Chapter 2

Spermatogenesis and Its Significance in Reproductive Medicine

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Additional information is available at the end of the chapter

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Abstract

As infertility rates across nations become a growing concern, the interest in the development of treatments, such as *in vitro* gametogenesis (IVG), increases. This is especially the case for male infertility. For instance, the average sperm count continues to decline across nations, while more adult and pediatric patients survive cancer only to be left with little to no options for fertility restorative therapies. Understanding the male reproductive system and the process of spermatogenesis, however, has proven to be a difficult task. Progress occurs slowly and inconsistencies remain in the literature while reports attempt to better understand spermatogonial stem cells (SSCs) in conjunction with spermatogenesis. Interestingly, stem cell behavior, the decision to self-renewal or commit to differentiation, has shown to be closely linked to the stem cell's microenvironment (i.e. niche). Perhaps the missing pieces required to better understanding spermatogenesis are found in the re-defined perspective of SSC niche dynamics.

Keywords: spermatogenesis, spermatogonial stem cells, stem cell therapy, *in vitro* gametogenesis, organoid engineering

1. Introduction

The Center for Disease Control (CDC)'s Division of Vital Statistics released a recent report titled *Births: Provisional Data for 2017*, which placed the provisional general fertility rate (GFR) at approximately 60.2 births per 1000 women aged 14–44. This rate is reported to be down 3% from 2016 and a record low for the United States. The provisional total fertility rate (TFR) which is based on the age-specific births in a given year, and estimates the number of births that a hypothetical group of 1000 women would have over their lifetimes, is also down 3% from the rate reported in 2016— the lowest TFR since 1978 [1]. Though fertility rates are a multifaceted

phenomenon, recent findings regarding reproduction and family planning may be contributing to the increasingly low rates. For instance, a recent populations report by the U.S. Census Bureau found that young adults, aged 18–34, not only believe that economic and educational accomplishments are far more important milestones than marriage and parenthood, but that they are also actively delaying parenthood [2]. Moreover, a study on the temporal trends in sperm count also found that the average sperm counts for men, unselected for their fertility status, has declined in western countries by approximately 59% since 1973 [3]. This is the most comprehensive study to date that not only shows the continuous nature of the decline but also calls for the urgency in male reproductive research, as such findings have significant public health implications. Combine this with the increasing number of cancer survivors (both post and pre-pubertal patients) that also require fertility preservation therapies [4]. Currently, the only option available for male cancer patients is based on successful sperm retrieval and sperm freezing for future use. This is an option not extended to prepubertal patients, as these individuals have not yet produced viable sperm, and are left with no other alternatives to conceive children for their entire life. Altogether, the overall cultural shifts in family planning alongside the declining rates of the general fertility has led to major market research reports expecting the global fertility services market to grow from the current multimillion dollar industry to upwards of $30 billion. Therefore, the advancements in fertility services are increasingly significant in our changing global populations.

The production of germ cells (i.e. gametogenesis), is a process that begins in embryos with the formation of primordial germ cells (PGCs) that continues differently in male and female reproductive systems. Only recently did studies show that mouse embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) can differentiate into PGC-like cells (PGCLCs) that upon transplantation gave rise to both functional sperm or oocytes [5, 6]. Importantly, the culture conditions and differentiation protocols of murine PGCLCs still require further optimization, as these cells differentiate inefficiently and lack well-defined long-term culture conditions [7]. This is especially the case with SSCs that constitute the male testis. Understanding how these stem cells initiate spermatogenesis within the seminiferous tubules of the testis is vital for the future of IVG applications, as such knowledge would lead to optimized differentiation protocols and long-term SSC culture conditions that could be implemented for the treatment of infertility [8].

The focus of this chapter is on the significance of mammalian spermatogenesis as it pertains to both the successes of IVG, and to the push for innovation in the general field of reproductive medicine. We will see how SSC fate decisions establish spermatogenesis through the multifaceted interaction of the stem cell and its niche. Furthermore, we discuss how novel technologies, which allow for SSC niche mapping and in vitro preservation of the seminiferous tubules, hold the key to therapeutic and diagnostic breakthroughs—and the challenges and ethical implications that follow.

2. The spermatogonial stem cell niche establish and regulate spermatogenesis

The variation in stem cell behavior, whether it is the decision to self-renew or to commit to differentiation, is strictly linked to the stem cell's niche [9]. The niche is highly dynamic and has

shown to direct such fate decisions in a variety of adult stem cells such as hematopoietic stem cells (HSCs) [10], intestinal [11], and epidermal stem cells [12]. The concept of the niche extends beyond the direct cell–cell contact and is comprised of other key components such as secreted factors (i.e. chemokines, hormones, growth factor receptors), inflammation (i.e. macrophages, T cells), physical factors (i.e. shear forces, topography, elasticity/stiffness), hypoxia (i.e. glycolysis-optimizing conditions), and cellular metabolism (i.e. glucose, lipids, calcium, calcium receptors) [9]. Importantly, the stem cell-niche communication not only occurs over short and long range distances, but is also reciprocal and significant in tissue homeostasis [13–15]. For instance, in mouse skin, the removal of the stem cell population (i.e. hair follicle stem cells) can result in niche cells dedifferentiating to replace them. In this case, the repopulation of epithelial cells (cells that do not contribute to hair growth), replace the stem cells and sustain hair regeneration [16]. Interestingly, with respect to the field of spermatogenesis, the key components of the SSC niche also appear to be as multifaceted as the examples provided above.

2.1. Direct and indirect cell contact: SSCs, Sertoli cells, and other key players

One key player in regulating the accessibility of SSCs to other components in the niche is the Sertoli cell. These cells extend from the basal compartment of the seminiferous tubule to the adluminal region. Sertoli cells take on a constantly evolving and irregular shape that is in a continuous three-dimensional relationship with not only the SSCs, but also the differentiating spermatogonia throughout spermatogenesis [17]. Due to the complexity of such a dynamic relationship, traditional experiments involving two-dimensional or stage-specific cell analysis may have portrayed an incomplete depiction of spermatogenesis and may have attributed to the large discrepancy found in today's literature [8]. Regardless, it is still worth discussing such findings because it provides a glimpse into the three-dimensional relationship between the SSC and Sertoli cells.

To begin, Sertoli cells have a large surface area that allows them to support germ cell development at a higher ratio of germ cells to Sertoli cells [17, 18]. Such a characteristic is critical for providing structural support to the germ cells, but also to germ cell movements throughout the tubule. Furthermore, the unique structural and signaling flexibility of Sertoli cells create two distinct environments within the tubules that are otherwise referred to as the blood-testis barrier (BTB). This is where the basal compartment, the region in close contact with lymph and blood, is speculated to maintain earlier staged cells of spermatogenesis, such as SSCs and early progenitors. Later stages that are committed and differentiating spermatogonia, however, appear to occur in the adluminal compartment, isolated from lymph and blood [17]. Importantly, the BTB is created by tight or gap junctions, and desmosomes that are present between Sertoli cells. The BTB does not exist between Sertoli cells and germs cells or between germ cells [19–22]. Additionally, the specialization of the BTB was first depicted in early studies of testicular transferrin. Cells located in the later staged adluminal region of the tubule (i.e. cells that do not have access to serum iron), gained access to testicular transferrin through Sertoli cells [17, 23, 24].

