# Identification of Proteins with Potentially Essential Functions in Sumoylation and Male Infertility

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**Abstract**

Of all the couples trying to conceive in the United States, it is reported that roughly 20% do not successfully conceive in the first year of trying. Problems with fertility can be attributed to both the male and female in the couple. About 33% of infertility issues are of unknown causes for men. Spermatogenesis is the development by which haploid sperm cells are produced from the maturation of germ cells. Many proteins and biological processes are involved in the creation of sperm cells, including Sertoli cells. The developing spermatogonia are imbedded within the recesses of the Sertoli cells, and the Sertoli cells function to secrete proteins and hormones that aid in the maturation of these germ cells. An integral mechanism within all testicular cells, including Sertoli cells, is sumoylation, a post transcriptional modification process that alters the function of proteins via the addition of a SUMO peptide. Sertoli cells were treated with Ginkgolic Acid to inhibit sumoylation and the secreted proteins were compared to the secreted proteins of the control cells. Protein expression was studied using two-dimensional gel electrophoresis and the proteins were identified using mass spectrometry. All secreted proteins that exhibited a change in expression should be studied to better understand their role in spermatogenesis and Sertoli cells. Two proteins, Insulin-Like Growth Factor Binding Protein 7 and Protein 14-3-3 Beta/Alpha, exhibited the biggest fold-change in protein expression upon the inhibition of sumoylation. Additionally, these proteins, and/or the protein family, have established roles relating to spermatogenesis, Sertoli cells, and cell signaling. Due to both the previous research and the difference in protein expression, I believe that Insulin-Like growth Factor Binding Protein 7 and Protein 14-3-3 Beta/Alpha should be the primary focus of future studies to determine their roles in sperm cell development and infertility.

# I. Introduction

## A. Male Infertility

According to the Center for Disease Control and Prevention, infertility is defined as "not being able to get pregnant (conceive) after one year (or longer) of unprotected sex" (Center for Disease Control and Prevention, 2023). Every 1 in 6 couples in the United States suffer from infertility to a certain degree, with roughly 50% of those issues stemming from some sort of male infertility (Azura Vascular Care, 2016). The percentage of infertility in couples increases markedly in developing countries, with the global estimate of infertility reaching almost 73 million couples struggling to conceive. Male infertility can be attributed to numerous causes, including genetic disorders, hormonal disorders, environment, and lifestyle choices (Kumar *et al*., 2015). Low sperm count as well as low sperm quality are common causes widely attributed to male infertility (Mayo Clinic, 2023). While many causes of infertility are known, and some even treatable, 33.3% of male infertility are of unknown cause (U.S. Department of Health and Human Services, 2017), as shown in **Figure 1**.

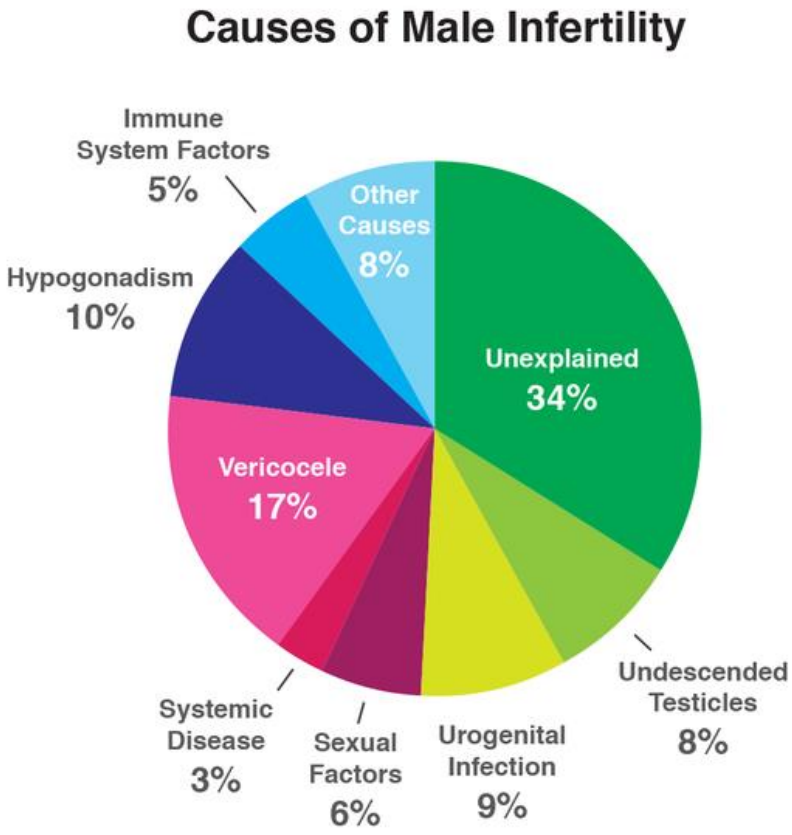


**Figure 1**: Causes of Male Infertility
https://www.fertilitysa.com/fertility-treatment/male-factor-infertility/

Male infertility can stem from complications in the formation of sperm or in the transportation of normal sperm from the testicles. The development of sperm starts in the testes, and if the testes do not function properly the sperm will not develop properly. Genetic and environmental disorders make up a large percentage of the known factors of male infertility (Yale Medicine, 2023). Klinefelter Syndrome is an example of a genetic disorder which has a direct correlation with male infertility. Typically, men are born with one X and Y chromosome, while women are born with two X chromosomes. Instead of the usual 46 chromosome, someone with Klinefelter Syndrome has 47 chromosomes, one Y and two X chromosomes. The extra X

chromosome does not allow for proper progression of puberty, decreasing the amount of testosterone and sperm produced. These symptoms of the genetic disorder often result in infertility (Cleveland Clinic, 2023). Hyperprolactinemia is an example of a hormone imbalance which causes excess stimulation in the pituitary gland and leads to an overproduction of prolactin. This hormone imbalance causes a low testosterone level and therefore a decrease in sperm formation. The only known risk factor for this condition is the genetic disorder multiple endocrine neoplasia (Center for Disease Control and Prevention, 2023). Another example of a disorder that affects male fertility is retrograde ejaculation. While not a genetic disorder in its own right, people with multiple sclerosis and diabetes have an increased risk of developing retrograde ejaculation. The condition causes sperm to travel into the bladder instead of the sperm following its normal route of exiting the body by moving from the testes to the penis (Mayo Clinic, 2023). Klinefelter Syndrome, Hyperprolactinemia, and retrograde ejaculation are just three of the many known disorders that affect sperm production and transport, and in turn lead to fertility issues.

