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The Sertoli Cell-Spermatid Junctional Complex: A Potential Avenue For Male Contraception

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by

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DISCLOSURE

Results from some of the experiments required the use of color in the technique to analyze the data. If color cannot be seen in this version of the dissertation, the color copy can be found at the Shimberg Health Sciences Library, 12901 Bruce B. Downs Blvd., Tampa, Florida 33612-4479, (813) 974-2243.

DEDICATION

Wir haben soviel Ungeklärtes auf dieser Welt, und damit dieses so bleibt, haben wir die Wissenschaft. - Otto Waalkes

The important thing in science is not so much to obtain new facts as to discover new ways of thinking about them. - William Lawrence Bragg

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ABSTRACT

The Sertoli cell ectoplasmic specialization is a specialized domain of the calcium-dependent Sertoli-spermatid adherens junction. Structurally abnormal or absent Sertoli ectoplasmic specializations are associated with spermatid sloughing and subsequent oligospermia in conditions associated with reduced fertility potential, although the junctional strength between these cells is not known. Adjudin is a potential male contraceptive agent thought to interrupt testicular binding dynamics of adherens junctions, resulting in controlled spermatid sloughing.

It was hypothesized that the mechanism of action of Adjudin, pertinent to its putative contraceptive effect, is the disruption of the Sertoli cell-spermatid junction. This was tested *in vitro* using primary isolates of germ cells and both primary and immortal Sertoli cells.

This dissertation presents the examination of Sertoli-germ cell interactions in three parts, which address the overall aims of this dissertation project: (1) measurement of the junctional strength between Sertoli cells and spermatids *in vitro*, (2) determination of the efficacy of sk Sertoli cell lines in Sertoli-germ cell binding studies *in vitro*, and (3) assessment of Adjudin as a potential male contraceptive, by measuring the junctional binding strength between Sertoli cells and spermatids exposed to this chemical *in vitro*.

For the first time, the strength of the Sertoli-spermatid junction has been measured, using a micropipette pressure transducing system (MPTS). Results reported in this dissertation demonstrate that the junctional strength between Sertoli cells and germ cells can be measured *in vitro*, support long held speculations regarding Sertoli-spermatid junctional interactions, and provide a technology to test proposed mechanisms of junctional binding dynamics between cells of the seminiferous epithelium (Chapter 2). Although the sk cell lines initially expressed mRNA for the FSH receptor, coculture results determined that these cell lines have limited value for investigating Sertoli-germ cell binding dynamics *in vitro* (Chapter 3). By utilizing the MPTS and primary cell isolates, Adjudin was determined to reduce the junctional strength between Sertoli cells and step-8 spermatids. In conclusion, results support the use of Adjudin as a potential reversible male contraceptive agent by a mechanism which alters the adhesion properties between the step-8 spermatid and the Sertoli cell (Chapter 4).

CHAPTER 1

Background

Spermatogenesis

The complicated process of spermatogenesis occurs throughout the reproductive life of the male. It is a remarkable process requiring many cellular, molecular, and biochemical events, in which diploid spermatogonia undergo division, chromosome reduction, and extensive morphological differentiation to result in haploid elongated spermatozoa [1, 2]. This process occurs in the seminiferous tubules of the testis, within the seminiferous epithelium – the cellular "lining" of the seminiferous tubule – which consists of the developing germ cells and the fixed population of Sertoli cells. The seminiferous epithelium is divided into two compartments – the basal and adluminal compartments – by the blood-testis barrier (see Sertoli Cells below). It is here that the male contribution to the perpetuation of all mammalian species begins.

Spermatogenesis can be divided into three stages, producing upward of 150 x 10^6 spermatozoa per day per man [3]. In stage one, the stem cell-like spermatogonia residing in the basal compartment either self-proliferate or undergo mitosis to give rise to spermatocytes. After entering meiosis and reaching the preleptotene/leptotene stage, these spermatocytes must migrate through the blood-testis barrier to the adluminal compartment. Stage two occurs as these spermatocytes continue through meiosis to result in round spermatids, which then enter stage three, a process identified as spermiogenesis, wherein no cell division occurs, only morphological cell differentiation. The goal of spermiogenesis is to turn round spermatids into elongated spermatids with condensed nuclear material (i.e., spermatozoa), which are then released into the lumen of the seminiferous tubule at spermiation. To reach this goal, many temporal and spatial

mechanisms occur. These include the crossing of the germ cells through the seminiferous epithelium, signaling events, and other interactions between the Sertoli cells and the germ cells during this epithelial translocation, collectively referred to as the "guiding hand" in the formation and disassembly of the Sertoli-germ cell junctions, However, there is limited understanding of the mechanisms involved in spermatogenesis.

At any given point in time, several generations of germ cells develop concurrently in the seminiferous tubule of the mammalian testis [4]. The seminiferous epithelium has been subdivided into various spermatogenic stages, in which new and old generations of germ cells are undergoing the spermatogenic process in close association with the Sertoli cells [4]. The seminiferous epithelial cycle is, therefore, made up of various stages in which new generations of germ cells are connected to older generations. Their development is coordinated via the presence of fixed cellular associations [4, 5]. A seminiferous epithelial cycle in the rat consists of 14 stages, identified by the unique morphology of the developing germ cells [5-7], and extends over 12 – 14 days. It takes a total of ~4.5 epithelial cycles, i.e., ~58 days, for a single spermatogonium to differentiate into 256 spermatozoa. At each stage, at least four different germ cells are present in the seminiferous epithelium. In the human, this cycle has six stages and extends over 16 days with 70 – 74 days required for completion of spermatogenesis [8, 9].

Hormonal control of spermatogenesis

The hormonal control of spermatogenesis begins before birth and continues through puberty and adulthood. This complex interplay within the hypothalmo-pituitary-testicular axis is driven by hypothalamic gonadotrophin-releasing hormone (GnRH), which induces gonadotrophin [follicle stimulating hormone (FSH) and luteinizing

hormone (LH)] secretion from the pituitary. Sertoli cells possess the FSH-receptor [10], whereas Leydig cells primarily express the LH receptor, though LH receptor staining has also been seen in spermatogenic cells [11]. In order to achieve full spermatogenic potential, both FSH and androgens are required.

LH stimulates testosterone secretion by the Leydig cells in the testis, which promotes spermatogenesis. FSH acts on the Sertoli cell, and while FSH has a key role in the development of the testis, a controversy over whether or not FSH is needed for adult spermatogenesis exists. Control of gonadotrophin release involves negative feedback of testosterone on LH and FSH, as well as inhibin B on FSH. Whereas an increase in testosterone reduces the amount of LH and FSH released from the pituitary, a decrease in inhibin B increases the amount of FSH released [12]

Four main steps comprise the effects of hormones on spermatogenesis: (1) the proliferation and differentiation of spermatogonia, (2) the development of spermatocytes, (3) spermiogenesis, and (4) spermiation. Strong evidence exists for the regulation of spermatogonial development by FSH through the prevention of apoptosis [13]. Moreover, testosterone requires the presence of FSH in order to influence the development of spermatocytes [14]. The prevention of apoptosis by exposure to FSH is also important in normal spermatocyte development in the rat [15], whereas in the human, spermatocytes still appear to enter meiosis upon withdrawal of FSH [16]. Acute withdrawal of FSH induces the apoptosis of round spermatids in the rat [13, 15, 17], and a chronic lack of testosterone causes spermatid sloughing, identified as a loss of adhesion between Sertoli cells and round spermatids [18]. However, in the human, no evidence exists to indicate that FSH is necessary for fertility, in that no large decrease in the number of spermiogenic cells is seen [16], and the number of round spermatids seen in the ejaculate (i.e., spermatid sloughing) of FSH-deficient men is relatively low,

therefore not accounting for the profound fall in sperm count [19]. Perhaps more important in the human is the retention of elongated spermatids in the seminiferous epithelium upon withdrawal of FSH [16, 20], as is also seen to some degree in the rat (reduction of spermiation by 15%) [21].

Hormonal disruption of spermatogenesis

In the adult, withdrawal of gonadotrophins stops sperm production, in the human halting at the spermatogonial stage [16], and in the rat at the primary spermatocyte stage [22]. It is clear that the key regulators of spermatogenesis and junctional dynamics in the seminiferous epithelium are FSH and testosterone [23-25]. Cameron and Muffly [26] showed maximal binding of round spermatids to Sertoli cells *in vitro* in the presence of FSH and testosterone. It appears that FSH regulates the Sertoli cell cytoskeleton associated with the Sertoli-germ cell junctions [27], while testosterone implements the adhesion process of round spermatids at these junctions [18]. It is proposed that testosterone also promotes and maintains the maturation of round spermatids in the rat [22]. The withdrawal of testosterone has been shown to result in the detachment of round spermatids (i.e. spermatid sloughing) between stages VII and VIII [18], the time when the Sertoli-spermatid junctional complex forms [28]. When both FSH and testosterone are withdrawn, spermatogenesis is severely affected [21, 29], which includes disruption of the Sertoli cell junctional dynamics within the seminiferous epithelium. A more detailed description of the roles of FSH, androgens, and estrogens on spermatogenesis is presented in Appendix 1.

Sertoli cells

Without the support of Sertoli cells, spermatogenesis could not be completed. The extensive functions of these cells include (1) providing structural support for the germ cells, (2) aiding in the translocation of the germ cells, (3) secreting many trophic

factors and nutrients for the germ cells, (4) phagocytosing dead/damaged germ cells, (5) forming the blood testis barrier (in part to define the polarity of the seminiferous epithelium and in part to create immunity), and other essential functions in spermatogenesis [30-35].

The Sertoli cell was discovered in 1865 by Enrico Sertoli and is also known as the “nurse cell” of the seminiferous epithelium. It is a tall, columnar, polar cell extending from the base to the lumen of the seminiferous tubule, with many cytoplasmic crypt-like extensions due to the reshaping of the cell by germ cells [28, 35-37]. They occupy approximately 17 – 19% of the volume in the seminiferous epithelium of the adult rat [38, 39]. Each Sertoli cell nurtures and protects anywhere from 30 to 50 germ cells [39, 40] at various stages of development. Around postnatal day 20 in the rat, Sertoli cells cease proliferation, and this crucial fixed number determines the amount of germ cells that will normally develop in spermatogenesis.

Some authors classify Sertoli cells into two categories: type A and type B [35-37]. Type A Sertoli cells are classified by the presence of mature spermatids wedged deep within Sertoli cell cytoplasmic crypts, whereas type B Sertoli cells are classified as having none or few, barely visible cytoplasmic crypts. In this context, type A Sertoli cells are thought to transform themselves into type B Sertoli cells in the course of a seminiferous epithelium cycle. These changes are understood to be necessary in order to support the developing germ cells and their movement through the seminiferous epithelium.

Sertoli cell cytoskeleton

Sertoli cells support developing germ cells physically by depositing extracellular matrix and by forming specialized cell junctions between themselves and germ cells in various stages of differentiation. The well-developed cytoskeleton of the Sertoli cell is

essential in maintaining the collective organization of the seminiferous epithelium [7, 28, 36, 41], in part, by (1) maintaining the shape of the cell; (2) stabilizing the cell membrane at sites of contact; (3) positioning, securing, and aiding in the movement of germ cells; (4) arranging and transporting organelles within the cell; and (5) participating in the release of mature spermatids from the seminiferous epithelium. Three major components – actin, intermediate filaments, and microtubules – make up the cytoskeleton and exhibit unique distribution patterns in each stage of the seminiferous epithelial cycle [35, 41, 42].

Actin plays a very important role in maintaining Sertoli cell structure and in providing the cell with properties of cell contractility. It also is thought to participate in motility-related processes, since it is found at sites of cell contact at the periphery of the Sertoli cell. Actin filaments are chief components of ectoplasmic specializations (see Ectoplasmic Specialization below) and tubulobulbar complexes, which exist between the Sertoli cell and germ cells in various stages of differentiation.

Intermediate filaments are found at the desmosome-like junctions between Sertoli cells and between Sertoli cells and spermatocytes/round spermatids [43]. The precise role of intermediate filaments in the Sertoli cell is not yet known, but their distribution indicates they play a part in retaining the integrity of the seminiferous epithelium [44, 45].

The role of microtubules in the Sertoli cell is much clearer than that of intermediate filaments. They participate in (1) the preservation of the cell's columnar shape; (2) the translocation of spermatids in the seminiferous epithelium; (3) the movement of intracellular organelles; and (4) the alteration of the cell membrane to the nearby spermatid heads [37, 41]. Microtubules are found at the ectoplasmic specialization [46, 47] and the most remarkable changes in the organization of

microtubules occurs as mature spermatids associate with Sertoli cell ectoplasmic specializations [48].

While these cytoskeletal components have been associated with the events of germ cell movement, the precise mechanisms underlying the regulation of the cytoskeleton during the different epithelial stages and in the movement of germ cells remains a mystery.

Blood-testis barrier

The blood-testis barrier, formed by a unique junctional complex between adjacent Sertoli cells, divides the seminiferous epithelium into a basal and an adluminal compartment [review [49]]. It serves as an immunological barrier for the highly antigenic adluminal compartment germ cells and in doing so creates a specialized adluminal environment for these germ cells. This is accomplished, in part, by controlling the passage of molecules between the two epithelial compartments [review [49]]. This dynamic barrier is comparable in strength to the blood-brain barrier. However, the blood-testis barrier must periodically "open" to allow the passage of germ cells from the basal to the adluminal compartment of the seminiferous epithelium, requiring disassembly and assembly of cell-cell junctions by mechanisms which are still unclear. The mechanisms for the formation and regulation of the blood-testis barrier are also unknown, though gonadotrophins have been suggested to play a role, as have cytokines and growth factors [50, 51].

Two important findings led to the development of the concept of the blood-testis barrier: (1) the radical differences in the composition of fluids and proteins obtained from the rete testis and seminiferous tubule lumen when compared to those of the testicular lymph and blood plasma [52] and (2) the variations in the rate in which radioactive tracers and dyes passed from the blood plasma to testicular fluids [53-59]. However,

concrete evidence for this barrier did not exist until the studies of Dym and Fawcett [60], who established its unique ultrastructure and its ability to occlude intercellular tracers, such as lanthanum.

The specialized environment created by the blood-testis barrier is crucial to germ cell development. Sertoli cells produce, secrete, and efficiently distribute products into the adluminal compartment, created by the blood-testis barrier, essential for growth and differentiation of the adluminal germ cells [32, 61-65]. Not only do these tight junctions limit the movement of nutrients and wastes into and out of the adluminal compartment, they deny adluminal access to immunoglobulins and lymphocytes [66], thereby creating a unique sequestered space for spermatids [67]. Since foreign antigens reside on the surface of these haploid germ cells, without the blood-testis barrier the body's immune system would recognize them and reject them.

Recently, the blood-testis barrier and the Sertoli cell tight junctions have been divided into two finer physiological distinctions – the occluding zonule and the occluding macule [68] – based on the actuality that *in vivo*, the assembly of the blood-testis barrier and the Sertoli cell tight junctions are not synchronous events [69-71]. The occluding zonule divides the seminiferous epithelium into its two compartments and is the basis of the blood-testis barrier. Formed at 15 – 18 days of age in the rat, the fibers composing this zonule are continuous and form an impervious barrier [72, 73]. In contrast, the occluding macule is a focal seal through which interstitial fluids or tracers can easily pass. The fibrils composing the macule are discontinuous and situated immediately above and below the occluding zonule [69-71].

Sertoli cell-germ cell junctions

There are two types of intercellular junctions between germ cells and Sertoli cells: (1) anchoring junctions and (2) gap communicating junctions. Both types are

believed to play crucial roles in spermatogenesis. Up- and down-regulation of these junctions occurs during spermatogenesis and the process of germ cell migration from the basal to the adluminal epithelial compartments [74]. However, the regulation of these junctions and their role in the completion of spermatogenesis is not yet fully understood.

Anchoring junctions

Four types of anchoring junctions exist between Sertoli and germ cells: (1) cell-cell actin based adherens junctions; (2) cell-matrix actin-based focal contacts; (3) cell-cell intermediate filament-based desmosomes; and (4) cell-matrix intermediate filament hemidesmosomes. All anchoring junctions link the cytoskeleton of one cell to another cell or to the extracellular matrix, in order to maintain tissue integrity [75], however, each of these is biochemically and structurally different from one another. Recent reports have suggested that these junctions also play a role in signal transduction [76-78].

Adherens junctions. Four protein complexes are identified with the actin based adhesion of adherens junctions: (1) cadherin-catenin; (2) nectin-afadin-ponsin; (3) integrin-laminin; and (4) vezatin-myosin [79] (Figure 1.1). Of these, three (1-3) have been found in the testis [79]. Two modified forms of the adherens junction are found in the testis – the ectoplasmic specialization and the tubulobulbar complex, which will be discussed later.

Cadherin-catenin complex. The best studied adherens junction proteins in the testis have been the cadherins. These 115 – 140 kDa transmembrane proteins consist of two cytoplasmic domains, one transmembrane domain, and five calcium-binding domains (EC1 – 5, with the most conserved region being a His-Ala-Val cell adhesion

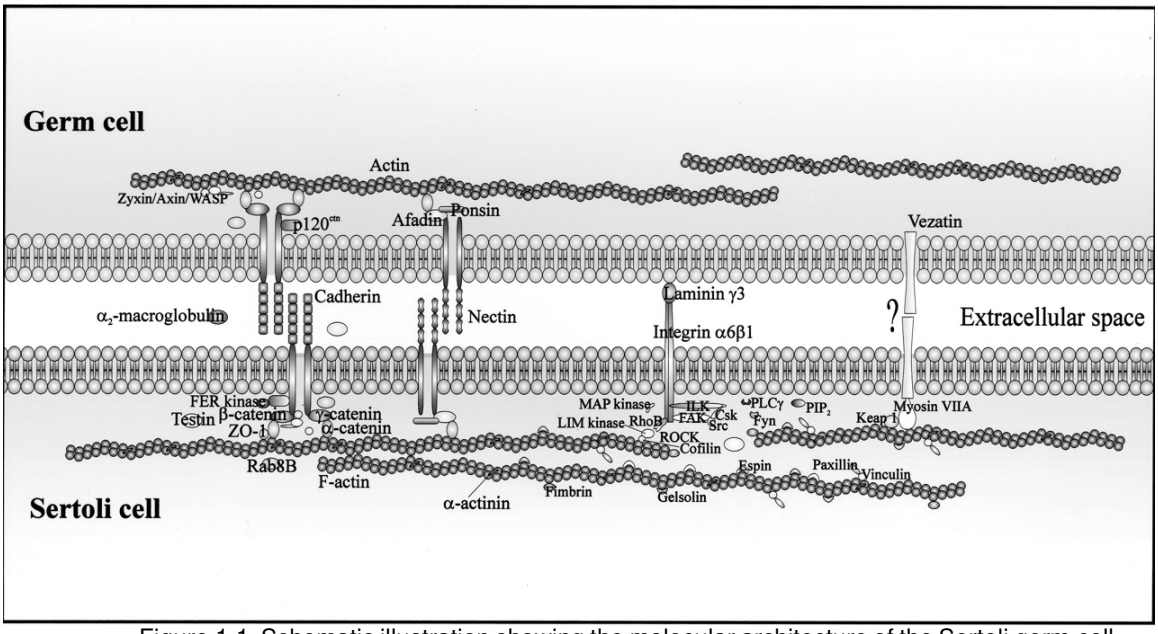

Figure 1.1. Schematic illustration showing the molecular architecture of the Sertoli-germ cell adherens junction [68].

recognition sequence [80] found within the EC1 domain) and bind homotypically with cadherins in adjacent cells in a calcium dependent manner [81-85]. The cytoplasmic domain binds to intracellular proteins, which is important in clustering cadherins to form functional anchoring junctions and in transmitting information from the cell surface to the nucleus to activate genes that maintain the integrity of the epithelium [81-84, 86].

