Triggers of Spermatogenesis

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Abstract

Of the 7% of men affected with infertility, about 54% suffer from pre-testicular and/or testicular factor induced azoospermia/oligospermia. This agenesis of spermatozoa has been the subject of much andrology research over the past 50 years, with a particular focus in the triggers of spermatogenesis. While much of their work is limited to murine populations, researchers have put a lot of emphasis on the spermatogonial stem cell (SSC) niche as the source of the trigger(s). By following physiological patterns exhibited in the seminiferous epithelium, researchers have been able to detect distinct morphological stages that correlate with spermatogonial germ-line action. Different niche cells appear to release different concentrations of active compounds, androgens, and receptors during different stages. Specifically, in the steps leading up to and during SSC differentiation, Sertoli cells and germ line cells release retinoic acid and retinoic acid receptors. Retinoic acid appears to trigger SSCs in vitro as well. Testosterone, released by Leydig cells and potentially testicular macrophages, appears to play an essential role in a spermiation-SSC differentiation axis, as well as a role in GDNF production by peritubular myoid cells, both required for proper maintenance of SSC populations and commitment to meiosis. With new and promising research being done on the whole of the SSC niche, as opposed to just Sertoli cells, scientists are closer than ever to uncovering the secrets of male fertility.

Introduction

The mechanisms of spermatogenesis, the process by which male gametes are produced in the testes, are held within the constant and timed divisions of spermatogonial stem cells (SSCs). In a healthy human adult male, to maintain adequate virility, about 1000 spermatozoa are produced per second (Johnson et al., 1980). About 7% of men suffer from infertility (Lotti & Maggi, 2015), whether idiopathic or organic. In as many as 90% of these cases, azoospermia, a lack of sperm in the ejaculate, or oligospermia, a low sperm count (below 15 million sperm cells per cc of semen), is typically observed (Sabra & Al-Harbi, 2014). Some of this data can be attributed to post-testicular factors, including anatomical blockages like tumors and congenital absence of the vas deferens; but much of this data, perhaps 60%, is due to pre-testicular and testicular issues. For example, a lack of anterior pituitary hormones such as Luteinizing hormone and Follicle stimulating hormone (FSH), deficiencies in SSC count or serum testosterone could all be factors (Bernie, et al. 2013). All of these factors appear to be of the utmost importance for the steps of spermatogenesis to be carried out properly, or to be carried out at all. Perhaps the most essential of these steps in the journey from SSC to spermatozoon is the first. What triggers commit undifferentiated spermatogonia to meiosis? Understanding the triggers of spermatogenesis can ultimately lead to uncovering the secrets of both male fertility and infertility. This review attempts to collect and analyze the relevant research on this subject.

Methods

Data was collected using ProQuest and PubMed databases through Touro College's online library. Among the key-phrases used were "triggers of spermatogenesis", "spermatogonium", "the cycle of seminiferous epithelium", and "testosterone and spermatogenesis."

The Spermatogonial Stem Cell

The testes are the male gonads, where both androgens and sperm are produced. Sperm is more specifically produced in the seminiferous tubules, which are tightly coiled throughout the testes. Sperm cells are developed within the walls of the tubules and, by the process of spermiation, are released into the lumen prior to their passage to the epididymis, where they are stored until released into semen. In humans, this timed and coordinated process, from type A pale spermatogonia to spermiation, takes 64 days (Heller & Clermont, 1963). In order to maintain virility, mature sperm production must be continuous, tightly coordinated, and staggered over the 64 days. Otherwise, instead of being released continuously, sperm would be released into the lumina of multiple tubules in a pulsatile fashion, resulting in staggered fertility (Griswold, 2016). All sperm cells stem from SSCs. They are the cell that gives rise to the rest of the germ-line.

Another necessary characteristic of spermatogonia is the ability to replenish SSC populations as they are continuously committed to meiosis. In this manner, an adequate sperm count is maintained. At the beginning of the germ-line in human testes, SSCs can be identified as two types: type A dark and type A pale. The exact functions of these cells, or whether they are actually different, are not completely agreed upon (Di Persio et al., 2017). However, most of the literature subscribes to the following theory. Pale cells transition into type B spermatogonia, which are committed to meiosis, as well as mitotically dividing to maintain the proper number of SSCs. Dark cells remain on reserve in case of damage to the testicle or SSC population. According to Clermont, dark cells can be considered the true, most undifferentiated, SSCs in the spermatogonial germ-line, while pale cells are best described as the actively dividing and self-replenishing progenitors of B cells (Clermont, 1969; Amann, 2008; Dym et al., 2009). Type A pale spermatogonia are the cells which are regularly triggered by some mechanism to enter the process of spermatogenesis (Gilbert, 2000).