With over 8 billion people in the world, the percentage of people dealing with infertility issues stemming from unknown causes is tremendous. The commonality of infertility highlights the importance of research in this widely undetermined field. Spermatogenesis is an extremely long, complicated process reliant upon numerous enzymes and proteins. However, studying the effect of certain changes to the environment, proteins, enzymes, and processes in spermatogenesis can uncover the root cause of infertility in men. While the underlying causes of male infertility are relatively unknown, the discovery and understanding of these causes offers both reprieve to the couples suffering from infertility as well as a breakthrough in a field filled with uncertainty.

## B. Spermatogenesis

Spermatogenesis is the process by which haploid spermatozoa sperm cells are produced from within the seminiferous tubules. The molecular regulation of spermatogenesis is not completely understood, and so the role of novel molecules in germ cells is important to characterize further.

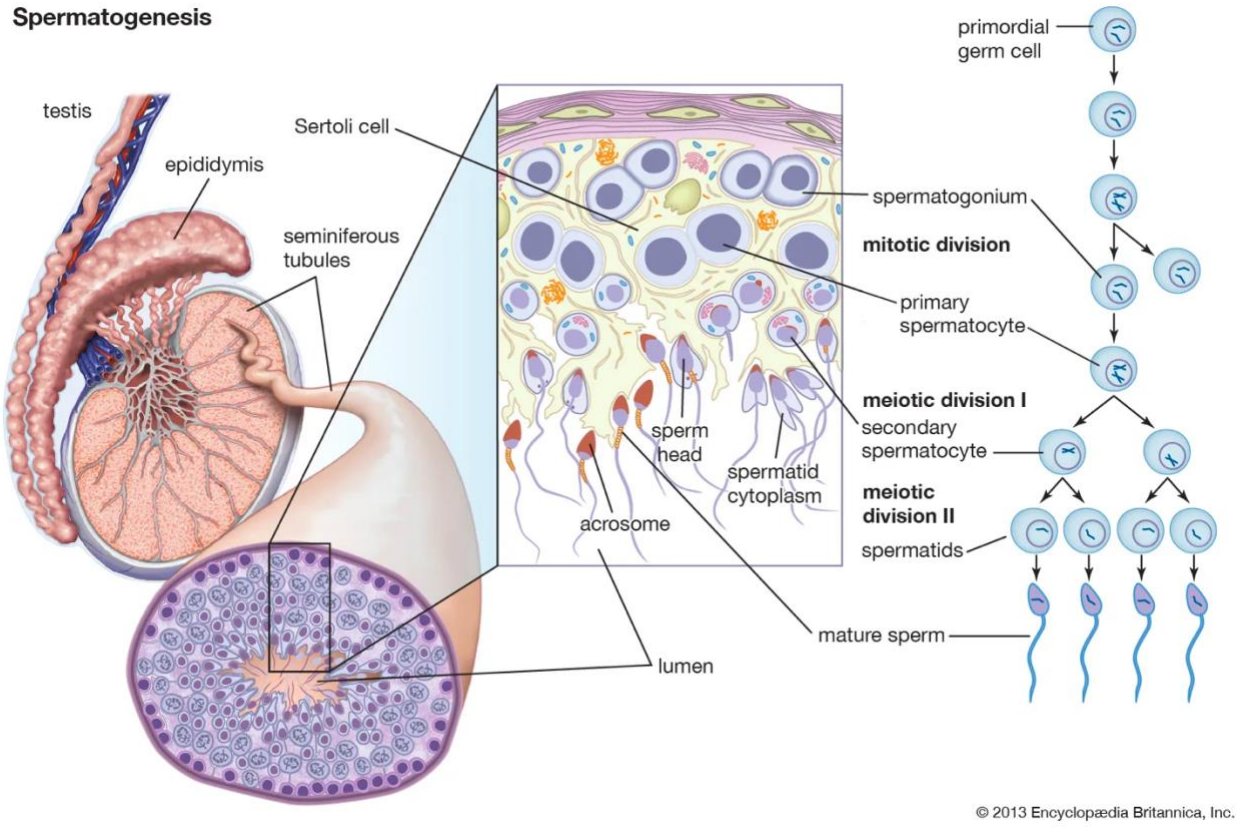


**Figure 2**: Graphic Showcasing Different Cells Involved in Spermatogenesis
https://www.britannica.com/science/spermatogenesis

Spermatogenesis begins with mitotic divisions of spermatogonia. Like all somatic cells in the human body, spermatogonia are diploid, containing a complete set 23 chromosome pairs. Spermatogonia divisions result in the creation of primary spermatocytes. With an increased

presence of testosterone, the primary spermatocytes will undergo meiosis I, which produces two secondary spermatocytes for every primary spermatocyte. The secondary spermatocytes are haploid cells, containing only one copy of each of the 23 chromosomes. Each chromosome in the secondary spermatocyte, however, is formed of two sister chromatids. As this means that there is double the amount of genetic material in each secondary spermatocyte, the secondary spermatocytes undergo meiosis II. Each secondary spermatocytes will give rise to two haploid spermatids. In total, four spermatids are produced for each starting spermatogonia. The spermatids continue differentiation and maturation through spermiogenesis, which allows for a complex metamorphosis resulting in spermatozoa, or mature sperm (ScienceDirect, 2023). The maturation process of sperm cells is illustrated in **Figure 2**.

While spermatogonia lie adjacent to the basement membrane of the tubules, as the germ cells mature, they move through the seminiferous epithelium, eventually releasing the spermatozoa into the lumen of the seminiferous tubules (ScienceDirect, 2023).

### C. Sertoli Cells

Somatic cells known as Sertoli cells aid in the development of the spermatozoa from spermatogonia. Sertoli cells are interspersed between germ cells in the seminiferous tubules and extend from the basement membrane of the tubule through to the lumen. Sertoli cells play an integral role in the maturation of germ cells (Griswold, 1998). The spermatogonia are bound to the Sertoli cells, and spermatogenesis occurs within the recesses of the cells, as shown in **Figure 3**. Sertoli cells provide nourishment and protection by secreting growth factors and hormones to

the developing germ cells (Johnson *et al*., 2007). For this reason, Sertoli cells have taken on the nickname of "nurse" cells.

