2015-06-12

Use of de novo Formation of Testis Tissue to Study Cell Interactions in the Neonatal Porcine Testis

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Use of *de novo* Formation of Testis Tissue to Study Cell Interactions in the Neonatal Porcine Testis

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE

DEGREE OF DOCTOR OF PHILOSOPHY

GRADUATE PROGRAM IN VETERINARY MEDICAL SCIENCES

CALGARY, ALBERTA

MAY, 2015

Abstract

The work presented in this dissertation employs the use of *de novo* formation of seminiferous tubules *in vivo* and *in vitro* to study the testicular microenvironment, with a focus on the role of Vascular Endothelial Growth Factor A (VEGFA) and the primary cilium organelle. First, we optimized the *de novo* morphogenesis assay by testing different methods to improve the spermatogenic efficiency of the bioassay. We showed that addition of Matrigel to the cell pellet prior to grafting results in a better efficiency than enrichment of germ cells, and when cells are grafted under the same conditions, the number of cells transplanted directly relates to the number of seminiferous tubules formed. Second, we evaluated the role of VEGFA on the reestablishment of the testicular microenvironment. We showed that *de novo* formed tubules developed from cell pellets treated with VEGFA contained higher number of proliferating cells per tubule and higher spermatogenic efficiency. These findings suggest that VEGFA has a protective role during the grafting process. Another study explored the stirred suspension bioreactor (SSB) as a novel method to enrich for germ cells on a large scale. We used the optimized *de novo* morphogenesis assay to functionally assess the physiological abilities of cells cultured under shear force stress in the SSB. Results of these experiments indicate that SSB is a practical alternative to attain enriched populations of germ cells on a large scale, which maintains the functional spermatogenic potential of the cells. Finally, the last chapter focused on the role of the primary cilium in the testicular microenvironment. This set of experiments used the small molecule inhibitor, Ciliobrevin D, to disturb the organelle. We demonstrated that lack of primary cilia blocks Hedgehog signaling and impairs tubule formation *in vitro*. Overall, the work presented in this dissertation contributes to the improvement of available models to study male reproductive biology and to our understanding of the role of VEGFA and primary cilia in the testicular microenvironment.

Acknowledgements

First and foremost, I would like to thank Dr. Ina Dobrinski for all the support over the last five years. I thank you for the opportunity and for believing in my potential to be a researcher.

I would like to acknowledge my family, Euan, Mum, Dad, my siblings, Carol and Marcel, and my dear Zeca for their unconditional love through this journey.

I also would like to show my deepest appreciation to my co supervisor, Dr. James Cross, thanks for the meetings and the guidance when I needed. And I would l like to thank my supervisory committee, Dr. Jeff Biernaskie, Dr. Jacob Thundathil, and Dr. Derrick Rancourt, for the technical guidance, support, teaching and advice over the past five years. Dr. Biernaskie, thank you for the extra meetings, the teaching opportunity, guidance and motivation.

I would also like to thank all members of the Dobrinski lab. In particular, I thank Whitney Alpaugh for being a great friend and confident, it was great having you around to share experiences as a student in the lab.

I would like to acknowledge UCVM for the Entrance Awards scholarship, Alberta Children's Hospital Research Institute for the Graduate Student Trainee scholarship, and the Alberta Innovates for Health Research for the Graduate student scholarship.

I would like to thank the employees from Sunterra Farms, Strathmore, especially Frank for always welcoming me with a great smile while providing material for our research.

Lastly, I would like to thank the friends I made through graduate school and during the last, life became better with you in it.

Dedication

To my husband Euan, for the devoted love and care, for always challenging my beliefs and reminding me of the greater purpose in life. To mum and dad, Val and Gerson, for your unconditional support through my academic endeavours. I love you.

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List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
RPM	Rotation per Minute
AMH	Anti-Müllerian Hormone
ART	Assisted Reproductive Technologies
BTB	Blood-Testis-Barrier
Dhh	Desert Hedgehog
DPC	Days post coitum
EMC	Extra Cellular Matrix
FGF	Fibroblast Growth Factor
FGF9	Fibroblast Growth Factor 9
FGFb	basic Fibroblast Growth Factor
FLT1	fms-related tyrosine kinase 1
GDNF	Glial cell line derived neurotrophic factor
GCT	Germ Cell Transplantation
ICSI	Intracytoplasmic Sperm Injection
Gli	Glioma
Hh	Hedgehog
IF	Immunofluorescence
IFT	Intraflagellar Transport
Ihh	Indian Hedgehog
KDR	Kinase domain receptor
NRP	Neuropilin
PCG	Primordial germ cells
PDG2	Prostaglandin D2

PDGF	Pellet derived Growth factor
PDGF	Placenta Growth Factor
PTC	Patched
PTM	Peritubular Myoid Cells
RPM	Rotation per minute
SCF	Stem Cell Factor
SDF-1	Stromal Cell Derived Factor 1
SMO	Smoothened
SRY	Sex Determining Region on Y chromosome
SSC	Spermatogonial Stem Cell
TGF-b	Basic Transforming Growth Factor
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor

1 Chapter I: Introduction

1.1 Introduction

The evolutionary goal of all living organisms is to pass their genes to further generations and perpetuate their existence. When we study developmental biology, from gonad morphogenesis to mature organs which are able to generate fertilization competent gametes, we are elucidating the core of life.

This dissertation focuses on the male gonad and the cellular interactions taking part in the testicular microenvironment. The detailed understanding of the communications between germ cells and their niche in the testis is essential to harness the power of germ cells and male fertility. This can lead to advances in reproductive biotechnologies, agriculture and medicine.

The development of the male reproductive system is an intricate process that starts early during development with the expression of the SRY gene by precursor of Sertoli cells, and will trigger a cascade of events building the male gonad [1].

Males differ from females, where germ cell development arrests before birth, in that they have a mitotic germ cell population, the spermatogonia stem cells (SSCs), which undergo multiple divisions providing a continuous supply of precursor cells for the beginning of spermatogenesis through adult life. For that to happen, SSCs rely on the testicular

microenvironment, the niche, to provide guidance and support, sustaining self-renewal in symmetrical and/or asymmetrical divisions [2, 3].

SSCs are scattered through the convoluted seminiferous tubules and constitute presumably 1 in every 3000 cells in the mouse testis population [4]. Previous studies provided evidence that SSCs are not randomly distributed, but restricted to specific sites of the basal compartment adjacent to blood vessels and interstitium, pointing towards possible interactions between the vasculature and the SSC testicular niche but there are still many undetermined factors [5, 6]

Numerous obstacles hamper the study of testicular morphogenesis, the SSC, and its niche, particularly in higher mammals. The majority of presented data is derived from murine species. In this chapter, concepts of male reproduction, current topics of the testicular niche, and the use of the pig as an animal model for human research will be presented.

1.2 **Male Gonad**

1.2.1 **Sex Determination**

In mammals, during initial stages of embryonic life, primordial germ cells (PGCs) are bipotent, meaning their surrounding environment determines their fate at later stages of development. Initially located in the epiblast, PGCs passively relocate to the extra-embryonic mesoderm as development progresses. In the mouse, at 9.5 day post coitus

(dpc), PGCs migrate, and colonize the genital ridge where they meet somatic precursor cells. [1, 2]

The process of PGC migration is still not entirely elucidated. From what is known, fibroblast growth factors (FGF) [7], transforming growth factor (TGF-b), stromal-cell-derived factor 1 (SDF-1), stem cell factor (SCF) and c-Kit are major players in a complex signaling network [8]. During this migration, PGCs are exposed to a dynamic interaction with components of the extracellular matrix (ECM) such as adhesion molecules (β1, α6 integrins, E-cadherin, and Ep-CAM) that are believed to facilitate interaction and signaling along the cell surface [9].

After PCGs enter the genital ridges, the initially strong PGC-fibronectin bond is weakened and their adherence to laminin increases [10], these changes in PCG-ECM interaction pattern indicates that the ECM helps modulating PCG migration and gonad formation. Once in the gonadal ridges, at 10.5 dpc, PCG fate is determined; the turning point for gender determination is the expression of the SRY transcription factor (sex determining Region on Y chromosome), by a subset of somatic cells [1, 11]

1.2.2 **Testis Morphogenesis**

SRY triggers the expression of SOX9, leading to the specialization of Sertoli precursor cells [11], initiating activation of transcription factors that will further promote differentiation of Sertoli and other cell lineages. This helps to orchestrate the formation of testis cords, interstitium, and vascularization [12].

These signaling pathways involve secreted ligands such as fibroblast growth factor 9 (FGF9) [13], prostaglandin D2 (PGD2) [14], anti-Müllerian Hormone (AMH) [15] and desert hedgehog (Dhh); While the expression of SRY and most of transcription factor genes is transitory [16], Dhh is expressed throughout the male's lifespan [17].

Sertoli cells will surround clusters of PGCs, followed by peritubular myoid cells, derived from the mesonephros, that will surround these aggregates of Sertoli cells and PGCs, forming the basement membrane and testicular cords. At Day 12.5, the gonad is fully assembled [18], PGCs stop proliferating, arrest in G0/G1 mitosis, occupy the center of the cords and become gonocytes. Gonocytes have a very distinct morphology as they are large circular cells with a prominent nucleus surrounded by a ring-like cytosol, containing one or two nucleoli [19]. Gonocytes will differentiate into Type A spermatogonia and initiate complete spermatogenesis in post-natal stages in close contact with the testicular niche.

1.2.3 **Spermatogonial Stem Cell Niche**

During the neonatal phase, the testis remains in the fetal stage configuration - gonocytes are at the lumen of the testicular cords, and proliferating Sertoli cells are surrounded by peritubular myoid cells which arrange the basement membrane and segregate the cords from the interstitial tissue where Leydig cells, vasculature, macrophages, lymphatics, and nerves are located. The migration of gonocytes to the basement membrane occurs, post-natal, at day 3 to 6 in mice [20], and in the pig, gonocyte migration begins during the first week of life and gonocytes can no longer be identified at the fourth month of life [21]. In

the course of the homing process towards the basement membrane, gonocytes differentiate into two different populations, spermatogonia stem cells (SSC), and Type A spermatogonia. The first, SSCs, will provide an infinite supply of cells for spermatogenesis by their differentiation into Type A spermatogonia. The first cohort of Type A spermatogonia that arises directly from gonocytes is committed to the first round of spermatogenesis at the onset of puberty [22].

1.2.4 **Spermatogenesis**

The testis produces a lifelong supply of fertilization competent gametes by a highly structured process called spermatogenesis. This process relies on the endocrine system and on Sertoli, Leydig, PTM cells, ECM and vasculature to coordinate germ cell proliferation and differentiation [23]. The generation of spermatozoa is one of the most productive biological process in the body [24] and the SSC are responsible for providing an unlimited pool of cells to replenish the gonad with the differentiating germ cells [25]. Spermatogenesis can be divided into three phases including proliferation, meiotic divisions, and spermiogenesis, the final phase of differentiation within the basal and adluminal compartments of seminiferous tubules resulting in functional spermatozoa. During proliferation SSC undergo a series of mitotic divisions, resulting in identical clones or daughter cells committed to further successive divisions involved in spermatogenesis. Spermatogonia in this stage can be A-single (As), A-paired [26] and A-aligned (Aal) spermatogonia [27], which differ in their topographical arrangement on the seminiferous tubule basement membrane. The Apr and Aal spermatogonia are

characterized by the presence of intercellular bridges that connect both cell types. It is uncertain how interchangeable the As and other states can be. An elegant manuscript used an OCT4-GFP reporter in cells and *in vivo* imaging and demonstrated that Aal can revert to the As state [28]. The following mitotic division will lead to differentiation into Type B spermatogonia.

The final division of B spermatogonia results in preleptotene spermatocytes, or primary spermatocytes. They will start meiotic division, pass the BTB, and switch their location to the adluminal compartment of the seminiferous tubule. Once protected by the immune-privileged site, the primary spermatocytes can synthetize DNA and complete the first round of meiosis going from preleptotene spermatocytes to leptotene, then zygotene, and finally achieving the pachytene spermatocytes stages. The pachytene primary spermatocytes undergo the first meiotic division, then become secondary spermatocytes and initiate meiosis II. This leads to the production of four haploid round spermatids from each primary spermatocyte [29]. After the second stage of spermatogenesis is completed, spermiogenesis follows.

Spermiogenesis involves round spermatids morphologically changing to become elongated spermatids. During this process, the acrosome is assembled, the nucleus condensates, the spermatid elongates with subsequent production of a flagellum (similar structure as the motile cilia), and finally the reabsorption of the cytoplasmic droplet characterizes the end of the process [30, 31].

1.3 Spermatogonial Stem Cells and their Niche

For decades Sertoli cells were the main somatic cell of interest in the testis, they dominated the investigation of SSC niche components and, as a consequence, there is a deeper understanding of the Sertoli cell's supportive role during spermatogenesis. Sertoli cells provide anchorage, nutrients, and can help determine the SSC's fate through the release of growth factors. Nevertheless, it should be emphasized that the niche comprises the group of Sertoli, peritubular myoid and Leydig cells together with the basement membrane and the vasculature and other interstitial cells, and the environment which they generate a complex interplay of cues and signaling [32, 33]. This environment provides nutrients and influences on SSC dictating their fate between self-renew or differentiation.

1.3.1 Sertoli cells and the Blood-Testis Barrier

Before puberty, Sertoli cells proliferate and mature, producing nutrients and fluids and turning cords into hollow structures, the seminiferous tubules. During puberty, proliferation ceases and the differentiation process begins. Sertoli cells are located along the basement membrane throughout the seminiferous tubules in intimate contact with SSCs. They provide growth factors such as glial cell line derived neurotrophic factor (GDNF), basic fibroblast growth factor (bFGF) and interaction with adhesion molecules influencing SSC fate. Sertoli cells form the blood-testis barrier (BTB) composed by desmosome-like, gap and tight junctions and ectoplasmic specializations formed by interactions of proteins from the cadherin, occludin, claudin and integrin families [34]. The BTB creates two compartments: basal and adluminal. The basal compartment is

where germ cells undergo mitosis and are in closer contact with the basement membrane; whereas the adluminal compartment controls the entrance of molecules and protects post meiotic germ cells from the host immune system. Leydig, PTM and other interstitial components of the niche reach SSC through indirect or secondary contact. PTM cells release GDNF [35] and, together with Leydig cells, they supply CSF-1 [36, 37]. Currently, it is believed that somatic components of the niche are homogenously distributed, Sertoli cells are located along the seminiferous tubules, PTM around them, and interstitial cells in the interstitium. Previous studies have provided evidence that, despite SSC being located at the basement membrane along the seminiferous tubules, their distribution is not random; they are asymmetrically distributed, clustered adjacent to the vascular branch points and interstitial cells which indicates a role for the blood supply in guiding the niche orientation in the testis [5, 6]. Data indicating the proximity of SSCs to the interstitial vasculature points to a possible role for the family of Vascular Endothelial Growth Factors (VEGF) factors as a key regulator of the SSC niche.

1.3.2 **Vascular endothelial Growth Factor and the SSC niche**

The VEGF family consists of VEGFA, VEGFB, VEGFC, VEGFD and Placenta Growth Factor (PGF). VEGFA has been heavily studied and is a major promoter of physiological and pathological angiogenesis [38, 39]. It is a mitogenic protein stimulated by hypoxia, required for angiogenesis, proliferation and migration, and it also has been demonstrated to have non-vascular targets [40, 41]

Up to this point, three VEGFA tyrosine kinases receptors (VEGFR) have been identified Flt1 (VEGFR1), Flk1 (VEGFR2), and VEGFR3. There is evidence supporting VEGFR-2 as the main receptor to VEGFA, being accountable to promote developmental angiogenesis and hematopoiesis; FLK-1 null mice fail to develop organized vasculature and result in embryonic lethality between 8.5 and 9.5dpc [42]. VEGFA can also interact with the soluble cofactors: neuropilin 1 (NRP1) and NRP2, they are able to facilitate or suppress VEGFA isoform-receptor interaction.

Activation of the pathway starts by receptor dimerization and phosphorylation, it activates several signal transductions proteins capable to elicit a myriad of responses by the cells, including activation of cell survival mechanisms through Akt/PKB signalling, proliferation through PI3k and ERK1/2, and cellular migration through the activation of p38 MAPK [43, 44] (see Figure1.1).

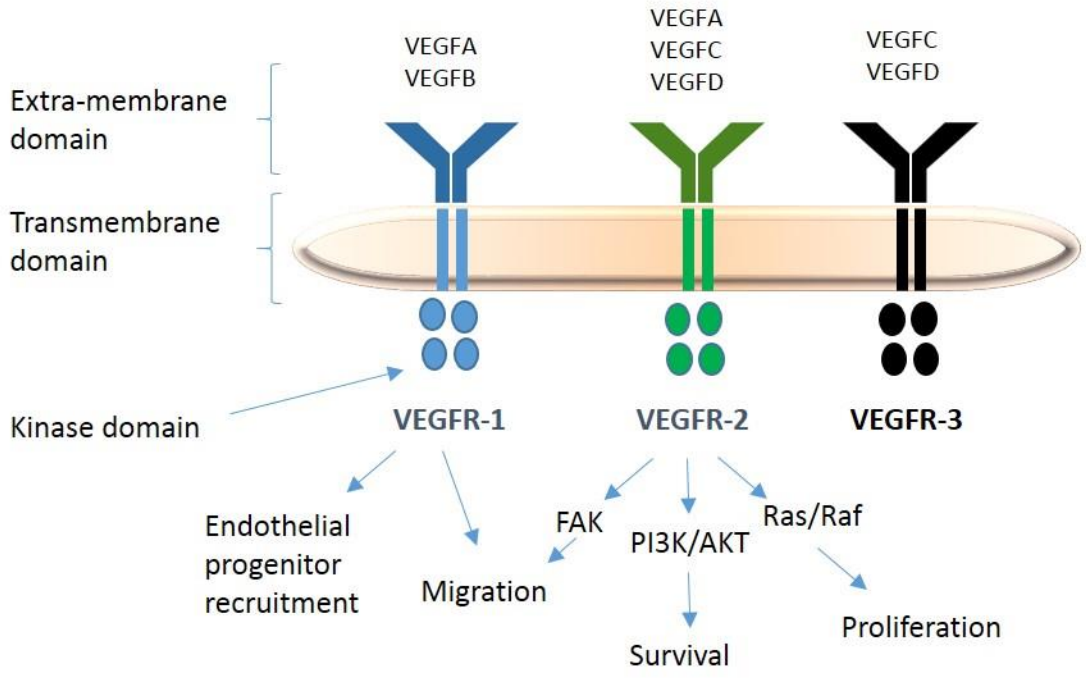

Figure 1-1. Illustration of the VEGF signalling pathway. VEGF ligands bind to the extra-membrane domain of the receptors trigger dimerization, and/or auto-phosphorylation of the tyrosine kinase domain of the receptor, and initiate activation of a cascade of downstream signaling transduction pathways.

VEGFA displays different isoforms, proangiogenic and antiangiogenic, which are generated through alternative splicing. Likely, antiangiogenic isoforms counterbalance proangiogenic isoforms and prevent unrestrained vascular growth [45, 46].

In the testis, VEGFA is important during early stages of development promoting formation of sex cords [47]. It has been demonstrated that this factor plays a role in testis differentiation- especially in cord formation and vascular development [48]. Inhibition of

VEGFA has been shown to induce aberrant endothelial cell migration inhibiting germ cell aggregation or formation of sex-specific vasculature [47].

In post-natal life, VEGFA is expressed in testicular endothelial, in proliferating germ, in Leydig and in Sertoli cells [49-51]. One manuscript using germ cell transplantation assay (GCT) demonstrated that in mice, the VEGFA angiogeneic isoform benefits SSC proliferation, in a contrasting manner the VEGFA anti- angiogeneic isoform promotes SSC differentiation [52]. Knockout mice displaying a conditional deletion of all VEGFA isoforms in the testis demonstrated a low number of undifferentiated spermatogonia and were infertile [53]. Nonetheless, up to this point, the non-vascular role of the VEGFA family in the postnatal testis is still poorly understood, and the exact roles of VEGFA isoforms during testis development and early postnatal stages have not been defined.

At postnatal stages there is no active angiogenesis under physiological conditions, nonetheless, Sertoli, Leydig, and peritubular myoid cells release VEGFA and display its receptors; Moreover SSC, in different stages of germ cell development, display different receptors: undifferentiated spermatogonia have VEGFR-2 while pachytene spermatocytes and round spermatids, express VEGFR-1 [54].

The elaborate assembly of the testis and the dynamic and fast paced development of different testis stages (fetal, prepubertal, and adult), challenge our current knowledge and raise questions regarding underlying signaling events that mediate these highly dynamic changes. This is particularly evident at the early stages, where there are gaps in the current understanding about how the gonads are assembled, and the niche is formed. In

an attempt to shed light to interactions amongst pathways and growth factors, our group started to investigate the role of primary cilia in the testicular microenvironment.

1.3.3 Primary Cilia and the Testis Microenvironment

There are two types of cilia organelles in eukaryotic cells, both are formed by an axoneme body made of microtubules, based on a basal body with a centrosome at its foundation. They protrude to the cytoplasm of the cell, and in a simplistic manner can be classified as motile and primary cilia [55, 56].

Motile cilia have been the object of extensive investigation. This organelle is assembled by a central pair of single microtubules, associated with dynein arms, radial spokes, and nine peripheral microtubules connected by nexin links which gives the organelle a recognizable ’9+2’ microtubule architecture [57]. Motile cilia are found in specialized sites, such as oviduct, trachea, efferent ductules and middle ear. Their main function is to promote movement and absorbance of fluid. For example, in the respiratory tract they absorb mucus, and in the oviduct they propel the embryo towards the uterus [58-60].

Primary cilia are present in virtually every eukaryotic cell. For many years they were considered a vestigial organelle and not given much consideration. For example, literature focussing on this organelle is scarce from the mid-1980s to late 1990s [61, 62], but it boomed, when in the early 2000s, human disorders were correlated to primary cilia abnormalities, and their role as a hub for signaling pathways has started to be revealed [63, 64]. This information has begun to shed light on the importance of the primary

cilium and could explain why the organelle was preserved in eukaryotic cells [65]. Ciliary deficiencies are now linked to a variety of diseases, such as anomalies in left-right asymmetry (situs inversus), polycystic kidney and genetic disorders like Jeune, and Bardet-Biedl syndrome [66], which are caused by mutations in different basal body genes. Primary cilia lack a central pair of microtubules, therefore the dynein arms display a '9+0' microtubular organization. This structure attaches to the mother centrosome and its presence is related to the cell cycle. Cells undergoing mitosis disassemble the structure for the completion of cell division. Different than other organelles in the body, primary cilia only develop when cells exit cell cycle from mitosis into a differentiated or quiescent state [67].

Primary cilia cannot synthesize proteins but promote communication between cell and environment via a unique intraflagellar transport system [68]. The IFT has a retrograde and anterograde traffic that mediates pathways like Hedgehog and platelet derived growth factor (PDGF) signalling [69, 70], and dictates decision between canonical or non-canonical WNT through Ca+ influx [71]. Such pathways are fundamental during embryo development, guiding cell patterning, differentiation and homeostasis [71-73].

1.3.4 **Hedgehog Signaling, Primary Cilia and the Testicular Microenvironment**

The activation of the Hh pathway is exclusively associated with the existence of primary cilia. Hh protein binds to transmembrane receptors called Patched (Ptc). Ptc inhibits a transmembrane protein called Smoothened (Smo), which will signal through glioma transcription factors [74], Gli1, Gli2, and Gli3 activating or inhibiting Hh gene targets in

the nucleus [69, 75, 76]. It has been extensively demonstrated in knockout mouse models, that disturbances in the IFT traffic complex lead to defects of Gli processing and blockage of the Hh pathway [77].

In mammals the Hh family consist of three genes: Sonic hedgehog (Shh), Desert hedgehog (Dhh) and Indian hedgehog (Ihh) [78]. In the male gonad, Dhh is expressed by precursors of Sertoli cells right after SRY expression and, in mice, the receptor Ptc1 is present in interstitial cells 12.5 days post coitus. Dhh-null animals are infertile, have anastomotic seminiferous tubules, peritubular cell abnormalities, and absence of adult-type Leydig cells [17]. Additionally, the receptor Ptc2 has been identified in spermatocytes of reproductively competent males [79]. Collectively, these data provide evidence that Dhh plays a role during testis morphogenesis and is important for the formation of a functional testicular microenvironment; however, the exact mechanisms by which cells interact and how this pathway orchestrate testicular morphogenesis and spermatogenesis remain to be elucidated.

Despite the intricate relationship between primary cilia and Hh, and Dhh and the testis, there are no in depth studies evaluating this interaction. Our group was first to describe the presence of primary cilia in testicular somatic cells and their absence in undifferentiated germ cells in neonatal porcine testis [80]. It was shown that primary cilia are abundant during the first two weeks of age in the porcine testis, and from the second week to the onset of adulthood their presence is reduced. This pattern was also observed in the *de novo* morphogenesis bioassay, when the development of the testis is

recapitulated in the host environment. The ubiquitous presence of Hh during testicular development, together with our previous study indicating the presence of primary cilia in testicular somatic cells, has prompted the exploration of this organelle function during testis development.

1.3.5 Models to study testicular morphogenesis and microenvironment.

The complexity associated with testicular morphogenesis and spermatogenesis make it difficult to elucidate the cellular interactions and environmental cues acting upon these processes. Most of the information available derives from genetically modified mouse models and *in vitro* culture systems studies. Unfortunately, the establishment of primary culture of germ and somatic cells from non-rodent species is challenging, and does not provide the spatial arrangement and network similar to their physiological environment.

The study of reproductive biology in higher mammals is also hindered by the difficult access to the testis environment, and lack of a systems that recapitulate initial stages of development. The scarcity of data leads researchers to extrapolate findings to fill the gap between livestock and laboratory animals which can lead to assumption-based mistakes.

1.3.6 Three-dimensional *in vitro* culture

Three-dimensional *in vitro* culture has been explored as an improved alternative to the two dimensional systems. It relies on providing a scaffold similar to the ECM to sustain and act as an anchorage site, able to provide sufficient contact between germ and somatic cells to portray signaling interactions more similar to the natural testicular environment.

First reported in 1985, disassociated mouse neonatal Sertoli cells were cultured in a reconstituted basement membrane matrix and were able to re-aggregate and form cord-like structures permissive for germ cell differentiation [81]. The technique was further explored and improved by testing different protocols and matrices such as collagen, soft-agar, and methylcellulose. Different research groups succeeded in differentiating undifferentiated spermatogonia through the primary spermatocytes stage in rodent [82, 83], bovine [26] and even humans [84]. It has to be pointed out that the existing manuscripts describing completion of full spermatogenesis, from undifferentiated Type A spermatogonia to elongated spermatids, display very low efficiency and most often, other research groups experience difficulties reproducing published results [85, 86]. Nonetheless, the method has been validated as an alternative to study testicular morphogenesis instead of only relying on animal experimentation [87]. Taken together, results from three-dimensional *in vitro* culture studies suggest that the ideal setting for germ cell maturation requires three-dimensional architecture, somatic testicular cells, and gonadotropin support.

De novo morphogenesis of the testis tissue

Based on the same concept of providing the pillars for germ cell development - spatial arrangement, resident niche cells, and gonadotropins, Honaramooz *et al.* [88] developed a novel *in vivo* bioassay. The technique dubbed "*de novo* morphogenesis of testis tissue" consisted of xenografting pellets of isolated testicular cells, obtained via enzymatic digestion from neonatal porcine testes, under the dorsal skin of immunocompromised

castrated male mice. These pellets were able to rearrange into a functional endocrine and spermatogenic unit, supporting complete maturation and development of functional haploid gametes [89]. Four weeks after grafting, somatic cells formed seminiferous tubule structures with germ cells lying in close contact with the basement membrane. Leydig cells located in the interstitial tissue were capable of responding to mouse gonadotropins and sustain testosterone production similar to the in situ environment; reestablishment of a functional niche was verified 30 weeks after grafting by the production of elongated spermatids. The ability to recapitulate development proved to be conserved amongst species, subsequent to the porcine report. *De novo* morphogenesis of testis tissue was described from a variety of donors including mice [90], rat [83], bovine [91], sheep [92] and fish [93].

This bioassay allows manipulation of different compartments of the testicular niche prior to tissue reassembly, giving this technique potential to be used to study signaling, orientation, and guidance of the cells when forming the testis and to elucidate factors controlling spermatogenesis [94]. Initial studies reported low spermatogenic efficiency within the *de novo* formed tissue. The major drawback for this technology to be more widely applicable, is the low efficiency of producing elongated spermatids [88, 90]. Percentages of tubules containing spermatids have been enhanced from the initial 10% to 40% when Matrigel was added to the transplanted pellet [94]. However, the variability within recovered specimens remains a challenge to be overcome. The bioassay has the advantage of allowing manipulation of cells prior to grafting, it uses mice as a bioreactor to support experimentation from higher mammals circumventing space and handling

issues, and it allows for the expansion of replicate numbers and experimental protocols. Overall, it is a promising tool to study male reproduction in larger animal species.

1.4 Pig as a model for human research

The ideal animal model must replicate the same conditions as the system which is being studied. Ideally diverse types of experimentation should be available including: spontaneous development of condition, easy experimental induction of the condition, and available transgenic individuals for the study of perturbations to the condition of interest. The species used must have a long life span, be multiparous, and be easy to handle and to keep in animal care facilities [95].

Rodents have been prevailing as the animal of choice used in research, mostly for convenient reasons including in depth knowledge of their physiology, minimal housing required, and availability of genetically engineered animals. Still, they are far from being an ideal model species. They display considerable differences with human physiology and genome, leading to an inevitable delay when translating the findings to higher mammals.