In addition to the role of the Sertoli cells, Leydig cells within the interstitial space of the seminiferous tubules produce and signal testosterone. Without the presence of testosterone, spermatogenesis does not proceed completely and results in male infertility. Therefore, if Leydig cells were to be removed, the germ cells that have initiated meiosis and completed

differentiation begin to improperly detach from the Sertoli cells and die [25]. Furthermore, mature sperm near the lumen of the tubule (i.e. the adluminal compartment) cannot properly release from Sertoli cells without testosterone signaling. Another major hormone, follicle-stimulating hormone (FSH) has shown to act synergistically with testosterone to increase and regulate spermatogenesis. It does this by binding to the FSH receptor (FSHR) on the surface membrane of Sertoli cells [25]. Peritubular myoid cells (PMCs) are also located in the interstitial space of the tubules, and express the androgen receptor for testosterone. Within the seminiferous tubules, however, only Sertoli cells express the androgen receptor. Therefore, Sertoli cells, in communication with other niche cells (i.e. Leydig cells and PMCs) regulate spermatogenesis indirectly and thus impact the initiation, development, and survival of germs cells [25, 26]. Furthermore, the surface of some seminiferous tubules associates with vascular endothelium and perivascular cells [27]. The role of such vasculature-associated cells may be involved in regulation of SSC niche dynamics. For instance, some studies found that in the prepubescent and adult testis, macrophages are closely associated with Leydig cells and play a role in the signaling and production of testosterone [28]. Another study found populations of macrophages near the surface of the basal compartment where enriched undifferentiated spermatogonia were found. Such testicular macrophages expressed SSC proliferative and differentiating factors such as enzymes involved in retinoic acid (RA) synthesis and colony stimulating factor 1 (CSF1) signaling [27].

2.2. The extracellular matrix, secreted factors, and their respective receptors

The extracellular matrix (ECM) and its role in the stem cell niche vary substantially in almost every tissue [9]. In some cases, the ECM is also involved in the maintenance of local concentrations of growth factors that direct stem cell fate or target niche cells involved in the regulation of those SC fate decisions [29, 30]. In the testis, the ECM located at the basal compartment, is made up of the basement membrane that is composed of proteins like laminin, type IV collagen, and entactin. Importantly, the basement membrane (a modified ECM) is speculated to not only interact with Sertoli cells, but also regulate SSC fate decisions. Sertoli cells even secrete components of the ECM (i.e. laminin) that is not only useful in short-term SSC culture conditions, but is also involved in Sertoli cell tight junctions, and in turn, the formation of the BTB [8, 17, 31–33]. It is the formation of tight junctions across Sertoli cells that is said to create a semipermeable barrier that restricts molecule movement based on either weight or chemical structure [34]. The exact involvement, however, of the basement membrane with key components like laminin and the mechanisms of junction formation still require further investigation with their role in SSC niche dynamics. Nevertheless, specialized junctions have been found throughout the seminiferous epithelium and include junctions such as adherens, desmosome-like, hemidesmosome, and gap junctions that are located throughout the tubule from the basal to adluminal compartment. These junctions not only appear to control germ cell movement, but are also involved in the regulation or perhaps local concentrations of secreted factors in SSC fate decisions [17].

Glial cell line-derived neurotrophic factor (GDNF) is a secreted factor produced by Sertoli cells and PMCs that is linked to SSC fate determination, Sertoli cell proliferation and short-term

SSC *in vitro* maintenance [35–37]. Furthermore, the *in vitro* GDNF expression promotes the self-renewal and proliferation of SSCs by activating the phosphoinositide-3 kinase (PI3K)/AKT signaling pathway [38]. Studies *in vivo,* however, show that ERK1/2 signaling pathway is activated in SSC self-renewal, while PI3K/AKT signaling is shown to be activated in SSC proliferation during stages where RA signaling is both low and high [39]. Based on the significance of Sertoli cells as a key player in the niche, one can suspect that such cells and their respective GDNF expression is a relationship that is sufficient for the recapitulation of niche dynamics for *in vitro* studies. The current discrepancy in the literature, however, suggests that once SSCs are removed from *in vivo* conditions, the multifaceted three-dimensional SSC niche is then disrupted in a significant way that causes SSCs to behave differently, and perhaps inconsistently. The establishment of a long-term and well-defined *in vitro* protocol for SSC maintenance would no longer be an open question if the maintenance of normal spermatogenesis were based on a fairly simplified stem cell niche model. In fact, recent reports appear to provide further support for a niche that is in a three-dimensional relationship with the SSCs. For example, a recently published report found that cyclic expression of GDNF is not only required for SSC homeostasis but also that GDNF cyclic expression is normally expressed during spermatogenesis [35]. Furthermore, the ectopic expression of GDNF during late staged spermatogenesis caused the accumulation of early-staged undifferentiated spermatogonia that was also positive for the GDNF receptor (GFRA1). Another study found that the lack of a RA target gene in mice called Stimulated by Retinoic Acid gene 8 (Stra8), caused the accumulation of undifferentiated spermatogonia and the depletion of differentiating spermatogonia. Furthermore, the capability of germ cells to begin differentiation or meiotic initiation in response to RA was distinct, periodic and limited to a particular seminiferous stage. This study suggests that properly timed differentiation depends on the intersection of cell intrinsic competence and extrinsic chemical cues. Such findings led to the conclusion that periodic RA-STRA8 signaling intersects with the periodic germ cell capability to regulate spermatogenesis [40].