The binding of the cytoplasmic domain of cadherins to either ß- or ?-catenin (plakoglobin) is crucial to its function [85-88]. This cadherin-catenin complex is linked indirectly to the actin cytoskeleton by a-catenin [89]. ß-catenin is also stimulated by the *Wnt* signal transduction pathway as a transcriptional cofactor [90-92]. Colocalization of ß-catenin with ezrin, which links the cell membrane and the actin cytoskeleton [93], and TGF-ß type II receptor [94], suggests a link between TGF-ß1 and adherens junction dynamics [94]. Another catenin, $p120^{ctn}$ has been identified as a putative Src-substrate critical to cadherin-mediated cell adhesion. One study indicates that $p120^{ctn}$ induces the clustering of E-cadherin [95], and in response to growth factors, such as EGF, this

catenin is phosphorylated on its tyrosine and serine residues, thereby entering the nucleus to interact with Kaiso, a newly identified transcription factor [96]. Though the interaction between E-cadherin and ß-catenin is required to begin the cascade of events leading to cell adhesion, it is the interaction between E-cadherin and $p120^{ctn}$ that is necessary for the formation of stable adherens junctions [97]. Changes in the phosphorylation of $p120^{ctn}$ also affect cell adhesion [98]. Tighter cell adhesion is associated with a decrease in phosphorylation and loss of adhesion is associated with an increase in phosphorylation [98, 99].

The regulation of cadherin-catenin may be related to the GTPases. RhoA, Rac1, and Cdc 42, all members of the GTPase family, have been shown to localize with the cadherin-catenin complex [100, 101]. Also, the over expression of $p120^{ctn}$ is thought to elicit an increase in cell motility via Rho GTPases [102, 103]. During junction assembly, E-cadherin and ß-catenin have been shown to be internalized and then recycled back to the cell membrane via Rab5 [104, 105], a member of the GTPase family. Tyrosine phosphorylation of ß- and/or ?-catenin has been shown to result in the loss of cadherin-catenin mediated cell adhesion [106-108].

Though the presence of adherens junctions in the testis have been demonstrated [28, 49, 72], their structure and function are poorly understood. The actin based cell-cell adherens junction between the Sertoli cell and the germ cell in the mammalian testis is important not only in mechanical adhesion of the cells, but also in the morphogenesis and differentiation of the germ cells. Turnover of these calcium dependent junctions occurs during the process of germ cell migration from the basal to the adluminal epithelial compartments [74]. However, their role in completion of spermatogenesis is not yet fully understood.

Both Sertoli cells and germ cells have cadherin/catenin complexes as identified by immunohistochemical analysis of related intracellular molecules such as N-cadherin, E-cadherin, a-catenin, ß-catenin, ?-catenin, and $p120^{ctn}$ [47, 109-113]. The N-cadherin/catenin complex has been shown to regulate cell adhesive function between Sertoli cells and germ cells [114]. The presence of cadherins and catenins has been identified in isolated Sertoli and germ cells via immunoprecipitation and immunoblotting. N-cadherin has been detected by Western blot analysis in the plasma membranes of both Sertoli cells and spermatogenic cells [114]. Lee et al [113] have detected N- and E-cadherin in Sertoli and germ cells via semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) and immunoblotting. During the assembly of adherens junctions between Sertoli cells and germ cells, Lee et al [113] observed a transient induction in the steady-state mRNA and protein levels of cadherins and catenins, indicating that the cadherin/catenin complex may be a functional unit of the Sertoli-spermatid junctional complex. Isolated germ cells have been shown to possess E-cadherin and ß-catenin in an almost equimolar ratio of 1:1 to that which occurs in Sertoli cells [113]. This suggests that both Sertoli and germ cells contain the cadherin/catenin complex, thereby interacting with each other in a homotypic fashion. However, it has not yet been localized directly to the ectoplasmic specialization.

Studies using cross-linking and immunoprecipitation in isolated seminiferous tubules and in Sertoli-germ cell cocultures show the N-cadherin-catenin complex attaching to the actin cytoskeleton [113]. This finding was in direct contrast to that of two previous studies, in which this complex was found to be associated with intermediate filaments [114, 115]. It is also possible that an intermediate filament based cadherin-catenin complex does exist, e.g., at the intermediate filament based desmosome, and is used in adhering developing germ cells to Sertoli cells. However, a thorough study of

desmosome proteins has not yet been conducted in the testis, although the results of such a study would be useful in order to delineate the composition of desmosome-like junctions in the testis.

At least 25 different cadherins have been identified in the testis by RT-PCR [114]. Each cadherin demonstrates a stage-specific staining pattern. For example, N-cadherin is seen to be localized at: (a) the basal inter-Sertoli junctions, (b) Sertoli-spermatocyte junctions, and (c) Sertoli-elongated spermatid junctions at spermatogenic stages I through VII [47]. N-cadherin immunostaining has been observed in and/or around the heads of elongated spermatids in spermatogenic stage VIII that was less intense following spermiation [110]. However, in these studies of N-cadherin, specific localization was not clear, and its localization to the ectoplasmic specialization could not be substantiated by subsequent studies in this lab or others. Abnormally retained spermatids at spermatogenic stage IX also have shown positive immunostaining for N-cadherin around the heads of elongated spermatids [47], whereas in a recent study by Beardsley and O'Donnell [116], abnormally retained spermatids did not show N-cadherin immunostaining in or around the heads, but it was present in the remaining residual bodies. However, Mulholland et al [115] and Johnson and Boekelheide [117] have not observed cadherin staining in relation to ectoplasmic specializations and deny the idea that N-cadherin, or any cadherins, are present at the ectoplasmic specialization. In these studies, N-cadherin has been found to be at the site of spermatocyte adhesion to the Sertoli cell. Coimmunoprecipitation and chemical cross-linking experiments by Lee et al [113] have shown that Sertoli cell N-cadherin interacts with actin and vimentin. Unfortunately, *in vivo* studies of such proteins are near impossible because mutations in N- or E-cadherin are lethal in the early stages of development [118-120].

N-cadherins are located in the Sertoli-germ cell junctional complex, and immunoneutralization of N-cadherin results in a reduction in spermatid adhesion [121]. Perryman et al [121] and Lampa et al [122] have shown that follicle stimulating hormone (FSH) and testosterone control N-cadherin expression and spermatid binding. Moreover, upregulation of N-cadherin mRNA has also been seen in isolated Sertoli cells when exposed to FSH and estrogen [123].

Immunohistochemical studies have localized ß-catenin and $p120^{ctn}$ to the luminal edge of spermatogenic stage VII and VIII tubules [47] and $p120^{ctn}$ at Sertoli-Sertoli junctions, as well as at sites of Sertoli cell-elongated spermatid contact [112]. After spermiation, ß-catenin relocalizes to a more basal position around groups of elongated spermatids [47]. N-cadherin antisera coprecipitates ß-catenin [47, 110, 113] and $p120^{ctn}$ [47] before and after spermiation, and ß-catenin antisera coprecipitates N-cadherin [47, 113] and $p120^{ctn}$ [47]. Finally, recent findings have suggested a necessary role of ß-catenin in the activity of N-cadherin related to signal transduction within the Sertoli cell [74, 109].

Nectin-afadin-ponsin complex. The nectin-afadin-ponsin complex has also been reported to be present at adherens junctions between Sertoli cells and round spermatids [124]. The calcium-independent cell adhesion protein nectin consists of one transmembrane domain, one cytoplasmic domain, and three Ig-like extracellular domains [125, 126]. Four nectins have been identified [127, 128], all of which have two or three splice variants (except nectin-4) [124-127, 129-133], and mediate cell adhesion by binding either homo- or heterotypically with another nectin on the adjacent cell membrane [124, 127, 133-135].

In the testis, Northern blots have identified weak expression of nectins-1 and -4, moderate expression of nectin-2, and strong expression of nectin-3 [127, 133]. Nectin-3

is almost exclusively expressed by spermatids [136]. Nectin-2 is found in Sertoli cells, germ cells, and Leydig cells [136]. Nectin-2d is found at adhesion sites between Sertoli cells and elongated spermatids, colocalizing with F-actin at what appears to be the ectoplasmic specialization (see Ectoplasmic Specialization below), with its highest levels at stages VI – VIII of spermatogenesis [137]. Nectin-$2^{-/-}$ mice show abnormal sperm morphology and infertility [137]. Because of these studies, it is possible that the nectin-afadin-ponsin complex may induce cell-cell adhesion in the seminiferous epithelium via a heterotypic nectin-based interaction [136].

Afadin, an F-actin binding protein, connects the cytoplasmic domain of nectin to the actin cytoskeleton [138, 139]. Two splicing variants of this molecule exist: 1-afadin, found ubiquitously in tissues, including cells of the testis, and s-afadin, found exclusively in cells of the brain [138, 139]. Recently it has been demonstrated that the entire binding site for 1-afadin is found on a-catenin [140], thereby giving rise to the idea of cross-talk between the cadherin-catenin protein and the nectin-afadin-ponsin complexes. For the localization of the nectin-afadin-ponsin complex to the adherens junction, a-catenin is needed [141].

Ponsin is a cytoplasmic protein that associates with 1-afadin [142] or vinculin [143-146]. As a result, vinculin may be the protein that links the cadherin-catenin and nectin-afadin-ponsin complexes to each other [142]. Very little is known about the function of ponsin, but its presence in the testis has been shown via Northern blots [142].

Integrin complex. Though the integrins a1 through a6 and ß1 through ß3 have been identified in the testis [115, 147, 148], the most studied of these is the integrin receptor a6ß1, which has been localized to the Sertoli cell-germ cell interface [115, 148-150]. This location is interesting, in that the traditional role of integrins is in cell binding to the extracellular matrix at hemidesmisomes and focal adhesions, interacting with

collagens and laminins [review [151]], not at adherens junctions, joining two cells. Laminin is a known binding partner for a6ß1 integrin [152], and it is the ?3 laminin chain (of laminin 12) that has been localized to the adluminal compartment of the seminiferous epithelium [153], thereby suggesting that laminin ?3 is a putative binding partner for a6ß1 integrin at the Sertoli-spermatid adherens junction. The a6ß1 integrin is also thought to be involved in signaling events in the Sertoli cell via integrin-linked kinase [115].

Integrins in the Sertoli cell convey bidirectional signals, prompting events such as tyrosine phosphorylation [154-156], activating downstream signal transducers, such as Rho GTPase and focal adhesion kinase (FAK), which affect cell adhesion [157, 158]. For example, integrins at the apical Sertoli-spermatid ectoplasmic specialization are partially regulated by the integrin/ROCK (Rho-associated protein kinase)/LIM (Lin-11, Isl-1, and Mec-3 kinase)/cofilin pathway [68, 157]. When cell-cell adhesion in the seminiferous epithelium was chemically perturbed using Adjudin (see Adjudin below), the proteins of this integrin/ROCK/LIM/cofilin pathway were shown to be upregulated, with changes in their phosphorylation status [159, 160]. In addition, when rats were pretreated with a ROCK inhibitor, the effects of Adjudin on the disruption of cell-cell adhesion were delayed [157]. Moreover, Adjudin has been shown to alter the integrin/FAK/phosphatidylinositol 3 (PI 3)-kinase/p130 Cas/ERK signaling pathway [158].

Integrins also play a role in the signaling events associated with extracellular matrix remodeling and cell movement [161], involving cycles of cell adhesion and de-adhesion [162, 163]. Several kinases that are found at the ectoplasmic specialization have been identified in the process of integrin-mediated cell signaling, including FAK [115, 158], Csk [47], integrin-linked kinase (ILK) [115], and Src [47, 158].

Ectoplasmic Specialization. The ectoplasmic specialization is the unique cytoskeletal structure of the Sertoli cell that forms the principal component of the Sertoli-spermatid adherens junction [31, 164, 165]. Abnormal or absent Sertoli ectoplasmic specializations have been associated with a reduction of mature sperm in semen [27, 117, 166] and is thought to a major contributing factor in oligospermia [27, 167]. The morphology of these junctions has been well described, but the molecular composition of them still is not well understood.

Ectoplasmic specializations are found basally in the Sertoli cell near Sertoli-Sertoli tight junctions and between Sertoli cells and round (step-8) and elongating spermatids. They consist of hexagonally packed bundles of actin filaments situated between the plasma membrane and a cistern of endoplasmic reticulum [168]. Ectoplasmic specializations are important in cell-cell adhesion in the seminiferous epithelium and are dynamic structures that remodel during spermatogenesis. The basal ectoplasmic specializations between Sertoli cells assemble and disassemble as germ cells move from the basal to the apical compartments of the seminiferous epithelium [review [7, 28, 150, 164, 168]. Apical ectoplasmic specializations are first seen in the rat at stage VIII of rat spermatogenesis, when the step-8 spermatid appears [164]. It is thought the ectoplasmic specialization forms in the Sertoli cell to strongly anchor the step-8 spermatid to the seminiferous epithelium [28, 164], since desmosome-like junctions no longer exist at this time between the Sertoli cell and the spermatid [28]. However, until now, the actual strength of this junction has not been determined. The ectoplasmic specialization is also thought to play a role in the positioning and the elongation of the spermatids, since throughout spermiogenesis, the ectoplasmic specializations remodel in adaptation to the morphological changes occurring to the spermatid heads in the Sertoli cell crypts [169-171]. The apical ectoplasmic

specialization is present until appropriate release of the step-19 spermatid [164], and inappropriate release of earlier stage spermatids (i.e., spermatid sloughing) is related to abnormal ectoplasmic specialization structure and oligospermia [18, 172].

The best studied protein complex of the apical ectoplasmic specialization is the a6ß1 integrin [115, 147, 148], but a recent study has also suggested the presence of a4ß1 integrin [110]. The proteins existing at the basal ectoplasmic specializations are not as well characterized as those at the apical ectoplasmic specializations. The nectin-afadin-ponsin complex appears to be restricted to the apical ectoplasmic specializations [136], whereas the cadherin-catenin complex is largely found at basal ectoplasmic specializations [110, 113, 114]. However, N-cadherin has also been localized to the Sertoli-elongated spermatid junctions [47, 173], and N-cadherin immunostaining has been observed in and/or around the heads of elongated spermatids at stage VIII of spermatogenesis and was less intense following spermiation [110]. According to Wine et al [47], abnormally retained spermatids at stage IX of spermatogenesis also show positive immunostaining for N-cadherin around the heads of the elongated spermatids, but Beardsley and O'Donnell [116] maintain that abnormally retained spermatids do not show N-cadherin, whereas the remaining residual bodies do. Mulholland et al [115] and Johnson and Boekelheide [117] contend that N-cadherin, or any other cadherins, are not present at the ectoplasmic specialization. However, immunoneutralization of N-cadherin demonstrates a reduction in spermatid adhesion [121]. Immunohistochemistry has also localized ß-catenin and $p120^{ctn}$ to the luminal edge of tubules at stages VII and VIII of spermatogenesis [47, 112]. After spermiation, ß-catenin relocalizes to a more basal position around groups of elongated spermatids [47]. N-cadherin antisera also coprecipitates ß-catenin [47, 110, 113] and $p120^{ctn}$ before and after spermiation [47], while ß-catenin antisera coprecipitates N-cadherin [47, 113] and $p120^{ctn}$ [47]. Nectin-3 is

expressed in germ cells and heterotypically binds to nectin-2 on the Sertoli cell membrane [136]. Nectin-3 is reported to be restricted to sites of Sertoli cell-elongating spermatid interaction in stages VII and VIII of spermatogenesis [137]. Other proteins also are reported to provide functional contributions at the ectoplasmic specialization. These include actin [review [41]], a-actinin [[174], vinculin [175, 176], myosin VIIA [177], gelsolin [178], fimbrin [168], espin [179], paxillin [115], testin [180], integrin-linked kinase (ILK) [115], and Kelch-like neurofilament-E2-related molecule-associating protein 1 (Keap1) [181, 182]. Keap 1 has been reported to be located at the ectoplasmic specialization [182], even though its binding partner, myosin VIIA [177], has not, indicating that Keap1 can associate with other molecules when at the ectoplasmic specialization.

"Free" ectoplasmic specializations are also found within the Sertoli cell [7], though the significance of these remains unknown. They may be part of left over attachment sites of adherens junctions not yet completely recycled.

Espin. Espin is an actin binding protein found in many organs but most abundantly in the testis [179], specifically the Sertoli cells, and shows no resemblance to other actin-binding proteins [183]. Espin in the seminiferous epithelium appears to be concentrated around the heads of spermatids from mid- to late spermiogenesis, as determined by immunoperoxidase immunocytochemistry [172, 179]; it is also seen near the base of the seminiferous tubules [179]. However, stages VI – VII of spermatogenesis demonstrate espin immunofluorescence primarily at the apical ectoplasmic specialization [68, 172, 179]. Sertoli cells surrounding step-8 spermatids, where an organized ectoplasmic specialization is first seen, demonstrate espin immunostaining in a C-shaped cap near the area where the spermatid meets the Sertoli cell [183]. This is not seen around step-7 spermatids [183]. Nearing spermiation, espin

immunostaining near the luminal edge of the seminiferous epithelium decreases and then disappears around the time of sperm release [172, 183]. This change in localization appears to reflect the disassembly of the ectoplasmic specialization [172, 183]. Through immunogold electron microscopy, espin has been localized to the parallel bundles of actin filaments present at the ectoplasmic specialization in Sertoli cells [179]. A smaller isoform of espin, termed “small espin,” has been seen associated with parallel actin bundles found in brush border microvilli in the kidney and intestine [184]. This further supports the hypothesis that espin is involved in the bundling of actin at the ectoplasmic specialization.