Pigs display numerous advantages as a suitable model for human research. They share more anatomic, genetic, and physiological traits with humans than rodents do; their immune system is well characterized and their variety in size and breeds provide a wide range of options when choosing animals to perform experiments and/or surgical procedures [95, 96]. They are all season breeders, acquire early sexual maturity (5-8 months), have short gestational periods (114d), are omnivorous, and have a multitude of

outbread and inbred breeds. There are other non-rodent large animal models used such as dogs and rabbits; however, the pig physiology similarities and advanced reproductive techniques added to fewer ethical concerns when compared to animals used as pets make the pig a preferred model.

Cardiovascular surgery [97], and upper respiratory tract immunology [98] are examples of fields that validated the use of the pig as a good model. Pigs can also be used for wound healing studies [99] given that their integumentary system, epidermal and dermal thickness ratios are comparable to humans [100]. Currently available reproductive biotechnologies for genetic modification such as DNA microinjection into the pronuclei of fertilized oocytes [101], lentiviral transgenesis [102], and somatic cell nuclear transfer of modified donor cells [103] combined with accurate genome editing techniques like zinc finger nuclease–mediated modification [104-106], and transcription activator-like effector nucleases (TALENs) [107, 108], were used to make transgenic pig models for Alzheimer's disease [109], retinitis pigmentosa [110], cystic fibrosis [111] and diabetes I [112]. However, development of these animals through SCNT is costly, an alternative to optimize the technique would be to genetically manipulate the male germ line and transmit the altered gene through the male gametes. The fast paced development of biotechnologies sets a promising scenario for the use of the pig as model; primarily because the species is so biologically similar to humans, it has been used as spontaneous and experimentally induced models, and there are new transgenic tools for more directed studies.

2 Chapter II: From *in vitro* culture to *in vivo* models to study testis development and spermatogenesis.

2.1 Manuscript details

Title: From *in vitro* culture to *in vivo* models to study testis development and spermatogenesis

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Cell Tissue Research. Sep; 349(3):691-702, 2012.

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2.2 Abstract:

The testis is a complex organ playing host to one of the most intricate mass cell divisions occurring in postnatal life. Since the beginning of the 20th century, great efforts have been made to recapitulate spermatogenesis and elucidate spermatogonial stem cell function. These efforts resulted in the development of a variety of model systems which provide invaluable knowledge regarding testis organogenesis, key cell types and their interactions, and signaling pathways controlling testis function. The goal of this review is to elaborate on the evolution of techniques available, from *in vitro* culture systems to *in vivo* bioassays, providing up to date information and weighing their particular strengths and weaknesses. Each technique offers a different approach to elucidating male reproduction, enhancing germ lineage genetic manipulation, preservation of gametes, restoration of fertility and improving our understanding of stem cell biology.

2.3 Introduction

Spermatogonial stem cells (SSCs) form the basis of spermatogenesis. They are able to divide symmetrically or asymmetrically, giving rise to daughter stem cells or committed progenitor cells ensuring the continual production of gametes and propagation of genetic material to the following generations during the life of the adult male.

SSCs are an essential component of reproductive biology and their genetic manipulation provides a powerful tool to produce transgenic animals and to elucidate mechanisms underlying germ cell development and differentiation. SSCs can also serve as a model to further our understanding of the interactions between stem cells and their niche in other stem cell-based organ systems.

Different methods have been developed and improved in the past decades to help us better understand the intricate process of spermatogenesis. Techniques such as *in vitro* culture, three-dimensional (3D) organ culture, germ cell transplantation and testicular grafting have led to milestone advancements in reproductive biology. The purpose of this review is to collectively consider and dissect existing technologies analyzing their potentials and limitations to the elucidation of testicular development and spermatogenesis, while highlighting the advances in testis tissue xenografting and *de novo* morphogenesis of testis tissue.

To date, most of the studies involving embryo development and organogenesis were performed in the mouse; therefore more data is required from other species to enable the

translation of knowledge and accurately extrapolate findings in rodents to higher mammals. For most of the discussion, this review we will consider the mouse as the model for mammalian spermatogenesis.

2.4 **Organogenesis and formation of the stem cell niche in the testis**

The formation of the bipotential gonad and determination of primordial germ cell (PGC) fate occurs during the early stages of embryo development. This fate decision is the cornerstone of a developmental process that will guarantee the lifelong production of gametes.

In mammals, early during gastrulation, PGC precursors form a cluster of alkaline phosphatase positive cells in the most proximal region of the epiblast. As gastrulation progresses, this area becomes the extraembryonic mesoderm and stays in close contact with the extraembryonic ectoderm [113]. It has been shown that position and exposure to signals released from adjacent tissues rather than genetic makeup determine germ cell fate in mammals [114]. Predisposing signals from the extraembryonic ectoderm will guide PGC precursors to their PGC fate [115]. Bone Morphogenic Proteins (BMPs) are the first molecular signals to promote PGC specification. Recently, it was demonstrated that the visceral endoderm also plays a role in PGC precursor proliferation and later in PGC formation by Wnt signaling via Wnt3 but the precise mechanism by which this occurs remains to be elucidated [116].

BMPs induce serine phosphorylation of transducer family SMAD4, specifically SMAD 2/5/8, and trigger up-regulation of Blimp1 and Stella [117], which promote the reestablishment of pluripotency related genes and suppression of somatic cell specific genes. Meanwhile, epigenetic modifications required for germ cell development such as erasure of parental imprinting, demethylation and reactivation of the silenced X chromosome are initiated [118].

The series of genetic reprogramming phases that PGCs undergo from early development turn the prenatal period into a sensitive time for environmental and drug induced abnormalities. Any disturbance during this time period can lead to fertility problems that can be transmitted to subsequent generations [119]. PGCs resume mitosis and migrate back to the embryo through the allantois, moving to the adjacent yolk sac. They then home caudally to the hindgut and up to the genital ridges [120]. The process of PGC migration relies on a complex signaling network not yet completely elucidated. However, migration is known to be regulated by fibroblast growth factors (FGF) [121], transforming growth factor (TGF-b), stromal cell derived factor 1 (SDF-1) and its G-protein coupled receptor CXCR4, c-Kit and stem cell factor signal transduction [122]. Adhesion molecules such as β1, α6 integrins, E-cadherin and Ep-CAM facilitate interaction and signaling along the cell surface [9, 123]

When in the gonadal ridges, PGCs remain bipotent but display a different phenotype from the starting population. They are connected by intracellular bridges and express PGC markers such as SSEA-1, Vasa, Plzf, Dazl, Nanos 3, Oct-4, Sox2 and Nanog [118,

124]. Sexual dimorphism will be dictated by the sex region Y encoded gene (SRY). SRY up-regulates Sox9 expression in pre Sertoli somatic cells leading to up-regulation of multiple testis specific genes guiding testicular development [11, 125].

Shortly after sex differentiation begins, PGC arrival stimulates Sertoli cell precursors to proliferate and migrate from the coelomic epithelium towards germ and mesenchymal cells. Sertoli cells orchestrate the development of primitive seminiferous cords by inducing endothelial and peritubular myoid cell migration from the mesonephros. The assembly of the gonads is a multistep event mediated by chemokines, cell-matrix interaction (mostly governed by fibronectin and laminin) and specific gene expression [126]. In females, germ cell proliferation ceases and development arrests before birth in meiosis. Conversely in males, PGCs develop into gonocytes remaining in mitotic arrest until after birth.

2.5 **The testicular stem cell niche**

The spermatogenetic process depends on an interactive network established by supporting testicular cells to guide SSCs through differentiation, and efficiently sustain the high turnover required to promote the daily production of millions to billions of spermatozoa. This supportive and instructive environment where the SSC reside is called the niche [127] and it is constituted by Sertoli, peritubular myoid and Leydig cells together with the basement membrane and the vasculature compartment. The development of the niche is a long process that begins in early stages of embryo formation and results in the ability to sustain spermatogenesis at puberty by creating an

intricate interplay of cues and signaling amongst supporting cells, SSCs and their differentiated progeny [128].

Sertoli cells continue to proliferate from birth to puberty, then proliferation ceases and differentiation begins. Mature Sertoli cells are homogenously distributed along testicular cords, and will fill the cords with fluid forming seminiferous tubules and setting the stage for SSC differentiation.

Sertoli cells provide anchorage, growth factors such as glial cell-derived neurotrophic factor (GDNF), bFGF and adhesion molecules to modulate SSC self-renewal and differentiation [129, 130]. They are also key players to form the blood-testis barrier (BTB) and segregate the seminiferous epithelium into basal and adluminal compartments. Through tight junctions, actin based adherens junctions and desmosome complexes, the BTB maintains the adluminal compartment as an immune protected site controlling the entrance of specific molecules [131, 132].

Primordial germ cells established in the testis during development become gonocytes after birth and further differentiate into type A spermatogonia which will promote the first wave of spermatogenesis and contain the subpopulation of spermatogonial stem cells (SSC). SSC presumably constitute only 1 in 3000 cells in the adult mouse testis [133]. During puberty germ cells resume mitosis, home to the periphery of the seminiferous tubules and undergo multiple divisions to provide a continuous supply of precursor cells for spermatogenesis [4].

Recent studies provided evidence that suggests the distribution of SSCs in the seminiferous tubule is not random but restricted to specific sites of the basal compartment adjacent to blood vessels and interstitium [6, 134].

The understanding of cytokine signals, cellular interactions and mechanisms that control SSC fate in the highly orchestrated spermatogenic process has been attained at a slow pace. This is mostly due to the difficulty in accessing and manipulating the testis environment, lack of a system which mimics initial stages of development, absence of exclusive markers to identify SSCs, poor viability of suitable transgenic mouse strains and the difficulty in mimicking the physiological interactions of the niche in an *in vitro* culture system.

2.6 Approaches to study mammalian spermatogenesis

Many methods have been developed to comprehend spermatogenesis and to study spermatogonia and their niche. The focus of this review is to provide up to date information regarding existing technologies available, with a major focus on testicular cell and tissue xenotransplantation. Advantages and limitations of every method will be addressed keeping in mind that each technique should be selected according to the specific need of the experimental question.

2.7 *In vitro* culture systems

An efficient and feasible method to culture and differentiate spermatogonia *in vitro* is extremely desirable. *In vitro* culture can be used as an approach to study cell proliferation, metabolism, and *ex vivo* interaction with hormones or growth factors. It

also promotes expansion of the scarce population of undifferentiated spermatogonia allowing for genetic manipulation and expanding the potential use of the male germ cell lineage. Additionally, culture and maturation of diploid germ cells into competent spermatids *in vitro* would bypass the current costly and time consuming process of keeping laboratory animals which may not even be an accurate model for the species of interest.

The discovery of GDNF as a major player in the niche to promote spermatogonial self-renewal [135] grounded the establishment of protocols to culture murine [122, 136], and bovine [137] SSCs for long periods. Pioneering *in vitro* culture methods shared the use of feeders and serum to support spermatogonial maintenance and proliferation. However, the establishment of a widely accepted *in vitro* culture system supporting the maintenance of SSCs remains a challenge due to discrepancies amongst protocols such as different types of feeder cells used (fibroblasts or Sertoli cells) [138], serum concentration and supplements added to the media.

Two years ago, an innovative procedure circumvented the variability associated with the use of feeders and serum by culturing mouse SSCs up to 6 months in the absence of both components. However, the report did not define fundamental conditions for SSC culture since the protocol was based on the use of a commercial media of which the exact content is not disclosed [139]. Therefore, studies are still required to determine the essential components to maintain these cells in culture.

In other species, SSCs could not be maintained for long periods without becoming senescent. Even in species where SSC culture is already established, the development of undifferentiated spermatogonia to fertilization competent gametes *in vitro* remains a challenge. Thus, cells must be transplanted to an *in vivo* organism when complete differentiation is desired. Cell culture methods are also not suitable to study organogenesis and niche formation.

Three dimensional *in vitro* culture

Three dimensional *in vitro* culture is based on the premise that germ cell position and contact with somatic cells within its niche are vital to sustain spermatogenesis. Therefore, efficient *in vitro* conditions need to provide similar anchorage and signaling cues emulating the physiological environment. The three dimensional culture system yields more promising results for the achievement of germ cell differentiation than the two dimensional counterpart, but the development of the entire spermatogenic process starting from undifferentiated spermatogonia remains to be reported.

The first published paper in 1985 described the ability of disassociated neonatal Sertoli cells cultured in a reconstituted basement membrane matrix to re-aggregate and form cord like structures permissive for germ cell differentiation [81]. Further studies elaborated on the technique by adding growth factors and hormonal supplementation [140]. These modifications enhanced proliferation and achieved the differentiation of spermatogonia to primary spermatocytes.

Different matrices such as Matrigel, methylcellulose and collagen were also tested to determine which would provide the best support for cell differentiation [141]. However, it has since been demonstrated that the type of matrix used does not affect establishment of spermatogenesis but rather the presence of Sertoli cells and a defined culture media are influential components. In 2009, promising results announced the completion of the entire spermatogenic cycle *in vitro*. Despite the initial excitement, no follow up data has been published regarding the functionality of derived elongated spermatids and all further experiments were performed exclusively using mouse derived cells [142].

Organ and three dimensional cultures have a few advantages over *in vivo* bioassays, such as better control of spermatogenesis in an *ex situ* environment, no cross contamination between donor and host tissues and that no invasive transplantation protocols are required. Conversely, *in vitro* conditions may lead to epigenetic mutations and may not provide the most suitable conditions to perform experiments for screening drugs and toxicants as an *in vivo* model system would be.

Organ Culture approaches

Organ culture sustains the testicular architecture and the paracrine environment, being a better portrait of the physiological scenario than other *in vitro* bioassays previously discussed. The first attempts to culture testis tissue *in vitro* appeared at the beginning of the century [143] and were further investigated in the 1960s. Steinberger *et al* (1964) [254] hypothesized that the three dimensional architecture *in situ* supports germ cell maturation and should therefore be preserved to attain spermatogenesis in a culture dish.

By culturing fragments of testis in a gas liquid interphase [144] the group conserved the tissue and succeeded in culturing rat prepubertal gonocytes up to the pachytene stage of meiosis.

Tissue architecture is not the only essential component to promote germ cell development. Testicular factors such as hormonal environment, temperature and nutrients should also correspond to *in vivo* developmental stages. For example, in mice testosterone levels rise during fetal development whereas *in vitro* they decreased; therefore, the media requires LH supplementation [145].

One caveat of the method is that sustaining the testicular environment for prolonged periods is challenging. The absence of a circulating, oxygenated blood supply in the organ culture will eventually lead to ischemia and limits the maintenance of the tissue for longer periods. For these reasons organ culture is an impractical system to promote spermatogenesis from species with lengthy spermatogenic cycles [146].

Recently, Sato *et al*. [147] used defined media conditions and neonatal testicular tissue from genetically modified Gsg2-GFP and Acr-GFP mice (which express GFP during meiosis or in haploid germ cells, respectively) to assess germ cell development and improve culture conditions. The group overcame meiotic blockage and achieved maturation of fertilization competent mouse sperm. This was a significant step forward to establish *in vitro* organ culture as a feasible method to obtain full spermatogenesis. Realistically, the path to extend the results to other species may be long, as different species will require tailoring of the culture to their different physiological needs.

2.8 **Genetically modified mouse models**

Genetically modified mice have been cardinal in elucidating gene expression and signaling pathways from multiple different organs systems including the testis. Moreover, the hallmark development of the immunodeficient mouse in the late 1960's [148] has enabled the development of novel technologies to study the testicular environment by providing a transplantation host that does not reject donor testis cells and tissues. These transplantation procedures have provided an avenue to manipulate the testicular environment before grafting in order to study signaling and development and will be further elaborated on in subsequent sections.

Specific knock out (KO) mouse strains were essential in deciphering the role of GDNF, produced by Sertoli cells, in supporting SSC self-renewal [149].

KO models were also used to reveal genes and transcription factors important in promoting spermatogonial differentiation such as: Sohlh2 [150], Sohlh1 [151], Sox3 [152], SPOC1 [153] and Neurogenin 3 [154, 155]; and in self-renewal such as Etv-5 [156]. Furthermore, the generation of a knock-in LacZ positive GPR-125 mouse led to the establishment of GPR-125 as a reliable surface marker for SSCs [157].

In multiple, independent experiments Naughton *et al* and Zeng *et al* [130, 159] grafted testis tissue from transgenic donors into immunocompromised hosts to circumvent the neonatal lethality in Hells, GDNF, Gfra1 or Ret14 KO mice. Since the KO animals were not long-term viable, allografts facilitated the development of testicular tissue enabling

the characterization of germ cell development and differentiation in the absence of specific gene expression.

The use of transgenic animal models is not without its limitations. For example, gene expression may not be exclusive to a particular tissue (especially during early stages of embryo development), resulting in a mutant animal with a confounding phenotype. Cell type-specific KOs may also not be available; offspring may not be viable or have shortened life span. Furthermore, as with other experiments performed in rodents, even when genes are highly conserved across species, findings may not always be applicable to higher mammals. Undoubtedly the study of organogenesis *in vivo* provides information for specific gene function at the level of the whole animal. When the cited limitations can be minimized the use of transgenic animals is the first choice to start evaluating gene specific roles.

2.9 *In vivo* bioassays

The efficiency of the *in vivo* model to support SSC proliferation and spermatogenesis has already been validated. Thus far, three methodologies can be used for this purpose:

2.9.1 Germ Cell Transplantation

In 1994, Brinster and Zimmermann revolutionized the study of spermatogenesis publishing the first report of germ cell transplantation (GCT) [160]. It was the first method to allow access to SSCs for *in vitro* manipulation. GCT involves transplanting germ cells isolated from a donor testis into the seminiferous tubules of a germ cell depleted host animal via

the efferent duct or rete testis. After transplantation, cells home retrogradely reaching the basement membrane of the seminiferous tubules, populate their new niche, resume proliferation and restore the recipient's fertility.

Initial experiments were performed using mouse donor germ cells and allogeneic recipients. Subsequently, viable sperm were recovered after transplanting SSCs from rats into mouse recipient testis [162, 163]. The success of xenogeneic development sparked interest to investigate the capacity of germ cells from larger mammals to develop in murine testis. Although transplanted germ cells were capable of colonizing host testes, full spermatogenesis was not achieved [164-166]. The phylogenetic distance between donor and host appears to limit the ability of the testis to support xenogeneic germ cell development.

To circumvent this phylogenetic block, researchers focused on the potential of allogeneic transplantation. Germ cell development has since been reported in pigs [167], goats [168], sheep [169], cattle [170], dogs [171] and cats [172] when germ cells were introduced into recipients of the same species.

Since its development almost 2 decades ago, GCT has served as a functional reconstitution assay to verify stem cell presence and function in the absence of exclusive SSC markers. The technique has helped to enhance and expand the knowledge of SSC function and signaling pathways involved in spermatogonial differentiation.

GCT was used to determine that timing of spermatogenesis is guided by the donor SSC spermatogenic cycle instead of being reliant on influences from the niche: after xenogeneic transplantations, SSC maintained the donor's developmental timeframe [173]. Additionally, genetic pathways such as c-Kit and its ligand SCF [174] and the requirement of PLZF for SSC self-renewal [175] were elucidated using germ cell transplantation and transgenic mouse strains.

The use of transgenic germ cells for transplantation would be ideal to improve the efficiency and decrease the time required to obtain transgenic animals and it bypasses the necessity for Intracytoplasmic Sperm Injection (ICSI) for fertilization. Differentiated transgenic germ cells undergo epididymal maturation in the host testis being produced in large enough numbers to be used in other Assisted Reproductive Technologies (ART) or natural mating. This has already been reported in mice [176], rats [177] and goats [168] Furthermore, it allows for the preservation and maturation of germ cells cultured *in vitro* or from donors who display spermatogenic impairment or require fertility preservation before puberty when sperm are not yet available.

The successful restoration of fertility after transplanting cryopreserved germ cells [178] raised interest from the scientific community and public to translate the findings to human medicine applications. Germ cell transplantation has a potential use for autologous transplantation to recover fertility in cancer patients after potentially sterilizing radiation or chemotherapy treatments. Before reaching clinical application several concerns have yet to be addressed. For example, it is still under debate whether

current available sorting methods are efficient to provide a healthy cell population to abolish the potential risk of reintroducing cancer cells in the patient [179-182]. Exclusive and robust markers to identify and purify the SSC population from contaminated cells as well as an efficient cell culture system to promote SSC multiplication need to be established before this technique can be used to safely and efficiently restore human spermatogenesis in a clinical setting.

Germ cell transplantation is not without its limitations. The technique requires laborious animal handling to manage donor and recipient availability, a large number of cells is needed for transplantation and there are risks associated with ablation of host germ cells. Moreover, the transplantation procedure is invasive and needs to be modified according to physiological differences amongst species [183].

2.9.2 **Ectopic Grafting**

Over the past decade, two alternative *in vivo* methods were developed and shown to support spermatogenesis in a potentially more practical and versatile approach than those previously described: ectopic grafting of testicular tissue or disassociated cells into immunocompromised mice hosts.

2.9.3 **Testicular Xenografting**

Xenotransplantation is the transplantation of organs, tissues or cells across species barriers [184]. Transplantation of reproductive tissues in both humans and animals has been described in the scientific literature for over 100 years. In the early 20th century

testicular transplantation was performed using monkey donors and human recipients. The practicing surgeons claimed potential rejuvenation in physical and intellectual abilities to their willing patients [185]. The practice has drastically evolved over the past century, taking a significantly more scientific approach to testis tissue transplantation.

In 1975, the first xenotransplantation of testicular tissue was reported using human fetal testis fragments implanted in the abdominal wall of an adult nude mouse [186]. The immunocompromised environment enabled tissue survival; however, germ cell development was not observed past the gonocyte stage. Much later, in 2002, came the first report of full spermatogenesis in xenotransplanted tissue [187]. Newborn goat and pig testis tissue transplanted into an immunodeficient mouse host achieved functional sperm development. Since this report, testis tissue of many species has been xenografted and achieved full spermatogenesis, including rhesus monkeys [188], cattle [189], sheep [190], horses [191], cats [192] and ferrets [193].

In 2010, viable offspring was generated using sperm obtained from an ectopic porcine xenograft [194]. The reproductive challenges associated with the use of gametes obtained from xenografted tissue need to be considered, fertilization requires the use of elaborate and costly assisted reproductive technologies (ART) such as intracytoplasmic sperm injection (ICSI) to generate progeny. Furthermore, studies performed in a variety of species demonstrated that *in vitro* embryo production success rates are widely variable [195]. However, as techniques are developed and optimized, the potential increases for generation of offspring from testicular grafts to become more widely feasible.

Xenografting of testicular tissue is typically performed by inserting small fragments, 1 to 2 mm^3, of tissue under the dorsal skin of an immunodeficient mouse. Other locations such as intra-scrotal [196] and intra-testicular grafting have also been explored using human tissue [197]. Human testis grafts do not survive well in the back skin [198] therefore it was hypothesized that orthotopic grafting (grafting to the natural environment) may yield better results. Thus far, these studies have produced promising but variable results [197, 199]. The back skin of the mouse is more frequently used for grafting due to its capacity to accommodate higher number of grafts per host compared to the limited grafting area of the scrotum or testis in host mice.

A variety of factors, such as donor species and age, influences the development of grafts. Interestingly, donor species such as rhesus monkeys [200-202] and pigs [203] displayed accelerated development of spermatogenesis in grafted tissue when compared to *in situ*. While cattle [189] and ferrets [204] had similar spermatogenic time frames as *in situ*, feline xenografts display accelerated initial development of spermatogenesis followed by delayed sperm production [205].

Another significant donor factor to consider in testis tissue grafting is developmental or functional stage of the tissue at time of grafting. After initiation of meiosis and appearance of haploid spermatocytes, donor tissue displays an impaired ability to adapt to the new environment [206]. Many factors have been proposed to influence this difference in post grafting survival and development such as decreased angiogenic potential of mature tissues and the post-proliferative state of mature Sertoli cells [207].

In order to validate testis tissue xenografting as a reliable model to study testis function and development, a study evaluated gene expression between porcine testis tissue xenografts and the tissue *in situ*. Comparable global gene expression was reported indicating that testis tissue xenografts, at least in porcine, are a representative model of the donor testis *in situ* [208]. It is reasonable to assume similar finding in other species.

Testis tissue xenografting provides a model system to evaluate developmental and endocrine functions by allowing further modulation of the *in vivo* testicular environment. In 2010, Schlatt *et al.* [209] used a modified xenograft model in which they augmented the amount of testicular tissue present (castrated, hemi-castrated and 2-8 grafts) in order to explore the underlying control mechanisms of testis development and endocrine function. The authors found that hamster testis tissue grafts grow to a similar size despite the total amount of testis tissue present indicating that testis size is under intrinsic control. However, seminal vesicle size, which can be used as a bio-indicator of host androgen levels, remained consistent regardless of amount of testis tissue present suggesting that steroid levels are under the extrinsic control of the hypothalamic-pituitary axis.

The xenograft model presents a logistically simplified system to study effects of toxicants and other agents on testicular tissue. It allows for larger scale, multi variable analysis from a single donor by dividing one donor testis into numerous grafts and distributing to multiple host mice. As a result, a small donor number can yield a larger sample size (number of grafts). Samples from the same testis can then be subjected to different treatments per mouse host decreasing donor variability. In a study used to determine the

dose dependent effect of irradiation causing long-term spermatogonial depletion in juvenile primate tissue, only 2 donor monkeys were used [210]. Donor testes were dissected into fragments allowing for valid comparison of 4 irradiation doses.

The current method available to preserve gametes is cryopreservation of sperm from donor species. Xenografting as an alternative bioassay conserves not only the gametes, but also the testis environment enabling the preservation and study of immature individuals, genetically valuable males and species that are ethically restricted or not feasibly obtained as a whole animal. Although many species have very low sperm yield from grafted tissue [198, 201, 209, 212] is tissue xenografting has promise of further optimization potentially leading to more efficient sperm production. It was found that exposure of prepubertal bull testis tissue to vascular endothelial growth factor (VEGF) for 5 days in culture resulted in significant increase in number of differentiating germ cells within grafted tissue [213].

Germ cells divide rapidly rendering them sensitive to irradiation used in anticancer treatments. Prepubertal boys undergoing anticancer therapy, or other potential gonadotoxic therapies, are not eligible for sperm collection and cryopreservation prior to cytotoxic intervention. Stored biopsies obtained before treatment could potentially be grafted at a later date to obtain sperm for assisted reproduction [214]. Currently this technique can only be considered a theoretical option due to remaining safety and ethical limitations surrounding the use of xenotransplantation for human applications

Overall, testis tissue xenografting provides an avenue for gamete preservation, and a representative model for the study of testis function and development. The system is not perfect however, and much work is still being done to optimize many of the applications cited.

2.9.4 *De novo* morphogenesis of testis tissue

Sertoli cells are the key players to confer testis immune-privilege. Different research groups harnessed their potential to evade immune rejection by combining Sertoli cells with cells from other organs to exploit the immune-protection and enable the reestablishment of organ specific cellular function in ectopic grafts. These studies noted the ability of Sertoli cells to rearrange into seminiferous tubule-like structures leading to the concept of *de novo* morphogenesis of testis tissue [215, 216]

This morphogenic potential was further explored to test if *de novo* formed seminiferous tubules were capable of supporting germ cell development sustaining spermatogenesis. Isolated testicular somatic and germ cells obtained by enzymatic digestion from neonatal porcine testes were transplanted under the dorsal skin of immunocompromised castrated male mice, and once in the host environment they rearranged into a functional endocrine and spermatogenic unit, supporting complete maturation and development of functional haploid gametes.

Four weeks after transplantation, seminiferous tubules were reassembled maintaining the same *in vivo* arrangement, where germ cells are in close contact with the basement

membrane and the interstitial cells sustain testosterone production through gonadotropin stimulation. The newly formed tissue created a permissive environment for SSC development into elongated spermatids after 30 weeks [88]. The principle of this method is illustrated in Figure 2.1.

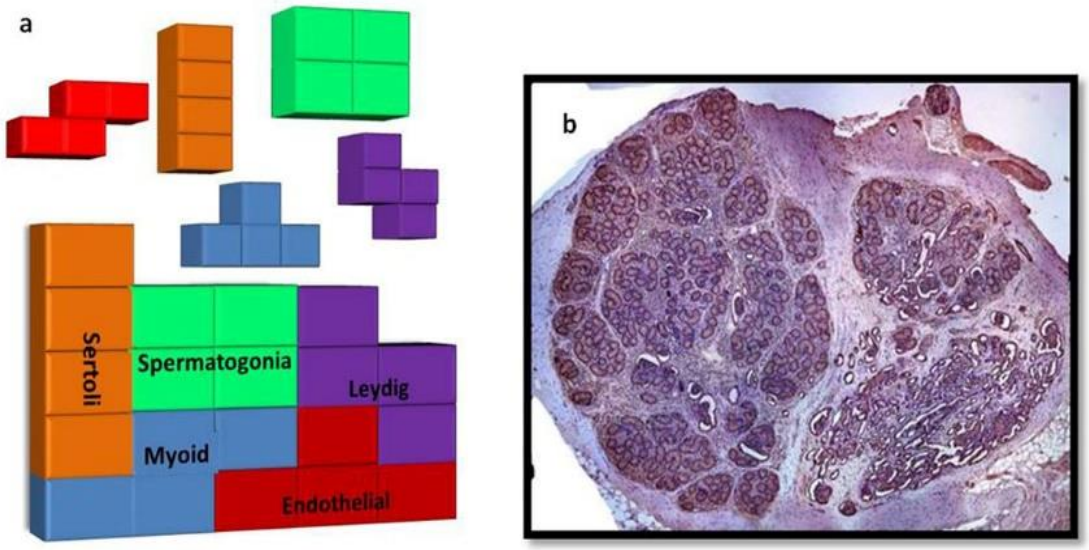

Figure 2-1 Schematic illustration of *de novo* morphogenesis of testis tissue. A. *De novo* morphogenesis can be compared to the game Tetris. In the game, different shapes composed of four square blocks each should be manipulated and arranged to achieve the goal: score by forming complete lines. In the testis, four major cell types: spermatogonia, Sertoli, peritubular myoid and Leydig cells should also be assembled to create a functional *de novo* formed tubule where the main goal is sperm production. Just like in the game, arrangements may vary according to given pieces, therefore *de novo* formed testis can be made from different cell donors, populations or even genetically

This emerging technique, termed *de novo* morphogenesis of testis tissue, represents a unique approach to study testis development and genetically manipulated testicular cells

prior to grafting. Subsequent to the initial report using donor pig cells, *de novo* morphogenesis of testis tissue was also described from isolated cells of rodents [217] and sheep [206]. In experiments using donor sheep testis cells, spermatogenesis occurred in an extended time frame compared to the normal *in vivo* timing, but *de novo* tubule formation was not delayed and could be observed after 4 weeks.