In terms of respective receptors, in the seminiferous epithelium, some major ECM-receptors are integrins. In fact, studies have claimed that integrin-α6 and integrin-β1 are key surface markers involved in the regulation of spermatogenesis. There is still much discrepancy, however, on whether such markers are exclusively expressed on SSCs or germ cell progenitors [8, 17]. It has been shown, however, that the deletion of integrin-β1 on Sertoli cells not only reduced SSC homing (i.e. the repopulation of SCs after the removal of endogenous SCs), but also that the adhesion receptor's association with laminin is critical for the several steps involved in SSC homing [41]. Again, though the integrins are significant in niche dynamics, it appears that integrins cannot be used to distinguish the sub-populations of early staged germ cells (i.e. SSCs and their progenitors). Importantly, such studies use marker-based techniques (i.e. Fluorescence-activated cell sorting (FACS)) to isolate cells positive for markers such as integrin-α6, integrin-β1 or GFRα1, to then transplant back into germ cell-depleted testes for further analysis [8, 42, 43]. Though the transplantation assay [44] is a great tool to gauge stem cell competency, the SSC niche dynamics must also be clearly defined since germ cells have shown to behave inconsistently from *in vivo* to *in vitro* conditions. Therefore, perhaps the discrepancy in such studies highlights the limitations or inability of marker-based techniques,

like FACS, to suffice for the understanding of the SSC-niche relationship. Such a variation in SSC behavior is further highlighted in a study that modeled the entire GFRα1+ population within the seminiferous tubules. Results showed that during steady-state spermatogenesis, the GFRα1+ population comprised of a single stem cell pool that continually interconverted between different states of equipotent singly or syncytial states [45]. The early example of the hair follicle stem cells further resonates with this study because self-renewal potential may be influenced by the position of the stem cell within the niche. Another recent publication also attempted to address the heterogenous expression of markers present on early staged germ cells comprise of stem cells and progenitors. More specifically, the isolation of GFRα1+ and GFRα1-spermatogonia, interestingly, both showed elevated transplantation activity. Furthermore, GFRα1-spermatogonia not only produced GFRα1+ spermatogonia when negative cells were transplanted into germ cell depleted testis, but also restored spermatogenesis. Such results indicated that a stem cell pool of GFRα1+ and GFRα1-cells could interconvert between the two states of positive and negative cells in a niche-dependent mechanism. Additionally, though these two populations may be closely related, they still differ in key cell-intrinsic components [46]. Altogether, both studies display the behavioral variation in not only GFRα1+ germ cells, but also GFRα1+/− populations in relation to the niche. The GFRα1+ only population interconverts between different stem cell states while the GFRα1+/− populations reveal a niche-dependent mechanism for fate determination. How these two studies come together for a more cohesive story requires further investigation and an overall better niche understanding.

2.3. Hypoxia, metabolism and the role of inflammation

Tissue specific cell populations such as HSCs, and cardiac progenitors are found to be in low oxygen (i.e. hypoxic) microenvironments that contribute to cell survivability and maintenance [9, 47]. During hypoxic conditions, cells favor glycolysis rather than mitochondrial oxidative phosphorylation. In terms of the SSC niche, one recent report found that the reduction in O_2 tension during *in vitro* conditions enhanced the *in vivo* maintenance of the SSCs' regenerative integrity. SSCs cultured long term in hypoxic conditions (10% O_2 tension rather than the standard 21% O_2 tension), had the capacity to continue spermatogenesis following transplantation in recipient tubules devoid of germ cells [48]. Previous work from the same group had also shown that key glycolysis regulating enzymes were elevated in cultured undifferentiated germ cells [49]. Furthermore, another study found that the inhibition of glycolysis (via the double-knockout of Myc/Mycn genes) decreased SSC activity and inhibited spermatogonial differentiation. The chemical stimulation of glycolysis, however, increased the frequency of the SSC self-renewal capacity [50]. On the contrary, another report found that the ablation of Max (a key partner for Myc function) induced differentiation of germ cells in culture conditions [51]. Interestingly, the same group that reported on SSC self-renewal through the chemical activation of glycolysis also reported on a lipid-rich medium that enhanced SSC self-renewal for long-term culture conditions [52, 53]. The excess of free fatty acids, however, promotes fatty acid catabolism (i.e. β-oxidation and oxidative phosphorylation) rather than glycolytic activity [48]. Together, there are possible explanations and takeaways from such discrepancies regarding hypoxia and metabolism. First, low oxygen-tension may be

involved in the regulation of both *in vitro* and *in vivo* SSC self-renewal. Furthermore, though examples of HSC-niche dynamics have shown that hypoxic microenvironments contribute to stem cell maintenance, this in some ways contradicts the role of the BTB in spermatogenesis. The regions where early staged spermatogenesis occurs are in the basal compartment of the tubule, near lymph and blood. One would then suspect that the basal environment favors oxidative phosphorylation over glycolysis. Another possibility, however, is that the Sertoli cell tight junctions may create a highly restrictive, thus semi-hypoxic, niche for the SSCs in order to tightly regulate self-renewal. Second, there is also the possibility that the connection of low oxygen-tension to SSC maintenance may only be an *in vitro* phenomenon. As previously stated, the three-dimensional relationship of the niche to the SSCs may be significant enough to cause these cells to behave differently once removed from *in vivo* conditions. For instance, the disruption of this multifaceted communication may cause SSCs to begin to favor glycolytic activity during *in vitro* conditions. Furthermore, reactive oxygen species (ROS) were shown to also influence the outcome of *in vitro* SSC maintenance. Moreover, modulating ROS levels demonstrated that moderate concentrations may promote SSC self-renewal and that achieving this may be possible by manipulating or reducing the use of β-oxidation as the primary bioenergetics pathway [48, 54]. Such speculations, however, only point to the need for further investigations of the three-dimensional relationship of the SSC and its niche. Overall, cellular metabolism is critical in determining whether stem cells proliferate, differentiate or remain quiescent. There is typically a balance that is established between oxidative phosphorylation, glycolysis, and oxidative stress for the adult stem cells [9]. Such a balance is further exemplified in the dynamic niche with the role of inflammation.

The general understanding regarding immune privilege (i.e. the capacity to tolerate the introduction of new antigens without the trigger of an inflammatory response) is the evolutionary adaptation to protect tissues from loss of functions due to their limited capacity for regeneration [55, 56]. In the testis, however, this protection against loss of function is for the tissue's reproductive capacity. The production and differentiation of male germ cells are unique in that sperm matures at puberty, which is long after the maturation of the immune system and systemic self-tolerance [55]. Due to these phenomena, the BTB plays a role in protecting the maturation of sperm from an autoimmune reaction. For instance, the various junctions and desmosomes between Sertoli cells have created such a limited access in the passage of other molecules that the composition of the basal, adluminal, and interstitial spaces differ significantly. Additionally, while the BTB's ability to isolate meiotic and postmeiotic germ cells from lymph and blood is significant in testicular immune privilege, there are also physical and immunological components required for the immunotolerance of the testis [55]. The expression of anti-inflammatory cytokines by immune and somatic cells and the role of androgens also play a role in the immunoprivileged niche.

