed in the interstitial structures of organoid. Lower row (control): Intact testis tissue from a 1-wk-old piglet with the corresponding cell types;* ***(E)*** *immature germ cells,* ***(F)*** *Sertoli cells,* ***(G)*** *peritubular myoid cells,* ***(H)*** *Leydig cells.*

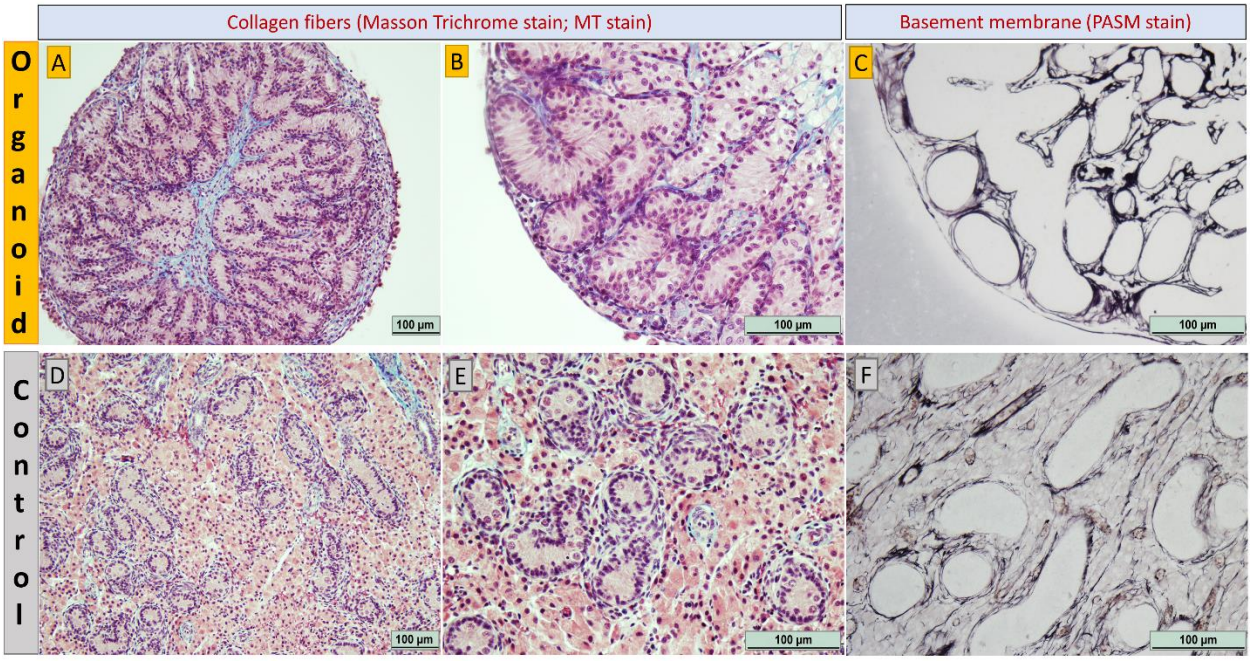

Figure 3.2. *Tissue-specific staining using Masson's trichrome (MT) and periodic Schiff-methenamine (PASM) staining for testis organoids and intact testes. Upper row (organoid): The presence of connective tissue* ***(A&B)*** *and basement membrane* ***(C)*** *was confirmed in testis organoids. Lower row (control): Intact testis tissue from a 1-wk-old piglet with the corresponding staining for connective tissue* ***(D&E)*** *and basement membrane* ***(F)****.*

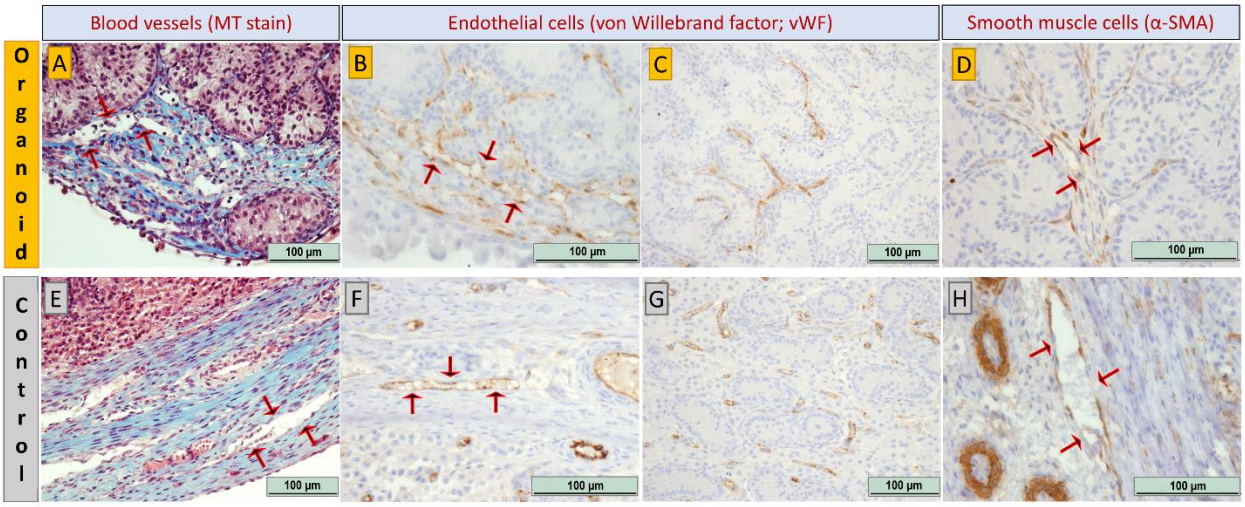

Figure 3.3. *Tissue-specific staining using Masson's trichrome (MT) to detect blood vessels, and immunohistochemistry for endothelial (vWF) and smooth muscle cells (α-SMA) to confirm blood vessels in testis organoids and intact testes. Upper row (organoid): vascular structures* ***(A)*** *constructed by endothelial cells* ***(B&C)*** *and vascular smooth muscle cells* ***(D)*** *were observed and confirmed in testis organoids. Lower row (control): intact testis tissue from a 1-wk-old piglet with the corresponding staining and immunohistochemistry for vascular structures* ***(E)*** *constructed by endothelial cells* ***(F&G)*** *and vascular smooth muscle cells* ***(H)***.

