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Cell Junction Dynamics in the Testis: Sertoli-Germ Cell Interactions and Male Contraceptive Development

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Cheng, C. Yan, and Dolores D. Mruk. Cell Junction Dynamics in the Testis: Sertoli-Germ Cell Interactions and Male Contraceptive Development. *Physiol Rev* 82: 825–874, 2002; 10.1152/physrev.00009.2002.—Spermatogenesis is an intriguing but complicated biological process. However, many studies since the 1960s have focused either on the hormonal events of the hypothalamus-pituitary-testicular axis or morphological events that take place in the seminiferous epithelium. Recent advances in biochemistry, cell biology, and molecular biology have shifted attention to understanding some of the key events that regulate spermatogenesis, such as germ cell apoptosis, cell cycle regulation, Sertoli-germ cell communication, and junction dynamics. In this review, we discuss the physiology and biology of junction dynamics in the testis, in particular how these events affect interactions of Sertoli and germ cells in the seminiferous epithelium behind the blood-testis barrier. We also discuss how these events regulate the opening and closing of the blood-testis barrier to permit the timely passage of preleptotene and leptotene spermatocytes across the blood-testis barrier. This is physiologically important since developing germ cells must translocate across the blood-testis barrier as well as traverse the seminiferous epithelium during their development.

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We also discuss several available in vitro and in vivo models that can be used to study Sertoli-germ cell anchoring junctions and Sertoli-Sertoli tight junctions. An in-depth survey in this subject has also identified several potential targets to be tackled to perturb spermatogenesis, which will likely lead to the development of novel male contraceptives.

I. CHANGES IN SERTOLI-GERM CELL INTERACTIONS AND JUNCTION DYNAMICS DURING SPERMATOGENESIS: AN OVERVIEW

In mammals, the functional unit of the testis is the seminiferous tubule. Each seminiferous tubule is about 1 m in length and 0.5 mm in diameter (for review, see Ref. 366). Figure 1 shows the cross-section of a typical seminiferous tubule from an adult rat testis. The close morphological association between Sertoli cells and germ cells at different stages of their development (such as spermatogonia, spermatocytes, round spermatids, and elongated spermatids) is clearly visible in the seminiferous epithelium (Fig. 1). As a result of such morphological intimacy between Sertoli and germ cells, it is conceivable that extensive interactions and communications take place between these cells throughout spermatogenesis both at the biochemical and molecular level. Indeed, morphometric analysis of the adult rat testis has shown that each Sertoli cell is associated with ~30–50 germ cells at each stage of the spermatogenic cycle in the epithelium (480, 497), illustrating not only that germ cell development relies heavily on the Sertoli cell but that extensive communications take place to coordinate the various events of spermatogenesis. Studies from the past two decades have indeed demonstrated that germ cells largely rely on Sertoli cells for structural and nutritional support (for reviews, see Refs. 105, 221, 379). For instance, in the

FIG. 1. Cross-section of a seminiferous tubule from an adult Sprague-Dawley rat showing the organization of testicular cells and the intimate relationships between Sertoli and germ cells. S, Sertoli cell nucleus; SG, spermatogonium; PS, pachytene spermatocyte; RS, round spermatid; ES, elongated spermatid. [Adapted from Mruk and Cheng (308); courtesy of Dr. Li-Ji Zhu.]

rat, the entire process of germ cell development, except for the early phase of spermatogenesis from type B spermatogonia up to preleptotene and leptotene spermatocytes, is segregated from the systemic circulation because of the blood-testis barrier (BTB) created by tight junctions (TJ) between Sertoli cells near the basal lamina (128, 129, 131, 380, 400, 401). As such, germ cells and Sertoli cells develop an intimate and elaborate cellular network for cell-cell communications via paracrine factors and signaling molecules, so that Sertoli cells can provide developing germ cells with the needed nutrients and biological factors (for reviews, see Refs. 61, 62, 133, 147, 221, 416). Indeed, in vitro studies have shown that there is bidirectional trafficking between Sertoli and germ cells and that each cell type regulates the function of the other (20, 21, 221, 416).

Throughout spermatogenesis, different biochemical, cellular, and molecular events take place in the seminiferous epithelium leading to the formation of eight spermatids (haploid) from a single type B spermatogonium (diploid) (for reviews, see Refs. 117, 379). Furthermore, preleptotene and leptotene spermatocytes must migrate progressively from the basal to the adluminal compartment of the seminiferous epithelium traversing the BTB, while differentiating into haploid spermatids (Fig. 1). Without this timely movement of developing germ cells across the seminiferous epithelium, spermatogenesis cannot go to completion, and infertility will result. Moreover, this event of cell movement is accompanied by extensive restructuring of cell-cell actin-based adherens junctions (AJs) between Sertoli and germ cells, such as ectoplasmic specializations (ES) (for reviews, see Refs. 329, 330, 402). Although the subject of spermatogenesis, in particular its morphological changes and hormonal regulation, has been extensively studied (for reviews, see Refs. 117, 221, 378, 379, 404), the subject of cell junction restructuring pertinent to spermatogenesis from a biochemical and molecular standpoint has largely been neglected. In this review, we attempt to provide an updated review in this subject area. However, it must be noted that much of the information discussed herein is derived from investigations in other epithelia, but a significant amount of work has also been done in the testis investigating molecules pertinent to junction dynamics in the past decade. As such, every effort was made to refer to recent studies in the testis.

In mammals, spermatogenesis is composed of three distinct phases of cellular and molecular changes (for reviews, see Refs. 117, 379). *1*) Mitosis is proliferation of type A spermatogonia, some of which will differentiate into type B spermatogonia (for reviews, see Refs. 123, 126). These in turn will differentiate into preleptotene and leptotene spermatocytes, which are the germ cells that will traverse the BTB entering into the adluminal compartment. This phase takes place in the basal lamina outside of the BTB (Fig. 2). *2*) The meiotic phase is when primary spermatocytes divide and differentiate into secondary spermatocytes and haploid spermatids. This phase largely takes place behind the BTB in the adluminal compartment (Fig. 2). *3*) Spermiogenesis is the morphogenesis of spermatids into spermatozoa, which is accompanied by extensive changes in the nucleus such as nuclear condensation (Fig. 2) (94, 105, 369). The fully developed spermatids (spermatozoa) will then leave the seminiferous epithelium via spermiation. In the mouse, the differentiation of haploid round spermatids into spermatozoa can be morphologically divided into 16 steps (379) (Fig. 2). During *steps 1–7* of spermiogenesis, round spermatids develop acrosomes and flagellae (Fig. 2). At *step 8*, the heads of spermatids orientate toward the basal compartment of the epithelium. From *steps 9* to *13*, spermatids undergo a series of morphological changes, which include nucleus condensation and elongation of the flagella. At *steps 14* and *15*, alignment of mitochondria along the elongating flagella takes place. At *step 16*, spermatids are translocated to the adluminal surface of the epithelium to be released into the lumen at spermiation. Throughout these steps (Fig. 2), developing germ cells remain attached to the epithelium via a modified type of cell-cell anchoring junction with actin filament attachment sites (i.e., AJs) specific to the testis known as the ES (Fig. 2) (for reviews, see Refs. 343, 374, 380, 470).

In the seminiferous epithelium, the association between Sertoli and germ cells throughout these three phases of development as described above are arranged into defined stages. In the rat, these stages follow one another giving rise to the wave of the seminiferous epithelium along the seminiferous tubule (for review, see Ref. 339). Using periodic acid-Schiff (PAS) staining to visualize changes in the shape of the nucleus and acrosome of germ cells during their development in the seminiferous epithelium, the spermatogenic cycle is divided into 14 stages in the rat (258) (Fig. 3) and 12 stages in the mouse (379). Interestingly, different stages of the cycle in the seminiferous epithelium can be readily visualized by the transillumination pattern of freshly isolated rat seminiferous tubules by stereomicroscopy largely because of the changes in nuclear condensation of the sperm head (for review, see Ref. 339) (Fig. 3). In the rat, one spermatogenic cycle takes ~12–14 days to complete (130, 258) and ~8–9 days in the mouse (379). Studies using [^{3}H]thymidine, however, have shown that it takes ~54 and ~35 days for a single spermatogonium to complete spermatogenesis and give rise to eight haploid spermatids in the rat (105, 117, 379) and the mouse (379), respectively. This is because developing germ cells must go through the cycle 4.5 times before they can become fully developed spermatids (spermatozoa) that are released into the tubular lumen at stage VIII of the cycle (403). Given this exten-

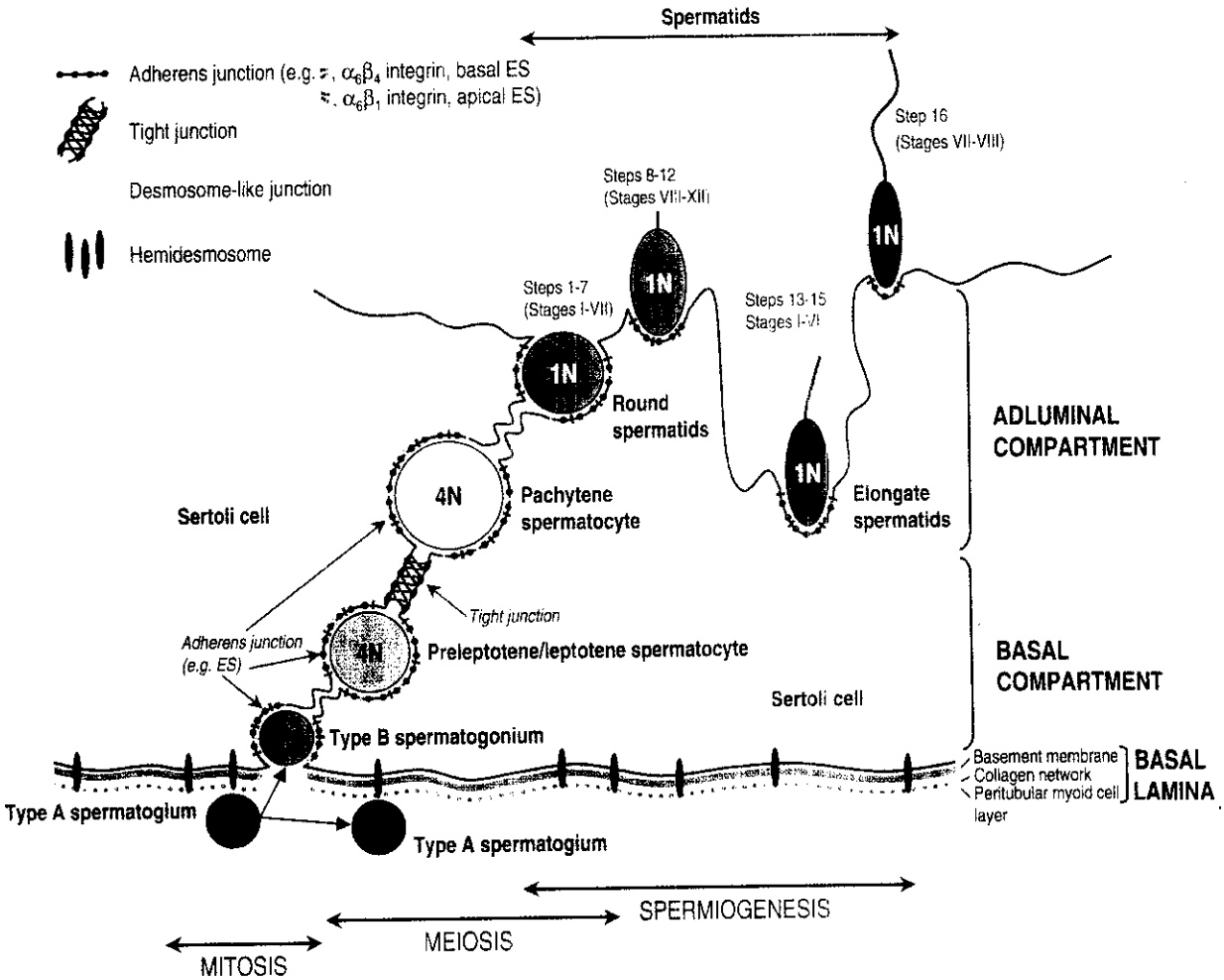

FIG. 2. A schematic drawing that illustrates extensive changes in tight junction (TJ) and cell-cell actin-based adherens junction (AJ) dynamics during spermatogenesis and spermiogenesis in the mouse. This figure was prepared based on reviews and reports cited in sections III and V. Among the AJs in the testis, four functional complexes are known to exist to date, which include cadherin/catenin complex, nectin/afadin complex, tubulobulbar complex, and ES (see sect. V). The ES is composed of basal and apical ES constituted possibly by $\alpha_6\beta_4$- and $\alpha_6\beta_1$-integrins, respectively (310, 470); however, their binding partner(s), if any, is not known. It is possible that laminin $\alpha_1\beta_1\gamma_1$-chains and γ_3-chains constitute the binding partners for the basal and apical integrins in the ES, respectively (244a) (see sect. V*C1*). While it is certain that $\alpha_6\beta_1$-integrins are found between Sertoli cells and developing spermatids in the apical ES (470), it remains to be determined if $\alpha_6\beta_4$-integrins can be found between Sertoli cells and developing spermatocytes and spermatogonia (type B) in the basal ES, or it is restricted only to the interface of Sertoli cells and the basement membrane. ES, ectoplasmic specialization, a modified testis-specific AJ.

sive cell movement across the epithelium, it is conceivable that there is extensive restructuring at the interface of Sertoli-germ cells at the adherens junction level. Furthermore, the biochemical, molecular, and cellular events pertinent to spermatogenesis are under endocrine control of the hypothalamic-pituitary-testicular axis (for reviews, see Refs. 146, 339, 403, 428). In this review, we limit our discussion on the biology and regulation of Sertoli-germ cell interactions to the level of cell junctions, in particular, occluding and anchoring junctions, and how current advances in these two areas have widened the possibilities of developing innovative male contraceptives.

II. JUNCTION DISASSEMBLY AND REASSEMBLY ARE PERTINENT TO GERM CELL MOVEMENT DURING SPERMATOGENESIS

As described in section I, extensive interactions between Sertoli and germ cells take place in the seminiferous epithelium to coordinate the intermittent events of disassembly and reassembly of Sertoli cell AJs and TJs and Sertoli-germ cell AJs to facilitate the movement of germ cells across the epithelium (see Figs. 1–3). In this section, as well as in sections III and V, we have reviewed the current status of research in

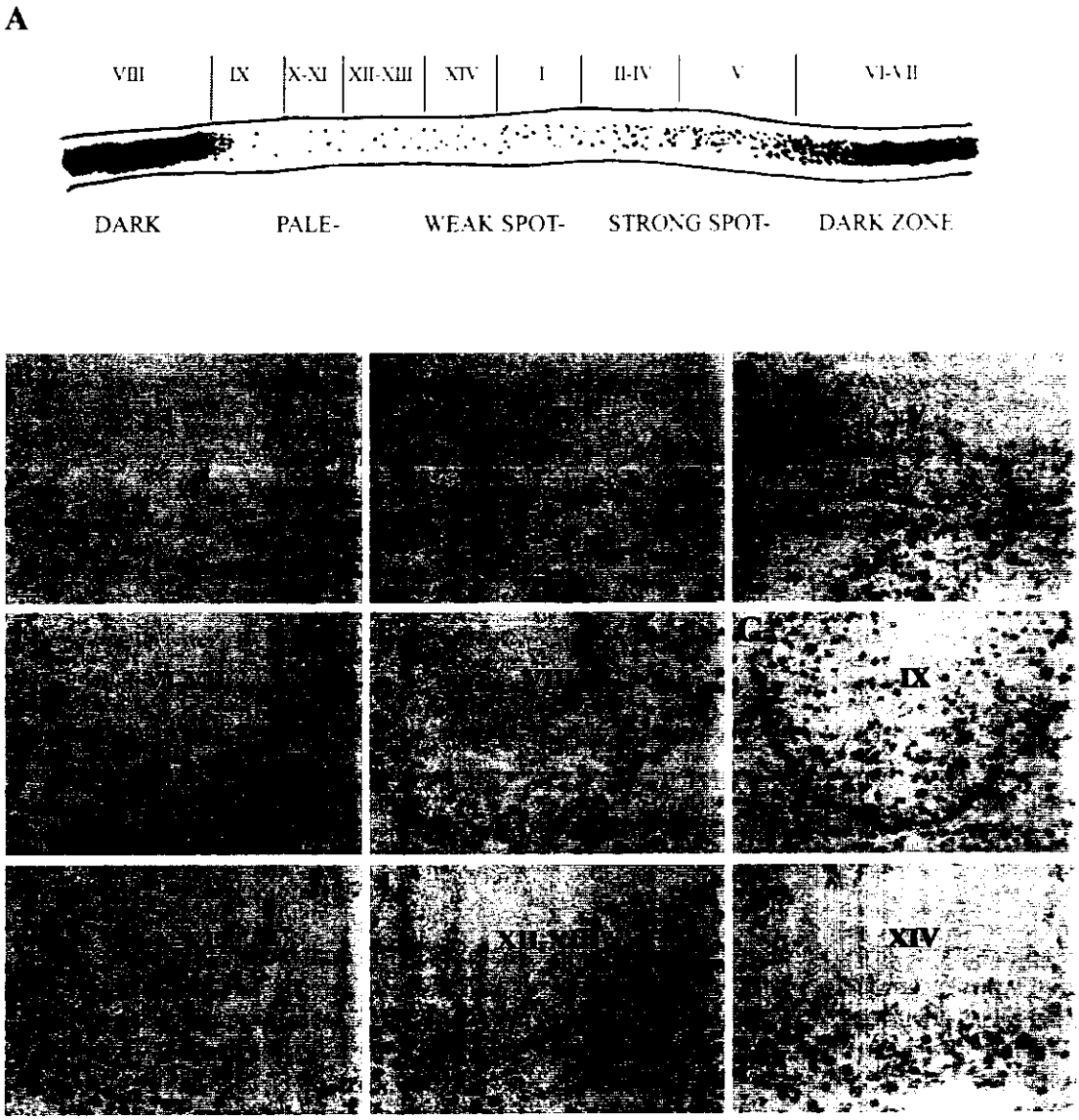

FIG. 3. Shown are stages I–XIV of the cycle of the seminiferous epithelium in the rat as determined by transillumination pattern of the freshly isolated seminiferous tubules from adult rats (*A*) and cross-sections of the tubules in the testis that show the unique association between Sertoli cells and developing germ cells in each stage of the cycle (*B–J*). *A*: this is the schematic drawing of the different zones of a seminiferous tubule from adult rats under a transilluminator stereomicrocope, which can be divided into different zones, namely, pale, weak spot, strong spot, and dark zone representing different stages of the spermatogenic cycle. The different zoning pattern is the result of different numbers of condensed nuclei in developing germ cells associated with Sertoli cells at different stages. [Modified from Parvinen (339).] *B–J*: these are micrographs of cross-sections, 8 μm, of frozen testes from adult rats stained with hematoxylin, corresponding to seminiferous tubules at stage I (*B*), II–IV (*C*), V (*D*), VI–VII (*E*), VIII (*F*), IX (*G*), X–XI (*H*), XII–XIII (*I*), and XIV (*J*). They illustrate the unique pattern of association of developing germ cells, such as spermatocytes, spermatids, and elongated spermatids, with Sertoli cells in the seminiferous epithelium that gives rise to the different zones under transillumination microscopy shown in *A*, representing different stages of the spermatogenic cycle from I to XIV. Frozen sections were fixed in modified Bouin's fixative and stained with hematoxylin using techniques as previously described (171, 512, 513, 516, 517). Bar = 20 μm.

the dynamics of cell junctions in the testis. Lastly, we have introduced two new innovative approaches for male contraception that are based on current research by perturbing the functions of TJs (see sect. IV) and AJs (see sect. VI) in the testis.