The Sertoli cells derive iron through serum, or blood, move it across the cells own cytoplasm, and secrete the iron, now bound to testicular transferrin molecules, to be delivered to receptors on the membrane of developing germ cells (Johnson *et al.*, 2007). Sertoli cells are responsible for secreting transport proteins such as $Fe^{+3}$, $Cu^{+2}$, and androgens. Proteins with other functions, such as proteins with enzymatic activity, are secreted by Sertoli cells. A couple of these proteins include plasminogen activator and alpha-lactalbumin-like activity. Lastly, Sertoli cells secrete proteins which aid in the upkeep and proper function of the basement membrane of the seminiferous tubules, including type IV collagen and laminin (Griswold, 1988). The proteins secreted, or secretome, by Sertoli cells assist in a variety of functions that allow for the normal development of sperm. However, the secretome of Sertoli cells has not been fully characterized.

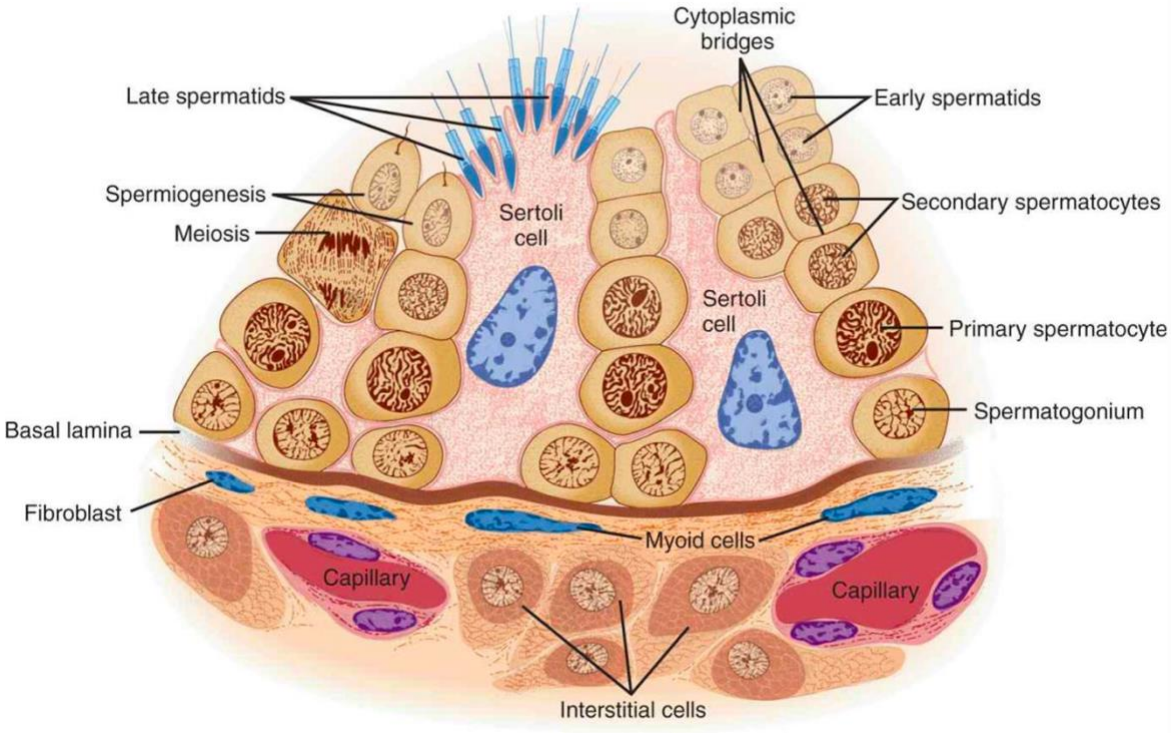


**Figure 3**: Relationship Between Sertoli Cells and Developing Sperm Cells in the Seminiferous Tubules
https://doctorlib.info/physiology/review/27.html

Another important role of Sertoli cells is to secure the blood-testis barrier, which separates the seminiferous epithelium into the basal compartment and the apical compartment. Meiosis I and II and many fundamental steps of spermatogenesis occur in the apical compartment, which has a highly organized and maintained environment. However, the renewal of spermatogonia and differentiation and cell cycle progression up to meiosis occurs in the basal compartment of the epithelium. This separation is regulated by the blood-testis barrier, which is necessary for the proper environments of each process to be maintained. The barrier provides immunological protection to the developing spermatocytes as they move from one compartment to another over the course of its development into fully developed sperm. The blood-testis barrier is not a

constantly static structure as spermatocytes must move from one side of the barrier to the other. However, the immunological barrier created must remain intact to circumvent the production of antibodies against meiotic and post-meiotic germ cells. To seamlessly accomplish the transfer of spermatogonia across the blood-testis barrier without breaking the immunological barrier, studies have shown that a "new" blood-testis barrier is formed behind the spermatogonia that must travel across the barrier, while the "old" barrier is degraded in a timely manner (Cheng *et al.*, 2012). This approach allows the spermatogonia the ability to be transferred while never breaking the ever-important immunological barrier.

The Sertoli cell also functions in hormonal regulation of spermatogenesis. The process of sperm development is regulated and operates under a feedback inhibition cycle of which the Sertoli cell plays a key role. For the process of spermatogenesis to begin, the brain releases the gonadotropin releasing hormone, or GnRH (Johns Hopkins Medicine, 2019). In turn, another area of the brain is stimulated to release follicle stimulating hormone, or FSH, and luteinizing hormone, or LH. FSH is directly involved with Sertoli cells, as FSH is the hormone that will inform the Sertoli cell to begin nourishment of the germs cell, thus allowing spermatogenesis to begin. LH is also necessary for spermatogenesis as it stimulates the production of testosterone from Leydig cells. When the levels of testosterone reach a critical threshold, which happens after spermatogenesis has begun, a signal is sent back to the brain to decrease the production of GnRH. As GnRH was responsible for facilitating the process as a whole, a decreased level of GnRH will in turn slow down the process of spermatogenesis. When the level of testosterone decreases, the process will begin anew with the release of GnRH (Ni *et al.,* 2019). As the hormones regulate cellular functions, the system is a feedback inhibition cycle as depicted in **Figure 4** below. Sertoli cells also secrete proteins such as AMH, somatomedin C, and EGF-like growth factor

(Griswold, 1988). These proteins also have important roles in the regulation of the developing sperm cells.