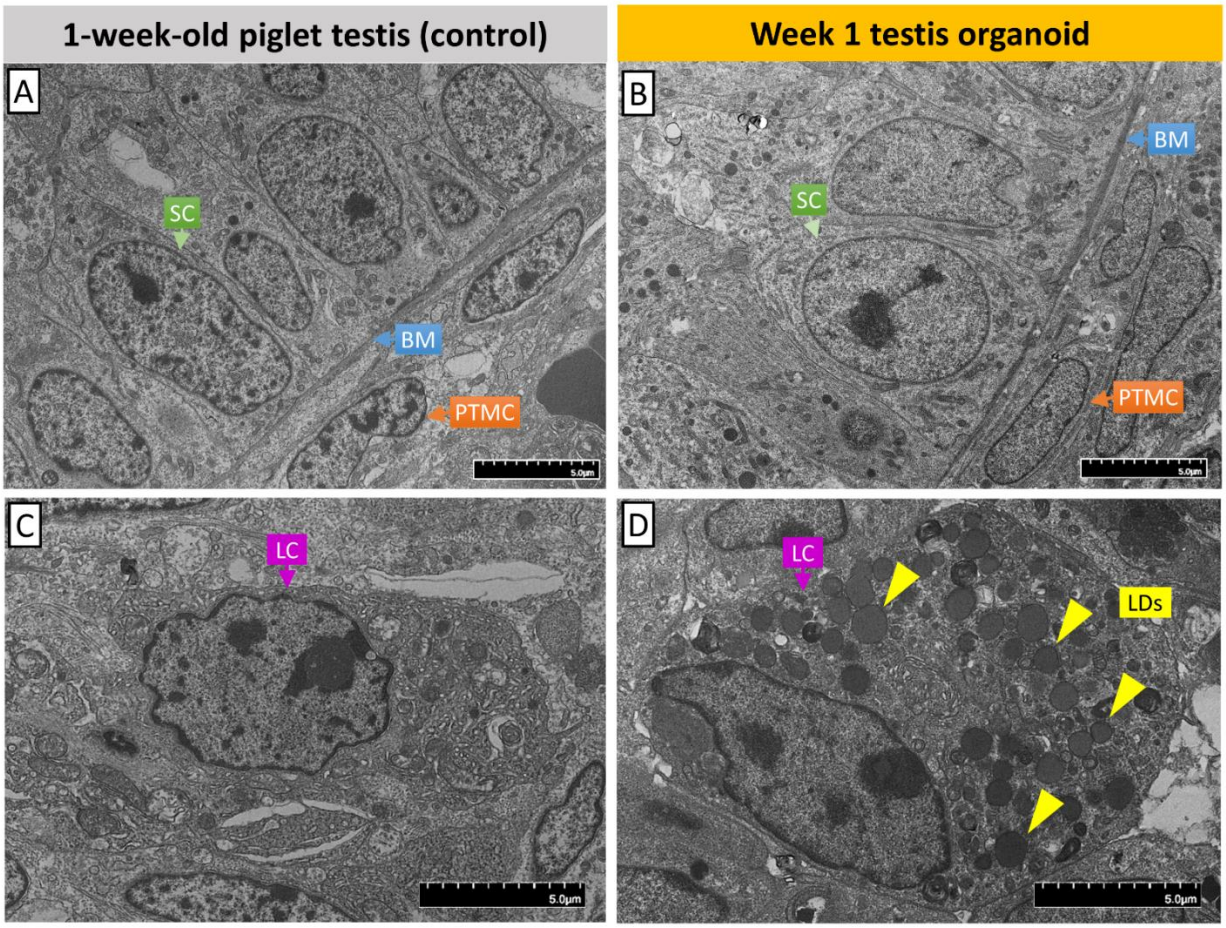

Figure 3.4. *Transmission electron microscope (TEM) to observe ultrastructure of testis organoids. Left column (control): Ultrastructure of testis tissue from 1-week-old piglet. Right Column: Ultrastructure of a week-1 testis organoid.* ***(A)*** *The native testis epithelium comprised of Sertoli cells (SCs), basement membrane (BM), and peritubular myoid cells (PTMCs).* ***(B)*** *A testis epithelium-like structures constructed by SCs, BM, and PTMCs was observed in testis organoids as early as 1 week of culture.* ***(C)*** *A Leydig cell (LC) in the control testis tissue.* ***(D)*** *Lipid droplets (LDs) were observed in LCs of a week-1 testis organoid, indicating initial androgen synthesis and functional maturation of LCs in the organoids.*