To validate the potential to produce functionally competent gametes, isolated cells from mouse newborn testes were combined with germline stem cells carrying a GFP marker prior grafting. GFP labeled round spermatids developed and were used for ICSI into mouse oocytes producing healthy transgenic pups. *De novo* morphogenesis has also been successfully achieved in species that are used as model organisms to study gene function such as zebrafish [219] and Xenopus [220]. Cells from both species were able to reconstitute testicular tissue and initiate spermatogenesis after grafting testicular cell aggregates in their respective recipient. In the zebrafish experiment, the fertility competence of produced gametes was confirmed by the production of viable offspring.

It became of interest to analyze if the immune deficiency level from the host mice would influence germ cell development from ectopic grafted tissue or disassociated cells. No significant differences were reported between severe immunocompromised (SCID), Nude and NOD-NOD/Shi-SCID,IL-2Rγcnull [221] recipients [222]. However, limited experimental size and low power might have affected the results. Therefore, further studies are needed to rule out any potential differences.

De novo morphogenesis of testis tissue offers a feasible approach to study the niche and the spermatogenic process in a wide range of species. Similarly to tissue xenografting, the technique overcomes the costly and time consuming process of maintaining large animal models in research while providing reliable results. Using mice as a bioreactor to support the maintenance of testis cells and subsequent formation of tissue circumvents space and handling issues. This allows increased numbers of replicates and experiments to be performed regardless of the species and number of donors being studied.

The opportunity to manipulate different cell types prior to grafting makes this technique unique. It enables not only the study of signaling, orientation and guidance of the cells when forming the testis, but also access to the developing germ cell. Furthermore, since complete spermatogenesis is achieved, this method can be an alternative to germ cell transplantation for producing gene-manipulated gametes from a wide range of mammals. Additionally, mixing cells from different sources may provide a feasible approach to mature germ cells from donors incapable of sustained spermatogenesis or who must undergo gene therapy to produce healthy gametes.

Despite the broad range of applications to study cell to cell interaction, this bioassay comes with limitations. *De novo* formed testis tissue has low spermatogenic efficiency with the presence of elongated spermatids ranging from 10 to 20% in *de novo* formed seminiferous tubules. This is a major restriction to making this technology more widely applicable [88, 90]. The low level of differentiation can be attributed to germ cell scarcity and impairment during the procedure. In their physiological environment, germ cell

division and development are governed by close cellular and paracrine interactions. The tissue disassociation prior to grafting breaks down the niche connections forcing testicular grafted cells to reinstitute their previous microenvironment under new conditions to maintain or re-establish germ cell development [212]. Consequently, strategies to attain higher recovery of haploid spermatids are needed.

Table 2-1 Summary table for the applications of each research method reviewed

	Research Model						
Application/ study of	Transgenic Animals	*In Vitro* Culture	3D *In Vitro* Culture	Germ Cell Transplantation	*De Novo* Morphogenesis	Organ Culture	Testis Tissue Xenografting
Testis Development	✓	✗	✗	✗	✓	✓	✓
Morphogenesis/ organogenesis	✗	✗	✗	✗	✓	✓	✗
Signaling	✓	✓	✓	✓	✓	✓	✗
Niche Interactions	✓	✗	✗	✓	✓	✓	✗
Germ Cell Homing	✓	✗	✗	✓	✓	✗	✗
Gene Expression	✓	✓	✓	✓	✓	✓	✓
Spermatogenesis	✓	✓	✓	✓	✓	✓	✓
Fertility Preservation	✗	✗	✗	✓	✓	✓	✓
Reproductive Toxicology	✗	✓	✓	✓	✓	✓	✓

✓ Represents ability of specific method to address corresponding application whereas ✗ represents inability of specific method to assess corresponding application

2.10 **Discussion**

In the last decade, a growing number of research groups working in male reproductive biology significantly extended our knowledge of testicular organogenesis and spermatogenesis. These experimental reproductive techniques may also be adapted for potential clinical applications.

Agriculture, biofarming and regenerative medicine are examples of areas that could benefit from these advances. Also, understanding how SSCs interact with their niche would contribute to understanding of stem cell-niche interactions from other stem cell types.

As mentioned above, all existing methodologies have limitations, which must be evaluated according to the pursued goal and availability of donor material. To make use of any of the described techniques, the investigator has to identify the most efficient system for the intended research considering the strengths and limitations of each approach and the information it can provide. For example, germ cell transplantation enables proliferation and development of competent germ cells. It serves as a standard functional assay for identifying SSCs and has been successfully performed in a range of species. However, if the number of transplanted cells is limited, or if there are issues regarding availability of matching recipients and/or animal maintenance, other methods should be considered.

Ectopic grafting arose as a promising methodology to easily access spermatogenesis and manipulate the testicular environment. It can also be used to preserve fertility, sustain spermatogenesis and enhance the number of experimental replicates. Nonetheless, since the testis architecture is preserved it is not possible to manipulate individual cells.

De novo formed tissue provides a versatile approach to manipulate each individual cell population; however, it currently has low spermatogenic efficiency and to date, there are a limited number of publications validating its use. Figure 2.2 and Table 2.1 summarize the potential applications of each described technique.

The complex signaling pathways, growth factors and niche interactions orchestrating spermatogenesis are still far from being entirely understood. Filling the existing gaps in knowledge will provide the foundation to develop safe and efficient protocols to culture, identify and genetically manipulate male germ cells.

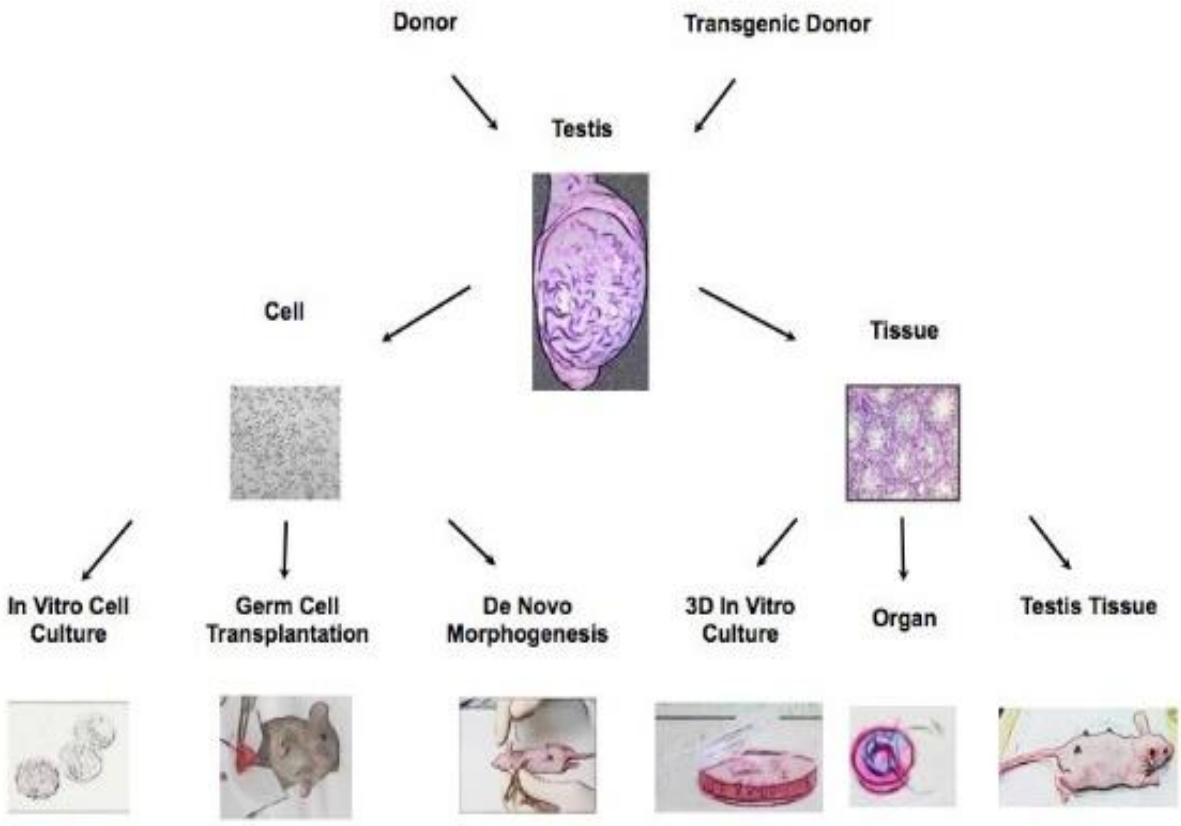

Figure 2-2 Flow chart depicting the different starting materials and techniques currently available to study testis development, spermatogenesis and the SSC niche

The highly structured process of spermatogenesis supports the lifelong production of male gametes. It relies on the testicular somatic cells to form an ideal microenvironment and guide SSCs' fate, supporting their further differentiation. The majority of existing information stems from rodents studies and, still, the process is not fully elucidated. The overall objective of this dissertation is to improve current models for experimentation in the pig, and to shed light on the niche interaction with the vasculature and the role of primary cilia.

Hypothesis

This dissertation explores the following central hypothesis: The *de novo* morphogenesis of testis tissue, *in vivo and in vitro*, is a functional bioassay to investigate cell interactions in the testis.

Specific Aims

1. Improve efficiency of germ cell differentiation in *de novo* formed testis tissue by enrichment of the germ cell population and test different enrichment methods.

2. Characterize the stem cell niche and homing of germ cells in relation to the vasculare by the supplementation of VEGFA165 and assess if it will hasten SSC development in the *de novo* formed tissue.

3. Characterize the role of the primary cilia during testicular morphogenesis and evaluate the relationship to the testicular microenvironment and the Hedgehog signalling pathway.

2.12 Chapter Overview

Overall, this body of work exploits the ability of isolated testis cells to rearrange into seminiferous tubule-like structures *in vitro or in vivo* to study testicular cell interactions within the testis microenvironment. As explained above, the experiments performed aimed to increase the knowledge in reproductive biology in the pig. Advances in this area

will lead to more translatable research findings and also harness the potential of the SSC niche in the species, making the production of transgenic pigs more efficient.

Chapter II reviewed the current available methods to study germ and somatic cells *in vitro and in vivo*. It is a comprehensive collection of currently available data, ranging from rodents to farm animals and primates, and the pros and cons of each method are described. Chapter II was published as a review article in Cell & Tissue Research September, 2012.

One major goal of this dissertation was to elaborate on the *de novo* morphogenesis bioassays, since the first studies reported low spermatogenic efficiency and presence of empty niches (seminiferous tubules). We hypothesized that the initial low number of SSC combined with the transplantation procedure were responsible for that finding. Chapter III explored different combinations of cells prior to grafting, then the effect of VEGF-165 in protecting the cells from the damaging effects of a new hypoxic environment. Chapter III was published as a manuscript in Reproduction July, 1, 2014.

Chapter IV addressed a major requisite faced when working with germ cells and large animal model experimentation: the need of large numbers of cells from a scarce cell population. We adapted a method largely used in the bio- industry and stem cell laboratories, the stirred suspension bioreactor, as a novel method to enrich for germ cells on large scale. We used this to improve the *de novo* morphogenesis method developed in Chapter III, as a functional bioassay to verify for testicular cell physiology. This Chapter is accepted and published ahead of print in Andrology, 2015.

Chapter V endeavored to further explore the testis microenvironment using our new bioassays, setting out to investigate the role of primary cilia during testis development and its relationship with the Hedgehog pathway. Our goal was made possible through the use of a small molecule inhibitor, CiliobrevinD, blocking cytoplasmic dynein complexes and eliminating the primary cilia in testicular cells. Then we evaluated the relationship between Hedgehog pathway and the ability to form *in vitro* tubules using the three dimensional culture bioassay.

3 Chapter III: *De novo* morphogenesis of testis tissue to investigate the role of VEGF-165 during testis formation.

3.1 Manuscript details

Title: *De novo* morphogenesis of testis tissue to investigate the role of VEGF-165 during testis formation.

Reproduction, 2014. 148(1), 109-117.

Authors: Camila Dores, Ina Dobrinski

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C.D. performed 100% of the experiments, analyzed 100% of the results and contributed to 95% of the writing. I.D. supervised the project and reviewed the manuscript.

3.2 Summary

The work provided in this chapter aimed to improve the efficiency of *de novo* morphogenesis of the testis tissue by evaluating if the number of germ cells available, rather than the limited niche, was the limitation for the study cellular and molecular mechanisms of spermatogenesis. This novel method was used to provide a better understanding of the Vascular Endothelial Growth Factor's role, and the importance of vasculature in the spermatogenesis of *de novo* formed testis tissue.

3.3 **Abstract**

De novo formation of testis tissue from single cell suspensions allows manipulation of different testicular compartments before grafting to study testicular development and the spermatogonial stem cell niche. However, the low percentages of newly formed seminiferous tubules supporting complete spermatogenesis and lack of a defined protocol have limited use of this bioassay. Low spermatogenic efficiency in *de novo* formed tissue could result from the scarcity of germ cells in the donor cell suspension, cell damage caused by handling or from hypoxia during tissue formation in the host environment. Here, we compared different proportions of spermatogonia in the donor cell suspension and the use of Matrigel as scaffold to support *de novo* tissue formation and spermatogenesis. Then, we utilized the system to investigate the role of Vascular Endothelial Growth Factor-165 during testicular morphogenesis on blood vessel and seminiferous tubule formation, and on presence of germ cells in the *de novo* developed tubules. Our results show that donor cell pellets with $10x10^6$ porcine neonatal testicular cells in Matrigel efficiently formed testis tissue *de novo*. Contrary to what was expected, the enrichment of the cell suspension with germ cells did not result in higher numbers of tubules supporting spermatogenesis. The addition of VEGF-165 did not improve blood vessel or tubule formation but it enhanced the number of tubules containing spermatogonia. These results indicate that spermatogenic efficiency was improved by the addition of Matrigel, and that VEGF-165 may have a protective role supporting germ cell establishment in their niche.

3.4 Introduction

The formation of the male gonad begins in the early stages of embryo formation and results in an environment capable to sustain spermatogenesis at puberty (Chen et al. 2005). This supportive and instructive environment is constituted of Sertoli, peritubular myoid and Leydig cells together with the basement membrane and the vasculature compartment and it guides Spermatogonia stem cells (SSC) to sustain a lifelong supply of gametes. Sertoli cells are major components of this environment, they are in intimate contact with germ cells inside seminiferous tubules, being homogenously distributed and providing growth factors such as GDNF, bFGF and adhesion molecules maintaining SSC self -renewal [129, 130] . In contrast, undifferentiated spermatogonia are asymmetrically distributed, clustered adjacent to the vascular branch points and interstitial cells [6], indicating a role for the blood supply guiding the niche orientation in the testis.

The proximity of SSCs to the interstitial vasculature indicated a role for the family of vascular endothelial growth (VEGF) factors, more specifically VEGFA, which is the major promoter of physiological and pathological angiogenesis [38, 39] and protects extra vascular cells from deleterious effects induced by hypoxia [223].

In the testis, VEGFA is important during early stages of development promoting formation of sex cords [47]. At post-natal stages there is no active angiogenesis under physiological conditions. Nonetheless Sertoli, Leydig and peritubular myoid cells release VEGFA and display its receptors in different stages of testis development [48].

The VEGFA synthesis by niche cells and the location of SSC closer to the vasculature make this factor worth further investigation for understanding the SSC niche. However, the study of SSCs and their niche *in vivo* is challenged by limitations in the available existing methods, especially in large mammalian species. To date, most of the knowledge was attained in rodents; and more data from other species is required to enable the accurate extrapolation of findings to higher mammals.

Testis tissue xenografting has been used to study male fertility in different mammalian species. It was the first method described to achieve full spermatogenesis from prepubertal donors after ectopic transplantation into immunocompromised mice [167] and since then it has been used to study or preserve male fertility [92, 201, 224]. When testis tissue was treated with VEGF164 prior to tissue xenografting or added during tissue culture for 7 days before xenografting, the addition of VEGF164 resulted in a higher percentage of seminiferous tubules supporting spermatogenesis [225]. This report was followed up by another manuscript investigating the role of VEGFA in testis development [213], where the addition of VEGF164 in tissue explants cultured *in vitro* increased the ratio of selected anti apoptotic genes versus pro apoptotic genes in the tissue.

In testis tissue xenografting, the architecture of the tissue is preserved and cell associations are maintained, therefore the ability to study individual cell types and their interaction within the SSC niche is limited.

We previously reported *de novo* morphogenesis of functional testis tissue form isolated testicular somatic and germ cells. Cells obtained by enzymatic digestion from neonatal porcine testes, when transplanted under the dorsal skin of immunocompromised mice, were able to rearrange into a functional endocrine and spermatogenic unit, supporting complete maturation and development of haploid male gametes [88]. Further reports described this morphogenic ability of isolated testis cells in different species such as rodents [90], ovine [92] and bovine [91] donors and also in species used as model organisms such as zebrafish [219] and *Xenopus* [220].

There are many differences between xenografts of testicular tissue and *de novo* morphogenesis of testis tissue after grafting of isolated cells. Grafting of cells more likely subjects all testicular cells to the same exposure to growth factors, such as VEGFA, while the existing structure and cell associations present in tissue fragments may limit growth factor uptake to deeper areas of the tissue.

De novo morphogenesis of testis tissue allows for manipulation of different compartments of the testicular niche prior to tissue reassembly, giving this technique potential to be used to study signaling, orientation and guidance of the cells when forming the testis and to elucidate factors controlling spermatogenesis. However, most of the *de novo* formed tubules contain only solely Sertoli cells leading to low spermatogenic efficiency, with the presence of elongated spermatids ranging from 10 to 20% in *de novo* formed seminiferous tubules [88, 90].

In this study our goals were to overcome the low spermatogenic efficiency of the system by testing different conditions when transplanting porcine testicular cells and to test the *de novo* morphogenesis as a functional assay to study aspects of testis function. We evaluated development of grafts with different cell numbers, percentage of germ cells and the use of Matrigel as a scaffold to maintain cells closer together. The improved method was then tested as a functional assay to study the effect of VEGF-165 on blood supply and reorganization of the testis tissue.

3.5 Materials and methods

3.5.1 Tissue enzymatic digestion

Testes from one week old piglets were donated by a commercial farm in Strathmore, Alberta, Canada. Cells were harvested by a two-step enzymatic protocol previously described [167]. The final cell population was dived in two groups. One was kept refrigerated at 4^{o}C for 72 hours until grafting surgery and the other one was submitted to differential plating for enrichment of germ cells.

3.5.2 Enrichment of germ cells

$50x10^{6}$ cells were plated on 100 mm tissue culture dishes (BD Bioscience, Mississauga, Canada) in 15 ml of Dulbecco Modified Eagle Medium (DMEM) (Life technologies, Burlington, Canada) with 5% fetal bovine serum (FBS) (Thermo Scientific, Rochester, USA) and incubated at 37^{o}C in 5% CO_2 in air. After 18 hours, cells remaining in suspension and those slightly attached were removed by trypsinization for 30 seconds

with 1:10 dilution of 0.25% trypsin-EDTA (Gibco, Life technologies), and plated in new flasks. This was repeated at 48 and 72 hours after the initial plating. Cell recovery and viability were recorded and immunocytochemistry using antibodies against UCHL1, an established marker to identify undifferentiated spermatogonia [226], was used to identify germ cells (UCHL1^{+}, vimentin^{-}) and somatic cells (UCHL1^{-}, vimentin^{+}; see below).

3.5.3 **Cell characterization**

Cells kept refrigerated and the enriched cell suspension used in all five replicates were characterized by immunocytochemistry with the following antibodies: mouse monoclonal anti vimentin Cy3 (1:500,Sigma –Aldrich), mouse monoclonal anti alpha-Smooth Muscle Actin FITC conjugated (1:500, Sigma –Aldrich), mouse monoclonal anti Gata4 (1:40;Santa Cruz Biotechnology, Dallas, USA), rabbit anti human protein gene product 9.5 (UCHL1) (1:500, AbDSerotec, Raleigh, USA), mouse monoclonal anti P450c17 (1:500, kindly donated by Dr. Alan Conley, University of California at Davis), primary antibodies incubations were followed by secondary antibodies: goat anti rabbit Alexafluor 488 (1:400, Sigma –Aldrich) or donkey anti mouse Alexafluor 555 (1:400, Sigma –Aldrich) to identify spermatogonia (UCHL1^{+}, Vimentin), Sertoli (Gata4^{+}, UCHL1^{-}), Leydig (P450c17^{+}, Vimentin^{+}) and myoid cells (alpha smooth muscle Actin^{+}, Vimentin^{+}).

After characterization of both cell populations, 4 different groups were created.

Effect of germ cell number and extracellular matrix on spermatogenic efficiency in *de novo* formed testis tissue:

The experimental design is outlined schematically in Figure 3.1. Four groups with different concentrations of spermatogonia and the scaffold Matrigel with reduced growth factors (MRGF) (BD Sciences, Bedford, United States) were created:

Group 1 (Control): $50x10^6$ cells not enriched for spermatogonia (containing 3 to 4% spermatogonia) without MRGF; group 2: $50x10^6$ cells not enriched for spermatogonia in 100µl of MRGF; group 3: $10x10^6$ cells containing 25% spermatogonia in 100µl of MRGF, group 4: $10x10^6$ cells not enriched for spermatogonia in 100µl of MRGF.

Immunodeficient recipient mice (26 NU/NCr, 6 SCID, Charles River, United States) were castrated and groups were randomly assigned to one of four injection sites located under the dorsal skin, spaced between the shoulder and the rump. Our previous studies demonstrated that the immunocompromised strains of the recipients do not influence the development of *de novo* formed tissue [88, 92].

The experiment was performed in five replicates with different pools of testicular cells, to minimize any influence from the cell suspension, surgical procedure or batch of mice used on the observed results.

The care and use of research animals were approved by the Institutional Animal Care and Use Committee of the University of Calgary,

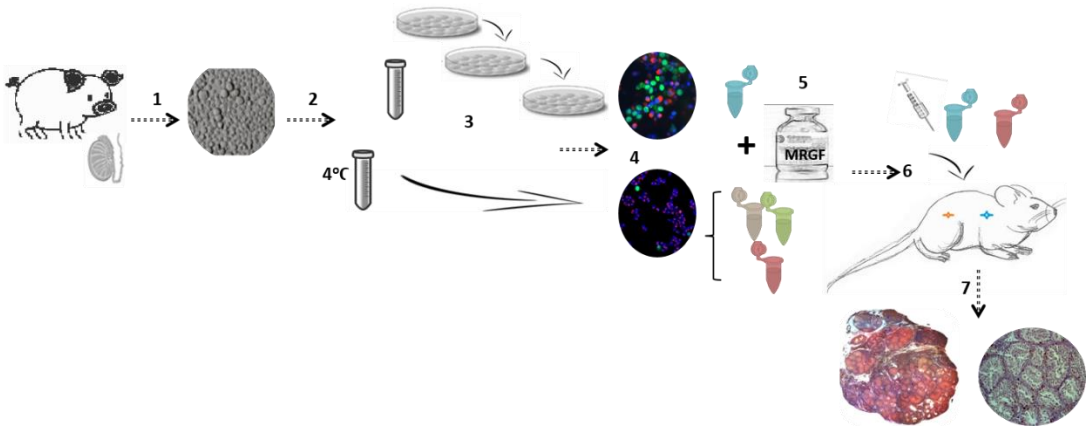

Figure 3-1 Experimental design. 1 One week old piglet testes were enzymatically digested into a single cell suspension. 2 Cell suspension was split into two fractions: one was kept at 4°C until characterization and the other was used for enrichment. 3 Enrichment for germ cells by 3 sequential platings. 4 Both cell fractions were characterized by immunocytochemistry. 5 Cell pellets with the defined concentrations of germ cells were created and Matrigel was added (groups 2, 3 and 4). 6 Surgical grafting was performed. 7 After 24 and 40 weeks, the *de novo* formed tissue was harvested from host mice and cross sections were analyzed for the quantification of *de novo* formed tubules, detection of UCHL-1^+ cells and development of spermatogenesis.

3.5.4 **Sample Recovery and Processing**

Mice from different surgery dates were randomly assigned to analysis at 24 and 40 weeks, data from both time points was combined to analyze each group. Mice were sacrificed by CO_2 inhalation; grafts were recovered, fixed in Bouin's solution, and stored in 70% ethanol until processing for histology. Grafts were embedded in paraffin and cut into 5µm sections.

The reconstitution of functional spermatogenic tissue was assessed in cross sections from the central area of formed grafts by immunohistochemistry. UCHL1 was used to identify

gonocytes and spermatogonia [226]. Paraffin sections were dewaxed and dehydrated through xylene followed by a graded series of graded ethanol washes (100%, 95% and 70% ethanol, 5 minutes each) and treated with 3% hydrogen peroxide in distilled H_2O to block endogenous peroxidase activity and were incubated at room temperature in CAS-Block (Invitrogen) for 20 minutes to block nonspecific antibody binding. Primary antibody incubation was performed overnight at 4^{o}C using anti UCHL1 (1:500, AbDSerotec), followed by peroxidase-conjugated donkey anti-rabbit IgG (1:400, Sigma - Aldrich) as the secondary antibody for one hour at room temperature. Peroxidase activity was detected using Vector NovaRED (Vector Laboratories, Burlington, Canada) and tissue was counterstained with haematoxylin (Vector Laboratories).

Effect of VEGF-165 on formation of testis tissue:

One week old piglet testicular cells were obtained as described above and the cell suspension obtained was immediately divided in pellets of $10x10^6$ cells mixed in 100µl MRGF and assigned to two groups: VEGF-165 treated: $10x10^6$ cells mixed in 100µl MRGF with 100ng/ml of recombinant human VEGF165 (R&D Systems,) and control: $10x10^6$ cells mixed in 100µl MRGF. Four cell pellets per animal (3 treated, 1 control) were randomly assigned to injection sites and ectopically grafted under the dorsal skin of 12 castrated immunocompromised mice (NU/NCr, Charles Rivers) in three sets of replicates.

3.5.5 **Sample recovery and analysis:**

Cell aggregates were recovered after 12 weeks of transplantation; at this time point seminiferous cords are organized and germ cells are located on the basement membrane. Recovered grafts were fixed and processed as described above.

An antibody to alpha smooth muscle actin was used to label blood vessels after comparison with other markers such as von Willerbrand Factor (vWf), VE cadherin and Cd31. VE cadherin is expressed in germ cells [227] and adhesion molecules from the cadherin family play a role in interaction amongst Leydig, Sertoli and germ cells [228], Cd31 is expressed in endothelial cells and leucocytes. Therefore, the use of these markers could lead to overestimate the number and location of blood vessel in xenografts recovered. vWf expression in porcine tissue is not consistent in all tissues and blood vessels [229, 230].

The reliability of alpha smooth muscle actin to identify blood vessels was verified by, immunohistochemistry in sequential cross sections from grafts recovered after 12 weeks comparing Cd31 and alpha smooth muscle actin. Paraffin sections from grafts recovered after 12 weeks were dewaxed and dehydrated as described above, incubated with CAS Block (Invitrogen) at room temperature for 20 minutes, followed by incubation overnight at 4^{o}C with mouse monoclonal anti alpha smooth muscle actin, FITC conjugated (1:500, Sigma –Aldrich) or mouse anti porcine CD31, FITC conjugated (1:400, AbD Serotec). Both antibodies labeled the same structures; however, staining for alpha smooth muscle actin provided a clearer staining pattern making blood vessel counts easier and more

reliable (Fig 3.2). Alpha smooth protein actin is also present in contractile apparatus such peritubular myoid cells of the seminiferous tubule, the positive staining pattern obtained by blood vessels and peritubular myoid cells made identification and measurement of newly formed seminiferous tubules and blood vessels more accurate.

Cross sections from the middle area of recovered grafts were analyzed by double staining immunohistochemistry for the detection of UCHL1 cells and alpha smooth muscle actin to identify undifferentiated spermatogonia and blood vessels, respectively (Figure 3.3). Slides were treated with Bloxall (Vector laboratories, Burlingame, USA) to block the endogenous peroxidase and alkaline phosphatase activity, then CAS-Block (Invitrogen) was added for 20 minutes to block nonspecific antibody binding. Samples were incubated with primary antibody UCHL1 (1:500, AbDSerotec) and anti-alpha smooth muscle actin (1:400, Sigma-Aldrich) overnight at 4^{o}C, followed by peroxidase-conjugated donkey anti-rabbit IgG (1:400, Sigma-Aldrich) as the secondary antibody. Peroxidase activity was detected using Vector NovaRED (Vector Laboratories), samples were then incubated with secondary alkaline-phosphatase conjugated goat anti mouse IgA (1:400, AbDSerotec). Alkaline phosphatase activity was detected using Vector Blue Alkaline Phosphatase kit (Vector Laboratories), followed by counterstaining with VECTOR Methyl Green (Vector Laboratories).