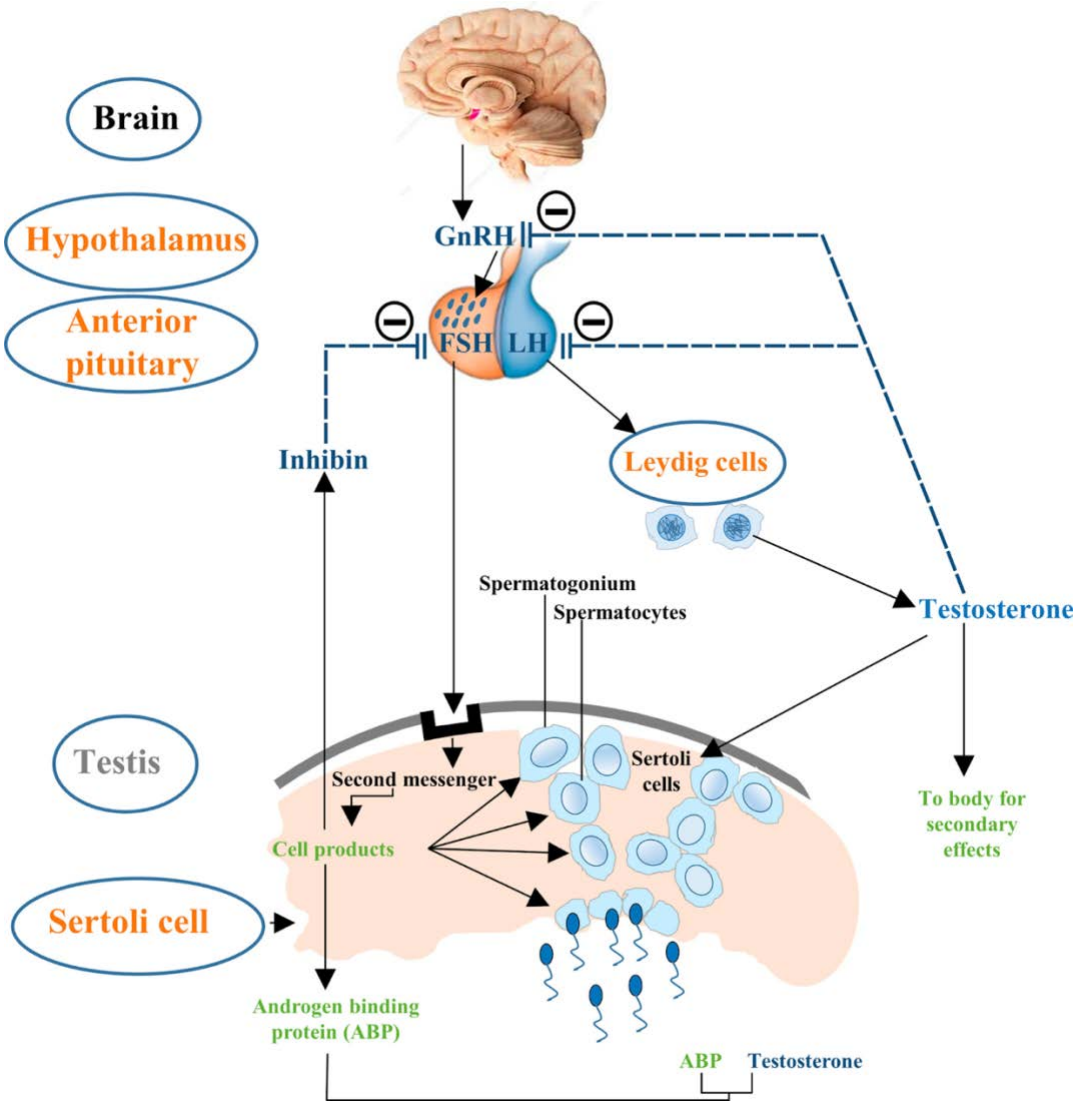


**Figure 4**: Schematic on the Inhibition Cycle's Relationship to Sertoli Cell Proliferation
https://www.frontiersin.org/articles/10.3389/fendo.2021.648141/full

The Sertoli cells must remain attached to the developing germ cells for spermatogenesis to be completed properly. The germ cells are connected to the outside of the Sertoli cells, and the space between the Sertoli cells are known as junctional complexes. The germ cells are attached

via testis-unique intermediate filament and actin-based cell junctions. Desmosome-like junctions and ectoplasmic specializations prevent the developing germ cells from being removed from the seminiferous epithelium (Kopera *et al.,* 2010). If these junctions fail, or are for some reason fragile, the shedding of the germs cells from the region could cause a low sperm count and infertility. The unique interaction of maturing sperm cells and Sertoli cells are reliant upon junctions and adhesions between them, which allows for the Sertoli cells to enact their various important roles in spermatogenesis (Khalili *et al.,* 2015).

### D. Sumoylation

Post translational modification refers to processes which involve adding peptides, proteins, or other molecules onto proteins that are already synthesized. The purpose of these attachments is to change or regulate its function. One example of post translation modification is the addition of a SUMO peptide chain, also known as Small Ubiquitin-like Modifier proteins, to other synthesized proteins. SUMO proteins have a specific amino acid sequence. SUMO proteins attach to other proteins via covalent bonds, modifying the function of other proteins (Wei *et al*., 2012). This process is called sumoylation. Previous studies have found SUMO proteins to be present across many somatic cell types, with a highly expressed presence in testicular cells. Sumoylation may affect protein localization, and act as an activator or deactivator of the protein. Some proteins interact with SUMO non-covalently (Sengupta *et al.,* 2021).

The SUMO proteins in Sertoli cells are found as free peptide chains and attached to other protein targets. As shown in **Figure 5**, three classes of enzymes are used to attach the SUMO peptide to other proteins. To activate a free SUMO peptide, a protease enzyme is used to cleave a

SUMO protein from an inactive precursor. Several c-terminal amino acids are removed as a result of this cleavage. Three proteins, SUMO-activating enzyme (E1), SUMO-conjugating enzyme (E2), and SUMO ligase protein (E3) are used to effectively bind SUMO proteins to target proteins (Sengupta *et al.,* 2021).

The SUMO-activating enzyme (E1) requires energy in the form of ATP to form a thioester bond with the cysteine residue of Uba2. SUMO-conjugating enzyme (E2), or Ubc9 alone, brings the SUMO to the target protein. An enzyme from the SUMO ligase protein (E3) family subsequently forms a covalent bond between the SUMO peptide and to the substrate. While sumoylation binds a SUMO peptide to a protein to modify the function of the protein, desumoylation allows for the protein to reverse its function. Protease acts as the enzyme to reverse sumoylation, promoting the removal of the SUMO peptide, or desumoylation (Sengupta *et al.,* 2021).