Tubulobulbar Complexes. The tubulobulbar complex is another modified adherens junction found between Sertoli cells at the level of the tight junction and between Sertoli cells and elongate spermatids that are ready for release into the tubule lumen [171, 185, 186]. This complex is a pair of tubular structures surrounded by actin, extending from the concave surface of the spermatid head [187]. It is believed tubulobulbar complexes aid in preventing the premature release of elongated spermatids [169], as well as remove cytoplasm from spermatids [185, 188], since in the presence of these complexes, the volume of cytoplasm is reduced by as much as 70% [185]. Interestingly, unique to tubulobulbar complexes, the apical tubulobulbar complexes are not visible until a few days before spermiation, at stage VIII of spermatogenesis. After spermiation, they are quickly internalized and degraded by the Sertoli cell [169, 187]. In contrast, basal tubulobulbar complexes form during stages II – V and are most abundant during stages IV – V of spermatogenesis, while being least abundant at stages VI – VIII [41]. Each mature testicular spermatid can also contain 4 – 24 tubulobulbar complexes, thereby proposing a role in germ cell movement [185, 186]. They also may play a role in

the internalization of junctions during germ cell movement [166, 186, 189], since basal tubulobulbar complexes interact with tight and gap junctions [186].

Focal Contacts. Also known as focal adhesions or adhesion plaques, these actin-based adhering junctions anchor cells to the extracellular matrix and are found in nearly every epithelia. They are largely composed of integrins that connect the actin within the cell to the extracellular matrix. These dynamic structures regulate cell movement and are known to participate in signal transduction [reviews [103, 190-194]. This junction type has not been investigated in any depth in the testis, but proteins found at focal contacts are often also found at the ectoplasmic specialization.

Integrins, belonging to a family of aß heterodimeric transmembrane proteins, provide a physical link between the cell cytoskeleton and the extracellular matrix, as well as providing a mechanism to move signals bidirectionally across the plasma membrane to regulate many cell behaviors [151]. For example, in response to cell adhesion, integrin cytoplasmic-domain-associated protein-1 can be phosphorylated by protein kinase C (PKC), cAMP-/cGMP-dependent kinases, and calcium/calmodulin-dependent protein kinase II [195].

The adaptor protein vinculin is functionally related to a-catenin [196] and is found at focal contacts in several epithelia, as well as cell-cell anchoring junctions. Vinculin is mostly found at the E-cadherin-catenin complex [197]. When vinculin expression was blocked in 3T3 cells, cell adhesion was disrupted and cell motility increased [198], whereas its overexpression decreased movement of the cell [199]. Vinculin, a substrate for tyrosine and serine/threonine protein kinases [200], can substitute for a-catenin in functional adherens junctions [197]. In the testis, this protein is found in both the apical and basal ectoplasmic specializations [175, 176] in the cytoplasm of the Sertoli cell [47, 172].

Another component of the focal contact is talin, which has actin-binding sites, as well as binding sites for focal adhesion kinase [201], phospholipids [202], vinculin [203], TES [204], and layilin [205]. Since talin can bind to the cytoplasmic tails of ß1, ß2, ß3, and ß7 integrins [206-209], talin and integrin together are required for focal adhesion assembly [210]. Cell adhesion and movement are disrupted when talin is blocked in fibroblasts and HeLa cells [211]. This protein is also found in the testis [212].

Desmosomes. Desmosomes are cell-cell anchoring junctions that attach to intermediate filaments. These highly organized spots between cells are situated between two plaques [review [213] which consist of two domains: (1) an extracellular core (desmoglea) and (2) several dense symmetrical cytoplasmic plaques that are parallel to the membrane.

Three protein families make up the desmosome: (1) cadherins (desmocollins and desmogleins), (2) armadillo proteins, and (3) plakins (review [214]). The desmosomal cadherins are connected to the intermediate filaments via proteins such as plakoglobin (?-catenin), desmoplakin, and plakophilin. Plakoglobin is common to both desmosomes and adherens junctions [215]. Other proteins are also thought to play roles, including intermediate filaments and associated proteins (IFAP) 300 [216], desmocalmin [217], periplakin [218], envoplakin [218], plectin [219], and pinin [220]. Though desmosomes have been most extensively studied in the epidermis, their architecture in the testis, except for the presence of plakoglobin [113], remains unknown [review [28, 43].

Hemidesmosomes. The hemidesmosome is found on the basal lamina in the testis, between the Sertoli cell and the basement membrane [221]. Similar to focal adhesions, these junctions connect the cytoskeleton of a cell to the basement membrane, but use intermediate filaments instead of actin. Integrins are the only protein of the hemidesmosome that has been characterized, with ß4 integrin as crucial [review

[222]. In the early 1990s, a monoclonal antibody (1-2B7B) raised against a component of hemidesmisomes reacted with a protein that localized to the basement membrane of the testis [223]. To this day, this protein has yet to be identified.

Gap communicating junctions

Gap communicating junctions allow the exchange of small molecules and ions between cells through intercellular channels formed by the noncovalent interaction of two hemichannels (connexons). Each cell contributes a connexon, and the connexon is usually found clustered with other connexons to form a plaque. These connexons are composed of six integral membrane subunits (connexins) that surround a hydrophilic pore [review [75, 224]. At last 20 connexins have been discovered in mammalian tissues [review [224].

Though the presence of gap communicating junctions between Sertoli cells has been known for several decades [60], it wasn't until 1996 [225] that stage-specific expression of connexins was determined. The best studied connexin, connexin43, is found at Sertoli cell gap junctions, which has the most intense staining detected during stages I – VIII of spermatogenesis, in addition to reported increases in staining intensity appearing during testicular development [226]. Other studies have shown that gap junctions exist between Sertoli cells and pachytene spermatocytes *in vitro* [227, 228]. Connexin33 is also a testis-specific and expressed mainly in germ cells [74]. At least 13 connexins have been identified thus far in the testis [229].

Gap communicating junctions appear to mediate signals between Sertoli cell and germ cells, thereby indicating an important role in the development of spermatids in the seminiferous epithelium.

Although a great deal has been learned about Sertoli cell junctions, especially from studies *in vitro*, these studies have all been accomplished almost entirely by using

primary cell isolates, limiting the practicability of these types of studies. Although several Sertoli cell lines exist, one exhibiting the capability of binding spermatids would greatly expedite these types of studies. A more detailed description of immortalized Sertoli cell lines is presented in Appendix 2.

Male infertility and Adjudin

A number of health-related conditions are associated with reduced fertility potential and oligospermia in men, including varicocele [230], hyperprolactinemia [231], diabetes [232], and idiopathic oligospermia [233]. These conditions all are associated with reduced sperm in the semen, i.e., oligospermia, and ultrastructural pathology unique to the junctional apparatus of the seminiferous epithelium [233]. Cap stage spermatids in the human (step-8 spermatids in the rat) are presumed to be tightly anchored to the seminiferous epithelium at a Sertoli cell adherens junction, which includes the unique Sertoli ectoplasmic specialization [31, 165]. In both *in vitro* and *in vivo* observations of experimental animal models, disruption of this junction resulted in spermatid sloughing and subsequent oligospermia [18, 117, 166, 172].

Due in part to both the lack of comprehension about the process and the complexity of spermatogenesis, the development of a safe, effective, and reversible (oral) contraceptive for men lags far behind that for women. Currently all that is available to men in this form of contraception is the condom or abstinence.

Early attempts to develop a male oral contraceptive pill were based on using testosterone to turn off sperm production via signals from the brain [234], as is seen with the female oral contraceptive. However, this has not been as successful as in the female and several side effects exist. Other methods of hormone delivery, such as the patch, have also not been highly successful.

Lonidamine (1-[2,4-dichlorobenzyl]-indazole-3-carboxylic acid) is an anticancer drug that disrupts the respiratory process of cells with condensed mitochondria, such as cancer cells and spermatids [235, 236]. Lonidamine also causes vacuolation and retraction of apical cytoplasm in the Sertoli cell in the rat, thereby releasing germ cells into the lumen of the seminiferous epithelium [237]. However, at high doses, lonidamine is toxic and irreversible [159]. Therefore, in an effort to produce a safe male contraceptive, Cheng et al [159] developed two analogs to lonidamine – 1-(2,4-dichlorobenzyl)-indazole-3-carbohydrazide (AF-2364, now known as Adjudin) and 1-(2,4-dichlorobenzyl)-indazole-3-acrylic acid (AF-2785), both with the potential to be male contraceptives.

Throughout recent years, it has been discovered that Adjudin is a better candidate for male contraception than AF-2785. Essentially, administration of Adjudin depletes seminiferous tubules of germ cells [159, 160]. Studies have shown that this compound has little or no effect on organs not involved in reproduction [160], in that the metabolic processes of organs such as the liver, kidney and brain are left undisturbed with the administration of Adjudin. By 24 – 48 hours >95% of Adjudin is removed from nearly all organs [238]. The hypothalamic-pituitary-testicular axis also appears to remain unaffected by Adjudin administration, in that negligible changes in serum FSH, LH and testosterone have been observed.

The rapid depletion of round and elongating spermatids, and the consequent ultimate depletion of spermatocytes, occurred in the rat with a weekly treatment of 50 mg Adjudin/kg body weight (bw; via gavage) for five weeks [160]. Twenty-nine days after the initial dose of Adjudin, the fertility efficacy fell to zero and remained at 0% for up to 90 days [160]. At 104 days post-treatment, fertility resumed, with 25% of the rats siring a normal litter size, and after 157 days, the fertility efficacy rose to 75% [160]. Only 25%

of the rats remained infertile at 197 days (the last mating period) [160]. After only six days from the initial dose of Adjudin, many seminiferous tubules were found to be devoid of elongated spermatids, and structural disorganization was seen via prominent spaces within the germinal epithelium [160]. At 40 days after initial treatment, 98% of the seminiferous tubules were devoid of spermatocytes and spermatids [160]. The diameter of the seminiferous epithelium was reduced by 30%, and no apparent changes were seen in the interstitium of the testis [160]. Regeneration of the germinal epithelium began at 100 days post-treatment, and at 130 days post-treatment, normal spermatogenic activity was seen [160]. By 210 days post-treatment, 95% of the seminiferous tubules demonstrated normal morphology [160]. Testicular weight decreased by 70% until day 210 post-treatment [160].

Most vulnerable to Adjudin treatment is the site of the apical ectoplasmic specialization and tubulobulbar complexes [239]. The desmosome-like junctions between Sertoli cells and spermatids and spermatocytes are also affected [157, 158, 239]. In as little as four hours after the administration of Adjudin (50 mg/kg bw, gavage), damage to the adherens junction between the Sertoli cell and spermatid was seen via electron microscopy [173], in that many distinct intercellular spaces existed, leading to spermatid sloughing.

Lui et al [157] have determined that Adjudin initially activates the integrin/cadherin/testin protein complex at the site of the ectoplasmic specialization. Downstream activation by Adjudin then occurs in the integrin/ROCK/LIM kinase/cofilin [157] and integrin/FAK/PI 3-kinase/p130 Cas/MAP kinase pathways [158]. In other epithelia Adjudin has no effect on cell adhesion [157]. These data suggested that Adjudin works at the level of the ectoplasmic specialization. A recent study confirmed this idea, in that it used a biotinylated UV-cross-linking analog of lonidamine (the parent

compound of Adjudin) and showed that lonidamine bound tightly to actin stress fibers in Sertoli cells [240]. In addition, Adjudin may cause germ cell loss in the seminiferous epithelium by disrupting the homeostasis of proteases and protease inhibitors at sites of cell adhesion [241]. Siu et al [241] showed the Adjudin-induced spermatid sloughing involved the activation of metalloproteases (MMP). MMP-2 and MMP-9 inhibitors delay the loss of elongating/elongate spermatids by Adjudin [241]. Further studies of Adjudin have demonstrated that the efficacy of this compound is quite poor when administered intramuscularly, as well as having a relatively low bioavailability when given by gavage in adult rats [238]. An important finding in the studies of Adjudin has been that only <7% of Adjudin administered orally is absorbed [238]. However, the dosage has been adjusted to induce effective and reversible infertility [238]. Doses of Adjudin at 37.5 – 50 mg/kg body weight every week for 2 – 3 doses via gavage are highly effective in inducing reversible infertility in male rats are [238], which is also maintained when given intraperitoneally but not intramuscularly. The most effective course of administration of Adjudin is two doses at 50 mg/kg bw every week [238]. Two or more doses are possible, but it takes longer to regain fertility, and in some instances, only partial fertility is then achieved, due to a loss of spermatogonia [238]. The bioavailability of Adjudin was increased 2 -3 fold with micronized Adjudin at <53 µm particle size (i.e., 16 mg /kg bw versus 50 mg/kg bw) [238]. Though the bioavailability of this compound is low, it may still be an appropriate and effective contraceptive in the male [238]. It is apparent that the efficacy of Adjudin can be improved with a finer version of the micronized drug, perhaps at a particle size of <5 µm [238]. Even though the absorption is low, microionized Adjudin can be prepared and does increase the bioavailability [238].

Currently, the understanding of the exact molecular mechanism by which Adjudin exerts its affects is limited. The protein complex that acts as a receptor for Adjudin or is

targeted by this compound is unknown [238]. What is understood is that the first step of the process is the activation of the integrin/laminin complex and the subsequent downstream activation of either the RhoB/ROCK/LIM kinase/cofilin [157] or the FAK/PI 3-kinase/p130Cas/MAP kinase pathway [242]. This results in a change in the polymerization/depolymerization of the actin cytoskeleton in the Sertoli cell, inducing spermatid sloughing [238]. Studies using androgen suppression-induced spermatid sloughing have also shown similar activation of protein kinases, as has been reported to occur after Adjudin administration [243]. Though the levels of N-cadherin and ß-catenin increased with the hormone suppression-induced spermatid sloughing (also reported to occur after the administration of Adjudin), tyrosine phosphorylation of ß-catenin increased, and the specific interaction between N-cadherin and ß-catenin decreased [243]. It has been suggested that this also may be a crucial mechanism for the action of Adjudin in spermatid sloughing [243].

Specific aims and general hypothesis

To develop efficient and reversible contraceptives in the male by manipulating the spermatogenic process in a definable manner, one popular approach today is the targeted and controlled disruption of the junctional dynamics within the seminiferous epithelium, in particular the Sertoli cell-spermatid adherens junction. The specific aims of this doctoral research were: (1) to measure the strength of junctions between germ cells and Sertoli cells *in vitro* and determine if the presence of the unique ectoplasmic specialization between Sertoli cells and step-8 spermatids actually results in an increase in the binding strength between these two cell types; (2) to determine if Sertoli cell lines (sk11, sk9, and sk11 TNUA5) are as effective as primary Sertoli cell isolates in Sertoli cell-spermatid binding studies *in vitro*; and (3) to determine if the potential contraceptive

agent Adjudin disrupts the junctional strength between the Sertoli cell and step-8 spermatid *in vitro*.

The specific aims of this dissertation project (Chapters 2 – 4) are based on the scientific prerequisite that potential contraceptive agent, such as Adjudin, can only be realized when its targeted mechanism of action, which in this case is the disruption of Sertoli-spermatid junctions, is clearly defined. It is hypothesized that the mechanism of action of Adjudin, pertinent to its putative contraceptive effect, is the disruption of the Sertoli cell-spermatid junction.

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CHAPTER 2

Strength measurement of the Sertoli-spermatid junctional complex.

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ABSTRACT

The Sertoli cell ectoplasmic specialization is a specialized domain of the calcium-dependent Sertoli cell-spermatid junctional complex. Its role is not only associated with the mechanical adhesion of the cells but also in the morphogenesis and differentiation of the developing germ cells. Abnormal or absent Sertoli ectoplasmic specializations have been associated with step-8 spermatid sloughing and subsequent oligospermia. The aim of this study was to determine if a modified micropipette pressure transducing system (MPTS) could determine the adhesion force between Sertoli cells and germ cells. Using the MPTS this study examined, for the first time, the strength of the junction between Sertoli cells and spermatids and between Sertoli cells and spermatocytes. The mean force needed to detach spermatocytes from Sertoli cells was 5.25×10^{-7} pN, pre-step-8 spermatids from Sertoli cells was 4.73×10^{-7} pN, step-8 spermatids from Sertoli cells was 8.82×10^{-7} pN, and spermatids + EDTA was 2.16×10^{-7} pN. These data confirm the hypothesis that step-8 spermatids are more firmly attached to Sertoli cells than are spermatocytes and pre-step-8 spermatids and that calcium chelation reduces binding strength between Sertoli cells and spermatids. The MPTS is a useful tool in studying the various molecular models of the Sertoli-germ cell junctional strength and the role of reproductive hormones and enzymes in coupling and uncoupling of germ cells from Sertoli cells.

INTRODUCTION

The complicated process of spermatogenesis occurs throughout the reproductive life of the male. It is a remarkable process in which spermatogonia undergo mitosis to become spermatocytes, which then undergo meiosis to become round spermatids, which then enter the process of spermiogenesis to differentiate into elongated spermatids (sperm) [1]. At any given point in time several generations of germ cells are present in the seminiferous epithelium but in different stages of maturation [2]. During spermatogenesis, germ cells form different types of junctions with Sertoli cells, including the specialized ectoplasmic specialization between Sertoli cells and spermatids [3].

Several types of intercellular junctions, including occluding junctions, adherens junctions, and gap communicating junctions, are believed to play crucial roles in spermatogenesis. The actin based cell-cell adherens junction between the Sertoli cell and the germ cell in the mammalian testis are important not only in mechanical adhesion of the cells, but in the morphogenesis and differentiation of the germ cells [4]. Turnover of these calcium dependent junctions occurs during the process of germ cell migration from the basal to the adluminal epithelial compartment [5].

The Sertoli ectoplasmic specialization, a cytoskeletal structure of the Sertoli cell, is associated with Sertoli-spermatid binding at the adherens junction [6,7]. Abnormal or absent Sertoli ectoplasmic specializations have been associated with a reduction of mature sperm in semen [8-11] and conditions associated with oligospermia [12]. ectoplasmic specializations are found basally in the Sertoli cell near Sertoli-Sertoli tight junctions and apically between Sertoli cells and spermatids. They consist of hexagonally

packed bundles of actin filaments situated between the plasma membrane and a cistern of endoplasmic reticulum [4]. The ectoplasmic specialization is an important cell-cell adhesion mechanism in the seminiferous epithelium to ensure the retention of spermatids as they mature into spermatozoa. ectoplasmic specializations are first seen in the rat at Stage VIII of rat spermatogenesis, when the step-8 spermatid appears. It is thought the ectoplasmic specialization forms in the Sertoli cell to strongly anchor the step-8 spermatid to the seminiferous epithelium; however, this has yet to be actually measured. The ectoplasmic specialization is present at the adherens junction until appropriate release of the step-19 spermatid and inappropriate release of earlier stage spermatids (i.e., spermatid sloughing) is related to abnormal ectoplasmic specialization structure and oligospermia [10,11].