A. Cell Junctions in the Testis

1. *Introduction*

Morphological studies of the testis performed in the 1970s and 1980s have identified many junction types com-

TABLE 1. *A functional classification of cell junctions in the testis and their component proteins*

Junction Type	Component Proteins
Occluding junctions	
Tight junctions	TJ-integral membrane proteins: **occludin**, **occludin-1B**, **claudin-1**, **-3**, **-4**, **-5**, **-7**, **-8**, **and -11** (at least 24 claudins have been identified as transmembrane proteins in other epithelia) and junctional adhesion molecules (JAM) TJ-associated proteins: **ZO-1**, **ZO-2**, ZO-3, **cingulin**, 7H6 antigen, **symplekin**, **actin**, ZAK, ZA-1 TJ, AF-6, fodrin, Gα, PKC, 19B1, BG9.1, Rab3B, **Rab8**, **Rab13**, c-*yes*, **Src**, VAP-33, Sec 6/8
Anchoring junctions or adhering junctions	
With actin filament attachment sites	
Cell-cell adherens junctions or adhesion belts	**N-cadherin**, **E-cadherin**, **α-** and **F-actin**, tensin, **α-actinin**, **α-**, **β-** and **γ-catenin**, **vinculin**, plakoglobin, **Src**, **CK-2**, **p120^{ctn}**, **FAK**, **Csk**, **paxillin**, radixin, **Fer kinase**, **GSK-3β**, **ILK**, **fimbrin**, **espin**, **nectins**, **afadins**, **testin**, **myosin VIIa**, **ponsin**, **gelsolin**, **Fyn**, **keap1**
There are two specialized *adherens junctions* unique to the testis: *1*) ectoplasmic specializations (can be found between Sertoli and germ cells, in particular spermatids) and *2*) tubulobulbar complexes (can be found between Sertoli cells and elongated spermatids)	
Cell-matrix adherens (e.g., *focal contacts*, can be found between testicular cells and extracellular matrix at the basal lamina)	**Integrins** (at least 20 different integrins are known to exist; but only **α_1-**, **α_3-**, **α_5-**, **α_6-**, **α_9-**, **β_1-**, and **β_4**-subunits have been positively identified in the testis), **actin**, **α-actinin**, **vinculin**, talin, **nexilin**, **paxillin**, zyxin, tensin, **p130cas**, Src kinase, **FAK**, Grb2, SOS, **Ras**, **collagen**, **fibronectin**, **laminin α_1**, **β_1**, **γ_1**, **γ_3**
With intermediate filament attachment sites	
Cell-cell (*desmosomes* can be found between Sertoli cells as well as between Sertoli and germ cells)	Desmocollins, desmogleins, desmoplakin, **plakoglobin**, plakophilin, **p120^{ctn}**
Cell matrix (*hemidesmosomes* can be found at the basal lamina)	**Integrin**, desmoplakin-like protein, **paxillin**
Communicating junctions	
Gap junctions (can be found between Sertoli cells, between Leydig cells, and between Sertoli and germ cells)	**Connexins 26**, 30.3, **31**, **31.1**, **32**, **33**, **36**, **37**, **40**, **43**, **45**, **46**, **50**, **57**

This table was prepared based on earlier reviews and reports (see Refs. 4, 12, 55, 61, 62, 67, 69, 77a, 133, 138, 177, 180a, 240, 274, 298, 328, 374, 377–380, 395, 416, 432, 447, 461, 493, 505, 515). **Underlined** and **boldfaced** component proteins indicate those that have been positively identified in the testis. This list is not intended to be exhaustive, and readers are encouraged to find additional information in the cited reviews and reports. TJ, tight junction.

mon to other epithelia (Table 1) (61, 62, 117, 221, 342, 377, 380). The three types of junctions found in the testis are as follows: occluding, anchoring, and communicating gap junctions (GJ) (see Table 1). Other structural modifications of AJs that use actin filaments as their attachment sites (see Table 1), such as ES and tubulobulbar complexes, are unique to the testis (Table 1). In general, the relative locations of these junctions in different epithelia are the same; TJs occupy the most apical portion of cells, followed by AJs, and then by a parallel row of desmosomes. Altogether these structures form the "junctional complex." On the other hand, GJs and additional desmosomes are not integrated tightly with TJs and AJs and can be scattered around the epithelium, which in turn anchor cells onto the extracellular matrix (ECM). As such, TJs are furthest away from the ECM (for review, see Ref. 4). In the testis, however, the location of TJs relative to anchoring junctions and GJs is different from other epithelia since Sertoli cell TJs that create the BTB are found at the basal compartment of the seminiferous epithelium adjacent to the basal lamina closest to the basement membrane (see Table 1 and Figs. 1–3), which is a specialized form of ECM in the testis (for review, see Ref. 127). The classification of the three junction types in mammalian tissues and their constituent proteins are summarized in Table 1. The only known occluding and communicating junction types are the TJ and GJ, respectively (Table 1). However, there is more than one type of anchoring junction, which are classified as follows (see Table 1). In this review, we limit our discussion on TJs and anchoring junctions (in particular the cell-cell actin-based adherens junctions, AJs), since a sufficient body of work has been conducted in these areas, forming the basis for future studies to understand the biology of junction dynamics in spermatogenesis and for contraceptive development. An excellent review on GJs in the testis can be found elsewhere (see Ref. 133).

2. *Classification of anchoring (or adhering) junctions*

Anchoring junctions are abundant in many tissues, in particular those subjected to mechanical stress (for review, see Ref. 4). Anchoring junctions physically connect cytoskeletal elements of one cell to a neighboring cell or to the ECM. Altogether, there are four types of anchoring junctions (for review, see Ref. 4): *1*) adhesion belts or adherens junctions (AJs), *2*) focal contacts, *3*) desmosomes, and *4*) hemidesmosomes (for review, see Ref. 4). Anchoring junctions that connect two cells together are known as adhesion belts (*or* adherens junctions, AJs) and

desmosomes; those that connect cells to the extracellular matrix are focal contacts and hemidesmosomes (see Table 1). AJs and focal contacts utilize actin filaments as attachment sites, whereas desmosomes and hemidesmosomes use intermediate filaments as attachment sites (Table 1). AJs, zonula adherens or adhesion belts, are located in the apical domains of cells below TJs and are constituted largely by Ca^{2+}-dependent cell adhesion molecules (CAMs), also known as cadherins (for reviews, see Refs. 4, 177, 443). Cadherins in turn attach to intracellular attachment proteins, such as β- and γ-catenin. This cadherin/catenin complex associates with the underlying actin filament bundles via its interactions with vinculin and α-actinin (for reviews, see Refs. 177, 443). It must be noted that in the testis, AJs between Sertoli and germ cells are morphologically different from those in other epithelia, and the best-described modified testis-specific AJ structures are ES and tubulobulbar complexes (62, 378, 470, 471). Herein, we focus our review largely on the cell-cell actin-based AJs. Because several excellent reviews on desmosomes, focal contacts, and hemidesmosomes in other epithelia can be found elsewhere (for reviews, see Refs. 4, 9, 45, 52–56, 229, 246, 247, 396, 397), we will not elaborate on these junction types in this review.

B. Technical Difficulties in Elucidating the Events of Cell-Cell Interactions During Spermatogenesis Pertinent to a Defined Physiological Phenomenon, Such as Germ Cell Movement

Although germ cell movement across the seminiferous epithelium is one of the most important and interesting biological phenomena during spermatogenesis, very few studies have been performed to examine the participating molecules and the mechanism by which this event is regulated. Our belief is that these studies are significant because not only can they expand our knowledge of spermatogenesis pertinent to junction restructuring, but a thorough understanding of the biology of germ cell movement should lead to the development of novel and safer male contraceptives. We have hypothesized that if germ cells are induced to translocate across the epithelium rapidly, even before they complete their development, germ cells found in the seminiferous tubular lumen will be immature and lack the ability to fertilize the ovum (308). This can also be achieved by perturbing cell adhesion in the testis prompting the depletion of germ cells from the seminiferous epithelium. Alternatively, if germ cell movement is hampered and germ cells are retained in the epithelium for a prolonged period of time, they will become "aged" and be removed by Sertoli cells via phagocytosis (308). In both instances, infertility will result. A disruption of fertility by this approach is likely to induce minimal side effects since the hypothalamus-pituitary-testicular axis is not disrupted. However, a model to study the events of germ cell movement is lacking. If such a model were available, it could be used to study the cascade of events leading to germ cell movement. This, in turn, could be used to identify target genes and/or proteins that perturb cell movement and/or cell adhesion. Once the technique to obtain staged tubules for in vitro studies became available (339), studies were performed to identify target genes that associated with germ cell movement, such as spermiation (61, 62, 147). For instance, it was shown that the expression and/or accumulation of proteases, such as cathepsin L (a cysteine protease), was highest in stages V–VII of the cycle preceding spermiation (499, 508), which occurs in stage VIII, illustrating its possible involvement in disrupting elongated spermatid-Sertoli cell junctions at spermiation. Subsequent studies by in situ hybridization have also confirmed the predominant but transient expression of cathepsin L at stages V–VII (86, 345). Interestingly, the expression of cathepsins D (an aspartic protease) and S (also a cysteine protease), in contrast to cathepsin L, was shown to be predominant not only at stage VII preceding spermiation but also high at stages VIII–XII (86). These results thus challenge the notion that cathepsin L is involved in spermiation. Needless to say, there are many biochemical and molecular events occurring at stages V–VII, some of which are for purposes entirely unrelated to the events of spermiation or cell movement. As such, it is crucial that in vitro functional studies be performed first to determine which target genes and/or proteins are involved in the dynamics of TJs or AJs. This should be followed by in vivo studies to examine if these changes indeed occur in the seminiferous epithelium during junction restructuring, such as analyzing junction-associated proteins in cross-sections of staged tubules in the testis during the spermatogenic cycle. This can be followed by additional studies to investigate if a specific cellular event can be perturbed by manipulating the function of a molecule or a group of molecules to assess whether this can disrupt spermatogenesis. In this context, it is noteworthy to mention that the expression of a target gene, such as occludin, that is not stage specific in the testis (110) does not necessarily preclude its involvement in junction dynamics. Indeed, occludin is an important regulator of TJ dynamics (138, 298). Thus a molecule that is crucial to junction restructuring may not necessarily be stage specific unless it is linked to a specific event, such as spermiation.

C. In Vitro Model to Study Sertoli Cell TJ Dynamics

Sertoli cells cultured in vitro in chemically defined serum-free medium have been used to study the biology and regulation of the TJ-permeability barrier for almost two decades (60, 167, 218–220, 329). This model was used

in our laboratory to identify several target genes that are implicated in the regulation of Sertoli cell TJ dynamics, such as occludin, claudin-11, ZO-1, α_2-macroglobulin, and others (82–86, 169, 263, 264, 270, 271, 389, 495). Furthermore, it was shown that transforming growth factor (TGF)-β3 perturbed the Sertoli cell TJ-permeability barrier in vitro via its effects on the expression of occludin, ZO-1, and claudin-11 (270), utilizing the p38-mitogen-activated protein (MAP) kinase signaling pathway (271). The novelty of this model to study Sertoli TJ dynamics is as follows. First, Sertoli cells are cultured in chemically defined serum-free medium; as such, it can be used to identify molecules that play a crucial physiological role in regulating TJ dynamics. Indeed, with the use of this model, studies from various laboratories have shown that testosterone, cAMP (82, 218, 219), and protease inhibitors (329) are important regulators of Sertoli cell TJ dynamics. Second, this model coupled with the use of other techniques, such as restricted diffusion of FITC-dextran, [^{3}H]inulin, ^{125}I-BSA; polarized secretion of Sertoli cell proteins, such as α_2-macroglobulin, testin, rat androgen binding protein, and clusterin; and the measurement of transepithelial electrical resistance (TER) across the Sertoli cell epithelium, can quantitatively assess the dynamics of the TJ barrier (82, 83, 166, 167, 270, 271). Indeed, this model was used in conjunction with $CdCl_2$ to study the Sertoli cell TJ dynamics (82, 219). Preliminary studies have illustrated that this is a reliable in vitro model to delineate the possible cascade of events pertinent to TJ disassembly and reassembly (82).

D. Current Concepts in the Regulation of Sertoli Cell TJ Dynamics

Three theories are found in the literature explaining the events of Sertoli cell TJ disassembly/reassembly during spermatogenesis. First, the "zipper theory," which proposes that inter-Sertoli TJs or occluding zonules, consisting of fibrils that completely encircle the basal domains of Sertoli cells, break down to accommodate the passage of preleptotene or leptotene spermatocytes while new occluding zonules reform under the migrating preleptotene spermatocytes (142, 342–344). However, there are no in vivo studies showing leakage of tracers into the tubular lumen even for a short period of time. Second, the "intermediate cellular compartment theory" proposed by Russell (375a) suggests the presence of a compartment occupied by germ cells in transit from the basal to the adluminal compartment. However, subsequent morphological studies have shown that there is only one occluding zonule per Sertoli cell at any one time, making it unlikely that such a compartment exists, because if it does, one should be able to visualize a preleptotene spermatocyte trapped in between two occluding zonules (343, 344, 380). Also, both theories do not explain what triggers the dissociation and association of TJ fibrils to facilitate the movement of preleptotene and leptotene spermatocytes across the BTB. For instance, if the disrupted fibrils, which are composed of TJ-associated proteins, such as occludins and claudins, can be repaired rapidly, a sophisticated signaling/trafficking system between Sertoli and germ cells must exist. How can this be achieved and regulated? Because germ cells do not possess an extensive cytoskeleton network, their movement is likely dependent on Sertoli cells. What is the mechanism(s) that signals Sertoli cells to facilitate germ cell movement? Are both Sertoli and germ cells equipped with the necessary signaling molecules to regulate their interactions that triggers cell movement? As such, there are many open questions that remain to be addressed. Third, the "stress theory" or "repetitive removal of membrane segments theory" proposed by Pelletier and Byers (342, 343) suggests that the continuous upward migration of a large number of germ cells creates a stress against the Sertoli cell occluding zonule. This may result in junction proliferation, changes in orientation, and disintegration in the fibrils composing the occluding zonule (344, 342). This theory, however, does not explain what triggers and facilitates the upward movement of germ cells. Also, how can stress induce changes in junction orientation and breakage in the TJ fibrils?

III. OCCLUDING JUNCTIONS: TIGHT JUNCTIONS AND THE BLOOD-TESTIS BARRIER

A. Functions of TJs in the Testis

The only known occluding junction type in mammalian tissues, including the testis, is the TJ (zonulae occludens) (see Figs. 2 and 4). Three functions can be ascribed to TJs. First, TJs formed between adjacent cells play an essential role in compartmentalization by creating a barrier to restrict the diffusion of solutes through the paracellular pathway (297, 432). Second, TJs also create a boundary between the apical and basolateral domains of a cell, which differ in protein and lipid composition. This in turn creates and maintains epithelial and endothelial cell polarity (367). These two roles of TJs are known as the "barrier" and "fence" functions, respectively. Third, the BTB also creates an immunological barrier that sequesters antigenic determinants residing on germ cell surfaces from the systemic circulation. This barrier also excludes the entry of circulating immunoglobulins and lymphocytes into the adluminal compartment (for reviews, see Refs. 131, 400, 401).

In the testis, TJs are different from those found in other epithelia in several ways. First, testicular TJs are not assembled until puberty, which is ~15 days after birth in the rat (117, 131, 379, 400). Second, the Sertoli cell-TJ

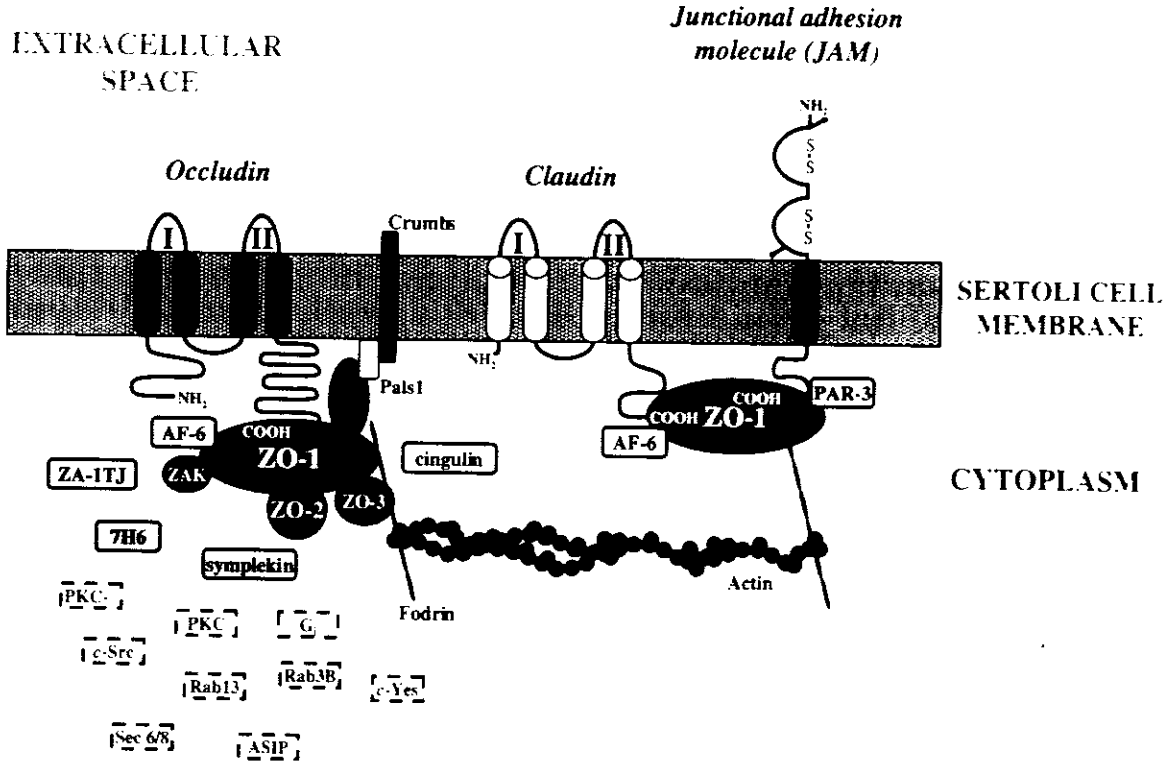

FIG. 4. Current model on the molecular structure of tight junctions and the constituent proteins in the testis. A schematic drawing that illustrates the molecular architecture of TJs is shown. Only three classes of TJ-integral membrane proteins, namely, occludin, claudin, and JAM, are known to date. Each TJ-integral membrane protein has distinctive transmembrane, extracellular, and intracellular domains. The COOH terminus of occludin is associated with ZO-1 intracellularly, which in turn associates with ZO-2, ZO-3, AF-6, ZAK, and others. Cytosolic proteins enclosed in dashed boxes are TJ-associated signal transducers whose functions are not yet known. This diagram was prepared based on earlier published models (for reviews, see Refs. 88, 138, 289, 298, 368, 431, 432, 458, 459, 472, 482).

complex in the testis has a unique architecture and location within the seminiferous epithelium, which is formed by the close apposition of adjacent Sertoli cell membranes at the basal compartment of the epithelium and designated the BTB (141, 401). Furthermore, the BTB is closest to the basal lamina in the testis. This is in sharp contrast to TJs found in other epithelia, which are furthest away from the basement membrane. The BTB in the testis also creates a specialized microenvironment for germ cell development because developing germ cells do not have access to the systemic circulation. As such, they must rely on Sertoli cells for the provision of nutrients and biological factors for their maturation. Third, the BTB is dynamic in nature since it must open (disassemble) and close (reassemble) periodically to allow preleptotene and leptotene spermatocytes to gain entry into the adluminal compartment for further development.

B. Molecular Constituents of TJs

Morphologically, TJs form a continuous circumferential seal near the apex of both epithelial and endothelial cells, farthest from the basal lamina (4, 139), whereas in the testis, they are located in the basal compartment of the seminiferous epithelium adjacent to the basement membrane, which is a modified form of ECM in the testis (127) (Figs. 1 and 2). Thus the relative location of TJs, AJs, and ECM in the testis is in reverse order compared with other epithelia. There are three classes of TJ-integral membrane proteins: occludins, claudins, and junctional adhesion molecules (JAM, a member of the immunoglobulin superfamily) (138, 285, 298).

1. *TJ-integral membrane proteins: occludins, claudins, and JAM*

A) OCCLUDIN FAMILY. Occludin is a 60- to 65-kDa single polypeptide and a Ca^{2+}-independent intercellular adhesion molecule (467). It is also a putative TJ-integral membrane protein that contributes to the barrier and fence functions of TJs (for reviews, see Refs. 68, 138, 298, 458, 459). Recently, a new variant of occludin designated occludin 1B has been identified in the mouse testis, which has an additional 193-bp insertion and a unique NH_2-terminal sequence compared with occludin (313). Each

occludin molecule consists of four transmembrane domains, two extracellular loops (loop 1, amino acid residues 90–138; and loop 2, residues 199–243 from the NH_2 terminus in the rat), one intracellular loop, a small NH_2-terminal cytosolic domain, and a large COOH-terminal cytosolic domain (Fig. 4) (for reviews, see Refs. 88, 138). Among these domains, the first extracellular domain is rich in Tyr and Gly (~60%), and these structural characteristics are well conserved among different mammalian species (12). Its cytoplasmic COOH-terminal domain associates with ZO-1 at a stoichiometric ratio of 1:1 (153). Overexpression of chicken occludin in Madin-Darby canine kidney (MDCK) cells increased the "tightness" of the TJ barrier as manifested by an increase in transepithelial electrical resistance across the cell epithelium, whereas truncation of occludin at the COOH terminus caused the influx of *N*-(4,4-difluoro-5,7-dimethyl-4-bora-3α,4α-diaza-*S*-indacene-3-pentanoyl)sphingosyl phosphocholine (BODIPY)-sphingomyelin across the TJ barrier in vitro (28). Furthermore, introduction of a peptide corresponding to the second extracellular domain of occludin can perturb the TJ-permeability barrier of *Xenopus* A6 epithelial cells in vitro (496) and Sertoli cells in vitro (83) and can disrupt the BTB in vivo (83). These results thus suggest the notion that the second extracellular loop of occludin is important to confer TJ functionality. However, occludin alone cannot generate a bona fide TJ barrier. For instance, occludin-deficient embryonic stem cells can still differentiate into polarized epithelial cells having TJs (384). Instead, studies have shown that occludins and claudins both contribute to maintain TJ function in epithelial cells (for review, see Ref. 459). Other studies have shown that the first extracellular loop of occludin contributes significantly to cell adhesive function (138, 298, 467). Studies by immuno-freeze fracture electron microscopy have also shown that occludin is concentrated within TJ fibrils (148) and is phosphorylated both at Ser/Thr and Tyr residues (386, 457). However, an additional minor pool of occludin is found in the basolateral membrane, which is less phosphorylated, and is not assembled into TJ fibrils (100, 386). This lateral pool of occludin is likely to serve as a reservoir of molecules for rapid expansion of TJs. For instance, earlier studies predating the discovery of occludin have shown that application of trypsin to the basolateral surface of MDCK cells could induce a rapid increase in the number of TJ fibrils and transepithelial electrical resistance (TER) (275), illustrating that mammalian cells possess a mechanism to rapidly assemble TJs in response to an external stimulus. With the use of peptide mass fingerprinting analysis coupled with electrospray ionization tandem mass spectroscopy, it has been shown that Ser-338 of occludin is the putative phosphorylation site induced by protein kinase C (PKC) in MDCK cells in vitro (13). Taking these observations collectively, it is possible that preleptotene and leptotene spermatocytes are the source of the stimulus that regulates TJ dynamics by inducing localized changes in the phosphorylation of occludin in the TJ fibrils. Before this hypothesis can be tested, one must first assess whether there are changes in occludin phosphorylation at the time of junction assembly both in vitro and in vivo. Other studies have shown that two adjacent occludin molecules are capable of lateral oligomerization, which in turn form interlocking TJ fibrils, perhaps within the membrane bilayer (72). However, claudin-1, a structural molecule that constitutes TJ fibrils by forming paired TJ strands in the apposing membrane of adjacent cells with claudin-2 or -3 (459), is largely unphosphorylated (71), seemingly suggesting that TJ dynamics are regulated by multiple signaling pathways, some of which are not regulated by protein phosphorylation. Also, different TJ-integral membrane proteins may be regulated by distinctly different signaling pathways. It has been reported that occludin and occludin-1B are found in the rat and mouse testis but not human and guinea pig Sertoli cells (110, 303), suggesting that the testis is equipped with other yet to be identified TJ-integral membrane proteins to maintain the BTB integrity and its function. With the use of immunohistochemistry, it was shown that the accumulation of occludin closely associated with the assembly of the BTB at the site of TJs in the mouse testis during postnatal development; however, its pattern of localization in the seminiferous epithelium was not stage specific (110). Between postnatal day 23 and adulthood, occludin was diffusely localized in the seminiferous epithelium (110). Furthermore, intratesticular injection of glycerol in adult rats that damaged the BTB by disrupting the organization of microfilaments and microtubules in Sertoli cells was also shown to disrupt the cellular occludin distribution (490). Taken collectively, these results illustrate that much work remains to be done to define the role of occludin in regulating TJ dynamics in the testis. A more recent study has demonstrated that expression of occludin mutants in transfected epithelial cells with a modified NH_2-terminal cytoplasmic domain (but not the COOH-terminal cytoplasmic domain since its deletion failed to perturb cell transepithelial migration) upregulated the migration of neutrophils across the TJ barrier without affecting TER and paracellular permeability (201). This report clearly illustrates that occludin regulates the transepithelial migration of cells across the TJ barrier utilizing its NH_2-terminal cytoplasmic domain. In transgenic occludin$^{-/-}$ mice, TJs in epithelia that were examined did not appear to be morphologically affected; for instance, the TJ barrier function of the intestinal epithelium remained intact (385). However, other phenotypic changes were detected in occludin$^{-/-}$ mice, such as testicular atrophy, calcification of the brain, and thinning of the bone (385). Furthermore, although the testes and seminiferous tubules of occludin$^{-/-}$ mice in their early postnatal stage appeared to be normal without

any detectable damage to spermatogenesis, the tubules displayed atrophy and the seminiferous epithelium was devoid of germ cells in adulthood (384). These findings thus suggest that the functions of occludin and TJs may be more complicated than originally conceived. Ironically, this is not entirely unexpected since TJs are implicated as the platform for signal transduction (for review, see Ref. 510).