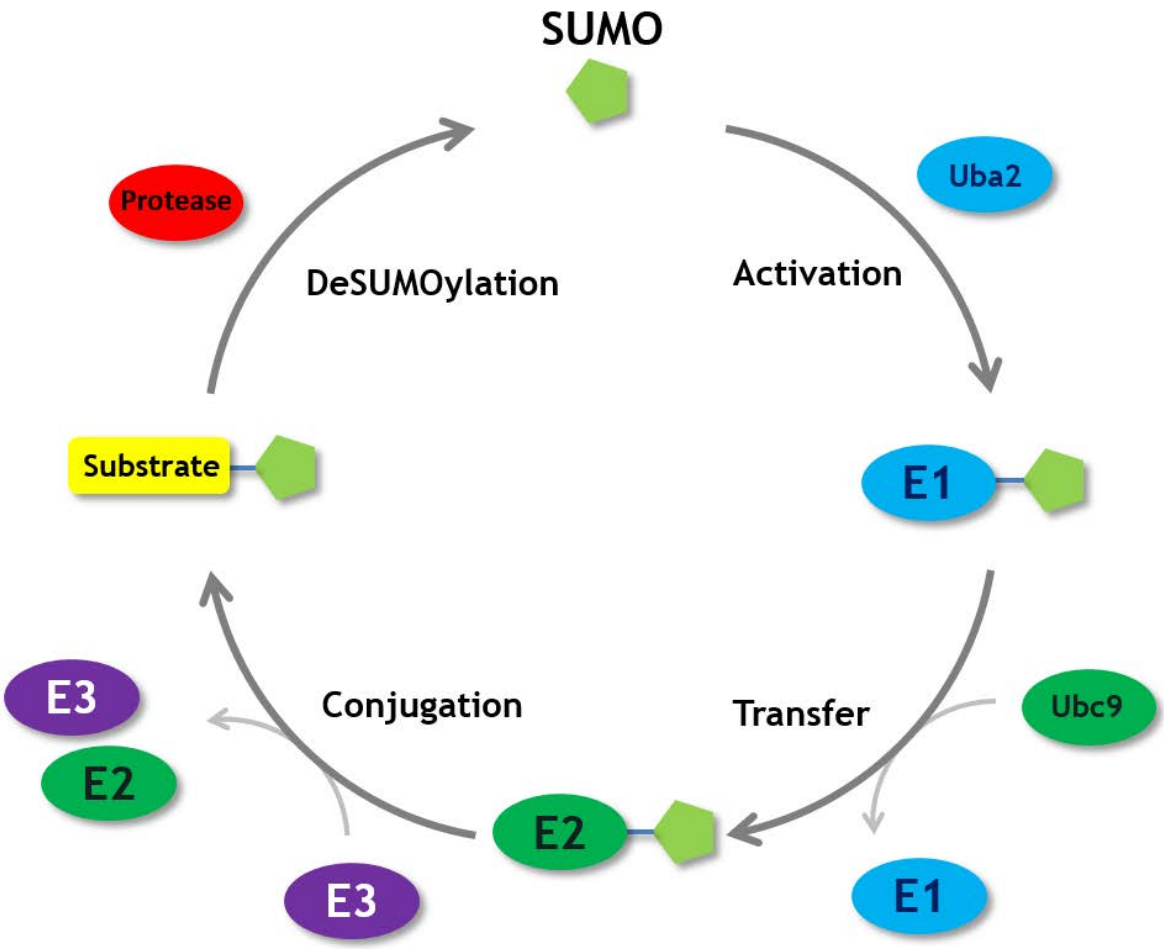


**Figure 5**: The Sumoylation Process, Highlighting Enzymes Utilized for Each Step

All testicular cells have been found to express SUMO peptides at a high level, and the role of sumoylation in spermatogenesis and Sertoli cells has been studied by Dr. Margarita Vigodner's lab (Sengupta *et al.,* 2021). Studies have shown that when sumoylation is inhibited in Sertoli cells, the cell can undergo oxidative stress and even programed cell death, or apoptosis (Fukuda *et al*., 2009). A major function of the Sertoli cell is known to be the secretion of proteins and hormones to support spermatogenesis and developing sperm cells. The role of sumoylation in the regulation of secretions of Sertoli cells is not yet understood.

## II. Goals

As previously mentioned, there is a gap in knowledge between the role of sumoylation in the regulation of Sertoli cell secretions. The goal of this research was to address this gap, and better characterize the role of sumoylation in regulation of Sertoli cell secretions. During my time in Vigodner's lab, a two-dimensional gel electrophoresis was run successfully to identify and quantify the proteins secreted by Sertoli cells when sumoylation occurred as normal in comparison to when sumoylation was inhibited. The first goal of my research was to characterize the secreted proteins, using two-dimensional gel electrophoresis. This serves to identify differing quantities in protein secretions. The second goal of my research was to analyze the results of mass spectrometry protein identification, and conduct literature research to pinpoint target proteins for further research based on their possible involvement in spermatogenesis.

## III. Materials and Methods

### A. Cell Lines

To understand the proteins involved in Sertoli cells and sumoylation, mouse cells were treated and analyzed. The mouse Sertoli cell line 15P-1 (ATCC, CRL-2618) was bought from ATCC (Manassas, VA). The cell line was grown in DMEM media with 5% fetal bovine serum (FBS, Life Technologies, 16140-071), 5% bovine growth serum (Fisher Scientific, SH30541.03), 1% penicillin/streptomycin (Life Technologies, 15140-122), and 0.5% Fungizone (Life Technologies, 15290-018) at 32 degrees Celsius with 5% $CO_2$. This Sertoli cell line was derived from primary

testicular mouse cells, and cultured in a way that allowed the cell line to survive for multiple days of studies. The cells were split twice a week and were treated with trypsin/EDTA in order to maintain the cell line (Sengupta *et al*., 2021).