Many labs regularly use murine SSCs for their studies. It is important to also be familiarized with the nuances of murine SSCs and how they differ from humans. In mice, type A spermatogonia (known in mice as type Asingle spermatogonia) only exhibit qualities found in human type A pale cells. Dark cells do not

exist in murine SSC populations. Before committing to meiosis, type Asingle cells divide into chains, or syncytia, of 2 (known as Apaired), 4, 8, or 16 (all three known as Aaligned). After this, the type A cells transition into A1, A2, A3, A4, B spermatogonia, and primary spermatocytes, in that order, before initiating meiotic division. Without a type A dark cell reserve, murine SSC populations run the risk of dwindling if commitment to meiosis is not properly regulated. This extensive germ-line, compared to the human model, is thought to be necessary for the proper maintenance of SSC populations. What hasn't been observed is by what mechanism these type A spermatogonia are triggered to commit to spermatogenesis.

There have been many reports that both human and mouse SSCs can be converted to an embryonic stem cell-like state without the addition of new genes (Shamblott et al., 1998; Donovan & de Miguel, 2003; Shim et al., 2008). The limit of the induced pluripotency of these cells appears to be indefinite (Mahapatra & Gallicano, 2014). This quality has led many researchers to look solely extrinsically from SSCs for the source of their differentiation to spermatids. This conclusion doesn't appear to be correct. Although the existence of an extrinsic signal seems likely, as the divisions of SSCs appear to be carefully timed and regulated by an outside mechanism, we cannot ignore the fact that intrinsic factors unique to SSCs, such as certain receptors, are required as well (Lin et al., 2008).

In recent years, the dogma of where to look for influences of SSC commitment to meiosis has shifted from just the seminiferous compartment toward the whole testicular environment of the stem cells, otherwise known as the SSC niche (Potter & DeFalco, 2017). This niche is made up of cells found in both the seminiferous compartment, such as Sertoli cells, as well as the interstitial compartment, such as Leydig cells and peritubular myoid cells.

The SSC Niche

Sertoli Cells, the Cycle of Seminiferous Epithelium, and Retinoic Acid

The most adjacent cells to SSCs, Sertoli cells (once referred to as nurse cells) are essential in assisting the successful maturation of spermatids from SSCs. They do so through direct contact with germ-line cells and by creating the specific environment needed for proper sperm development. These triangular cells are bound by tight junctions, and are seen extending from the basement membrane to the lumen of a tubule, with SSCs, developing luminally directly between them. These branching cells are also responsible for forming the blood-testis barrier through tight junctions, which prevents unwanted substances from entering the seminiferous tubules, as well as regulating which tubular substances can enter the bloodstream (Anatomy and Physiology). This makes them an obvious candidate for influencing SSC differentiation.

A murine spermatogonial transplant study, designed to correct genetic male infertility in mice, observed an interesting relationship between Sertoli cells and SSCs. Whether host, foreign, or rat stem cells were injected into the lumen of a mouse seminiferous tubule, Sertoli cells were observed transporting them to the basal layer, a movement that requires the breaking and making of tight junctions between adjacent Sertoli cells (Brinster & Avarbock, 1994; Russel & Brinster, 1996). It can be inferred that Sertoli cells have a specific mechanism for recognizing SSCs, perhaps through cadherins (Griswold, 1998). It follows that the relationship between Sertoli cells and SSCs may be more essential than previously thought.

The number and size of murine Sertoli cells is potentially regulated by FSH released by the pituitary during the pubertal stage (Steinberger & Jakubowiak, 1993; Meachem et al., 1996). These conclusions continued to be confirmed by later studies performed with prepubertal murine Sertoli cells but proved difficult to be repeated with cells from older mice (Griswold, 2018b). The studies of the effects of FSH on murine Sertoli cells in culture also exhibitted a positive correlation between the exposure of FSH and the production of Androgen Binding Protein (ABP) by Sertoli cells. There was evidence that spermatogenic cells took up ABP, and that overexposure to ABP led to an increased number of SSCs in vitro and in vivo (Gerard et al., 1994; Jeyaraj et al., 2002). A homologue to ABP was isolated in human Sertoli cells (Hammond, 2011). Further studies on this chemical may be able to confirm an undeniable link between FSH production, the number of Sertoli cells, and the number of SSCs in vivo. A reasonable conclusion is that the number of Sertoli cells has a high correlation to the number of SSCs in vivo.