3.5.6 **Detection of apoptosis by TUNEL (terminal deoxynucleotidyl transferase dUTP nick end label) assay:**

Paraffin embedded cross sections from *de novo* formed tissue recovered after 12 weeks and 10 week old testis tissue (positive control) were subjected to DeadEnd™ Fluorometric TUNEL System assay according to manufacturer's instructions (Promega, Wisconsin, USA) to identify apoptotic cells.

3.5.7 **Cell proliferation assay:**

Cross sections from the middle area of recovered grafts were analyzed by immunohistochemistry for the detection of Ki67 to identify proliferative cells. Slides were subjected to heat induce epitope retrieval in citrate buffer for 20 minutes at 95^{o}C, then endogenous peroxidase was quenched by incubation for 20 minutes in 3% H_2O_2 followed by CAS-Block (Invitrogen) for 20 minutes. Samples were incubated with Ki67 mouse monoclonal antibody (1:200, Dako, Glostrup,Denmark) overnight at 4^{o}C, followed by peroxidase-conjugated donkey anti-mouse IgG (1:400,Sigma-Aldrich) as the secondary antibody. Peroxidase activity was detected using Vector NovaRED (Vector Laboratories), followed by counterstaining with Gill's haematoxylin (Vector Laboratories);

3.5.8 **Statistical Analysis:**

Number of *de novo* formed tubules and percentage of tubules supporting spermatogenesis per group (mean ± SD) was compared using Kruskal-Wallis, $P<0.05$ was considered

significant. Student t test with $p<0.05$ was used to test for statistical significance between groups from the VEGFA experiment.

Image Analysis:

All sections were analyzed using a Zeiss microscopy AxioImager M2 equipped with a CCD camera and controlled with AxioVision 4.8 software.

3.6 Results

Effect of germ cell number and extracellular matrix:

Based on immunocytochemistry for the detection of UCHL1, GATA 4, P450c17 and alpha smooth-muscle actin, the cell suspension used in Groups 1, 2 and 4 contained a mean ± SD of 3.1±1.1% undifferentiated spermatogonia, 72.8±14.6% Sertoli, 5±0.3% Leydig and 4.4±2.8% peritubular myoid cells, in Group 3 the germ cell population was adjusted to 25% undifferentiated spermatogonia, the other cell types were present in a concentration of 33.7±11.8% Sertoli, 10.7±2 Leydig and 3.3±2.2% peritubular myoid cells. The method used to attain enrichment of spermatogonia also provided a two fold increase in the Leydig cell population, probably due to the lower adherence of this cell type to plastic [231].

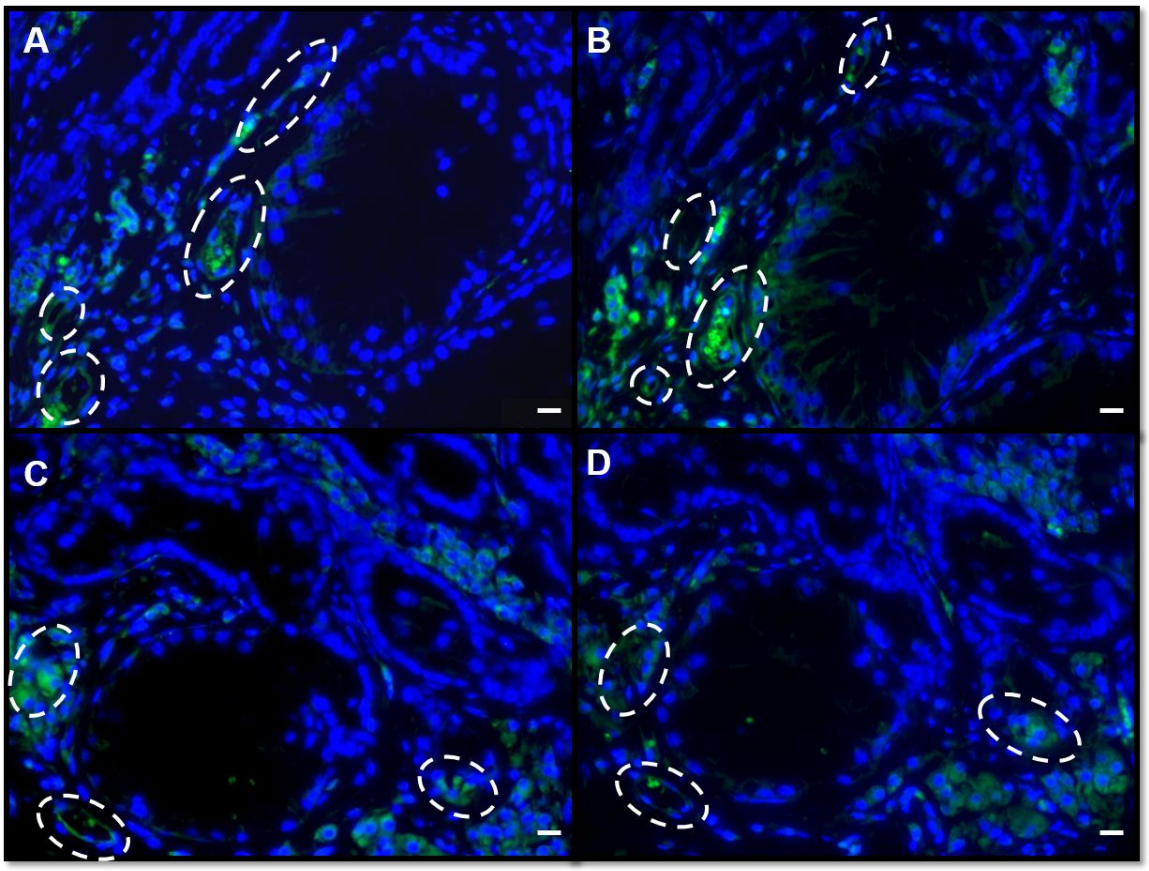

Figure 3-2 Validation of alpha smooth muscle actin as a marker to identify blood vessels. Immunofluorescence from sequential cross sections from grafts recovered after 12 weeks labeled against alpha smooth muscle actin and CD31, punctuated white lines indicates blood vessels. A, C Grafts stained against alpha smooth muscle actin. B, D Grafts stained against CD31. Bar=50µm.

From 32 recipients grafted, 3 did not survive up to the final end point of analysis at 40 weeks, thus grafts were not recovered. The percentage of grafts recovered from both time points that developed *de novo* formed tubules was 79% for Group 1 (control), 70% for group 2, 67% group 3 and 58% in group 4 (Table 1). Recovered grafts that did not develop into organized tubules were not included into further analysis.

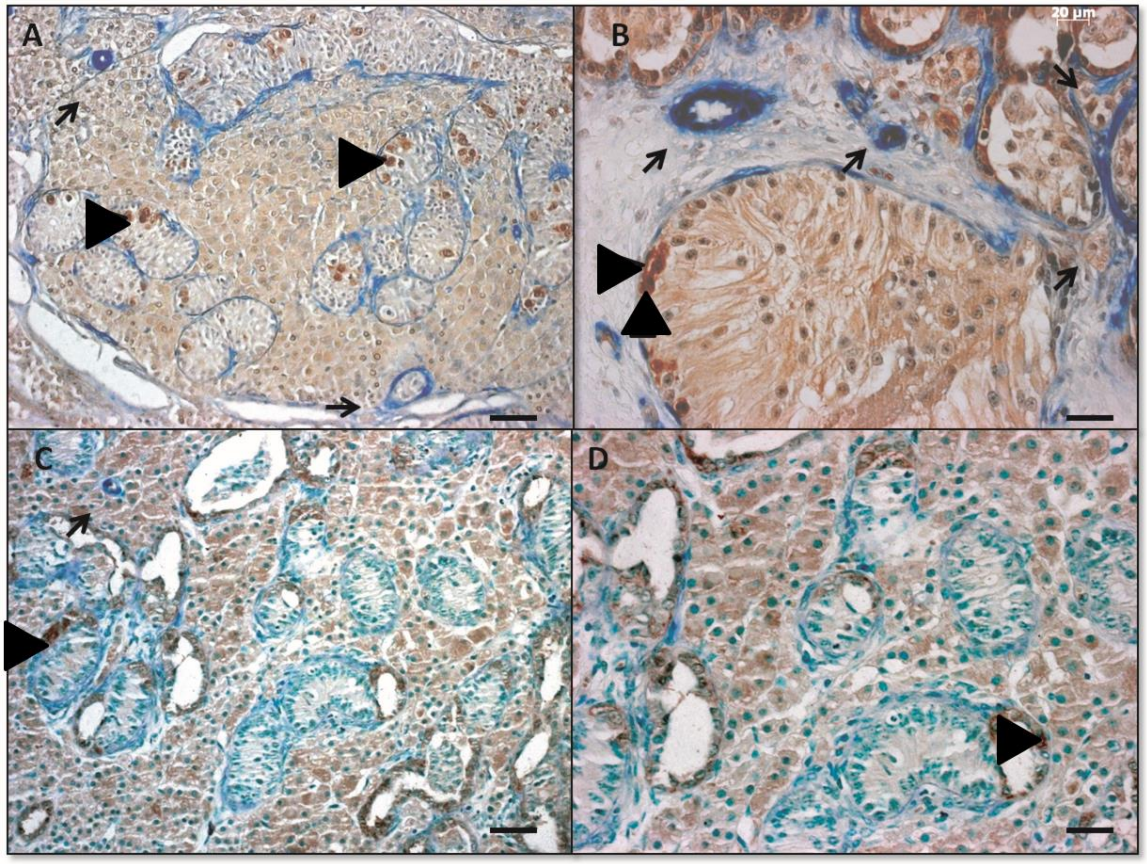

Figure 3-3 Morphology and immunohistochemistry to identify UCHL1^{+} (brown, black arrow heads) and alpha-smooth muscle actin $^{+}$ (blue, black arrows) cells in *de novo* formed testis tissue from VEGF experiment, A VEGFA treated tissue bar=50µm, B: VEGFA treated tissue bar=20µm, C Control tissue, bar=50µm; D Control tissue, bar=20µm.

Immunohistochemical identification of undifferentiated spermatogonia, seminiferous tubules formed and the presence of a functional niche supporting proliferation and development of spermatogenesis was performed in cross sections at two time points of analysis. After 24 weeks of grafting, *de novo* formed seminiferous tubules resembled the morphology of prepubertal testis and the spermatogenic efficiency was determined as percentage of *de novo* formed tubules containing spermatogonia along the basement

membrane per graft (Figure 3.4). After 40 weeks of cell grafting, spermatogenic efficiency was determined by percentage of seminiferous tubules supporting germ cell differentiation (Figure 3.5).

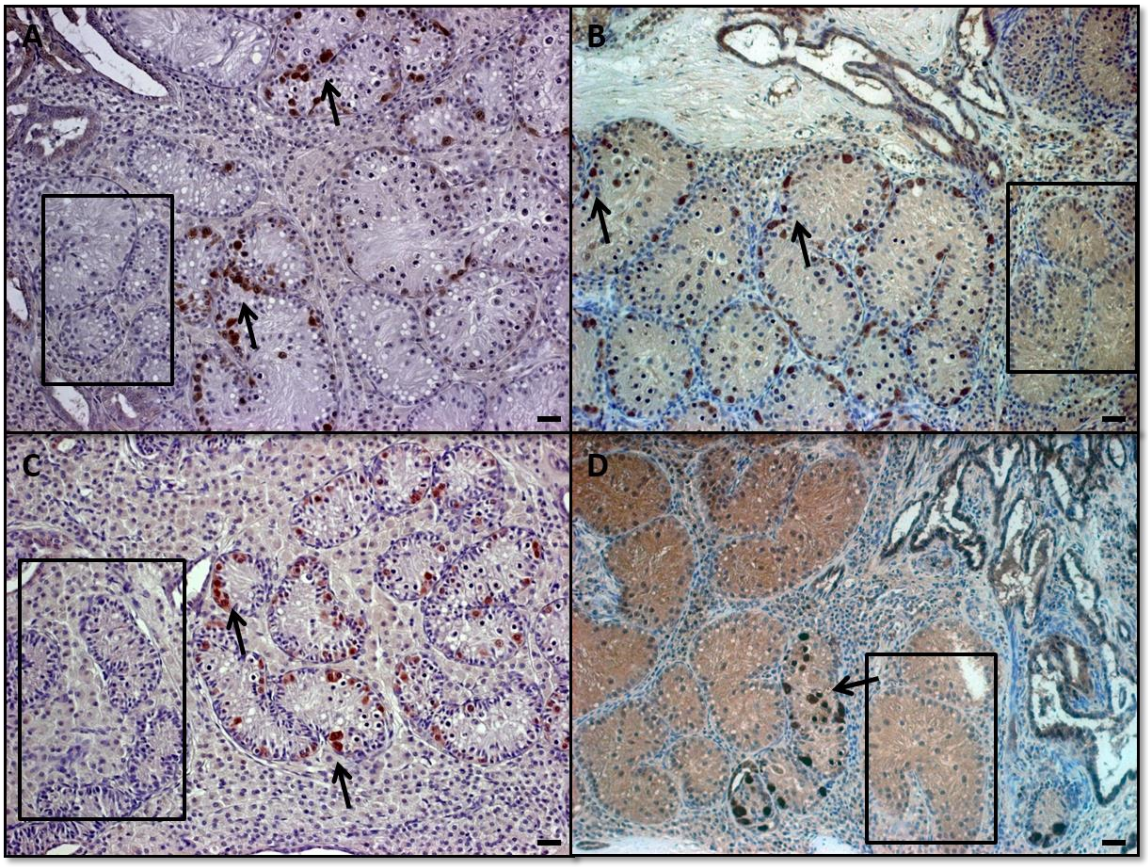

Figure 3-4 Morphology of *de novo* formed tubules and spermatogenesis 24 weeks after transplantation. A group1, control: $50x10^6$ cells, B group 2: $50x10^6$ cells in Matrigel, C group 3: $10x10^6$ enriched (25% spermatogonia) in Matrigel, D group 4 $10x10^6$ in Matrigel. Spermatogenesis was detected by the presence of spermatogonia and UCHL1+ germ cells [232], black arrows indicate examples of positive cells. Black rectangles demonstrate areas where tubules contain only Sertoli cells. Bar=20µm

Grafts recovered from all groups displayed large variance within group. The mean ±SEM of *de novo* formed tubules per graft were group1 (control): 142 ± 49, group 2: 267± 70, group 3: 183±51 and group 4: 67±16. The number of *de novo* formed tubules was significantly lower ($p<0.05$) in group 1 (control; $50x10^6$) than group 2 ($50x10^6$ in MRGF), when the same number of cells were grafted but without the addition of Matrigel as a supporting scaffold. Group 2 ($50x10^6$ in MRGF) and group 4 ($10x10^6$ in MRGF) were also significantly different, both groups had cells grafted under the same conditions mixed in Matrigel but they differed in number of cells injected, so we inferred that the amount of cells influenced number of tubules formed. No significant difference was detected between group 1 (control) and groups 3 and 4, and between group 3 ($10x10^6$ cells containing 25% spermatogonia) vs group 4 ($10x10^6$ in MRGF).

The mean ±SEM of spermatogenic efficiency measured in groups 1, 2, 3 and 4 was: 15±2%, 32±3%, 31±5% and 39±7% respectively. The control group was significantly less efficient ($p<0.05$) than all other groups when formation of a functional niche was measured, no significant difference was detected amongst the other groups. Based on our results, the enrichment of germ cells in the cell suspension grafted did not increase the number of tubules supporting spermatogenesis (Table 3.1).

Table 3-1 Summary of results for effects of cell number, germ cell concentration and Matrigel on *de novo* formed testis tissue

Group*	# of developed/ recovered grafts (%)	# of Tubules/graft (X±SEM)	# of tubules with spermatogenesis/graft** (X±SEM)
Control	23 /29 (79)	142 ± 49[a]	15±2%[a]
50x10^6 M	20/ 29 (70)	267± 70[b]	32±3%[b]
10x10^6 ME	19/ 29 (67)	183±51[a]	31±5%[b]
10x10^6 M	17/ 29 (58)	67±16[a]	39±7%[b]

* Cell #,M=Matrigel,,E=enriched for germ cells

a,b: values with different superscripts within columns are significantly different ($p<0.05$)

**Presence of UCHL1$^+$ spermatogonia at 24 weeks and spermatogonia and spermatids at 40 weeks.

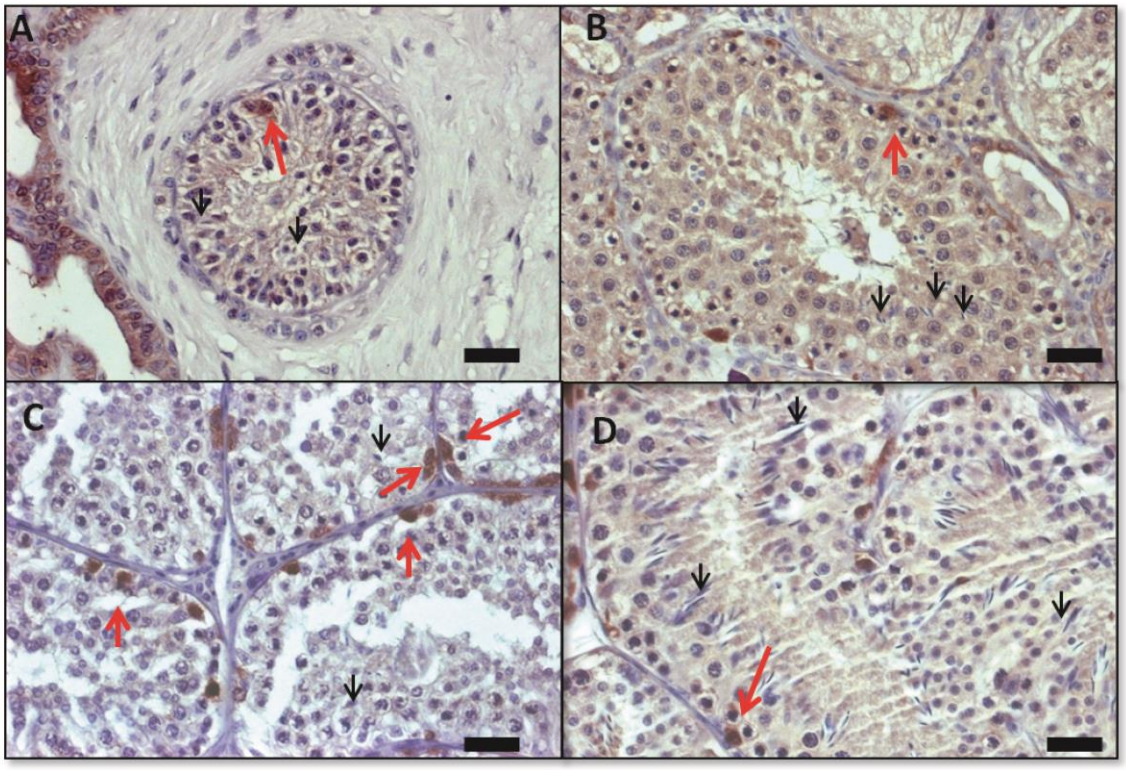

Figure 3-5 Morphology of *de novo* formed tubules and spermatogenesis by immunohistochemistry for the detection of UCHL1 40 weeks after transplantation. A Control, group 1: $50x10^6$ cells, B group 2: $50x10^6$ cells in Matrigel, C group 3:$10x10^6$ enriched with spermatogonia in Matrigel, D group 4:$10x10^6$cells in Matrigel. Red arrows indicate undifferentiated spermatogonia and black arrows point to elongated spermatids. Bar=20µm

Effect of VEGF-165:

Based on the results of the first set of experiments we established Group 4 ($10x10^6$ cells in MRGF) to be used as the standard protocol to investigate the effect of VEGF-165 in *de novo* morphogenesis of the testis tissue.

35 out of 36 grafts were recovered from the VEGF-165 treated group and 12 out of 12 were recovered from control grafts. From all grafts recovered, 92% of grafts from the

VEGF-165 treated group and 75% from the control group (p = 0.13) were organized as *de novo* formed testis tissue; grafts which did not develop into testis tissue were constituted mainly of Matrigel and were not analyzed.

Immunohistochemistry for UCHL1 and alpha smooth muscle actin from all grafts recovered demonstrated that the number of blood vessels or seminiferous tubules formed did not differ significantly between treated and control groups: 50.0±3.7 vs 49.8±6.2 and 78.2±9.7 vs 100.8±23 (mean ± SEM), respectively. However, when analyzing the spermatogenic efficiency between *de novo* formed tubules, VEGF-165 treated grafts displayed higher percentage of seminiferous tubules containing spermatogonia per graft than the control: 18.6±2.2% vs 11.3±3.3% ($p<0.05$) (Figure 3.6). Table 3.2 summarizes the results.

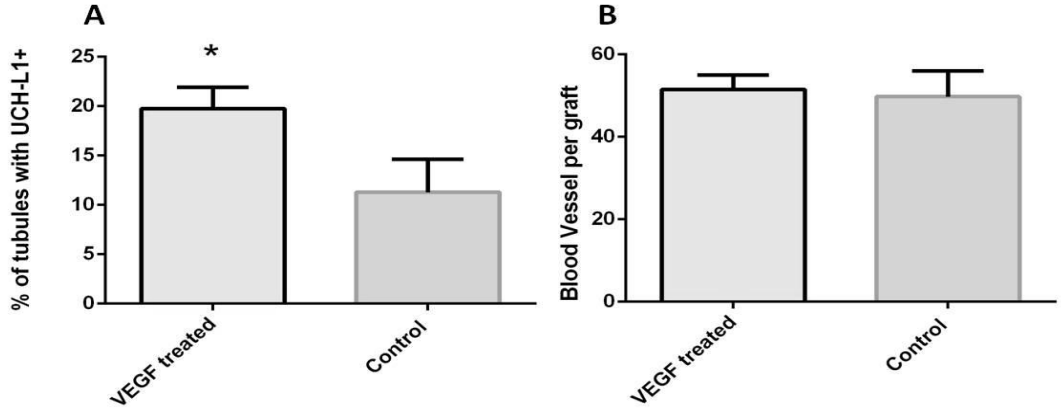

Figure 3-6 Germ cells and number of blood vessels in VEGF treated and control grafts after 12 weeks. A Percentage of tubules *de novo* formed containing UCHL1^{+}germ cells; B Number of blood vessels formed in the recovered tissue. All data is presented as mean ±SD per graft, *$p<0.05$.

To investigate how the addition of VEGF-165 benefited the spermatogenic efficiency in the treated group, apoptosis and proliferation assays were performed. TUNEL assay labeled very few apoptotic cells in recovered cell aggregates and in 10 week old testis tissue used as a positive control, and there was no difference in the percentage of *de novo* tubules with apoptotic cells between the Control and VEGF-165 treated groups.

To analyze proliferation in VEGF-165 treated versus Control groups we measured percentage of *de novo* formed tubules supporting proliferation (51.6±7.9 vs 39.5±12.7), and ratio of proliferating spermatogonia to Sertoli cells (0.40±0.11 vs 0.12±0.06). Both parameters did not differ significantly; however, there was a trend with the VEGF-165 treated group having a higher ratio of proliferating spermatogonia to proliferating Sertoli cell ($p = 0.09$).There was a significantly higher number of proliferating cells per tubule (2.3±0.2 vs 1.8±0.1; $p<0.05$) in the VEGF-165 treated group compared to the control group (Figure 3.7).

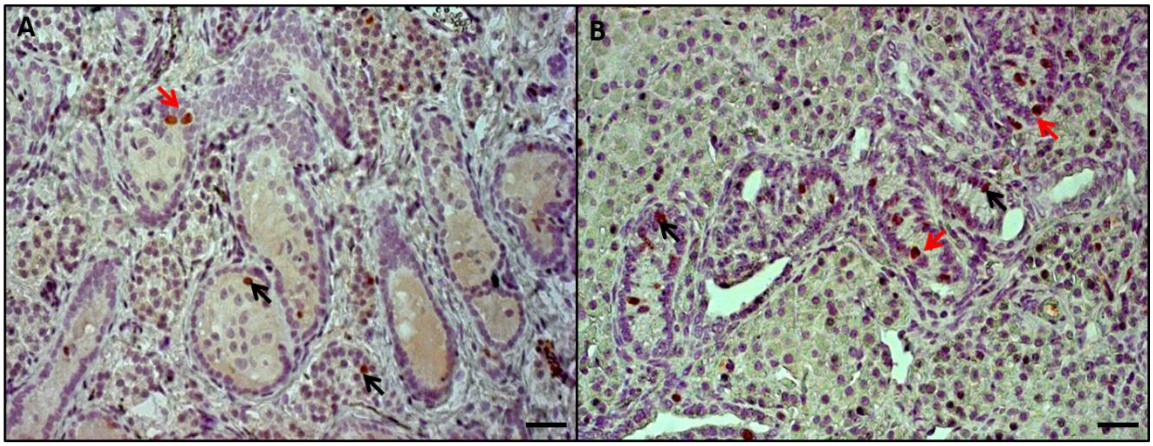

Figure 3-7 Immunohistochemistry against Ki67^{+} in *de novo* formed testis tissue from VEGFA experiment. A Control tissue, B VEGFA treated graft recovered after 12 weeks. Ki67^{+} cells are labeled in brown, bar=50µm

Table 3-2 Summary of results obtained from VEGFA treatment

Group	# of developed/ recovered grafts (%)	# of blood vessels/graft (x±SEM)	# of tubules/graft (x±SEM)	# of tubules with spermatogenesis/graft* (x±SEM)
10x10^{6}	32/35(92)	49.8±6.2^{a}	100.8±23^{a}	11.3±3.3^{a}
10x10^{6} VEGFA	9/12(75)	50.0±3.7^{a}	78.2±9.7^{a}	18.6±2.2^{b}

a,b: values with different superscripts within columns are significantly different ($p<0.05$)
*Presence of UCHL1^{+} spermatogonia at 12 weeks

3.7 Discussion:

De novo morphogenesis of testis tissue is a unique assay which allows manipulation of cells prior to grafting and enables the recovery of cells in further phases of spermatogenesis. This bioassay can overcome existing challenges to study testicular

morphogenesis and the SSC niche such as the difficult access to the testis during different stages of development and to maintain and study larger animal models. However, the majority of existing reports are descriptive and protocols used differ in number of cells grafted, recovery times and the use of cell pellets or combination with extracellular matrix.

Despite the disparity amongst protocols, all groups described low numbers of tubules with spermatogenesis in the *de novo* formed tissue. Most of the tubules formed were constituted only by Sertoli cells. The low efficiency observed can be attributed to several reasons: the testicular tissue contains low number of spermatogonia and SSC under physiological conditions [146] resulting in scarcity of spermatogonia in the transplanted pool of cells, the procedure is elaborate from the enzymatic digestion of the tissue to surgical grafting creating a harsh environment prone to cell damage and loss. Then, after transplantation, cells must adapt and reconstitute their previous microenvironment and reestablish germ cell development. Therefore, our first aim was to test different protocols and different conditions in a large number of samples to characterize and optimize this bioassay. Specifically, we evaluated the role of the extracellular matrix, number of cells grafted and the enrichment of undifferentiated spermatogonia in the development of *de novo* formed testicular tissue.

The comparison between control (established from our previous report by Honaramooz *et al.* 2007) and all other groups provided insight about the influence of Matrigel and the quantity of cells in the system. We concluded that addition of Matrigel to the mixture

enhances tubule formation and decreases the requirement for large numbers of donor cells, which is beneficial for future studies in animal models where the availability of donor tissue may be limited. The positive influence of Matrigel on the development of tissue from a smaller number of testicular cells is in accordance to what has been reported [90, 233]. Similarly, this matrix has been used to support tumor growth or maintenance of pancreatic islets in xenogenic hosts [234, 235].

The influence of number of cells grafted was determined comparing tubule formation when the same conditions were provided to different number of cells grafted; our results supported what has been previously hypothesized [233], that the number of cells grafted are correlated with tubule formation.

Different from what was anticipated, however, the fivefold enrichment of spermatogonia did not enhance number of tubules supporting spermatogenesis. We must consider that this bioassay faces a large variability in development of the recovered tissue and this might have hampered the detection of a significant difference. Nonetheless, we report that the limited availability of SSCs is not primarily responsible for the system's lower efficiency. However, addition of Matrigel to the cell pellet enhanced tubule formation and promoted the efficiency of *de novo* formed testis tissue, possibly by contributing to cell to cell contact while diminishing cell loss.

Optimization of the method was the first step to validate *de novo* morphogenesis as functional bioassay to study morphogenesis and spermatogenesis as demonstrated with

the experiment investigating the effects of VEGF-165 on de novo formation of testis tissue.

It has already been demonstrated that during morphogenesis, VEGFA amongst other growth factors orchestrates cord and vasculature formation by promoting migration of cells including pre endothelial cells from the mesonephros to the developing gonads [47, 51, 54]; in our results we did not detect any improvement in neovascularization or number of tubules formed by exogenous supplementation of VEGF-165; we noticed a tendency in overall development and organization as *de novo* formed testis tissue from recovered grafts in the treated group but it was not significantly different. However, the higher percentage of tubules with spermatogonia in the VEGF-165 treated grafts suggested a role for VEGF-165 in testicular development and a protective effect against the transient hypoxia and/or excessive handling by which cells are exposed during the grafting process.