A number of health-related conditions are associated with reduced fertility potential and oligospermia in men, including varicocele, hyperprolactinemia, diabetes, and idiopathic oligospermia [12]. These conditions all are associated with reduced sperm in the semen, i.e., oligospermia, and ultrastructural pathology unique to the junctional apparatus of the seminiferous epithelium [12]. Cap stage spermatids in the human (step-8 spermatids in the rat) are presumed to be tightly anchored to the seminiferous epithelium at a Sertoli cell adherens junction, which includes the unique Sertoli ectoplasmic specialization [6,7]. In both *in vitro* and *in vivo* observations of experimental animal models, disruption of this junction results in spermatid sloughing and subsequent oligospermia [8-11].

This project was designed to measure the strength of junctions between germ cells and Sertoli cells and to determine if the presence of the unique ectoplasmic specialization between Sertoli cells and step-8 spermatids actually results in an increase in the binding strength between these two cell types. To do this, we have modified a

micropipette pressure transducing system for the purpose of testing junctional strengths between cells in a Sertoli-germ cell coculture model optimized for cell-cell binding [13]. It is hypothesized that the junctions between step-8 spermatids and Sertoli cells are stronger than those between pre-step-8 spermatids and Sertoli cells and between spermatocytes and Sertoli cells.

MATERIALS AND METHODS

Sertoli and germ cells were isolated from Sprague-Dawley rats, as previously described [14]. Sixteen- to seventeen-day-old rats were used for Sertoli cell isolation, and adult rats were used for germ cell isolation.

Sertoli cell isolation, culture, and pre-treatment

Briefly, testes were excised from prepubertal male rats, and the parenchyma was digested using routine sequential enzymatic treatments with trypsin (0.25%, Sigma) and collagenase (0.20%, BD). Isolated cells were plated to confluence on 13 mm round plastic coverslips coated with undiluted Matrigel® in 24 well cell culture dishes. Cultures were incubated in DMEM:F12 medium (supplemented with 0.01 mol/L retinol and 1000 µl/100 ml ITS) at 39°C in a humidified incubator with 5% CO_2-95% air for 48 hrs to expedite the removal of contaminating germ cells. After the 48 hr pre-incubation, the cultures were exposed to a 20 mM Tris-HCl buffer for 2.5 min to hypotonically lyse remaining germ cells, then incubated in supplemented DMEM:F12 at 33°C in a humidified incubator with 5% CO_2-95% air for 24 hrs. After the 24 hr incubation, the media was replaced with supplemented DMEM:F12 containing 0.06 µg/ml FSH (NIDDK-oFSH-20, AFP7028D, 175xNIH-FSH-S1) and 100 nM testosterone (Sigma). These pre-treated Sertoli cell cultures were used in all coculture experiments.

Spermatocyte and round spermatid isolation and unit gravity velocity sedimentation

Spermatocytes and pre-step 9 spermatids (round spermatids) were isolated from an adult male rat testis. Briefly, the decapsulated adult testis was digested with 0.10%

collagenase (Gibco; 37°C, 80 oscillations/min, 30 min) to separate seminiferous tubules from the testicular interstitial tissue. The washed seminiferous tubules then were digested with 0.25% trypsin (Sigma; 37°C, 90 oscillations/min, 15 min) to separate the peritubular cells from the seminiferous epithelium and to expedite the release of germ cells from the seminiferous epithelium. A 0.20% trypsin inhibitor solution (Sigma) was added to terminate the trypsin reaction. The resulting cell suspension (mixed germ cells and Sertoli cells) was resuspended in 25 ml McCoy's media + 0.5% BSA.

Using sterile technique, the gradient chambers on a STA-PUT velocity sedimentation cell separator were filled with the appropriate McCoy's + BSA medium (2% and 4% BSA), and a linear gradient (2-4%) was built under the cell suspension, at the loading rate initially at 10 ml/min. After 20 minutes, the rate was increased to 40 ml/min. Eighty minutes prior to the end of the collection time (4 h), media with germ cell fractions were collected using a Fractomat automatic fraction collector (10 ml/vial at 160 drops/min). Spermatocytes and round spermatids (pre-step 9) were identified by phase contrast microscopy and pooled, washed, and resuspended in McCoy's media. The number of cells in the spermatocyte and spermatid fractions were counted by hemocytometric analysis and assayed for viability by trypan blue exclusion.

Sertoli-germ cell coculture

Approximately 400,000 isolated germ cells (spermatocytes and round spermatids) were added directly to the pre-treated Sertoli cell enriched monocultures. The Sertoli-germ cell cocultures were incubated with 0.06 µg/ml FSH + 100 nM testosterone in a humidified chamber at 33°C with 5% CO_2-95% air for 36 hrs.

Measurement of junctional strength using a micropipette pressure transducing system (MPTS)

The Sertoli-germ cell cocultures were imaged on an inverted interference contrast microscope (Axiovert 100, Zeiss) with a 20x objective. The microscope was fitted with the MPTS, which consisted of a 3-D water robot micromanipulator (Narishige Scientific Instruments Lab), a micropipette holder, a glass micropipette, a water reservoir system to control the micropipette pressure, and a video system to record experiments (Figure 2.1).

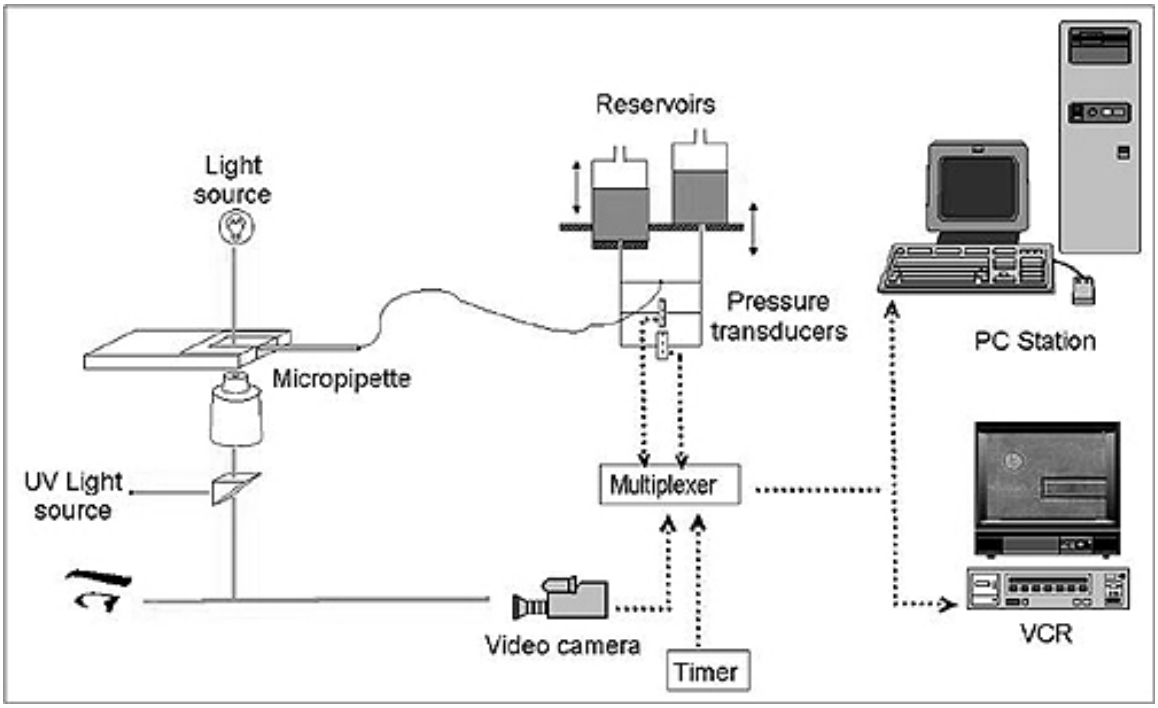

Figure 2.1. A schematic drawing of the micropipette pressure transducing system (MPTS).

The micropipette

Micropipettes were created from 1mm-outer diameter, 0.5mm-inner diameter glass capillary tubes (A-M Systems, Inc.). The capillary tube was mounted onto a pipette puller (Model PB-7, Narishige Scientific Instruments), heated, and pulled into a pipette with a tip of a few microns. To ensure a flat tip, this pipette was then mounted onto a microforge (Model MF 83, Narishige Scientific Instruments). The microforge consists of a horizontal microscope, a micromanipulator, and a glass bead on a platinum

wire. Upon heating of the wire, the glass bead melted and the tip of the micropipette was inserted into the melted glass. The bead/micropipette was allowed to cool. The micropipette was then pulled up, and the tip was broken by quick fracture, leaving a flat tip. The tip was filled with a saline solution to avoid plugging. In order to prevent rupture of the cells on the glass surface, the micropipette was coated with plasma proteins. The diameters of the pipettes used were 13.32 µm for spermatids (diameter 10 µm) and 16.65 µm for spermatocytes (diameter 15 µm).

The water reservoir system

The pressure at the tip of the micropipette was controlled by a system consisting of two water reservoirs and a pressure transducer (Model DP15-30, Validyne) connected between the two reservoirs. One reservoir was a reference reservoir and the other one was an adjustable reservoir. The reference reservoir was adjusted so that no pressure would be applied at the micropipette. This was achieved by connecting the reference reservoir directly to the micropipette and positioning it at the same level as the micropipette. As a result, there were no movements from particles or cells in front of the micropipette. The adjustable reservoir was then positioned to create the desired pressure, as read by the pressure transducer. A valve switch was used to connect the micropipette either to the reference reservoir or the adjustable reservoir. The pressure transducer output signal was decoded via a carrier demodulator (Model CD 280-2, Validyne). The pressure range of the transducer was 80,000 dyn/cm^2 with an accuracy of 400 dyn/cm^2.

Cell measurements

Cover slips containing Sertoli-germ cell cocultures were carefully removed from the wells and placed in an engineered cover slip holder for use with the MPTS-fitted inverted microscope. The detachment of germ cells from Sertoli cells was measured

and analyzed. In some experiments, 2mM or 4mM EDTA was added to the cultures immediately before the measurements, as controls. To detach germ cells from Sertoli cells, the micropipette was brought near the individual spermatocyte or the individual spermatid at 200x magnification. The pressure required to detach the germ cell from the underlying Sertoli cell monolayer was then recorded. Each detachment event (a maximum of 4) consisted of a 5 second suction pressure interval. If the germ cell did not dissociate, it was abandoned, and the last pressure reading was recorded. The recorded pressure (in cm-H_2O) was used to calculate force via the equation $F = \Delta P \cdot \pi R^2 \pi$, where F (pN) is the force on a static cell, ΔP is the suction pressure ($N/\mu m^2$), and $\pi R^2 \pi$ is the cross sectional area of the pipette (μm^2). To convert the pressure reading received in cm-H_2O to $N/\mu m^2$, for use in the above equation, the conversion factors 1 cm-H_2O = 98.06 Pa and 1 Pa = N/m^2 were used, since the international unit of force is Newtons (1 N = 1 kg•m/s^2), and the international unit of pressure is Pascal (Pa).

Statistics

To determine statistical significance of the mean force (set at the 0.05 level), a one-way ANOVA was performed, followed by Tukey HSD.

RESULTS

The mean force required to detach spermatocytes, pre-step-8 spermatids, and step-8 spermatids from Sertoli cells in the optimized Sertoli-germ cell *in vitro* binding model was determined following multiple measurements acquired from the modified MPTS. The mean force necessary to detach spermatocytes from Sertoli cells was 5.25×10^{-7} pN (SE= 3.43×10^{-8}, n=38), pre-step-8 spermatids from Sertoli cells (i.e., Sertoli-spermatid junctions with no ES) was 4.73×10^{-7} pN (SE= 2.17×10^{-8}, n=38), step-8 spermatids from Sertoli cells (i.e., Sertoli-spermatid junctions with ES) was 8.82×10^{-7} pN (SE= 3.37×10^{-8}, n=33), and spermatids + EDTA was 2.16×10^{-7} pN (SD= 3.12×10^{-8}, n=6). These results are presented in Figure 2.2.

A one-way ANOVA determined a significant difference between the mean force of spermatocytes and the mean force of step-8 spermatids, between the mean force of pre-step-8 spermatids and the mean force of step-8 spermatids, and between the mean force of spermatids versus spermatids + EDTA, where $p<0.05$. There was no significant difference between the mean force of spermatocytes and the mean force of pre-step-8 spermatids.

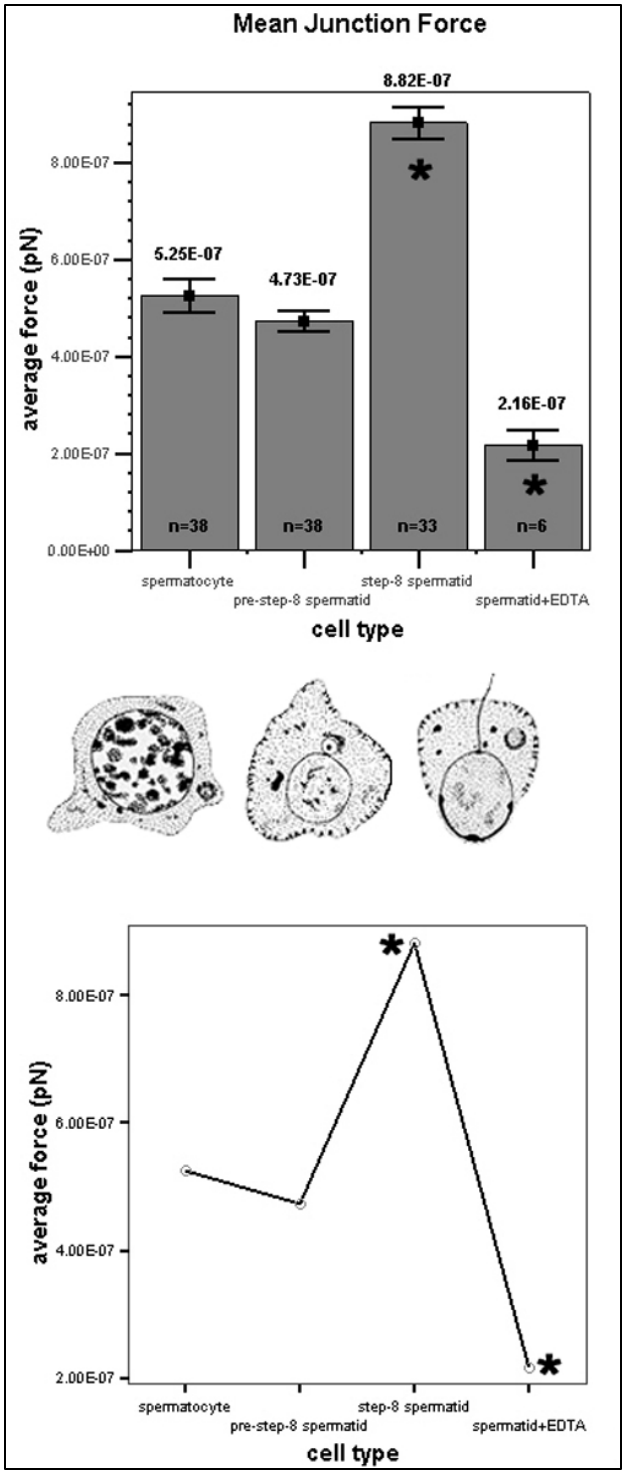

Figure 2.2. Bar graph and line chart displaying the mean force (pN) required to detach spermatocytes, pre-step-8 spermatids, and step-8 spermatids from Sertoli cells in vitro with FSH+T. * indicates a significant difference, as determined by one-way ANOVA.

DISCUSSION

It is theorized that spermatids associated with ectoplasmic specializations adhere to Sertoli cells more strongly than all other germ cells and that this is essential for anchoring spermatids in the seminiferous epithelium during the final stages of spermiogenesis. Additionally, complete spermiogenesis is not observed in the absence of these unique Sertoli-spermatid junctions, which when disrupted lead to spermatid sloughing and oligospermia [8-10]. However, the actual junctional strength between Sertoli cells and spermatids has never been measured to verify this unsubstantiated dogma central to the successful completion of spermatogenesis.

We have, for the first time, determined the actual strength of junctions between germ cells and Sertoli cells *in vitro*. The data presented confirm the hypothesis that step-8 spermatids are more firmly attached to Sertoli cells than are spermatocytes and pre-step-8 spermatids.

Of the cells tested, the ectoplasmic specialization is only present between Sertoli cells and step-8 – step-19 spermatids and is conspicuously absent between Sertoli cells and spermatocytes and pre-step-8 spermatids [4]. This suggests that the structural nature of the ectoplasmic specialization contributes to the actual junctional strength between these two cell types, ensuring that elongating spermatids (post-step-8 spermatids) are securely anchored to the seminiferous epithelium during the final stages of spermiogenesis. This also supports the hypothesis that when the ectoplasmic specialization does not form properly between the Sertoli cell and the periluminal step-8

spermatid, or is otherwise abnormal, the junction strength is significantly lessened, thereby leading to spermatid sloughing and oligospermia [12].

Several molecular models of the structure and regulation of the ectoplasmic specialization at the Sertoli cell-spermatid junction have been proposed. One such model includes the controversial and most studied cadherin-catenin complex. In this model, it is proposed that the presence and regulation of the multi-protein cadherin-catenin complex at the ectoplasmic specialization controls the coupling and uncoupling of spermatids to Sertoli cells [15-17]. Disruption of this protein complex via phosphorylation of $p120^{ctn}$ [18], tyrosine phosphorylation of ß- and/or γ-catenin [18], and/or the addition of an anti-N-cadherin antibody [17,19] results in the loss of germ cells from the seminiferous epithelium. This step-8 sloughing, as described by O'Donnell et al [10], is also related to testosterone reduction and possibly, therefore, N-cadherin expression [17,20,21]. *In vitro*, testosterone and DHT with a fixed concentration of FSH causes a dose-related increase in N-cadherin levels [19]. Increasing doses of FSH in the presence of a fixed concentration of testosterone also creates a dose-related increase in N-cadherin protein levels [19]. In the models studied, the ectoplasmic specialization is still present, therefore indicating that testosterone has an effect on the cell adhesion molecules at this junction and not the ectoplasmic specialization structure itself [11, 20,21]. The effects of reproductive hormones on cell adhesion and coupling and uncoupling dynamics between Sertoli cells and germ cells, as defined above, can be tested utilizing the MPTS.