Meiotic and postmeiotic germ cells express a large variety of neoantigens that emerge during puberty and long after self-tolerance is already established. Furthermore, once spermatogenesis begins, the BTB is established and immediately isolates post pubertal germ cells from the immune system [55]. Interestingly, germ cell neoantigens are also present on SSCs and progenitors that are located in the basal compartment, and not isolated from lymph and blood, unlike the adluminal compartment of the BTB [55, 57, 58]. This suggests that other

components of the testes also play a role in the immunoprivileged niche. For instance, accumulating evidence suggests that PMCs secrete cytokines like transforming growth factor-β (TGF-β), leukemia inhibitory factor (LIF), and macrophage chemoattractant protein 1 (MCP-1) that directly affect leukocytes in the interstitial space of the testis [59, 60]. Furthermore, the local high concentrations of testosterone appear to play an important role in the immunoprivileged niche within the testis. For example, when testosterone was incubated with stimulated human macrophages, monocytes and non-immune cells, the suppression of cytokines and adhesion molecules occurred while the production of anti-inflammatory cytokines increased [61, 62]. In transplantation studies, rats that were treated with estrogen to suppress Leydig cell production of testosterone immediately rejected allotransplanted cells within the seminiferous tubules. This directly contrasted the untreated control group where no rejection occurred in allotransplanted cells [55]. Though this provides evidence that the local high concentrations of testosterone plays an important role in the immunoprivileged niche, the exact mechanism testosterone and its anti-inflammatory function on testicular leukocytes, still remains unknown. There may be an indirect regulatory mechanism involved in the balance between the expression of pro and anti-inflammatory cytokines in Sertoli cells, Leydig cells, and PMCs [55]. Such a balance between pro and anti-inflammatory cytokines may also play a significant role in the protection and maintenance of SSCs that are not isolated from lymph and blood. For instance, *in vitro* studies have shown that rat testicular macrophages exhibit immunosuppressive characteristics [63]. Interestingly, a heterogeneous macrophage population resides in the rat testis' interstitium where one population participates in the inflammatory response while the other is thought to have a role in the immunoprivileged niche [63, 64]. This suggests that the testis is capable of initiating both a normal inflammatory response and maintaining an immunoprivileged niche. Such a balance appears to be crucial in the SSC niche, especially since there is mounting evidence of immune-related male infertility [65, 66]. Importantly, the microvasculature in the interstitium not only contains macrophages, lymphocytes, and mast cells but also dendritic cells (DC). Studies have shown, thus far, that testicular DCs may play a significant role in maintaining the balance of testis tolerance and intolerance [63, 67], but still require further investigation. The role of inflammation and the extent of its connection to SSC function are still unclear. One would speculate, however, that because the immune privileged testis protects the tissue's reproductive capacity, that this may also directly connect to SSC fate decisions.

2.4. Physical factors

The physical surroundings such as the three-dimensional physical shape, shear forces, and topography (i.e. the physical arrangement of cells) all contribute to the stem cell and its niche [9]. For example, shear forces such blood flow, have shown to play a role in either the acceleration or reduction of *in vivo* development of zebrafish embryonic HSCs [68]. Distinct niche topographies have also shown to have an effect on signaling pathways and the regulation of differentiation on mesenchymal stem cells [69]. Testicular blood flow, and its connection to niche dynamics and spermatogenesis, is largely an under-investigated area. Interestingly, older reports showed that a reduction of approximately 70%, for 5 hours, from normal blood flow led to varying degrees of damage to the seminiferous tubules and

resulted in an inflammatory-like response (i.e. increased number of leukocytes within the testis interstitial space was found) [70]. Additionally, one important suggestion from this study was that vasomotion, the smooth muscle oscillations of the blood vessel walls (independent of heartbeat), contributed to testicular function. Some studies even found that vasomotion was directly regulated by testosterone, and indirectly by Sertoli cells [71, 72]. More specifically, vasomotion was not detected in the testes where Leydig cells were removed but was induced with a dose of testosterone [72]. More recently, however, one report found that Sertoli cells may play a direct role in supporting the testicular vascular network. Results showed that loss of germ cells had no effect on testicular vasculature while loss of Sertoli cells led to the reduction of total vascular branches, volume, and the number of small micro-vessels [73]. The mechanism by which Sertoli cells influence vasomotion still remains unclear. Perhaps the multifaceted communications between Sertoli cells, Leydig cells and PMCs also work to regulate vasomotion.

Furthermore, the biophysical cues involved in the *in vivo* ECM, including the basement membrane and the formation of the BTB, are all significant factors in the SSC niche topography. Equally importantly, however, is the distribution of germ cells within the seminiferous tubules. One study found that the distribution of early mouse germ cells, typically termed type A spermatogonia that are either a single cell, paired or aligned, within the spermatogenic cycle is nonrandom [74]. Interestingly, the transplantation of a single SSC first results in asymmetrical spermatogenesis followed thereafter by uniform spermatogenesis (i.e. spermatogenesis around the entire tubule) [74, 75]. What this suggests is that SSCs are capable of movement. Further evidence provided by Chiarini-Garcia *et al.* indicated that spermatogonia were not only mobile, but also cyclically positioned themselves at periodic intervals along the tubules to ensure uniform spermatogenesis. Such a nonrandom distribution of germ cells was made possible because tubules remained in somewhat of a constant relationship with each other. This contact with one another also allowed the same group to map the topography of these tubules [74]. Together, one can speculate that the three-dimensional physical shape and the biophysical cues of the testis may be significant in the SSC niche dynamics. The physical factors involved in the SSC niche, however, appear to be undervalued in the field of spermatogenesis as many questions remain to be unanswered.

2.5. Niche mapping and its significance in the seminiferous tubule microenvironment

Modulating the SSC niche requires well-defined and reproducible studies. Since SSCs appear to be highly interconnected with their niche, the *in vivo* dynamics must be clearly defined, and more importantly, encompass all the key components of the stem cell-niche interactions. Just as Handel *et al.* made the necessary case for applying 'gold standards' (i.e. benchmarks) to *in vitro*-derived germ cells, we make the case for extending and expanding such benchmarks in the studies involving spermatogenesis. Handel *et al.* made the vitally important argument for the scientific community to apply the highest standards in evaluating and conducting IVG research. To further emphasize their point, we believe that much of the discrepancy in the literature today is due the lack of consensus or a laid out rigorous criteria by which

to evaluate SSC identity, SSC fate decisions, and the general understanding of mammalian spermatogenesis, as it is the foundational knowledge for future IVG practices. Moreover, Lane *et al.* effectively organized the composition of the general stem cell niche into reciprocal interactions of different cellular components, secreted factors, ECM, immunological control, metabolic control and physical factors that we sought out to re-evaluate and highlight some of the literature in the field of spermatogenesis, under that same style of organization. As the literature shows, SSC niche dynamics appear to include all of those reciprocal interactions. Our understanding of spermatogenesis, however, still remains incomplete due to perhaps the lack of aerial or three-dimensional perspective with regards to not only the types of experiments conducted, but also the interpretation of those results. SSCs, and niche cells alike, appear to behave much differently during *in vitro* conditions than *in vivo* dynamics as much of the inconsistencies in the literature suggests. Aside from the variability in culture protocols that may contribute to this dilemma, we believe two actions are required for the push forward in the field of spermatogenesis. First, it is imperative for a set of benchmarks to be put into place when evaluating the *in vitro* recapitulation of spermatogenesis. Perhaps this involves the modification of Lane *et al.'s* organization of the stem cell niche to the SSC niche dynamics as summarized pictorially in **Figure 1**. Second, emerging methodologies and technologies may be required to facilitate the implementation of better *in vitro* analyses of spermatogenesis.