"This process [spermatogenesis] has been simplified morphologically by recognizing cellular associations or 'stages' and 'phases' of spermatogenesis, which progress through precisely

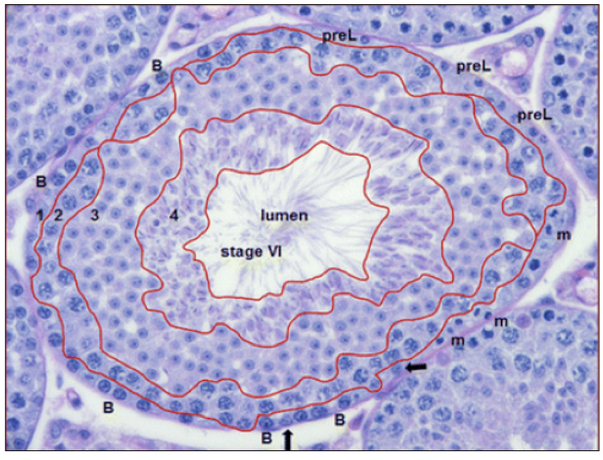

Figure 1: A cross section of a seminiferous tubule highlighting the cellular associations between cells in the epithelial layers, maturing luminally (de Rooij, 2017).

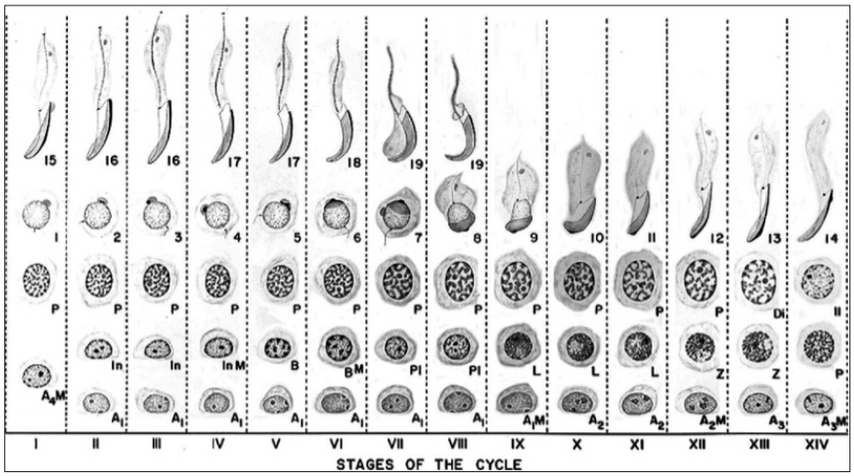

Figure 2: A representation of the linear associations between certain germ cell types at each stage of a cycle (Amann, 2008)

timed and highly organized cycles. These cycles of spermatogenesis are essential for continuous sperm production, which is dependent upon numerous factors, both intrinsic (Sertoli and germ cells) and extrinsic (androgens, retinoic acids), as well as being species-specific." (Hess & de Franca, 2007) Observations of Sertoli cells during different stages of this cycle of the seminiferous epithelium has given insight to possible triggers of the all-important first step of spermatogenesis.

Simply, the cycle of the seminiferous epithelium is the asynchronous cycle through which SSCs, located basally, eventually develop into spermatids and are released into the lumen (Figure 1). An SSC must go through four complete cycles to become an elongated spermatozoon. After every cycle (cycles begin at stage IX and end at stage VIII), a germ cell graduates into a more luminal layer of epithelium; at stage IX after the fourth cycle, the spermatid is released into the lumen. Every cycle has fourteen stages. Each stage in a cycle is characterized by the linear association (luminally to basally) between certain germ cell types (Figure 2), as well changes in neighboring Sertoli cells.

Understanding stages VII-IX is essential for understanding what triggers differentiation in SSCs. Stage VII seems to be the start of some obvious morphological changes in type A1/type A pale spermatogonia. At stage IX, type A pale spermatogonia appear to be officially committed to meiosis, by differentiating into the next cells in the germline. Neighboring Sertoli cells appear to go through many changes with the cycle, with notable changes occurring at stages VII-IX as well (Parvinen, 1982; Parvinen, 1993; Johnston et al., 2008).

