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THE APICAL ECTOPLASMIC SPECIALIZATION-BLOOD-TESTIS BARRIER FUNCTIONAL AXIS IS A NOVEL TARGET FOR MALE CONTRACEPTION

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Abstract

The blood-testis barrier (BTB), similar to other blood-tissue barriers, such as the blood-brain barrier and the blood-retinal barrier, is used to protect the corresponding organ from harmful substances (e.g., xenobiotics) including drugs and foreign compounds. More importantly, the BTB allows postmeiotic spermatid development to take place in an immune privileged site at the adluminal (or apical) compartment to avoid the production of antibodies against spermatid-specific antigens, many of which express transiently during spermiogenesis and spermiation. The BTB, however, also poses an obstacle in developing nonhormonal-based male contraceptives by sequestering drugs (e.g., adjuvin) that exert their effects on germ cells in the adluminal compartment. The effects of these drugs include disruption of germ cell cycle progression and development, apoptosis, cell adhesion, metabolism and others. Recent studies have demonstrated that there is a functional axis that operates locally in the seminiferous epithelium to co-ordinate different cellular events across the Sertoli cell epithelium, such as spermiation and BTB restructuring during the seminiferous epithelial cycle of spermatogenesis. Components of this functional axis, such as the apical ectoplasmic specialization (apical ES, a testis-specific atypical anchoring junction type) and the BTB, in particular their constituent protein complexes, such as $\alpha 6 \beta 1$ -integrin and occludin at the apical ES and the BTB, respectively, can be the target of male contraception. In this chapter, we highlight recent advances regarding the likely mechanism of action of adjuvin in this functional axis with emphasis on the use of molecular modeling technique to facilitate the design of better compounds in male contraceptive development.

INTRODUCTION

Male contraception is an important alternative in family planning.¹⁻³ An ideal male contraceptive, such as a male pill, must be safe, effective, accessible, inexpensive,

preferably long-acting yet reversible and culturally acceptable by couples in both developing and industrialized nations, similar to the “pills” for women. Unfortunately, this ideal has proven to be elusive after decades of research and efforts in the field. One of the major obstacles for the development of male contraceptives, unlike pills for women, is our incomplete understanding of spermatogenesis, namely the biology of sperm production in the testis. Even though the cellular events pertinent to spermatogenesis⁴⁻⁸ and the hypothalamic-pituitary-testicular axis that regulates spermatogenesis⁹⁻¹² are well understood, the underlying mechanisms that regulate spermatogenesis at the molecular and biochemical levels remain largely unexplored.^{8,13-17} Based on a recently completed Phase III multicenter trial in China,¹⁸ it seems that the most promising lead of putting a male contraceptive on the market at present is via the use of monthly testosterone injections based on testosterone undecanoate^{19,20} (a long-acting testosterone) that suppresses spermatogenesis. Since the level of intratesticular testosterone in men and rodents is about 100-fold higher than the peripheral concentration in the systemic circulation to maintain spermatogenesis,^{21,22} administration of testosterone undecanoate to men via injections thus suppresses the release of GnRH from the hypothalamus, which in turn reduces pituitary LH production, disrupting Leydig cell steroidogenesis in the interstitium in the testis. The net result of these interactions lowers the proper intratesticular testosterone level to sustain spermatogenesis. However, in a recent Phase II clinical study based on the use of a combination of long-acting progestin (norethisterone enantate) and testosterone undecanoate conducted in the United States sponsored by World Health Organization (WHO) and CONRAD, the review panel in early 2011 has recommended discontinuation of this trial since risks of possible side effects outweigh the potential benefits to the male study participants (see www.conrad.org/news-pressreleases-63.html). This latest episode thus represents a major setback in the development of testosterone injection or hormonal-based “male pills” as male contraceptives since different ethnic groups, Asian *versus* Caucasian men, can have different response to exogenous testosterone to suppress spermatogenesis, in which Asian men were found to be more susceptible to testosterone-based contraception.^{23,24} Yet the use of nonhormonal contraceptives also has its own obstacles.²⁵ First and foremost, the blood-testis barrier (BTB) in the testis poses a major obstacle to contraceptives^{26,27} if these drugs exert their effects behind the BTB in the immune privilege site in the testis, namely the adluminal (apical) compartment (Fig. 1). The BTB also confers poor bioavailability to most nonhormonal contraceptives, such as adjuvin.^{28,29} Thus, even though adjuvin is highly potent in the testis by exerting its effects behind the BTB to induce “premature” spermiogenesis, this low bioavailability thus narrows the margin between efficacy and safety as illustrated in a subchronic toxicity study.³⁰ Second, the molecular and biochemical mechanisms that regulate the cellular events of spermatogenesis remain poorly understood. For instance, the regulation of spermatogonia/spermatogonial stem cell (SSC) renewal and their differentiation, germ cell cycle progression (such as via the actions of cyclins, vitamin A and its metabolites), meiosis, spermiogenesis and spermiogenesis are not entirely known^{15,31-35} even though major advances have been made in the past decade. However, several new targets that could become the prime candidates for male contraception are forthcoming. One of these candidates is a newly identified local functional axis in the seminiferous epithelium known as the apical ES-BTB axis that co-ordinates the events of spermiogenesis and BTB restructuring during spermatogenesis.^{36,37} Given that

adjudin, 1-(2,4-dichlorobenzyl)-*1H*-indazole-3-carbohydrazide, is a potential male contraceptive that exerts its effects by perturbing cell adhesion at the apical compartment behind the BTB in rats,³⁸ rabbits³⁹ and beagle dogs,⁴⁰ we review herein recent findings in the field regarding the possible use of adjudin to disrupt the apical ES-BTB axis and hence spermatogenesis. We also highlight the significance of putting more resources and efforts in research in order to understand the biology and regulation of the BTB, so that better approaches can be developed to deliver male contraceptives (and perhaps therapeutic drugs to treat germ cell tumors) behind this blood-tissue barrier in men. Obviously, these findings will also benefit the development of nonsurgical approach to control fertility in pets and even wildlife, thereby protecting our environment.