B) CLAUDIN FAMILY. Claudins are another family of TJ-integral membrane proteins with an apparent molecular mass of ~22 kDa that confer TJ functionality and cell adhesiveness to epithelial cells (151, 153a, 154, 458, 459). Claudins share a similar molecular topology with occludin. Each claudin molecule consists of one short NH_2-terminal cytoplasmic domain, two extracellular loops, four putative transmembrane domains, and a long COOH-terminal cytoplasmic domain (Fig. 4). Nevertheless, the primary amino acid sequences of claudins do not share any significant homologies with occludins, illustrating that these two classes of TJ proteins are distinct. At least 24 claudins have been identified in TJs in different epithelia (for reviews, see Refs. 138, 151, 154, 155, 298, 301, 302, 459). With the use of immunogold freeze-fracture labeling, claudins have been shown to localize within TJ fibrils (310, 302). Recent studies have shown that claudins are the principal TJ-component proteins primarily responsible for constructing the seal-forming elements in TJs (for reviews, see Refs. 458, 459). In contrast to occludin, claudins, such as claudin-1, found in TJ fibrils are largely unphosphorylated at the site of TJs in MDCK cells (71), illustrating that protein phosphorylation, while it plays an important role in TJ assembly, is possibly not the only determining factor that regulates TJ assembly. Seven different claudins (i.e., claudin-1, -3, -4, -5, -7, -8, and -11) are found in the testis. For instance, claudin-11 is found exclusively in the brain [also known as oligodendrocyte-specific protein (OSP) restricted to the myelin sheaths of oligodendrocytes in the central nervous system], the testis (restricted to Sertoli cells) (301, 302), the choroid plexus, and the collecting ducts in the kidney (164). The expression of claudin-11 in the testis appears to be limited to the Sertoli cell, since it was not found in germ cells (190). Moreover, the expression of claudin-11 by mouse Sertoli cells in vitro is inhibited by follicle stimulating hormone (FSH) and tumor necrosis factor (TNF)-α with an ED_{50} at 4 and 4.5 ng/ml, respectively (190). Its expression in the mouse testis in vivo was induced at postnatal days 6–16, coinciding with the assembly of the BTB that occurs at ~10–16 days postnatally (190). These results suggest that the intricate interactions between claudin-11, FSH, and cytokines play a crucial role in the assembly of the BTB and the regulation of TJ dynamics in the testis. Furthermore, gene knock-out experiments showed that null mice lacking claudin-11 are sterile and have hindlimb weakness and retardation of nerve conduction (164). TJ intramembranous strands were absent in the central nervous system myelin and between Sertoli cells in the testis in claudin-11 knock-out mice (164). These results thus illustrate the significance of claudin-11 in spermatogenesis. A recent report has also demonstrated the significance of claudin-1 in TJ function since claudin-1 mice died within 1 day after birth with wrinkled skin (151a). Although both occludin and claudin-4 were found in the TJs in these claudin-1 mice, the epidermal TJ barrier could not form (151a), seemingly suggesting that claudin-1 is primarily responsible for the integrity of the epidermal TJ barrier.

Different members of the claudin family are expressed in different tissues and/or organs. For instance, claudin-2 is predominantly expressed in the kidney and liver, whereas claudin-5 can be found in virtually all tissues examined to date (151, 154, 298). Claudins-4, -7, and -8 are present in both lung and kidney (150, 301, 302). Recently, a new member of the claudin family designated paracellin-1/claudin-16 was identified. Its mutation results in renal hypomagnesemia in humans (413). Two different claudins can also be coexpressed in a single cell, raising the possibility that heterogeneous claudins can form heteromeric TJ strands (155). Indeed, recent studies have demonstrated that claudins in one cell can associate heterotypically and homotypically with claudins in the apposing cell (155). When occludin was transfected into claudin-expressing fibroblasts, it was recruited to claudin fibrils, suggesting that claudins are the major contributor of fibril formation. Furthermore, claudins appear to confer stronger cell adhesion between cells than occludin (244), indicating they form transcellular contacts required to seal the intercellular space.

C) OCCLUDIN AND CLAUDINS: PARTNERS IN BEATING SEMEN TJS. Claudins and occludin can be organized into oligomers and hetero-oligomers. Freeze-fracture analysis has shown that TJ fibrils are composed of particles of ~10 nm in diameter, which is similar in size to the connexons found in GJs (148). Interestingly, the folding topology of connexons is similar to claudins and occludin, and these molecules have similar M_r. By analogy, it is likely that the ~10 nm TJ particles are composed of more than one claudin or occludin molecule. For instance, each connexon in the GJ is composed of a ring of six identical protein subunits called the connexin at the periphery with a central pore, which permits the passage of chemical signals between two cells (for reviews, see Refs. 133, 250, 340). When claudin and occludin are coexpressed in fibroblasts, claudin recruits occludin to fibrils (154), suggesting they can form a functional protein complex. With the use of claudin L cells transfected with claudins, it was found that claudin-1 and -3 and claudin-2 and -3 can form a TJ complex but claudin-1 and -2 cannot (for review, see Ref. 459). Thus, among the 24 known claudins, only some claudins can interact with selected members.

Indeed, it has recently been proposed that the homotypic or heterotypic claudin-claudin complexes formed between apposing cells are composed of aqueous TJ pores to permit transport of small molecules across the TJ barrier (for review, see Ref. 459). Still, the detailed molecular architecture that forms these TJ pores in the paracellular space and their regulation is unclear. Additional mutational and structural studies are likely to resolve the issue of how these proteins interact with their partner molecules in apposing cells. It has been reported that the number of TJ strands correlate with the tightness of the TJ barrier (93, 278, 350). It is also known that the Sertoli cell TJ is one of the tightness in the mammalian body (128, 131, 159), suggesting that the number of junctional strands in Sertoli cell TJs is relatively high (343). However, the Sertoli cell TJ-permeability barrier in vitro was shown to have an electrical resistance of ~100 $\Omega \cdot cm^2$ (28, 82, 169, 218, 219, 270, 496), which is only about one-tenth of that observed in MDCK cells and keratinocytes in vitro (28, 179, 496). These results seemingly suggest that the tightness of the BTB is approximately one-tenth of that of TJs found in the kidney and other epithelia. Janecki et al. (219) first reported that the tightness of the Sertoli cell TJ-permeability barrier in vitro can be significantly increased in the presence of testosterone. Furthermore, a recent report (82) has shown that testosterone at 1×10^{-7} M, which is ~100 times higher than the level of androgen found in the systemic circulation, but similar to the level detected in the testis, such as in the rete testis fluid (462), can protect the Sertoli TJ-permeability barrier in vitro from the disruptive effects of $CdCl_2$ (82, 219). More important, it was shown that testosterone could stimulate the expression of occludin by Sertoli cells in these same cultures (82). These results further demonstrate that the dynamics of TJs in the testis are regulated by androgens. In addition, proteases and protease inhibitors also appear to play a role in the BTB function, since chloroquine, a protease inhibitor, has been shown to facilitate the assembly and maintenance of Sertoli TJ in vitro (329), consistent with another study reporting that the assembly of junctions between testicular cells in vitro is regulated by both proteases and protease inhibitors (309).

D) JAM. JAM is the third type of TJ-integral membrane protein identified in TJs by using monoclonal antibodies against endothelial cells (285) and is localized to the intercellular boundaries of both epithelial and endothelial cells (334). To date, at least three JAM molecules are known, designated JAM-1, JAM-2, and JAM-3 (22, 23, 108, 334, 491). Although it is not known if JAM-3 is present in the testis, JAM-1 and JAM-2 have recently been identified in the mouse testis by Northern blot analysis (23). The molecular mass of JAM found in different tissues ranges from 36 to 41 kDa, possibly the result of carbohydrate heterogeneity. JAM-2 binds to JAM-3 (22). Unlike occludins and claudins, JAM exhibits a distinct topographic structure (Fig. 4). Each JAM molecule consists of a putative intracellular domain, a transmembrane domain, and an extracellular domain. The extracellular domain is composed of two V-shaped immunoglobulin-like loops with two interchained disulfide bonds (285) (Fig. 4). JAM has also been reported to facilitate homotypic cell-cell adhesion (285). When JAM was transfected into Chinese hamster ovary (CHO) cells, cell permeability to dextran (molecular mass ~38.9 kDa) was reduced by 50%, increasing the tightness of the TJ barrier (285), which is similar to results obtained when cadherin was transfected into CHO cells. However, it is not known if JAM is indeed a component of TJ fibrils and if it can form a functional TJ barrier. The use of anti-JAM monoclonal antibody, however, can prohibit the migration of monocytes through endothelial cells when chemotaxis assays were conducted both in vitro and in vivo (118). Also, administration of anti-JAM monoclonal antibody inhibited leukocyte accumulation in the cerebrospinal fluid and infiltration in the brain parenchyma. Furthermore, the tightness of the blood-brain permeability barrier was reduced in the presence of anti-JAM monoclonal antibody. On the basis of these observations, it has been concluded that JAM is a new target in limiting the inflammatory response accompanying meningitis (118). On the other hand, combined treatment of human endothelial cells with TNF-α and interferon (IFN)-γ induced the redistribution but not the amount of JAM promoting transmigration of leukocytes across endothelial cells (331). It has been suggested that this redistribution of JAM is crucial for the transendothelial migration of leukocytes at inflammatory sites in response to proinflammatory cytokines (331). Another striking feature of JAM is that its mRNA is found in megakaryocytes, which do not possess TJs; also, the expression of JAM is either absent or at a very low level in hepatocytes, which contain well-developed TJs (285), suggesting JAM may have other yet-to-be identified physiological functions. A recent study has shown that JAM associates with ZO-1 when assessed by in vitro binding and coprecipitation experiments. More recent studies using immunofluorescent microscopy have shown that the COOH terminus of JAM binds to the PDZ3 domain of ZO-1 (214). JAM also coprecipitates with cingulin, another cytoplasmic TJ protein, through its interaction with the NH_2 terminus of cingulin (31). Although the role of JAM in tissues without TJs is still unclear, studies using immunoreplica electron microscopy have shown that JAM has an intimate spatial relationship with TJ strands in epithelial cells, such as fibroblasts (214). These results seemingly suggest that aggregates of JAM are tethered to claudin-based TJ strands through ZO-1, contributing to the overall architectural integrity of TJs in epithelia (214). Results of these studies clearly illustrate that JAM is an important TJ candidate protein. However, it remains to be determined if JAM-1 and JAM-2, which are found in the

testis (23), can indeed facilitate the transmigration of preleptotene and leptotene spermatocytes across the BTB in the testis. In light of their unusual properties in regulating transendothelial migration of neutrophils and monocytes, this class of molecules should be aggressively studied in the testis to understand their role in TJ dynamics during spermatogenesis.

2. *Cytoplasmic proteins linking TJ-integral membrane proteins to cytoskeleton*

A) ZO-1 AND OTHERS. ZO-1 is the first TJ-associated cytoplasmic protein subjected to extensive investigation (433). ZO-1, ZO-2, and ZO-3 are members of the membrane-associated guanylate kinase (MAGUK) protein family of signal transducers. It is characterized by a PSD-95-Discs Large-zona occludens-1 (PDZ) domain, a Src homology 3 (SH3) domain, and a guanylate kinase (GUK) homology region (for reviews, see Refs. 297, 432). ZO-1, a 225-kDa polypeptide, was first identified in the membrane fraction of hepatocytes. It was also localized to Sertoli cell junctional complexes (59, 433). In the mature testis, ZO-1 is associated with Sertoli cell occluding and nonoccluding junctional membrane specializations, such as the subsurface filamentous specializations facing germ cells (59), suggesting that this protein is not restricted to TJs. In two MDCK cell strains, which exhibit high and low electrical resistance, the level of ZO-1 is similar, indicating the abundance of ZO-1 in a given tissue does not correlate with the tightness of TJs (433). ZO-1 is an asymmetric phosphoprotein that is peripherally associated with the cytoplasmic surface of the plasma membrane (10) at the sites of TJs (433). ZO-1 is also implicated in creating a mechanochemical linkage between the submembrane cytoskeleton and integral membrane components of the TJ instead of contributing to the TJ sealing properties (for review, see Ref. 432). Recent studies have shown that the PDZ domain of ZO-1 is the binding site for the COOH termini of claudins and occludins (153, 213), indicating ZO-1 is a linker protein coupling the transmembrane TJ proteins to the cytoskeleton. ZO-1 coexists with occludin in a stoichiometric ratio of 1:1 (155) (Fig. 4). The expression of ZO-1 becomes progressively restricted to the developing junction in the testis at the site of TJs between *days 7* and *14* in the rat and mouse (59), correlating with the assembly of the BTB that takes places at ~13–15 days of age in rats. In addition, two ZO-1 isoforms are known: the α^- and α^- forms, which are predominantly expressed at puberty and adulthood in the guinea pig testis, respectively, implicating the significance of α^- in the BTB assembly at puberty (344). Under conditions designed to preserve protein-protein interactions, ZO-2 (160 kDa) and ZO-3 (130 kDa) were shown to coimmunoprecipitate with ZO-1 (178, 188, 223). Both ZO-2 and ZO-3 share strong sequence homology with ZO-1 (for review, see Ref. 432). Both ZO-1 and ZO-2 are found in the testis; however, it is not known if ZO-3 is present in the testis. A recent study has identified a new MAGUK protein designated Pals1 in TJs in the kidney (368), which functions as an adapter protein linking the mammalian homologs of Crumbs and Discs Lost (the mammalian homolog of *Drosophila* Discs Lost is Patj). Both are proteins crucial for epithelial cell polarity in *Drosophila*, and this complex, the Patj-Pals1-Crumbs complex, in turn anchors to the TJ by interacting with the PDZ domain of ZO-1 (368, 405) (Fig. 4). Again, it remains to be determined if such complex-regulating cell polarity exists in the testis.

B) CINGULIN. Cingulin is the first TJ protein identified by using monoclonal antibodies (90, 91) and is found in the rat and mouse testis (see Table 1). Cingulin is a 140-kDa phosphoprotein found at the cytoplasmic plaque of TJs from epithelial and endothelial cells (90) and is further away from the site of the TJ than ZO-1 (431). Nonetheless, cingulin is essential to conferring TJ function by maintaining an impermeable barrier (88, 89). Cingulin shares structural homology with other cytoskeletal proteins, such as myosin rod and paramyosin (88, 91). Recent in vitro immunoprecipitation studies have shown that cingulin interacts with other TJ proteins, in particular ZO-1, ZO-2, ZO-3, AF-6, and myosin (100), suggesting an in vivo interaction of cingulin with ZO-1 and ZO-2 may occur. In addition, cingulin interacts with occludin in vitro (100). Studies by immunofluorescent microscopy have localized cingulin to the same site of ZO-1 at the basal compartment near the BTB in the rat and mouse testes (62). Furthermore, cingulin is also found at the spermatid-Sertoli cell contact sites consistent with its localization at the ectoplasmic specialization (62).

C) SYMPLEKIN. Symplekin is a 150-kDa protein initially identified by the use of monoclonal antibodies raised against lateral membrane junctional extract (for review, see Ref. 432). It has been localized to the site of TJs in polarized epithelia, as well as in Sertoli cells by immunohistochemistry and immunogold electron microscopy, but not at TJs in endothelial cells (236). In addition to its localization in TJs, symplekin is a ubiquitous component of the nucleoplasm in both epithelial and nonepithelial cells (236). Molecular cloning of symplekin has shown that its cDNA consists of a well-conserved Kozak sequence; however, symplekin does not share any homology with ZO-1, ZO-2, or any of the members of the MAGUK family of proteins (463).

D) OTHERS. With the use of electron microscopy techniques in conjunction with specific antibodies, protein kinases, heterotrimeric $G\alpha$ proteins, small GTP-binding proteins (such as Rab3B and Rab13), AF6, Src substrate, and *c-yes* have all been localized to the cytoplasmic surface of TJs (for review, see Ref. 510). However, the functions of many of these molecules are not known for

TABLE 2. *Tight junction proteins*

TJ Proteins	Function(s)	Molecular Mass, kDa	Partner Protein(s)	Reference Nos.
TJ-integral membrane proteins				
Occludins	TJ barrier	65–82	ZO-1	12, 152, 213, 313
Claudins	TJ barrier	22	ZO-1, ZO-2, VAP33, actin	151, 154, 213, 254, 298, 301, 302, 494
JAMs	Monocyte transmigration	36–41	ZO-1	22, 214, 285
Peripheral proteins				
ZO-1	Scaffold protein/signaling Cell polarity	210–225	Occludins, claudins, ZO-2, ZO-3, cingulin, actin, Patj-Pals1-Crumbs complex	10, 27, 99, 212, 213, 368, 405, 433, 492, 494
ZO-2	Scaffold protein	160	ZO-1, actin	32, 178, 494
ZO-3	Scaffold protein	130	ZO-1, occludin	26, 188, 494
Cingulin	Scaffold protein	140–160	ZO-1, -2, -3, occludin, myosin	90, 99
7H6		155–175		511
AF-6	Scaffold protein	180–195	Ras, ZO-1	504
Fodrin	Scaffold protein	240		212
TJ-associated signaling proteins				
ASIP/Par3		150–180/100	aPKC (e.g., PKC-ζ, PKC-λ)	215, 225, 266, 436
Symplekin		150		236
Src substrate		120		357
c-*yes*		~60		460
ZO-1-associated kinase			ZO-1	28
Gα		39–41	ZO-1	121, 124, 383
PKC-ξ		81		124
PKC-α		81		370
19B1		210		295
Trafficking proteins				
Rab3b	Intracellular traffic	25		202
Rab8	Intracellular traffic	24	G/C kinase, exocyst subunits	172, 202, 356
Rab13	Intracellular traffic	22–24	δ-PDE	286, 509
Sec6/8	Intracellular traffic	70–110		510

TJ, tight junction; JAM, junctional adhesion molecules; PKC, protein kinase C; PDE, phosphodiesterase.

review, see Ref. 120). Furthermore, these protein complexes eventually anchor to actin. Although actin is not exclusively found at the site of TJs, actin is known to regulate TJ permeability since agents that disrupt the actin cytoskeleton can induce influx through the paracellular space (for review, see Ref. 432). More recent studies have shown that the TJ is a platform for trafficking and signal transduction because many of the proteins necessary for membrane trafficking and signal transduction are found at TJs (see Table 2). Indeed, recent studies have identified a new family of proteins designated junctophilins (JP), which consists of JP-1, -2, and -3 (322), in the junctional complexes in excitable cells, such as heart and skeletal muscle (446). Although JP-1 and JP-2 were not found in the testis but apparently confined to heart and skeletal muscle, JP-3 is largely confined to the brain and testis, localized in the junctional complexes between the plasma membrane and endoplasmic/sarcoplasmic reticulum (446). JP proteins appear to play an important role in facilitating functional cross-talks between cells through the ionic channels on the plasma membrane and endoplasmic/sarcoplasmic reticulum in excitable cells (211, 322).