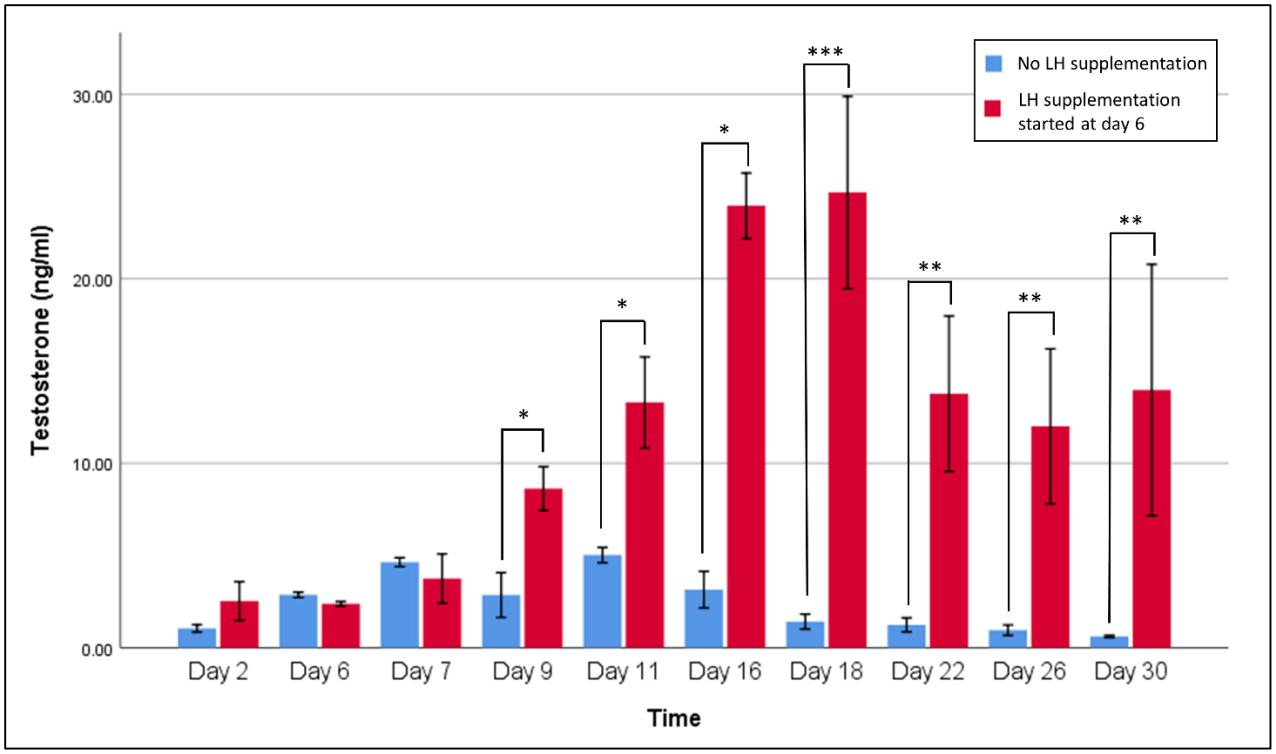

Figure 3.5. *Testosterone levels in the organoid culture media were measured via ELISA. Red columns: Luteinizing hormone (LH) supplementation in organoid culture media started at day 6 of culture. Blue columns: Control groups without LH supplementation. Testosterone levels (ng/mL) in the organoid culture media were measured over time. Data are presented as mean ± SEM. Data with asterisks differ significantly among groups (P: * <0.05, ** <0.01, *** <0.005). n=3 replications*

3.5 Discussion

The general objective of this chapter was to examine the structural and endocrine characteristics of the porcine testis organoid system. We first hypothesized that porcine testis organoids will have various testis cell types and structural components which would resemble intact porcine testis tissue *in situ*. Based on the IHC and tissue-specific staining results, our testis organoids consisted of tubular and interstitial compartments resembling innate testis tissue. Namely, gonocytes were observed within the testis tubular compartments reconstructed by Sertoli cells, PTMCs, and peritubular basement membrane. Also, reconstruction of the inter-tubular interstitial compartments containing Leydig cells, collagen fibers and vascular structures was observed in testis organoids. As described in Section 3.2, native testis tissue consists of both interstitial and tubular compartments, each with their own native cell populations and spatial orientation. The cellular and acellular components in both compartments contribute to the SSC niche that regulates the proliferation and/or differentiation of SSCs (Kubota and Brinster 2018). Therefore, the presence of major testis cell types and biomimetic cell polarity are keys to fabricating a functional testis organoid that recapitulates the SSC niche (Li, et al. 2017). As mentioned previously, the criteria for organoid formation include (a) testis cell reassembly, (b) the inclusion of major testis cell types, and (c) the compartmentalized architectures. One of the challenges in interpreting the findings of established testis organoid culture systems is that not all criteria are assessed in all studies. For example, immunostaining for Leydig cells and PTMCs was not performed in some studies leading to incomplete interpretation of the overall structures, cell types, and cell orientation in the culture systems (Lee, et al. 2007, Lee, et al. 2011, Yokonishi, et al. 2013, Yu, et al. 2009, Yu, et al. 2005). To our knowledge, only 3 testis organoid studies have achieved the criteria of forming organoids with biomimetic, compartmentalized, and complete testis tubular/interstitial structures comprising at least 3 major testis cell types (Edmonds and Woodruff 2020, Yokonishi, et al. 2013, Zhang, et al. 2014).

It is worth nothing that Yokonishi, *et al.* (2013) observed irregular architecture of the reconstructed testis tubular structures in mouse testis organoids (Yokonishi, et al. 2013). Namely, these tubular structures had uneven diameter and a maze-like configuration. In addition, only a few Leydig cells and germ cells were observed in these organoids. Similar cellular components and architectures were observed in current study and other organoid studies (Edmonds and Woodruff 2020,

Vermeulen, et al. 2019). The cause and consequence of unbalanced cell proportion and deformed testis tubular structures remain to be investigated. Nevertheless, these *in vitro* reconstructed structures are comparable to the *in vivo* reconstructed testis tubular structures using ectopic testis cell implantation, indicating that *in vivo* microenvironment is not necessary for *de novo* testis tubulogenesis (Kita, et al. 2007). On the other hands, proper cell maturation in the organoids is crucial for spermatogenesis and testis development. The deformation of seminiferous tubule and the absence of Leydig cell maturation were reported to hinder spermatogenesis in *Desert hedgehog* (*Dhh*) null mice (Clark, et al. 2000). Also, the maturation of Sertoli cells, especially the formation of blood-testis barrier (BTB), plays a critical role in regulating spermatogenesis (Setchell 2009). Therefore, the maturation of testis cells in organoids should be determined in future studies. Furthermore, strategies such as the addition of growth factors may potentially promote cell maturation. For instances, activin A was reported to support Sertoli cell proliferation and testis cord expansion (Ungewitter and Yao 2013); while, fibroblast growth factor 2 (FGF2) was reported to promote Leydig cell maturation and testosterone secretion (Ye, et al. 2017).