THE APICAL ES-BTB FUNCTIONAL AXIS

Spermatogenesis takes place in the seminiferous epithelium of the seminiferous tubule, which is the functional unit that produces spermatozoa and estrogen, whereas Leydig cells in the interstitial space produce testosterone and estrogen. All these hormones together with FSH and LH released from the pituitary gland support and maintain spermatogenesis.^{9-12,41,42} Interestingly, the seminiferous epithelium (see Fig. 1) is composed of only a monolayer of Sertoli cells, with each Sertoli cell supports up to ~30-50 germ cells at different stages of their development.³⁷ The seminiferous epithelium is anatomically segregated into the basal and the adluminal (apical) compartments by the BTB (Fig. 1) such that meiosis I and II, postmeiotic spermatid development, namely spermiogenesis, as well as spermiation all take place in the apical compartment behind the BTB. However, during the seminiferous epithelial cycle of spermatogenesis, distinctive cellular events take place across the epithelium at the opposite ends of the Sertoli cell. For instance, at Stage VIII of the epithelial cycle, spermiation that allows the release of fully developed spermatids (i.e., spermatozoa) takes place concurrently with the restructuring of the BTB that facilitates the transit of preleptotene spermatocytes at the site from the basal to the apical compartment, so that they can differentiate to zygotene, pachytene and then diplotene spermatocytes which enters meiosis I to be followed immediately by meiosis II at Stage XIV of the epithelial cycle.⁷ Recent studies have shown that a functional axis operates locally in the seminiferous epithelium to co-ordinate cellular events such as spermiation and BTB restructuring.³⁶ Ectoplasmic specialization (ES) is a unique testis anchoring junction type^{37,43} which is typified by the presence of actin filament bundles sandwiched in between cisternae of endoplasmic reticulum and the apposing plasma membranes of Sertoli cells. In the seminiferous epithelium, ES is found at the BTB and the apical compartment and is known as basal ES and apical ES, respectively. Basal ES is restricted to the Sertoli-Sertoli cell interface at the BTB^{37,44} with the typical features of actin filament bundles and the cisternae of endoplasmic reticulum present on *both* sides of the two adjacent Sertoli cells. On the other hand, apical ES at the apical compartment is responsible for anchoring spermatids (step 8-19) at the Sertoli-spermatid interface.^{37,44,45} Once it appears during spermiogenesis, apical ES is the only anchoring device that adheres spermatids (step 8-19) to the Sertoli cells in the epithelium and to confer spermatid polarity (orientation) until spermiation.^{8,13,45,46} Unlike basal ES, apical ES has the typical actin filament bundles ultrastructure restricted to the Sertoli cell side and these ultrastructural features are not

visible in the developing spermatids. These actin-based filament bundles are known to confer the unusual adhesive strength of cell adhesion at the apical ES.¹⁷ $\alpha 6\beta 1$ -Integrin which is restricted to the Sertoli cells and laminin-333 (laminin $\alpha 3$, $\beta 3$ and $\gamma 3$; note: a functional laminin ligand is a trimeric protein composed of an α , β and γ chain that can bind to an integrin receptor) which is limited to the spermatid constitute one of the best studied adhesion protein complexes at the apical ES.^{43,47,48} This protein complex, together with N-cadherin- β -catenin and nectin-afadin complexes that use actin-based cytoskeleton are three of the known adhesion protein complexes that confer apical ES function to anchor developing spermatids to the Sertoli cell in the seminiferous epithelium.^{8,14,16}

Matrix metalloproteinase-2 (MMP-2), a product of Sertoli cells, is highly expressed at Stage VIII of the epithelial cycle, and mostly restricted to the apical ES in the rat testis.⁴⁹ Laminin chains are known substrate of MMP-2, and fragments of laminin chains following proteolytic cleavage by MMPs are also known to act as biologically active peptides to regulate various cellular functions in multiple epithelia.⁵⁰ Indeed, recombinant laminin fragments of laminin chains added to Sertoli cell epithelium with an intact tight junction (TJ)-permeability barrier that mimics the Sertoli cell BTB in vivo was found to perturb the TJ-barrier function dose-dependently. It was found that laminin fragments was able to reduce the steady-state level of occludin at the BTB, possibly mediated by an increase in occludin internalization via endocytic vesicle-mediated endocytosis.³⁶ Furthermore, overexpression of specific laminin fragments in Sertoli cell epithelium in vitro also displayed the same phenotype with a disrupted TJ-barrier function.³⁶ These findings thus illustrate the presence of a functional axis in the seminiferous epithelium between the apical ES and the BTB. In short, release of laminin chains mediated by the action of MMP-2 on the $\alpha 6\beta 1$ -integrin-laminin-333 complex at the apical ES during spermiation can promote BTB restructuring, and thus co-ordinating the events of spermiation and BTB restructuring that occurs at the opposite ends of the seminiferous epithelium at Stage VIII of the epithelial cycle.

These findings, besides their physiological significance, are critical to male contraceptive development. If the biology of this apical ES-BTB axis is fully elucidated, components in this axis can become the prime targets for male contraceptive development. For instance, a blockade or an activation of MMP-2 action at the apical ES can “delay” or induce “unwanted/premature” spermiation, respectively. This can lead to malfunctioning sperm in the epididymis or spermatids being phagocytosed by Sertoli cells in the epithelium to disrupt spermatogenesis. Also, the underlying signaling mechanism(s) by which the biologically active laminin fragments exert their effects to perturb the BTB function, if known, can also be “blocked”, which would disrupt BTB restructuring necessary for the timely transit of preleptotene spermatocytes to enter the apical compartment for further development. In both approaches, spermatogenesis will be disrupted, resulting in infertility. In the section below, we illustrate how adjuvin is being developed as a candidate male contraceptive that exerts its effects on some of the components in this functional axis.