Morphologically, Sertoli cells experience dramatic changes with every stage of the cycle. Perhaps the two stage ranges that expressed the most dramatic changes were stages VII-IX and stages XIII-III. Stages VII-IX appeared to show an increase in retinoic acid receptors, retinoic acid itself, and ABP. Stages XIII-III showed an increase in FSH and androgen receptors (Linder et al., 1991). These findings "fit the known functional modes of the cycle with spermiation, onset of spermatogonial differentiation and meiosis, and tight junction dynamics in mode A [stages VII-IX], and spermatogonial expansion in mode B [stages XIII-III]." (Griswold, 2018b)

It has been known since 1925 that vitamin A, more specifically retinol (a precursor of retinoic acid), is needed for spermatogenesis. Sertoli cells were also found expressing retinol uptake and retinoic acid production (Hogarth & Griswold, 2010). Many vitamin A depletion studies have found dramatic cessations of sperm production, while reintroduction of vitamin A led to an almost equally dramatic, synchronous, start of spermatogenesis throughout the whole length of every tubule (Morales & Griswold, 1987). The vitamin A depletion would usually last for weeks and would often leave rats and mice in very unhealthy states, so unequal function of SSCs at reintroduction was expected. The true magnitude of the need for retinoic acid for spermatogenesis may need a different model to be properly observed.

Since stages VII-IX, the period in which Sertoli cells have been observed producing retinoic acid and its receptors, directly coincide with the commitment of SSCs to meiosis, a connection between retinoic acid levels and type A pale spermatogonial differentiation was proposed.

Subsequent experiments found that both drug-induced and diet-induced vitamin A depletion led to an accumulation of type A spermatogonia in mice, with no progression into later cells in the spermatogonial germline. WIN 18,446, a drug previously used in studies aimed at developing a male birth control pill, was found to directly interfere with the aldehyde dehydrogenase that converted retinal to retinoic acid. When mice, who were given this drug for 9 days, were finally injected with retinoic acid, expression of Str8 mRNA and proteins (which were previously known to be stimulated by retinoic acid), was observed in both Sertoli cells and SSCs. After 24 hours, differentiation in these cells was observed (Hogarth et al., 2013). The drug-induced studies are more valid than the diet induced studies, because total body effects of vitamin A depletion was avoided by the short periods the drug was given in.

In short, these studies laid incredibly strong evidence that Sertoli cells, a source of retinoic acid, triggered the commitment of undifferentiated spermatogonia to meiosis. An obvious limitation to these studies is the fact that all of the test subjects were murine. Human studies of retinoic acid with equal specificity have yet to be conducted.

Leydig Cells, Testicular Macrophages, and Testosterone

Adult Leydig cells, located in the interstitial space between seminiferous tubules, are responsible for the production of testosterone. Luteinizing hormone binds to receptors on the Leydig cell. This activates LDL cholesterol displacement from the cell membrane and its conversion to pregnenolone in the mitochondria. Pregnenolone is then transported to the smooth endoplasmic reticulum to be converted to testosterone through a series of enzyme mediated reactions (Mechanisms in Medicine., 2011).

Interstitial and peritubular testicular macrophages protect SSCs and other testicular structures from both foreign and auto-immune attacks (Mossadegh-Keller et al, 2017). Other mechanisms of these macrophages are not completely known or understood, but some researchers speculate that they might indirectly influence SSC differentiation by producing small amounts of testosterone. More plausible is the hypothesis that interstitial testicular macrophages interact with Leydig cells to affect the production of Leydig cell steroidal agents, like testosterone (Trpimir, 2014).

The average serum testosterone for men over 30 years of age is 350-750 ng/dL (Davis, 2018). In the testicle, the concentration of testosterone can be up to a factor 125 times that amount; this is due to the local production of testosterone (Comhaire & Vermeulen, 1976). A study on rats found that spermatogenesis decreased significantly when testicular testosterone concentrations dropped below 70mM, five times average serum concentrations (Zirkin et al., 1989). Due to an unusually high concentration of testosterone in the testes required for proper spermatogenesis, some point to testosterone as a major extrinsic signal in SSC differentiation.