C. Interactions of Signaling Molecules in TJ Dynamics

Recent studies on TJ-associated signaling molecules (see Tables 1 and 2; Fig. 4) have shown that the TJ has emerged as a platform for signal transduction to coordinate different cellular functions, in particular the dynamics of TJ functionality to permit the timely passage of cells, such as preleptotene and leptotene spermatocytes and monocytes across the BTB and epithelium, respectively. However, the precise regulation of these events is not yet known. For instance, small GTPases, Cdc42 and Rac, known to be involved in actin cytoskeleton dynamics and cell polarity, bind to a protein complex containing Par6, Par3/ASIP, and PKC that is found in TJs (for review, see Ref. 510) (Table 2, Fig. 4). When either Par6, Par3, or Cdc42 is overexpressed in epithelial cells, it causes dislocation of ZO-1 from the site of TJs, rendering the loss of cell polarity, indicating the Cdc42/Par6/Par3/PKCζ complex, along with ZO-1, may be critical to maintain cell-cell contact and cell polarity at the site of TJs (224, 266). Furthermore, small GTPases, such as Rho, Rac, and Cdc42, are important molecules regulating TJ dynamics

via their effects on F-actin (for reviews, see Refs. 181, 182). For instance, overexpression of RhoV14 and RacV12 causes disruption of the TJ strands due to a chaotic redistribution of ZO-1 and occludin (230). It is known that these GTPases, such as RhoB, regulate the events of actin organization by targeting vesicles to the appropriate sites in the membranous structures within a cell including the sites of TJs and AJs (for reviews, see Refs. 182, 510). More recent studies have shown that both Sertoli and germ cells express small GTPases, such as Cdc42, N-Ras, Rac2, and RhoB (271–273), indicating both Sertoli and germ cells are likely to take part in the reorganization of their cytoskeleton network to facilitate germ cell movement. It is obvious that studies of small GTPases in the testis should be expanded to understand the precise regulatory pathway(s) by which TJ dynamics are regulated utilizing these GTPases (for review, see Ref. 273).

D. Molecular Mechanisms That Regulate TJ Dynamics

Although major advances were made in the past two decades identifying many constituent components of TJs in different epithelia including the testis (Tables 1 and 2 and Fig. 4), the factors and pathway(s) that regulate TJ dynamics in the testis are poorly understood. Nevertheless, extensive studies have been performed using epithelial cells, such as MDCK cells and keratinocytes, cultured in vitro to identify and investigate the regulatory molecules and signaling pathway(s) that modulate TJ dynamics (for review, see Ref. 120). To date, different signal transduction pathways are implicated in the regulation of TJ dynamics. These include protein kinases, protein phosphatases (26, 87, 262, 264, 317, 319), intracellular Ca^{2+} (318, 434, 435), G proteins (25, 212), calmodulin, cAMP, and phospholipase C (25, 218). On the basis of these earlier in vitro findings, two biochemical models have been proposed attempting to explain how small molecules, such as fatty acids, amino acids, glucose, and IgG, can traverse epithelial TJs during food absorption and inflammatory responses.

1. The Ca^{2+} switch model

This model is based on the observation that depletion of Ca^{2+} from MDCK cells cultured in vitro induces immediate disruption of the TJ barrier, which is manifested by a plunge in the TER across the cell epithelium (for review, see Ref. 120). Upon addition of $[Ca^{2+}]$ to the media, cell polarity restores and the TJ barrier reseals. It is also known that the Sertoli cell TJ barrier can be disrupted and resealed by manipulating $[Ca^{2+}]$ in the culture medium (169). For instance, depletion of $[Ca^{2+}]$ from the spent media of Sertoli cell cultures can perturb the Sertoli cell TJ barrier within 15 min, and its replacement induces the disrupted TJ barrier to reseal within 90 min, making it indistinguishable from control cultures (169). These results unequivocally demonstrate that $[Ca^{2+}]$ plays a very critical role in the regulation of TJ dynamics in the testis. Indeed, calcium is a known cell signaling molecule that regulates a variety of cellular events (for review, see Ref. 37).

2. The ATP depletion-repletion model

During TJ assembly or disassembly, the actin cytoskeleton undergoes extensive polymerization and depolymerization, which is an ATP-dependent event per se. For instance, treatment of cells with cytochalasin, a drug that disrupts actin filaments, can perturb the paracellular barrier (430), suggesting the significance of the cytoskeleton network in TJ functionality. This model hypothesizes (for review, see Ref. 120) that when ATP is depleted from the system, ZO-1 becomes associated with cytoskeletal proteins, such as fodrin. This, in turn, pulls ZO-1 away from TJ sites causing TJ leakiness. Upon repletion of ATP, the association between ZO-1 and fodrin becomes disrupted, allowing ZO-1 molecules to move back to TJ sites, resealing the TJ (457). These models, however, apparently can only explain how TJs become leaky in vitro to allow passage of small molecules and ions. This is in contrast to the dynamics of Sertoli cell TJs that constitute the BTB, which must disassemble to allow for the passage of preleptotene and leptotene spermatocytes. These models also do not take into consideration how the recently identified TJ-integral membrane proteins, such as occludins, claudins, and JAM, take part in these processes.

E. Regulation of TJ Dynamics

1. Regulation by protein phosphorylation: the interplay of kinases and phosphatases

In mammalian cells, as much as 30% of the cellular proteins are phosphorylated (122), illustrating the regulatory roles of protein phosphorylation. Indeed, tyrosine phosphorylation of junction-associated proteins is known to play a crucial role in junction assembly (373). For instance, tyrosine kinases of the Src family are found at the sites of TJs and AJs (see Tables 1 and 2 as well as Figs. 4 and 7) (460). β-Catenin, an AJ-associated protein, becomes highly phosphorylated in Src-transfected cells and is a putative substrate of protein kinases (33). Recent studies from our laboratory have demonstrated the presence of myotubularin, a putative protein tyrosine phosphatase (PTP) in Sertoli and germ cells (262, 264). Its expression is induced when the Sertoli cell TJ barrier is being assembled in vitro (264). Furthermore, a testis-specific serine/threonine protein kinase is also found in the mouse (38). Thus both AJs and TJs apparently consist

of the necessary proteins for signaling via tyrosine phosphorylation. With the use of various PTP inhibitors (PTPi), it was shown that both β-catenin and ZO-1 are tyrosine phosphorylated and are putative substrates of tyrosine kinases (424). Also, vanadate (a specific PTPi) can induce a rapid increase in TJ permeability in MDCK cells in vitro as revealed by reduced TER and increased permeability to [^{3}H]inulin by increasing the cellular phosphoprotein content (97). This observation is consistent with our recent observations demonstrating that sodium orthovanadate can perturb the Sertoli cell TJ-permeability barrier in vitro (263, 264). These changes in TJ permeability coincided with an increase in phosphotyrosine immunofluorescence at the site of the TJ and with a redistribution of F-actin, E-cadherin, and ZO-1 when examined by confocal microscopy (97). More importantly, these changes can be blocked in MDCK cells and Sertoli cells by the use of a protein tyrosine kinase (PTK) inhibitor (PTKi), such as tyrphostin A25 (263), but to a significantly lesser extent when a serine/threonine protein kinase inhibitor, such as staurosporine, was used (97). Studies in MDCK cells have shown that the assembly, opening, and resealing of TJs correlate with the phosphorylation of occludin on serine/threonine residues (140). Although the physiological significance of these studies remains to be elucidated, they strongly indicate that the assembly and maintenance of TJs are regulated by the phosphorylation status of cellular proteins whose identities remain to be uncovered. Taking these results collectively, it is apparent that a decline in cellular phosphoprotein content favors the assembly and maintenance of the Sertoli cell TJ barrier, whereas an increase in cellular phosphoprotein content perturbs the Sertoli cell TJ-permeability barrier. On the other hand, these results present a biological dilemma: if occludin molecules found in the TJ fibrils are highly phosphorylated (140), why would the use of a PTPi, such as vanadate, which supposedly increases cellular phosphoprotein content, perturb TJ-permeability barrier as seen in MDCK and Sertoli cells (262, 264)? Other studies have shown that tyrosine phosphorylation of AJ-associated proteins, such as β-catenin, γ-catenin, and E-cadherin, can also reduce AJ stability and perturb cell adhesion, which in turn perturbs the TJ-permeability barrier (351). Taking these results collectively, it seems logical to speculate that an increase in the overall phosphoprotein content of epithelial/endothelial cells reduces the stability of TJs and AJs (based on protein phosphatase inhibitor studies). Furthermore, a local mechanism and a compartmentalized microenvironment may exist at the level of cell membrane, such as the one between preleptotene/leptotene spermatocytes and Sertoli cells, where reduced phosphorylation of occludin can open up the Sertoli TJ barrier, and an increase in occludin phosphorylation can reseal the TJ. Also, in studies using a specific PTPi, such as vanadate, to block tyrosine protein dephosphorylation, PTPi can also inhibit and/or activate other signal transduction pathways, which in turn contribute to the observed junction assembly/disassembly events. Also, the assembly of TJs in MDCK cells is regulated by G proteins, phospholipase C, PKC, and calmodulin (25). Figure 5 depicts a hypothetical molecular model by which the Sertoli cell TJ barrier is regulated by changes in phosphorylation of selected target proteins, such as occludin.

2. *Regulation by small GTPases*

The Rho family of small GTPases belongs to the Ras GTPase superfamily of 20- to 30-kDa GTP-binding proteins, which regulates a wide spectrum of cellular functions (465). Like Rho and Cdc42, Rac cycles between a GDP-bound inactive state and a GTP-bound active state (for reviews, see Refs. 440, 465). GTP-bound active GTPases account for only ~0.5–5% of the total GTPases in a mammalian cell (19, 465). These small GTPases also provide a link between growth factor signaling and reorganization of the actin cytoskeleton (277, 483, 515). They are implicated in the signaling pathways that regulate the initiation and turnover of cell-cell adhesion and cell-substratum contact, which are essential for *1*) cell movement and *2*) junction assembly (181, 182, 196, 320). Although many of the earlier studies were performed in fibroblasts, recent studies reveal that their roles are not limited to fibroblasts and not restricted to the actin filament network (181, 182). GTPases are regulated by at least four types of proteins. These include *1*) guanosine nucleotide exchange factors (GEF), which facilitate the activation of GTPases by promoting the binding of GTP onto GTPases (for reviews, see Refs. 231, 237, 465); *2*) GTPase-activating proteins (GAP), which stimulate the intrinsic hydrolysis of GTP in GTP-bound GTPases, enabling GTPases to execute the desired biological function, this in turn renders them inactive (for reviews, see Refs. 44, 252); *3*) guanosine nucleotide dissociation inhibitors (GDI), which sequester GDP-bound GTPases prohibiting GDP to dissociate from GTPases thereby inactivating GTPases (for reviews, see Refs. 39, 187, 330); and *4*) effector proteins, which interact with the GTP-bound GTPases to activate the downstream signaling events (440). To date, at least five GAPs have been found in the testis (for review, see Ref. 273). For instance, the expression of β-chimaerin, a 34-kDa GAP that activates Rac GTPase, parallels with the acrosomal assembly of spermatids during spermatogenesis in the rat testis (261). α2-Chimaerin, a 45-kDa GAP specific to Rac GTPases, is associated with pachytene spermatocytes in the rat testis (183), whereas MgcRacGAP, a 58-kDa GAP interacting with both Rac 1 and Cdc42 GTPases, is expressed by spermatocytes and spermatids (454, 455). Rho, Rac, and Cdc42 GTPases have also been shown to regulate the c-Jun NH_2-terminal kinase (JNK)

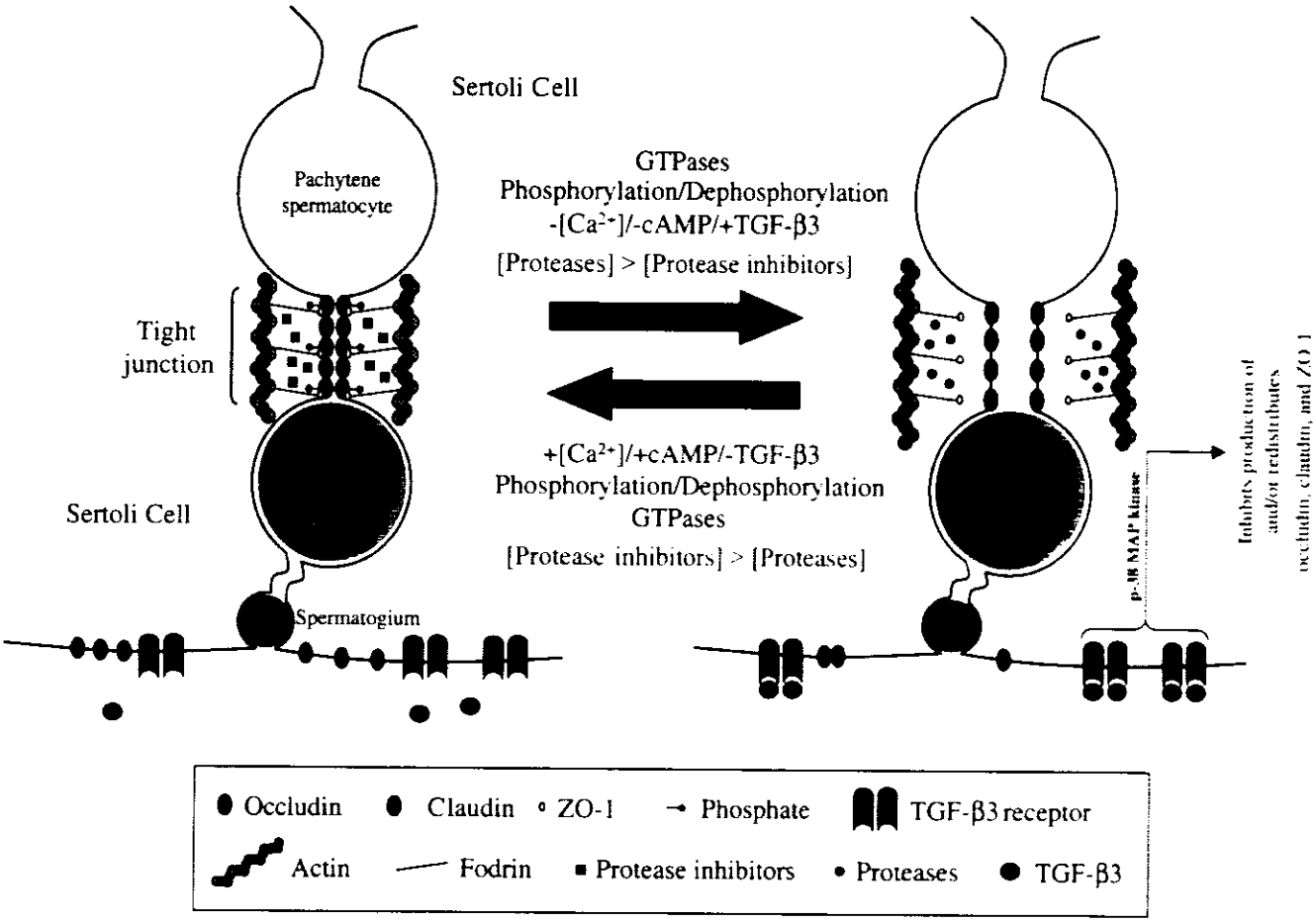

FIG. 5. A molecular model of the Sertoli cell TJ dynamics in which multiple molecules that interact with each other to regulate the opening and closing of the Sertoli TJ barrier that constitutes the BTB. *Left*: Sertoli cell TJ barrier that is closed. *Right*: TJ barrier that is opened. The tight junction dynamics in the testis are regulated by different sets of molecules and/or functional status of proteins, which include GTPases, proteases protease inhibitors, protein phosphorylation, intracellular calcium and cAMP levels, and cytokines, such as TGF-β3. For instance, reduced phosphorylation of occludin causes the dephosphorylated occludin molecules to move to the basolateral region of the membrane, away from the TJ sites, disrupting the TJ-barrier (see *right panel*). Likewise, an increase in proteases also favors the TJ disruption (see *right panel*) but an increase in protease inhibitors favors the closing of the TJ barrier (see *left panel*). This model was prepared based on reports and reviews discussed in section III*E*. Readers are encouraged to seek additional information from published reports and reviews cited in section III, *B–E*.

and the p38 MAP kinase cascades, thereby implicating their involvement in the regulation of Sertoli cell dynamics (270, 271), cell adhesion, and junction assembly via specific gene transcription pathways (102, 296, 448). The Rho family of GTP-binding proteins is also known to regulate TJ assembly and maintenance via their effects on perijunctional actin organization in polarized epithelia (320). Moreover, Rho GTPases can bind to phosphoinositide kinases (452), illustrating that there is a functional linkage between GTPases and phosphatases/kinases, which in turn can affect Sertoli cell TJ dynamics (263, 264). The mRNA encoding Rac, Rho, and Cdc42 GTPases have recently been identified in both Sertoli and germ cells (271), illustrating these cells are equipped with the machinery to link cytokine surface receptors to the underlying actin cytoskeleton network. Recent immunohistochemistry studies have also identified the presence of Ras, RalA, Rac1, and Cdc42 GTPases in the testis, associated both with Sertoli and germ cells in the seminiferous epithelium (69).

3. *Regulation by cAMP*

It is known that dibutyryl cAMP (DbcAMP) has a biphasic effect on the assembly and maintenance of TJs in Sertoli cells in vitro (218). At 4–20 μM, DbcAMP stimulates the assembly of Sertoli cell TJ-permeability barrier, whereas at 100–500 μM, it inhibits Sertoli cell TJ assembly and maintenance, illustrating TJ functionality in the testis is regulated, at least in part, by a cAMP-dependent pathway (218). Similarly, treatment of capillary endothelial cells isolated from human and rodent brains can in-

crease TJ resistance in vitro (372). When the cAMP level returns to its basal level, TJ resistance rapidly declines (323). Taken collectively, these results (for review, see Ref. 373) suggest that cAMP apparently induces phosphorylation of proteins by protein kinase A (PKA) through the cAMP/PKA signal transduction pathway. Such changes in the phosphorylation status of proteins may trigger changes in TJ functionality directly or may mediate their effects via the interactions between the cell surface and the cytoskeleton. Furthermore, intracellular cAMP levels are regulated by G proteins, which are also localized at the site of TJs (see Table 2), suggesting there is a local mechanistic pathway at TJs to alter cAMP level. Studies by immunofluorescent microscopy have identified $G\alpha_{i\text{-}2}$, $G\alpha_0$ and $G\alpha_{12}$ colocalized with ZO-1 alongside the lateral cell-cell contact areas at the site of TJs (121, 124), suggesting their possible involvement in regulating cell polarity. Also, overexpression of $G\alpha_5$ in MDCK cells induces a rapid accumulation of occludin and ZO-1 to the site of developing TJs without affecting E-cadherin distribution (383). These studies clearly demonstrate that this is an emerging area of research that deserves attention.

4. *Regulation by cytokines, proteases, and protease inhibitors*

Sertoli and germ cells are known to produce cytokines, such as TGF-α, TGF-β, nerve growth factor (NGF), fibroblast growth factor (FGF), IFN-α, IFN-β, IFN-γ, and TNF-α (for reviews, see Refs. 173, 221, 253, 308, 415–417). Immunolocalization of TGF-β1, - β2, and - β3 in staged tubules in the boar (66) and the developing rat testes (449) have shown that these cytokines are localized in both Sertoli and germ cells. In the boar, TGF-β1 appears to be predominant in stages IV–V, but TGF-β2/β3 does not appear to be stage specific (66). In the adult rat testis, studies by immunohistochemistry have demonstrated a plummeting in TGF-β1 at stages VIII–IX but a surge of TGF-β2 at stages V–VI (449). TGF-β1 is localized in spermatocytes and round spermatids in adult rat testes, and although TGF-β2 is not found in spermatocytes and round spermatids, there is an intense accumulation of TGF-β2 in elongated spermatids at stages V–VI (449). TGF-β in the rat testis is also stage specific as reported in a recent immunohistochemistry study, being low in stages I–VI, highest at stages VII–VIII, and virtually undetectable at stages IX–XIV (271). In the rat testis, TGF-β3 is localized predominantly near the basal compartment of the seminiferous epithelium in both Sertoli cells and primary spermatocytes (271), consistent with earlier studies suggesting that this cytokine possibly regulates the translocation of preleptotene and leptotene spermatocytes across the BTB by perturbing Sertoli cell TJs (270, 271). The fact that TGF-β is not stage specific in the boar seems to preclude the significance of TGF-β in regulating junction-restructuring events during spermatogenesis in this species. Needless to say, there are many biochemical and molecular events occurring in a given stage of the cycle, some of which are for purposes entirely unrelated to a specific event, such as spermiation. Thus the expression of a target gene, such as occludin, which is not stage specific (66, 110), does not necessarily exclude its involvement in the events of junction restructuring pertinent to germ cell movement. Indeed, occludin is known to play a prominent role in the regulation of TJ dynamics (for reviews, see Refs. 138, 298). Furthermore, the movement of germ cells across the seminiferous epithelium is a progressive cellular event, which takes place in virtually every stage of the cycle. Thus a molecule that is crucial to the regulation of junction restructuring, such as TGF-β and occludin, may not necessarily be stage specific unless it is linked to a specific event, such as spermiation, which takes place at stage VIII. Furthermore, INF-γ, TNF-α, and epidermal growth factor (EGF) have also been shown to affect TJ functionality in vitro and in vivo by reducing the levels of ZO-1, occludin, N-cadherin, and actin, or altering protein tyrosine kinases and/or phosphatases using epithelial cells derived from the small intestine (for review, see Ref. 476). Also, TGF-β can perturb the Sertoli cell TJ-permeability barrier in vitro, possibly via its effects on occludin, claudin-11, and ZO-1 by inhibiting the de novo synthesis of these TJ-associated molecules during TJ assembly (270). For instance, addition of recombinant TGF-β3 to Sertoli cells cultured in vitro during the assembly of the TJ barrier could abolish the transient induction of ZO-1 and occludin expression (270). Earlier studies have shown that this transient induction of ZO-1 and occludin apparently is needed for the assembly of the Sertoli TJ barrier by providing the necessary TJ building blocks (82–84). More recent studies have illustrated that the TGF-β-induced disruption of the Sertoli cell-TJ-permeability barrier is mediated via the MEKKs/p38-MAP kinase pathway (271). Vascular endothelial growth factor, on the other hand, has been shown to induce phosphorylation of occludin and ZO-1, which in turn affects the TJ-permeability barrier in diabetic retinopathy and tumors (15), suggesting cytokines affect TJ function via their effects on the phosphorylation of TJ-associated proteins. Moreover, a recent study has demonstrated that the human occludin promoter is negatively regulated by TNF-α and INF-γ (281). Furthermore, these two cytokines have a synergistic effect to block the expression of human occludin gene (281). Also, these results are consistent with earlier findings, which showed that both cytokines can perturb the TJ-permeability barrier in HT-29/B6 cells, a subclone of the human intestinal cell line HT-29 (248), when the integrity of the TJ barrier was monitored by TER measurement (281). However, the downstream transcription factor(s) that cytokines, such as TGF-β, utilize to affect TJ functionality in the testis remains to be identified. This

information will be crucial to identify innovative targets or sites to perturb TJ function, which can block the movement of germ cells across the seminiferous epithelium. Other studies have shown that proteases and protease inhibitors are implicated in TJ/AJ assembly (for reviews, see Refs. 147, 308). For instance, it was shown that the presence of a protease inhibitor, such as chloroquine, indeed facilitated the assembly of a "tighter" Sertoli cell TJ barrier in vitro when assessed by TER across the cell epithelium (329). In addition, basic FGF and TGF-β can affect Sertoli cell plasminogen activator (a serine protease) expression and production in vitro (217, 316). Furthermore, a recent study has shown that PCI$^{-/-}$ (PCI, protein C inhibitor, a serine protease inhibitor) mice are infertile with impaired spermatogenesis apparently caused by a disruption of the Sertoli cell TJ barrier, which is likely the result of unchecked proteolytic activity in the seminiferous epithelium (464a). This observation is consistent with recently completed studies showing that the assembly and disassembly of Sertoli cell TJ barrier is associated with transient induction of proteases and protease inhibitors (82, 495), and their activities must be coordinated to maintain the integrity of the BTB (309). Taken collectively, these results illustrate the significance of cytokines, proteases, and protease inhibitors in TJ function. Likewise, they also demonstrate a tight physiological relationship between cytokines and proteases/protease inhibitors in the regulation of TJ dynamics.