ADJUDIN

Adjudin, 1-(2,4-dichlorobenzyl)-*1H*-indazole-3-carbohydrazide, is an analog of lonidamine.²⁸ Lonidamine, however, is an anti-cancer drug that exerts its effects primarily by inhibiting hexokinase activity most potently in cancer cells following their exposure to radiation.⁵¹ During the course of investigation to develop lonidamine as an anti-cancer drug in the 1970s,⁵¹ it was found to possess potent anti-spermatogenic activity by depleting germ cells from the seminiferous epithelium in rodents.^{52,53} Lonidamine was found to disrupt the actin filament bundles in Sertoli cells that are restricted almost exclusively to the ES in the seminiferous epithelium when examined by electron microscopy.⁵⁴ Thus, various analogs of lonidamine were synthesized and tested in our laboratory almost two decades ago to select the most potent analog with minimal toxicity, and having the activity to disrupt apical ES based on an assay that would induce testin expression at the site.^{38,55} Subsequent studies have shown that adjudin disrupts actin filament bundles preferentially at the apical ES,^{56,57} inducing premature spermiation, leading to defoliation of virtually all germ cells except spermatogonia/spermatogonial stem cells.⁵⁸ Thus, the anti-fertility effect of adjudin is *reversible* since the remaining SSC can repopulate the epithelium similar to the initiation of spermatogenesis in immature rats.²⁸ In light of the potent effects of adjudin on cell adhesion in the testis, adjudin-treated rats have been used as an *in vivo* model to study anchoring junction regulation in the seminiferous epithelium by delineating the signaling pathways that regulate germ cell adhesion as well as the components of the adhesion protein complexes at the apical ES.^{48,59-63} For instance, it is now known that adjudin initially affects the integrin-based adhesion protein complex at the apical ES, and it also has effects on other signaling and/or adaptor proteins at the site including FAK, p130Cas, DOCK180, RhoA, vinculin, LIMK, ROCK, Arp2/3 complex, Eps8, and PKB,^{48,60,64-66} which in turn induces ERK1/2 downstream^{48,60} that leads to the apical ES degeneration. This process likely mimics the events of spermiation that take place at Stage VIII of the epithelial cycle.^{15,67-69}

DRUG TRANSPORT AT THE BTB

The BTB, similar to other blood-tissue barriers (e.g., blood-brain barrier, blood-retinal barrier) and the intestinal TJ-barrier, poses an important barrier to limit the entry of environmental toxicants (e.g., cadmium, mercury, bisphenol A, phthalates), drugs and other xenobiotics into the apical compartment behind the barrier (Fig. 1). This is mediated by the presence of efflux (ATP-dependent) and influx (ATP-independent) drug transporters at these barriers.⁷⁰⁻⁷² The BTB, constituted by *co-existing* TJ, basal ES, gap junction and desmosome between Sertoli cells near the basement membrane,^{17,73,74} being one of the tightest blood-tissue barriers besides the BBB, limits the access of male contraceptives to the apical compartment. For instance, it was shown that >95% of adjudin administered to rats orally by gavage failed to reach the testis^{28,30} even though adjudin was exceedingly potent to induce apical ES disruption in the seminiferous epithelium.^{28,75,76} Recent studies have shown that the entry of adjudin was neither mediated by diffusion nor by paracellular transport, such as during the epithelial cycle at Stage VIII when the BTB is undergoing restructuring to facilitate the transit of preleptotene spermatocytes.^{77,78} Instead, the entry of adjudin to the apical compartment is mediated by transcellular transport as a result of the combined efforts of multiple influx and efflux pump drug transporters.^{77,78} For instance,

even the knockdown of a major efflux drug transporter, such as P-glycoprotein, by RNAi failed to make the BTB freely permeable to [³H]-adjudin,⁷⁷ because of the presence of the multiple efflux and influx drug transporters at the Sertoli cell BTB.^{72,77,79} Interestingly, recent studies have shown that P-glycoprotein, besides serving as an efflux drug transporter, is an integrated component of occludin-based complex at the BTB.⁷⁹ Its expression is significantly induced following the exposure of testes and/or Sertoli cells to adjudin.^{77,79} More importantly, P-glycoprotein was found to regulate occludin-ZO-1-based adhesion protein complex at the BTB via its effects on FAK, which altered phosphorylation status of occludin to confer changes in the kinetics of protein endocytosis, thereby destabilizing or stabilizing the BTB integrity.⁷⁷ Collectively, these findings demonstrate the complexity of drug transport at the BTB, illustrating much research is needed to understand the regulation of drug entry at the BTB for nonhormonal contraceptives in particular when these drugs exert their effects on germ cell adhesion, apoptosis, and/or development behind the BTB.

INTERACTION OF ADJUDIN WITH INTEGRIN AT THE APICAL ES AND OCCLUDIN AT THE BTB

Introduction

In earlier studies using adjudin-treated rats (e.g., adjudin at 50 mg/kg b.w., by gavage) or Sertoli cell epithelium, it was shown that adjudin perturbed apical ES^{48,59,60} by compromising the adhesive function of the $\alpha 6\beta 1$ -integrin-laminin-333 complex initially, to be followed by desmosome.^{75,80} This in turn led to an activation of PKB (protein kinase B) and ERK1/2 (extracellular regulated kinase 1/2) downstream,⁶⁰ causing “unwanted” premature release of spermatids from the seminiferous epithelium, mimicking spermiogenesis.^{47,81} Subsequent studies have shown that the $\alpha 6\beta 1$ -integrin-based adhesion protein complex is composed of p-FAK (phosphorylated focal adhesion kinase)-Tyr³⁹⁷ and p-FAK-Tyr,⁵⁷⁶ p-130Cas (p130 Crk-associated substrate), DOCK180 (dedicator of cytokinesis 180), RhoA (Ras homolog gene family, member A, a small GTPase), and vinculin (an adaptor at the apical ES). These proteins create an $\alpha 6\beta 1$ -integrin-pFAK-p-130Cas-DOCK180-RhoA-vinculin protein complex at the apical ES,⁴⁸ and it is one of the primary targets of adjudin. Collectively, these findings illustrate that $\alpha 6\beta 1$ -integrin may serve as a “receptor” protein complex for adjudin. To explore this possibility, molecular modeling was used to examine the presence of a docking pocket in $\alpha 6\beta 1$ -integrin for adjudin binding. Since adjudin is likely to enter the apical compartment through the BTB via drug transporters, and occludin is one of the major TJ-fibril building blocks in the testis, we also elected to perform this docking study between occludin and adjudin.