Other proposed molecular models of the Sertoli-spermatid ectoplasmic specialization consist of the nectin-afadin-ponsin complex and the integrin complex. Nectins are found in both Sertoli cells (nectin-2) and spermatids (nectin-3), with the strongest expression at stages IX-IV and decreasing at stage VIII [22,23]. 1-Afadin,

found in the testis, connects to the actin cytoskeleton [24], and studies using afadin$^{-/-}$ mice have shown that afadin is essential in proper structural organization of tight junctions and cadherin-based AJs [25]. Ponsin, of which mRNA is found in the testis [26], binds to afadin and allows it to colocalize with nectin to the cadherin-based adherens junction [24]. However, no biochemical or functional studies on the nectin-afadin-ponsin complex have been conducted.

The most studied integrin receptor in the testis is a6ß1, which is found in the Sertoli cell membrane [for review 27]. The binding partner of a6ß1 is not yet known, but recent studies have indicated that the laminin γ3 chain is a putative binding partner [28-30]. The expression of ß1-integrin has been shown to be affected by hormones. Testosterone, in the presence of FSH, increases ß1-integrin levels in a dose-dependent manner [31], as do increasing doses of FSH in the presence of testosterone. Integrins are important in cell adhesion not only structurally, but also in that they transmit signals to trigger events that activate signal transducers, such as Rho GTPase [32], FAK [29,33], Src [16], Csk [16], and ILK [29], to affect Sertoli-germ cell AJ dynamics [5]. Again, the effects of reproductive hormones on the integrin-based model of the Sertoli-germ cell junction and its role in coupling and uncoupling of germ cells from Sertoli cells can be tested with the MPTS.

Results from this study show that the junctional strength between Sertoli cells and germ cells can be measured *in vitro*, support long held speculations regarding Sertoli-spermatid junctional interactions, and provide a means to actually test proposed mechanisms of junction dynamics between cells of the seminiferous epithelium.

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CHAPTER 3

The Sertoli-spermatid junctional complex adhesion strength is affected *in vitro* by Adjudin.

In Press: Journal of Andrology

ABSTRACT

The actin based cell-cell adherens junction between the Sertoli cell and the germ cell in the mammalian testis is important not only in mechanical adhesion of the cells, but in the morphogenesis and differentiation of the germ cells. The Sertoli ectoplasmic specialization, a specialized type of adherens junction, is associated with Sertoli-spermatid binding and is important in cell-cell adhesion in the seminiferous epithelium. Abnormal or absent Sertoli ectoplasmic specializations have been associated with step-8 spermatid sloughing and oligospermia in conditions associated with reduced fertility potential. The reproductive hormones follicle stimulating hormone (FSH) and testosterone also have been shown to play a role in the regulation of binding of spermatids at the Sertoli-spermatid junctional complex. Adjudin [1-(2,4-dichlorobenzyl)-1*H*-indazole-3-carbohydrazide] is a potential male contraceptive and is thought to exhibit its contraceptive effects by interrupting the Sertoli-spermatid junctional complex. It has been shown that this compound induces reversible germ cell loss from the seminiferous epithelium, particularly elongating/elongate/round spermatids and spermatocytes. The aim of this study was to determine if Adjudin disrupts the junctional strength between the Sertoli cell and step-8 spermatid *in vitro*. Using a micropipette pressure transducing system (MPTS) to measure the force needed to detach step-8 spermatids from Sertoli cells, this study examined the strength of the Sertoli-spermatid junctional complex in Sertoli-spermatid cocultures in the presence of Adjudin (1 ng/ml, 50 ng/ml, 125 ng/ml, or 500 ng/ml in EtOH) and hormones [FSH (0.1 µg/ml, NIDDK-oFSH-20, AFP7028D, 175xNIH-FSH-S1), testosterone (100 nM)] to optimize *in vitro* binding. The average

forces required to detach the spermatids from the underlying Sertoli cells in the presence of 1 ng/ml, 50 ng/ml, 125 ng/ml and 500 ng/ml Adjudin were $18.2x10^{-10}$ pN, $14.3x10^{-10}$ pN, $7.74x10^{-10}$ pN and $6.51x10^{-10}$ pN, respectively. The average force required to detach step-8 spermatids in the presence of vehicle only (control) was $19.0x10^{-10}$ pN. A significant difference for Adjudin concentrations at or above 125 ng/ml was determined by one-way ANOVA ($p<0.05$). These data confirm that Adjudin is effective in reducing the strength of the Sertoli-spermatid junctional complex, identifying Adjudin as a potential contraceptive agent in the male by inducing spermatid sloughing and therefore oligospermia.

INTRODUCTION

Spermatogenesis, the process in which germ cells undergo mitosis and meiosis to become elongated spermatids (sperm) [1], occurs throughout the reproductive life of the male. Present in the seminiferous epithelium at any given point in time are several generations of germ cells in different stages of maturation [2]. Various types of junctions between these developing germ cells and Sertoli cells exist throughout spermatogenesis, including the highly specialized ectoplasmic specialization found between Sertoli cells and germ cells [3].

The ectoplasmic specialization is an apical cytoskeletal structure of the Sertoli cell associated with Sertoli-spermatid binding at the adherens junction [4,5]. This structure is important in cell-cell adhesion in the seminiferous epithelium, ensuring the retention of spermatids as they mature into spermatozoa. It is believed that the ectoplasmic specialization forms to strongly anchor the step-8 spermatid to the seminiferous epithelium. In the rat, ectoplasmic specializations are first seen at Stage VIII of spermatogenesis, the time when the step-8 spermatid appears, and are present at the adherens junction until appropriate release of the step-19 spermatid. Inappropriate release of spermatids (i.e., spermatid sloughing) is related to abnormal ectoplasmic specialization structure and oligospermia [6,7]. A reduction of mature sperm in semen [6-9] and conditions associated with oligospermia are associated with structurally abnormal or absent Sertoli ectoplasmic specializations [10].

A number of health-related conditions are associated with reduced fertility potential and oligospermia in men, including varicocele [11], hyperprolactinemia [12],

diabetes [13], and idiopathic oligospermia [10]. These conditions all are associated with reduced sperm in the semen, i.e., oligospermia, and ultrastructural pathology unique to the junctional apparatus of the seminiferous epithelium [10]. Cap stage spermatids in the human (step-8 spermatids in the rat) are presumed to be tightly anchored to the seminiferous epithelium at a Sertoli cell adherens junction, which includes the unique Sertoli ectoplasmic specialization [4,5]. In both *in vitro* and *in vivo* observations of experimental animal models, disruption of this junction results in spermatid sloughing and subsequent oligospermia [6,7,8,9].

Adjudin, formerly known as AF-2364 (1-(2,4-dichlorobenzyl)-1*H*-indazole-3-carbohydrazide), depletes seminiferous tubules of germ cells [14,15]. By day 14 of administration of Adjudin, adult seminiferous tubules are found nearly devoid of elongated and round spermatids and spermatocyte numbers have been reduced, with no significant effect on reproductive hormone levels [15]. Although speculated, it is not yet been determined whether Adjudin works at the level of the adherens junction or the ectoplasmic specialization.

This project was designed to measure the strength of junctions between step-8 spermatids and Sertoli cells in the presence of various concentrations of Adjudin. To do this, a micropipette pressure transducing system was used to measure the force needed to detach step-8 spermatids from Sertoli cells [16] in the presence of Adjudin (0 ng/ml, 1 ng/ml, 50 ng/ml, 125 ng/ml, or 500 ng/ml in EtOH) [17] and reproductive hormones (follicle stimulating hormone and testosterone). It is hypothesized that Adjudin at higher concentrations will disrupt the Sertoli-spermatid junctional complex and cause reduced binding strength between the Sertoli cell and step-8 spermatid.

MATERIALS AND METHODS

Sertoli and germ cells were isolated from Sprague-Dawley rats, as previously described [18]. Sixteen- to seventeen-day-old rats were used for Sertoli cell isolation, and adult rats were used for germ cell isolation.

Sertoli cell isolation, culture, and pre-treatment

Briefly, testes were excised from prepubertal male rats, and the parenchyma was digested using routine sequential enzymatic treatments with trypsin (0.25%, Sigma) and collagenase (0.20%, BD). Isolated cells were plated to near confluence on 13 mm round glass coverslips coated with 1:3 Matrigel (BD) in culture medium in 24-well cell culture dishes. Cultures were incubated in DMEM:F12 medium [supplemented with 0.01 mol/L retinol (Sigma), 1000 µl/100 ml ITS (BD), 500 µl/500 ml gentamicin (Sigma) and 5 ml/500 ml antibiotic/antimycotic (Cellgro)] at 39°C in a humidified incubator with 5% CO_2-95% air for 48 hrs to expedite the removal of contaminating germ cells. After the 48 hr pre-incubation, the cultures were exposed to a 20 mM Tris-HCl buffer for 2.5 min to hypotonically lyse any remaining germ cells, then incubated in supplemented DMEM:F12 at 33°C in a humidified incubator with 5% CO_2-95% air for 24 hrs. After the 24 hr incubation, the media was replaced with supplemented DMEM:F12 containing 0.06 µg/ml follicle stimulating hormone (FSH; NIDDK-oFSH-20, AFP7028D, 175xNIH-FSH-S1) and 100 nM testosterone (T; Sigma) to optimize *in vitro* Sertoli-spermatid binding. These pre-treated Sertoli cell cultures were used in the coculture experiments.

Round spermatid isolation and unit gravity velocity sedimentation

Pre-step 9 spermatids (round spermatids) were isolated from an adult male rat testis. Briefly, the decapsulated adult testis was digested with 0.10% collagenase (Gibco; 37°C, 80 oscillations/min, 30 min) to separate seminiferous tubules from the testicular interstitial tissue. The washed seminiferous tubules then were digested with 0.25% trypsin (Sigma; 37°C, 90 oscillations/min, 15 min) to separate the peritubular cells from the seminiferous epithelium and to expedite the release of germ cells from the seminiferous epithelium. A 0.20% trypsin inhibitor solution (Sigma) was added to terminate the trypsin reaction. The resulting cell suspension (mixed germ cells and Sertoli cells) was resuspended in 25 ml McCoy's media + 0.5% BSA.

Using sterile technique, the gradient chambers on a STA-PUT velocity sedimentation cell separator were filled with the appropriate McCoy's + BSA medium (2% and 4% BSA), and a linear gradient (2-4%) was built under the cell suspension, at the loading rate initially at 10 ml/min. After 20 minutes, the rate was increased to 40 ml/min. Eighty minutes prior to the end of the collection time (4 h), media with germ cell fractions were collected using a Fractomat automatic fraction collector (10 ml/vial at 160 drops/min). Round spermatids (pre-step 9) were identified by phase contrast microscopy and pooled, washed, and resuspended in McCoy's media. The number of cells in the spermatocyte and spermatid fractions were counted by hemocytometric analysis and assayed for viability by trypan blue exclusion.

Sertoli-germ cell coculture

Approximately 400,000 isolated germ cells (round spermatid-enriched) were added directly to the pre-treated Sertoli cell enriched monocultures. The Sertoli-germ cell cocultures were incubated in a humidified chamber at 33°C with 5% CO_2-95% air for

36 hrs with 0.06 µg/ml FSH + 100 nM T to optimize Sertoli-spermatid binding as previously described [19, 20].

Addition of Adjudin to the coculture

After 30 hrs of incubation, Vehicle 1 (2.5 µl EtOH) was added to one column of the 24-well plate and incubated for 1 hr at 33°C. After the 1 hour incubation time, the next column received 1 ng/ml Adjudin in 2.5 µl EtOH and incubated for 1 hr at 33°C. This continued with the remaining concentrations of Adjudin (50 ng/ml, 125 ng/ml and 500 ng/ml) [17] and ended with Vehicle 2 (same as Vehicle 1) to ensure that time was not the factor affecting the junction.

Measurement of junctional strength using a micropipette pressure transducing system (MPTS)

The Sertoli-germ cell cocultures were imaged on an inverted interference contrast microscope (Axiovert 100, Zeiss) with a 20x objective. The microscope was fitted with the MPTS, as previously described [16]. After the 1 hr incubation with the treatment, the cover slips were washed 5x by gentle pipetting with supplemented DMEM:F12 + FSH and T (without Adjudin or EtOH). Cover slips containing the Sertoli-germ cell cocultures were carefully removed from the well. A step-8 spermatid were identified as a 10 µm round cell containing an eccentric nucleus, as previously described [19, 21]. The detachment of individual step-8 spermatids from Sertoli cells and subsequent force measurement and analysis was performed as previously described [16]. Briefly, pressure at the tip of a 10 µm-diameter micropipette was controlled by a system consisting of two water reservoirs and a pressure transducer connected between the two reservoirs. To detach spermatids from Sertoli cells, the glass micropipette tip was brought into close proximity to the unbound cell surface of the spermatid, and the hydrostatic pressure required to detach it from the underlying Sertoli cell monolayer was

recorded on the transducer. The recorded pressure (in cm-H2O) was used to calculate force via the equation F = ?P • pR2p, where F (pN) is the force on a static cell, ?P is the suction pressure (N/µm2), and pR2p is the cross sectional area of the pipette (µm2). To convert the pressure reading received in cm-H2O to N/µm2, for use in the above equation, the conversion factors 1 cm-H2O = 98.06 Pa and 1 Pa = N/m2 were used, since the international unit of force is Newtons (1 N = 1 kg•m/s2), and the international unit of pressure is Pascal (Pa). Each detachment event (a maximum of 4) consisted of a 5 second suction pressure interval. If the germ cell did not dissociate, the detachment effort was abandoned, and the last pressure reading was recorded.

Viability/cytotoxicity assays

Sertoli cell cultures were prepared from 20-day-old rat testes by sequential enzymatic treatments as described [18], and cells were plated at high-density (0.5×10^6 cells/cm^2) on Matrigel™ (diluted 1:7 with Ham's F-12 Nutrient Mixture and Dulbecco's Modified Eagle's Medium [F-12/DMEM], 1:1; Sigma)-coated Nunclon 24-well dishes in F-12/DMEM supplemented with 10 µg/ml bovine insulin, 5 µg/ml human transferrin, 10 µg/ml bacitracin, 2.5 ng/ml EGF, 0.06 µg/ml FSH and 100 nM T. To obtain Sertoli cells with a purity greater than 98%, cultures were hypotonically treated. Media were replaced every 24 hr thereafter, and Sertoli cells were incubated for an additional 3 days. This was followed by the isolation of germ cells from 90-day-old rat testes as previously described [22,23]. In this experiment, germ cell preparations were exposed to successive glass wool filtration steps, and thus, consisted of spermatogonia, spermatocytes, round and elongating spermatids when examined microscopically. Germ cells were added directly to Sertoli cell cultures at a Sertoli:germ cell ratio of 1:3 and cocultured for 36 hr [24]. Thereafter, cocultures were rinsed twice with media to remove unbound germ cells and increasing concentrations of Adjudin (1, 50, 125 and

500 ng/ml, and 1 µg/ml) were added. Sertoli-germ cell cocultures were incubated for 0, 1, 3, 6 and 12 hr. These cocultures were then used for viability/cytotoxicity assays. Because viable cells are characterized by the presence of intracellular esterase activity, this assay measured the ability of cells to enzymatically convert non-fluorescent, cell-permeable calcein AM to fluorescent calcein. Briefly, media was removed from Sertoli-germ cell cocultures and cells gently rinsed with media. Calcein AM (~2-5 µM, prepared in media or PBS, pH 7.4 prior to immediate use to prevent hydrolysis; Invitrogen) was added to Sertoli-germ cell cocultures and incubated briefly at 37 ºC or room temperature. Fluorescence was quantified at 10-15 min intervals for up to 60 min at 485 nm_{EX} and 535 nm_{EM} using a Tecan GENios fluorescence plate reader. Controls consisted of Sertoli-germ cell cocultures cultured in the absence of Adjudin and in the presence of vehicle (ethanol:DMSO, 1:1 dilution). Non-viable Sertoli-germ cell cocultures, which lacked the ability to enzymatically convert calcein AM to calcein, were prepared by treating cells with 75% ethanol (30 min) or 0.5% saponin (10 min).

RESULTS

The mean force required to detach step-8 spermatids from Sertoli cells in the optimized Sertoli-germ cell *in vitro* binding model in the presence of various concentrations of Adjudin was determined following multiple measurements acquired from the modified MPTS. The mean force necessary to detach step-8 spermatids from Sertoli cells (i.e., Sertoli-spermatid junctions with ectoplasmic specializations) in the presence of Vehicle 1 (at the start of the measurement process) and Vehicle 2 (at the end of the measurement process) was 18.03×10^{-10} pN (SE=1.263×10^{-6}, n=16) and 19.92×10^{-10} pN (SE=1.149×10^{-6}, n=16). The average for the two Vehicle groups was 19.0×10^{-10} pN. In the presence of 1 ng/ml, 50 ng/ml, 125 ng/ml and 500 ng/ml Adjudin, the mean forces required to detach step-8 spermatids from the underlying Sertoli cell monoculture were 18.2×10^{-10} pN (SE=1.383×10^{-6}, n=16), 14.3×10^{-10} pN (SE=1.412×10^{-6}, n=16), 7.74×10^{-10} pN (SE=1.122×10^{-6}, n=16) and 6.51×10^{-10} pN (SE=1.750×10^{-6}, n=10), respectively (Figure 3.1). A one-way ANOVA determined a significant difference for Adjudin concentrations at or above 125 ng/ml, where $p<0.05$.

Viability/cytotoxicity assays demonstrated that the viability of Sertoli-germ cell cocultures was not affected when these cells were incubated with increasing concentrations of Adjudin for up to 12 hr (Figure 3.2). Time points beyond 12 hr were not examined because higher doses of Adjudin (500 ng/ml and 1 µg/ml) perturb Sertoli-germ cell adhesion, resulting in a decrease in cell number in these wells.