5. *Regulation by ECM and peritubular myoid cells*

As described in section II.A.1, TJs in the testis are closest to, instead of furthest away from (vs. other epithelia), the basal lamina, which consists of several layers of matrices and cells. First, the basement membrane is a modified form of ECM of ~0.15 μm thick largely composed of proteins, such as collagen, laminin, heparan sulfate proteoglycan, entactin, and fibronectin (for review, see Ref. 127). In the testis, the basement membrane is adjacent to the Sertoli cell epithelium. Second, immediately outside of the basement membrane, there is a thin clear zone composed of collagen fibrils. Third, adjacent to the collagen layer is a layer of peritubular myoid cells with some scattered fibroblasts (for reviews, see Refs. 117, 127, 418). Due to such morphological intimacy between TJs and ECM in the testis, it was postulated that the ECM in the testis regulates the Sertoli cell TJ dynamics (415). Indeed, peritubular myoid cells in the basal lamina are known to regulate Sertoli cells via their secretory products (418, 518). Furthermore, collagen was shown to facilitate TJ assembly in A6 cells, a kidney epithelial cell line, by inducing phosphorylation of ZO-1 (216) and could stimulate occludin expression and production in human brain endothelial cells (391). A recent report has demonstrated that an antibody against collagen IV can indeed perturb the Sertoli cell TJ barrier in vitro, illustrating the significance of ECM in regulating Sertoli cell TJ dynamics (414, 415). Furthermore, it was demonstrated that the ECM-mediated effects on Sertoli cell TJ dynamics are possibly mediated via the interactions of ECM proteins, such as cytokines, collagen, proteases, and protease inhibitors, through a yet-to-be defined scheme of events (414, 415).

6. *A molecular model of TJ regulation in the testis*

Figure 5 depicts a hypothetical pathway by which Sertoli cell TJ dynamics are regulated in the rat testis. This model is prepared based on the recent findings that Sertoli cell TJ dynamics are regulated by *1*) protein phosphorylation, *2*) small GTPases, *3*) cAMP, *4*) proteases and protease inhibitors, *5*) calcium, and *6*) cytokines, as reviewed above.

IV. MALE CONTRACEPTION BY PERTURBING THE DYNAMICS OF SERTOLI CELL TIGHT JUNCTIONS

A. Introduction

The various devices, products, and approaches for male contraception currently available for men and/or under development are shown in Figure 6 and reviewed earlier (5a). These include approaches to perturb the function of the hypothalamus, pituitary, testis, epididymis, and spermatozoa per se as well as the barrier method (Fig. 6) (5a). Amongst these, the most widely used device for male contraception today is the condom, a barrier method that was developed in the 18th century (for review, see Ref. 157) (Fig. 6). While the condom is being used in industrialized nations, such as Japan, which consumed approximately one-fifth of the world's condoms, it has a relatively high failure rate versus female methods, such as the contraceptive pill for women (157). Another widely used irreversible male method is vasectomy, but it is associated with possible unwanted immunological consequences, such as the persistence of high-titer antisperm antibodies for up to 10 years, along with changes in testicular morphology (for review, see Ref. 113). For the past several decades, development of new and safer contraceptives for human males has largely focused on *1*) interfering the hypothalamus-pituitary-testicular axis to disrupt spermatogenesis and *2*) preparing vaccines against specific antigens residing on spermatozoa and male reproductive tract, or produced by the hypothalamus, such as gonadotropin releasing hormone (for reviews, see Refs. 50a, 341), with some success. For instance, administration of either high doses of testosterone or a combination of testosterone and synthetic progestins

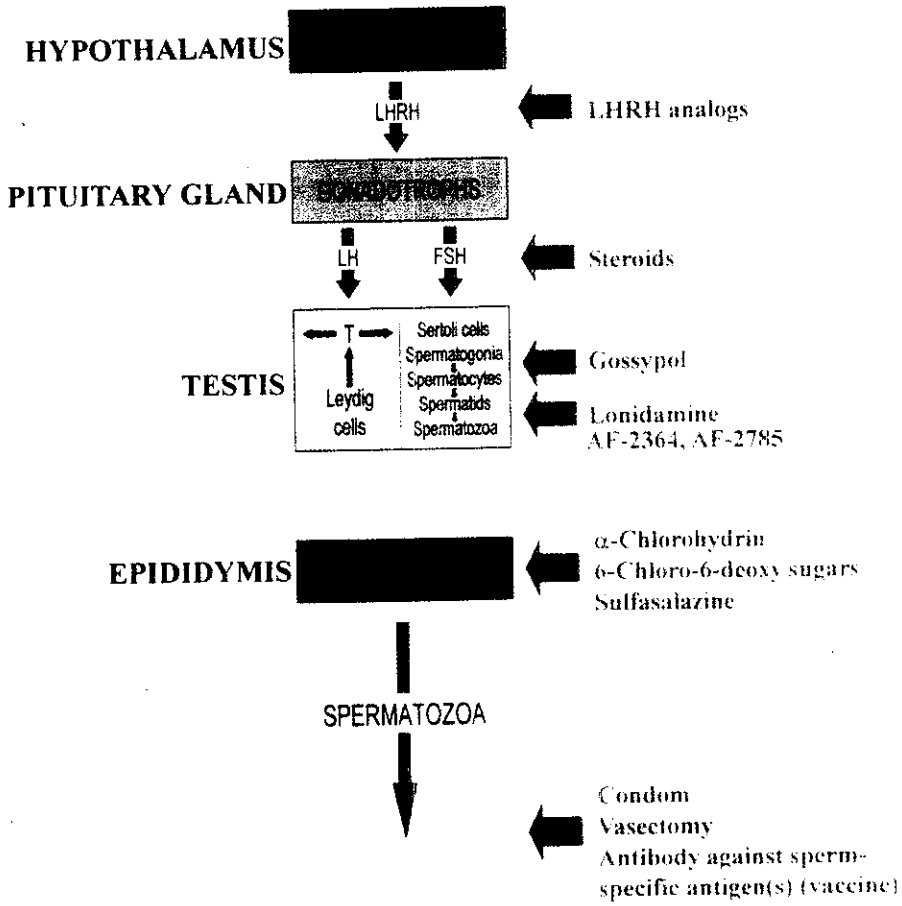

FIG. 6. Current approaches for male contraception. This figure depicts the current approaches in the field utilizing different routes to perturb male fertility at the level of the hypothalamus, pituitary gland, testis and epididymis, and spermatozoa per se. [Modified from Alexander and Bialy (5a).]

can inhibit pituitary gonadotropin secretion, which leads to oligospermia or azoospermia (for reviews, see Refs. 50a, 341). While its effects on spermatogenesis are reversible, exogenously added hormones can perturb the hormonal homeostasis of the treated human subjects. Because these hormones, such as testosterone, affect other physiological events in addition to their effects on the testis, their prolonged use may have undesirable side effects. On the other hand, while a safe vaccine against specific germ cell antigen(s) may be developed, its use in either man or woman could potentially have unwanted immunological consequences. Taking this information collectively, it is obvious that more innovative approaches should be developed. A safer approach may be to perturb the functionality of the BTB, such as blocking the migration of preleptotene and leptotene spermatocytes across the BTB to induce reversible aspermatogenesis. Recent studies have shown that TGF-β3, a Sertoli and germ cell product (for review, see Ref. 416), can perturb the Sertoli TJ-permeability barrier in vitro (270) possibly via its effects on the p38-MAP kinase pathway (271). These results thus suggest that if one can perturb the production of this cytokine by testicular cells, this could perturb the TJ barrier to permit the passage of developing spermatocytes across the BTB. Another alternative approach is to perturb TJs in the testis and the underlying associated AJs causing depletion of germ cells from the seminiferous epithelium. Indeed, two models are found in the literature with the use of either glycerol or cadmium chloride ($CdCl_2$) in which infertility can be induced except that the antispermatogenic effects of both compounds are irreversible. Furthermore, a potentially innovative approach is to perturb one of the key TJ proteins. Because occludin is a Ca^{2+}-independent intercellular adhesion molecule (399) known to confer to cell-cell adhesion likely via its first external loop (the second external loop confers the TJ functionality) (for review, see Refs. 138, 298), a disruption of the occludin can also lead to a loss of cell adhesiveness. In addition, occludin associates with ZO-1 at a stoichiometric ratio of 1:1 (153). This, in turn, associates with the underlying AJ molecule cadherin via fodrin (for reviews, see Refs. 68, 351). It is anticipated that any changes in occludin can likely disrupt AJs because there are cross-talks between components of the TJ and the cadherin-catenin system in the AJ (243, 456), such as those between Sertoli and germ cells. The disruption in TJs can possibly induce a cascade of events leading to germ cell release from the epithelium because of the disrupted AJs. Thus occludin apparently is one of the prime targets to perturb BTB function in the testis to

induce reversible infertility. In this section and section VI, we only review and discuss approaches targeted at perturbing junction dynamics in the testis "potentially" useful for male contraceptive development since extensive reviews on other approaches, such as hormonal contraception, vaccine development and vasectomy, have already been covered in several excellent recent reviews and books (for reviews, see Refs. 50a, 101a, 292, 341). Readers are strongly encouraged to seek additional information on these subject areas from these articles.

B. Perturbing the BTB by Glycerol or $CdCl_2$

1. *The glycerol model*

The use of glycerol to damage the BTB, which in turn inhibits spermatogenesis, is an important in vivo model for studying the biology of the BTB. Furthermore, it is a useful model to investigate the phenotypic consequences in the testis when Sertoli cell TJs are compromised (136, 486–489). When glycerol is applied intratesticularly, it causes long-term aspermatogenesis in rats without any apparent effects on Leydig cell steroidogenesis, serum FSH, luteinizing hormone, and testosterone levels, and secondary sexual characteristics (486, 487). Its antispermatogenic effects have also been shown in rabbits and squirrel monkeys (488, 489). On the other hand, when glycerol was administered intratesticularly at a lower concentration, it induced much less structural damage to the seminiferous tubules when examined microscopically (206). It also alters the permeability of the BTB in the rat (136). These results thus illustrate that its antispermatogenic effect may be largely mediated via its effects by compromising the functionality of Sertoli cell TJs. Indeed, recent fluorescent microscopy studies have shown that the network of occludin, actin microfilaments, and microtubules in the seminiferous epithelium in glycerol-treated rats are damaged (490). Such structural damage to the BTB is likely to induce an immunological response from the systemic circulation of treated animals, which in turn causes germ cell loss. However, the precise mechanism of action of glycerol in inducing male infertility is not entirely known. The major drawback of this model is that glycerol-induced germ cell loss and damage to the tubules are irreversible.

2. *The $CdCl_2$ model*

Cadmium toxicity and its associated damage to the testis have been known since the early 1900s (7). Cadmium-induced damage to the testis includes a loss of the endothelial TJ barrier, damage to the vascular system, edema, ischemia, and tissue necrosis (16, 156, 180, 287), which can be prevented by zinc (337). $CdCl_2$ also induces apoptosis in testicular tissue (501), characterized by an activation of endonucleases as a result of the loss of intracellular calcium (269). Subsequent studies have shown that cadmium causes damage to Sertoli cell TJs that constitute the BTB both in vivo, which occurs before vascular damage (194, 406). Cadmium can also perturb the Sertoli cell TJ barrier in vitro (82, 219). At low doses, cadmium can selectively cause failure of spermiation in the rat (193), suggesting that this model can be used to study selective features of spermatogenesis. Although cadmium-induced damage to the BTB in vivo is irreversible, $CdCl_2$-induced Sertoli cell TJ-permeability barrier disruption in vitro can be reversed after cadmium is removed from medium and cells are exposed to testosterone (82), suggesting the presence of androgens can protect Sertoli cells from $CdCl_2$-induced damage (82). Indeed, the presence of testosterone at 2×10^{-7} M, a concentration that is found in the testis (~200 nM), such as in the rete testis fluid and seminiferous tubular fluid (462), which is ~100-fold higher than the level of testosterone in the systemic circulation (~2 nM), can protect Sertoli cells from the $CdCl_2$-induced damage to the TJ-permeability barrier (82). Immunofluorescent confocal microscopy studies have shown that $CdCl_2$ causes disorganization of TJ-associated microfilaments in Sertoli cells in particular in stages VIII, as well as stages II–III tubules (194). However, microfilament bundles in Sertoli cells in stages before stage VIII, and in peritubular myoid cells are not affected by the $CdCl_2$ treatment (194). These results thus suggest that microfilament bundles in Sertoli cells are the primary target of $CdCl_2$, whose effect is stage dependent. Nevertheless, the precise mechanism by which $CdCl_2$ induces BTB disruption is not entirely known. Recent in vitro studies have shown that $CdCl_2$ also causes a reduction in occludin expression in Sertoli cells. Its removal, coupled with the presence of testosterone, however, can induce occludin expression causing the resealing of the perturbed Sertoli TJ-permeability barrier (82). Other studies have shown that E-cadherin is also one of the primary targets of $CdCl_2$ (for review, see Ref. 352). The significance of E-cadherin in TJ functionality has been known for more than two decades. For instance, addition of anti-E-cadherin antibody to MDCK cells in vitro can impair resealing of the Ca^{++} depletion-induced TJ permeability barrier disruption (179). The presence of $CdCl_2$ can cause redistribution of E-cadherin in Caco-2 cells (for review, see Ref. 352). It was also shown that Cd^{++} competed with the binding of Ca^{++} to the putative calcium binding motif of E-cadherin (353). Furthermore, studies using MDCK and LLC-PK_1 cells (354) cultured in vitro have shown that treatment of these cells with cadmium can cause the redistribution E-cadherin, causing it to move away from the site of AJs at the cell-cell border and becoming diffusely localized in the cytoplasm (for review, see Ref. 352). This is possibly aided by small GTPases. These results thus illustrate that a chemical entity that

can perturb TJ functionality can indeed induce male infertility. Again, the major drawback of this model is that the $CdCl_2$-induced BTB damage and male infertility are irreversible, although this is a useful model to study the functionality of the BTB and the cascade of events leading to the disassembly of TJs and AJs (82, 166, 194).

C. Other Toxicants That Disrupt Male Reproductive Function and Induce Infertility

In this context, it is noteworthy to mention that there are other models of male infertility induced by environmental toxicants other than $CdCl_2$ and glycerol. These other models are not being discussed in detail herein because either their mechanisms of action are not mediated at the site of TJs and/or AJs or they are not entirely clear. For instance, Sokol and colleagues (419–421) have shown that lead exposure can result in reduced fertility and sperm counts in male rats, but this effect is mediated via the hypothalamic-pituitary-testicular axis since serum testosterone and FSH levels (but not luteinizing hormone) of lead acetate-treated rats were significantly reduced verus control rats. These results suggest that the mechanism of action of lead-induced infertility is not mediated via an interference of junction dynamics. Other studies have shown that 1,2-dibromo-3-chloropropane (DBCP), phthalates [e.g., di-*n*-butyl phthalate (DBP), di-(2-ethylhexyl)phthalate (DEHP)] can also affect male reproductive function, thereby inducing male infertility (for reviews, see Refs. 42, 43, 199). DEHP is a Sertoli cell toxicant, which possibly exerts its effect on the Sertoli cell membrane by inhibiting FSH-mediated signal transduction. This reduces Sertoli cell proliferation activity (189), similar to other toxicants that affect signal transduction (for review, see Ref. 103). A subsequent study has shown that mono-(2-ethylhexyl)phthalate (MEHP) can disrupt vimentin filaments at the site of Sertoli-germ cell AJs (358), which reportedly also induces a surge in the expression of testicular Fas receptor and germ cell apoptosis (358). This latter study (358) thus suggests that some of these toxicants, such as phthalates, which induce germ cell loss from the seminiferous epithelium may indeed exert their effects by perturbing junction dynamics in the testis. However, the damaging effects for many of these toxicants, such as phthalates and 2,5-hexanedione, are irreversible unless the exposure of rats to such toxicants, such as phthalates, is over a relatively brief period of time and at very low doses (for review, see Ref. 42). If such compounds can be adequately modified so that their action is limited to the site of the ES or a testis-specific AJ component, they may become useful to induce male infertility by perturbing junction dynamics.

D. Disruption of Sertoli Cell TJ Dynamics In Vitro and In Vivo by a Synthetic Occludin Peptide

1. *Induction of occludin expression during the assembly of the Sertoli cell TJ-permeability barrier*

When freshly isolated Sertoli cells from 20-day-old rat testes were cultured in vitro on Matrigel-coated bicameral units or dishes, it was shown that the Sertoli cell TJ-permeability barrier was formed by *days 3–4* when assessed by either TER across the Sertoli cell epithelium or other parameters, such as the restricted diffusion of [^{3}H]inulin or ^{125}I-BSA across the cell epithelium (82, 166, 167, 169, 270, 495). This assembly of the Sertoli cell TJ barrier was also accompanied by a transient but significant induction in the expression of occludin and ZO-1 (82–84, 270, 495). Such an increase in TJ protein production may be used to provide the necessary building blocks for assembling TJs. This postulate is further strengthened by the observation that a change in ZO-1 or occludin expression was not detected when Sertoli cells were cultured at low cell density at which TJs could not form due to a lack of proximity between cells (84, 495). Although these results are corroborative in nature, and the actual involvement of occludin in Sertoli cell TJ assembly is still lacking, they suggest that a disruption in the functionality of one of the TJ proteins, such as occludin, may perturb TJ dynamics in the testis.