Molecular Modeling of $\alpha 6\beta 1$ -Integrin and Occludin with Adjudin

In rats, amino acid sequence of the two known $\alpha 6$ integrin variants, namely $\alpha 6$ integrin variant 1 [XP_001059353.2] and $\alpha 6$ integrin variant 2 [XP_215984.4], as well as $\beta 1$ integrin [NP_058718.2] and occludin [NP_112619.2] of *Rattus norvegicus* were obtained from NCBI-Protein sequence database (<http://www.ncbi.nlm.nih.gov/guide/proteins/>) for molecular modeling. The amino acid sequence of $\beta 1$ integrin was appended into $\alpha 6$ integrin variant 1 sequence for homology modeling of heterodimeric form of $\alpha 6\beta 1$ integrin variant 1. Similarly, $\beta 1$ integrin amino acid sequence was also appended into amino acid sequence of

$\alpha 6$ integrin variant 2 for homology modeling of heterodimeric form of $\alpha 6\beta 1$ integrin variant 2. The crystal structure of integrin $\alpha V\beta 3$ [PDB ID: 3IJE] was found as a suitable template for the homology modeling of both $\alpha 6\beta 1$ integrin variant 1 and variant 2 by NCBI-BLASTP search. We have used four different steps in our molecular modeling approach: (i) target-template sequence alignment, (ii) model building, (iii) loop refinement, and (iv) model validation. The programs of “Homology Modeling” modules such as Align123 and MODELER automodel of Discovery Studio 3.0 (Accelrys Software, <http://accelrys.com/>) were used for sequence alignment and model building, respectively. The alignment results show that $\alpha V\beta 3$ integrin matches with $\alpha 6\beta 1$ integrin variant 1 by 32.2% sequence identity and 52.5% sequence similarity. Similarly, $\alpha V\beta 3$ integrin shows 32.5% sequence identity and 53.3% sequence similarity with $\alpha 6\beta 1$ integrin variant 2 (Fig. 2). A total of five models were generated for each of the model building of integrin proteins. Among these, the model of lowest DOPE was selected for further process such as loop refinement by MODELER loop-model and energy minimization by Smart Minimizer algorithm in Discovery Studio 3.0. The minimization was carried out in 500 steps by applying CHARMM force field. Occludin protein sequence was modeled based on multiple-threading alignments by I-TASSER server⁸² since there is a lack of availability of suitable template structures for homology based modeling in PDB database. The confidence score (C-score) of the modeled occludin structure was -1.57 . Finally, the predicted models were validated by analyzing stereo chemical properties by Ramachandran plot.⁸³ The plots (Fig. 3) illustrate that 99.6% amino acid residues of $\alpha 6\beta 1$ -integrin variant 1, 99.7% residues of $\alpha 6\beta 1$ -integrin variant 2 and 98.1% residues of occludin are distributed in the allowed region of the Ramachandran plot which are in the inside, hardsphere and overlap regions. This thus confirms that the modeled structures of $\alpha 6\beta 1$ -integrin for both variants and occludin are reliable.

The modeled structures of $\alpha 6\beta 1$ integrin variant 1, $\alpha 6\beta 1$ integrin variant 2 and occludin were used for the preparation of proteins by Maestro 9.0 protein preparation wizard. In which bond order assigned, and hydrogen added appropriately. These structures were optimized by exhaustive sampling and minimization methods by applying OPLS2005 force field. The structural co-ordinate of adjudin (CID: 9819086) was obtained from NCBI-PubChem database. LigPrep (version 2.3, Schrödinger, LLC, New York, NY, 2009) was used to generate 3D structure of adjudin. The active site residues of each of $\alpha 6$ integrin variant 1, $\alpha 6$ integrin variant 2 and $\beta 1$ integrin were identified by performing EMBOSS global pairwise sequence alignment (<http://www.ebi.ac.uk/Tools/emboss/align/index.html>) with $\alpha V\beta 3$ integrin amino acid sequence. The alignment outputs show that ligand binding residues from Gly172 to Gly181 of αV integrin⁸⁴ matched with amino acid residues of both $\alpha 6$ integrin variant 1 and 2 from Gly183-Gly192 (Fig. 4A,B). Similarly, the ligand binding residues Asp119, Ser121, Ser123, Asp217 and Glu220 of $\beta 3$ integrin⁸⁴ matched with Asp130, Ser132, Ser134, Asp226 and Glu229 of $\beta 1$ integrin (Fig. 4C). Hence, the identified matching residues, namely from Gly183.A to Gly192.A, Asp130.B, Ser132.B, Ser134.B, Asp226.B and Glu229.B of both $\alpha 6\beta 1$ integrin variant 1 and variant 2, were selected as centroid of the active residues for the generation of receptor grid. For occludin, the extracellular loop region from Ala88 to Ser108 was defined as active site residues. The Standard Precision (SP) mode of Glide was used for the flexible ligand docking. UCSF Chimera⁸⁵ was used for the visualization and analysis of the docking results.

The docking results of adjudin into $\alpha 6\beta 1$ integrin variant 1, $\alpha 6\beta 1$ integrin variant 2 and occludin are summarized in Table 1. The molecular docking of adjudin into $\alpha 6\beta 1$ integrin variant 1 shows the formation of single hydrogen bond by Ser263.A in β -propeller domain of $\alpha 6$ integrin chain (i.e., in A-chain) and large number of hydrophobic contacts by Trp190.A, Tyr234.A, Leu235.A, Arg259.A, Ala260.A, His262.A, Ser292.A, Tyr175.B, Asp226.B, Pro228.B, His263.B, PHE264.B, Gly266.B, Asp267.B, Leu270.B residues in β -propeller domain of A-chain and βA domain of B-chain. It is interesting to note that adjudin molecule is interacting at the interface of both propeller domain and βA domain (Fig. 5). Generally, the biologically active integrin molecule is formed by the network of interactions of α and β integrin subunits. In this, N-termini of both α and β subunit (i.e., β -propeller domain and βA domain) form globular head of ligand binding domain for extracellular region.⁸⁴ Hence, the binding of adjudin in interface residues of α and β subunit would likely inhibit the signal transduction of this integrin protein across the cell membrane.