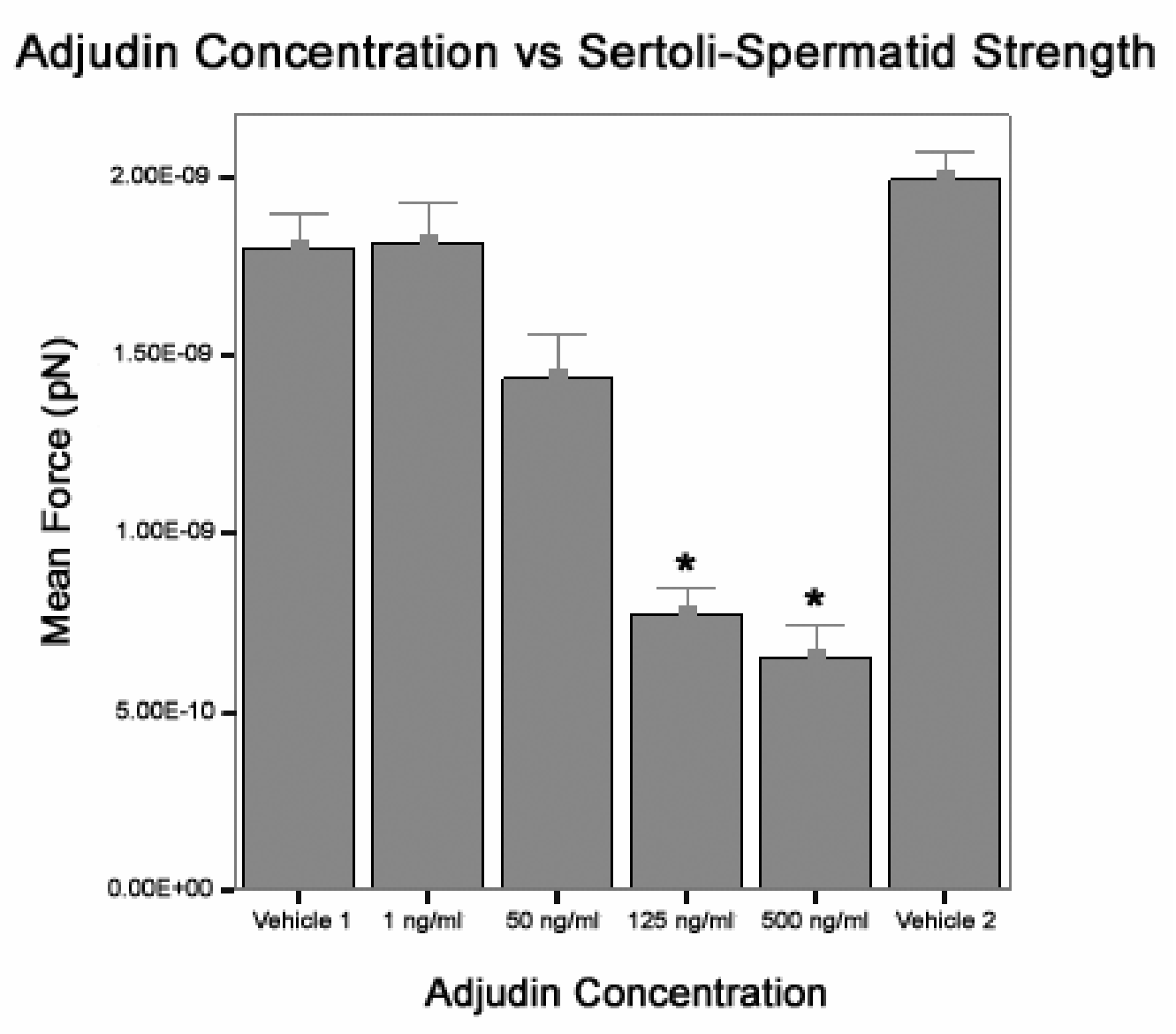

Figure 3.1. Bar graph displaying the effect of Adjudin on the mean force (in piconewtons, pN) required to detach step-8 spermatids from Sertoli cells in the optimized Sertoli-spermatid coculture binding model. * indicates a significant difference, as determined by one-way ANOVA.

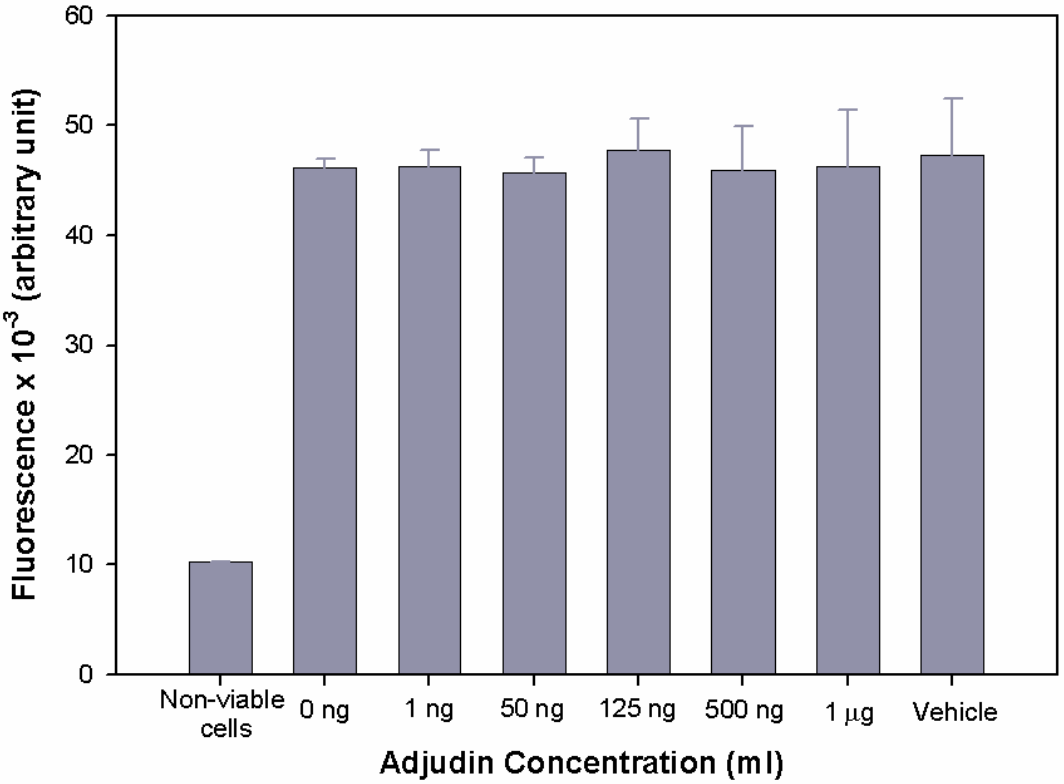

Figure 3.2. Bar graph illustrating that increasing concentrations of Adjudin (0, 1, 50, 125 and 500 ng/ml, and 1 μg/ml) at 12 hr post-treatment had no effect on the viability of Sertoli-germ cell cocultures when compared to controls (0 ng/ml Adjudin and vehicle).

DISCUSSION

It is hypothesized that Adjudin works at the level of the adherens junction and possibly the ectoplasmic specialization in the seminiferous tubules, since depletion of round and elongated spermatids is seen in rats after administration of this potential male contraceptive [14,15,25]. However, cell adhesion was not compromised in other organs such as the brain, liver and kidney when this drug was administered by gavage, inter-peritoneal or intramuscular injection [25] nor was the hypothalamus-pituitary-testicular axis affected at doses that were effective to induce male infertility [14]. Additionally, administration of Adjudin (50 mg/kg b.w., 3 doses administered every 2 days) to pups (*e.g.,* 5-25 days of age) did not effect the integrity of Sertoli-germ cell adherens junctions [26], suggesting that the apical ectoplasmic specialization is an initial target. Upon metabolic removal of Adjudin from virtually all organs by 48 hr, spermatogenesis began to resume progressively, and by day ~100 the testes of treated animals were indistinguishable from controls [14]. However, it was not known whether this compound actually affects the strength of the Sertoli-spermatid junctional complex.

We have, for the first time, determined that the strength necessary to detach step-8 spermatids from a Sertoli cell monolayer is reduced by specific doses of Adjudin *in vitro*, indicating a functional alteration of the Sertoli-spermatid junctional complex. The data presented confirm the hypothesis that this compound, at higher concentrations, disrupts the Sertoli-spermatid junctional complex, causing weaker binding between the Sertoli cell and step-8 spermatid. Only the Sertoli-spermatid junctional complex between step-8 spermatids and Sertoli cells, which contains the ectoplasmic specialization, was

tested. It is not known whether Adjudin affects the strength of desmosome-like junctions that are present, for example, between Sertoli and pachytene spermatocytes. Recent studies have suggested that Adjudin affects the ectoplasmic specialization by activating RhoB within hours of administration. This in turn activated ROCK, LIMK1 and cofilin, which perturbed actin cytoskeleton dynamics and resulted in germ cell detachment [27]. Moreover, ß1 integrin, which is predominantly located at the apical ectoplasmic specialization, was also shown to be up-regulated following Adjudin treatment, further activating the FAK/PI 3-kinase/p130Cas/MAP kinase [17] pathway. Though the exact protein complex acting as the receptor for Adjudin has not yet been defined, it is thought that through these signaling pathways, changes in the polymerization and depolymerization of actin at the ectoplasmic specialization lead to a depletion of germ cells from the seminiferous epithelium, in particular round and elongating spermatids [25]. It should be noted that Adjudin is a chemical entity that shares structural similarities with lonidamine [1-(2,4)-dichlorobenzyl-1H-indazole-carboxylic acid], which is known to severely damage stress fibers (*e.g.,* actin filaments) in Sertoli cells [28,29]. Likewise, preliminary studies have shown that Adjudin can induce extensive remodeling of the actin cytoskeleton in these cells (Mruk and Cheng, unpublished observations). What remains to be determined, however, is why Sertoli cell actin at the apical ectoplasmic specialization is sensitive to Adjudin's effects when this protein is a constituent of virtually all cell types. Certainly, other upstream regulators of RhoB activity, as well as additional signaling cascades, are likely to be involved, and their identification will help in determining why the apical ectoplasmic specialization is a primary target for Adjudin-mediated restructuring in the testis.

Results from this study show that the junctional strength between Sertoli cells and step-8 spermatids is reduced by Adjudin *in vitro*, supporting the potential use of this chemical as a male contraceptive.

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CHAPTER 4

Immortalized Sertoli cell lines sk11 and sk9 and binding of spermatids *in vitro*.

(In Review)

ABSTRACT

Current studies of Sertoli cell-germ cell binding dynamics *in vitro* require the use of primary Sertoli cell isolates. The aim of this study was to determine the effectiveness of the sk11, sk9, and sk11 TNUA5 Sertoli cell lines in binding germ cells *in vitro.* These immortalized cell lines were utilized in coculture experiments with germ cells in media with/without reproductive hormones and incubated for 44 h, 32°C. The number of germ cells bound to Sertoli cells was then determined and statistically analyzed. Western Blot analysis and RT-PCR studies were employed to investigate the presence of cell adhesion proteins and FSH receptor, respectively. No statistical difference between the number of bound step-8 spermatids and bound pre-step 8 spermatids on Sertoli cells from any of the cell lines existed. After the addition of germ cells, Sertoli cells showed more lipid accumulation in their cytoplasm, indicating active phagocytosis. Western Blot analysis in the sk11 TNUA5 line indicated the expression of N-cadherin. FSH only and testosterone only treatments increased N-cadherin expression, regardless of germ cell addition. The addition of germ cells to the sk11 TNUA5 Sertoli cells increased the expression of espin, as did the addition of FSH with germ cells. RT-PCR studies of the sk11 TNUA5 cells indicated that the mRNA for FSH receptor decreased with successive passages. *In vitro* binding between isolated germ cells and sk9, sk11 or sk11 TNUA5 Sertoli cells, in a manner similar to that when using primary isolated Sertoli cells, is not feasible, and therefore these cell lines are not useful for the investigation of Sertoli-germ cell interactions.

INTRODUCTION

Spermatogenesis is a complicated process occurring throughout the reproductive life of the male. It is a remarkable process in which germ cells undergo mitosis, meiosis and cellular differentiation to produce spermatozoa [1]. At any given point, several generations of germ cells develop at the same time in the seminiferous tubule of mammals [2]. The seminiferous epithelial cycle is made up of various stages, in which new generations of germ cells are connected to older generations, with their development coordinated via the presence of fixed cellular associations [2].

Occluding junctions, adherens junctions and gap communicating junctions are thought to play crucial roles in spermatogenesis. Not only important in mechanical adhesion, the actin-based cell-cell adherens junctions between the Sertoli cell and the germ cell in the mammalian testis is also important in the morphogenesis and differentiation of the germ cells [3]. During the process of germ cell migration from the basal to the adluminal epithelial compartments, these junctions turnover [4]. However, their role in complete spermatogenesis is not yet fully understood. The Sertoli ectoplasmic specialization, a cytoskeletal structure of the Sertoli cell, is associated with Sertoli-germ cell binding [5,6]. The morphology of testicular junctions has been well described, but their molecular composition still is not well understood. ectoplasmic specializations are found basally in the Sertoli cell near Sertoli-Sertoli tight junctions and between Sertoli cells and germ cells and consist of hexagonally packed bundles of actin filaments situated between the plasma membrane and a cistern of endoplasmic reticulum [3]. A reduction of mature sperm in semen has been associated with abnormal

or absent Sertoli ectoplasmic specializations [7-11]. To ensure the retention of spermatids as they mature into spermatozoa, the ectoplasmic specialization is an important cell-cell adhesion mechanism in the seminiferous epithelium.

Espin is an actin binding protein found in the testis, specifically the Sertoli cells and shows no resemblance to other actin-binding proteins [12]. In the seminiferous epithelium, espin appears to be concentrated around the heads of spermatids from mid- to late spermiogenesis, as determined by immunoperoxidase immunocytochemistry [13]. It is also seen near the base of the seminiferous tubules [13]. Sertoli cells surrounding step-8 spermatids, where an organized ectoplasmic specialization is first seen, demonstrate espin immunostaining in a C-shaped cap near the area where the spermatid meets the Sertoli cell [12]. This is not seen around step-7 spermatids [12]. Nearing spermiation, espin immunostaining near the luminal edge of the seminiferous epithelium decreases and then disappears around the time of sperm release [12]. This change in localization appears to reflect the disassembly of the ectoplasmic specialization [12]. Through immunogold electron microscopy, espin has been localized to the parallel bundles of actin filaments present at the ectoplasmic specialization in Sertoli cells [13]. A smaller isoform of espin, termed “small espin,” has been seen associated with parallel actin bundles found in brush border microvilli in the kidney and intestine [14]. This further supports the hypothesis that espin is involved in the bundling of actin at the ectoplasmic specialization.

Reproductive hormones have been shown to play a role in the regulation of binding of spermatids at the Sertoli-spermatid junctional complex. The key regulators of spermatogenesis are follicle stimulating hormone (FSH) and testosterone [15-17]. FSH is thought to induce the binding competence of the Sertoli cell [18-20], whereas testosterone is believed to stimulate the actual binding between the two cell types

[18,20-22]. Cameron and Muffly [19] showed maximal binding of round spermatids to Sertoli cells *in vitro* in the presence of FSH and testosterone. Testosterone is also known to promote and maintain the maturation of round to elongated spermatids in the rat [23]. The withdrawal of testosterone has shown detachment of round spermatids between spermatogenic stages VII and VIII [8], the time when the ectoplasmic specialization forms.

Several Sertoli cell lines have been established from 10-day-old H-2Kb-tsA58 transgenic mice carrying a temperature inducible SV40 T-antigen, including the sk11 and sk9 cell line [24,25]. At a culture temperature of 33°C these cells divide, and by switching the temperature to 39°C, division stops. Little is known about the molecular phenotype of these cells, however, they have been reported to express mRNAs for a-inhibin, Steel factor, SGP-2, transferrin, androgen receptor, SF-1 and FSH receptor [25]. Though the mRNA for the FSH receptor is found in these cells, it was down-regulated compared to *in vivo* levels, and the level of functional FSH receptor protein remains unknown. The sk11 cells were later transfected with human wild type FSH receptor, which allowed for continuously active FSH receptor expression [26]. These cells, sk11 TNUA5, showed a dose-dependent increase in cAMP production when stimulated with FSH [26].

This project was designed to determine the effectiveness of the sk11, sk9, and sk11 TNUA5 Sertoli cell lines in binding germ cells *in vitro*. To do this, an established Sertoli-germ cell coculture system was used [19], and the number of spermatids bound to Sertoli cells was determined by morphometric analysis and correlated with the hormone treatments. The ectoplasmic specialization protein espin was also assayed in the cocultures by immunocytochemistry and Western blot analysis, as was the cell adhesion protein N-cadherin. It was hypothesized that the sk11 TNUA5 Sertoli cell line

would be suitable to study FSH effects on Sertoli-germ cell coculture, as defined in the coculture model utilizing primary Sertoli cell isolates [19], and that the sk11 and sk9 cell lines would not.

MATERIALS AND METHODS

Germ cells were isolated from adult male mouse testes and cocultured with the immortalized mouse Sertoli cells in the presence of FSH, testosterone (T), and a combination of these two reproductive hormones [19]. The number of spermatids bound to Sertoli cells was determined by morphometric analysis and correlated with the hormone treatments [19]. Espin, N-cadherin, and FSH receptor were also assayed in these cocultures.

Sertoli cell isolation, culture, and pretreatment

Immortalized mouse sk9, sk11, and sk11 TNUA5 Sertoli cells were cultured on a Matrigel® substrate (BD) in 24-well cell culture trays at either 32ºC or 39ºC, 5% CO_2-95% air and treated with FSH (NIDDK-oFSH-20, AFP7028D, 175xNIH-FSH-S1), T (Sigma) or FSH+T 24 h prior to the addition of mouse germ cells. The culture medium was DMEM (high glucose+L-glutamine) supplemented with 10% fetal calf serum (PAA), 0.01 µl/ml penicillin/streptomycin (Sigma), 0.01 µl/ml antibiotic/antimycotic (Sigma), and 5 µg/ml Plasmocin (Cayla). Cultures were not allowed to grow to confluence, since there was lack of contact inhibition, and the cells did not maintain a monolayer configuration.

Mouse germ cell isolation and coculture

Mouse germ cells were isolated from adult mice using a series of enzymatic treatments [0.5 mg/ml collagenase (Sigma) and 0.25 mg/ml trypsin (PAA)] [19,27] and then filtered through a 74 µm nylon mesh. A total of 400 000 mouse germ cells were added to the Sertoli cell monocultures and incubated for 44 h at 32ºC, 5% CO_2-95% air.

Most Sertoli cell cultures were near confluence at the time of plating. Controls included no hormone treated cocultures and germ cells preincubated for 30 min with the various hormone treatments before being added to no hormone treated Sertoli cells.

Germ cell viability and coculture fixation

Following 44 h of incubation, the cocultures were washed 5 times with warm medium, and the viability of the germ cells in the cocultures was estimated using the Trypan Blue assay. Cocultures were fixed with 4% paraformaldehyde for 20 min at room temperature. Cocultures for immunostaining were fixed with ice cold methanol:acetone (1:1) for 10 min at -20ºC and then allowed to air dry at room temperature.

Morphometry and statistics

Five digital images were taken in a systematic pattern from each well using 20x and 40x objectives. The number of germ cells was determined for each digital image using ImageJ. Germ cells were classified and counted based on size and appearance. Means of germ cell numbers for each treatment group for the three Sertoli cells lines utilized were statistically analyzed using one-way ANOVA followed by Scheffe's multiple-range analysis.