### B. Ginkgolic Acid Treatment

Sumoylation in Sertoli cells is a fundamental process that allows the cells to nurse germ cells into haploid spermatozoa, or sperm. Ginkgolic acid has been identified as a sumoylation inhibitor, and thus treating a cell line with Ginkgolic acid both *in vitro* and *in vivo* will hinder sumoylation. Ginkgolic acid is effective by binding directly to Enzyme 1, the first enzyme necessary for sumoylation to occur. Without Enzyme 1, the formation of the Enzyme 1-SUMO intermediate is inhibited, and the process cannot continue, effectively inhibiting sumoylation (Fukuda et al., 2009). Treating cells with Ginkgolic acid does not affect the *in vivo* ubiquitination, ensuring that the rest of the cell functions as normal and allowing results to be directly linked to the sumoylation inhibition. Ginkgolic acid was diluted at DMSO, or dimethyl sulfoxide, to concentrations of 20 to 30 micromolars. The cells were treated with the diluted Ginkgolic Acid for two hours. After this Ginkgolic Acid treatment, the supernatants were collected from the control and the treated cells. The protein concentrations were determined using a dye-free medium to ensure there was no interference with protein concentration analysis. The dye-free medium was a solution of 4.5 grams per liter D-Glucose, L-Glutamine, 25 millimolar HEPES, and Sodium Pyruvate. Protein samples were then run on two-dimensional gel electrophoresis (Sengupta *et al*., 2021).

## C. Two-Dimensional Gel Electrophoresis

To identify the different proteins secreted from the Sertoli cells, or the secretome, a two-dimensional gel electrophoresis (2-D DIGE) and a subsequent Protein Identification was performed by Applied Biomics Incorporated, located in Hayward, California (Applied Biomics, 2023). Two protein samples were prepared: one containing the secretomes from Sertoli cells allowed to complete normal cell functions and the other containing the secretomes from Sertoli cells in which sumoylation was inhibited by treatment with Ginkgolic acid. The protein sample buffer was exchanged into a two-dimensional lysis buffer, made of 30 millimolar Tris-HCl, pH 8.8, containing 7 molar urea, 2 molar thiourea, and 4% CHAPS. Protein concentration was measured using Bio-Rad Protein Assay Kit II #500-0002 according to the manufacturer's protocol (Sengupta *et al*., 2021).

Two-dimensional gel electrophoresis operates by fluorescent labeling of the protein sample. For each sample, 30 micrograms of protein were mixed with 1.0 microliters of diluted CyDye and kept in the dark on ice for 30 minutes. The labeling reaction was stopped by adding 1.0 microliter of 10 millimolar Lysine to each sample and incubating in the dark on ice for an additional 15 minutes. The labeled samples were then mixed. The 2X 2-D Sample buffer (8 molar urea, 4% CHAPS, 20 milligrams/milliliter DTT, 2% pharmalytes, and trace amounts of bromophenol blue), 100 microliters streak solution, and Rehydration buffer (7 molar urea, 2 molar thiourea, 4% CHAPS, 20 milligrams/milliliter DTT, 1% pharmalytes, and trace amounts of bromophenol blue) were added to the labeling mix to make the total volume of 250 microliters. The samples were mixed and spun before being loaded into the strip holder IEF and SDS-PAGE. After loading the labeled samples, IEF (pH3-10 Linear) was run following the

protocol provided by Amersham BioSciences. Upon finishing the IEF, the IPG strips were incubated in the freshly made equilibration buffer-1 (50 millimolar Tris-HCl, pH 8.8, containing 6 molar urea, 30% glycerol, 2% SDS, trace amount of bromophenol blue, and 10 milligrams/milliliter DTT) for 15 minutes with gentle shaking. Then the strips were rinsed in the freshly made equilibration buffer-2 (50 millimolar Tris-HCl, pH 8.8, containing 6 molar urea, 30% glycerol, 2% SDS, trace amounts of bromophenol blue, and 45 milligrams/milliliter DTT) for 10 minutes with gentle shaking. Next the IPG strips were rinsed in the SDS-gel running buffer before transferring into 12% SDS-gels. The SDS-gels were run at 15 degrees Celsius until the dye front ran out of the gels. Gel images were scanned immediately following the SDS-PAGE using Typhoon TRIO (GE Healthcare). The protein ratio analysis was performed using DeCyder software version 6.0 (GE Healthcare). The results of the two-dimensional gel electrophoresis were then analyzed by mass spectrometry for protein identification (Sengupta *et al*., 2021).

## IV. Results

### A. Protein Analysis

Secreted proteins from control and from Ginkgolic Acid-treated cells were compared using a two-dimensional gel electrophoresis analysis and equal amounts of the proteins. The secreted proteins from the control Sertoli cells were labeled using a green fluorescent dye while the proteins from Sertoli cells treated with Ginkgolic Acid were labeled using a red fluorescent dye (Applied Biomics, 2023). The results of both fluorescence signals were then superimposed to compare the proteins intensity, as shown in **Figure 6**.

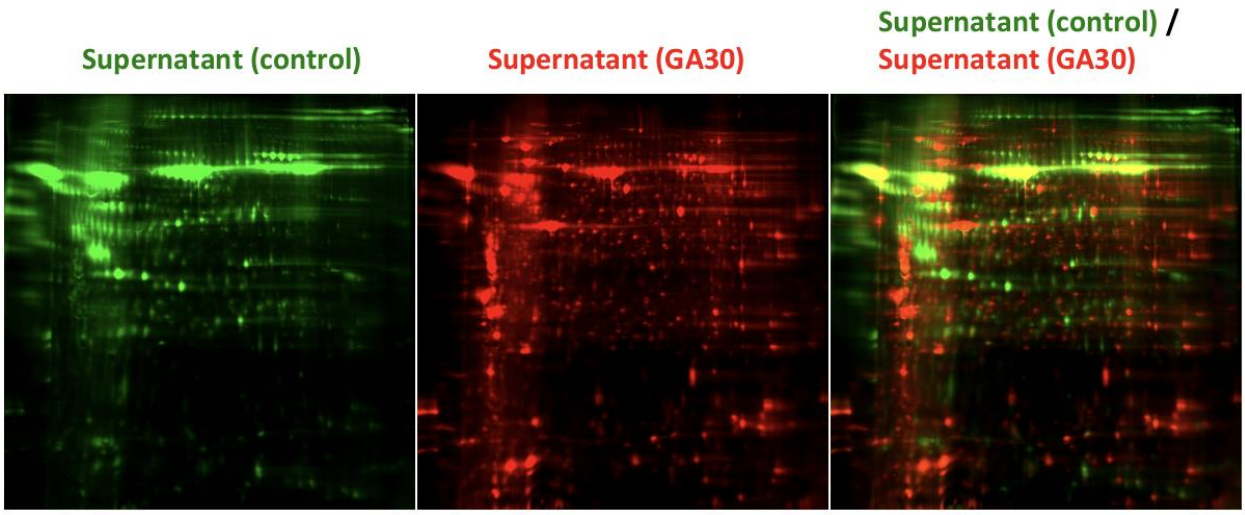


**Figure 6**: Two-Dimensional Gel Electrophoresis Results of Protein Secretions from Control Sertoli Cells and Ginkgolic Acid-Treated Sertoli Cells