Similarly, the molecular docking of adjudin into $\alpha 6\beta 1$ integrin variant 2 resulted in the formation of two hydrogen bonds and large number of hydrophobic contacts (Table 1). The amino acid residues of β -propeller domain such as Leu226.A and Arg259.A are involved in hydrogen bond formation. In addition, Val227.A, Pro230.A, Ala231.A, Ser233.A, Ala260.A, Asn261.A, His262.A and Ser263.A of β -propeller domain and Ser227.B, Pro228.B, Gly261.B, Glu320.B, and Phe321.B of βA domain are also involved in hydrophobic contacts to make more stable interactions. Hence, it is observed that adjudin also makes strong interactions at the β -propeller- βA domain interface residues of $\alpha 6\beta 1$ integrin variant 2 (Fig. 6). It is significant to note that the amino acid residues such as Arg259.A, Ala260.A, His262.A, Ser263.A and Pro228.B are the common residues involved in interactions with adjudin in both $\alpha 6\beta 1$ integrin variant 1 and $\alpha 6\beta 1$ integrin variant 2. In addition, $\alpha 6$ integrin is the only chain involved in hydrogen bond formation and makes more number of hydrophobic contacts in both $\alpha 6\beta 1$ integrin variant 1 and $\alpha 6\beta 1$ integrin variant 2. Hence, we conclude that adjudin molecule is interacting at the main inter-subunit interface residues lying within the head between the β -propeller of $\alpha 6$ integrin and βA domain of $\beta 1$ integrin.

The 'adjudin-occludin' docked complex shows three hydrogen bonds by Gly121, Gly123, and Lys380 residues, in addition to the active participation of Tyr93, Gly94, Gly96, Phe98, Phe117, Gly119, Tyr122, Gly125, Tyr126, Pro378, Thr384 and Glu400 residues for hydrophobic contact (Fig. 7). This docking result thus illustrates that adjudin molecule interacts with the extracellular residues such as Tyr93, Gly94, Gly96, Phe98, Pro378, Lys380, Thr384 and Glu400, in addition to the transmembrane helical residues such as Phe117, Gly119, Gly121, Tyr122, Gly123, Gly125 and Tyr126. Based on this molecular modeling study, adjudin molecule can alter the functional activity of occludin protein at the BTB.

Remarks and Summary

In short, the molecular modeling findings shown in Figures 5-7 clearly demonstrate that adjudin serves as a ligand that interacts with $\alpha 6\beta 1$ -integrin receptor at the apical ES and occludin at the BTB. These findings, in particular data shown in Figures 5 and 6, also yield

some new insights on the mechanism by which adjudin induces signaling function involving ERK1/2 downstream of the $\alpha 6 \beta 1$ -integrin-p-FAK-p130Cas-DOCK180-RhoA-vinculin complex. It is likely that the coupling of adjudin with the $\alpha 6 \beta 1$ -integrin receptor (see Figs. 5 and 6) induces activation of the FAK-p130Cas-DOCK180-RhoA-vinculin complex, which relates the signaling function to ERK1/2, which in turn perturbs the integrin-based adhesive function at the apical ES.

Adjudin is also found to bind to occludin at the BTB (Fig. 7), and this coupling can likely lead to a disruption of the TJ-permeability barrier function at the BTB. However, the BTB is composed of *co-existing* basal ES, desmosome and gap junction, and recent studies have shown that the BTB has a unique mechanism in which a transient disruption of TJ does not lead to the widespread disruption of the other co-existing junctions (e.g., basal ES, desmosome) in order to maintain BTB integrity⁸⁶⁻⁸⁸ during spermatogenesis unless it is exposed to an unusual acute concentration of a toxicant.⁵⁸ It was postulated that these co-existing junctions are usually “engaged” to maintain the BTB integrity, however, when the BTB is under assault, such as when exposed to toxicants, these co-existing junctions can be “disengaged” so that remaining junctions can maintain the immunological barrier transiently even though the TJ-barrier is temporarily disrupted.^{86,89} These findings also illustrate the uniqueness of the BTB among other blood-tissue barriers in mammals.

THE APICAL ES-BTB AXIS IS A PRIME TARGET FOR MALE CONTRACEPTIVE DEVELOPMENT

The apical ES-BTB functional axis in the seminiferous epithelium as depicted in Figure 1, which coupled with the molecular modeling data shown in Figures 5-7 thus illustrate that many component proteins in this axis can be the target of male contraception. The modeling data shown in Figures 5-7 also illustrate that a second generation adjudin analogs can likely be synthesized to improve: (i) its potency at the apical ES, and perhaps (ii) its bioavailability in the apical compartment behind the BTB by disrupting the BTB integrity transiently for its “transit” at the BTB, mimicking the transit of preleptotene spermatocytes at the site at Stage VIII of the epithelial cycle. This possibility is supported by recent findings that when rats were treated with adjudin at an acute dose (i.e., 250 mg/kg b.w.), BTB was *irreversibly* disrupted.⁵⁸ On the other hand, the optimal dose (i.e., 50 mg/kg b.w.) that effectively induced male fertility *reversibly* in rats by depleting virtually all germ cells from the epithelium except spermatogonia/spermatogonial stem cells (SSC), BTB was found to remain intact when germ cells began to dislodge from the epithelium within 6-hr following its administration until all the tubules were devoid of germ cells except spermatogonia/SSC by ~2-wk.⁵⁸ Interestingly, the BTB was found to be transiently disrupted at this low but effective dose by ~6-wk and it was “re-sealed” by 20-wk.⁵⁸ Thus, adjudin can be chemically modified based on findings in Figure 7, such that the appropriately modified adjudin can be rapidly “docked” to occludin to induce BTB disruption within hours following treatment. This thus promotes its bioavailability in the apical compartment behind the BTB to exert its effects at the apical ES to dislodge spermatids from the epithelium.