A recently published report characterized a three-dimensional multilayer model (termed the Three-Layer Gradient System (3-LGS)) that allowed for the reorganization of dissociated rat testicular cells into testicular organoids with the formation of a functional BTB and germ cell maintenance. This system used three concentric layers of Matrigel to not only increase

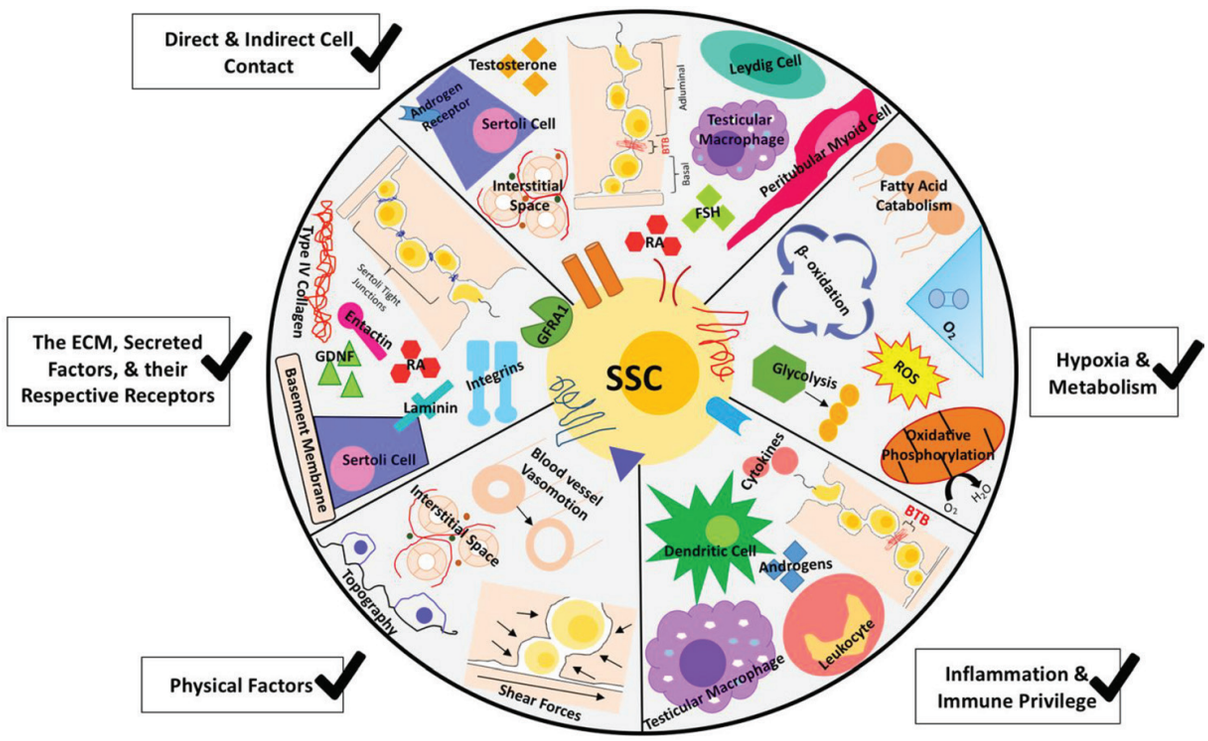

Figure 1. The spermatogonial stem cell and its niche, reimaged.

the area for factor exchange but also for testicular cell reorganization into organoids to take place [76]. Furthermore, the cellular organization of this *in vitro* three-dimensional multilayer model more closely represented the *in vivo* stem cell-niche interactions. This group also proposed that the 3-LGS is a new platform to better investigate the SSC niche *in vitro* [76, 77]. Interestingly, such an *in vitro* three-dimensional multilayer model represents an emerging methodology that not only signified the three-dimensional relationship of the SSC-niche interactions, but also provided a potential avenue to further study the SSC niche in a working *in vitro* model. Alongside the more appropriate development of three-dimensional *in vitro* models, methodologies that can image three-dimensional *in vivo* conditions still remain either undervalued in the field or lack the technological capabilities for in-depth analysis. For instance, though some work has been done using time-lapse imaging and histological staining of the seminiferous tubules, high quality data and clear analysis of the undisturbed *in vivo* environment is still a clear hurdle [78]. This same challenge remains in general biology, where obtaining high-resolution information from a complex system without losing the global perspective required to understand how the system functions, is still lacking [79]. Tissue clearing technologies, however, a technique developed for brain mapping [80] may address such challenges, especially in spermatogenesis where three-dimensionality appears to be highly significant. Through chemical transformations, this method (i.e. CLARITY) passively or actively transitions the whole tissue into a lipid-extracted, thus clear, and structurally stable tissue that is fully intact with its native biological molecules [81]. Brain mapping has significantly advanced due to technological innovations in tissue clearing methodologies like CLARITY [81]. The uniqueness of such a technique not only enables clear accessibility, but also maintains a global perspective in a three-dimensional analysis. Perhaps the mapping of complex systems through tissue-clearing technologies can also be expanded to the SSC niche dynamics (i.e. SSC niche mapping).

3. Recapitulating *in vivo* spermatogenesis through *in vitro* preservation

The *in vitro* propagation and maturation of germ cells is especially necessary for the development of therapies for prepubertal pediatric patients undergoing chemotherapy. Typically the storage of sperm for cancer patients has become routine since therapeutic agents, like radiation and chemotherapy, either directly or indirectly affect the SSC pool and ultimately patient fertility. Such options, however, do not extend to pediatric patients, as those individuals have not yet produced viable sperm [4]. Therefore, an alternative approach to studying spermatogenesis has shown great promise through the use of organoid static conditions or three-dimensional fluidic conditions. For instance, the *in vitro* production of functional sperm from neonatal mouse testes, using a well-defined organ culture protocol, was significant in providing a new avenue for *in vitro* spermatogenesis [82]. This study not only maintained organoids for approximately 2 months to obtain spermatids and sperm, but also produced healthy and competent offspring through micro-insemination. The system, however, still lacks the controlled monitoring necessary for the manipulation and study of SSC niche dynamics [83]. Furthermore, the use of testis fragments (i.e. testicular organoids) in static

conditions has limitations on the tubule maturation and tubule viability since the diffusion of nutrients and oxygen are constrained by the lack of a functional vascular system [84]. In order to address such limitations, the same group developed a different approach to *in vitro* spermatogenesis. A simple microfluidic device with a porous membrane was created to mimic the microvascular system of the testis [85]. The porous membrane separated the tissue from the flowing medium and successfully maintained spermatogenesis for approximately 6 months. Furthermore, the seminiferous tubules of testis were spread flat in the microfluidic chamber and in direct contact with the porous membrane. Interestingly, germ cell count, and thus, differentiation decreased over time in the long-term microfluidic culture. This may be the result of poor homeostasis between the tissue and the medium [83]. Tissue homeostasis is key in modulating the stem cell niche and the homeostatic imbalance may have also resulted from the distortion of the tubule-to-tubule contact found *in vivo.* Furthermore, perhaps the tubules spread flat in the chamber led to the loss of the interstitial fluid and niche cells, such as Leydig cells and PMCs that could have also contributed to the decrease in germ cells over time.