One of the most exciting findings in this study was the presence of vascular structures in testis organoids. There were also sinusoid-like structures near the capsule of organoids, constructed by endothelial cells and smooth muscle cells. Vascular structures were also observed in the interstitium between testis tubular structures. To date, the presence of vascular structure has not been reported in any of the previously reported testis organoid culture systems. The requirement or significance of a vascular network in an *in vitro* culture system is still unknown, especially given the absence of red blood cells. At the very least, it shows that the presence of blood is not a prerequisite for formation of blood vessels. Nevertheless, the presence of vascular structures in organoids might provide a previously unavailable *in vitro* model to examine hypotheses related to the SSC niche and embryonic testis organogenesis. For example, the SSC niche is said to require contributions from nearby blood vessels to function (Bhang, et al. 2018). However, it has been difficult to test this theory in an *in vitro* testis organoid system where the vascular network was lacking. This theory is also difficult to investigate using *in vivo* systems due to the interference of host-derived factors. Next, vasculature is also important for another elusive mechanism, the formation of testis cords during embryonic testis organogenesis. One theory proposes that migrating endothelial cells are responsible for signaling the patterning of testis cords during this period (Brennan, et al. 2003, Yao, et al. 2006). In other words, testis cords follow the pattern of

blood vessels and not vice-versa. Again, this theory has been difficult to be examined using the currently available *in vitro* or *in vivo* systems. Therefore, our organoid culture system provides a promising platform for various studies on the role of blood vessels in the SSC niche and testis organogenesis.

In vitro maturation of Leydig cells in fetal gonads was reported using an air-liquid interface culture method (Yuan, et al. 2020). To our knowledge, *in vitro* maturation of Leydig cells in neonatal testis cell-derived organoids has not yet been studied. In our studies, the presence of numerous cytoplasmic lipid droplets was observed in Leydig cells of organoids, indicating functional maturation of Leydig cells after one week of culture. In addition, LH-supplemented organoids secret significantly higher testosterone than control groups, suggesting that the Leydig cells in the organoids possess endocrine functionality and LH responsiveness. Stable secretion of testosterone for at least a month was reported in other neonatal testis cell-derived organoids in response to human chorionic gonadotropin (hCG) using mouse (Edmonds and Woodruff 2020) and pig models (Vermeulen, et al. 2019). It is worth noting that Edmonds and Woodruff (2020) showed that the testosterone secretion of organoids can be maintained for at least 12 weeks in response to hCG. hCG possesses α- and β-subunits that display homologies with LH and FSH (Nwabuobi, et al. 2017), and thus it has LH-like action on Leydig cells to induce testosterone secretion (Kerr and Sharpe 1986). In addition, since hCG has a longer *in vivo* half-life (33 hours) in comparison to LH (23 hours), it might also remain active longer than LH in the organoid culture media (Ezcurra and Humaidan 2014). Furthermore, combined supplementation of both FSH and hCG has also been reported to induce *in vitro* differentiation of SSCs to elongated spermatids in soft agarose- and methylcellulose-based testis cell culture (Stukenborg, et al. 2009). Therefore, more studies are required to compare the testosterone secretion in groups supplemented with hCG or LH.

Moreover, testosterone is a crucial hormone in supporting testis development and spermatogenesis, especially spermatogenic differentiation at meiosis stage (Preston, et al. 2012, Walker 2011); hence, it has been commonly supplemented in testis cell culture (Hadley, et al. 1985, Hadley, et al. 1990, Lee, et al. 2006b, Legendre, et al. 2010). However, based on the results in this study, the necessity of combined supplementation of both testosterone and LH in testis cell culture is uncertain due to the fact that testosterone can be produced by Leydig cells in response to LH induction (Edmonds and Woodruff 2020, Vermeulen, et al. 2019). More studies are required to

compare the degree of organoid formation and *in vitro* spermatogenesis in groups with or without testosterone supplementation. Next, dihydrotestosterone (DHT), a 5α-reduced metabolite of testosterone, is a more potent androgen that supports spermatogenesis and androgen-mediated events at puberty (Auchus 2004, Holdcraft and Braun 2004). The exact mechanisms as to why DHT has a higher potency are unclear, but it binds more tightly to androgen receptors leading to amplification of downstream gene transcription at lower concentration than testosterone (Wilbert, et al. 1983). The ELISA kit used in this study has only 27.4% cross reactivity with DHT; also, to our knowledge, no testis organoid study has specifically measured the DHT produced from the organoids. Hence, it will be interesting to investigate whether the organoids can secret DHT.

3.6 Conclusions

Our testis organoids consist of tubular and interstitial compartments resembling native testis tissue. Importantly, germ cells were observed within the testis tubular compartments reconstructed by Sertoli cells, PTMCs, and peritubular basement membrane. Also, reconstruction of the inter-tubular interstitial compartments containing Leydig cells, collagen fibers and vascular structures was observed in the organoids. Furthermore, the testis organoids possess endocrine characteristics of testosterone secretion and LH-responsiveness.

CHAPTER 4

GENERAL DISCUSSION AND FUTURE DIRECRIONS[5,6]

4.1 General discussion

Previously, our laboratory demonstrated that the *in vivo* implantation of neonatal piglet-derived primary testis cell aggregates under the skin of immunodeficient mice can induce *de novo* testis tubulogenesis and form a compartmentalized testis tissue (Honaramooz, et al. 2007). In the present studies, an *in vitro* testis organoid culture system derived from neonatal porcine testis cells has been established. Briefly, we cultured testis cells from 1-week-old piglets in ultra-low attachment U-bottom wells to form cell spheroids. The cell spheroids were then cultured on top of agarose gels immersed in media in an air-liquid interface system. In this way, the testis tissue could benefit from sufficient gaseous exchanges while receiving nutrients from the agarose gel below (Sato, et al. 2011, Yuan, et al. 2020). We also showed that cryopreserved testis cells can be utilized in this culture system and the reconstructed testis tubular structures can be maintained for at least 4 weeks. To our knowledge, this is the first report of building a porcine testis organoid using a scaffold-free spheroid-forming low attachment well using an air-liquid interface system.