Effectors of testosterone can be identified by the presence of androgen receptors on their cell surfaces. In the testicle, almost all cell types express androgen receptors and thus respond to testosterone. Knocking out (KO) this receptor in isolated cell types shows how testosterone acts in these cells. Surprisingly, androgen receptors have not been found on germ cells, indicating that testosterone must have an indirect effect on SSC differentiation, if any effect at all. "Germ cells from testicular feminized mice that lacked testosterone receptors were transplanted into the seminiferous tubules of azoospermic mice expressing functional androgen receptors. When recipient testes were analyzed between 110 and 200 days following transplantation, multiple colonies of qualitatively normal donor-derived spermatogenesis were seen in each recipient testis."(Sorkitis et al., 2003) Studies on androgen receptor KO Leydig cells seem to show spermatogenesis arresting at spermiation, but the protocols with which these experiments were carried out are questionable (Xu et al., 2007, Smith & Walker, 2014). The agent used to knock out androgen receptors was anti-Mullerian hormone. Later attempts at mimicking these methods found that not all Leydig cells were androgen receptor free, while some Sertoli cells were affected.

In three independent studies, androgen receptor KO Sertoli cells exhibited normal growth and proliferation in all subjects, showing that testosterone isn't required for those functions(De Gendt et al., 2004; Chang et al., 2004; Stanton et al., 2012). However, analysis of the seminiferous epithelium found that the blood-testis barrier under-expressed necessary cadherins, resulting in a loss of integrity in its structure.

Further analysis found that spermatogenesis slowed at the formation of elongated spermatids and arrested completely at spermiation, suggesting that testosterone's interaction with Sertoli cells is required for these actions. Additionally, the differentiation of spermatogonia also ceased shortly after spermiation did; this suggests that the mechanism of spermiation is involved directly with the proper maintenance of the cycle of seminiferous epithelium.

From the data presented, it seems likely that testosterone is at least indirectly involved in the differentiation of SSCs. Although it's not fully understood, other studies suggest that testosterone is essentially involved in a regulatory axis of spermatogenesis.

Peritubular Myoid Cells and GDNF

Peritubular myoid cells (PMCs) make up the smooth muscle-like layer that surrounds the seminiferous tubules. Some main functions of these contractile cells are to provide support as well as some peristaltic movement throughout the tubule. A few studies have suggested that PMCs may indirectly affect SSC differentiation.

Glial cell line-derived neurotrophic factor (GDNF), thought to affect the maintenance of SSC populations and SSC renewal, is present in both murine and human Sertoli cells (Fouchécourt et al., 2006). When GDNF levels are depleted, SSC populations dwindle while SSC differentiation is agitated. When GDNF levels are elevated, SSC populations are much higher than normal, while differentiation is arrested completely (Meng et al., 2000).

It has been suggested that FSH stimulates the production of GDNF by Sertoli cells, as observed by one study. Another study attempting to recreate the previous study's data was unsuccessful (Crépieux, 2001). But what was generally agreed on was that, by some unknown mechanism, Sertoli cell production of GDNF was essential for maintaining SSC populations and differentiation.

An androgen receptor KO study on peritubular myoid cells incidentally found that SSC populations decreased significantly. Further analysis found that GDNF levels were severely lowered, suggesting that testosterone-stimulated peritubular myoid cells synthesized GDNF. This was later confirmed by a study conducted in 2014 (Figure 3). The results of which bring up questions about which cell plays a more significant part in SSC population maintenance. Why original stains missed GDNF concentrations

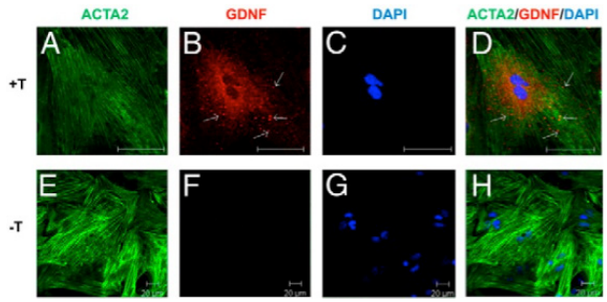

Figure 3: Stains of peritubular myoid cells and SSCs in culture with and without testosterone (Chen et al, 2014). A-D are grown with Testosterone. E-H are grown without. The molecule denoted at the top of each column is stained for

in peritubular myoid cells is probably due to the fact that these cells are very thin and don't stain as well as Sertoli cells.