Immunocytochemistry

Sk11 TNUA5 Sertoli cell monocultures and Sertoli-germ cell cocultures were fixed in methanol:acetone (1:1) for fluorescent immunostaining of the ectoplasmic specialization protein espin or fixed in 95% ethanol:5% acetic acid for fluorescent immunostaining of the cell adhesion protein N-cadherin. The fixed cocultures were incubated for 1 h at room temperature with espin (10 µg/ml; Transduction Laboratories) or anti-N-cadherin (2 µg/ml; Zymed) followed by a 1 h incubation at room temperature with Cy3 (1:100; Jackson) as the secondary antibody. The antibody complex was visualized using a fluorescent microscope.

Gel electrophoresis and Western blot

Some Sertoli cell monocultures and Sertoli-germ cell cocultures were collected for Western Blot analysis. After 44 h of incubation, the cultures were washed 5 times with medium and the cells were lysed using a cell scraper and pooled in the various treatment groups. The protein was extracted using homogenization in Buffer A (10 mM HEPES (KOH) pH 7.9, 10 mM KCl, 1 mM DTT, 0.2 mM EDTA, 0.1% NP-40, protease inhibitors (Roche "Complete"), 0.5 mM PMSF) or Crash Buffer (1 M Tris HCl pH 6.8, 20% SDS, 1 M DTT, protease inhibitors (Roche "Complete"), 0.5 M EDTA pH 8). The proteins were separated by SDS-PAGE gel electrophoresis and transferred onto 0.45 µm PVDF membrane. The blots were then stained for espin using the espin antibody mentioned above (1:1000, 1 h room temperature), followed by a 1 h incubation with Cy5 (1:500; Jackson). Membrane bound antibodies were detected using a fluoroimager (Storm 860; Molecular Dynamics) with a laser diode and emission filter for Cy5 (650 nm – 670 nm). The image was viewed using Image Quant 5.0 (Molecular Dynamics).

RNA Isolation

Monocultures of sk11 TNUA5 cells were washed once in serum-free medium and then lifted using a cell scraper and RNAPure (PeqLab). The cells were vortexed for 30 sec and incubated at room temperature for 5 min, after which 600 µl chloroform was added and mixed well. The cell lysate was then centrifuged for 30 min at 4000 rpm, 4ºC. The supernatant was collected and added to 1.5 ml isopropanol. This was then placed on a shaker for 1 h at -20ºC, followed by centrifugation for 1 h at 4000 rpm. The supernatant was aspirated and discarded, and the total RNA was then further purified using the RNeasy Mini Kit (Qiagen), as per manufacturer's instructions. After isolation, RNA integrity was assessed using agarose/GITC gels. The purity was checked by UV-spectrometry in 10 mM Na_2HPO_4/NaH_2PO_4-buffer (pH 7.0).

Real-time RT-PCR

Real-time RT-PCR was used to examine the mRNA expression of FSH receptor, N-cadherin and espin in sk11 TNUA5 Sertoli cells and was performed on a LightCycler™ instrument (Roche). cDNA was synthesized from 1000 ng of total RNA using oligo $dT_{(12-18)}$ (Invitrogen) with Superscript II reverse transcriptase (Invitrogen). PCR was performed using a PCR cocktail containing 10 pmol each gene specific primers (Table 4.1), 2 µl dNTP mix (25 mM each; Takara Bio), 0.5 µl SybrGreen I (1:1000 in DMSO; Molecular Probes), 0.25 µl BSA (20 mg/ml; Sigma), and 0.2 µl Ex-Taq HS (5 U/µl; Takara Bio) in a total volume of 20 µl. Cycling conditions were as follows: denaturation (95°C for 5 min), amplification and quantization (95°C for 10 sec, 60°C for 10 sec, and 72°C for 30 sec, with a single fluorescence measurement at the end of the 72°C segment) repeated 40 times, a melting curve program (60 – 95°C with a heating rate of 0.2°C/sec and continuous fluorescence measurement) and a cooling step to 40°C. The threshold cycle (crossing point) in which the fluorescence rises appreciably above background level was determined by a second derivate maximum method with the use of the LightCycler™ Quantification Software. For exact comparison of mRNA transcription in the different samples the ribosomal gene RPS27a was used as reference gene. In addition to the verification of a single PCR product by the presence of only one melting peak, the PCR products were resolved by electrophoresis on a 1% agarose/TAE gel and checked for correct molecular size.

Table 4.1. Sequences for real-time RT-PCR primers.

Sequences for real-time RT-PCR primers		
Gene	**Sequence**	**TM [ºC]**
RPS27a-5'	CCA GGA TAA GGA AGG AAT TCC TCC TG	64.8
RPS27a-3'	CCA GCA CCA CAT TCA TCA GAA GG	62.4
FSHR-5'	GTG GTC ATC TGT GGT TGC TAC ACC	64.4
FSHR-3'	AAG GAT TGG CAC AAG AAT TGA TGG	59.3
N-Cadherin-5'	CTG CCA ACT GGC TGA AAA TAG ACC	62.7
N-Cadherin-3'	AGT TGG GTT CTG GAG TTT CAC AGG	62.7

RESULTS

Three cell lines reported to express mRNA for the FSH receptor – the sk9, sk11, and sk11 TNUA5 Sertoli cell lines – were used for Sertoli-germ cell coculture. After the addition of germ cells to subconfluent layers of sk9, sk11, and sk11 TNUA5, the lipid accumulation in these cells increased (figure not shown), indicating an increase in phagocytic activity. The sk11 cells appeared to contain the most lipids, although this was not quantified. Upon the addition of hormones and germ cells, the sk9 and sk11 cells reorganized *in vitro* to form tubule-like aggregates (Figure 4.1).

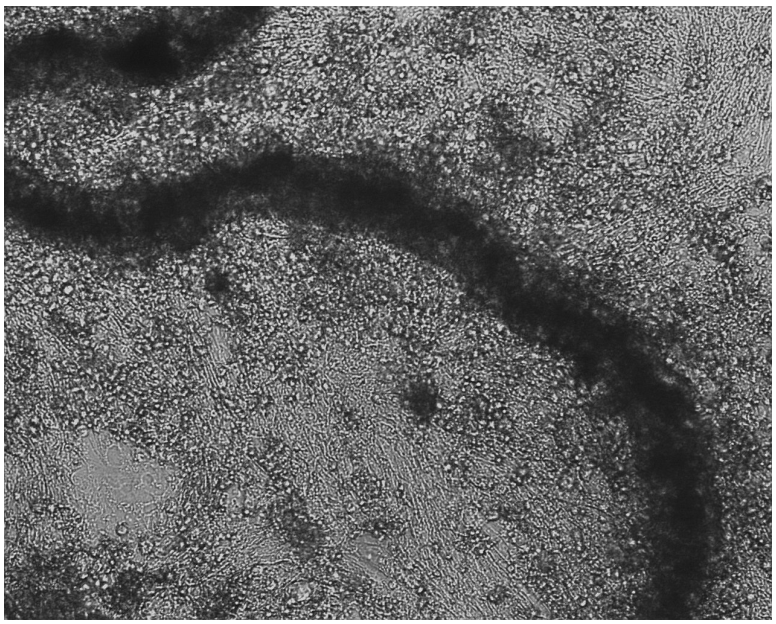

Figure 4.1. Light micrograph of tubule formation in vitro by the sk cell lines after the addition of germ cells and hormones. A light micrograph of an example of the tubule formation observed in the sk cell lines 44 h after adding germ cells and FSH and testosterone (image taken from sk11 cell line).

The number of bound spermatids per hormone treatment can be seen in Table 4.2. A one-way ANOVA ($p<0.05$) determined no significant difference between the hormone treatments and number of pre-step-8 spermatids or step-8 spermatids bound to Sertoli cells from any of the cell lines. No difference was seen in the number of bound spermatids on Sertoli cells incubated prior to germ cell addition at 32ºC or 39ºC.

Table 4.2. Total number of spermatids bounds to immortalized mouse Sertoli cells. Total number of spermatids bound to sk9 cells from 7 cocultures (24 well plates), bound to sk11 cells from 5 cocultures (24 well plates), and bound to sk11 TNUA5 cells from 9 cocultures (24 well plates).

Total number of spermatids bounds to immortalized mouse Sertoli cells				
sk9	**No Hormone**	**FSH**	**T**	**FSH+T**
Pre-step-8	51	38	35	43
Step-8	19	19	16	18
sk11	**No Hormone**	**FSH**	**T**	**FSH+T**
Pre-step-8	1	0	3	1
Step-8	1	0	0	2
sk11	**No Hormone**	**FSH**	**T**	**FSH+T**
Pre-step-8	1	2	6	3
Step-8	3	2	5	5

Since the most promising cell line was thought to be the sk11 TNUA5 line, immunocytochemistry and Western Blot was utilized to identify the presence of specific proteins involved in cell adhesion and the ectoplasmic specialization. The expression of espin in sk11 TNUA5 cells appeared to increase with the addition of FSH+T, as indicated by immunofluorescence staining intensity (Figure 4.2). Western Blot analysis of this protein in these cells is inconclusive. Immunofluorescence of N-cadherin in the sk11 TNUA5 cells has thus far been unsuccessful. However, via Western Blot analysis, N-cadherin was shown to be expressed in the sk11 TNUA5 cell line. Whereas the FSH and T treatments alone appeared to increase N-cadherin expression in these cells, the

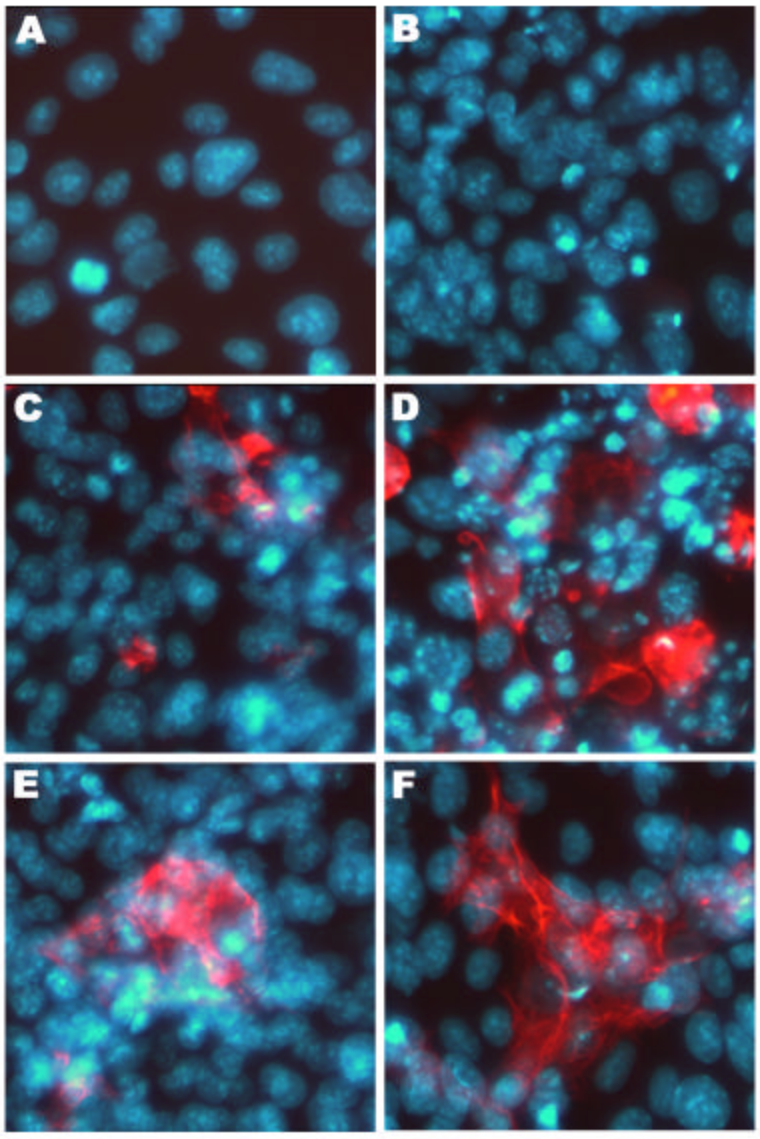

Figure 4.2. Fluorescent immunostaining of espin in immortalized mouse rat Sertoli cell-sperm atid cocultures plated on Matrigel®. (A) Sk11 TNUA5 monoculture immunostained for espin. (B) Sertoli-germ cell coculture using sk11 TNUA5 cells. Negative control for espin immunostaining. (C) Sertoli-germ cell coculture using sk11 TNUA5 cells immunostained for espin in the absence of hormones. (D) Sertoli-germ cell coculture using sk11 TNUA5 cells immunostained for espin in the presence of FSH. (E) Sertoli-germ cell coculture using sk11 TNUA5 cells immunostained for espin in the presence of testosterone. (F) Sertoli-germ cell coculture using sk11 TNUA5 cells immunostained for espin in the presence of FSH and testosterone. Blue=nucleus. Red=espin.

combination of the two hormones, as well as the addition of germ cells, did not appear to affect the expression of this protein (Figure 4.3).

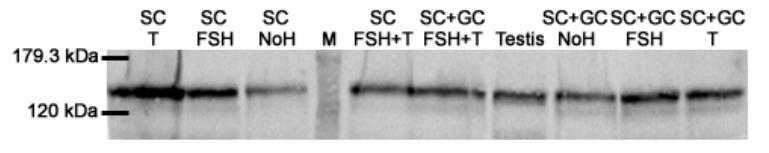

Figure 4.3. Immunodetection of espin in Sertoli cell, germ cell and Sertoli-germ cell coculture lysates. Western Blot for N-cadherin in sk11 TNUA5 cell cultures. SC=sk11 TNUA5 Sertoli cells, GC=germ cells, M=marker, NoH=no hormone, FSH=follicle stimulating hormone, T=testosterone.

Real-time RT-PCR was used to examine the mRNA expression of FSH receptor and N-cadherin in sk11 TNUA5 Sertoli cells in comparison to a control sample (C14 – mice testis, day 30). The crossing points (CP) for FSH receptor and N-cadherin in the cell line are shown in table 2.3. Briefly, the CP for the FSH receptor in sk11 TNUA5 passage 3 cells is 26.82, whereas in passage 17, it is 28.76, and for N-cadherin, sk11 TNUA5 passage 3 the CP is 17.79 and 16.22 in passage 17. The CP is defined as the point at which the fluorescence rises appreciably above the background fluorescence and is therefore a measure for the mRNA amounts.

For exact comparison of mRNA levels in the different samples the ribosomal gene RPS27a was used as reference gene. The CP values for the RPS27a show that this gene is expressed at a constant level in the sk11 TNUA5 Sertoli cells and in the control sample C143 what indicates that there are nearly equal amounts of mRNA starting material (Table 4.3). Figure 4.4 demonstrates the LightCycler PCR results of the sk11 TNUA5 Sertoli cells for the FSH receptor (a) and the N-Cadherin gene (b). Whereas N-cadherin could be detected, it was not possible to quantify mRNA levels for the FSH receptor gene in these cells.

Table 4.3. Crossing points for the FSH receptor and N-cadherin transcripts in the sk11 TNUA5 cell line. The crossing points for the investigated genes in the sk11 TNUA5 cells, using Real Time RT-PCR and performed on a LightCycler™ instrument. The crossing point is defined as the point at which the fluorescence rises appreciably above the background fluorescence and is therefore a measure for the mRNA amounts. FSH-R= FSH receptor; p=passage.

Crossing points for each transcript			
Cell	**RPS27a**	**FSH-R**	**N-cadherin**
sk11 TNUA5 p3	10.44	26.82	17.79
sk11 TNUA5 p17	10.37	28.76	16.22
sk11 TNUA5 p17 +FSH	10.66	28.93	16.20
control C143	9.69	22.65	20.71

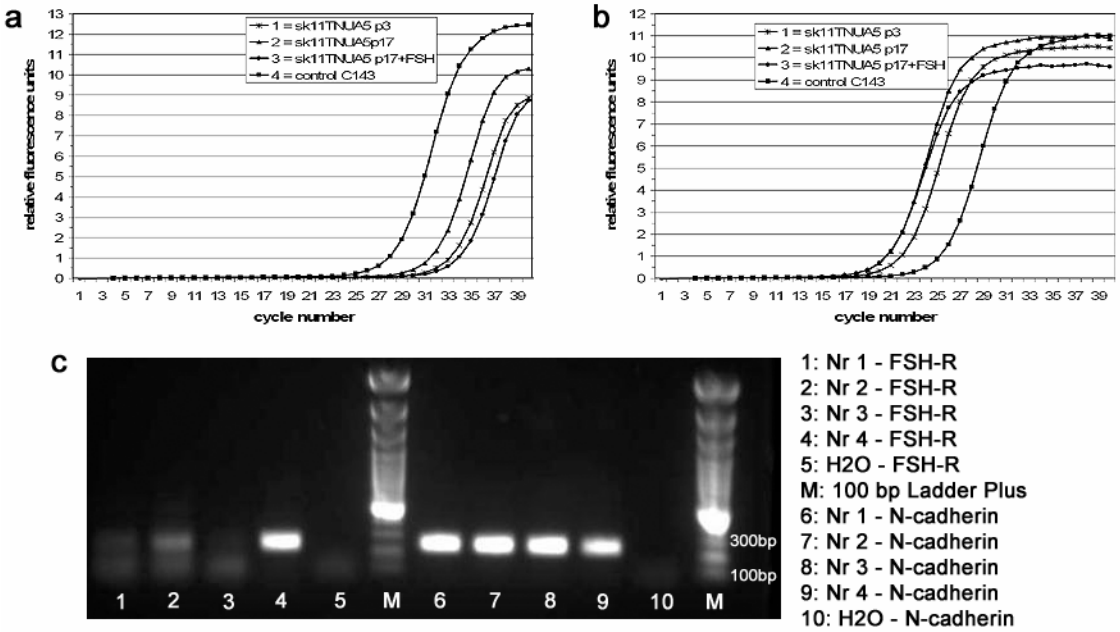

Figure 4.4. RT-PCR results for the FSH receptor and N-cadherin genes. RT-PCR results for sk11 TNUA5 Sertoli cells and a control (C143) for the FSH receptor (a) and the N-cadherin gene (b). (c) 1% agarose/TAE gel of the RT-PCR products. M=100 bp molecular weight marker, lane 5 and 10=no cDNA, water control. p=passage.