In the 2nd chapter, we showed that the air-liquid interface system is crucial for cell maintenance and reorganization. Also, we found that a cell density of 0.8×10^6 cells/organoid improved *de novo* tubulogenesis in the organoids. Another important condition in this study was the use of neonatal testis cells as the cell sources. Many studies have shown that the *de novo* tubulogenic ability of primary testis cells is age-dependent; primary testis cells derived from neonatal/prepubertal animals retain the tubulogenic ability, whereas pubertal/adult-derived primary testis display a delayed or absent tubulogenic potential. As mentioned previously, Zenzes and Engel (1981), using a rotation culture, reported the ability of neonatal and juvenile rat-derived testis cells to reconstruct testis tubular structures. Under these same culture conditions, adult rat testis cells were unable to achieve the same results (Zenzes and Engel 1981). Alves-Lopes *et al.* (2017) also observed that 5 to 8- and 20-day old primary rat testis cells were able to reconstruct the spherical-tubular structures

[5] Several parts of future directions have been published as a review paper. Tat-Chuan Cham, Xiongbiao Chen, and Ali Honaramooz (2021). "Current Progress, Challenges, and Future Prospects of Testis Organoids". *Biology of Reproduction* (*in press*). The document has been reformatted from the original version for inclusion in the thesis.

[6] T.C. contributed to conceiving the concept, wrote the first draft of the manuscript, summarized tables, and prepared the figures.

in their 3D multilayered Matrigel model, but 60-day-old primary rat testis cells were not (Alves-Lopes, et al. 2017). Moreover, Edmonds and Woodruff (2020) found that reassembly of testis organoids in a non-adherent microwell is considerably delayed for pubertal mouse testis cells and completely absent for adult testis cells in both mouse and human models. Interestingly, they found that co-culturing immature and adult mouse testis cells concurrently allowed re-assembly to resume (Edmonds and Woodruff 2020). Primary human testis cells derived from adults or adolescents have been reported to form cell aggregates instead of testis cord-like/tubular structures within alginate (Lee, et al. 2006a), collagen (Lee, et al. 2007) and DECM scaffolds (Baert, et al. 2017). As mentioned in Section 2.2, only a single study has demonstrated the ability of primary testis cells from 6-month-old and 5-year-old prepubertal children to reconstruct a compartmentalized testis organoid (Sakib, et al. 2019b). This latter testis organoid was reported to have a reversed architectural structure with its seminiferous tubules described as 'inside-out'. Nevertheless, this provides evidence that prepubertal human testis cells do in fact conserve the ability to self-reassemble and become polarized autonomously. Furthermore, Yuan *et al.* (2020) was able to successfully recapitulate *in vitro* testis organogenesis from human fetal gonads to produce functional haploid sperms using an organ culture system (Yuan, et al. 2020). These results indicate that the immature testis cells within the fetal human gonad maintain their organogenic potential to reconstitute mature seminiferous epithelium and achieve complete spermatogenesis. That being said, it will be interesting to test the tubulogenic potential of fresh or frozen human testis cells using our system in future studies.

Next, as stated previously, there is no consensus on appropriate media supplementation for testis organoid cultures (Richer, et al. 2019). Therefore, we examined various combinations including 10%KSR only, 10%FBS only, 10%KSR + 5%FBS, and 5%KSR + 10%FBS, to find the optimal supplements for efficient tubulogenesis and germ cell viability in organoids. In our results, combined supplementation of 5% KSR + 10% FBS in media improved *de novo* morphogenesis of testis cord-like structures. Importantly, the relative number of gonocytes in organoids does not significantly decrease over time or differ among different supplementation groups. Besides serum-based supplements, hormone and growth factor-based supplements should also be tested in future studies. For instances, FSH was reported to promote SSC self-renewal, Sertoli cell aggregation, and proliferation *in vitro* (Mäkelä, et al. 2014, Ruwanpura, et al. 2008, Schlatt, et al. 1996). Growth factors, such as GDNF and activin A, are also reported to regulate neonatal Sertoli cell

proliferation, and promote SSC survival and self-renewal (Mendis, et al. 2011, Sadri-Ardekani, et al. 2009, Ungewitter and Yao 2013). Together, hormonal and growth factor supplements may potentially improve organoid formation and *in vitro* spermatogenesis; thus, further studies may aim to test these supplements using our system.

The reconstructed testis organoids consisted of immature germ cells (gonocytes), Sertoli cells, PTMCs, peritubular basement membrane, and inter-tubular interstitium containing Leydig cells, collagen fibers and vascular structures. To our knowledge, our study represents the first report of the presence of vascular structures in testis organoids which opens many possibilities for future research. The low number of germ cells has been an issue in testis organoids (Edmonds and Woodruff 2020, Vermeulen, et al. 2019). Based on the results by Sakib, *et al.* (2019b) and our study, enriching the germ cell proportion in starting cell source may not necessarily be an ideal solution to increase the number of germ cells in organoids due to the fact that insufficient testis somatic cells impair organoid formation. To address this issue, Edmonds and Woodruff (2020) suggested that germ cells can be injected into the reconstructed tubular structures in organoids using similar techniques in germ cell transplantation (Edmonds and Woodruff 2020, Tang, et al. 2012). Also, strategies such as reducing the thickness and size of tissue, organ-chip cultures, or a microfluidic device, should be attempted in future studies to improve nutrient diffusion and germ cell survival in organoids (Baert, et al. 2020, Kojima, et al. 2018, Komeya, et al. 2016, Yamanaka, et al. 2018).

4.2 Future directions

4.2.1 *In vitro* spermatogenesis

Successful *de novo* testis organogenesis requires not only the reconstruction of compartmentalized testis architecture, but also the *in vitro* recapitulation of spermatogenic differentiation from SSCs to morphologically and genetically diverse spermatozoa. Induction of successful meiosis with correct DNA content and chromosomal recombination is one of the major challenges for *in vitro* spermatogenesis (Handel, et al. 2014, Komeya, et al. 2018). Testis organ culture systems have shown promising results in inducing meiosis *in vitro* and even generating spermatids with correct chromosomal recombination that produce euploid embryos (Yuan, et al. 2020). These findings suggest that testis microenvironment, including cell-to-cell interactions, structural support of seminiferous epithelium, and the biological signals from extracellular matrices, plays an important