Together, the controlled monitoring of a fluidic *in vitro* system that uses testicular organoids is significant. Preserving the complete SSC niche in a fluidic platform, however, is something that has not yet been successfully achieved. The static organoid culture is limited to oxygen and nutrient diffusions and cannot maintain overall tubule viability (i.e. organoid necrosis typically found in the center of testis fragments during static culture). Furthermore, the recent publications on microfluidic culture protocols also distort the three-dimensional environment of the testis that ultimately affects SSC maintenance [85, 86]. An optimized fluidic platform that will not only recapitulate the SSC niche, but also allow for the easy manipulations of SSC niche dynamics may be the next step for *in vitro* spermatogenesis.

4. Initiating innovation: promises, challenges, and the ethical implications for the push toward IVG

Human embryogenesis and gametogenesis is crucial to our understanding of reproduction, development, disease and evolution [87, 88]. The recent successes in the generation of human PGCLCs from human ESCs and human iPSCs solidified the prospects that the reconstitution of human IVG may be near [7]. In the future, IVG combined with IVF can allow infertile couples trying to conceive to generate their own gametes through iPSC technology [7, 89]. Interestingly, IVG has the potential for even broader implications in reproductive medicine. With the increasing cultural shift in family planning, such as the delay in marriage and parenthood from young adults, is a shift that will most likely impact the GFR and the replacement fertility rate in any given country. Countries that have a low GFR have a larger aging population that is not only positioned to shrink, but also has fewer populations in the working age to support the older dependents. Therefore, the replacement fertility rate, highly dependent on the GFR, is the rate in which women give birth to enough babies to sustain population levels in any given country [90]. With the availability of contraceptives, young adults can now easily delay parenthood to ages where fertility begins to decline and conceiving may become more difficult and at times no longer possible. IVG/IVF therapy combined with iPSC technology

can potentially provide a solution to the significant implications in the new cultural shifts that will affect the GFR and the replacement fertility rate across countries. Until such a promising therapy can come into fruition, there are many scientific and ethical challenges that we must undertake. The need for robust and reproducible studies in both spermatogenesis and oogenesis are crucial for the future in IVG practices. It is imperative to evaluate claims of IVG and germ cell meiosis through the use of standardized benchmarks and the demonstration of the 'gold standards' for meiosis [91]. Furthermore, the advancement in both knowledge (i.e. understanding SSC niche dynamics) and technology (i.e. creating comprehensive fluidic devices and viable *in vitro* platforms) are still clear challenges that need to be addressed.

In terms of the ethical implications of IVG, it is imperative for us to revisit the relevant regulatory measures. For instance, 10 countries permit human embryo research under the 14 day culture rule [87]. Of these countries, not including the United States, only seven include regulations to human IVG for medical or scientific applications. Interestingly, though the United States has no federal laws or regulations to prohibit IVG for research, the Dickey-Wicker amendment (signed in 1995) forbids federal funding for human embryo research [87, 92]. Even though the National Institutes of Health (NIH) recognized the value of it, and the CDC continues to report the GFR at record lows. Combine this with the 2017 provisional CDC report that birth rates are declining for nearly all age groups of women under 40, but rising in women aged 40–44 by 2% from 2016 [1]. Furthermore, just as the development of IVF was initially highly controversial, it has now become a widely accepted treatment for infertility, and more commonly used among women with declining fertility. Therefore, if the scientific and societal value of human IVG research is agreed to be significant, IVG research should then be conducted under balanced regulations with careful ethics review and close oversight [87]. Scientists, appropriate policy-makers and the public should all be included for all future discussions regarding human IVG research.

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References

[1] Hamilton BE, Martin JA, Osterman MJK, Driscoll AK, Rossen LM. Births: Provisional data for 2016. National Vital Statistics Rapid Release. 2017;**2**:1-21

[2] Population Characteristics. The Changing Economics and Demographics of Young Adulthood: 1975-2016. 2017. pp. 1-23

[3] Levine H et al. Temporal trends in sperm count: A systematic review and meta-regression analysis. Human Reproduction Update. 2018;**23**:646-659

[4] Medrano JV et al. Basic and clinical approaches for fertility preservation and restoration in cancer patients. Trends in Biotechnology. 2018;**36**:199-215

[5] Hikabe O et al. Reconstitution in vitro of the entire cycle of the mouse female germ line. Nature Publishing Group. 2016;**539**:299-303

[6] In vitro derivation and propagation of spermatogonial stem cell activity from mouse resource in vitro derivation and propagation of spermatogonial stem cell activity from mouse pluripotent stem cells. Cell Reports. 2016;**17**:2789-2804

[7] Saitou M, Miyauchi H. Gametogenesis from pluripotent stem cells. Cell Stem Cell. 2016;**18**:721-735

[8] Guo Y, Hai Y, Gong Y, Li Z. Characterization, isolation, and culture of mouse and human spermatogonial stem cells. Journal of Cellular Physiology. 2013;**229**:407-413. DOI: 10.1002/jcp.24471

[9] Lane SW, Williams DA, Watt FM. Modulating the stem cell niche for tissue regeneration. Nature Publishing Group. 2014;**32**:795-803

[10] Morrison SJ, Scadden DT. The bone marrow niche for haematopoietic stem cells. Nature. 2014;**505**:2-9. DOI: 10.1038/nature12984

[11] Tan DW, Barker N. Intestinal stem cells and their defining niche. Stem Cells in Development and Disease, Vol. 107. Cambridge, MA, USA: Elsevier Inc.; 2014

[12] Fuchs E. Minireview finding one' s niche in the skin. Stem Cells. 2009;**4**:499-502

[13] Driskell RR et al. Architecture in skin development and repair. Nature. 2014;**504**:277-281

[14] Fujiwara H et al. The basement membrane of hair follicle stem cells is a muscle cell niche. Cell. 2011;**144**:577-589

[15] Festa E et al. Adipocyte lineage cells contribute to the skin stem cell niche to drive hair cycling. Cell. 2011;**146**:761-771

[16] Rompolas P, Mesa KR, Greco V. Spatial organization within a niche as a determinant of stem-cell fate. Nature. 2013;**502**:513-518