THE ROLE OF BTB IN THE DIFFERENTIATION OF SPERMATOGONIA/SPERMATOGONIAL STEM CELLS (SSC)

Treatment of adult rats (~270-300 gm b.w.) with adjuvin at 50 mg/kg b.w. by gavage is known to induce *reversible* infertility since the remaining spermatogonia/SSC in the seminiferous tubule gradually repopulate the entire epithelium, re-initiating spermatogenesis.²⁸ A recent study, however, has shown that by treating adult rats (~270-300 gm b.w.) with an acute dose of adjuvin at 250 mg/kg b.w. by gavage, the infertility induced by adjuvin in these rats was *irreversible*, yet this was *not* the result of adjuvin-induced spermatogonia/SSC depletion from the seminiferous epithelium since the population of spermatogonia/SSC in the tubules of rats from this high-dose adjuvin treated group remained similar to rats in the low-dose treated and normal control groups.⁵⁸ Detailed histological/morphological and functional analysis of the testes from these animals showed that the BTB in rats from the high-dose treated group was *irreversibly* disrupted whereas the BTB in low-dose treated group was only *transiently* disrupted by adjuvin.⁵⁸ These findings thus demonstrate unequivocally that an intact BTB is critical for differentiation of spermatogonia/SSC to re-initiate spermatogenesis. This conclusion is also supported by genetics studies in which the knockout of connexin43 (Cx43) [note: Cx43-based GJ is an integrated component of the BTB^{34,90} which co-exists with TJ, basal ES and desmosome to constitute the BTB¹⁷] led to infertility in mice.⁹¹⁻⁹⁴ Furthermore, Cx43 knockout also produced a number of phenotypes shared by rats from the adjuvin-treated high dose group.⁵⁸ For instance, it was found that although adult Cx43^{-/-} mice had similar population of spermatogonia/SSC in the tubules as compared to the wild type, the Cx43^{-/-} mice displayed meiotic arrest in which spermatogonia/SSC failed to differentiate beyond Type A spermatogonia.⁹¹ Also, Sertoli cells in these mice remained actively mitotic and proliferative such that clusters of Sertoli cells were shed from the epithelium and Sertoli cell clusters were found in the tubule lumen.^{93,94} This thus contributes to the inability of the BTB to “seal” properly to maintain a functional BTB. Additionally, even though ultrastructures of TJ were visible at the BTB in these mice, the steady-state level of ZO-1 (a TJ-associated adaptor at the BTB) was significantly reduced and ZO-1 was also found to be mis-localized in the seminiferous epithelium.⁹² This in turn led to mis-localization of TJ- and basal ES-associated proteins at the BTB since many of these proteins (e.g., occludin, claudins, JAM-A, catenins, cadherins) interact directly or indirectly with ZO-1 at the BTB.^{56,61,86} These findings thus suggest that the BTB in these Cx43^{-/-} mice is not fully functional and intact. In short, an intact and fully functioning BTB is necessary for differentiation of spermatogonia/SSC to Type A and Type B spermatogonia, and spermatocytes, to initiate spermatogenesis.

CONCLUSION AND FUTURE PERSPECTIVES

Based on recently published findings in the field as summarized above, which coupled with the molecular modeling findings described here thus lead us to conclude that the $\alpha 6\beta 1$ -integrin receptor at the apical ES is one of the primary targets of adjuvin. Moreover, adjuvin can also form a docking complex with occludin at the BTB. It is of interest to know note that the occludin-ZO-1-based protein complex that regulates the Sertoli cell TJ-permeability

function can be modulated by FAK.^{95,96} Additionally, this occludin-ZO-1-FAK complex was found to structurally interact with P-glycoprotein, an efflux drug transporter, at the Sertoli cell BTB.⁷⁸ Collectively, these findings thus illustrate that several component proteins along the apical ES-BTB functional axis³⁶ (e.g., $\alpha 6\beta 1$ -integrin and occludin) can be the target of a male contraceptive, such as adjudin. Perhaps the most important of all, the molecular modeling findings as reported herein also illustrate that more potent adjudin analogs can be designed to enhance its “penetrability” at the BTB and its affinity to the $\alpha 6\beta 1$ -integrin receptor, thereby activating its associated regulatory proteins (e.g., p-FAK, DOCK180, PKB) and the ERK1/2 signaling pathway downstream. This, in turn, perturbs germ cell adhesion more effectively in the seminiferous epithelium, leading to infertility. This also requires lesser amount of drug to be administered because of the better BTB penetrability, and since the hypothalamic-pituitary-testicular axis is not disrupted, side-effects should be minimal.

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Figure 1.

A schematic drawing illustrating the apical ES-BTB functional axis in the seminiferous epithelium of adult rat testis. The micrograph shown in the left panel is the cross-section of a Stage VIII seminiferous tubule from an adult rat testis illustrating the occurrence of spermiation at the luminal edge of the seminiferous epithelium. BTB also undergoes restructuring at this stage so that preleptotene spermatocytes (see “dark gray/green” arrowheads) that are differentiated from spermatogonia (see “light gray/orange” arrowhead) at this stage can be in transit at the BTB to enter the adluminal (apical) compartment to differentiate into pachytene spermatocytes (PS) to prepare for meiosis I and II which occur at Stage XIV of the epithelial cycle. Also shown are the Sertoli cell (see “medium gray/pink” arrowhead). RS, round spermatid; ES, elongated spermatid (step 19). The schematic drawing shown on the right panel illustrates that the BTB physically divides the seminiferous epithelium into the basal and apical compartment. However, as described in text, a functional axis exists between the apical ES and the BTB to co-ordinate the cellular events, namely spermiation and BTB restructuring, that occur at opposite ends of the epithelium at Stage VIII of the epithelial cycle. A color version of this figure is available online at www.landesbioscience.com