2. *Disruption of the Sertoli cell TJ barrier by a synthetic occludin peptide in vitro*

To verify the physiological significance of the above in vitro studies, which suggest that an induction of occludin is required for the assembly of Sertoli cell TJs, a 22-amino acid peptide was synthesized, which corresponded to part of the second external loop of rat occludin (Figs. 4 and 7) between residues Gly-209 and Asp-230, NH_2-GSQIYTICSQFYTPGGTGLYVD-COOH (Fig. 7) (12), and is known to confer the TJ function (for reviews, see Refs. 138, 298). Sequence alignment of this second extracellular loop reveals that it displays extensive homology among different species (Fig. 7). When this 22-amino acid peptide at a concentration of either 0.4 or 4 μM was added to bicameral units when the Sertoli cell TJ barrier was being assembled, its presence significantly reduced the Sertoli cell TJ-permeability barrier dose dependently (83). When this peptide was removed from the Sertoli cell epithelium, the perturbed TJ-permeability barrier gradually resealed, making it indistinguishable from control cultures (83). In contrast, the inclusion of another synthetic peptide at 4 μM, NH_2-TKVNERYELCDTYPALLAV-PAN-COOH, corresponding to residues Thr-156-Asn-177 of yet another rat Sertoli cell product, myotubularin (rMTM) (262, 264), failed to affect the assembly of Sertoli cell TJs in vitro (83). These results clearly illustrate that

```
Human   NH2-...PTAQ-SSGSLYGSQIYALCNQFYTPAATGLYVDQYLYHYCVVDPQE...-COOH  199-243
Mouse   NH2-...PTAQ-ASGSMYGSQIYMICNQFYTPGGTGLYVDQYLYHYCVVDPQE...-COOH  197-241
Rat     NH2-...PTAQ-ASGSMYGSQIYTICSQFYTPGGTGLYVDQYLYHYCVVDPQE...-COOH  199-243
Chicken NH2-...PQAQM-SSGYYYSPLLAMCSQ---AYGST-YLNQYIYHYCTVDPQE...-COOH  187-227
Dog     NH2-...PTAQ-ASGSLYSSQIYAMCNQFYASTATGLYMDQYLYHYCVVDPQE...-COOH  198-242
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FIG. 7. Sequence alignment of the second extracellular loop of rat occludin versus that of human, mouse, chicken, and dog. Sequence alignment of the entire second extracellular loop of occludin among five different species (12, 297, 298; GenBank accession numbers AB016425, NP-032782, AAC50451, and A49467). This extracellular domain (see Fig. 4) spans residues 199–243 in the rat versus 187–227 in the chicken. For rat occludin, the underlined stretch of amino acid sequence represents the 22-amino acid peptide that had been synthesized to study its antispermatogenic effects (83).

the assembly of the Sertoli TJ barrier is a dynamic event requiring de novo synthesis and assortment of TJ component proteins, such as occludin, into the sites of TJs.

These results are also consistent with an earlier study by Wong and Gumbiner (496) who had used two 44-amino acid synthetic peptides based on chick occludin, which covered the entire first and second external loops, to perturb TJ assembly in *Xenopus* kidney A6 epithelial cells in vitro. However, only the 44-amino acid synthetic peptide based on the entire second external loop could perturb TJ assembly in this cell line (496). This disruption of the TJ barrier possibly resulted from the use of the synthetic occludin peptide by the cultured cells to construct TJs. While they are homologous to a specific segment of the extracellular domain of occludin, they do not have the structural conformation of the entire molecule to reinforce TJ functionality. As such, the TJ permeability barrier becomes disrupted. Alternatively, these peptides may form a homotypically interlocking association with the corresponding intact occludin molecule between apposing Sertoli cells preventing the assembly of "tight" and "functional" TJs.

3. *Reversible disruption of spermatogenesis in vivo by intratesticular injection of a 22-amino acid occludin peptide*

To define the in vivo physiological relevance of the in vitro observation described above, which illustrates that the occludin peptide can perturb the Sertoli cell TJ barrier assembly, studies were performed to examine whether this peptide can indeed perturb spermatogenesis and the BTB when administered in vivo. Two synthetic peptides, NH_2-GSQIYTICSQFYTPGGTGLYVD-COOH, corresponding to residues Gly-209-Asp-230 in the second extracellular loop of rat occludin (see Fig. 7), and NH_2-TKVNERYELCDTYPALLAVPAN-COOH, corresponding to residues Thr-156-Asn-177 of rat myotubularin (262, 264), were used. Adult rats received either saline or vehicle without (control) or with (test) 1.5 mg of the corresponding peptide per testis at three sites (136, 169, 381) by intratesticular injection. It was shown that intratesticular injection of this purified occludin peptide caused a reduction in testicular size and weight by as much as 50% within 4 wk compared with control rats receiving no treatment, vehicle alone, and myotubularin peptide (83). Morphological analysis of the treated testis revealed that more advanced germ cells, such as elongated spermatids, began to deplete from the epithelium between 8 and 16 days after occludin peptide treatment. Massive depletion of germ cells from the epithelium was detected in virtually all the tubules examined by 27 days after occludin peptide treatment. In addition, the seminiferous tubules of the occludin-treated testes shrunk significantly, with the tubular diameter being reduced by as much as 20–30% compared with control rats or testes receiving vehicle or myotubularin peptide. Germ cells began to repopulate the epithelium after 27 days postoccludin peptide treatment. By 47 days, spermatocytes were visible in all the tubules examined, and the morphology of the seminiferous epithelium appeared indistinguishable from control rats by 68 days, showing full recovery from the occludin peptide-induced testis damage. The fact that the testes recovered almost completely within 40 days suggests that spermatogonia were not destroyed by the occludin peptide treatment (83).

4. *Can the occludin peptide reversibly disrupt the BTB in the rat testis in vivo?*

Recent studies using ^{125}I-BSA administered to the jugular vein to monitor the diffusion of this tracer from the systemic circulation across the BTB into the rete testis fluid (RTF) and the seminiferous fluid (STF) to assess the integrity of the BTB by micropuncture technique after occludin peptide treatment have yielded some important observations. First, a disruption of the BTB indeed had occurred following an intratesticular injection of occludin peptide, since there was an accumulation of ^{125}I-BSA in the STF and RTF in the peptide-treated testis between 2 and 6 wk posttreatment compared with the untreated testis in the same rat after infusion of ^{125}I-BSA through the jugular vein (83). Second, the peptide-induced damage to the BTB was reversible, since there was a drastic decline in ^{125}I-BSA accumulation in both STF and RTF by 12 wk, coinciding with the recovery of the epithelium (83). Furthermore, the level of radioactivity in the STF and RTF collected from treated rats became indistinguishable from controls (83). Damage to the BTB

appears to be occludin peptide specific, since the rMTM peptide failed to perturb the BTB function.

5. *Mechanism of occludin peptide-induced germ cell loss from the seminiferous epithelium*

It was postulated that the loss of germ cells from the epithelium is mediated by the occludin peptide-induced disruption of TJs and the associated AJs, since it is known that TJ disruption can damage AJs, possibly via changes in E-cadherin (243, 456). These results also suggest that there is cross-talk between TJs and AJs. For instance, it has been shown that $CdCl_2$-induced damage to TJs can reduce the expression of E-cadherin and/or its redistribution at the site of AJs (243, 352). Other studies have also illustrated a functional linkage between TJs and AJs. For instance, when TJs in MDCK cells were disrupted by $[Ca^{2+}]$ depletion, addition of anti-E-cadherin antibody along with the replacement $[Ca^{2+}]$ could prevent the resealing of TJs (179), illustrating that a disruption of AJs can prevent TJ reassembly. Moreover, it has been shown that E-cadherin expression was highest in the rat testis at 14 days of age just before the establishment of BTB (500), suggesting the assembly of TJs requires the recruitment of E-cadherin to AJs as well. It remains to be determined, however, whether other cellular changes occur when the BTB was disrupted by the occludin peptide in vivo. Also, the observed germ cell loss can be mediated by an immunological mechanism similar to orchitis, which should be investigated in future studies. The fact that a disruption of Sertoli cell TJs can cause infertility is not without precedence. It has been shown that claudin-11 knock-out mice, in which a complete loss of TJ fibrils in Sertoli cells was detected, were sterile (164). Taken collectively, these results suggest that new approaches can be developed to disrupt spermatogenesis by manipulating the functionality of TJ proteins in the testis.

6. *Future perspectives*

It is obvious that the use of the peptide approach relies on the availability of an effective delivery system without the use of intratesticular injection, which would be highly uncomfortable to treated animals. This can possibly be achieved by the use of a recombinant modified FSH having only the receptor-binding domain but lacking the hormonal activity. If a contraceptive peptide can be conjugated to such a recombinant FSH protein, it can be efficiently delivered to the testis since FSH receptors are restricted to Sertoli cells in human males. This possibility should be explored as an alternative delivery system. A second approach is to use an adenovirus to deliver the peptide to the testis, which should also be explored in future studies. Alternatively, this peptide can be conjugated to protein transduction domains (PTDs), small peptides of ~10–16 residues having numerous positively charged lysine and arginine residues (185, 195, 398, 399, 453), for delivery. Recent studies have shown that a functional protein as large as β-galactosidase (120 kDa) can be delivered to cells intracellularly via an intraperitoneal injection coupled to Tat-PTD (Tat, transactivation protein from lentiviruses) with a sequence of NH_2-RKKRRQRRR (398, 399). In this respect, it is noteworthy to mention yet another approach for male contraception based on the existing knowledge of TJ dynamics is to hamper the translocation of preleptotene and leptotene spermatocytes across the BTB by shuting down the Sertoli cell TJ barrier. For instance, cytokines are known to perturb the TJ barrier in many epithelia (for reviews, see Ref. 476 and sect. III*E4*). Other studies have shown that the assembly of the Sertoli cell TJ barrier is associated with a transient plummeting of TGF-β3 mRNA expression and protein production (270, 271). Indeed, inclusion of TGF-β3 to Sertoli cells cultured on Matrigel-coated bicameral units can also perturb the Sertoli cell TJ barrier by affecting the expression of occludin, ZO-1, and claudin-11 (270) possibly via the MEKKs/p38 MAP kinase pathway (271), suggesting that TGF-β3, at the very least, is one of the molecules inducing the timely opening of the BTB. Ironically, these results suggest that if this TGF-β3-mediated regulatory pathway(s) can be delineated, the BTB can be shut down by blocking TGF-β3 action using SB202190, a specific p38 MAP inhibitor (354a). A recent study has demonstrated that SB202190 can indeed block the TGF-β3-induced perturbing effects on Sertoli cell TJ barrier (271).

In this context, it is noteworthy to mention that the use of a goitrogen, such as 6-propyl-2-thiouracil (PTU), to induce transient neonatal hypothyroidism in rats can increase testis weight and size and germ cell production, but not body weight, in adulthood (98a, 98b, 98c). PTU-treated rats also display delayed expression of Sertoli cell products, such as androgen binding protein, clusterin, inhibin-β_B, and hemiferrin, a spermatid-specific protein (52a). Furthermore, rats treated with PTU since birth until postnatal *day 25* displayed delayed BTB assembly (116a). While some TJ structures were detected by electron microscopy, extensive TJ development consistent with the assembly of the BTB that took place on *day 15* in normal rats was not detected in PTU-treated rats on *days 15* and *25* (116a). It is not known from these reports (98c, 116a) if the BTB was ever formed in PTU-treated rats. Also, the maturation of Sertoli cells was delayed in PTU-treated rats since Sertoli cells continued to divide after *day 15* (116a). This also contributed to the increase in overall Sertoli cell and germ cell number, as well as testicular weight (98a, 98b, 98c). These results thus suggest that a transient disruption of the BTB at puberty can lead to changes in Sertoli cell function and testicular development. Furthermore, these studies also demonstrate the significance of Sertoli cells to the development and func-

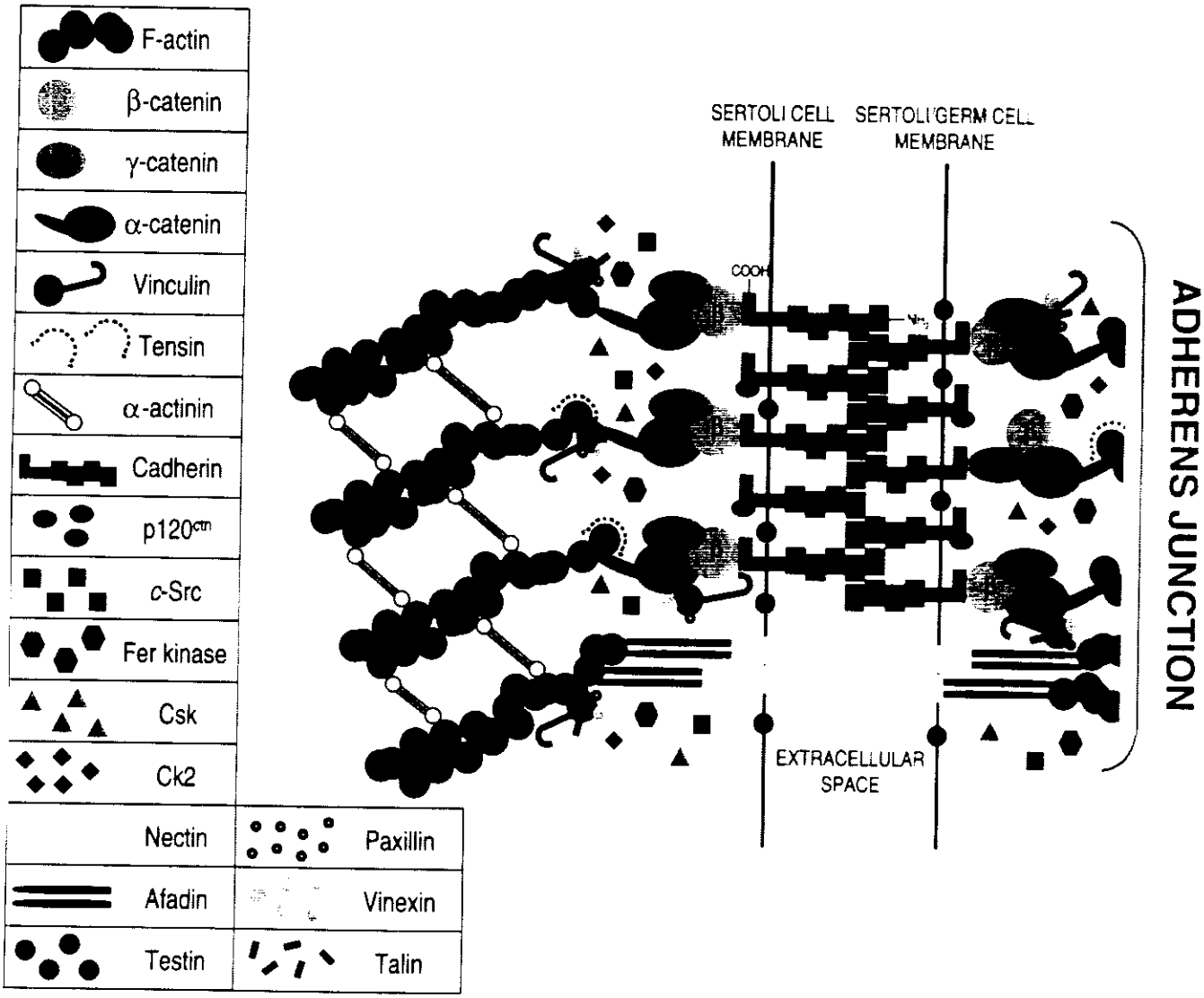


FIG. 8. A schematic drawing that depicts the component proteins at the sites of actin-based cell-cell adherens junctions (AJ) between Sertoli and germ cells and the associated signaling molecules in AJs. This figure was prepared based on the following articles and reviews (9, 34, 35, 52, 64, 111, 177, 265, 308, 382, 427, 451, 469, 503).

tioning of the testis. Yet, it is not known from these reports whether spermatogenesis was affected when the BTB was perturbed in adult rats. PTU apparently can only exert its effects in neonatal rats during the first postnatal week (*days 4–8*). This time frame appears to be the critical period in which rats respond to this treatment (98c). For instance, if PTU treatment begins on *day 8* or later, it fails to affect testis weight and daily sperm production, but it is not known if the BTB can be damaged (98c).

In summary, recent advances in studying TJ dynamics have provided an unprecedented opportunity not only to understand the biology of germ cell movement during spermatogenesis, but they will also be helpful in designing innovative approaches for male contraception.

V. ANCHORING (OR ADHERING) JUNCTIONS IN THE TESTIS

A. Functions of Anchoring Junctions

Anchoring or adhering junctions (Table 1, Fig. 8) link cytoskeletal elements from one cell either to another cell or to the extracellular matrix creating a network that maintains tissue integrity. In other tissues, such as the epidermis, the anchoring junction also acts as a first line of defense against the external environment (for review, see Ref. 210). Furthermore, their component proteins (e.g., cadherins, catenins, and p120^{ctn}) are signal transducers and can induce transcriptional activation (9, 437), acting as the platform for signal transduction by relating

an extracellular stimulus to nuclear activation of genes (for reviews, see Refs. 18, 468). More importantly, the dynamic nature of anchoring junctions in the testis permits the timely passage of developing germ cells across the seminiferous epithelium from the basal to the adluminal compartment during spermatogenesis. For instance, it is known that the half-life of E-cadherin is ~5–10 h in confluent epithelial cells (291, 407). This short half-life of an anchoring junction structural protein illustrates that Sertoli-germ cell contacts are subject to extensive alteration and remodeling, which are regulated by some yet-to-be defined mechanisms and signal transduction pathways. The classification of different anchoring junctions can be found in section II*A2* and Table 1. In the following section, most of the discussion is focused on cell-cell actin-based AJs (Fig. 8), one of the four types of anchoring junctions (see Table 1). This is the only anchoring junction type in the testis that has been subjected to intense investigations.

B. Proteins of Anchoring Junctions

1. *Cadherins*

Cadherins are transmembrane proteins of 115–130 kDa belonging to the family of Ca^{2+}-dependent CAMs, which affect cell morphology, architecture, and function (443–445, 502) (Fig. 8). More than 30 cadherins have been identified to date. Two different classes of cadherins are found in AJs and desmosomes (a cell-cell intermediate filament-based anchoring junction, see Table 1 and Fig. 2). Cadherins found in cell-to-cell AJs link to actin, whereas those found in desmosomes link to intermediate filaments (2, 191, 205, 234, 235, 244, 314, 443–445).

A) CLASSICAL CADHERINS. These include epithelial cadherin (E-cadherin, 120 kDa), neural cadherin (N-cadherin, 130 kDa), placental cadherin (P-cadherin), and liver-cell adhesion molecule (L-CAM) (80, 132, 176, 234, 235, 299, 423). The intracellular COOH-terminal domains of cadherins associate with β- or γ-catenins at the site of AJs forming the cadherin/catenin complex, whereas the extracellular NH_2-terminal domains of two cadherins residing in adjacent cells interact homotypically (Fig. 8). These complexes are the functional cell adhesive units (177). α-Catenin in turn interacts with β- or γ-catenin-bound cadherin, linking this complex to the actin cytoskeleton (365). The interaction of cadherin and catenin also requires Ca^{2+} (1, 2, 191, 203, 485) and is regulated by GTPases (177, 232). In the absence of Ca^{2+}, cadherins are inactive and susceptible to proteolysis. Furthermore, the cadherin cytoplasmic domain contains consensus sequences for phosphorylation by casein kinase-2 (CK-2) and glycogen synthase kinase (GSK) (265). Indeed, phosphorylation of cadherin in vitro increases its ability to bind to β-catenin (265), raising the possibility that the cadherin-mediated cell adhesiveness can be regulated by the extent of its β-catenin binding. However, evidence is lacking that a modulation of β-catenin binding can affect cell adhesion in vivo (for review, see Ref. 177). Recent studies have also shown that both E-cadherin and β-catenin are recyclable proteins, which can become internalized and recycled back to the plasma membrane by Rab 5 GTPase (257).

B) DESMOSOMAL CADHERINS. The major desmosomal cadherins are the desmogleins (Dsgs) and desmocollins (Dscs), which are known to participate in cell adhesion (58, 245, 246) in the cell-cell intermediate filament-based anchoring junctions called desmosomes. Three Dsgs and three Dscs are known to exist: Dsgs 1, 2, and 3 as well as Dscs 1, 2, and 3. Other desmosomal proteins are desmoplakins I and II (58, 245, 246). Recent immunohistochemistry studies have demonstrated the presence of desmoglein around spermatids (493). Still, this class of anchoring junction remains to be characterized biochemically and functionally in the testis.

2. *Catenins*

There are four types of catenins, namely, α-catenin (~100 kDa), β-catenin (85–88 kDa), γ-catenin (80–82 kDa), and $p120^{ctn}$ (90–120 kDa) (332), which share high homologies with cytoskeletal proteins (246, 485) (Fig. 8). The β- and γ-catenins interact with the cadherin cytoplasmic domain in a stretch of sequence of ~100 amino acids (200), and α-catenin (also an actin-binding protein) links the cadherin to the actin cytoskeleton (177, 235) via its interactions with a number of actin-binding proteins, such as α-actinin, vinculin, ZO-1, or directly binds to actin itself (209, 365, 477, 482). It is also known that the binding of α-catenin to ZO-1 can affect the strength of E-cadherin-mediated adhesion in nonepithelial cells; however, this effect is not detectable in epithelial cells (209), suggesting the role of catenins in different cell types could be different. Also, β-catenin is known to form complex with LEF/TCF family of transcription factors and the tumor suppressor product APC (204, 474). Recent mutation studies have revealed clusters of amino acid residues found in β-catenin that are used specifically for binding to LEF/TCF, APC, axin/conductin, and cadherin (165, 474). Based on this evidence, it is clear that β-catenin, in addition to its structural role in AJs, also functions as a signal transducer. In this regard, it is of interest to note that overproduction of cadherins inhibits the transcriptional activity of β-catenin (137), suggesting the cadherin/catenin complex is an important platform for signal transduction. Furthermore, catenins also exist as a free pool of intracellular proteins and are substrates of protein kinases (191, 485). For instance, tyrosine phosphorylation of β-catenin and/or γ-catenin (also known as plakoglobin) (106, 332, 514) correlates with the loss of cadherin-medi-

ated cell adhesiveness (33, 288, 406), possibly by causing the cadherin/catenin complexes between apposing cells to move away from each other (111, 177). Indeed, recent studies have shown that β-catenin is the putative substrate of Src (348a). However, how such changes in the phosphorylation status of the cadherin/catenin complex lead to the loss of adhesiveness and how they affect the catenin-cadherin binding are not known? It was recently proposed that there are pools of active and inactive forms of β-catenin in mammalian cells, regulating the cadherin/catenin and the APC-axin complex and the downstream transcription events (163). However, it remains to be determined if such active and inactive pools of catenins indeed exist in vivo. If they do, what then triggers the conversion of active from inactive form, and vice versa?

3. *Nectins and afadins*

In addition to the cadherin/catenin complex, nectins belong to an emerging new family of AJ-integral membrane proteins found in most AJs. The intracellular domain of each nectin molecule interacts with an afadin (formerly called AF6 and was originally found at TJs) molecule, an F-actin binding protein, to form the afadin/nectin complex (46, 207, 390, 438, 439) (Fig. 8). Afadin in turn interacts with ZO-1, α-catenin, and ponsin (ponsin is a putative afadin-binding protein) (507a) (see Fig. 8) in the cytoplasm at the site of AJs. A recent study has shown that a short stretch of sequence of α-catenin, residues 385–651, contains the putative afadin binding site (348b). As such, afadin can bind the nectin/afadin complex directly to actin or via α-catenin (see Fig. 8). Both nectins and afadins are immunoglobulin-like cell adhesion molecules (46, 207, 390, 439). Two variants of afadin are known to date designated l- and s-afadin (207), which are variants of AF-6 and are putative effector proteins of Ras GTPase (440). The afadin/nectin complex is highly concentrated in AJs and is found at the same site of the cadherin/catenin complex (439) (Fig. 8). While the afadin/nectin complex does not interact directly with the cadherin/catenin complex structurally (439), nectin and cadherin interact with each other via their cytoplasmic domain-associated proteins (438) (Fig. 8). For instance, nectin was shown to recruit ZO-1 and α-catenin to the nectin/afadin complex (507a), possibly enhancing cell adhesion. Yet, the precise mechanism by which afadin/nectin affects cell adhesiveness is not known. Presumably, it exerts its effects via the cytoskeletal organization and polarization of epithelial cells. Also, it is not known if the testis is using this AJ functional unit side by side with the cadherin/catenin complex to regulate AJ dynamics. Recent studies have shown that in afadin$^{-/-}$ mice, the organization of AJs is severely impaired (207). Furthermore, mouse embryos lacking afadin display disrupted structural organization of cadherin-based AJs and TJs in polarized epithelia (207). These results seemingly suggest that there is cross-talk between the nectin/afadin and cadherin/catenin complexes and TJs and that both complexes may interact cooperatively at the functional level. Furthermore, the nectin/afadin complex may play a role in the communication between AJs and TJs, since a deletion of the afadin gene affects both AJ and TJ functionality (207). Nectin is a growing family of AJ integral membrane proteins with four members known to date, namely, nectin-1, -2, -3, and -4, all of which are immunoglobulin-type CAMs (46, 95, 300, 304, 390). Nectin-1 and -2 are also members of the poliovirus receptor gene family (95, 96), whereas nectin-3 (83 kDa) is a member of the alpha herpes virus receptor family (356a). Nectin-1 is abundantly expressed in the brain, and nectin-2 and -3 are found in virtually all tissues, including the testis (390). The expression of nectin-2 is stage specific in the mouse testis, and immunohistochemistry studies have shown that it is largely restricted between spermatids and Sertoli cells at stages V–VIII (46), likely to be at the site of apical ES. It has recently been reported that nectin-2 is present exclusively in Sertoli cells and nectin-3 in elongated spermatids (331a), allowing the heterotypic interactions of nectin-2/afadin and nectin-3/afadin at the site of apical ES. These results also suggest that the nectin-afadin complex is one of the three constituent protein complexes of the ES besides $\alpha_6\beta_1$-integrin/laminin (e.g., laminin-12 $\alpha_2\beta_1\gamma_1$) (244a, 310, 470) and the cadherin/catenin complex (69, 260, 277, 493). Furthermore, an increase in tyrosine phosphorylation of nectin-2δ is detected in response to nectin-afadin-mediated cell-cell adhesion (237a). For instance, the use of an anti-E-cadherin antibody to disrupt AJs can reduce the tyrosine phosphorylation of nectin-2δ, possibly at Tyr-505 (237a). These results further support the postulate that there are cross-talks between the cadherin/catenin and nectin/afadin complexes. Nectin-3 is known to have three splicing variants of nectin-3α, -3β, and -3γ (390). Nectins interact with each other homo- or heterotypically confering cell-cell adhesion activities between adjacent cells (300, 390). Transgenic male mice lacking nectin-2 are infertile with abnormal cytoskeleton and defects in nuclear morphology in spermatids with mitochondria found in the head of spermatids from steps 10–16 instead of confining to the midpiece (46). These results thus illustrate the crucial function of the nectin/afadin complex in male reproductive function.