role in unblocking the barriers of *in vitro* meiosis. Despite tremendous efforts, the establishment of a testis organoid featuring physiologically accurate architecture and a complete spermatogenic cycle remains a major challenge in current organoid studies. Similar to this study, Yokonishi *et al.* (2013) used low attachment wells to form a highly compartmentalized murine testis organoid that resembles native testis tissue. In their results, the spermatogenic differentiation from SSCs to the round spermatid stage was detected by expression of meiotic markers (SYCP1 and *Acr*-GFP) and the presence of PAS-positive acrosomal cap (Yokonishi, et al. 2013). Since germ cells with PAS-positive cap were rarely observed in their study, the authors suggested that the degree of spermatogenesis in their culture systems remained to be determined. Recently, Edmonds and Woodruff (2020) used similar non-adherent microwells to build multicellular testis organoids possessing distinct tubular and interstitial compartments that had physiologically relevant cell orientations and endocrine functions (Edmonds and Woodruff 2020). Although gonocytes and SSCs were present in their organoids, they were rare, and no expression of meiotic markers and spermatogenic differentiation was detected. The cause of *in vitro* meiotic blockage in testis organoids remains to be determined. Strategies such as the use of scaffolds, addition of different supplements, and prolonging the culture duration may potentially unblock the barrier of *in vitro* spermatogenesis in organoids. In this study, the number of germ cells was very low and spermatogenic differentiation was not observed based on cell morphology. However, we only maintained the organoids for a few weeks which is likely not sufficient to observe extensive germ cell development from neonatal pig donor cells. Nevertheless, the spermatogenic differentiation in current study needs to be further investigated by genetic or protein markers.

4.2.2 Fabrication of a bioengineered testis organoid

Various tissue engineering techniques have been used to create and study bioengineered reproductive tissues. Examples of such tissues include the testis, uterus, and ovary (Gargus, et al. 2020, Jahanbani, et al. 2020). However, building a multilayered and compartmentalized scaffold-based testis organoid remains a challenge. Fortunately, newly developed tissue engineering techniques such as 3D printing provide potential tools in unblocking this barrier. Different scaffold designs and 3D printing methodologies can be potentially used to build a testis scaffold, many of which are based on pre-existing tissue engineering techniques. For example, the utilization of neonatal testis cells in a scaffold-based tubular sheath printed by the coaxial extrusion system is an appealing approach for building a self-assembling testis tissue strand (Akkouch, et al. 2015,

Yu, et al. 2016, Zhang, et al. 2015) **(Fig. 4.1a)**. Additionally, since multi-tubular scaffolds can be printed horizontally and by layer to fabricate a tubular scaffold in extrusion-based printing (Owens, et al. 2015), similar printing strategies can be used to build a scaffold-based seminiferous tubule. To achieve this, one could print a tubular scaffold with testis somatic cells (*e.g.* Sertoli cells and PTMCs) in the external layer and the spermatogenic cells within the core (**Fig. 4.1b)**. The design and fabrication of testis scaffolds must be comprehensively studied. For examples, the shape, external and internal architecture, pore size, porosity, and the topography of a testis scaffold must be optimized. Taken together, the combination of scaffolds and different fabrication techniques allow the development of a compartmentalized testis organoid that resembles the native testis microenvironment and favors spermatogenesis.

4.2.3 Testing alternative cell sources

Theoretically, primary testis cells with a mixed cell population are the ideal cell sources for making testis organoids. This is due to their high physiological relevance to the testis tissue composition and low risk of cell mutation compared with cell lines or induced pluripotent stem cells (iPSCs). However, obtaining primary testis cells requires fresh testis tissue samples, which are not always readily available. Fresh human testis samples are particularly difficult to obtain, due to the various legal and ethical implications in human research. Also, the amount of useable tissue from testis biopsies is often scarce. Thus, testis cell line and iPSC-derived testis cells can also be considered as an alternative cell source with which to fabricate a testis organoid in the future (**Fig. 4.2**). Here, we discuss the biological characteristics of these two potential cell sources in testis organoid culture systems, including testis cell lines and iPSC-derived testis cells.

Testis cell lines are immortalized cells derived from repetitive cell passaging or a tumorigenic source. The phenotypic and genotypic homogeneity of cell lines provides consistency throughout trials to the benefit of one's results. For these reasons, cell lines of various tissues and organs are commonly used and have been reported in many different fields such as *in vitro* toxicology testing (Hung, et al. 2016). However, excessive cell passaging can cause cell lines to lose their natural cellular characteristics, which compared with primary cell populations, renders them less biologically relevant to the tissue of origin. Excessive cell passaging can also result in genotypic mutation and phenotypic variation, leading to false findings when using cell lines in research. Hofmann *et al.* (1992) established murine testis cell lines of PTMCs, Sertoli cells, Leydig cells,