[17] Mruk DD, Cheng CY. Sertoli-Sertoli and Sertoli-germ cell interactions and their significance in germ cell movement in the seminiferous epithelium during spermatogenesis. Endocrine Reviews. 2004;**25**:747-806

[18] Weber JE, Russell LD, Wong V, Peterson RN. Three-dimensional reconstruction of a rat stage V Sertoli cell. II. Morphometry of Sertoli-Sertoli and Sertoli-germ-cell relationships. American Journal of Anatomy. 1983;**179**:163-179

[19] Introduction I. The mammalian blood-testis barrier: Its biology and regulation. Endocrine Reviews. 2015;**36**:564-591

[20] Mruk DD, Cheng CY. Tight junctions in the testis: New perspectives. Philosophical Transactions of the Royal Society B. 2010:1621-1635. DOI: 10.1098/rstb.2010.0010

[21] Defamie N, Segretain D, Pointis G. Gap junctional communication in the male reproductive system. 2005;**1719**:102-116

[22] Lie PPY, Cheng CY, Mruk DD. The biology of the desmosome-like junction: A versatile anchoring junction and signal transducer in the seminiferous epithelium. In: International Review of Cell and Molecular Biology. Vol. 286. New York City, NY, USA: Elsevier Inc.; 2011

[23] Thorbecke GJ, Cox K. Sites of formation of the serum proteins transferrin and hemopexin find the latest version: Sites of formation of the serum proteins transferrin and hemopexin. Journal of Clinical Investigation. 1973;**52**:725-731

[24] Aravindan GR, Pineau CP, Bardin CW. Ability of trypsin in mimicking germ cell factors that affect Sertoli cell secretory function. Cellular Physiology. 1996;**133**:123-133

[25] Walker WH. Non-classical actions of testosterone and spermatogenesis. Philosophical Transactions of the Royal Society B: Biological Sciences. 2010;**365**:1557-1569

[26] Wilson EM, Hill C, Carolina N. Immunohistochemical localization of the androgen receptor in rat and human tissues. Endocrinology. 1990;**127**:3180-3186

[27] Defalco T et al. Macrophages contribute to the spermatogonial niche in the adult testis article macrophages contribute to the spermatogonial niche in the adult testis. Cell Reports. 2015;**12**:1107-1119

[28] Requirement for testicular macrophages in Leydig cell proliferation and differentiation during prepubertal development in rats. 1994

[29] Brizzi MF, Tarone G, Defilippi P. Extracellular matrix, integrins, and growth factors as tailors of the stem cell niche. Current Opinion in Cell Biology. 2012;**24**:645-651

[30] Watt FM, Huck WTS. Role of the extracellular matrix in. Nature Publishing Group. 2013;**14**:467-473

[31] Dym M. Basement membrane regulation of Sertoli cells. Endocrine Reviews. 1994;**15**: 102-115

[32] Tung PS, Fritz IB. Interactions of Sertoli cells with laminin are essential to maintain integrity of the cytoskeleton and barrier functions of cells in culture in the two-chambered assembly. 1993;**11**:1-11

[33] Dym M. Commentary spermatogonial stem cells of the testis. Proceedings of the National Academy of Sciences. 1994;**91**:11287-11289

[34] Madara JL. Regulation of the movement of solutes across tight junctions. Annual Review of Physiology. 1998;**i**:143-159

[35] Sharma M, Braun RE. Cyclical expression of GDNF is required for spermatogonial stem cell homeostasis. Development. 2018. DOI: 10.1242/dev.151555

[36] Meng X et al. Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. Science. 2000;**287**:1489-1493

[37] Chen L, Willis WD, Eddy EM. Targeting the Gdnf gene in peritubular myoid cells disrupts undifferentiated spermatogonial cell development. Proceedings of the National Academy of Sciences. 2016;**113**:1829-1834

[38] Lee J et al. Akt mediates self-renewal division of mouse spermatogonial stem cells. Development. 2007;**1859**:1853-1859

[39] MEK/ERK signaling directly and indirectly contributes to the cyclical self-renewal of spermatogonial stem cells. Stem Cell Reports. 2013:2517-2527. DOI: 10.1002/stem.1486

[40] Endo T, Romer KA, Anderson EL, Baltus AE, De Rooij DG. Periodic retinoic acid–STRA8 signaling intersects with periodic germ-cell competencies to regulate spermatogenesis. 2015. DOI: 10.1073/pnas.1505683112

[41] Kanatsu-Shinohara M et al. Homing of mouse spermatogonial stem cells to germline niche depends on β1-integrin. Cell Stem Cell. 2008;**3**:533-542

[42] Rinster RA. beta1- and alpha6-integrin are surface markers on mouse spermatogonial stem cells. Proceedings of the National Academy of Sciences. 1999;**96**:5504-5509

[43] Valli H et al. FACs and MACS sorting strategies to isolate and enrich human spermatogonial stem cells. Fertility and Sterility. 2015;**102**:566-580

[44] Brinster RL, Zimmermannt JW. Spermatogenesis following male germ-cell transplantation. Developmental Biology. 1994;**91**:11298-11302

[45] States S et al. Mouse spermatogenic stem cells continually interconvert between equipotent singly isolated and syncytial states. Stem Cells. 2014;**14**:658-672

[46] Garbuzov A et al. Purification of GFRa1+ and GFRa1–spermatogonial stem cells reveals a niche-dependent mechanism for fate determination. Stem Cell Reports. 2018;**10**:553-567

[47] Kimura W, Sadek HA. The cardiac hypoxic niche: Emerging role of hypoxic microenvironment in cardiac progenitors. Cardiovascular Diagnosis and Therapy. 2012;**2**:278-289

[48] Helsel AR, Oatley MJ, Oatley JM. Glycolysis-optimized conditions enhance maintenance of regenerative integrity in mouse spermatogonial stem cells during long-term culture. Stem Cell Reports. 2017;**8**:1430-1441

[49] Chan F. Functional and molecular features of the Id4+germline stem cell population in mouse testes. et al., Genes & Development. 2014:1351-1362. DOI: 10.1101/gad.240465.114.4

[50] Kanatsu-shinohara M et al. Myc/Mycn-mediated glycolysis enhances mouse spermatogonial stem cell self-renewal. 2016:2637-2648. DOI: 10.1101/gad.287045.116.6

[51] Suzuki A et al. Loss of MAX results in meiotic entry in mouse embryonic and germline stem cells. Nature Communications. 2016;**7**:1-15

[52] Kanatsu-Shinohara M et al. Serum- and feeder-free culture of mouse germline stem cells. Biology of Reproduction. 2011;**84**:97-105

[53] Kanatsu-shinohara M et al. Improved serum- and feeder-free culture of mouse germline stem cells. Biology of Reproduction. 2014;**91**:1-11