A

integrin 3DE
FNLDTREINIVKRGSDPSSVLFPGFSIAMHWOLPEDKRLLLVGAPRAEALPLKANRKTGS-LYSCDSTISRGPTFEDNDIA-----DPMSESKKE
FNLDV--DSPAEYGGPESSYEGFADVFPVSPASSRMFLLVGAPKANIT--APGIVEGGVFLKCDVSSSTRRCOPTIEDATGNRDYAKDPLS

integrin 3DE
DOWHGAVTVOSGPGGGKVVTCARHREKROHVNTKOEKDFIFGRVYLSQNLRIEDDDGGDWSF-DGRLRGHGKFFSGOEEVAATETDHFYVVF6
HOWFGVTSVRKMD--KILACALMLHWR--TEMKOE-NEPVETFLDODGTKTSVLYAP-----RSQDIDADGGKFFGOGFSDITETADR-VLLG

integrin 3DE
APGTYNKKGIIRVVE--KNNTFFPMNIFEDGPEYVGGEIDHDESLVPPVANSYLGSLDSGKGIKSKDDITFVSGAPRANHSAGAVLLKRDMSA
GPGSFYVMOGOLISDOVAEIVBKVYPPVYS-1KYNQLAARTAAIF--DSDVLYGYSVAVG-DFNFGDDIGDFVSGVPRARTLGMVVIYD--G-K

integrin 3DE
HLLLENIIDGEGGLASSFGDYVAATVDLNAAGWODIVIGAPQYFDR--DGEVGGAVVYINQGRWSGVFPVRLNGTKD-SMFGIVKKNIGDINDOG
MSSLLHITDGGMAAYEGFSVAITIGDDHAGDIFVIGAFMDRSGKQLGQVGGWMSLFRASGDFOTTKLNGFEVFAFGSLAPLGDLDODG

integrin 3DE
YPDIAVAGPY--NDLKVFIYVHSPTGIIVKPTDVLG-----TTFEGYSIANMMLDRSPYDVAVYGL-SDSVTFIRSRAPVNIILKTIIT
FNDAIATVAGPGEKKKGIIVYIFNRSITGLNAVPTISGLIGOWAARSMPGSGYSMKIATDIDKNGYDPLIVGAGVGRRAILYARAPVITVNAVLE

integrin 3DE
TPSRIDLROKSMCGSP---SGILCKVKKACFEYTKAPSSQYNIPISILGLEAEKERKRSKSLSSVQVRNQGSEKYTOETLTNOKORACMEETL
YPSILINDONK-TESLIGTALKVSGFNNAFKKADGG--SVLRKLNIFOWELLDKOKRAIRALILYSRS-SHSHKSTISLGGMLGEEFLTA

integrin 3DE
VLOEN--IRDKLRPTITASVEVLPSSRRVNSLPEVILPINSNEAKTIVTQVHFLKSGCGDNNVCNSNKLLEYKFGITREGNOKFSYLPIDNG
YLRODESEFRDKLPTITFMEYRLD--YR-TAADTTGLOPILNOFTPANISQDAAHTILD-CGGDNNVCKP--KLEVSVDSE-----D-----OK-

integrin 3DE
IPEVLVKDOKDAIEITVTGPSDDPRNPKRDKDHEAKLTIATFDTLTYS-AYRELAFPEKOLCVANONGSDADCELGNNPGRNKSQVTFYFI
--K-IYIGDGNPLTILYKAAQ--D-----EGEYEAELVITLLOADIGVVRNLEAKFLRLSCLAFKTEHQDCELGNNMAGLNOVLGAGR

integrin 3DE
LSTTVEOBDTDLIDNLKLETSITNOINDLAPIT-IAKAKVYIELLSVSGVAKPSOVYFGGTVGEENAKM-EDEFGVSLIEYEFIRLILKPLKNLG
FSVHVOBFEMDSVKFDLOIOSSNLFDKPSVPVSHKVDVLAALVEIRGKADQDHVFLIPINWEEKANPETEEDDGPVVOHIVYELRNNGP--SSFS

integrin 3DE
TATLNIOWPKEISNGKWLILYLMKVEKLELEOVCEPHNEINFLKLESHNSRKKRELPEKOIDDSRKSFLFSEKRYDTLNGSNVRCVNTRCPLR
KAMHLHOWPYNYNNLTLTYLILYHIDB--PMTNSDMETNPLRILIS-----LID--IHTLGGM-AODLKLVIYONG

integrin 3DE
GLS--KASVLRRLWNSLTLEEYSKLYLDLVRASIDTAARAAIKLPLAGTOVRVTVFSPKTVAGVSGIAWVILLVAGILMALLVFL
RLRGKSAIILYVKSLILTEFTFMNKENONHSYLSKSSASFNV-----TEFPKKNLPJEDITNSTLVTNTV---WGLOPAMP--

integrin 3DE
LWKCGFFKRSRYDSDVPVRYHAVRIKKEEREINDEKHTDNPEKKPVPKWNENESYSITDKNRLTKANAKSGCECLOAGPNCWGTNTTFLDGGMT
-----VYVIVL-----GP--RLTGAVSSGOOLVAGSMWACS-----DEALML

integrin 3DE
-SARCDOLAEKKKKGGOSDLENRGSGOTLKKNNVITRSKGMAKSLRPEDITOTPOOULLKLRSGPKQKTLFKFRKAEEDYPDILLYLMDLSYS
GSPACDLKENLLDNCAESETFVSEARVLEDRPLSD--KSGSDKQ--VTOVSOPRIALRLRPDSSKNTFSIROVREDDYVDIYYLMDLSYS

integrin 3DE
MKDDLNVNKS LGTLMNEMRRITISDFRIGFSFVEKTMVPIYSTP-AKLRNPCTSEON-CTSPFSYKNVLSLTDGREFNELLGQORISGNLDS
MKCDLWSITONLGTIATOMRKLTSLNLTGFGGAFVDKPSGYMKYISPEALENCPGYDMKTLTCLPMFEGKHVLTITDQTVRFNEEKKKSVSRIRDA

integrin 3DE
FEGGFDAIMQAVAGSLGWRN-VTRLVFSYDAKTFHFGGDKGLBIVPNDGOCHELV--VMTMHHYDYPSPHIAVOKLSENNIQTIFAVTE
FEGGFDAIMQATVDEKIGWRNDASHLLVFTDAKTFHIALDGRLAGIIVPNDGOCHELVSDNHMSAETTMQYPSLGLMTEKLSOXKNILIFAVTE

integrin 3DE
FOPVYKELKNLIPKSAVGTLSGNSSNVOLITDAYNSLSSEYILNSKLPDGVITNYKYSKGVNGVTGENGKKSCTNISTGDEMOEISITANK
VVNLYOYSELITPTGTGVGLSSDNNVOLTVDYVGKIRKWELEVRDLPEGLTYSFNATCLW--NEVIPGLKSCMLGLKTGDETSSEAKVRG