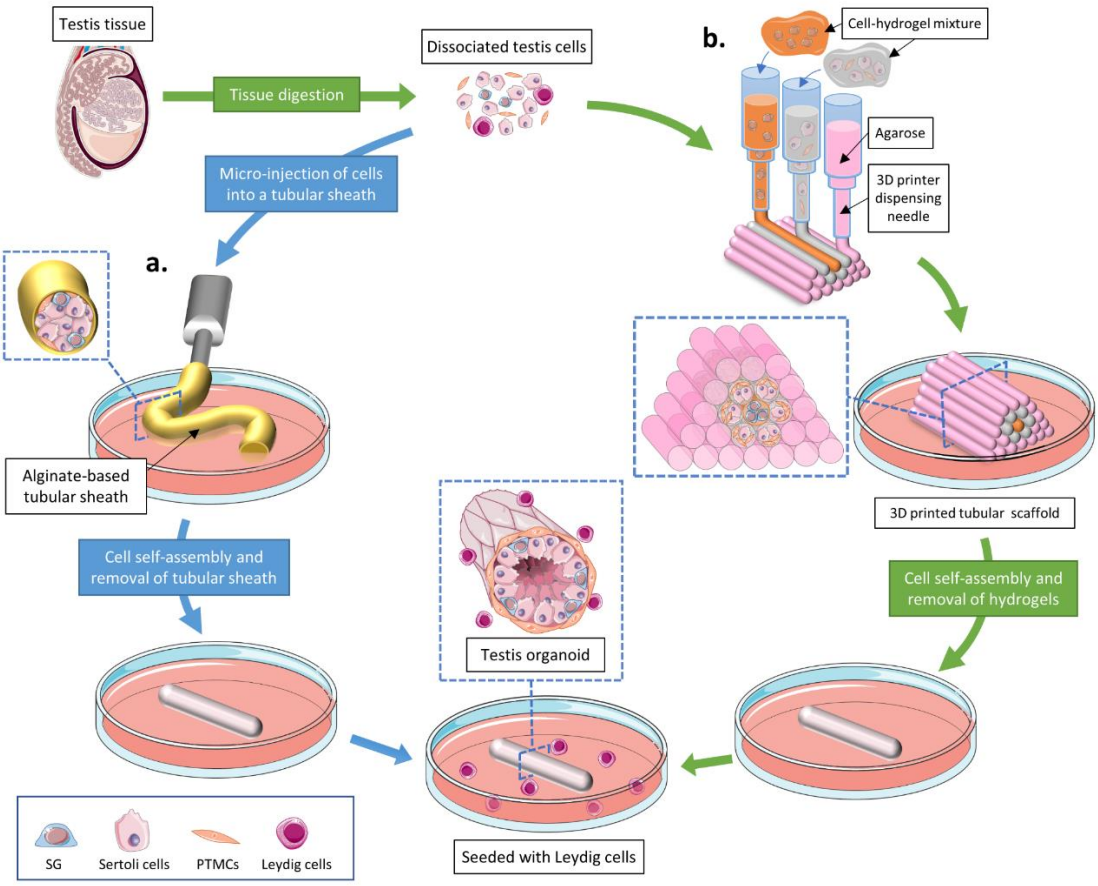

Fig. 4.1 *A schematic summary of potential fabrication strategies to build testis organoids, including the use of* ***(a)*** *Alginate-based tubular sheaths or* ***(b)*** *Multi-tubular scaffolds.* ***(a)*** *Testis cells are isolated from testis tissues and then aggregated and micro-injected into an alginate-based tubular sheath created by the coaxial extrusion system. Once the testis cells have occupied the tubular sheaths, the testis cells are then cultured in the bioreactor to promote cellular self-assembly and de novo reconstruction of the testis tissue strands. The alginate-based tubular sheath is removed once tissue reorganization is completed.* ***(b)*** *Agarose-based cylindrical strands are printed to provide a supportive first layer or base. Hydrogel cylindrical strands containing different testis cell populations are then printed and stacked on the supportive base to build a tubular scaffold; Sertoli cells and PTMCs are printed onto the external layer of the tubular scaffold, whereas spermatogenic cells are printed in the core of the tubular scaffold. The scaffolding biomaterials used for the cell-laden cylindrical strand should be cytocompatible to promote cell adhesion and de novo tissue formation. Natural hydrogels (e.g. solubilized DECM, collagen, Matrigel) are good options for this purpose. The printed tubular scaffold is then*

incubated in the bioreactor while the testis cells reorganize into a testis tissue strand. The agarose-based sacrificial strands are disintegrated once tissue reorganization is completed. The testis tissue strands can be further seeded with the Leydig cells to establish a compartmentalized testis organoid that resembles native testis tissue. (Cham, et al. 2021)

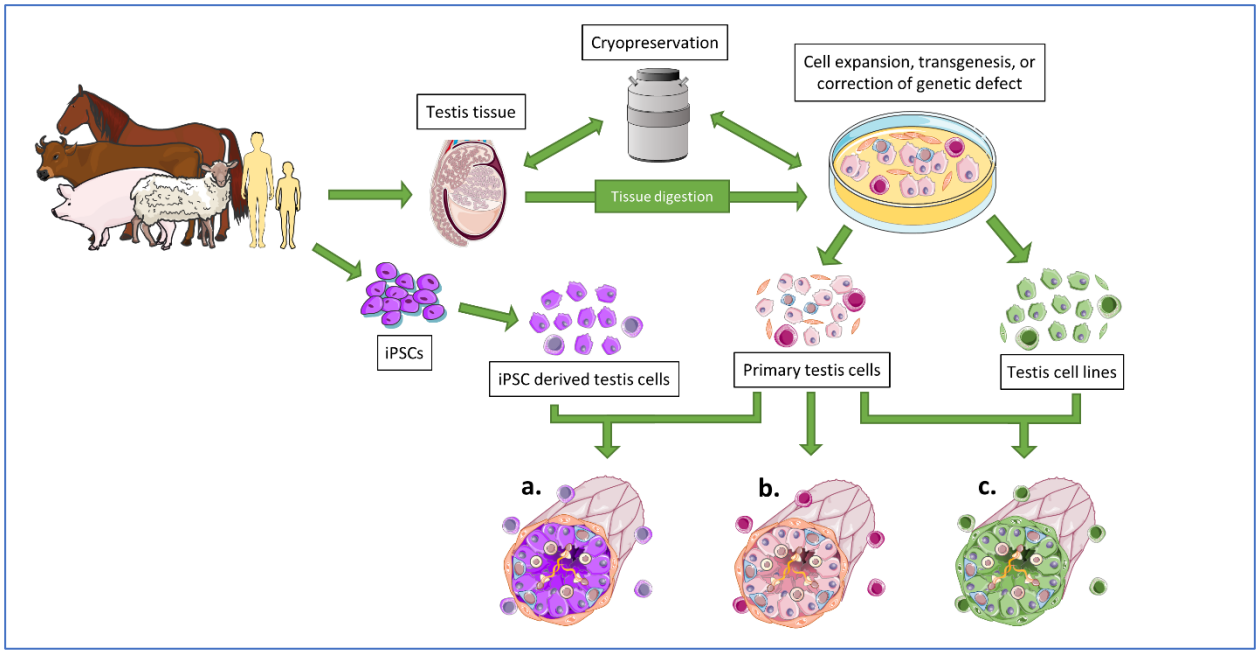

Fig. 4.2 *A schematics representation of using different cell sources including primary testis cells, testis cell lines, and induced-pluripotent stem cell (iPSC) derived testis cells to build testis organoids. To begin, testis tissues are collected from animals with high genetic value or from prepubertal cancer patients prior to receiving their treatment. The tissues can be digested to isolate the testis cells or cryopreserved for future use. Isolated testis cells can also be cryopreserved or expanded in vitro to increase the cell number. Strategies such as the removal of carcinogenic cells, the correction of genetic defects, or the production of transgenic cells/cell lines can be performed during cell culture. Primary testis cells, testis cell lines, and iPSC derived testis cells are then used as cell sources from which to build the testis organoids.* ***(a-c)*** *illustrate testis organoids that are constructed by cell sources such as* ***(a)*** *iPSC-derived Sertoli and Leydig cells + primary SSCs and PTMCs;* ***(b)*** *Primary testis cells only;* ***(c)*** *PTMCs, Sertoli, and Leydig cell lines + primary SSCs. (Cham, et al. 2021)*