The protective mechanism of VEGFA has been described in the central nervous system and retina when cells face ischemic environments [161, 236]. The ability to improve the adaptation of cells to a new environment by over expression of VEGFA has been reported in xenotransplantation of pancreatic islet cells and when testis tissue was treated prior to xenografting [213].

Analysis of apoptotic (TUNEL) and proliferative cells (Ki67) was used to detect a potential mechanism underlying the observed differences in tubules with germ cells formed with or without VEGF-165 treatment. Only few cells were labeled by TUNEL

and there was no difference in the percentage of tubules with apoptotic cells between groups. It is possible that after 12 weeks, when grafts were recovered, cells that were damaged by the procedure may be no longer detectable. Also, sensitivity of TUNEL analysis in paraffin embedded tissue may be limited [237].

On the other hand, the significant increase in numbers of proliferative (Ki67 positive) cells inside *de novo* formed tubules from VEGF-165 treated grafts indicated that VEGF-165 protected cells against negative effects associated with transplantation; the ratio between proliferative spermatogonia and Sertoli cells indicates that this benefited all cell types and not exclusively spermatogonia.

Recently, a study using Sertoli-germ cell specific VEGFA null transgenic mice reported similar findings to those reported here [53]. The Knockout mouse testis had morphologic impairment, seminiferous tubules were disorganized and contained less undifferentiated spermatogonia, yet impairment or decrease in number of blood vessels was not detected. The knockout animals showed different expression levels of genes responsible for cell survival (*Bcl2*) as reported by Caires *et al*. [213] for bovine testis xenografts.

The similarity of finding by Lu *et al* [53] supports our conclusion that *de novo* morphogenesis can be used as a functional bioassay to study gonadal development, the spermatogonial cell and its niche. However, this bioassay displays large variability in the recovered grafts, therefore larger number of samples should be used to obtained meaningful results. The low spermatogenic efficiency initially reported can be improved by the addition of Matrigel and VEGF-165 to the grafted cell suspension.

4 Chapter IV: Stirred suspension bioreactors as a novel method to enrich germ cells from pre-pubertal pig testis

4.1 Manuscript Details

Title: Stirred suspension bioreactors as a novel method to enrich germ cells from pre-pubertal pig testis

Andrology Epub Ahead print 2015/ DOI: 10.1111/andr.12031

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C.D. performed 100% of the experiments, analyzed 100% of the results and contributed to 95% of the writing. D.R. assisted with the stirred suspension bioreactor assays and review of the manuscript. I.D. supervised the project and reviewed the manuscript

4.2 Summary

The work provided in this chapter investigated the effect of shear stress upon testicular cells through culture in stirred suspension bioreactors. We hypothesized that shear forces promote adherence amongst Sertoli cells, cause somatic cell aggregation, and enable germ cell enrichment by negatively opting for cell aggregate formation. This chapter

helps to address the difficult task of selecting useful quantities of a cell population from large animal samples.

4.3 Abstract

To study spermatogonial stem cells the heterogeneous testicular cell population first needs to be enriched for undifferentiated spermatogonia, which contain the stem cell population. When working with non-rodent models, this step requires working with large numbers of cells. Available cell separation methods rely on differential properties of testicular cell types such as expression of specific cell surface proteins, size, density or differential adhesion to substrates to separate germ cells from somatic cells. The objective of this study was to develop an approach that allowed germ cell enrichment while providing efficiency of handling large cell numbers. Here we report the use of stirred suspension bioreactors to exploit the adhesion properties of Sertoli cells to enrich cells obtained from pre-pubertal porcine testes for undifferentiated spermatogonia. We also compared the bioreactor approach with an established differential plating method and the combination of both: stirred suspension bioreactor followed by differential plating. After 66 hours of culture, germ cell enrichment in stirred suspension bioreactors provided 7.3±1.0 fold (n=9), differential plating 9.8±2.4 fold (n=6) and combination of both methods resulted in 9.1±0.3 fold enrichment of germ cells from the initial germ cell population (n=3). To document functionality of cells recovered from the bioreactor, we demonstrated that cells retained their functional ability to reassemble seminiferous tubules *de novo* after grafting to mouse hosts and to support spermatogenesis. These results demonstrate that the stirred suspension bioreactor allows enrichment of germ cells in a controlled and scalable environment providing an efficient method when handling large cell numbers while reducing variability due to handling.

4.4 **Introduction:**

Spermatogonial stem cells (SSCs) are the foundation of male fertility providing a lifelong supply of progenitor cells that will develop into functional gametes. Located at the basement membrane throughout the seminiferous tubules, they constitute a very small population, estimated at 1 in 3000 cells in the total mouse testis cell population [238], within the pool of undifferentiated spermatogonia.

The heterogeneity of testicular cells, SSCs' low abundance, and the lack of reliable and exclusive surface markers are limitations to their isolation and culture. SSC research will benefit from an efficient protocol to yield significant enrichment of spermatogonia with less handling where scalability is possible, especially in larger animal models where the number of cells handled is much higher than when working with rodents.

Current methods used to enrich for specific germ cell types rely on physical and biochemical properties of testicular cells, such as cell size, density, and expression of surface proteins. These properties have been validated for germ cell enrichment in post-pubertal animals. It is currently more challenging to generate a concentrated germ cell population from pre-pubertal donors. At that developmental stage, Sertoli cells, gonocytes, and undifferentiated spermatogonia are the only cell types present in the seminiferous tubules [21]; therefore, the only distinctive physical characteristics that can be exploited are the differential adhesion properties of testicular cells to each other and to matrices.

In the testis, Sertoli cells form the blood testis barrier (BTB) dividing seminiferous tubules into two different compartments: basal and adluminal, thereby protecting post meiotic germ cells from the host immune system. The BTB is composed of desmosome-like, gap and tight junctions and ectoplasmic specializations formed by stable interaction between proteins from the cadherin, occludin, claudin and integrin families [239]

This plethora of membrane proteins likely forms the basis for the adhesive properties of Sertoli cells. Differential adhesion of germ and somatic cells to tissue culture surfaces (differential plating) has been extensively used to achieve an enriched spermatogonia population in different species. In pre-pubertal and adult rodents, this method has been widely used since Hamra *et al.* [240] reported a high yield of enrichment after a sequential platting technique where Sertoli cells were eliminated by their adherence to plastic, followed by culture in collagen coated plates. Subsequently, the germ cells were positively selected by short culture in laminin coated plates. However, the preferential attachment to laminin, observed in germ cells from rodent species is not observed in the pig [241].

Somatic cells readily adhere to treated plastic surfaces whereas germ cells float or attach slightly. This technique has been adopted in multiple studies with satisfactory results [137, 242]; however, when working with large animal models this approach becomes very labor intensive and carries the risk of introducing variability due to extensive handling of cells. Therefore, a protocol that allows scalability and yields consistent results is needed.

Stirred suspension bioreactors (SSB) were first described in the 1950s as an efficient method to culture and maximize large scale expansion of cells in a standardized and controlled environment [243]. Since then, the method has been applied to culture cells from different organisms for production of high titers of protein, virus and bacteria [244]. SSB have gained additional attention since the mid-1990s for their ability to culture different stem cell types such as hematopoietic, embryonic and neural stem cells in the form of cell aggregates, which maintain cells in an undifferentiated state for prolonged culture periods [245-248].

The objective of this study was to exploit the adhesive properties of Sertoli cells in an environment permissive to their aggregation that is provided by the stired suspension culture. We hypothesized that, in suspension, Sertoli cells would attach to one another and eventually to other cell types, and the subsequent removal of cell aggregates formed from the mixed cell suspension would result in relative germ cell enrichment.

We analyzed different time points for the enrichment of germ cells, total cell recovery and viability, and compared this new methodology with the currently standard method of differential plating; we also combined both methods. Based on reports in the literature, that shear stress can be detrimental to cells, we investigated if exposure to shear forces present in stirred suspension culture disturbed testicular cell characteristics. We monitored cell morphology and marker expression, and studied functionality of recovered cells by cell xenografting to assess *de novo* reconstitution of testis tissue. This approach is a bioassay where cells from a neonatal donor are xenografted ectopically to

immunocompromised mice and are capable to re-establish seminiferous tubules, the spermatogonial niche and complete spermatogenesis [94].

Here, we report the use of stirred suspension in bioreactors as a method to enrich testicular germ cells on a large scale in a controlled environment with reproducible results.

4.5 Material and Methods:

4.5.1 Tissue enzymatic digestion

Testes, from 1 week old piglets, were donated by a commercial farm in Strathmore, Alberta, Canada. Cells were harvested using a two-step enzymatic protocol previously described [167]. Briefly, seminiferous tubules were isolated after digesting neonatal pig testes with collagenase IV (2mg/ml) and hyaluronidase (2.5 μg/ml, Sigma). Later, individual cells were isolated by digesting seminiferous tubules with 2 mg/ml trypsin (Sigma) and 7 mg/ml DNase. The final cell population was subject to experimental groups.

Enrichment of germ cells:

4.5.2 Stirred suspension bioreactor culture

Testicular cells from five piglets ($5x10^6$ cells/ml) were cultured in 100 ml stirred suspension bioreactors (Corning Style Spinner Flask, NDS Technologies Inc.) as previously reported [247] at 100 r.p.m. in high-glucose DMEM (Invitrogen) supplemented with 50U per ml penicillin, 50U per ml streptomycin and 5% Fetal bovine

serum (FBS) (Thermo Scientific, Rochester, USA). After 18, 44 and 66 hours the cell suspension was filtered through a 40µm mesh to remove large cell aggregates and poured back into the bioreactor bottle. At the final time point, collected cells were filtered again and concentrated as a cell pellet after 5 minutes centrifugation at 300g. The cell pellet was resuspended in 0.25% trypsin-EDTA (Gibco, Life technologies, Burlington, Canada) for five minutes to obtain a single cell suspension from the remaining cells by breaking up aggregates smaller than 40 µm. Trypsin was neutralized by addition of 10% FBS and the cell suspension was washed two times in PBS and resuspended in high-glucose DMEM. Cell recovery and viability were recorded and immunocytochemistry was used to characterize cell populations recovered at the last time point. This experiment was repeated 9 times with cell suspensions obtained from different pools of testes at different dates.

4.5.3 **Differential plating:**

Testicular cells from five piglets ($5x10^6$ cells/ml) were plated on 100 mm tissue culture dishes (BD Bioscience, Mississauga, Canada) in 10 ml of Dulbecco Modified Eagle Medium (DMEM) (Life technologies, Burlington, Canada) with 5% FBS (Thermo Scientific) and incubated at 37ºC in 5% CO_2 in air. After 18 hours, cells remaining in suspension and those slightly attached were removed by trypsinization for 30 seconds with 1:10 dilution of 0.25% trypsin-EDTA (Gibco, LifeTechnologies), and plated in new flasks. This was repeated at 18, 44 and 66 hours after the initial plating. Cell recovery and viability were recorded and immunocytochemistry was used to characterize the cell

populations recovered at the last time point. This experiment was repeated 6 times with different cell suspensions, acquired at different dates from different pools of testes

4.5.4 **Stirred Suspension bioreactor followed by differential plating:**

Testicular cells from five piglets ($5x10^6$ cells/ml) were subjected to enrichment by SSB as described above for 44 hours, recovered cells were centrifuged at 300 x g for 5 minutes, concentrated as pellets and resuspended in 0.25% Trypsin EDTA until a single cell suspension was observed. Trypsin was neutralized by addition of 10% FBS and the cell suspension was washed two times in PBS and resuspended in high-glucose DMEM with 5%FBS. $5x10^6$ cells/ml were plated overnight for differential plating under the same conditions as described above. Cells in suspension and slightly attached cells were collected and analyzed for enrichment and cell type by immunocytochemistry. This experiment was repeated 3 times with different cell suspensions acquired at different dates from different pools of testes.

4.5.5 **Cell characterization:**

Cells (before and after enrichment) used in all experimental groups were characterized by immunocytochemistry. Germ cells were characterized by their expression of UCHL1 [241, 249, 250], Sertoli cells by Gata4 [251], Leydig by P450c17 [252] and peritubular myoid cells by alpha-smooth muscle actin [253]. Cells were fixed in 4% paraformaldehyde for 15 minutes and washed three times in PBS, then cell suspensions ($8x10^4$ cells/ml) were attached to slides by cytocentrifugation (400g for 5 minutes). Cells

attached to slides were permeabilized in 0.1% Triton-X solution for 15 minutes followed by three consecutive washes in PBS. To avoid non-specific staining, cells were incubated in CAS-Block (LifeTechnologies), then were incubated overnight at 4ºC with the following antibodies: mouse monoclonal anti vimentin Cy3 (1:500, Sigma Aldrich), mouse monoclonal anti alpha-smooth muscle actin FITC conjugated (1:500, Sigma Aldrich), mouse monoclonal anti Gata4 (1:40; Santa Cruz Biotechnology, Dallas, USA), rabbit anti human protein gene product 9.5 (UCHL1) (1:500, AbDSerotec, Raleigh, USA), mouse monoclonal anti P450c17 (1:500, kindly donated by Dr. Alan Conley, University of California at Davis). Primary antibody incubations were followed by secondary antibodies: goat anti rabbit Alexafluor 488 (1:400, Sigma Aldrich) or donkey anti mouse Alexafluor 555 (1:400, Sigma Aldrich) to identify spermatogonia ($UCHL1^{+}$, $vimentin^{-}$), Sertoli ($Gata4^{+}$, $UCHL1^{-}$), Leydig ($P450c17^{+}$, $vimentin^{+}$) and peritubular myoid cells (alpha-smooth muscle $actin^{+}$, $vimentin^{+}$).

4.5.6 *De novo* formation of testis tissue:

Two groups were created: Group 1 (control): 10^7 cells in 100µl of Matrigel reduced growth factors (BD Sciences, Bedford, United States) and Group 2: 10^7 cells containing 25% of undifferentiated spermatogonia enriched by stirred suspension culture (approximately 50% of the cell pellet content derived from the stirred suspension bioreactor) in 100µl of Matrigel reduced growth factors, cell pellet mass was 32±2.3mg.

Cell grafting was performed essentially as described [94]. Immunodeficient recipient mice (9 NU/NCr, Charles River, Wilmington, United States) were castrated and two cell

pellets from each group were randomly assigned to one of four injection sites located under the dorsal skin, spaced between the shoulder and the rump. The experiment was performed in three replicates with different pools of testicular cells, to minimize any influence from the cell suspension, surgical procedure or batch of mice used on the observed results.

The care and use of research animals were approved by the Institutional Animal Care and Use Committee of the University of Calgary.

4.5.7 Sample Recovery and Processing

Mice from different surgery dates were randomly assigned to analysis at 24 and 40 weeks. At those time points *de novo* formed seminiferous tubules resemble the morphology of prepubertal and adult testes reflecting the spermatogenic potential of the cells used in the transplants [88, 94].

Mice were sacrificed by CO_2 inhalation; grafts were recovered, fixed in Bouin's solution, and stored in 70% ethanol until processing for histology. Grafts were embedded in paraffin and cut into 5µm sections.

Number of tubules formed, tubules supporting spermatogenesis and support of complete spermatogenesis by identification of elongated spermatids were quantified and compared between groups. Data from both time points were pooled to analyze each group.

The reconstitution of functional spermatogenic tissue was assessed in cross sections from the middle area of formed grafts by immunohistochemistry. UCHL1 was used to identify gonocytes and spermatogonia [241]. Paraffin sections were dewaxed, dehydrated through xylene followed by a graded series of ethanol washes (100%, 95% and 70% ethanol, 5 minutes each), treated with 3% hydrogen peroxide in distilled H_2O to block endogenous peroxidase activity and incubated at room temperature in CAS-Block (Invitrogen) for 20 minutes to block non-specific antibody binding. Primary antibody incubation was performed overnight at 4^oC using UCHL1 (1:500, AbDSerotec), followed by peroxidase-conjugated donkey anti-rabbit IgG (1:400, Sigma - Aldrich) for one hour at room temperature. Peroxidase activity was detected using Vector NovaRED (Vector Laboratories, Burlington, Canada) and tissue was counterstained with Gill's hematoxylin (Vector Laboratories).

4.5.8 **Statistical analysis:**

Repeated measures two way ANOVA was used to analyze results from stirred suspension bioreactors and differential plating between different time points. One way ANOVA was used to evaluate enrichment results from stirred suspension bioreactor, differential plating and stirred suspension bioreactor combined by differential platting. Student T test was used to evaluate results from *de novo* morphogenesis assay and cell population before and after every enrichment method (Prism 6, GraphPad Software, La Jolla, CA, USA). Results were considered significantly different when $p<0.05$.

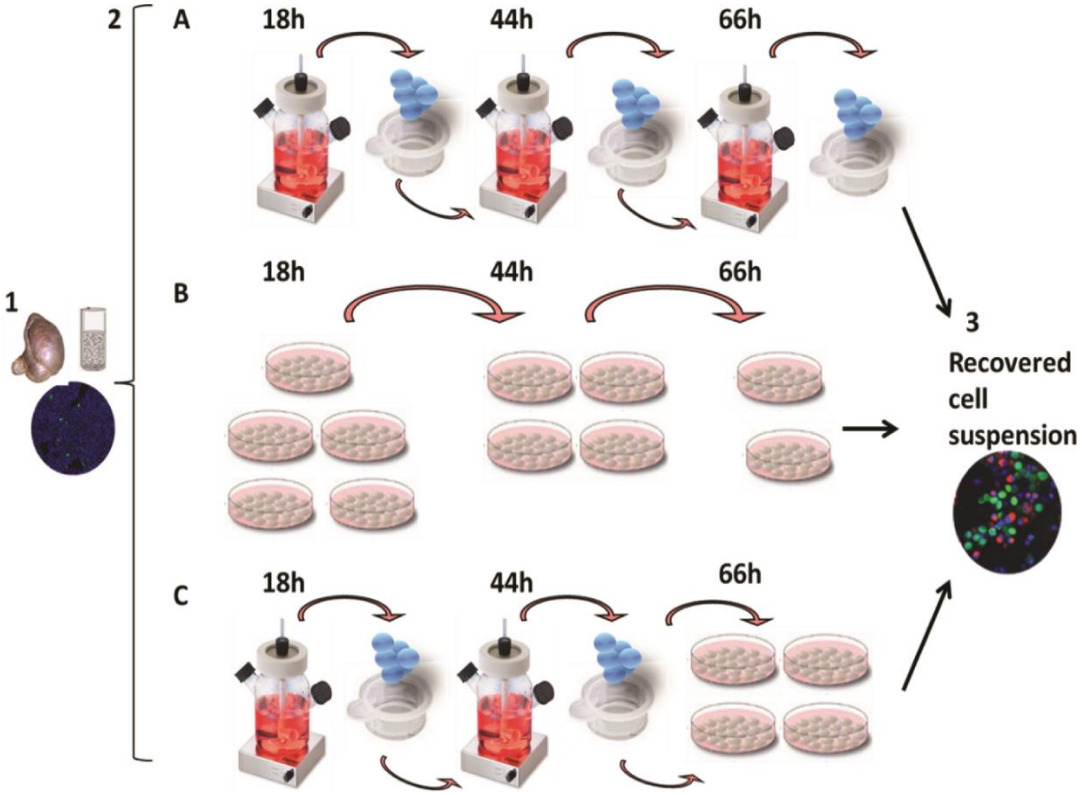

Figure 4-1 Experimental design. 1 One week old piglet testes were enzymatically digested into a single cell suspension. 2 Cell suspensions were split into three fractions and assigned to enrichment method A, B or C. 2A. Cell suspensions were placed in stirred suspension culture, after 18, 44 and 66 hours cell suspension was filter through a 40µM strainer to remove larger cell aggregates and poured back into culture until the next time point, 2B Enrichment for germ cells by differential plating, 2C. Cell suspensions were placed in stirred suspension culture for 44 hours then subjected to one round of differential plating. 3 Germ cell enrichment was measured at the end point of all three methods by immunofluorescence for the detection of germ cells (green: UCHL-1^{+}, vimentin^{-}) and somatic cells (red: UCHL-1^{-}, vimentin^{+}).

4.6 **Results:**

A flow chart describing the experimental design is shown in Figure 4.1. We compared enrichment, recovery, viability and characterized the cell population recovered from stirred suspension bioreactor and differential plating at time points: 18, 44 and 66 hours of culture.

Total cell recovery was significantly higher in the SSB method after 18h when compared to differential plating: 51.7±18.4 vs 34.9±17.0 ($p<0.05$). At 44h, recovery was similar between methods: 19.1±4.1 vs 17.9± 8.8. Finally, at 66h the DP method yielded superior recovery: 9.7±5.0 vs 6.3±2.0 from SSB (Fig. 2). Cell viability did not differ between SSB and differential plating at any time point. After 66h it was 72±3.9% and 75±4.5%, respectively (see Table 4.1).

Fold enrichment of germ cells obtained from SSB and DP at 18, 44 and 66 hours was: 2.2±0.7 vs 2.9±0.9, 4.6±0.9 vs 6.2±1.2, and 7.3±1.0 vs 9.8 ±2.4, respectively. Differential plating provided significantly better enrichment after 44 and 66 hours than SSB culture (Figure 4.2).

When both methods were combined, culture in stirred suspension bioreactors for 44 h followed by differential plating for 18 h, enrichment obtained at the final time point was similar to differential plating and SSB: 9.1±0.3. Total cell recovery was similar between

SSB and SSB followed by DP and superior in the DP group (Figure 4.2).

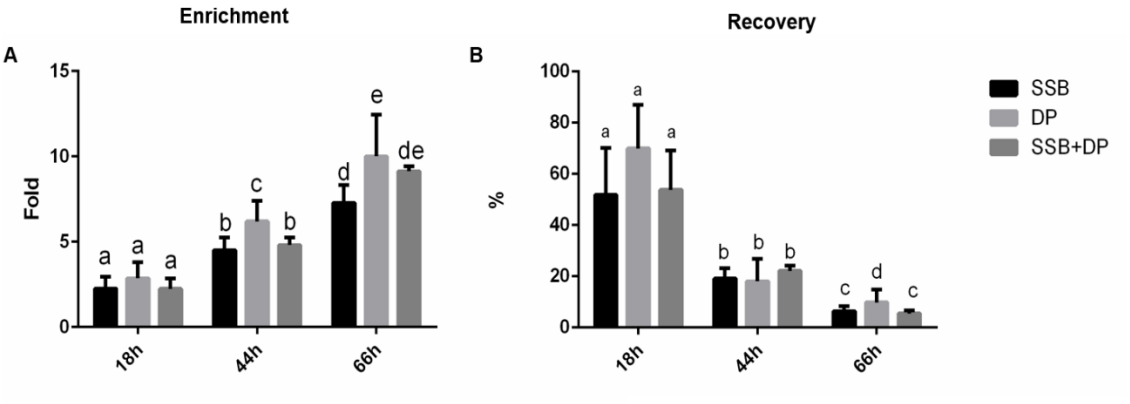

Figure 4-2 Comparison of cell recovery and enrichment between SSB, DP, and SSB followed by DP. A Total cell enrichment attained at different time points, B Germ cell recovery at different time points, different letters indicate significant difference.

Table 4-1 Cell viability through time points

Time point	SSB (×±SD)	DP (×±SD)	SSB+DP (×±SD)
0h	98 ± 1.0^{a}	94.3 ± 3.3^{a}	97.5 ± 1.2^{a}
18h	87 ± 4.4^{b}	82.1 ± 3.3^{b}	89.2 ± 2.4^{b}
44h	77 ± 2.0^{c}	79.5 ± 3.4^{bc}	78.9 ± 1.7^{bc}
66h	72 ± 3.9^{c}	75 ± 4.5^{c}	74.2 ± 3.0^{c}

Different letters indicate significant difference ($p < 0.05$). There was no significant difference in cell viability between methods within time points

Cell characterization demonstrated that both methods enrich the germ cell population by removing Sertoli cells. Cell aggregates were formed mostly by somatic cells (Figure 4.3), the percentage of spermatogonia, Sertoli and Leydig cells were significantly different from the initial testis cell suspension (0h) to the last time point when cells were collected (66h) (Figure 4.3 and 4.4).

To verify if shear forces that cells are subjected to during the bioreactor's stirred suspension culture affected testicular cell function, we used the *de novo* morphogenesis of testis tissue bioassay [88, 94]. Cells from SSB and control developed into functional seminiferous tubules, capable of supporting spermatogenesis. Both groups displayed similar percentages of seminiferous tubules supporting spermatogenesis following grafting: 34.3±7% vs 34.6 ±6%; SSB vs. control, respectively. These results showed that both groups had similar morphogenic and spermatogenic capacity and that the shear forces from the SSB enrichment did not negatively affect the physiological potential of the enriched testicular cells (Figure 4.4 and 4.5).

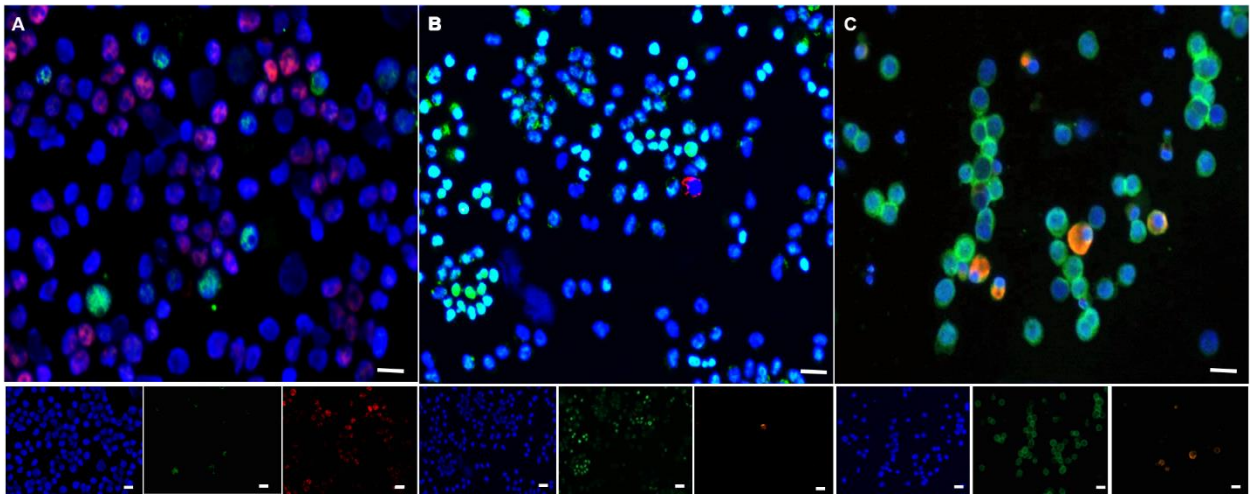

Figure 4-3. Immunohistochemistry analysis for the presence of germ (UCHL1^{+}) and Sertoli (Gata4^{+}) cells in testicular cell suspension after enzymatic digestion of tissue (0h), recovered cell aggregates and from the cell suspension at the last time point (66h) during SSB enrichment. A Cell suspension after testicular cell digestion (0h), (germ cells are labelled green, Sertoli cells in red, DAPI is blue), bar = 20µm.B cell aggregates recovered after 18h SSB enrichment (germ cells are labelled red, Sertoli cells in green, DAPI is blue), bar = 50µm, C cell suspension recovered after 66h of SSB enrichment (germ cells are labelled green, Sertoli cells in red, DAPI is blue), bars = 20µm.

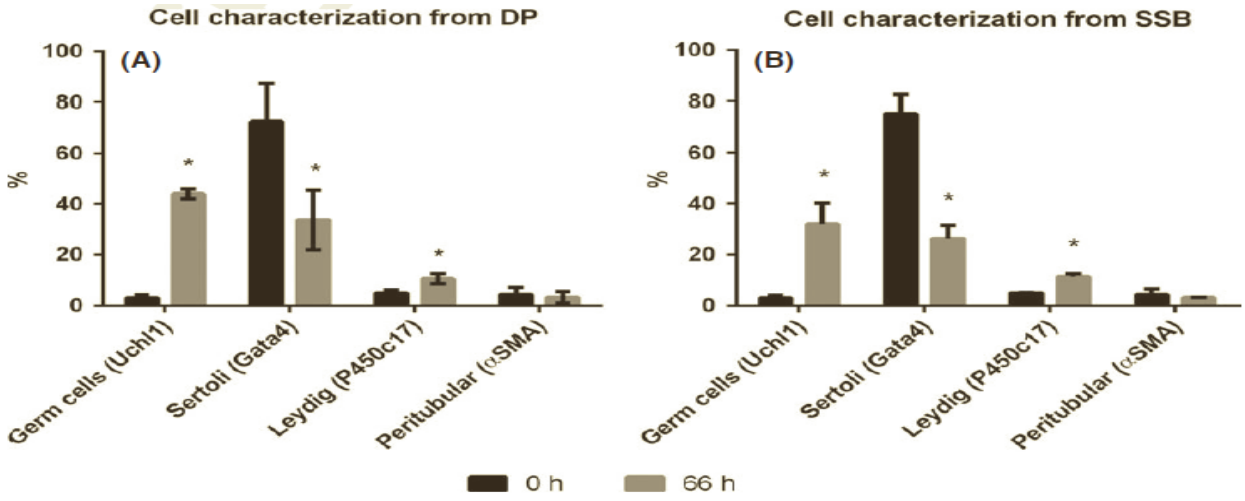

Figure 4-4 Characterization of the testicular cell suspension after enzymatic digestion of tissue (0h) and at the last time point (66h). Cell population from (A) SSB method and (B) DP. *Significant difference within cell type.