The differing amounts of protein secretion from normal Sertoli cells and sumoylation inhibited Sertoli cells was recorded, and a ratio was created. Mass spectrometry analysis of secreted proteins resulted in a total of 11 proteins identified with significant changes in quantity. The results can be found in **Table 1**.

| | Supernatant (GA20) / Supernatant (Control) | Supernatant (GA30) / Supernatant (Control) |
|---|---|---|
| ***Insulin Signaling*** | | |
| Insulin-Like Growth Factor Binding Protein 7 | -49.2 | -56.1 |
| | | |
| ***Oxidative Stress*** | | |
| Heat Shock Protein Beta-1/HSP27 | 9.7 | 8.4 |
| Peroxiredoxin-1 | 6.5 | 6.5 |
| Protein Disulfide-Isomerase | -2.8 | -6.1 |
| | | |
| ***Sertoli Cell Junctional Complexes*** | | |
| Plasminogen Activator Inhibitor | 4.7 | 4.5 |
| 14-3-3 Protein Beta/Alpha | 20.0 | 18.9 |
| Vimentin | 5.6 | 5.3 |
| Transgelin | 24.0 | 14.7 |
| Coactosin-Like Protein | -11.5 | -20.1 |
| Pyruvate Kinase PMK | -3.1 | 8.4 |
| Phosphoglycerate Mutase 1 | -8.4 | -11.0 |

**Table 1**: Ratios of Protein Secretions Between Control Cells and Cells Treated with 20 and 30 Micromolar Ginkgolic Acid

The larger the difference in protein secretion between the two treatments, the larger the number recorded in the data. Proteins which did not exhibit a change in their secretions when sumoylation was enacted as normal and when sumoylation was inhibited were not reported. Changes in protein expression are displayed on **Table 1** with positive numbers indicating an increase in protein secretion when sumoylation was inhibited and negative numbers indicating a decrease in protein secretion when sumoylation was inhibited. The proteins were tabulated into three large groups based on the processes the proteins primarily function in: insulin signaling, oxidative stress, and Sertoli cell junctional complexes.

Insulin, while commonly thought of in relation to diabetes, is an essential hormone in the human body. Insulin is synthesized and secreted by the pancreas and aids in the body's use of

sugar for energy. Glucose from the bloodstream must make its way into the cell in order to allow for the cell to participate in normal cellular activity and reactions (Cleveland Clinic, 2023). However, insulin does not work alone. Insulin-like growth factors are small single-chain polypeptides that are necessary in the signaling pathways responsible for the control of growth, metabolism, and reproductive functions. The insulin/insulin-like growth factor system plays a crucial role in the regulation of cell growth, differentiation, and survival of cells throughout the body, but is specifically important in this discussion in that it directly affects the development and function of the testes. The relationship between insulin and insulin-like growth factors is of such importance to the regulation of the reproductive process that males who lack insulin-like growth factor 1 (IGF1) in its entirety are infertile and exhibit an 80% reduction in spermatogenesis and serum testosterone levels (Lewitt *et al*., 2019). Insulin and insulin-like growth factors play a critical role in male reproduction. It is therefore logical that the proteins involved in helping insulin and insulin-like growth factors also play a role in spermatogenesis and male fertility.

Oxidative stress is a cellular imbalance between an accumulation of reactive oxygen species, or free radicals, and the ability of the cell to detoxify these harmful molecules. Reactive oxygen species play important roles in cell signaling pathways and are produced by the cell as by-products of oxygen metabolism. Free radicals also aid in synthesizing cellular structures and fighting pathogens in the cell. Over production of reactive oxygen products, however, are harmful and can lead to the imbalance known as oxidative stress, causing cell and tissue damage (Pizzino *et al*., 2017). Oxidative stress has been proven to be a participating factor in numerous diseases such as cancers, chronic obstructive pulmonary disease, Alzheimer disease, and more (Forman *et al.,* 2021). Oxidative stress has also been found as a mediator in of male infertility by sperm dysfunction. As with cells all over the body, small amounts of reactive oxygen species are required

for normal cell function, but too many free radicals negatively impact both the quality of and fertilization ability of sperm cells (Agarwal *et al.,* 2014).

Sertoli cells are also responsible for facilitating tight junctions and adhesion junctions between two Sertoli cells and Sertoli cells and germ cells, respectively (Kopera *et al*., 2010). These relationships are important for Sertoli cells to nurse the germ cells through maturation. When the Sertoli cell is affected by the inhibition of the sumoylation, it is possible that the adhesion junctions and tight junctions are being affected or broken down, causing the proteins inside those junctions to be released.

All 11 proteins identified as being regulated by sumoylation should be studied further. However, our attention was drawn to two specific proteins: Insulin-Like Growth Factor Binding Protein 7 and Protein 14-3-3 Beta/Alpha. These two proteins documented the greatest difference in secretions, a change of 50-fold and a change of 20-fold, respectively. These proteins, and/or members of their broader protein family, have already established functions suggested in spermatogenesis and Sertoli cells.

### B. Insulin-Like Growth Factor Binding Protein 7

The hormone insulin works in tandem with insulin-like growth factors I and II to provide cells with essential signals for growth, metabolism, and reproductive functions. Insulin is specifically expressed in beta pancreatic cells, however insulin-like growth factors I and II are produced by almost every cell. The commonality of these peptides highlights their importance in the homeostasis and proper functioning of the human body. Insulin-like growth factors are found in serum, and the quick degeneration of these peptides is curbed by high-affinity binding proteins

which serve, among other things, to increase the half-life of the growth factors (Lewitt *et al.,* 2019). The binding proteins of insulin-like growth factors also serve to prevent the overstimulation of cell growth, prevent unnecessary programmed cell death, and regulate the transport of and interaction between insulin-like growth factors and their receptors. Insulin-like growth factor I has proven to have continuous function throughout the development of humans, past their embryonic state and into the maturation of the body, unlike its counterpart insulin-like growth factor II. Insulin-like growth factor I plays a significant role in sustaining the development of men, specifically relating to reproductive functions. Past studies have suggested that cell localized deficiencies in insulin-like growth factor I and/or insulin-like growth factor I receptor production exhibit abnormal cell development (Griffeth *et al.,* 2014).