[54] Morimoto H, Kanatsu-Shinohara M, Shinohara T. ROS-generating oxidase Nox3 regulates the self-renewal of mouse spermatogonial animals and cell culture. Biology of Reproduction. 2015;**92**:1-10

[55] Fijak M. The testis in immune privilege. Immunological Reviews. 2006;**213**:66-81

[56] Filippini A et al. Immunology and immunopathology of the male genital tract: Control and impairment of immune privilege in the testis and in semen. Human Reproduction Update. 2001;**7**:444-449

[57] Yule TD, Montoya GD, Russell LD, Williams TM, Tung KSK. Autoantigenic germ cells exist outside the blood testis barrier. Journal of Immunology. 1988;**141**:1161-1167

[58] Teijo S, Jahnukainen Kirsi PP. Autoantigenicity of the basal compartment of seminiferous tubules in the rat. Journal of Reproductive Immunology. 1996;**31**:65-79

[59] Piquet-pellorce C et al. Leukemia inhibitory factor expression and regulation within the testis. Endocrinology. 2000;**141**:1136-1141

[60] Müller R, Klug J, Rodewald M, Meinhardt A. Macrophage migration inhibitory factor suppresses transforming growth factor-β2 secretion in cultured rat testicular peritubular cells. Reproduction, Fertility, and Development. 2005;**17**:435-438

[61] Hatakeyama H et al. Testosterone inhibits tumor necrosis factor-alpha-induced vascular cell adhesion molecule-1 expression in human aortic endothelial cells. Federation of European Biochemical Societies. 2002;**530**:129-132

[62] Liva SM, Voskuhl RR. Testosterone acts directly on CD4 + T lymphocytes to increase IL-10 production. Journal of Immunology. 2001;**167**:2060-2067

[63] Fijak M, Bhushan S, Meinhardt A. The immune privilege of the testis. Immune Infertility. 2009:69-77. DOI: 10.1007/978-3-642-01379-9

[64] Gerdprasert O et al. The response of testicular leukocytes to lipopolysaccharide-induced inflammation: Further evidence for heterogeneity of the testicular macrophage population. Cell and Tissue Research. 2002:277-285. DOI: 10.1007/s00441-002-0547-6

[65] Naz RK. Modalities for treatment of antisperm antibody mediated infertility: Novel perspectives. Reproductive Immunology. 2004:390-397

[66] Fraczek M, Kurpisz M. Cytokines in the male reproductive tract and their role in infertility disorders. Journal of Reproductive Immunology. 2015;**108**:98-104

[67] Pollanen P, Maddock S. Macrophages, lymphocytes and MHC II antigen in the ram and the rat testis. Journal of Reproduction and Fertility. 1988;**82**:437-445

[68] North TE et al. Hematopoietic stem cell development is dependent on blood flow. Cell. 2009;**137**:736-748

[69] Kilian KA, Bugarija B, Lahn BT, Mrksich M. Geometric cues for directing the differentiation of mesenchymal stem cells. PNAS. 2010;**107**:4872-4877

[70] Bergh A, Collin O, Lissbrant E. Effects of acute graded reductions in testicular blood flow on testicular morphology in the adult rat. Biology of Reproduction. 2001;**64**:13-20

[71] Colin O, Damber J, Widmark A. Control of testicular vasomotion by testosterone and tubular factors in rats. Journal of Reproduction and Fertility. 1993;**97**:115-121

[72] Damber J-E, Maddocks S, et al. Testicular blood flow and vasomotion can be maintained by testosterone in Leydig cell-depleted rats. International Journal of Andrology. 1992;**15**:385-393

[73] Rebourcet D et al. Sertoli cells modulate testicular vascular network development, structure, and function to influence circulating testosterone concentrations in adult male mice. Endocrinology. 2016;**157**:2479-2488

[74] Chiarini-Garcia H, Raymer AM, Russell LD. Non-random distribution of spermatogonia in rats: Evidence of niches in the seminiferous tubules. Reproduction. 2003;**126**:669-680

[75] Brinster RL. Germline stem cell transplantation and transgenesis. Science. 2002;**296**: 2174-2177

[76] Alves-lopes P, Olle S, Stukenborg J. Testicular organoid generation by a novel in vitro three-layer gradient system. Biomaterials. 2017;**130**:76-89

[77] Alves-lopes JP, Söder O, Stukenborg J. Use of a three-layer gradient system of cells for rat testicular organoid generation. Nature Protocols. 2018;**13**:248-259

[78] Yoshida S, Sukeno M, Nabeshima Y-I. A vasculature-associated niche for undifferentiated spermatogonia in the mouse testis. Science. 2007;**317**:1722-1726

[79] Chung K. CLARITY and beyond: Tools for integrated brain mapping. 2014:27-34

[80] Chung K, Deisseroth K. CLARITY for mapping the nervous system. Nature Methods. 2013;**10**:508-513

[81] Tomer R, Ye L, Hsueh B, Deisseroth K. Advanced CLARITY for rapid and high-resolution imaging of intact tissues. Nature Protocols. 2014;**9**:1682-1697

[82] Sato T et al. In vitro production of functional sperm in cultured neonatal mouse testes. Nature. 2011;**471**:504-507

[83] Alves-lopes JP, Stukenborg J. Testicular organoids: A new model to study the testicular microenvironment in vitro? Human Reproduction Update. 2018;**24**:176-191

[84] Lancaster MA, Knoblich JA. Organogenesis in a dish: Modeling development and disease using organoid technologies. Science. 2014;**345**

[85] Komeya M et al. Long-term ex vivo maintenance of testis tissues producing fertile sperm in a microfluidic device. Nature Scientific Reports. 2016;**6**

[86] Komeya M et al. Pumpless microfluidic system driven by hydrostatic pressure induces and maintains mouse spermatogenesis in vitro. Nature Scientific Reports. 2017;**7**

[87] Ishii T, Saitou M. Promoting in vitro gametogenesis research with a social understanding. Cell Press: Trends in Molecular Medicine. 2017;**23**:985-988

[88] Tang WWC, Kobayashi T, Irie N, Dietmann S, Surani MA. Specification and epigenetic programming of the human germ line. Nature Reviews. Genetics. 2016;**17**:585-600

[89] Hirota T et al. Fertile offspring from sterile sex chromosome trisomic mice. Science. 2017;**357**:932-935

[90] Espenshade TJ, Guzman JC, Westoff CF. The surprising global variation in replacement fertility. Population Research and Policy Review. 2003;**22**:575-583

[91] Handel MA, Eppig JJ, Schimenti JC. Applying 'gold standards' to in-vitro-derived germ cells. Cell. 2014;**157**:1257-1261

[92] Rodriguez S, Campo-Engelstein L, Tingen C, Woodruff T. An obscure rider obstructing science: The conflation of parthenotes with embryos in the Dickey-Wicker amendment. The American Journal of Bioethics. 2011;**11**:20-28

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