DISCUSSION

In vitro studies of the interactions between Sertoli cells and germ cells are time-consuming and expensive, in that the current established method consists of using primary Sertoli cell isolates. Very few Sertoli cell lines exist and most do not possess the receptor for FSH and are therefore insufficient for studying how FSH is involved in the binding dynamics between germ cells and Sertoli cells. The development of a Sertoli cell line that expresses functional FSH receptor protein and supports germ cell binding is of great interest. Three cell lines have been reported to express the mRNA for the FSH receptor – the sk9, sk11, and sk11 TNUA5 Sertoli cell lines [24-26], all established from H-2Kb-tsA58 transgenic mice. Sneddon et al [28] have demonstrated that the sk11 Sertoli cell line maintains the Sertoli cell phenotype in relation to androgen and estrogen receptors, in that the expressed androgen receptor and estrogen receptor-ß induces expression of reporter gene constructs in the presence of a range of steroid ligands [28]. As a result of these studies, these cells would appear to be good candidates for use in Sertoli-spermatid binding studies.

The accumulation of lipids in the sk9, sk11, and sk11 TNUA5 cells was evident following the addition of germ cells, indicating, therefore, the retention of the well-defined phagocytic activity of Sertoli cells. Results from the current study, however, indicate that these cell lines have limited value for the investigation of Sertoli-germ cell binding dynamics *in vitro*.

Although the addition of hormones and germ cells to the sk9 and sk11 cells resulted in the formation of tubule-like structures, there was no apparent binding *in vitro*

between these cells and the added germ cells, resulting in an inappropriate membrane binding domain for the germ cells [19]. It is also possible that the presence of serum in the culture medium, necessary to maintain the viability of the immortalized Sertoli cells, inhibited FSH binding and/or receptor activation [29]. This being the case, there would not likely be FSH-induction of Sertoli cell binding competency, and therefore an inability to bind germ cells [19]. When cultured in medium without serum, but supplemented with retinol, insulin, transferrin and selenium, the cells appeared fusiform and not suitable for binding studies. The RT-PCR results indicate that the sk11 TNUA5 Sertoli cells have almost non-existent levels of FSH receptor mRNA with each passage, thereby providing an explanation for the lack of specific spermatid binding. This is in contrast to Strothmann et al [26], who claim continuous active FSH receptor expression [26] in these cells, showing a dose-dependent increase in cAMP production when stimulated with FSH [26].

With the addition of germ cells, the expression of espin in the sk11 TNUA5 cells, as analyzed via immunofluorescence, appeared to increase in the presence of FSH, but the organization of this protein appeared to be random. Still, in the presence of both FSH and T, espin appeared to be at the periphery of the Sertoli cells, suggesting that this actin binding protein was not involved in cell-cell binding activity in our coculture model.

Via Western Blot analysis, N-cadherin was also detected in the sk11 TNUA5 cell line. Whereas FSH or T treatment alone appeared to increase N-cadherin expression in these cells, the combination of the two hormones, as well as the addition of germ cells, did not appear to affect the expression of this binding protein.

Although the sk11 TNUA5 cells were stably transfected with a human FSH receptor construct [26], these cells lines have limited value for the investigation *in vitro* of

Sertoli-germ cell binding interactions. First, the mRNA for the FSH receptor decreases in amount with successive passages, so that by passage 17 the message is almost non-existent. Second, the actin binding protein espin was expressed with the addition of germ cells, appeared to increase in the presence of FSH and became peripheralized in the presence of both FSH and T together. Still, this response was not associated with germ cell binding. Finally, the sk11 TNUA5 Sertoli cell line also expressed the binding protein N-cadherin, which appeared enhanced by the presence of either FSH or T alone. The combination of these hormones, as well as the addition of germ cells, did not appear to affect the expression of this binding protein, and, like espin, was not associated with binding of germ cells.

Although the sk9 and sk11cells originally expressed mRNA for the FSH receptor, and the sk11 TNUA5 cells are thought to have a functional FSH receptor, these cells are not useful for *in vitro* investigation of Sertoli-germ cell interactions. However, they should not be ruled out for *in vitro* work, such as Sertoli cell biology and/or Sertoli cell interactions with non-germ cell types not requiring FSH.

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CHAPTER 5

Summary

It is theorized that spermatids associated with ectoplasmic specializations adhere to Sertoli cells more strongly than all other germ cells and that this is essential for anchoring spermatids in the seminiferous epithelium during the final stages of spermiogenesis. Additionally, complete spermiogenesis is not observed in the absence of these unique Sertoli-spermatid junctions, which when disrupted, lead to spermatid sloughing and oligospermia [1-3]. However, the actual junctional strength between Sertoli cells and spermatids has never been measured to verify this unsubstantiated dogma central to the successful completion of spermatogenesis.

In this dissertation, for the first time, the actual strength of junctions between germ cells and Sertoli cells *in vitro* has been determined. The data presented confirm the hypothesis that step-8 spermatids are more firmly attached to Sertoli cells than are spermatocytes and pre-step-8 spermatids. Of the cells tested, the ectoplasmic specialization is only present between Sertoli cells and step-8 – step-19 spermatids and is conspicuously absent between Sertoli cells and spermatocytes and pre-step-8 spermatids [4]. This suggests that the structural nature of the ectoplasmic specialization contributes to the actual junctional strength between these two cell types, ensuring that elongating spermatids (post-step-8 spermatids) are securely anchored to the seminiferous epithelium during the final stages of spermiogenesis. This also supports the hypothesis that when the ectoplasmic specialization does not form properly between the Sertoli cell and the periluminal step-8 spermatid, or is otherwise abnormal, the junction strength is significantly lessened, thereby leading to spermatid sloughing and oligospermia [5].

Results from this dissertation show that the junctional strength between Sertoli cells and germ cells can be measured *in vitro*, support long held speculations regarding Sertoli-spermatid junctional interactions, and provide a means to actually test proposed mechanisms of junction dynamics between cells of the seminiferous epithelium.

One such disruptor of cell junctions in the seminiferous epithelium is Adjudin, which is hypothesizes to work at the level of the adherens junction and possibly the ectoplasmic specialization, since depletion of round and elongated spermatids is seen in rats after administration of this potential male contraceptive [6-8]. This dissertation tested the strength of the junction between the step-8 spermatid and the Sertoli cell in the presence of Adjudin *in vitro*. The data presented confirm the hypothesis that this compound, at higher concentrations, disrupts the Sertoli-spermatid junctional complex, causing weaker binding between the Sertoli cell and step-8 spermatid. Though the exact protein complex acting as the receptor for Adjudin has not yet been defined, it is thought that through various signaling pathways (such as the RhoB activation of ROCK, LIMK1, and cofilin [9] and/or the ß1 integrin activation of the FAK/PI 3-kinase/p130Cas/MAP kinase pathway [10]), changes in the polymerization and depolymerization of actin at the ectoplasmic specialization lead to a depletion of germ cells from the seminiferous epithelium, in particular round and elongating spermatids [8]. Results from this dissertation show that the junctional strength between Sertoli cells and step-8 spermatids is reduced by Adjudin *in vitro*, supporting the potential use of this chemical as a male contraceptive.

In vitro studies of the interactions between Sertoli cells and germ cells, such as those in the first and third chapters of this dissertation, are difficult and expensive, in that the current established method consists of using primary Sertoli cell isolates. Very few Sertoli cell lines exist and most do not possess the receptor for FSH and are therefore

insufficient for studying how FSH is involved in the binding dynamics between germ cells and Sertoli cells. The development of a Sertoli cell line that expresses functional FSH receptor protein and supports germ cell binding is of great interest. Three cell lines have been reported to express the mRNA for the FSH receptor – the sk9, sk11 and sk11 TNUA5 Sertoli cell lines [11-13], all established from H-2Kb-tsA58 transgenic mice. As a result of the aforementioned studies and others, these cells would appear to be good candidates for use in Sertoli-spermatid binding studies.

This dissertation explored the potential use of these cells for *in vitro* Sertoli cell-spermatid binding studies. Results indicate that these cell lines have limited value for the investigation of Sertoli-germ cell binding dynamics *in vitro*. Although the addition of hormones and germ cells to the sk9 and sk11 cells resulted in the formation of tubule-like structures, there was no apparent binding *in vitro* between these cells and the added germ cells, resulting in an inappropriate membrane binding domain for the germ cells [14]. It is also possible that the presence of serum in the culture medium, necessary to maintain the viability of the immortalized Sertoli cells, inhibited FSH binding and/or receptor activation [15]. This being the case, there would not likely be FSH-induction of Sertoli cell binding competency, and therefore an inability to bind germ cells [14]. The RT-PCR results indicate, however, that the sk11 TNUA5 Sertoli cells have almost non-existent levels of FSH receptor mRNA with each passage, thereby providing an explanation for the lack of specific spermatid binding. Although the sk11 TNUA5 cells were stably transfected with a human FSH receptor construct [13], these cells lines have limited value for the investigation *in vitro* of Sertoli-germ cell binding interactions, as do the sk11 and sk9 cell lines.

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APPENDICES

APPENDIX 1 – HORMONES

Follicle stimulating hormone

Evidence exists for the idea that spermatogenesis can be completed, and that fertility is possible, without the presence of FSH. Although the testis size and sperm count are reduced in FSHß subunit knock-out mice, they mature normally sexually and fertility is present [1]. The same is seen when the FSH-receptor is disrupted [2, 3]. In the human, a mutation of the FSH-receptor results in a reduction of sperm count but fertility still remains [4]. Spermatogenesis can be induced and restored by testosterone in the absence of FSH, as seen in mice with a GnRH deficiency [5].

However, primate studies have demonstrated a need for FSH in the maintenance of spermatogenesis in both the human and the monkey. Immunization to FSH in the monkey and the human induces impairment in sperm production and quality [6, 7]. An essential role for FSH in man is further supported by the finding of azoospermia in a man with an inactive FSHß subunit [8], which could indicate either the necessity of FSH for the testicular development required for spermatogenesis and/or the establishment and maintenance of spermatogenesis in the adult. The requirement of exogenous FSH in hypogonadotrophic men in order to establish spermatogenesis identifies the need for FSH in the induction of permanent maturational effects on the seminiferous epithelium.

Androgens

Testosterone plays a critical role in normal germ cell development and maintenance of reproductive potential in the male. However, it is the function of the androgens and androgen receptor (AR) that are vital to sexual differentiation and normal spermatogenesis. The binding of testosterone and dihydrotestosterone (DHT) initiates nuclear translocation of the AR-steroid complex, which then regulates the transcriptional function of AR [9]. In the human, DHT has an important role in male reproductive tract development [10], but in the mouse, there is little importance in development for this

hormone [11]. Through autocrine feedback on the Leydig cell, the AR is important in regulating the level of testosterone by endocrine effects of GnRH production and inhibition of LH synthesis and secretion by the pituitary [12]. For example, humans and mice with a hemizygous null mutation in the *Ar* gene demonstrate pseudohermaphroditism and infertility [13, 14], in which an XY individual displays an external appearance of a female [14], with an incomplete formed vagina and small, abdominal testes with early meiotic arrest [13, 15]. Mice homozygous for a mutation in the GnRH gene have considerably lowered serum testosterone levels [5], as well as meiotic arrest of spermatogenesis [16]. However, spermatogenesis can be rescued with androgen replacement [5], whereas FSH alone fails in doing so [17, 18], supporting the idea that it is the androgens, not FSH, that are the major regulators of spermatogenesis. Furthermore, classic hormone withdrawal studies in rats also help verify the requirement of androgens in spermatogenesis. Acute, stage-specific regression of the seminiferous epithelium occurs when androgens are removed from adult rats by hypophysectomy [19, 20]. The manifestation of testosterone loss is seen as a loss of mid-stage round and elongated spermatids, suggesting androgens affect spermiogenesis and spermiation. Loss of mid-stage meiotic spermatocytes is also seen. With long-term hypophysectomy and residual testosterone activity removed, spermatogenesis hardly progresses past meiosis [21, 22]. Replacement of FSH has little effect on the recovery of spermatogenesis, but replacement of LH or androgens does [19, 23, 24]. Similar results are seen with the suppression of GnRH activity [25] and destruction of Leydig cells with EDS [21, 26].

Although androgens have a regulatory positive effect on differentiating germ cells, a negative effect of androgens also exists for the differentiation of spermatogonial

stem cells. Studies in humans and rats that have had therapeutic radiation or chemotherapy for cancer demonstrate a failure of proliferation/differentiation of spermatogonia, but upon treatment with a GnRH agonist or testosterone (both of which decrease intratesticular testosterone concentration), the germ cell population was enhanced by induced spermatogonial mitotic activity and apoptosis inhibition [27, 28].

Androgen receptors (AR) are found in the Sertoli cell, peritubular myoid cell and the Leydig cell [29]. Androgens are necessary for spermatogenesis, and since the AR is not found in the germ cell [29], it is speculated that testosterone works through the Sertoli cell, as does FSH. The concentration of testosterone in the testis is greater than that needed to fully saturate the AR. When this level is reduced experimentally, spermatogenesis is interrupted, but at a testosterone concentration far in excess of that needed for the maintenance of androgen effects elsewhere in the body [30].

Androgen receptor

AR expression in the testis is limited to the somatic cells (Leydig, peritubular myoid, and Sertoli cells) [29, 31-33], but some authors have described the AR in human spermatogonia [34], mouse fetal and postnatal germ cells [33], and rat spermatids [31]. Work with AR-null chimeric mice and from germ cell transplantation studies demonstrate that AR in male germ cells is not necessary for normal fertility [35, 36]. Continuous expression of AR is seen in Leydig and peritubular cells, while a stage-specific expression of AR is seen in the Sertoli cell [29], with testosterone supporting this expression [37]. A DHT responsive promoter is also found upstream of the *Ar* gene [38], thereby further illustrating the idea of androgen/AR auto-regulation of AR expression in the testis. Stages VII – VIII of spermatogenesis demonstrate the highest expression of

AR in the Sertoli cell, corresponding with the stages most affected by androgen withdrawal [19-21].

Estrogens

Surprisingly one of the metabolites of testosterone, estradiol (E_2), also plays a role in the maintenance of fertility in the male, though the effects appear to be secondary and indirect. Estrogens are derivatives of androgens, synthesized by the aromatase complex, which contains the cytochrome p450 enzyme (encoded by the *cyp19* gene) [39]. Though aromatase has been found to be expressed in the Leydig cells [40], elongated spermatids [41], immature Sertoli cells [42], and ejaculated spermatozoa [43, 44], the role of estrogens in spermatogenesis is still not clear.

Estrogen receptor (ER) a has been detected in the Leydig cells and the peritubular myoid cells in the adult rat and mouse testis [33, 45], as well as in the efferent ductules [33, 45, 46]. ERß, however, is found in many cell types, including the Sertoli cell [47-49], early round spermatids, late spermatocytes [48-50], efferent ductules, epididymis, and vas deferens [33, 46, 48, 49]. Within the human testis, there has been a failure to immunolocalize ERa [51, 52], but high levels of ERa are found in the efferent ductules [53]. In contrast, ERß has been detected in the human testis, with the highest level of ERß1 being in round spermatids and the highest level of ERß2 in the Sertoli cells [51-53]. Both ERa and ERß bind E_2 with high affinity.

In mouse studies, targeted disruptions of the ER receptors have been undertaken in order to study the effect of estrogens on reproductive function (knock-out models: ERaKO, ERßKO, and ERaßKO) [54-56]. Aromatase knockout mice (ArKO) have also been created by knocking out *Cyp19* [57]. Other studies have used knockout

mice for estrogen sulfotransferase enzymes [58] and the administration of anti-estrogens [59-61].

Studies of ERaKO mice demonstrate a decline in testicular function in the adult, due to a pressure build-up in the seminiferous tubules as a result of impairment of fluid absorption in the efferent ductules [46]. Germ cell maturation does not require ERa; germ cells transplanted from ERaKO mice produce sperm capable of fertilizing eggs [62]. ERßKO mice have no disruption of testicular function or fertility [54]. ArKO male mice are unable to convert C_{19} steroids (androgens) to C_{18} steroids (estrogens) and exhibit disrupted spermatogenesis as they develop, with the specific defect in spermatid development, manifested as spermatogeneic arrest at early spermiogenesis and increased apoptosis [63, 64]. A large number of round spermatids are found in the semen (spermatid sloughing) of ArKO mice, indicating that the conversion of testosterone to estrogen may be necessary for the formation of the Sertoli-spermatid junctional complex [63].

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APPENDIX 2 – IMMORTALIZED SERTOLI CELL LINES

Currently, the best way to obtain Sertoli cells for *in vitro* studies is via primary isolation. Although this method is very effective, it can also cause a delay in investigation, since several hours are lost during the isolation. Moreover, use of an established cell line could effectively and immensely assist research Sertoli cells function. Currently several lines exist, including the widely used TM4 cells from the mouse [1]. Other cell lines that have been established are the mouse Sertoli cell 1 (MSC-1) [2], 15P-1 (mouse) [3], 45T-1 (mouse) [3], 42GPA-9 (mouse) [4], SF-7 (mouse) [5], TR-ST (rat) [6], ASC-17D (rat) [7], and the TTE-3 (mouse) [8]. Many other Sertoli cell lines have been established, but little has been reported on them. None of these cell lines have been suitable for the study of Sertoli cell-spermatid interactions, mainly due to a lack of FSH-receptor expression.

Several Sertoli cell lines (labeled sk) have been established from 10-day-old H-2Kb-tsA58 transgenic mice carrying a temperature inducible SV40 T-antigen and exhibit a genetic phenotype like those *in vivo* [9, 10]. As a result of this antigen, these cells divide at a culture temperature of 33°C and stop division at a temperature of 39°C [9]. Within two days at the higher, nonpermissive temperature, a change in cell morphology is observed [9]. No indication of massive apoptosis is apparent in the culture [9]. When returned to the lower, permissive temperature, the cells resume the morphology of dividing cells and proliferate [9]. Protein synthesis is not affected by the shift to the nonpermissive temperature [9].

Little is known about the molecular phenotype of these cells. However, they have been reported to express mRNAs for a-inhibin, Steel factor, sulfated glycoprotein-2, transferrin, androgen receptor, GATA-1, and FSH-receptor [9, 10]. Immunocytoskeletal analysis of cytoskeletal proteins demonstrated the presence of a-

smooth muscle actin, neurofilament protein 200, and vimentin in a pattern exactly corresponding to that of Sertoli cells *in vivo* [9], indicating further that these cells are of Sertoli cell origin. Although the mRNA for the FSH-receptor was found in these cells, it was down-regulated compared to *in vivo* levels, and the level of functional FSH-receptor protein remains unknown. Sneddon et al [11] have demonstrated that the sk11 Sertoli cell line (one of the sk cell lines) maintains the Sertoli cell phenotype in relation to androgen and estrogen receptors, in that the expressed AR and ERß induced expression of reporter gene constructs in the presence of a range of steroid ligands [11]. These sk11 cells were later transfected with human wild type FSH-receptor, which allowed for continuously active FSH-receptor expression [12]. These cells, sk11 TNUA5, showed a dose-dependent increase in cAMP production when stimulated with FSH [12]. These cells may provide a possible alternative to primary isolations in Sertoli cell studies *in vitro*.

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