To determine the presence of insulin-like growth factor I in human testes, an immunostaining protocol was performed by Dr. Richard Griffith (Griffeth *et al.,* 2014). Immunostaining, the means by which specific proteins in tissues may be identified through various staining and antibody binding (Idleburg *et al.,* 2020), established the frequency of insulin-like growth factor I expressed in Sertoli cells. A decreased population of the peptide was found in spermatocytes. Alternatively, insulin-like growth factor I receptors were highly expressed in secondary spermatocytes and early spermatid, with a decreased presence in Sertoli cells. A similar study found that male mice completely lacking insulin-like growth factor I were infertile due to a severe decline in spermatogenesis and testosterone production (Griffeth *et al.,* 2014). Importantly, after inactivating insulin receptors and insulin-like growth factor I receptors in Sertoli cells, sperm production dropped by a staggering 79% while the testes size shrunk a similarly impressive 72%. These results were attributed to the reduced proliferation of immature Sertoli cells (Griffeth *et al.,* 2014).

When analyzing the results of the secretome performed in Dr. Vigodner's lab, there was a notable reduction in the secretion of Insulin-Like Growth Factor Binding Protein 7 when sumoylation was inhibited. This specific binding protein in a part of the insulin-like growth factor binding protein family. Binding proteins 1 through 6 are relatively more well known, and they function as a high-affinity binding agent to insulin-like growth factors, aiding in the regulation and availability of insulin-like growth factor I and II. It has been suggested that binding protein 7 has a slightly lower affinity for insulin-like growth factors, but its function may be of a similar nature (U.S. National Library of Medicine, 2023). Although the exact role of Insulin-Like Growth Factor Binding Protein 7 in Sertoli cells is unknown, given that the deactivation of insulin-like growth factor I and its receptors result in detrimental effects on male fertility, such large changes in protein secretions of Insulin-Like Growth Factor Binding Protein 7 makes the protein a strong candidate to study further to understand its function in spermatogenesis and Sertoli cells.

### C. 14-3-3 Protein Beta/Alpha

The many functions of Sertoli cells render them essential in the proper development of sperm cells (Johnson *et al.,* 2007). Sertoli cells have been given the nickname of nurse cells due to the nutrients and signals passed between the Sertoli cells and the maturing germ cells. The communication between Sertoli cells and sperm cells as well as between Sertoli cells and other Sertoli cells are reliant upon a complex system of junctions. Sertoli cells are interconnected with germ cells by testis-unique intermediate filament and actin-based cell junctions. The areas in which germ cells reside in the space between Sertoli cells are known as junctional complexes (Kopera *et al.,* 2010). The developing germ cells remain in the seminiferous epithelium for a

majority of their differentiation, and desmosome-like junction and apical ectoplasmic specialization are responsible for developing sperm remaining anchored in place for the necessary period (Pelletier *et al*., 1983).

Cell-cell communication is reliant upon cell-cell adhesions. The adhesions that allow cells to be held together are also essential in the regulation, development, and maintenance of tissues throughout the human body. Cell adhesions allow for normal mechanical interactions between cells, which can influence and control cell behavior and function. Adherens junctions are important to the initiation of cellular adhesions and are essential to their continuation. The regulation of adherens junctions has been linked to numerous proteins, which maintain the junction's connections and allow for proper cell communication and function (Hartsock *et al.,* 2008).

The anchoring of developing sperm to Sertoli cells is similarly regulated by many proteins. Apical ectoplasmic specialization are testes specific adherens junctions, working to optimize the function of Sertoli cells in their protein secretions, cellular communication, and the maintenance of the blood-testis barrier. Apical ectoplasmic specialization aides in the correct positioning of the developing spermatids, ensuring that the head of the sperm is facing toward the basement membrane (Wan *et al.,* 2013).

It is known that many proteins are involved in the maintenance of cellular junctions and adhesions. Importantly, a recent study highlighted the importance of a class of proteins in the role of Sertoli cells. 14-3-3 references a family of proteins, comprising at least 7 isoforms. The 14-3-3 proteins are highly acidic and always found in all mammal cell tissues. This class of proteins plays a key role in the transport of proteins as well as aiding in either the maintenance or the degradation of junctions (Shengyi *et al*., 2009). A recent study found that certain isoforms of the

14-3-3 proteins were highly expressed in the testes and brain. Protein 14-3-3 Theta was detected in the entirety of the seminiferous epithelium, with the greatest population existing at the interface between the developing sperm and the Sertoli cells, which is the apical ectoplasmic specialization junction area (Wong *et al.,* 2009). The high presence of 14-3-3 proteins in these junctions, while not yet understanding their exact role, suggests they may be essential for the normal development of and communication between Sertoli cells and germ cells.

The secretion of Protein 14-3-3 Beta/Alpha in Sertoli cells increased significantly upon the inhibition of sumoylation. Given that the inhibition of sumoylation causes oxidative stress and apoptosis of Sertoli cells (Sengupta *et al*., 2021), it is possible that the increased presence of Protein 14-3-3 Beta/Alpha in the supernatant is indicative of the destruction of the apical ectoplasmic specialization and adherens junctions. This could be suggestive of a key role of protein 14-3-3 Beta/Alpha in the communication and the maintenance of the junctions between Sertoli cells and developing germ cells. Protein 14-3-3 Beta/Alpha may play an essential role in the process of spermatogenesis and its exact functions should be explored further.

## V. Conclusion

Proteins secreted from Sertoli cells upon inhibition of sumoylation were compared to those secreted from the control Sertoli cells. Sumoylation was inhibited by treatment of the cells with Ginkgolic Acid, and the resulting changes were analyzed successfully using two-dimensional gel electrophoresis. The results of the secretome analysis identified differential secretion of 11 proteins. All the identified proteins should be studied further; however, I suggest starting the studies with Insulin-Like Growth Factor Binding Protein 7 and Protein 14-3-3 Beta/Alpha. These

two proteins have shown the highest fold-changes and were previously implicated in signaling and regulation of junctional complexes. Insulin-Like Growth Factor Binding Protein 7 and Protein 14-3-3 Beta/Alpha should be researched for their role in Sertoli cells, spermatogenesis, and overall male infertility.

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