4. *Testin*

Testin, an AJ-associated protein (Fig. 8), was originally identified in Sertoli cell-enriched culture medium (77), which was subsequently purified to apparent homogeneity (73, 75), and its full-length cDNA was cloned and sequenced (171). Analysis by SDS-PAGE and protein microsequencing have demonstrated that testin consists of

two molecular variants of 37 kDa (testin I) and 40 kDa (testin II), which differ by only three amino acid residues of TAP found in testin II versus testin I from the NH_2 terminus (75, 78, 171). As such, testin I is possibly a product of testin II, which is formed as the result of posttranslational processing of the mature protein, and these two variants are now referred to as testin (75, 78, 167, 169–171). Since the late 1980s, this Sertoli cell glycoprotein has been extensively studied, and the following features can be ascribed to this protein.

While testin is a Sertoli cell secretory and testosterone-responsive glycoprotein (73, 75, 167), it becomes quickly associated with the Sertoli cell surface at the site of Sertoli-germ cell AJs, in particular between Sertoli cells and late spermatids, following its secretion (169, 170). Immunogold electron microscopy (EM), fluorescent microscopy (169, 516), and other biochemical analyses have shown that testin binds to the Sertoli cell surface via its interaction with a receptor-like protein, possibly the cadherin/catenin and/or nectin/afadin complex and an AJ-associated protein, particularly at the site of ectoplasmic specializations (169, 170, 513, 516, 517).

In adult rats, testin is confined almost exclusively to the gonad (171, 513). Sertoli cells are the principal source of testin in the seminiferous epithelium, and germ cells isolated from adult rat testes do not express or secrete testin (171, 513).

In the adult rat testis, testin is associated with the Sertoli cell membrane. Its level in the cytosol is virtually undetectable and is not found in the epididymis, fluids of the rete testis, and the seminiferous tubule (73, 75, 169, 170). This is in sharp contrast to other Sertoli cell products, such as androgen binding protein, α_2-macroglobulin, and transferrin, which are concentrated in the luminal fluids of the male reproductive tract (73, 75, 169, 170).

Studies by immunofluorescent microscopy, immunohistochemistry, and immunogold EM have shown that testin is found at the sites of Sertoli-germ cell AJs, such as ectoplasmic specializations and tubulobulbar complexes (169, 516, 517). Its pattern of localization is stage specific, being highly expressed in stage VII and early stage VIII, just before spermiation (517). However, testin is neither a protease nor protease inhibitor (171). The fact that there is an intense but transient accumulation of testin between elongated spermatids and Sertoli cells in vivo at the site of ectoplasmic specializations just before spermiation strongly suggests its signaling role in AJ dynamics.

A surge in testin expression is not detected when either the Sertoli cell TJ-permeability barrier in vitro is perturbed, such as by depleting Ca^{2+} from the spent medium, or when the blood-testis barrier in vivo is disrupted by $CdCl_2$ or by glycerol treatment (166, 169). However, its expression is greatly induced when AJs are perturbed both in vitro and in vivo (78, 166–170, 222). These results thus demonstrate that testin can be used to monitor the integrity of AJs, but not TJs, in the testis.

While testin is virtually expressed exclusively in the adult rat testis, its level declines during testicular maturation (73, 75, 170). However, depletion of germ cells from the adult rat testis in vivo induced by X-irradiation (222) or by treatment with either busulfan (78) or lonidamine (170), which disrupts AJs, can induce a surge in testin. When germ cells repopulate the epithelium, the level of testin declines rapidly. Removal of germ cells from Sertoli-germ cell cocultures by hypotonic treatment, which disrupts AJs between Sertoli and germ cells in vitro physically, also induces a surge in testin expression (170). These results illustrate that a disruption of AJs induces testin expression, but when AJ functionality is rebuilt, its level subsides. These observations, however, are not the result of downregulation of Sertoli cell testin expression by germ cells, since neither germ cells nor their conditioned media per se affect Sertoli cell testin expression (169, 170).

Taken collectively, these results clearly illustrate that the secretion of testin by Sertoli cells is in response to the disruption of Sertoli-germ cell AJs, suggesting that this may be an AJ-associated signaling molecule. Based on these unusual features of testin, this protein was used to screen more than two dozen new compounds related to lonidamine [1-(2,4-dichlorobenzyl)-indazole-3-carboxylic acid], which is a molecule previously shown to perturb the Sertoli cell actin filament network (119, 412). The goal is to select those that specifically disrupt Sertoli-germ cell AJs, such as ES, thereby inducing germ cell loss from the epithelium by monitoring their effects on the testicular testin expression (79, 168). We have identified two candidate compounds having potent antispermatogenic effects without any noticeable toxicity using adult Sprague-Dawley rats, which is based on the unusual features of testin as summarized herein (79, 168).

5. *AJ-associated signaling molecules*

Several signaling molecules are found in AJs, which include Src, Csk, CK2, GSK, and $p120^{ctn}$ (9, 51, 177, 284, 450). Protein phosphorylation of these molecules in turn regulates the cadherin-catenin complex altering cell adhesiveness (1, 112, 177, 265, 349, 361, 394, 450, 468). Indeed, these signaling molecules are implicated in spermiation (493). $p120^{ctn}$ and β-catenin can also induce gene activation upon entering into the nucleus, illustrating their physiological significance (9). However, the precise biochemical mechanism by which phosphorylation of the cadherin/catenin complex induced by the AJ-associated signaling molecules, such as Src, CK2, and $p120^{ctn}$, that can perturb the AJ function is entirely unknown. Furthermore, $p120^{ctn}$ is known to bind to the membrane proximal segment of cadherins, and this interaction is required for

adhesive activity (17, 326, 451, 506). However, the mechanism by which this interaction contributes to changes in cell adhesiveness is not yet clear unless the structural interactions of p120^{ctn} and the membrane proximal segment of cadherin are known. Previous morphological studies have suggested that p120^{ctn} is possibly a component of the intermediate filament-based desmosome-like AJ in the rat testis (375). A more recent study by immunoprecipitation has demonstrated the presence of at least four p120^{ctn} isoforms in the testis (226). Immunofluorescent microscopy using a monoclonal antibody against p120^{ctn} has shown that it is localized between Sertoli cells and round spermatids in a stage-specific pattern in the rat testis, being expressed between stages I–IX, but not XII–XIV, and highest at stage VIII (226) coinciding with spermiation.

6. Others

Studies by immunohistochemistry have demonstrated the presence of fimbrin (175) and espin (30) in cell-cell AJs using actin filaments as attachment sites in the testis (Table 1). Plakoglobin is also detected in cell-cell desmosomes using intermediate filaments as attachment sites (106) in the testis. However, virtually no studies can be found in the literature to probe the physiology function of these molecules in the regulation of testicular AJ dynamics.

C. Current Status of Research of AJs in the Testis

Three major families of CAMs including the cadherin family (Ca^{2+} dependent), the immunoglobulin (Ca^{2+} independent), and the integrin superfamilies are found in rodent seminiferous tubules during development and in adulthood (61, 62, 343). Among these three families, cadherins are the most extensively studied CAMs in the testis (63, 109, 321, 346, 500). In addition, N-cadherin plays an important role in regulating cell-cell interactions in the seminiferous epithelium (11, 63, 276). For instance, the binding of round spermatids to Sertoli cells is mediated by N-cadherin (321, 346). Immunohistochemistry analysis has shown that the recruitment of β-catenin to the Sertoli cell junctional complex in the testis correlates with the formation of the blood-testis barrier (61), illustrating the tight functional relationship between AJs and TJs in the testis. In the rat, β_1-integrin, a signal transducer and a cell-matrix AJ protein (Table 1), is found at the site of ES and at the Sertoli cell-spermatogonia interface at stages I–VIII (63, 335, 336), illustrating the testis is utilizing this cell surface receptor for signal transduction. The best-studied and characterized transmembrane adhesion molecule in the testis are $\alpha_6\beta_1$- and $\alpha_2\beta_1$-integrins (336, 387, 388), which are members of the cell-matrix anchoring junction (see Table 1). However, the signaling pathway(s) that regulate the dynamics of anchoring junctions are entirely unknown. A recent study reports that the downstream signaling events pertinent to the regulation of ES dynamics are mediated by the $\alpha_6\beta_1$-integrin receptor via integrin-linked kinase (ILK) (310). Furthermore, nectin afadin appears to be an important AJ functional unit in the testis since homozygous null male mice lacking nectin-2 are sterile and their spermatozoa display defects in cytoskeletal and nuclear morphology (46).

1. ES: the role of integrins

The best-characterized cell-to-cell anchoring junction type using an actin filament attachment site in the testis (i.e., AJ) between Sertoli and germ cells is the ES (see Table 1 and Figs. 2 and 8) (for reviews, see Refs. 177, 374, 471). In the testis, there are two types of ES, designated apical and basal ES (374). Apical ESs are found between Sertoli cells and the adjacent heads of developing spermatids; basal ESs are present between Sertoli cells as well as between Sertoli cells and spermatocytes at the base of the seminiferous epithelium. This permits the passage of spermatocytes across the epithelium via cyclic disassembly and reassembly of these junctions (469, 471). To date, the molecules that have been positively identified in the ES are as follows: actin, α-actinin (145), fimbrin (175), espin (an actin binding protein) (30), vinculin (174, 348), β_1-integrin (310, 387, 388), paxillin, gelsolin (188a), myosin VIIa (188a), and ILK (310). It was postulated that ILK, a serine-threonine protein kinase (116, 186), is an important signaling molecule regulating ES dynamics (for review, see Ref. 470). ILK, but not FAK (208, 392), was found to colocalize with β_1-integrin at the site of ESs in the testis (310). Because it was shown that phosphotyrosine proteins are highly concentrated at the sites of the ES (310), these observations seem to suggest that the dynamics of ES are regulated, at least in part, by protein phosphorylation. Indeed, the phosphorylated form of FAK was shown to localize primarily at the site of apical ES at the adluminal compartment of the seminiferous epithelium adjacent to the seminiferous tubular lumen (415). This pattern of localization is in sharp contrast to FAK, which was found largely at the site of basal ES (310, 415). Also, it was proposed that the $\alpha_6\beta_1$- (in apical ES) and $\alpha_6\beta_4$-integrins (basal ES) found in the ES in the testis are the primary CAMs, whereas the cadherin-catenin complexes are likely used for cell adhesion between Sertoli cells (310) (Fig. 2). If $\alpha_6\beta_1$-integrin found in Sertoli cells at the site of ES at the adluminal compartment of the seminiferous epithelium (310) is the cell adhesion molecule, what is the corresponding binding partner in germ cells? It must be noted that the conventional partner of integrin in anchoring junction is laminin, which is usually restricted to ECM (see Table 1). A recent study indeed has reported the localization of laminin γ-chain as a nonbase-

ment membrane laminin chain in the testis at the adluminal compartment (244a), consistent with its localization at the ES. This is in contrast to laminin $\alpha_1\beta_1\gamma_1$-chains, which are confined to the basal lamina in the testis (244a). Ironically, this study thus suggests that laminin γ_3-chain is one of the three laminin chains at the site of ES that constitute the binding partner of $\alpha_6\beta_1$-integrin. Moreover, these integrin complexes are being used as the platform to transduce signals downstream via ILK, instead of FAK, to regulate AJ dynamics (310). Much work remains to be done to define the biochemical and molecular composition of ES in the testis. Once this is completed, studies can be performed to investigate the mechanism(s) by which these modified AJs are being regulated in spermatogenesis and spermiogenesis.

D. The Functional Unit of AJs in the Testis

1. *Does the cadherin/catenin complex exist in the testis?*

In the classical actin-based AJ [one of the anchoring (or adhering) junction types between cells, see Table 1] found in epithelia, the functional unit is the cadherin-catenin complex, in which the cytoplasmic tail of E-cadherin interacts with β-catenin. This, in turn, binds to α-catenin, vinculin, and the actin cytoskeleton (for review, see Ref. 177) (Fig. 8). However, the presence of E-cadherin and α-catenin in AJs in the testis has been an issue of controversy (11, 63, 109, 177, 267), and the coexistence of cadherin and β-catenin at the same site in the testis between Sertoli and germ cells, in particular, has been the subject of discrepancy (63, 228, 310, 312, 500). For instance, immunohistochemistry studies also failed to localize N-cadherin at Sertoli-spermatid junctions (11, 63). Although the presence of β-catenin in AJs between Sertoli and germ cells is controversial (310, 470, 493), more recent studies suggest that this may likely be the result of antibody specificity used by different investigators (260). For instance, the presence of E-cadherin and catenins has been reported in the rat testis using Northern blot and semiquantitative RT-PCR (84, 228, 312, 500), as well as by immunohistochemistry and immunoblotting techniques (500). There is also a transient induction of N-cadherin, E-cadherin, and β-catenin mRNAs during the assembly of Sertoli TJ-permeability barrier in vitro (84), as well as during the assembly of Sertoli-germ cell AJs in vitro (260). Further studies by immunoblottings using antibodies specific to cadherins and catenins and lysates of Sertoli-germ cell cocultures also confirm these earlier observations (260). More importantly, studies by immunoprecipitation have pulled out β-catenin with an anti-N-cadherin antibody using cell lysates from Sertoli-germ cell cocultures (260). Similarity, N-cadherin can be pulled out using an anti-β-catenin antibody in the same experiment. Furthermore, the relative concentration of N-cadherin and β-catenin in these lysates is present in a stoichiometric ratio of almost 1:1 when visualized by immunoblotting (260). As such, these results demonstrate unequivocally that AJs in the testis are utilizing the same cadherin/catenin complex as the functional unit found in other epithelia (for review, see Ref. 177). A recent immunofluorescent microscopy study has demonstrated N-cadherin at the Sertoli-Sertoli as well as Sertoli-spermatocyte junctions near the basal compartment consistent with its localization at AJs (227). Furthermore, it is also found between Sertoli-elongated spermatid AJs at stages I–VII and appears to be stage specific (227). The inability of detecting the cadherin/catenin complex at the site of ES between Sertoli cells and elongated spermatids in the adluminal compartment of the seminiferous epithelium by immunohistochemistry can be of several reasons. First, the ES can be constituted largely by $\alpha_6\beta_1$- (in apical ES) and $\alpha_6\beta_4$-integrins (in basal ES) as reported earlier (310) or by the nectin/afadin complex (see sect. v*B3*) (Figs. 2 and 8). And the cadherin/catenin complex may only function as a signaling component at the site of apical ES. Second, if the above postulate is true, the cadherin/catenin complex will likely have a short half-life and low abundancy at ES rendering it undetectable by conventional technique. Indeed, the half-life of E-cadherin is only ~5 h in confluent epithelial cells (291, 407). Third, the titer of the anti-cadherin and anti-catenin antibodies used in these earlier studies were low or cross-reacting with unwanted epitopes making data difficult, if not possible, to interpret. However, it must be cautioned that additional functional AJ units other than those that have been described may be present in the testis at the sites of Sertoli cell-spermatids in light of the existence of testis-specific AJ, such as the ES (for reviews, see Refs. 374, 378, 470), which can be operating alongside with the cadherin/catenin complex to ensure rapid changes in cell adhesiveness to accommodate the timely event of germ cell movement. Indeed, an afadin-nectin complex has recently been found in cell-cell AJs adjacent to the cadherin/catenin complex in epithelia (439), which may be one of the yet-to-be confirmed ES-functional units in the testis. Furthermore, deletion or reduction of the classical cadherin/catenin complex cannot eliminate the morphologically normal AJ structures (104, 464) in nonmammalian species. Indeed, the afadin-nectin complex was found to colocalize and interact with the cadherin/catenin complex (280, 438), and afadin, an actin-binding protein, is known to interact structurally with the F-actin (380). However, it must be noted that E-cadherin$^{-/-}$ mice died at the embryo stage around the time of implantation (255a). Also, E-cadherin$^{-/-}$ null mouse embryos display severe abnormalities particularly in the cells that constitute the morula, which become dissociated soon after compaction has occurred, losing their adhesiveness and cell polarity

(364a). Yet, intact desmosomes and TJs are still found in the blastomeres at the site of damaged cell-cell contacts (364a). Similar defects are also detected in N-cadherin$^{-/-}$ mice (354a) with severe cardiac defects (273a, 354a). Furthermore, β-catenin$^{-/-}$ mice also display abnormalities during the embryo stage at gastrulation; in particular, cells are detached from the ectodermal cell layer (180b).

E. Regulation of AJ Dynamics

The events of AJ dynamics and their regulation in the testis are one of the most intriguing phenomena in spermatogenesis (62, 63, 308, 379, 380), since germ cells must migrate from the basal to the adluminal compartment of the seminiferous epithelium while they also remain attached to the epithelium (see Figs. 2 and 3). How these events are regulated in the testis is largely unknown. At present, it is accepted that AJ dynamics are regulated by several possible pathways and/or mechanisms.

1. Changes in the functionality of the cadherin/catenin complexes: the functional unit of AJs

Recent studies have shown that the AJ functional unit is regulated largely by the AJ-associated signaling molecules, such as Src, Csk, CK2, and p120^{ctn}. For instance, recent studies have shown that selective uncoupling of p120^{ctn} from E-cadherin can induce the loss of cadherin-dependent cell adhesiveness (451). This is largely mediated by changes on the phosphorylation status of the cadherin/catenin complex mediated by these signaling molecules, many of which are also putative protein kinases (Table 1, Fig. 8). Indeed, putative Src phosphorylation sites have been identified in p120^{ctn} (283). Recent studies have shown that the downstream signaling pathways by the adhesion receptors, such as cadherins and integrins, which regulate AJ dynamics, converge on the MAP kinase cascade (141). As such, an unprecedented opportunity to study the regulation of AJ dynamics is now available since the MAP kinase cascade can modulate actin polymerization and cell migration via their action on the myosin light-chain kinase (242). Others, such as the phosphatidylinositol-5-kinase (PI-5K) can regulate the conformation and scaffolding of the focal adhesion protein vinculin (158). Surprisingly, very few studies have been performed in the field of male reproductive physiology to study these events (for reviews, see Refs. 61, 62, 308). For instance, the presence of cadherins in the testis was not known until the early to mid 1990s (63, 109), which is more than two decades after the discovery of the cadherin/catenin complex (for reviews, see Refs. 443–445). It is increasingly clear that AJ proteins, such as cadherins and catenins, are responsible for the attachment of germ cells onto the epithelium, since antibodies against cadherins can perturb the attachment of germ cells onto Sertoli cells (321, 346), which is a prerequisite of subsequent AJ assembly. Ironically, if the functionality of these proteins can be disrupted or compromised, germ cells can no longer attach onto the epithelium and will be depleted from the testis inducing infertility (79, 83, 168). Other studies have shown cytokines may also play a significant role in the regulation of AJ dynamics. For instance, EGF and hepatocyte growth factor/scatter factor can reduce cell-cell contacts causing cell dissociation and scattering from each other without apparent effects on the E-cadherin catenin complex in vitro (406, 481). However, the underlying mechanism that mediates these changes is not known. Recent studies have shown that cytokines can also affect TJ function. For instance, TGF-β3 can perturb the assembly of the Sertoli cell TJ-permeability barrier (270), which is mediated via the MEKKs/p38-MAP kinase pathway, by affecting the timely expression of occludin and ZO-1 needed to assemble TJs (271). Because there is a tight functional linkage between TJs and AJs (reviewed in sect. III*E*), the reported effects of cytokines on cell-cell contact may be a result of disruption on TJs rather than a direct effect on AJ functionality.

2. Protein phosphorylation