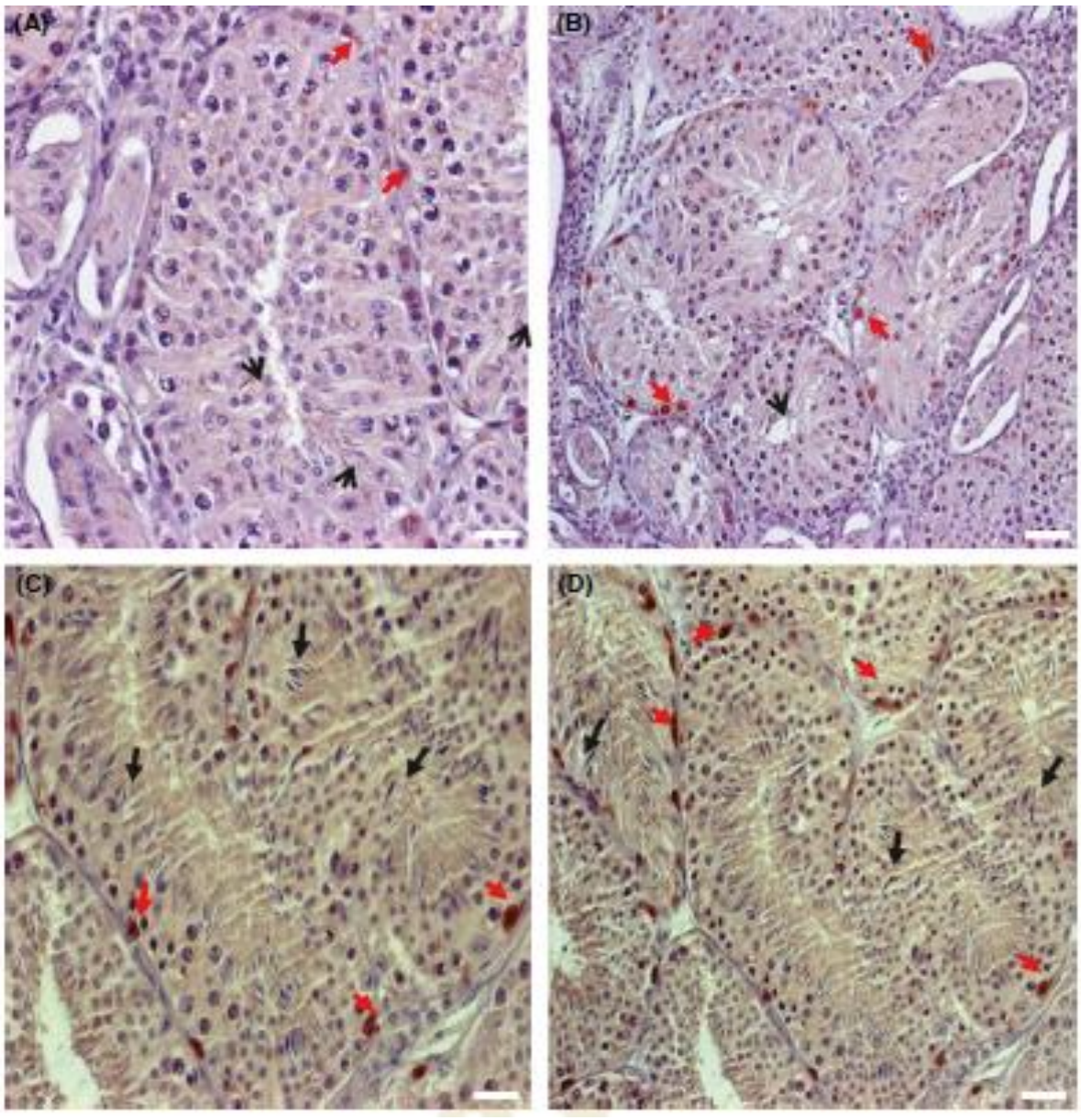

Figure 4-5 Histology of seminiferous tubules formed *de novo* 40 weeks after cell transplantation. A, B: *De novo* formed tubules developed from cells obtained by SSB, bars=20μm (A) or 50μm (B). C, D: *De novo* formed tubules developed from control cells, bars=20μm (C) or 50μm (D). Seminiferous tubules supporting spermatogenesis are identified as tubules with germ cells (UCHL1+, red arrows) and presence of elongated spermatids (black arrows).

4.7 **Discussion:**

Murine models have been indispensable to help decipher the intricate processes of gonadogenesis and spermatogenesis. To expand the use of current knowledge and enhance its relevance for research and medicine, it is necessary to translate findings to higher mammals. This creates a need to adapt existing techniques and address technical hurdles related to managing larger numbers of cells.

To date, most of the available data related to testicular cell morphology, adhesive properties, and how they can be exploited to achieve germ cell enrichment, derives from studies performed in rodents. Methods like Sta-put, centrifugal elutriation, and the use of plates coated with matrix components maybe subject to particularities of each developmental stage and species. We used, as our control method, a technique proven to be effective in a broad range of species [241, 242, 255]: the differential plating. We investigated an alternative system that is able to support scalability and enrich for cells based on common characteristics of Sertoli cells: being highly adhesive.

Differential plating, as also shown in this report, allows effective enrichment of germ cells from testicular cell suspensions with the advantage of not being limited by cell number; however, it becomes less practical when scalability is required as it requires extensive handling carrying the risk of increased chances of contamination, and variability of results. Our data revealed a large variability in germ cell enrichment by DP.

To find an alternative to DP we investigated a technique broadly used in the biotechnology industry – the use of stirred suspension bioreactors. This method allows scalability in a controlled environment, thus reducing problems related to DP. SSB did not result in superior germ cell enrichment compared to DP but due to lower variability it proved more suitable to enrich for germ cells when scalability is required.

The method demonstrated less variability, and sufficient enrichment with substantial reduction in handling time. Consistent results are of critical importance when dealing with experimentation requiring large cell numbers. All enrichment methods result in substantial cell loss, likely due to the lack of specificity of the approach and number of steps necessary to enrich for germ cells. This has to be taken into account when calculating cell numbers required for experimentation.

The most variable stage of DP is the first round of plating, because at this step the number of Petri dishes and cells handled are at the highest and mostly cell debris and Sertoli cells are eliminated. The use of SSB at this time point provided better total cell recovery with the same enrichment efficiency, making it a practical alternative to substitute this step of DP. For comparison, when using one petri dish, 117.3cm^2 of area for every 5 x10^7 cells, which is equal to 10 petri dishes for 5 x10^8 cells and a final volume of 1173cm^3. When working with SSB, the same number of cells was enriched using only 410cm^3 of incubator space, the volume of the 100 ml stirred bottle.

Follow up time points demonstrated that SSB still provided enrichment but DP maintained its superiority in fold enrichment. However, variability was higher with DP

than with SSB. Combination of both methods capitalized on benefits from both methodologies: reasonable enrichment and repeatable results. Enrichment obtained by the combination was not significantly different than DP, and the variability was reduced.

The ultimate goal of germ cell enrichment is to obtain a larger yield of germ cells for use in genetic manipulation and/or germ cell transplantation; therefore, functionality of the enriched germ cell population attained by SSB is essential. The cell grafting experiments allowed us to verify that both components of the testicular niche (somatic and germ cells) maintained their function, developing functional *de novo* formed tubules. *De novo* formed testicular tissue developed from pellets containing cells from SSB and Control group had similar development of seminiferous tubules, and maintained their spermatogenic efficiency.

It has been demonstrated by several groups that SSB culture can sustain, and maintain the undifferentiated state of pluripotent stem cells under feeder-free conditions for prolonged periods [256, 257]; additionally, shear force stress on cells during the stirred suspension culture can promote cell proliferation [246, 258]. *In vivo* and *in vitro*, cells can respond to the stress promoted by shear forces in diverse ways, such as proliferation, differentiation or undifferentaitation [259, 260]. Prolonged culture, and the influence of shear and gravitational forces can result in variations in cell adherance, and size of cell aggregates formed [261].

Since stired supension bioreactors have not been used previously to maintain testicular cells in culture, we evaluated if shear forces affected cell potency, and physiological

characteristics of somatic and germ cells by using the *de novo* morphogenesis assay. SSB culture did not have an effect on cell function after enrichment for 24-66h, cells were capable to restablish the spermatogonial stem cell niche and support germ cell proliferation in the *de novo* formed tubules.

Considering that discrepancies in rpm, shear stress, cell density and culture time may influence germ cell enrichment [262]; we will continue to investigate the optimal conditions for the use of SSB in germ cell enrichment.

In conclusion, this report describes a new method to attain germ cell enrichment from pre-pubertal porcine testes. Overall, the SSB is a simple experimental approach because of limited cell handling required, and homogenous culture conditions. These traits lead to a reduced risk of contamination, and support large-scale scalability. Additionally, this method provides reduced variability. Our data suggest that the use of the SSB can provide the first step of germ cell enrichment, when separating the somatic cell population. Stirred suspension bioreactors can then be combined with other techniques, such as differential plating, to enhance efficiency and reduce experimental variability.

4.8 **Supplementary Information:**

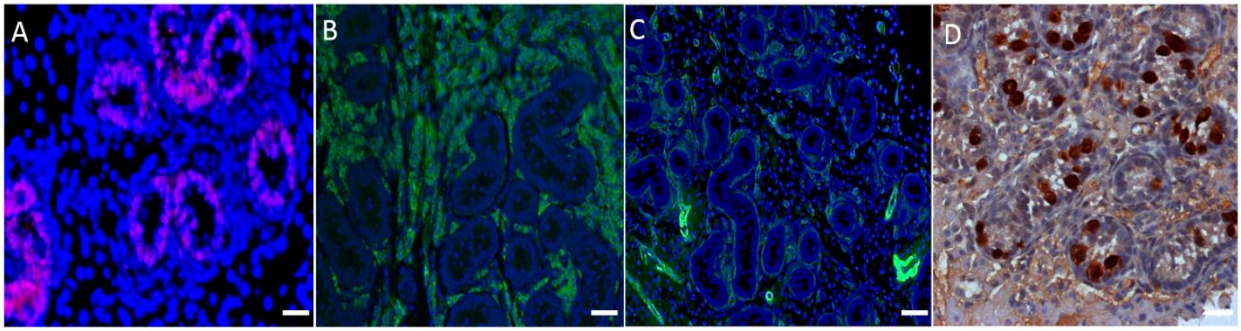

Figure 4-6: Positive controls for antibodies used. A–C: Immunofluorescence staining in neonatal testis tissue. A. Gata4 (labeled in red), bar=20 µm. B. P450c17 (labeled in green), bar=20 µm. C. Alpha-smooth muscle actin (labeled in green) bar=50 µm, D. Horseradish Peroxidase staining to detect UCHL1 (labeled in brown), bar=20 µm.

5 Chapter V: Primary cilia on porcine testicular somatic cells play a role in hedgehog signaling and tubular morphogenesis *in vitro*

5.1 Manuscript details

Primary cilia on porcine testicular somatic cells play a role in hedgehog signaling and tubular morphogenesis *in vitro*

Manuscript submitted to Biology of Reproduction

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CD. performed 95% of the experiments, analyzed 95% of the results and contributed to 95% of the writing. E.R. performed flow cytometry, W.A. performed qPCR, J. B. reviewed the manuscript, ID supervised the project and reviewed manuscript.

5.2 Summary

Our group has described the presence of primary cilia in the somatic compartment of testicular cells. In this chapter we continue the investigation of the testicular microenvironment by exploring the role of primary cilia using the *de novo* formation ability of the testis cells in an *in vitro* system. We examine if known roles of primary cilia in other cell types hold in testicular cells.

5.3 Abstract

In vertebrates, the primary cilium is an organelle nearly ubiquitously present in somatic cells but little is known about its function in the testis. We investigated the role of primary cilia in testis cells using *in vitro* formation of seminiferous tubules, *in vitro* culture of testicular somatic cells and by inhibiting formation of the primary cilium with Ciliobrevin D, a reversible chemical modulator that inhibits ATPase motor cytoplasmic dynein. We analyzed primary cultured cells for the presence of primary cilia, localization of the centrosome based on γ-tubulin staining, cell proliferation and the activation of hedgehog signaling through translocation of Gli2 to the nucleus. *In vitro* tubule formation was evaluated by length, width, amount of primary cilia between control and Ciliobrevin D treated cells. Our results indicate that the inhibition of ATPase motor cytoplasmic dynein perturbs formation of primary cilia in testicular somatic cells, blocks Hedgehog signaling and impairs tubule formation *in vitro*, providing evidence for a role of cilia in cell signaling and testicular morphogenesis.

5.4 Introduction:

Two types of cilia are present in eukaryotic cells, motile cilia and primary cilia. Motile cilia are present only in specialized tissues such as trachea, oviduct, middle ear, whereas primary cilia are formed on the majority of cells. Defects in primary cilia lead to several complex syndromes called ciliopathies, development of abnormal growths and cancer [263]

The function of motile cilia in the female and male reproductive tract is well understood; in females they are localized in the oviduct, have steroid hormone receptors and synchronize their ciliary beating to the estrous cycle phases to help propel the oocyte towards the uterus [264, 265,60]. In males, the sperm flagellum is considered a motile cilium, and the organelles are also found in the efferent ducts, where they are responsible to maintain continuous uptake of fluid [266]. The role of primary cilia in male reproduction has only recently been investigated [80]. In the last decade, the expansion of scientific knowledge surrounding the primary cilium promoted this organelle from an accessory addendum of the cell to a highly important structure playing a role in orchestrating development and homeostasis [267].

Primary cilia function is pivotal during embryo development. The microtubular structure of the primary cilium sustains an intraflagellar trafficking system, which is essential for signaling pathways such as hedgehog [68], platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), canonical and non-canonical planar cell polarity WNT [71]. These pathways are responsible for guiding cell patterning and differentiation leading to the evolvement of all systems of the body [72, 73, 268].

Knockout mice engineered to have a deficiency or absence of primary cilia do not complete fetal development, display central nervous system defects and randomization of left-right asymmetry of organs [269]. These defects are mostly likely due to the disruption in the Hh and Wnt pathways.

Hh signaling via primary cilia has been well characterized. Hh ligand binds to the transmembrane protein Patched, this allows the protein smoothened (Smo) to flow to the primary cilium and activate the family of Gli transcription factors [270, 271].

Bitgood *et al*. [17] showed that one of three Hh genes, desert hedgehog (Dhh), is one of the first indicators of sex differentiation during early stages of embryo development, being expressed by precursors of Sertoli cells right after Sry. They also demonstrated that Leydig cells contain the transmembrane protein Patched, which may be a target for Dhh. Barsoum *et al*. [272] further confirmed that activation of Dhh in somatic cell precursors of fetal ovaries lead to differentiation of Leydig cells. Clark *et al*. [273] also revealed that peritubular myoid cells rely on Dhh to fully develop. Despite the evidence supporting the importance of Dhh in male gonadogenesis and testis maturation, characterization and function of primary cilia and Hh signaling in postnatal gonads has not yet been explored.

We recently described the presence of primary cilia in the somatic compartment of testicular cells from mammals and their absence in undifferentiated germ cells [80]. We reported that primary cilia are abundant during the first two weeks of age in the porcine testis after which their numbers are reduced. This pattern could be replicated in testicular tissue formed *de novo* after transplantation of porcine testis cells to mouse hosts.

To explore the role of hedgehog signaling and primary cilia on testicular somatic cells during seminiferous tubule formation, we exploited the ability of neonatal testicular cells to reconstruct testicular cords *in vitro* [274] and used a small molecule inhibitor, Ciliobrevin D [275], a cell-permeable reversible chemical modulator of cytoplasmic

dynein, to test the hypothesis that the inhibition of primary cilia blocks hedgehog activation and affects the ability of testicular somatic cells to form *de novo* cord-like structures *in vitro* .

5.5 Material and Methods

Tissue enzymatic digestion

Testes from 1 week old piglets were donated by a commercial farm in Strathmore, Alberta, Canada. Cells were harvested using a two-step enzymatic protocol previously described. Briefly, seminiferous tubules were isolated after digesting neonatal pig testes with collagenase IV (2mg/ml) and hyaluronidase (2.5µg/ml, Sigma). Then, individual cells were isolated by digesting seminiferous tubules with 2 mg/ml trypsin (Sigma) and 8 mg/ml DNase.

Selection of testicular somatic cells by differential plating

Testicular cells ($5x10^6$ cells/ml) were plated on 100 mm tissue culture dishes (BD Bioscience) in 10 ml of Dulbecco Modified Eagle Medium (DMEM) (Life Technologies) with 5% Fetal Bovine Serum (FBS) (Thermo Scientific) and incubated at 37ºC in 5% CO_2 in air. After 18 hours, cells remaining in suspension and those slightly attached (containing the majority of germ cells (Luo, 2006 #112); were removed after several washes with Dulbecco's Phosphate-Buffered Saline (DPBS) (Life Technologies). The remaining attached cells were collect by using 2ml of 0.25% trypsin-EDTA (Gibco, Life Technologies) per dish and incubated for five minutes at 37^0C, Trypsin action was neutralized by addition of 10% of FBS, and the cell suspension was washed two times in

PBS and resuspended in high-glucose DMEM. The recovered cell population was characterized by immunohistochemistry and subjected to two different experimental treatments.

5.5.1 **Experimental design:**

Attached cells recovered from one round of differential platting were subjected to two different treatments: *in vitro* culture (A) or *in vitro* tubule formation (B), 5 replicates per treatment were performed at different dates with different pool of cells (n=5).

Treatment A - *in vitro* culture:

Somatic cells, $200x10^3$, were plated in each well of a 24 well plate, cultured in DMEM with 5% FBS and 50 I.U. of Penicillin and incubated at 37ºC in 5% CO_2 in air overnight to promote cell attachment. Then cells were serum deprived for 48 hours to attenuate proliferation and promote primary cilia growth ((Schneider, 2005 #5). After that, media was changed: For the treated group, 10µM of Ciliobrevin D (EMD Millipore) was added for 48 hours. Preliminary experiments testing different concentrations (5µM to 40µM) showed that 10µM was the minimal amount required to eliminate cilia in more than 90% of cells. For the control group the equivalent amount of DMSO used as vehicle in the treated group was added for 48 hours. After 48 hours, attached cells from control and treated groups were fixed in 4%PFA and characterized by immunocytochemistry for ARL13B, vimentin, Gata4, γ-tubulin, Ki67, Gli2.

Treatment B - *in vitro* tubule formation

In vitro tubule formation was performed as described by Gassei *et al* [83]. Briefly, 24 well plates were pre-coated with 1:1 diluted Matrigel with DMEM, then $1x10^6$ cells were added in 300ul in DMEM/F12 (Life Technologies), 1% Insulin-Transferrin-Selenium, 1%Penicilin, and 0.1% Epidermal Growth Factor (Sigma Aldrich). The treated cells received 20µM of Ciliobrevin D at Day 0 and control cells the equivalent amount of DMSO. Preliminary experiments testing different concentrations (10µM to 40µM) showed that 20µM was the minimal amount required to disturb tubule-like assembly in three dimensional culture conditions. At day 4, *in vitro* formed tubules were measured and characterized for the presence of primary cilia by immunohistochemistry against ARL13B and evaluated by confocal imaging.

Propidium Iodide (PI) staining was performed in wells from control and treated groups to evaluate cell viability after treatment with Ciliobrevin D. Propidium Iodide (Sigma-Aldrich), 20ul of 500nM was added to each well, incubated for 10 minutes, rinsed in PBS and evaluated under the microscope.

Ciliobrevin D reversibility assay

Somatic cells, $200x10^3$, were plated in glass coverslips attached to 24 well plates, cultured in DMEM with 5% FBS and 50 I.U. of Penicillin and incubated at 37ºC in 5% CO_2 in air overnight to promote cell attachment. Then media was changed for DMEM/F12, 1% Insulin-Transferrin-Selenium, 1% Penicillin, and 0.1% Epidermal Growth Factor for 24 hours to enhance primary cilia growth. Subsequently, 10µM of Ciliobrevin D was added for 48 hours and the same amount of DMSO was added to the

Control group. After 48 hours, Ciliobrevin D glass coverslips were collected and cells fixed in 4% PFA. Remaining wells, had their media removed through sequential washes with DPBS. Media without Ciliobrevin D, was added to the wells, (control and previously treated) and incubated at 37ºC in 5% CO_2 for 48 hours, then cells were fixed in 4%PFA for further characterization by immunocytochemistry against ARL13B.

Cell Characterization:

Cells used in all experimental groups were characterized by immunocytochemistry. Germ cells were characterized by their expression of VASA [276], Sertoli cells by Gata4 [251], Leydig by P450c17 [252] and peritubular myoid cells by alpha-smooth muscle actin [253]. Primary cilia were identified by ARL13B [277], hedgehog pathway activation was evaluated by nuclear localization of Gli2 [278]. Centrosomes were identified by γ-tubulin, and cell proliferation by Ki67 staining. Vimentin was used to identify somatic cells.

Cells were fixed in 4% paraformaldehyde for 10 minutes and washed three times in PBS, then cell suspensions ($8x10^4$ cells/ml) were attached to slides by cytocentrifugation (400g for 5 minutes). Cells attached to slides were permeabilized in 0.1% Triton-X solution for 15 minutes followed by three consecutive washes in PBS. To avoid non-specific staining, cells were incubated in CAS-Block (Life Technologies) for 10 minutes, then were incubated overnight at 4ºC with the following antibodies: mouse monoclonal anti vimentin Cy3 (1:500, C9080 Sigma Aldrich), mouse monoclonal anti alpha-smooth muscle actin FITC conjugated (1:500, F3777,Sigma Aldrich), mouse monoclonal anti

Gata4 (1:40; sc25310,Santa Cruz Biotechnology), mouse monoclonal anti P450c17 (1:500, kindly donated by Dr. Alan Conley, University of California at Davis), mouse monoclonal anti human ki67 (1:200, m7240, Dako), rabbit anti human protein gene product 9.5 (UCHL1) (1:500, 7863-0504, AbDSerotec), rabbit polyclonal antibody to ARL13B (1:500, 17711-1-AP , Proteintech,), rabbit polyclonal antibody to Gli2 (1:200, ab7181, Abcam). Primary antibody incubations were followed by secondary antibodies: goat anti rabbit Alexafluor 488 (1:400, A-11008, Sigma Aldrich) or donkey anti mouse Alexafluor 555 (1:400, A-31570 Sigma Aldrich) to identify spermatogonia ($UCHL1^{+}$, $vimentin^{-}$), Sertoli ($Gata4^{+}$, $UCHL1^{-}$), Leydig ($P450c17^{+}$, $vimentin^{+}$) and peritubular myoid cells (alpha-smooth muscle $actin^{+}$, $vimentin^{+}$).

The hedgehog pathway was considered active when Gli2 was nuclear, primary cilia were $ARL13^{+}$, and proliferative cells were $Ki67^{+}$.

5.5.2 **Image Analysis:**

Confocal image analysis and quantification was performed using a Leica SP8 spectral confocal microscope and ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2014.).

Analysis and measurement of *in vitro* tubules was done with a Zeiss inverted microscope AxioImager M2 equipped with a CCD camera and controlled with AxioVision 4.8 software.

5.5.3 **Statistical Analysis:**

Student *t*-test (PRISM software version 6; GraphPad) was used to assess group differences and $p<0.05$ was considered statistically significant.

5.6 **Results:**

Testes cell compositions were characterized prior to each experiment. Sertoli cells comprised 89.8±2.4% of all cells, followed by peritubular myoid cells (6.8±0.2%), Leydig cells (2.6±0.7), and germ cells (2.1±1.5) (Fig.5.1).

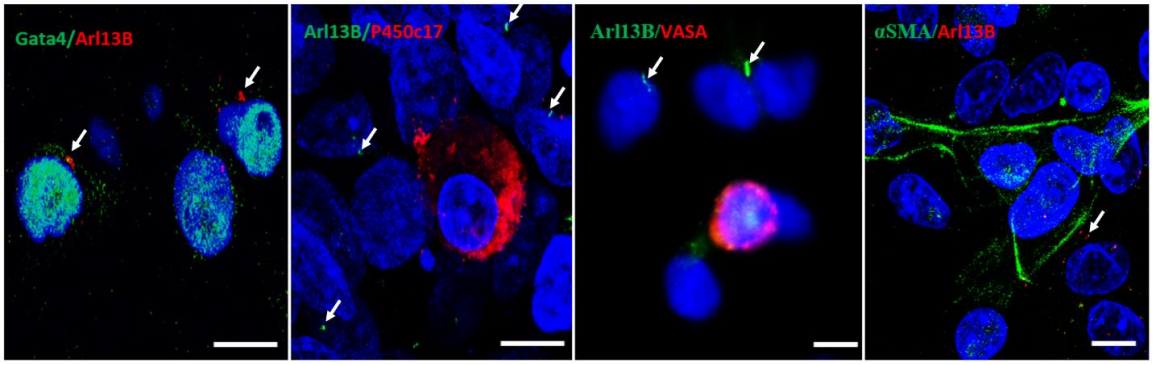

Figure 5-1 Characterization of the somatic cell population used in experiments and localization of primary cilia by immunofluorescence staining. A. Sertoli cells (Gata4, ARL13B and Dapi). B. Leydig cells (P450c17, ARL13B and Dapi). C. Germ cells (Vasa, ARL13B and Dapi). Germ cells do not display primary cilia (Ou et al., 2014). D. Peritubular myoid cels (smooth muscle actin, ARL13B and Dapi). White arrows indicate primary cilia. Bars=10μm.

After 48 hours of serum starvation, primary cilia were present in 89.3±2.3% of cells cultured in the control group but showed an almost complete loss following application

of 10 μM Ciliobrevin D (3.1±2.5%; $p<0.05$). Similarly, the Hh transcriptional activator Gli2 was located in the nucleus in 90.2±1.2% of cells from the control group indicating an active hedgehog pathway. In the treated group only 3.9±0.7% of cells showed nuclear localization of Gli2 (Fig.2 and Fig.3).

To evaluate possible off target effects driven by the inhibition of dynein, such as abnormal mitotic spindle arrangement or centrosome positioning, we evaluated centrosome localization and cell proliferation. After 48 hours, neither cell proliferation as assessed by % $Ki67^+$ cells, (9.1±1% vs 9.3±1.4%, respectively; Fig.4), or distance between the centrosome and nuclear membrane (2.16±0.76μm in control vs 2.4±0.8μm) was changed by Ciliobrevin D treatment.

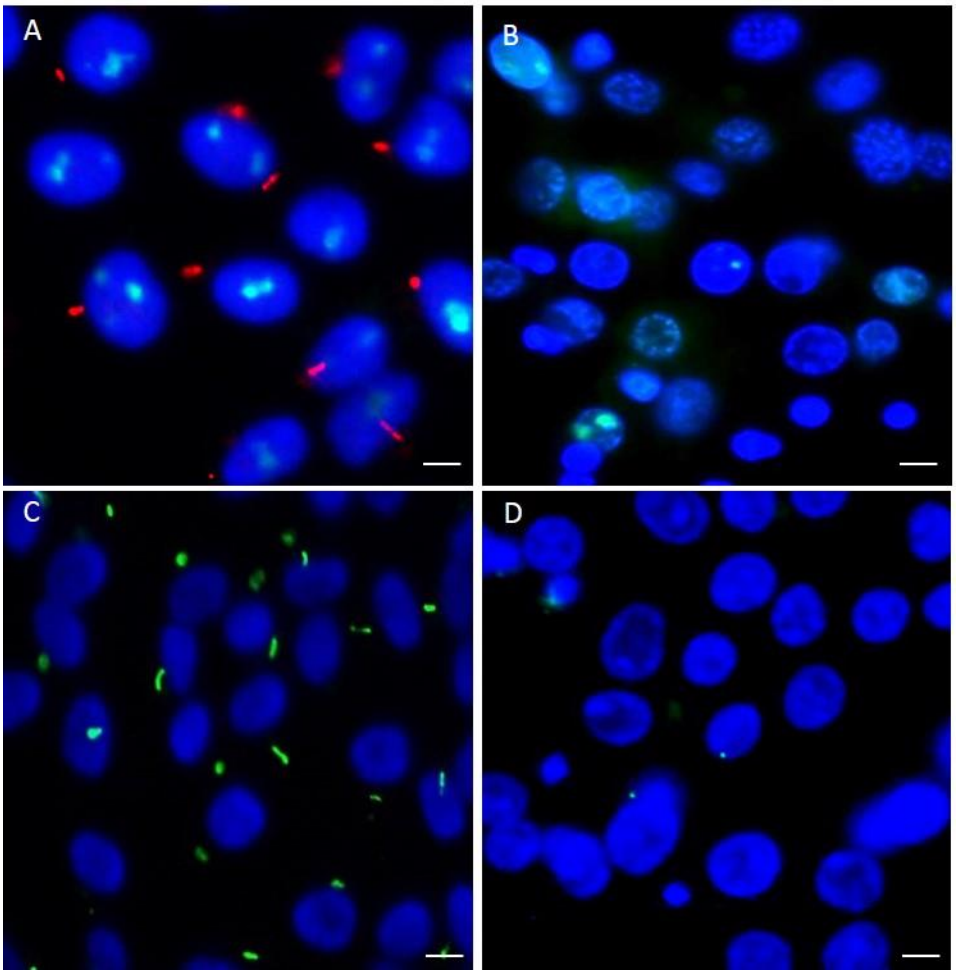

Figure 5-2 Analysis of hedgehog signaling and primary cilia by immunocytochemistry. A-B. Immunofluorescence against Gli2 and ARL13B, (Gli2 labelled in green and primary cilia in red). A. Control cells B. Ciliobrevin D treated cells. C-D Immunofluorescence against ARL13B (primary cilia labelled in green). C. Control. D. Ciliobrevin D treated cells. DAPI indicates nuclear staining Bars=10µm

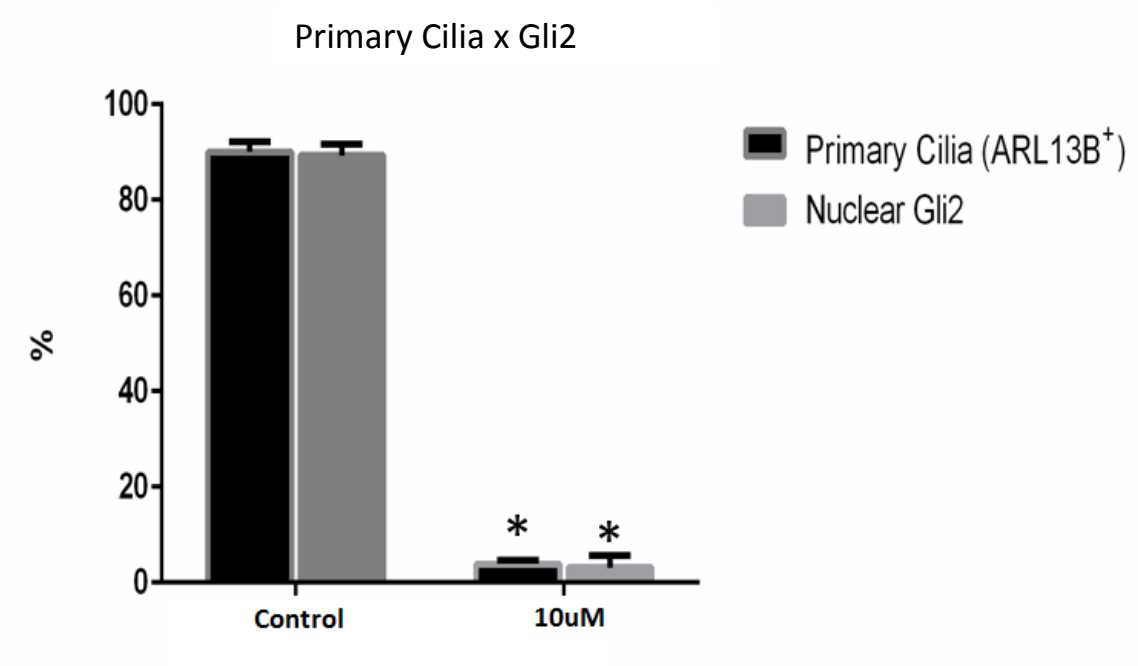

Figure 5-3 Comparison of presence of primary cilia and nuclear localization of Gli2 between control and 10uM Ciliobrevin D treated cells after 48hours *in vitro*.* indicates $p<0.05$

Inhibition of primary cilium impedes *in vitro* tubule formation

After 48 hours in culture, tubule like structures formed in all wells from control and treated groups. Wells treated with 20µM Ciliobrevin D displayed smaller and more delicate looking structures (Fig.5.5). By day 4, we measured *in vitro* tubule like structures from both groups and found that tubules from the control group were significantly longer (9.91 ± 0.85 vs 5.54 ± 1.08mm) and wider (339.8 ± 55.78 vs 127.2 ± 11.9µm) than those formed in the presence of Ciliobrevin D ($p<0.05$).