Germ-Line Cells

Spermiation, the process of mature spermatids being released into the lumen prior to their passage to the epididymis, takes place at the apical surface of the seminiferous epithelium. However, the interruption of this process further impedes the basal processes of SSC differentiation and commitment to meiosis. Interestingly, the process of spermiation directly coincides with this SSC differentiation. This suggests evidence of a regulatory axis between the tightly coordinated processes of spermiation and SSC differentiation.

As spermatids are elongated, in preparation for spermiation, all nonessential organelles and cytoplasm are collected into a section attached to the head of the spermatid. Through a series of Sertoli cell interactions with these elongated cells, and through testosterone stimulation of a Src pathway (see Figure 4), Sertoli cells break and remake tight junctions, mechanically severing this residual body from the elongated spermatid and releasing the sperm into the lumen. At the same time, this pathway allows for conformational changes throughout the entire length of the Sertoli cell, allowing basal germ-line cells to move apically (Yan et al., 2008a; Yan et al., 2008b; Cheng & Mruk, 2010).

Studies on germ-line cells have also found that descendent cells from SSCs exhibit a mechanism of taking up retinol and producing retinoic acid, a mechanism that is very similar to the mechanism of Sertoli cells. This discovery led to the manufacturing of a Sertoli cell specific variant of the drug WIN 18,446, called WIN 14,446. When administered before the first wave of retinoic acid could be initiated in mice, at 2-3 days old, the effects of WIN 14,446 were very similar to WIN 18,446. However, when administered at day 9, after the first wave, there appeared to be no effect (Griswold, 2018c). This suggests that Sertoli cell production of retinoic acid may be essential for starting the cycle of spermatogenesis, while germ-line cells may be the primary source of retinoic acid afterward.

Discussion and Conclusion

Much of the research presented has been done strictly on murine populations, with few parallels found in humans. Almost the entirety of the research on retinoic acid has been done in mice. Future studies must be done in human populations in order to obtain a true understanding of spermatogenic processes in humans. Other topics that require more insight are (1) the concentration of intra-testicular testosterone needed for proper spermatogenesis, (2) the specific mechanisms of the regulatory axis between spermiation and SSC differentiation, particularly if the conformation of the Sertoli cell sets off differentiation, and (3) how the cycle of seminiferous epithelium in murine populations differs from humans.

Additionally, a topic that goes hand-in-hand with triggers of spermatogenesis is the asynchronous nature of the cycle of seminiferous epithelium. Throughout the length of the tubule, sperm are released into the lumen constantly, in an uninterrupted fashion. This is due to the fact that the stage (of the cycle) at one portion of the tubule is not the same as the stage being carried out in adjacent portions. All portions of every tubule are releasing sperm into the lumen at a different time. In retinoic acid depletion studies, after spermatogenesis was completely halted, injections of retinol stimulated an almost synchronous restarting of the cycle in every part of every tubule. After about six months, the cycles had returned to their asynchronous nature. Obviously, this is a very confusing phenomenon. When asked directly about it, Dr. Michael Griswold, who is currently researching this issue states that "the key to the cycle is the conversion of A spermatogonia to A1 spermatogonia. Any variance in the timing of this conversion would ultimately lead to asynchrony." (Griswold, 2018a) Perhaps the carefully coordinated cycle of seminiferous epithelium isn't as carefully coordinated as researchers once believed. This evolutionary phenomenon proves to be incredibly important in the maintenance of mammalian fertility.

The ideas presented here is the relevant research done on the topic of triggers of spermatogenesis. Obviously, the research is heavily focused on the Sertoli cell. A reason for this is the fact that the field is mainly contributed to by those that can be called "Sertolists". The Sertoli cell, because of its adjacency to germ cells, has been the obvious focus of these researchers. An example of dangers of this bias is the peritubular myoid cell and the late discovery of its extremely important role in GDNF production. Yes, peritubular myoid cells are small and hard to stain, but many published micrographs didn't even include the interstitial space. Perhaps the original researchers weren't expecting the interstitial space to be involved.

In February 2018, researchers successfully observed human induced pluripotent stem cells (hiPSCs) transforming into actively differentiating SSCs in vivo in mice (Fang et al., 2018). When these results were attempted to be recreated in vitro with just hiPSCs and Sertoli cells, there was no change. The entirety of the

niche must be present for proper functioning. As more research is done on the rest of the SSC niche, the field is beginning to experience a rise in extra-Sertoli cell understanding. And perhaps, with this understanding, the mysteries of non-obstructive azoospermia and oligospermia will be uncovered.

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