Protein phosphorylation of AJ-associated proteins plays a crucial role in the regulation of AJ dynamics (14, 111, 177, 198, 311). For instance, tyrosine phosphorylation of the cadherin-catenin complex has been implicated in the regulation of AJ dynamics (111), since tyrosine phosphorylation of this complex affects their association (371). Src, a putative protein tyrosine kinase, is an AJ-associated signaling molecule (51, 284; see also Table 1; Fig. 8) that becomes concentrated at the site of AJs (460). Src can also become heavily phosphorylated during AJ assembly (460). Src is also known to regulate cell-cell adhesion in keratinocytes (64). Indeed, Src-induced tyrosine phosphorylation of the N-cadherin-catenin complex can lead to the loss of cell adhesiveness (184). A more recent immunohistochemistry study has shown that Src is a stage-specific protein in the rat testis, being highest at stage VIII at the site of ES between Sertoli cells and elongated spermatids (493). Furthermore, Csk and Fer kinase (Table 1), both of which are AJ-associated signaling molecules and putative protein tyrosine kinases, are also found in the rat testis (493). Indeed, a testis-specific form of Fer kinase designated *ferT* was found to be restricted to spermatocytes at the pachytene stage of meiotic prophase (237). Other studies have shown that Fer kinase is associated with p120^{ctn} at the site of AJs (238). Surprisingly, Fer kinase$^{-/-}$ null mice are fertile with apparently normal spermatogenesis but display reduced cortactin phosphorylation (107), suggesting other

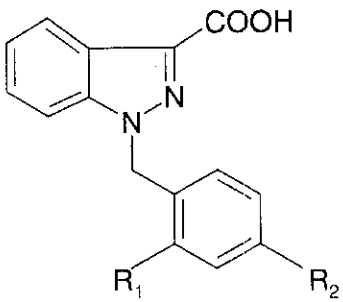


FIG. 9. Structural formula of lonidamine [1-(2,4-dichlorobenzyl)-indazole-3-carboxylic acid] where $R_1 = R_2 = Cl$.

testis by causing depletion of germ cells from the seminiferous epithelium. While the precise mechanism by which gossypol induces germ cell loss from the epithelium is not yet known, lonidamine apparently exerts its effects by perturbing the adhesion of germ cells onto the Sertoli cell, since the cytoskeleton network is one of the subcellular targets of lonidamine (279). Furthermore, electron microscopy studies have shown that lonidamine can induce damage to the Sertoli cell cytoskeleton and microfilament network (119). However, the efficacy dose range and safety margin of gossypol, similar to lonidamine, are very narrow. Also, both compounds are nephrotoxic after prolonged use (for reviews, see Refs. 36, 412, 475). Furthermore, the antifertility effect of gossypol is irreversible in a large proportion of men after prolonged exposure to gossypol (98, 475). These compounds, however, raise new hopes of developing safer male contraceptives, since their mode of action apparently is at the site of Sertoli-germ cell AJs, perturbing cell adhesive function. Indeed, neither compound affects the hypothalamus-pituitary-testicular axis nor Leydig cell steroidogenic function (for reviews, see Refs. 412, 475). Ironically, lonidamine does not affect the assembly of the Sertoli cell TJ barrier in vitro (166) but can perturb the assembly of Sertoli-germ cell AJ in vitro (unpublished observations) using a Sertoli-germ cell coculture assay with fluorescein-labeled germ cells (309). Taken collectively, if a chemical entity based on these known structures can be properly modified and synthesized to perturb AJ functionality between Sertoli and germ cells, in particular between Sertoli and late spermatids, such as the ES, it can become an ideal male contraceptive. For instance, earlier studies with indazole-3-carboxylic acid derivates (101, 412) have shown that the general formula shown in Figure 9, which is not steroidal in nature, is a potent antispermatogenic agent.

Our recently completed studies (79) have yielded the following observations. First, the presence of a substituted benzyl group in position 1 of the indazole ring (see Fig. 9) is essential for the antispermatogenic activity. Furthermore, it seems necessary that R_1 should either be a halogen or a methyl group. Second, the antispermatogenic activity increases rapidly when R_1 and R_2 in positions ortho and para are $R_1 = R_2 = Cl$ or $R_1 = CH_3$ and $R_2 = Cl$ (see Fig. 9). If the two substituents are in a different position, then the activity considerably decreases. Third, the following two analogs are shown to have potent antispermatogenic activity.

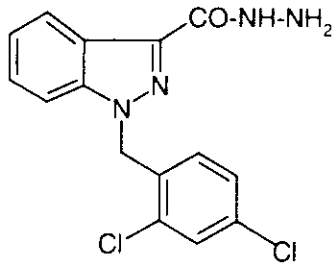
B. Disruption of Cell Adhesion by Suppressing Intratesticular Testosterone Level

Recent studies by suppressing the testicular testosterone in adult rats with the use of an estradiol/testosterone implant (293, 294, 323) have shown that this can also become a model to study the AJ dynamics in vivo while inducing reversible infertility in male rats receiving treatment (for review, see Ref. 292). For instance, chronic testosterone withdrawal can cause a stage-specific detachment of round spermatids from the seminiferous epithelium (293, 324). When adult rats receive low doses of testosterone and estradiol via Silastic implants, this induces serum testosterone and estradiol levels suppressing pituitary luteinizing hormone but not FSH release (293, 294, 323, 324). As such, intratesticular testosterone level reduces by >90% versus normal rats. While round spermatids continue to mature from *steps 1* to *7*, the conversion of *step 7* to *8* round spermatids fails to occur as a result of their early detachment from the epithelium (293, 323). When the intratesticular testosterone level is restored, a rapid restoration of spermatid attachment is detected within 4 days alongside with the normal spermatid elongation process (323). At present, it is not known if this androgen depletion-induced germ cell loss is mediated via a disruption of AJ dynamics between Sertoli and germ cells. A recently completed study has demonstrated that at the time of androgen suppression, the actin-containing domain at the site of ES between spermatids and Sertoli cells appeared to be damaged (325), suggesting that the cadherin/catenin- or afadin/nectin-induced cell adhesion function may be disrupted (for review, see Ref. 292). Needless to say, this is an emerging and potentially important in vivo model to study AJ dynamics in the testis and a novel male contraceptive approach.

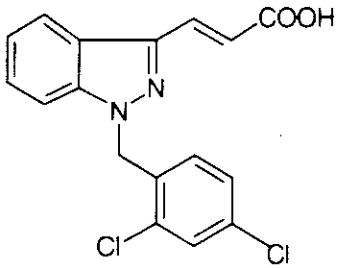
C. 1-(2,4-Dichlorobenzyl)-indazole-3-carbohydrazide (AF-2364) and 1-(2,4-Dichlorobenzyl)-indazole-3-acrylic acid (AF-2785)

1. *Background: discovery of AF-2364 and AF-2785, two potential male contraceptives*

Lonidamine [1-(2,4-dichlorobenzyl)-indazole-3-carboxylic acid], a derivative of 1-indazole-3-carboxylic acid (Fig. 9), is a nonsteroidal and nonhormonal anticancer drug



AF-2364
[1-(2,4-dichlorobenzyl)-indazole-3-carbohydrazide]



AF-2785
[1-(2,4-dichlorobenzyl)-indazole-3-acrylic acid]

FIG. 10. Structural formulas of AF-2364 and AF-2785.

(279, 408–410) having a potent antispermatogenic effect (412). Administration of lonidamine to adult rats by gavage at a dose of 20–50 mg/kg body wt (119, 170, 268) induced a massive depletion of germ cells from the seminiferous epithelium. By 2–4 wk after a single dose of lonidamine at 50 mg/kg body wt, the tubules were devoid of virtually all elongated spermatids, round spermatids, and some spermatocytes (119, 170), and rats became infertile (268). Lonidamine apparently exerts its effects by disrupting and rearranging the microfilament elements in the Sertoli cell plasma membrane (279), which is part of the cell adhesion junctional complex necessary to allow the anchorage of germ cells onto Sertoli cells in the epithelium. At doses ranging between 1 ng/ml and 10 μg/ml, lonidamine was not toxic to Sertoli cells in vitro (170); however, its administration in vivo at 25–50 mg/kg body wt induced morphological changes in Sertoli cells at the ultrastructural level as manifested by the vacuolation and retraction of the apical cytoplasm at the site where immature spermatids attach onto Sertoli cells (119). This was followed by the enlargement of the intercellular space between Sertoli and germ cells, eventually leading to the release of immature spermatids into the tubular lumen (119). This action of lonidamine on Sertoli cells mimics those observed in lonidamine-treated epithelial squamous carcinoma (A431) and melanoma (M14) cells by disrupting the cytoskeletal network (279, 282). On the other hand, lonidamine was also shown to inhibit respiration in condensed mitochondria found in spermatids and spermatocytes or tumor cells previously sensitized by irradiation, but not in Sertoli cells or other somatic cells both in vivo and in vitro (143, 144). Such an effect on cellular respiration may also confer to the unwanted cytotoxicity on lonidamine. Although the antispermatogenic effects of lonidamine are worthy of further investigation, it was not developed into a male contraceptive due to its nephrotoxicity and irreversibility; instead, it was used as an antitumor drug in chemotherapy (410, 412). Needless to say, this compound, if properly modified to eliminate side effects, could become a novel male contraceptive. Unfortunately, all efforts to develop new derivatives based on the core structure of lonidamine for male contraception ceased because of the high costs of screening by conventional methods (409, 412). In addition, a screening assay targeted to identify new analogs of lonidamine by depleting advanced germ cells, such as late spermatids but not spermatogonia, from the seminiferous epithelium was lacking. In an early study from this laboratory, lonidamine was shown to induce a surge in testicular testin expression within 24 h (170), long before germ cells were seen to deplete from the epithelium, which became visible only by *days 4–6* after a single oral dose treatment of lonidamine at 25–50 mg/kg body wt (170). These results thus suggest that when lonidamine exerts its effects on the Sertoli cell cytoskeletal network, it somehow activates the expression of testin. These observations also illustrate that testin is a useful marker to identify new male contraceptives exerting their effects specifically at the site of Sertoli-germ cell AJs, such as ES, causing AJ cleavage and germ cell loss from the epithelium. Indeed, we have utilized this unusual feature of testin in an in vivo assay to screen over 20 new analogs of lonidamine and have identified two new chemical entities, AF-2364 and AF-2785, which specifically deplete spermatids from the epithelium (79, 168) (Fig. 10). While it remains to be determined if AF-2364 and AF-2785 are indeed suitable for contraceptive use for human males without the side effects of either lonidamine or gossypol, recently completed toxicity studies on AF-2364 performed by licensed toxicologists according to Food and Drug Administration guidelines have indicated that this is a promising candidate compound. For instance, the recently completed acute toxicity study using the Irwin dose range (100–1,000 mg/kg body wt) by intraperitoneal injection or gavage in mice and rats performed by licensed toxicologists revealed that AF-2364 did not influence any of the neurological or autonomic parameters. Moreover, both AF-2785 and AF-2364 did not induce reverse mutation in *Salmonella typhimurium* or *Escherichia coli* by standard mutagenicity tests (unpublished observations). Also, chro-

mosomal aberration tests in CHO cells revealed that AF-2364 did not cause DNA damage (unpublished observations). In addition, neither compound is nephrotoxic nor hepatotoxic at doses that are effective to induce reversible infertility in male rats (79, 168).

2. *Biological effects of AF-2364*

A) ANTIFERTILITY EFFECTS: EFFICACY AND REVERSIBILITY. When adult Sprague-Dawley rats were treated with AF-2364 (Fig. 10) by gavage, AF-2364 could not suppress fertility by 100% during the first 2–3 wk after the first dose was administered because of the epididymal sperm reserve, since this compound neither induced any apparent changes in the epididymis when examined microscopically (79) nor killed epididymal sperm (79, 168). However, two or three consecutive doses of AF-2364 between 25 and 50 mg/kg body wt are effective to induce infertility in male rats (79). On the basis of these earlier studies, it is apparent that at least two consecutive doses of AF-2364, which must be administered at least 1 wk apart, are needed to induce complete infertility in the rat (79, 168). More importantly, the antifertility effects of AF-2364 are reversible.

B) EFFECTS ON TESTICULAR MORPHOLOGY, TESTICULAR WEIGHT, AND BODY WEIGHT. When the testes of the treated rats were examined histologically using a regimen that was effective to induce reversible infertility, germ cells began to deplete from the seminiferous epithelium as early as 4–6 days after the first dose of AF-2364 (79); >80% of the tubules were devoid of germ cells by *day 28*. The tubules were virtually devoid of germ cells by *day 40* posttreatment, and this morphological change persisted until *day 79*, coinciding with the loss of fertility. When the testes were examined on *day 128*, germ cells began to repopulate the seminiferous epithelium, and by *days 211–254*, virtually 100% of the tubules appeared indistinguishable from control rats, consistent with results of the mating studies, illustrating the reversibility of this treatment (79). AF-2364 also induced a decline in testicular weight as a result of germ cell loss; however, testicular weight returned to normal when fertility rebounded (79, 168). Furthermore, AF-2364 had no effects on body weight (79, 168).

C) EFFECTS OF AF-2364 ON THE HYPOTHALAMUS-PITUITARY-TESTICULAR AXIS, AND ON LIVER AND KIDNEY FUNCTION. When the serum testosterone, FSH, and luteinizing hormone levels from the AF-2364-treated rats using different treatment regimens were quantified and compared with control rats, no significant changes were detected throughout the entire treatment period versus controls (79, 168). These data thus demonstrate that the hypothalamus-pituitary-testicular axis was not impaired by AF-2364 when the fertility of treated rats was affected. Moreover, serum microchemistry analysis revealed that the serum levels of serum glutamic oxaloacetic transaminase, serum glutamic pyruvic transaminase, and alkaline phosphatase (for liver function tests) as well as blood urea nitrogen, glucose, creatinine, albumin, γ-globulins, sodium, and potassium (for kidney function tests) were not altered in AF-2364-treated rats compared with control animals using the treatment regimens that were effective to induce reversibly infertility, illustrating that neither liver nor kidney function was affected by AF-2364 (79, 168).

3. *Effects of AF-2785 on rat fertility; its efficacy; serum FSH, luteinizing hormone, and testosterone levels; and other parameters*

With the use of five different treatment regimens of AF-2785 (Fig. 10) to study its effects on the fertility of adult rats, it was shown that AF-2785 is also a potent antispermatogenic compound (79). However, this chemical entity needs to be administered more frequently than AF-2364, preferably on a daily (or every other day) basis, to unleash its efficacy. In contrast to AF-2364, rats fed with AF-2785 recovered more rapidly. Also, all treated rats recovered to full fertility in each treatment regimen. Similar to AF-2364, AF-2875 induced germ cell depletion, virtually all elongated and round spermatids, from the seminiferous epithelium (79). Testes were indistinguishable from control rats histologically when fertility was restored in the AF-2785-treated rats (79). Germ cells also repopulated more rapidly in AF-2785-treated rats than in AF-2364-treated rats. Furthermore, AF-2785, similar to AF-2364, did not alter the serum levels of testosterone, FSH, and luteinizing hormone in treated rats compared with controls (79), illustrating AF-2785, similar to AF-2364, did not interfere with the hypothalamus-pituitary-testicular hormonal axis. Furthermore, serum microchemistry analysis revealed that neither the liver nor the kidney function was affected by AF-2785 at doses that were effective to induce reversible infertility. AF-2785 also induced a decline in testicular weight in treated rats, which returned to normal more rapidly than AF-2364-treated rats (79). Also, AF-2785 had no apparent effects on body weight (79).

D. Molecular Mechanism of Action of AF-2364

The mechanism by which these drugs induce germ cell loss from the seminiferous epithelium is not entirely known. Nevertheless, both compounds induce a progressive loss of germ cells from the epithelium, which begins with the latest stages, such as elongated spermatids, to earlier stages, such as round spermatids and some spermatocytes. This drug-induced, stage-dependent germ cell loss can be explained as follows. It was postulated that the two analogs of lonidamine exert their effects by activating the cascade of events leading to the cleavage of AJs

between late spermatids and Sertoli cells through a yet-to-be-defined mechanism. For instance, lonidamine is known to induce rearrangement and disruption of the cytoskeleton network in Sertoli cells, A431 epithelial squamous carcinoma cells, and M14 melanoma cells (119, 279, 282). Given the fact that these two new analogs share similar structural features with lonidamine (see Figs. 9 and 10), they may indeed utilize the same mechanism to induce changes in the Sertoli cell cytoskeleton network. Indeed, both compounds failed to perturb the assembly and maintenance of the Sertoli cell-TJ barrier in vitro when the TER and the influx of FITC-labeled dextran from the apical to the basal compartment on bicameral units across the Sertoli cell epithelium were assessed (unpublished observations). These data are consistent with an earlier report from this laboratory showing that lonidamine has no effects on the Sertoli cell TJ barrier (166). More importantly, with the use of an in vitro assay to monitor the binding of fluorescein-labeled germ cells onto Sertoli cell epithelium as described (309), which is a prerequisite of the subsequent AJ assembly that is known to complete within 24–48 h in vitro (65, 135), both compounds were shown to perturb the Sertoli-germ cell AJ assembly (unpublished observations). A recently completed morphological study has shown that the AF-2364- or AF-2785-induced AJ-disruptive activity between Sertoli cells and late spermatids is more efficient than that between early spermatids/spermatocytes and Sertoli cells (79, 168), and they had no effects on depleting spermatogonia from the seminiferous epithelium (79, 168). If they did, these effects would have been irreversible. It is possible that the testis-specific AJs, such as ES and tubulobulbar complexes, and their constituent proteins between Sertoli cells and late spermatids, are more susceptible to these analogs. Indeed, rats treated with AF-2364 displayed a drastic induction in cadherin and catenin (260), and by 7–14 days posttreatment, the entire basal compartment of the seminiferous epithelium was encircled by an intense immunostaining of cadherin and catenin (unpublished observations), suggesting its site of action is at the level of AJs. Also, these compounds were selected in the initial screening for their ability to induce testin (79, 168), which is an AJ-associated signaling molecule structurally and functionally linked to AJs (169–171). The relative long lag of response of germ cell loss, ~4–6 days (79, 168) after either the AF-2364 or AF-2785 treatment, seemingly signifies that the AF-2364-induced AJ disruption consists of a cascade of events that triggers an induction of testin before germ cell loss from the epithelium can be histologically detected. Indeed, a recent report has shown that AF-2364 initially activates the cell adhesion molecule β_1-integrin, a component of the apical ES (310), which in turn tranduces the signal downstream via the RhoB GTPase/ROCK/LIMK signaling pathway at the site of AJs (272).

While these compounds appear to be potential candidates for male contraception, an intriguing but important question remains to be answered: Would these compounds also disrupt AJs in other epithelia that are present in virtually all organs? It is obvious that this question must be addressed carefully and thoroughly. Several observations suggest that these compounds indeed target their effects exclusively on testis-specific AJs between Sertoli cells and spermatids in the seminiferous epithelium, such as ES (343, 378–380, 470). First, the screening assay that was used to select these two candidate compounds is based on their ability to induce testin expression (79). Testin is a Sertoli cell product predominantly accumulated at the site of specialized AJs, such as ES, between Sertoli and late spermatids (based on biochemical and immunogold electron microscopy analysis) (169, 170) whose expression is known to be induced when Sertoli-germ cell AJs are disrupted (166, 169, 170). It is known that ESs and tubulobulbar complexes (62, 379, 470) are specialized AJs restricted only to the testis. More recently completed studies have also illustrated that such an induction of testin is limited to the disruption of AJs but not the Sertoli cell TJs in vivo and in vitro (79, 166, 168). For instance, treatment of Sertoli cells in vitro with lonidamine failed to perturb the assembly and maintenance of the Sertoli cell TJ barrier in vitro (166). Thus the screening assay has limited our initial search to identify candidate compounds that would specifically disrupt testicular AJs, such as ES, but not those found in nongonadal tissues. Second, with the use of a treatment regimen that can induce reversible infertility in adult rats by AF-2364, it was shown that the gross morphology of the kidney and liver was not damaged by AF-2364 during routine histological analysis, which is in sharp contrast to the drug-induced germ cell depletion from the epithelium in the testis (79, 168). These results thus illustrate that the AJs in both the kidney and liver are apparently unaffected by AF-2364. Third, results of serum microchemistry have shown that both the kidney and liver functions are unaffected by both compounds using a treatment regimen that can induce reversible infertility in the rat (79, 168), suggesting these compounds are neither nephrotoxic nor hepatotoxic, at least at the doses that are effective to induce reversible infertility in the rat. Work is now in progress to investigate the signal transduction pathway(s) by which AF-2364 is utilized to perturb AJs between Sertoli cells and late spermatids, such as the ES. And this AJ structure likely consists of testin and integrin.

VII. CONCLUDING REMARKS

In this review we have summarized some of the recent developments in the study of junction dynamics in the testis and their physiological significance to testicular

function and spermatogenesis. Most of the studies performed in the past two decades investigating Sertoli-germ cell interactions (for reviews, see Refs. 173, 221, 416) largely focus on the secretory function and activity of either Sertoli or germ cells. It is increasingly clear that these cell-cell interactions and the subsequent changes in secretory activity of either Sertoli or germ cells initially take place at the level of cell junctions. Ironically, a huge disparity exists between the study of TJ and AJ dynamics in the testis versus epithelia in other organs at the biochemical and molecular levels. For instance, there is an almost 25-year delay between the initial identification of cadherins as CAMs in other epithelia (204a, 235a, 442a) and in the testis (63, 109, 276). Until now, the biochemical and molecular structure and architecture of AJs, such as ES, and TJs in the testis, remain unclear. We have provided some intriguing evidence and several models in the field illustrating that a compromise of the junction dynamics in the testis can lead to a plunge in fertility and aspermatogenesis. This information also provides a framework upon which innovative male contraceptives could be developed. It is obvious that possible side effects and/or toxicity, such as AF-2364, could have been overlooked in some of the recent reports, which will not be known until the full battery of toxicity (both acute and subchronic) and safety pharmacology studies are completed. Although we have only reviewed some recent developments in the field of contraceptive development using approaches to perturb junction dynamics, this is not to say that recent advancements in hormonal contraceptives or vaccine development should be overlooked. Instead, it is our belief that these different approaches of male contraception (see Fig. 6) should be investigated side by side not only for the sake of contraceptive development, but for a thorough understanding of spermatogenesis.

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