and germ cells to study testis cell interactions within 2D culture conditions. They found that testis cell lines formed cell aggregates which histologically resembled the 2D cross-section of the *in situ* seminiferous tubules. They further found that co-culturing murine primary pachytene spermatocytes with the testis somatic or interstitial cell lines resulted in reconstruction of testis cord-like structures, where pachytene spermatocytes were thought to integrate within the cords (Hofmann, et al. 1992). Van Der Wee and Hofmann (1999) cultured the same cell lines on a growth factor-supplemented layer of Matrigel to find that the Sertoli cell lines reconstructed a lumen-containing tubular structure that resembled mature seminiferous tubules *in situ*. They also reported that certain ECM components (*i.e.* laminin and collagen IV) play crucial roles in the formation testis cord-like structure and that hepatocyte growth factor (HGF)/*c-met* is necessary for the tubular remodeling of the testis cord-like structures (Van Der Wee and Hofmann 1999). Moreover, Pendergraft *et al.* (2017) co-cultured the lentiviral transduced Leydig and Sertoli cell lines with the primary SSCs using the hanging drop culture system. The resulting human testis organoid produced testosterone actively and supported the transition of diploid to haploid spermatogenic cells (Pendergraft, et al. 2017). Taken together, these studies demonstrate that a mixture of primary spermatogenic cells and testis somatic and interstitial cell lines provides an appropriate alternative cell source for the fabrication of a testis organoid. However, the major concern of carcinogenicity within testis cell lines remains. To remedy this, the nuclear DNA/chromosome contents in the integrated primary spermatogenic cells should be carefully examined.

On the other hand, iPSCs possess the 'stemness' characteristics to either self-renew or differentiate into other specialized cell types such as testis cell types (Bilic and Belmonte 2012, Takahashi and Yamanaka 2006). The *in vitro* differentiation of iPSCs into Sertoli cells (Rodríguez Gutiérrez, et al. 2018), Leydig cells (Chen, et al. 2019, Li, et al. 2019), and germ cells (Hayashi, et al. 2018) has been reported in different species, including mouse and human models. Similar to cell lines, the major drawbacks of iPSCs include the safety concern of genetic modification and resumption of pluripotency, both of which may lead to neoplastic development. Different strategies, such as the use of plasmid vectors or non-integrating viral factors, have been attempted to minimize the risk of viral or oncogenic activity in iPSCs (Medvedev, et al. 2010). Although the utilization of iPSCs in fabricating a testis organoid has not been attempted yet, the plasticity, differentiation potential, and the unlimited self-renewal capacity of the iPSCs make them an appealing putative cell source.

4.2.4 Pharmacotoxicology tests

Drug development requires toxicology screening which often results in large numbers of candidate components being rejected due to their side effects such as renal, hepatic, cardiovascular, nervous, or reproductive toxicity. On the other hand, environmental toxicants and their potential effects on human/animal health have been a subject of interests in toxicology research (Cheng, et al. 2011, Faustman, et al. 2000, Lippmann 2000, Wong and Cheng 2011). Especially, testicular toxicity has become one of the crucial parameters in assessing potential drugs and environmental toxicants given the potential effects on fertility (Gao, et al. 2015, Mathur and D'cruz 2011).

Various environmental toxicants have been reported to have negative effects on spermatogenesis leading to male infertility. For examples, heavy metals, such as cadmium, are reported to impair germ-Sertoli cells junction, blood-testis barrier (BTB), sperm production, and Sertoli cell viability (El-Demerdash, et al. 2004, Hew, et al. 1993, Sakib, et al. 2019b). These metals ubiquitously exist and have long biological half-life, leading to long-term environmental pollution and gradual accumulation in the body (Wirth and Mijal 2010). Next, since pesticides have been commonly used in agriculture and food production, their possible toxicological effects on male reproduction have been widely studied. For instance, organochlorine pesticides, such as lindane (γ-hexachlorocyclohexane), have been reported to have negative impacts on testis cell function including transient inhibitory effects on testis steroidogenesis, impairing gap junction communications between Sertoli cells, and causing abnormal germ cell maturation (Batias, et al. 2000, Defamie, et al. 2001, Saradha and Mathur 2006, Traina, et al. 2003). Moreover, chemotherapeutic drugs such as cisplatin (cis-diamminedichloroplatinum-II, CP) have been reported to cause oxidative stress and reactive oxygen species (ROS) formation leading to CP-induced testicular toxicity (Ateşşahin, et al. 2006, Azu, et al. 2010, Sherif, et al. 2014, Türk, et al. 2008). The pathological mechanisms of most aforementioned toxicants are poorly understood; in addition, unknown toxicants are still universally present around us and remain to be investigated (Cheng, et al. 2011, Gao, et al. 2015, Lippmann 2000, Wong and Cheng 2011).

In vivo toxicological tests cost a lot and are time-consuming, and their use raises ethical concerns about animal research. In comparison, *in vitro* models are less expensive and less time consuming but since toxicants generally affect more than one cell type, *in vitro* toxicological tests on single types of testis cells in culture are not ideal (Reis, et al. 2015). Additionally, most *in vitro* cell

culture models do not provide a physiologically relevant testis structure and cellular orientation. Our organoid culture system includes more than four major testis cell types, and the reconstructed testis organoid has cellular orientation and architectural structures that resemble native testis tissue. Moreover, the experimental environment in the organoid culture is more standardized and consistent without unwanted interference from host animal's uncontrolled conditions *in vivo*. Therefore, our testis organoid system provides a new platform for pharmacotoxicology tests in a controlled and consistent culture environment without the use of animals.

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