integrin 3DE
FNKSEKISIKIPLGTFEVEVLODICKNCSHGIAPAPKCHEGNGTFECGACRNEVVRVGRHCEGSDTEVNSDQDAYRKENSSEICSNHGE
POEKESFTIKPVGFKSLIMOTVTDQACQAQAEARHNRNNGNGTFECGACRNGPPEVSGCEGSEEDYRPSDQ--SREPGQVCSGORE

integrin 3DE
CVCGOCVCRKRENTNEIYSKGFCECDNFENQSNGLIGGNGVGRVREVEYPNYTGSAEDCSLDVVPVATNGQIENGRRGICEGGAACKTDPKF
CLCGOCVCHSSDFG-KITGKYCECDSDSVRYKGEKMSGNGHOGSGDGLDQSDWTGVTGNCITRDTDCSSNGLLCSGRGKCECCGSCVCTOPGS

integrin 3DE
GQPTCECTOTGLGCAHKEKCVOCARFNKGEKKDTCASHSFNLLKVESREKLPPOVOVDVTHCKEKDIDCWFYFTTSSNGEAAHVHVEET
YDCECTCTOTGPDATFKKECGEKKDORGLALD--ENTNRYCRDTEISVKELKDTG--KDAVNTYNEDDVVRVOMYEDSSGKSLIYVVE

integrin 3DE
PDCITGPDPIPIVAGVVGAGIVLIGLALLIWKLIMIHDRREFAKFEKEMNAKWDTGENPIYKSAVTTTVVNPKYEGK
RECKMKGPDILV-----

Figure 2.

The sequence alignment results used for building 3-D models in heterodimeric form for the target sequences of (A) $\alpha\beta 1$ -integrin variant 1 and (B) $\alpha\beta 1$ -integrin variant 2 with template sequence of $\alpha V\beta 3$ -integrin [PDB ID: 3IJE]. In short, the $\alpha 6$ -integrin sequence shown in (A) and (B) corresponds to variant 1 and variant 2 of $\alpha 6$ -integrin of *Rattus norvegicus*, respectively; but the $\beta 1$ -integrin of *Rattus norvegicus* has no molecular variant. The “gray/green” line in (A) or (B) denotes the N-terminal amino acid residue of $\beta 1$ -integrin of the $\alpha\beta 1$ -integrin receptor, where the “black/red” line in (A) or (B) denotes the N-terminal amino acid residue of $\beta 3$ -integrin of the functional $\alpha V\beta 3$ -integrin receptor. A color version of this figure is available online at www.landesbioscience.com

Figure 3.
The Ramachandran plot for (A) $\alpha 6\beta 1$ -integrin variant 1, (B) $\alpha 6\beta 1$ -integrin variant 2 and (C) occludin. These Ramachandran diagrams are used to visualize backbone dihedral angles ψ against ϕ of amino acid residues in the corresponding protein structure of $\alpha 6\beta 1$ -integrin variant 1 and 2 and occludin.

Figure 4.

Pairwise sequence alignment results for predicting active site residues of (A) α 6-integrin variant 1, (B) α 6-integrin variant 2, and (C) β 1-integrin. Active amino acid residues are tagged by red box. A color version of this figure is available online at

www.landesbioscience.com

Figure 5.

Illustration of docked complex for adjudin into $\alpha 6\beta 1$ -integrin variant 1. A) Ribbon drawing of full-length docked complex [shown in cyan ($\alpha 6$) and green ($\beta 1$)], (B) ribbon drawing of $\alpha 6\beta 1$ -integrin interface domains that show the interacting adjudin molecule and (C) stick representation of interacting interface residues [shown in cyan ($\alpha 6$), green ($\beta 1$) and orange (adjudin)]. Hydrogen bond are depicted by red dotted lines along with the bond length. This picture was generated by using the UCSF Chimera software package. A color version of this figure is available online at www.landesbioscience.com

Figure 6.

Illustration of docked complex for adjudin into $\alpha 6\beta 1$ -integrin variant 2. (A) Ribbon drawing of full-length docked complex [shown in cyan ($\alpha 6$) and green ($\beta 1$)], (B) ribbon drawing of $\alpha 6\beta 1$ -integrin interface domains that show the interacting adjudin molecule and (C) stick representation of interacting interface residues [shown in cyan ($\alpha 6$), green ($\beta 1$) and orange (adjudin)]. Hydrogen bonds are depicted by red dotted lines along with the with bond length. A color version of this figure is available online at www.landesbioscience.com

Figure 7.

Illustration of docked complex of adjudin into occludin. (A) Ribbon drawing of full-length docked complex of adjudin into occludin. The interacting residues are tagged by a circle and (B) stick representation of interacting residues. Adjudin is shown in orange color. Hydrogen bonds are depicted by red dotted lines. A color version of this figure is available online at www.landesbioscience.com

Table 1

Molecular interactions of $\alpha\beta$ 1-integrin and occludin with adjudin

Docking Complex	H-bond (D-H...A) ^a	H-Bond Length (Å)	Hydrophobic Contact Residues	Docking Score	Glide Energy (kcal/mol)
$\alpha\beta$ 1-Integrin variant 1 and adjudin	OH(Ser263.A)...O	1.967	Trp190.A, Tyr234.A, Leu235.A, Arg259.A, Ala260.A, His262.A, Ser292.A, Tyr175.B, Asp226.B, Pro228.B, His263.B, PHE264.B, Gly266.B, Asp267.B, Leu270.B	-6.133	-43.739
$\alpha\beta$ 1-Integrin variant 2 and adjudin	NH...O (Leu226.A) NH...O (Arg259.A)	2.480 1.812	Val227.A, Pro230.A, Ala231.A, Ser233.A, Ala260.A, Asn261.A, His262.A, Ser263.A, Ser227.B, Pro228.B, Gly261.B, Glu320.B, Phe321.B	-5.459	-38.214
Occludin and adjudin	NH...O (Gly121) NH...O (Gly123) NH (Lys380)...N	2.181 2.283 2.334	Tyr93, Gly94, Gly96, Phe98, Phe117, Gly119, Tyr122, Gly125, Tyr126, Pro378, Thr384, Glu400	-3.987	-34.741

^aD- Donor; H- Hydrogen; A- Acceptor.