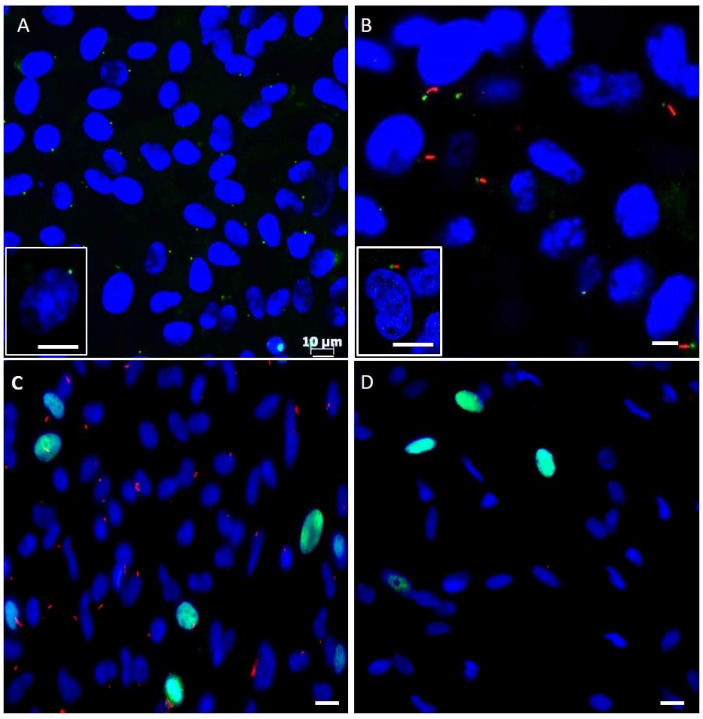

Figure 5-4 Immunocytochemistry to identify centrosome location and cell proliferation in control and Ciliobrevin D treated cells. A-B. γTubulin (green) and ARL13B (red) immunofluorescence staining. A. Control, B. Ciliobrevin D treated cells. C-D. Ki67 (green) and ARL13B (red) immunofluorescence staining. C. Control. D. Treated cells. Bars=10µm

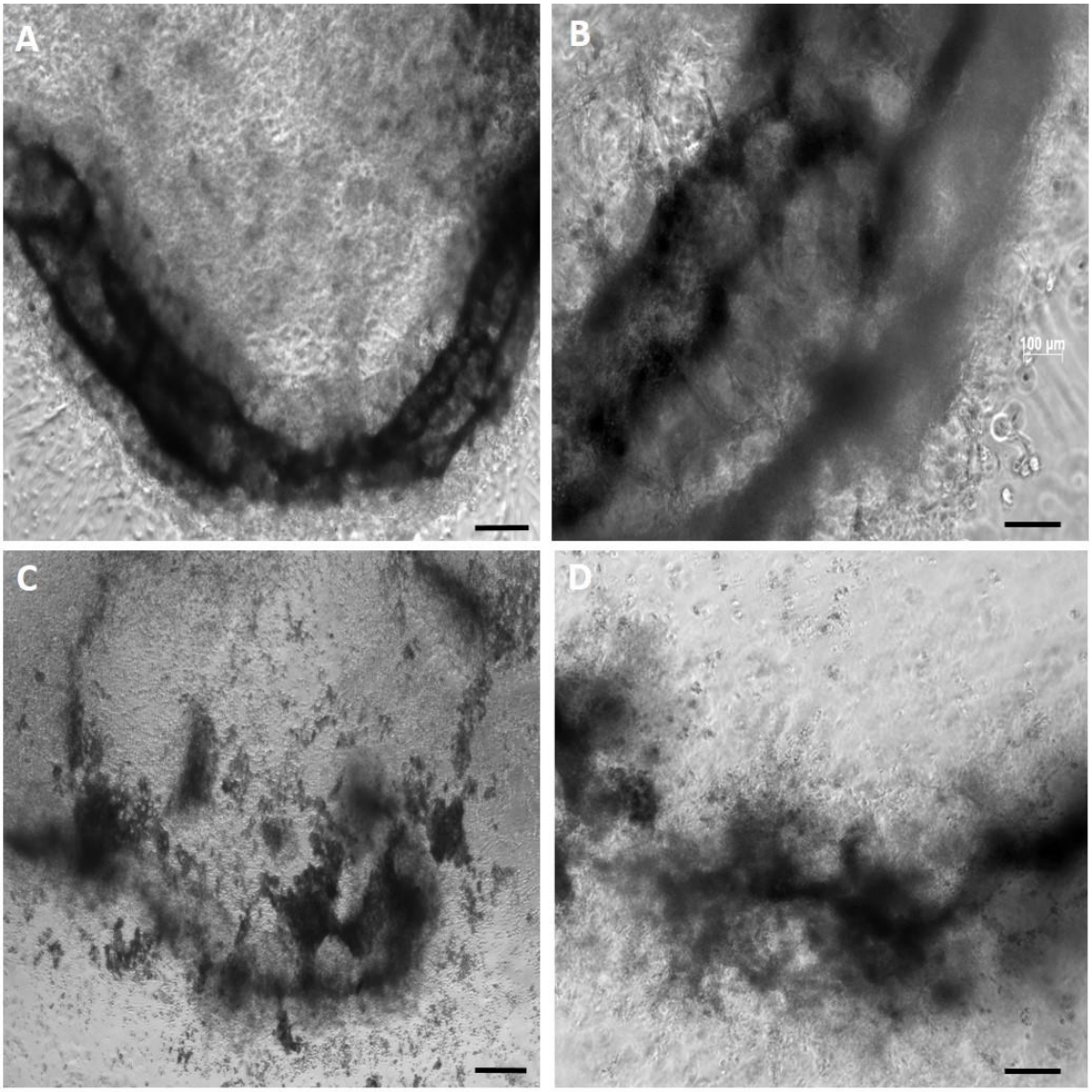

Figure 5-5 Morphology of *de novo* formed tubules *in vitro* A-D. Phase contrast images of tubule like structures at day 4. A-B Control, C-D. Ciliobrevin D treated group. A-C bars= 200µm. B,D bars=100µm.

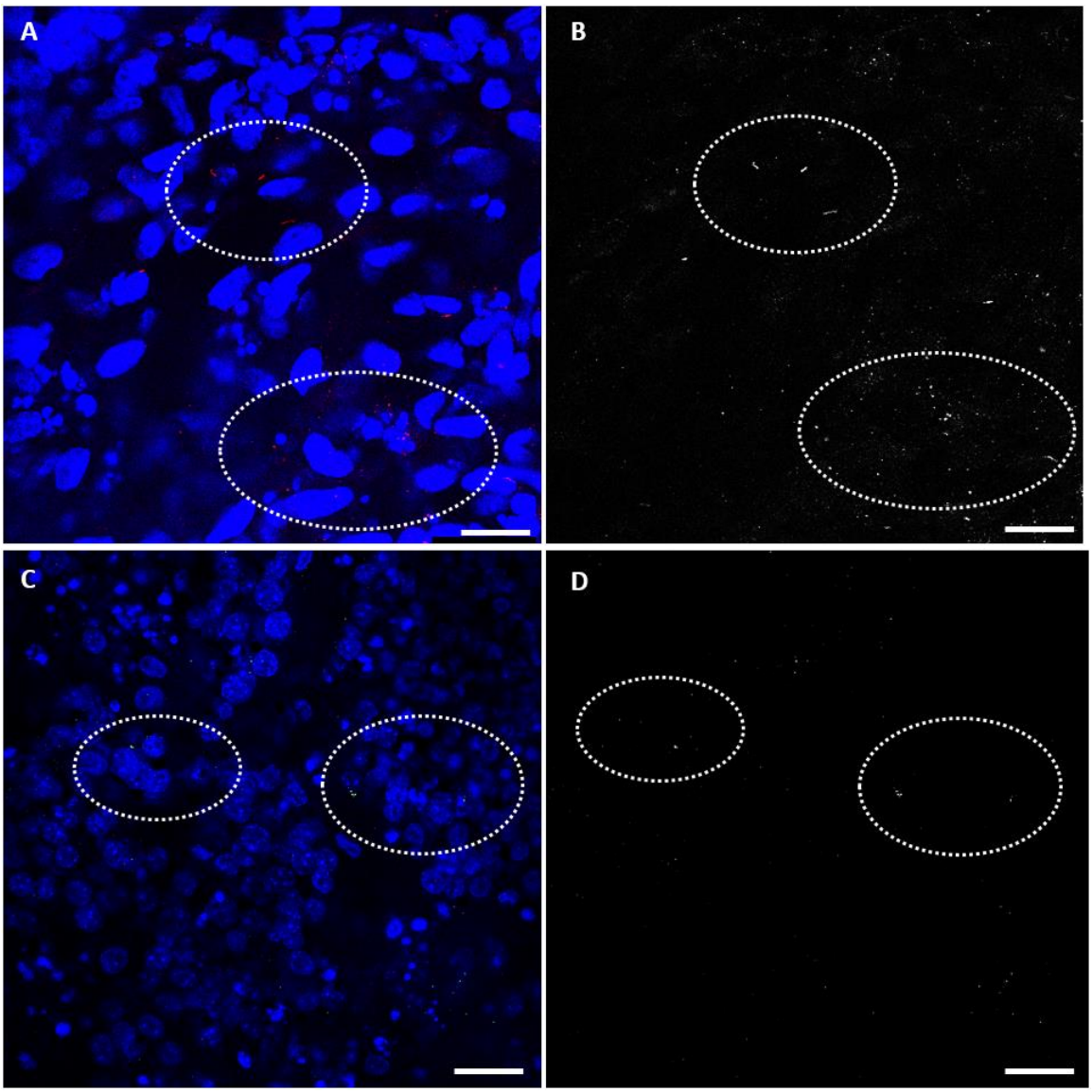

Figure 5-6 Analysis of primary cilia in tubule like structures formed *in vitro* at day 4 by immunohistochemistry against ARL13B. A. Control group, overlay images, Dapi and ARL13B (red). B. Control. ARL13B channel. C. Ciliobrevin D group, overlay images Dapi, ARL13B (green). D. Ciliobrevin D group, ARL13B channel. White lines indicate areas with primary cilia. Bars= 25µm.

To determine whether loss of cilia might underlie these functional deficits observed

following Ciliobrevin D treatment, we performed confocal imaging analysis of tubule-

like structures formed in the presence and absence of the inhibitor. Immunostaining of primary cilia with ARL13B showed that addition of 20µM Ciliobrevin D decreased the percentage of cells displaying primary cilia. In control group tubules, 43.2±2% of cells had primary cilia, whereas in the treated group a significantly lower ($p<0.05$) percentage of cells (21±3%) had the organelle (Fig.6). Addition of Ciliobrevin did not affect cell viability. In both groups, only a few floating cells were PI^+, and none of the cells contained within the tubules or aggregated into small clusters were PI^+. To verify if PI was able to penetrate the tubules and could be used as an accurate method to detect viability, tubules were heat treated to promote cell death and were used as positive control.

Ciliobrevin D reversibility assay

Testicular somatic cells treated with 10 µM of Ciliobrevin D for 48 hours displayed reversible inhibition of cilia formation. Primary cilia was present on 5.25±1% of 10µM treated cells vs 81.1±2.2% in rescued cells and 83±1.1% in control cells.

5.7 Discussion:

The past 15 years have yielded great progress in the understanding of how primary cilia orchestrate development by commanding important pathways. This has led to a better understanding of a multitude of complex human syndromes such as Joubert syndrome, polycystic kidney disease, Jeune syndrome, and others [56]; it also promoted increased interest from the pharmaceutical industry to develop drugs targeting cilia and hedgehog proteins.

The role of hedgehog pathways during embryo development has been established from Drosophila to vertebrates. In vertebrates, most of the available data about the male reproductive system and its coordinating pathways is taken from studies using rodents, nonetheless, no studies were performed to understand the role of primary cilia in male reproductive organs. The only information available is that most patients with ciliopathies have hypogonadism and are infertile [279].

Our group was the first to describe the presence of primary cilia in the testis [80], and the present study demonstrates a link between primary cilia and the hedgehog pathway in neonatal testicular somatic cells. To study the effect of primary cilia in testicular somatic cell function we used Ciliobrevin D, a selective ATPase motor cytoplasmic dynein antagonist. We were able to perturb cilia formation and consequently block hedgehog signaling. Given the importance of ATPase motor cytoplasmic dynein processes in cell viability, we used the minimal effective concentration to inhibit cilia formation and provided a serum free environment to inhibit proliferation and avoid that other cellular processes dependent on ATPase motor cytoplasmic dynein were affected [275]. Our data shows that viability and centrosome positioning were not disturbed by addition of 10µM of Ciliobrevin D, and proliferation rates were low and similar in both groups for more than 48 hours in culture.

Inhibition of primary cilia in testicular somatic cells blocked the Hh pathway and prevented Gli2 localization to the nucleus. Additionally, by using the *in vitro* tubule

formation, we demonstrated that reduction in primary cilia impaired the ability of neonatal testicular cells to rearrange into testicular like tubules.

The findings reported in this manuscript establish a relationship between primary cilia and hedgehog signaling in the neonatal testis. Given the recognized role of primary cilia as a signaling hub for important pathways in many other systems, it will be interesting to further investigate the role of this organelle and the interplay between pathways that establish and maintain the spermatogonial stem cell niche. The ability to manipulate the primary cilia in the testis could also serve as a new model for studying cilia function, elucidate reproductive defects in patients with ciliopathies and test new treatments.

In summary, we demonstrated that inhibition of ATPase motor cytoplasmic dynein inhibits formation of primary cilia in testicular somatic cells and thereby inhibits hedgehog signaling. Furthermore, we could show that primary cilia are required for robust *in vitro* formation of tubules, indicating that primary cilia on testicular somatic cells appear to be important for testicular morphogenesis.

5.8 **Supplementary Information**

This supplementary section describes additional experiments following the work presented in Chapter V. The aim of the experiemnts described here was to employ *de novo* morphogenesis of testis tissue from grafted cells, presented in chapter III, to explore the role of primary cilia in tubule assembly and the maintinance of a functional testicular microenviroment *in vivo*. We employed a targeted siRNA knockdown of the ODF2/Cenexin1 gene, given its exclusive role in sustaining the distal appendages of the basal body of the cilia [275], via siRNA lipofection. This was followed by immunocytochemistry, Western blot, and qPCR to monitor if Odf2/Cenexin1 siRNA sequences used effectivly knocked down ODF2. However, the Odf2/Cenexin1 protein could still be detected by Western blot 5 days post- transfection, indicating that the protein has a long half life in the cell and may sustain the foundation of the primary cilia despite the knockdown. Genes related to the IFT will be target in future experiments.

5.8.1 **Materials and Methods**

Cell culture

Neonatal porcine testicular cells enriched for somatic cells as described in Methods 5.2.

Lipofection of testicular somatic cells with ODF2 siRNA

Three Validated Stealth RNAi™ siRNAs specifically recognizing different sequences of porcine ODF2 mRNA were selected using BLOCK-iT™ RNAi Express (Life

technologies) labelled with Alexa 488 (Supplementary Table 5.1). As a negative control Stealth RNAi Negative Control Duplexes (LifeTechnologies) with medium GC content labelled with Alexa555 was used as recommended by the manufacturer. Transfection with RNAiMax (Invitrogen) was used to introduce siRNAs into cells according to the manufacturer's recommendations. Briefly, somatic cells, $1x10^6$ per well were plated in a 6 well plate and cultured in DMEM, 5%FBS with 50 I.U. Penicillin. When 80% confluence was achieved the media was changed to OptiMen (Gibco) and 75pM of siRNA combined with 9ul of RNAiMax with was added and incubated for 48 hours at 37^{o}C. The efficiency of the lipofection protocol was evaluated by FACS sorting after 48 hours of lipofection. Presence of cilia was evaluated by immunohistochemistry against ARL13B at days 2, 3, 4, 5, 6 after lipofection, expression of ODF2 was quantified by qRT-PCR and ODF2 protein detected by Western Blot at day 5 after lipofection. Lipofection with siRNAs was repeated independently five times. IF was performed all times, WB two times and qPCR once.

Table S 5-1 Sequences of ODF2 Validated Stealth RNAi™ siRNAs

Name	Sequences
siRNA_821	5'-GAGAAACAGAUGACCUGCACGGAUA-3'
	5'-UAUCCGUGCAGGUCAUCUGUUUCUC-3'
siRNA_1413	5'UGCAACUUGCUGACAAGGAUCUUUA-3'
	5'-UAAAGAUCCUUGUCAGCAAGUUGCA-3'
siRNA_2131	5'-GAAGGUGGGUGAACUGGAGAGGAAA-3'
	5'UUUCCUCUCCAGUUCACCCACCUUC-3'

Quantitative Real-Time PCR (qRT-PCR)

The expression of ODF2 was evaluated by qRT-PCR. Total RNA was isolated using RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. RNA (2ug) was reverse transcribed using High Capacity cDNA synthesis kit [40]. RT-PCR products for Odf2 and Rpl4 (pig endogenous control) primers were separated on a 1.3% agarose gel and visualized using ethidium bromide. Products were also sequenced using an Agilent 2100 bioanalyzer. Primer sequences for are: Odf2 forward 5'-AATGAACGCGGTTGGTTGTC-3' and reverse 5'-CGTACTCCTCGCTCTTCTCG-3' and RPL4 forward 5'-GCCTTCAGAAACATCCCTGGAATTACTCTG-3' and reverse 5'-GCATGGGAAGGTTGTAGTTACTCTTGAG-3'. Quantitative PCR was performed using SsoFastTM EvaGreen Supermix with Low Rox (Bio-Rad) and samples were run using 7500 Fast Real Time PCR system (Applied Biosystems). The expression level of

ODF2 was normalized to the expression of Rpl4 in each sample and was presented as a fold-change in expression compared to control sample.

5.8.2 **Western Blot**

Testicular somatic cells lipofected with siRNA for ODF2 and siRNAscrambled were culured in serum free media for five days after transfection to dimish cell division and optimize cilia growth. Cells were washed with PBS and 300µl RIPA buffer was added directly to the wells in order to induce lysis and immediately denature proteins. Protein was quantified using DC Protein Assay (Bio-Rad) and 20 µg of protein from each sample was separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA) previously activated in Methanol. Membranes were blocked in 5% skim milk (BSA) dissolved in Tris Buffered Saline with 0.1% Tween-20 and probed overnight with primary antibodies against Cenexin1/ODF2 (rabbit polyclonal; 1µl/ml, ABCAM), and against actin (mouse monoclobal; Sigma-Aldrich) . Secondary antibody detection was performed using appropriate HRP-conjugated secondary antibodies and bands were visualized by film-ECL detection.

5.8.3 **Immunofluorescence staining**

Coverslips containing attached testicular cells lipofected with siRNAs for ODF2 and scrambled siRNA from days 2, 3, 4, 5, 6 after lipofection were fixed in 4% paraformaldehyde for 10 minutes and washed three times in PBS, then permaebilized in

0.1% Triton-X solution for 15 minutes followed by three consecutive washes in PBS. To avoid non-specific staining, cells were incubated in CAS-Block (Life Technologies) for 10 minutes, afterwards they were incubated overnight at 4^{o}C with mouse monoclonal anti vimentin Cy3 antibody (Sigma Aldrich), and rabbit polyclonal antibody to ARL13B (Proteintech). Primary antibody incubations were followed by donkey anti rabbit Alexafluor 488 (Sigma Aldrich) and the nucleus couterstained with DAPI.

5.8.4 **Three dimensional *in vitro* tubule assays**

The capacity of neonatal somatic cells to assemble tubule like structures when in culture in Matrigel (BD Bioscience, San Jose, CA, USA) coated wells was evaluated 72 hours after lipofection. An *in vitro* system for evaluation of tubule formation was described in methods section 5.2. Three siRNA were tested and cells transfected with scrambled siRNA sequence were used as a control for this experiment (Supplemental Figure 5.8)

At day 4, *in vitro* formed tubules were measured and characterized for the presence of primary cilia by immunohistochemistry against ARL13B and evaluated by confocal imaging.

Figure 5-7 Flow chart describing the time line of the *in vitro* tubule formation assay

Statistical analysis

Data obtained from immunocytochemistry for detection of primary cilia were tested using Tukey's test method after one-way ANOVA. Prism 6, GraphPad Software, (La Jolla, CA, USA) was used for statistical analysis, $p < 0.05$ were considered to be significant.

5.8.5 Results and Discussion

Immunohistochemistry analysis of cells lipofected with one of the three siRNAs recognizing different sequences of the Odf2 gene indicated that cells still had cilia (see table S 5.1), there was no significant difference between cells with primary cilia among control and siRNA_ODF2 lipofected groups at any time point (Figures 5.8-5.11).

Table S 5-2 Primary cilia present at day 5 post transfection

	Not transfected	Control	siRNA821	siRNA1413	siRNA2123
ARL13B$^+$	87.5± 1.0%*	61.1 ± 2.2%	65.2± 1.3%	62.8± 3.1%	62.8± 2.8%

*Indicates $p<0.05$

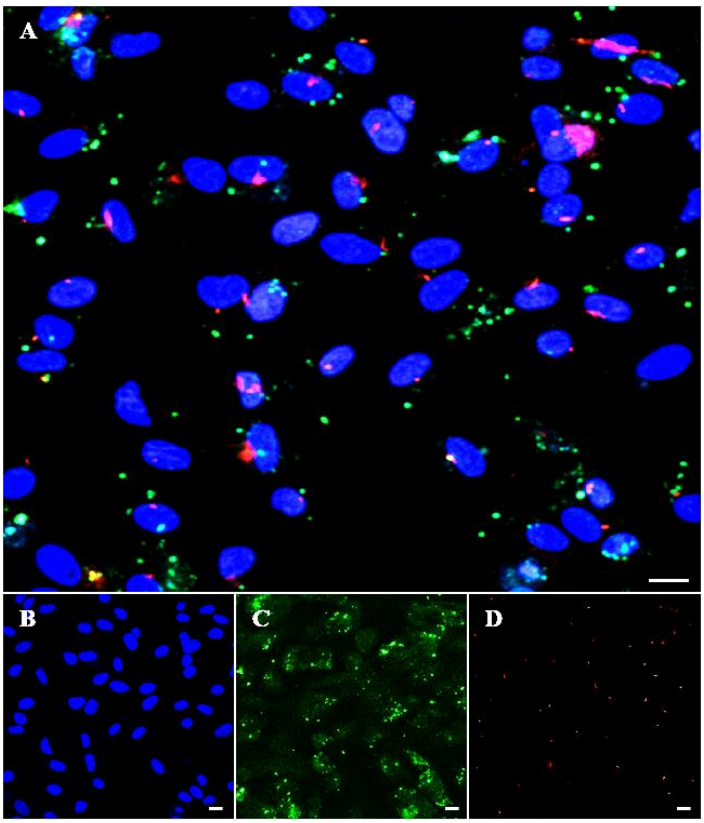

Figure 5-8 Immunocytochemistry to identify primary cilia in the control group at day 4 post lipofection. A. Overlay image of cells, DAPI, Alexa 488 and ARL13B channel (transfected cells labelled in green, primary cilia in red and nuclei in blue). B. Dapi nuclear staining. C.Alexa 488 channel (transfected cells). D. ARL13B channel (primary cilia in red). Bars=20µm

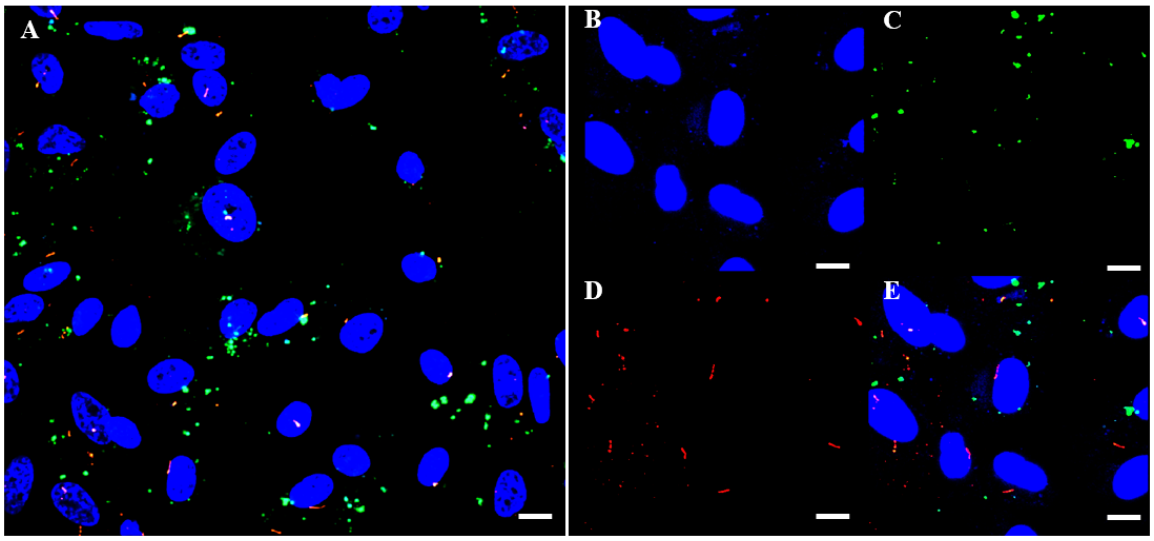

Figure 5-9 Immunocytochemistry to identify primary cilia in cells transfected with siRNA851 at days 4 and 6 after lipofection. A. Overlay image of cells at day 4, DAPI, Alexa 488 and ARL13B channel (transfected cells labelled in green, primary cilia in red and nuclei in blue), bars=10µm B. DAPI nuclear staining. C.Alexa 488 channel (transfected cells in green). D.Immunostaining against ARL13B (primary cilia in red). D. Overlay image of cells at day 6, DAPI, alexa 488 and ARL13B channel (transfected cells labelled in green, primary cilia in red and nuclei in blue). Bars= 5µm.

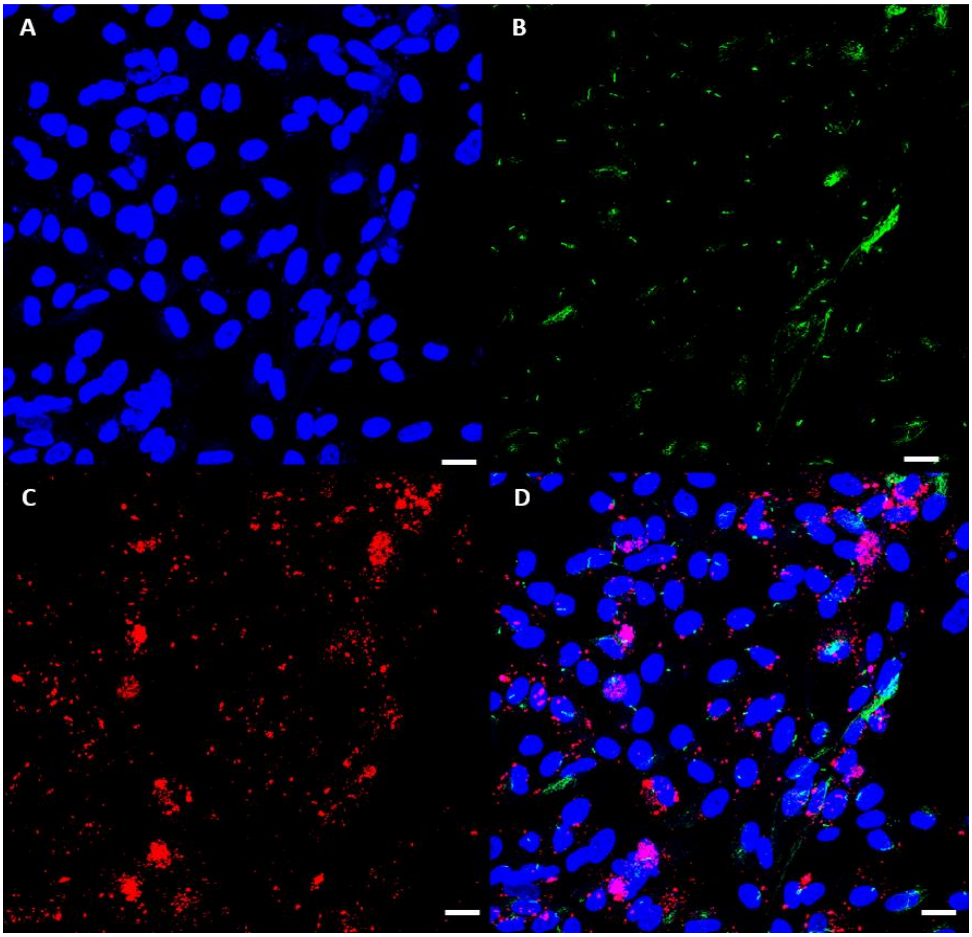

Figure 5-10 Immunocytochemistry to identify primary cilia in the control group at day 4 post lipofection. A. Dapi nuclear staining. B. Immunostaining against ARL13B (primary cilia in green). C Alexa 555 channel (transfected cells in red). D. Overlay image. Bars=10µm

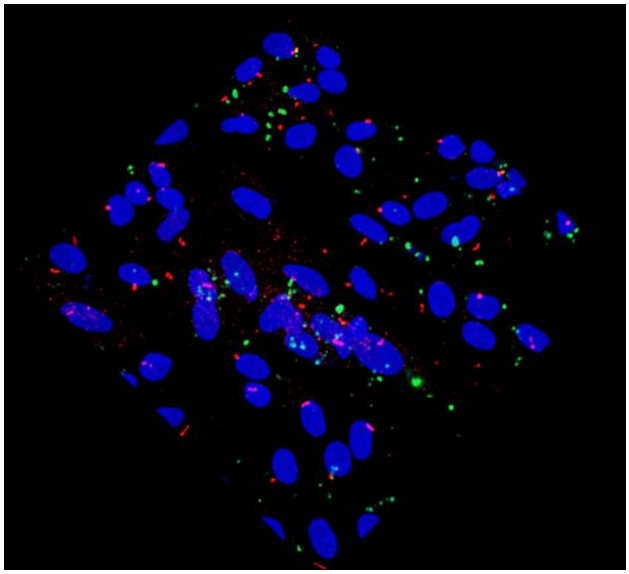

Figure 5-11 Three dimension image of immunocytochemistry against ARL13B to identify primary cilia in cells transfected with siRNA1413 at day 6 after lipofection. Overlay image of cells, DAPI, Alexa 488 and ARL13B channel (transfected cells labelled in green, primary cilia in red and nuclei in blue). Bars=20µm

Q-PCR indicated sequences used effectively knocked down ODF2 as shown in figure 5.12 and Western blot demonstrated that the protein was reduced between control and cells transfected with siRNA821 and siRNA1413 at day five (Figure5.12). *In vitro* tubule formation showed that cells lipofected with ODF2 siRNAs maintained their ability to aggregate and form tubule-like structure *in vitro* (Figure 5.14).

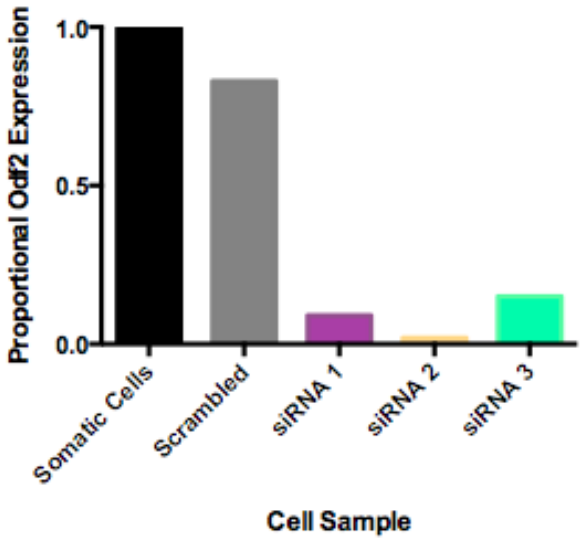

Figure 5-12 qPCR results of Odf2 knockdown for siRNA 1, 2, 3 and scrambled RNA in comparison to

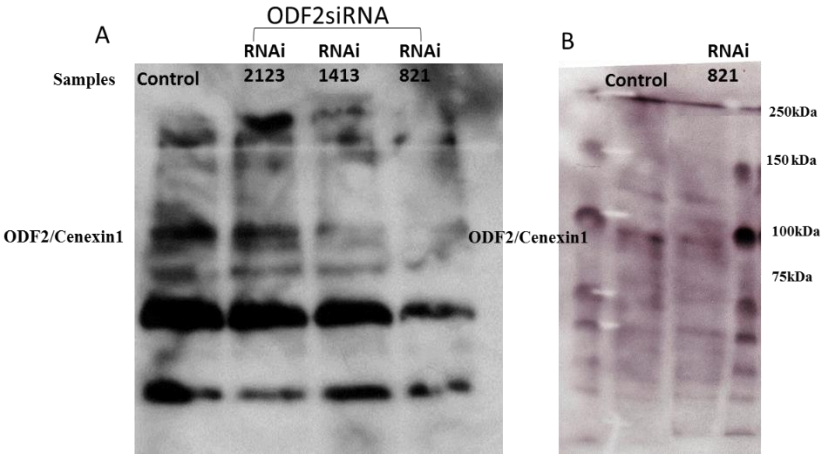

Figure 5-13 Western blot analysis of ODF2/Cenexin1 (93kDa) levels in cells after knockdown with siRNAs after 5 days after lipofection. A. Samples from three siRNAs were analyzed for ODF22/Cenexin1 levels. B. Repeat analysis of siRNA 821.

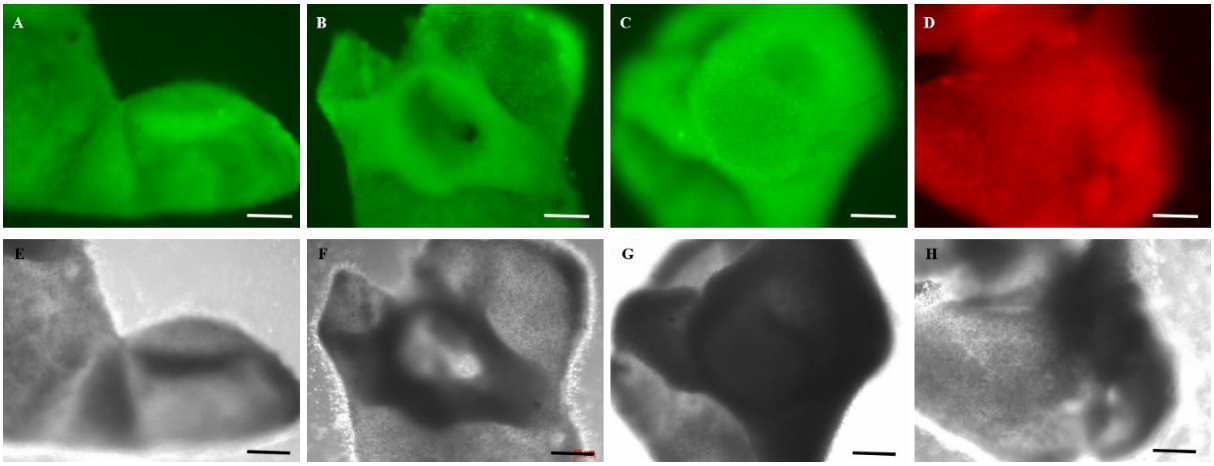

Figure 5-14 Morphology of *in vitro* formed tubule like structures from cells lipofected with Stealth siRNA. A,E: Cells lipofected with siRNA851. A .Alexa 488 channel, E, Phase contrast. B,F: Cells lipofected with siRNA1413. B Alexa 488 channel, F. Phase contrast. C, G: cells lipofected with siRNA2131. C Alexa 488 channel. G Phase contrast. D, H: Cells lipofected with siRNA control. Bars=100µm.

Our results indicate that the ODF2/Cenexin1 protein has an extended half-life in the cell, our serum free culture conditions aiming to reduce the proliferation rate may have contributed as a factor to to slow down the Odf2/Cenexin1 turn over, nonetheless the existing amount of protein was able to sustain the foundation of the primary cilia despite the knockdown. Future experiments will target genes related to the IFT for their continuous turnover of microtubule proteins [288].

6 Chapter VI: Discussion and Future Directions

Overall, the work completed in this dissertation focused on building knowledge on *de novo* morphogenesis of testis tissue to study the testicular microenvironment, and on investigating the role of primary cilia during testicular development. In summary, first, we optimize the existing *de novo* morphogenesis of the testis system by testing a multitude of factors. Second, we showed that the method is a useful bioassay to assess testicular cell viability and function. Lastly, we exploit the ability of testicular cells to assemble tubule-like structures *in vitro* and followed up previous work from our group investigating the role of primary cilia in the testis. We provided evidence that inhibition of primary cilia in testicular somatic cells negatively affects tubule formation *in vitro* and eliminates Hedgehog signalling.

6.1 Summary and Highlights

The use of *de novo* morphogenesis of testis tissue as a functional bioassay

One of the major goals of this thesis was to optimize the *de novo* morphogenesis of testis tissue and use the method as a functional bioassay to study male reproductive biology. The opportunity to manipulate different proportions of cells prior to grafting, combined with the further development of a functional SSC niche (as reviewed in Chapter II) makes this technique appealing for researchers in the field.

The method can be used as a research tool in a wide range of species as the capability of somatic cells to reassemble functional tissue appears to be preserved across species.

However, to be broadly adopted by other research centers, the technique would benefit from a standardized protocol, and should achieve improved spermatogenic efficiency.

In order to optimize the method and tackled exiting hurdles, our experiments tested different combinations of cell groups during grafting. Our initial hypothesis was based on the premise that the scarce spermatogonia population in the cell pellet is the limiting factor for achieving a higher efficiency [212]. Our results did not support our hypothesis; however, while testing different variations to improve the system we verified that the addition of Matrigel to the cell pellet prior to grafting significantly improved the efficiency of recovered grafts; we added this approach to the experimental design based on reports from other groups [90, 233]. The superiority of the Matrigel when compared to the enrichment of undifferentiated spermatogonia was an unexpected benefit, and it turned the improvement of the technique into a feasible and simpler task.

In summary, we demonstrated that the *de novo* formation of tissue benefited from addition of Matrigel, and the number of cells grafted also influences the number of tubules formed. The combinations of Matrigel and large numbers of cells is essential for the improvement of this bioassay. This tool is the first of its kind and has proved useful as a functional bioassay to study the testicular niche and assess functionality of testicular cells as shown in the data presented in Chapters III and IV.

Challenges of the *de novo* morphogenesis bioassay

The earliest studies using the *de novo* morphogenesis assay stated that the low spermatogenic efficiency, elongated spermatids present in 10-20% of the seminiferous

tubules formed, was the major challenged faced by the method. Additionally, our results indicated large variability within groups as an additional limitation. This variably demands a larger number of replicates to allow for a reliable analysis of the data, and increases the chance of a type II error, i.e. false negative results occurring due to the noise in the system. For example, if the objective is to study the function of a gene or a growth factor during spermatogenesis, there is a chance of not detecting an existing phenotype because of the variation within the results.

Vascular Endothelia Growth Factor 165

The VEGF165 was chosen to test the efficacy of *de novo* morphogenesis as a functional bioassay given its intriguing presence in the SSC niche. VEGF and its receptors, Flt-1 and KDR (or VEGF1 and VEGF2), are found in Leydig and Sertoli cells, and in germ cells during different stages of development, Flt-1 is present on elongated spermatids and KDR on spermatogonia [48, 54, 281]. Furthermore, we aimed to elaborate on data from previous reports that indicated an existing relationship between SSC location and vasculature sites [6].

Before the completion of this manuscript, existing data from larger mammals derived only from bovine species, and the experiments performed investigating the role of VEGF used the testicular xenografting method to measure the effects of VEGFA treatment on spermatogenesis. As reviewed in Chapter 2.10.3, the testicular xenografting bioassay does not allow manipulation of cells, or even reliable exposure to the growth factor by all

components of the tissue. Therefore, the use of the *de novo* morphogenesis technique may be a better model to enhance the current knowledge in the field.

Using this bioassay we corroborated results from reports that used tissue xenografting [225], we also described the presence of more tubules with spermatids in the treated group than in the control. Additionally, the findings presented by our group also showed that Sertoli cells were more proliferative when treated with VEGFA. The later report using Sertoli-germ cell specific VEGFA null transgenic mice [53] not only supported our findings but also underlined the efficacy of the *de novo* morphogenesis approach as a method to study male reproduction.

This work could not verify an existing relationship between the location of spermatogonia and vasculature. Moreover, the bioassay did not support the premise that spermatogonia are located adjacent to vasculature sites, or that VEGFA increases vascularization in the newly formed tissue. Initial reports linking SSC location to vasculature sites [5, 6] have not been supported by other groups. Most recently, a paper using a marker specific for Asingle spermatogonia (Id4) using GFP-Id4 labelled cells, revealed a limited association between Asingle spermatogonia and vasculature [282].

Overall these findings further contribute to our understanding of the protective role of VEGFA in the testis, and the results indicated that this effect is conserved among different mammalian species.

The use of VEGFA in the *de novo* formed grafts also shed light on our initial hypothesis regarding the low spermatogenic efficiency present in the *de novo* formed tissue. The protective effect of VEGFA improved efficiency indicating that the low efficiency is probably due to cell damage caused by handling, or from hypoxia during tissue formation in the host environment.

Future directions:

The experiments described in chapters III and IV required 57 mice, grafted with 4 cell pellets each, resulting in the analysis of 200 grafts. The extensive graft analyses, combined with the preliminary studies performed in our lab, lead to novel hypotheses regarding the reduction of cell variability. First, by standardizing the surgical protocol, preliminary experiments were performed grafting 6 cell pellets per recipient. We observed that graft location influenced its development because tissue recovered from transplants located in the dorsal area of the ribcage did not display the same efficiency in number of *de novo* formed tubules as their counterparts located closer to humeral and femoral vascular branches. Consequently, we reduced the number of sites to four per animal. Secondly, the surgery date and surgical technician may have influenced the results. It may aid in reducing experimental variability to increase the number of animals for a given surgery date, and only assign one experienced researcher to perform all surgical procedures.

The protective and proliferative effect of VEGFA on testicular cells, demonstrated through the use of the *de novo* morphogenesis assay, corroborated previous work

performed with bovine tissue [225, 283]. However, this is only one observation regarding the role of VEGFA in the testis. A multitude of experiments could follow from the data presented in Chapter III. The use of the *de novo* morphogenesis assay could help address experimental questions such as: the role of anti-angiogeneic isoforms of VEGFA 165 during testis tissue reassembly, and the role of FLT-1 and KDR receptors in different cell types. Together with a genetic screen of apoptotic and anti-apoptotic genes during different developmental stages of *de novo* formed tissue, these future experiments could expand on our previous findings.

Stirred suspension bioreactor

Chapter IV addresses methodological limitations in the field of large animal model experimentation. We addressed the need for large numbers of a germ cell enriched cell population from large mammals. The SSB is a novel method to enrich for germ cells on a large scale, with less handling, and therefore is a practical alternative to the standard method of differential plating. Our data demonstrated that the method is effective to achieve enrichment, and appeals as an enrichment technology. To optimize germ cell enrichment, we suggest a combination of the SSB with the standard DP method. Combining these methods can utilize the strengths of each protocol. Beginning with the SSB allows one to reduce variability, enrichment time, physical handling, and incubator space needed. Following this protocol with DP can enhance the final enrichment.

Future Directions:

The data present at Chapter IV was acquired using experimental settings adapted from earlier studies performed in embryonic stem cells [284] [285] using SSB culture. The current advance in knowledge in the stem cell field shows that shear forces and aggregate sizes are used to manipulate and to enhance efficiency of culture or differentiation to specific cell lineages [286]. Our findings suggest that the protocol used for germ cell enrichment could be improved by testing different combinations of shear stress upon the cells by trying different RPM, cell concentration and time under SSB culture. An important next step is to explore these variables, with the ultimate goal of tailoring the formation of aggregates, in order to achieve higher germ cell enrichment than the combinational method described above.

Primary Cilia in the developing testis

Our group was the first to describe the presence of primary cilia in neonatal testicular somatic cells and its absence in undifferentiated germ cells. In Chapter V we followed this work by investigating the role of primary cilia in the neonatal testis. We demonstrate that the hedgehog pathway requires primary cilia in these cells.

The hedgehog pathway was chosen for investigation because of its intricate relationship with primary cilia in many other cell types [56], and its conserved evolutionary role during testis development which is present from flies [287] to humans [288]. Additionally, previous studies demonstrated that Dhh (a gene from the hedgehog family) is expressed in Sertoli cells and targets the receptor Ptc in Leydig cells during their

maturation phase [289], making the hedgehog pathway an ideal initial pathway to focus our experiments.

This dissertation established the relationship between testicular somatic cells, primary cilia, and the hedgehog pathway. It also demonstrated the direct effect that the inhibition of primary cilia had on tubule formation. However, many questions still remain, and from all of the presented chapters, Chapter V opens the door to many more interesting future directions. The supplemental information in Chapter V endeavours to strengthen the findings from our *in vitro* studies. The knock down of the ODF2 gene was sufficient to inhibit transcription but the protein persisted in the cell for an extended period of time. Thus we were unable to inhibit primary cilia formation with this strategy and limited experiments to using inhibitors. The negative results, nevertheless provided us with insights on how to improve future experiments.

The direct mechanism that inhibits primary cilium formation is unclear. We directly blocked the hedgehog pathway by eliminating the primary cilia, but other pathways can signal through the organelle, which opens up a plethora of future experimental avenues. Furthermore, we have to consider off target effects of the small molecule inhibitor used. We attempted to limit those effects by using the smallest effective concentration and a highly selective inhibitor [275].

Future Directions:

Chapter V showed that primary cilia are active in neonatal testicular somatic cells, the knock down experiments also demonstrated that the use of siRNA and the lipofection

delivery are an efficient method to genetically manipulate these cell types. Future experiments would make use of these protocol to test new gene targets to block primary cilia formation. Future candidate genes will likely be affiliated with IFT and microtubules such as IFT88 and kinesins. These have been shown to be effective targets for the disruption of primary cilia via siRNA knock down [290, 291]. Different than genes associated to structural organelles such as ODF2, these genes have a higher turnover of their coded protein. We expect to exploit the advantage of manipulating cell types prior grafting in the *de novo* morphogenesis assay to advance the current knowledge on the role of primary cilia during testis development.

6.2 **Closing remarks**

Improving the current understanding of the testicular microenvironment has many applications. As more tools and techniques become available for the study of male reproductive biology in larger mammals, the translational value of these protocols for veterinary and human medicine will accelerate. This dissertation has contributed several improvements to the field by improving the *de novo* morphogenesis assay, investigating the role of VEGFA and primary cilia in the testicular microenvironment in pigs, and providing a novel alternative to enrich testis cell suspensions for germ cells on larger scale. I hope that this work will also provide the foundation for further studies at Dr. Dobrinski's lab to continue to unravel the dynamic role of primary cilia in the testicular niche

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Appendix **A**

Publications and Presentations

Publications

The following appendix covers publications relevant to and completed during this thesis project.

Publications:

The following appendix covers publications relevant to and completed during this thesis project.

Accepted/Submitted

Dores C, Alpaugh W, Raharjo E, Biernaskie J & Dobrinski I. Primary cilia on porcine testicular somatic cells play a role in hedgehog signaling and tubular morphogenesis *in vitro.* Submitted *to* Biology of Reproduction.

Dores C, Rancourt D, Dobrinski I. Stirred suspension bioreactors as a novel method to enrich germ cells from pre-pubertal pig testis. Andrology. Epub ahead print.

Dores C, Dobrinski I. Role of ATPase motor cytoplasmic dynein and primary cilia in testicular morphogenesis. Reproduction Fertility and Development; 27(1):223, 12/2014

Dores C, Dobrinski I. De novo morphogenesis of testis tissue to investigate the role of VEGF-165 during testis formation. Reproduction: REP-13-0303. 2014.

Ou Y, **Dores C,** Rodriguez-Sosa J-R, van der Hoorn F, Dobrinski I. Primary cilia in the developing pig testis. Cell and Tissue Research: 358 [292] 597-605, 2014.

Zeng W, Tang L , Honaramooz A, Bondareva A, Tanco V, Megee M, Modelski M, Rodriguez-Sosa R, Paczkowski M, **Dores C**, Silva E, Wheeler M, Krishner R. &

Dobrinski I. Viral transduction of male germline stem cells in transgene transmission after germ cell transplantation. Biology of Reproduction. 88(1): 27, 2013.

Robbins H, **Dores C**, Coyle K, Dobrinski I. Germ cells and testicular somatic cells have different sensitivity to cryopreservation. Reproduction, Fertility and Development.25 (1):184, 2012.

Dores C, Alpaugh W, Dobrinski I. From *in vitro* culture to *in vivo* models to study testis development and spermatogenesis. Cell Tissue Res. Sep; 349(3):691-702, 2012.

Zeng W, Baumann C, Honaramooz A, Tang L, Bondareva A, **Dores C**, Fan T, Xi S, Geiman T, Rathi R, de Rooji D, De La Fuente R, Muegge K, Dobrinski I. Lymphoid-specific helicase (hells) is essential for meiotic progression in mouse spermatocytes. Biology of Reproduction 84, 1235-1241, 2011.

Presentations:

Platform presentations

C Dores, I Dobrinski Stirred suspension bioreactors as a novel method to enrich germ cells from prepubertal pig testis. Platform presentation at the 2014 University of Calgary Veterinary Medicine Research Day.

C Dores, L Tang, I Dobrinski The use of stirred suspension bioreactors as a novel method to enrich germ cells from prepubertal pig testis. International conference on pig reproduction, Olsztyn, Poland. 2013.

Dores C, Alpaugh W, Dobrinski I Setting the stage to use de novo morphogenesis of testis tissue for germ lineage stem cell differentiation. Platform presentation at the 2012 University of Calgary Veterinary Medicine Research Day.

Dores C. Stem Cells and regenerative medicine seminar. Let's talk about Science workshop to 12th grade students held at Foothills Campus from university of Calgary. 2012.

Posters Presentations:

C Dores, L Tang, I Dobrinski The use of stirred suspension bioreactors as a novel method to enrich germ cells from pre-pubertal pig testis.. Alberta Children's Hospital Research Institute Symposium in Epigenetics in Health and Disease. Calgary, Canada, 2014.

Dores C. and Dobrinski I. Investigating the role of Vascular Endothelial Growth Factor in testicular morphogenesis using porcine de novo formed testicular tissue. XXII North American Testis Workshop, San Antonio, United States, 2013.

Dores C, Alpaugh A and I Dobrinski Setting the stage to use de novo morphogenesis of porcine testes tissue for germ lineage stem cell differentiation. 10th Annual Meeting of the International Society for Stem Cell Research. Yokohama, Japan. 2012.

Dores C, Alpaugh W & Dobrinski I. Setting the stage to use de novo morphogenesis of testis tissue for the study of testicular morphogenesis and germ cell development . Alberta Children's Hospital Research Institute 2012 Symposium in Epigenetics, Health and Disease, Calgary, Canada, 2012.

Dores C, Behboodi E, Bondareva A, Alpaugh A. and I. Dobrinski. Enrichment of porcine germ cells from neonatal and prepubertal donors by differential adhesion. XXI North American Testis Workshop, Montreal, Canada, 2011.

Scholarships:

2012-2015: Alberta Innovates Health Solutions (AIHS) Graduate Studentship.

2012-2015: Canadian Institute of Health Research (CIHR) Training Program in Genetics, Child Development and Health Studentship Support.

2010-2014: University of Calgary Faculty of Veterinary Medicine Professional Degree Top-up Award.

Appendix **B**

Staput Protocol

Velocity Sedimentation Separation

Medium

Prepare 2% and 4% BSA in DMEM with 25mM HEPES (pH7.4):

Dulbecco's Modified Eagle Medium (D-MEM) containing 4,500 mg/L D-glucose and L-glutamine (Invitrogen Corporation, Cat No. 12100-046) + 25 mM HEPES + 3.7g/L NaHCO3, 0.022g/L sodium pyruvate, 0.060g/L streptomycin and 0.050g/L penicillin.

Cell preparation:

Measure out 7-8 x 10^8 cells and resuspend in 0.5% BSA in DMEM/F12 (about 20mls for regular single staput, about 10 ml for small staput).

Filter through 40 micron mesh and keep them on ice till ready to use. (Keep some cells used to make total RNA/protein at this time)

Set up the Staput chamber:

Wipe down the deli with EtOH

Silconize the glass platter cover

Put stir plate on suspended shelf, put platter cover in hole with metal baffle in center

Clean the tube pincher part of the fractionator very well with EtOH.

Put fractionator on the floor.

Hook up tubing connecting the gradient chamber with a 60cc syringe attached to a 3 way stop cock.

Put 120 tubes in the holes of the fractionator (reuse these multiple times)

Gradient making and sample loading:

Pour 550 ml 4% BSA into outer chamber first, then pour 550 ml 2% BSA into inner chamber. Put stir bar in bottom of inner chamber.

After loading BSA solutions, evacuate all air bubbles from tubing by opening the stop cock and letting them run out. Also partially open valve at back of gradient chamber to get rid of bubbles. Hook gradient maker to syringe tube.

Turn on the stir plate.

Pour 30-70 ml (50ml) of DMEM with HEPES (Overlay) into the syringe barrel and adjust flow rate with stop cock, turned at 45 degree angle to the right on bottom of glass platter lid. Ideally, want a rate of about 1 drop per second (about 1100 drops in 15 min). No air in line. Use wheel on back of stop cock to adjust rate. Stop when syringe is empty and tubing is full.

Pour the cell suspension into the syringe and open the stopcock at the bottom of the syringe so that the cells flow into the tubing. Follow the cells immediately with the BSA gradient by opening the appropriate stopcocks. Loading should take about 15 minutes.

Cells settlement

Time for a single staput: 2.5 hr, add another hour for a Small Staput; It is important not to go over time on this incubation.

Collect cells

For single staput: collect 10 ml per tube at the rate of about 1 tube every 44 sec (there are 170 drops in 10 ml). For small staput, collect 5 ml per tube at rate of 1 tube every 22 sec.

To set the fractionator, turn it on, set count to 170. Adjust flow rate using screw at back of stop cock beneath glass platter lid. Open stop cock to 45 degree angle.

Cell fraction analysis

After fractions are collected or while they are collecting (after 60 tubes or so have filled)

Set aside the 1-20 tubes.

Spin the second 80 tubes for 10 min at 1500 rpm, 4 degree C. Remove supernatant , leaving just enough to resuspend the pellet.

Pool the appropriate fractions as follows:

Pachytene typically found in fractions 30-40 (large, round, clear cells with clear cytoplasm)

Round spermatids usually in fractions 60-70 (small, round cells, clear cytoplasm)

Condensing cells usually in fractions 70-75 (denser, smaller, often with tails, irregular)

Sertoli cells (branch like cells, rarely seen)

Leydig cells (about same size as pachytene, brownish and vacuolated cytoplasm)

Clean all the parts only with water and deionized water. Don't use any soap or detergent.

Brief manual:

1. 7-8 x 10^8 Cells/25ml resuspended in 0.5% BSA of DMEM with Hepes, keep them on ice

2. Prepare the gradient: 550 ml 4% BSA in outer barrel, open the cock to the inner barrel and then turn off; 550 ml 2% BSA in inner barrel, open the cock to the outlet to keep the bubbles out, and turn off.

3. Hook the 60 CC syringe barrel to the gradient maker, add 30-70 ml (50 ml) DMEM with Hepes without BSA (Overlay) to the syringe, open the 2 cocks to let the DMEM just enter the platter lid and then turn off the cock next to the platter. Adjust the flow rate with the cock at the bottom of glass platter lid: about 1 drop per sec (1100 drops in 15 min).

4. Loading the cells suspension (25 ml)and open the cock at the bottom of the syringe and cells flow into the tubing (about 15 min)

5. Follow the cells, immediately with the BSA gradient

6. Settle the cells for 2.5hr

7. Collect the cells (10 ml fraction)

8. Spin to condense the cells

9. Check the fraction and combine the same cells fractions.

10. Combine the fractions containing the desired population in 50ml tube, spin at 800xg for 10min, remove the DMEM + BSA supernatant, fill pre-warmed DMEM + 10% FBS.