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Extracellular Matrix and Its Role in Spermatogenesis

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Abstract

In adult mammalian testes, such as rats, Sertoli and germ cells at different stages of their development in the seminiferous epithelium are in close contact with the basement membrane, a modified form of extracellular matrix (ECM). In essence, Sertoli and germ cells in particular spermatogonia are “resting” on the basement membrane at different stages of the seminiferous epithelial cycle, relying on its structural and hormonal supports. Thus, it is not entirely unexpected that ECM plays a significant role in regulating spermatogenesis, particularly spermatogonia and Sertoli cells, and the blood-testis barrier (BTB) constituted by Sertoli cells since these cells are in physical contact with the basement membrane. Additionally, the basement membrane is also in close contact with the underlying collagen network and the myoid cell layers, which together with the lymphatic network, constitute the tunica propria. The seminiferous epithelium and the tunica propria, in turn, constitute the seminiferous tubule, which is the functional unit that produces spermatozoa via its interaction with Leydig cells in the interstitium. In short, the basement membrane and the underlying collagen network that create the acellular zone of the tunica propria may even facilitate cross-talk between the seminiferous epithelium, the myoid cells and cells in the interstitium. Recent studies in the field have illustrated the crucial role of ECM in supporting Sertoli and germ cell function in the seminiferous epithelium, including the BTB dynamics. In this chapter, we summarize some of the latest findings in the field regarding the functional role of ECM in spermatogenesis using the adult rat testis as a model. We also highlight specific areas of research that deserve attention for investigators in the field.

Introduction

Spermatogenesis is a precisely regulated process by which one spermatogonium (diploid, 2n) divides and differentiates into 256 spermatids (haploid, 1n) via 14 stages of the seminiferous epithelial cycle with six mitotic and two meiotic divisions in adult rat testes.¹ In order to complete these intriguingly regulated events, there are extensive junction restructuring in the seminiferous epithelium at both the blood-testis barrier (BTB; note: BTB is a testis-specific structure composed of side-by-side arranged tight junctions [TJ], the basal ectoplasmic specialization [ES], the basal tubulobulbar complexes [TBC], both are testis-specific adherens junction [AJ] types, and the desmosome-like junctions [DJ]) between adjacent Sertoli cells; and anchoring junctions, such as apical ES, apical TBS, DJs and gap junctions (GJ), between Sertoli and germ cells (see Fig. 1). This thus permits developing germ cells, such as preleptotene and leptotene spermatocytes, traverse the BTB at stage VIII

of the seminiferous epithelial cycle for further development into round, elongating, and elongated spermatids, yet these cells must remain attached to the nourishing and supporting Sertoli cells.^{2,3} In light of these extensive junction restructuring events during spermatogenesis to accommodate the timely migration of germ cells across the epithelium, it is not entirely unexpected that the morphological layouts of TJ and anchoring junctions in the testis are relatively unique versus other epithelia. Furthermore, unlike other blood-tissue barriers, such as the blood-brain and the blood-retina barriers, which are constituted by endothelial TJs of the microvessels in the corresponding organs namely brain and eyes, respectively, the BTB is contributed almost exclusively by adjacent Sertoli cells near the basement membrane of the seminiferous tubules, and the TJ-barrier in the microvessels in the interstitium contribute little, if any, to the BTB function. Interestingly, the peritubular myoid cell layer in rodent testes was shown to prevent the penetration of electron dense markers, such as lanthanum, colloidal carbon or thorium, into the seminiferous epithelium in almost ~85% of the tubules examined,^{4,5} even though myoid cells in primate testes were much less effective to restrict the penetration of these markers across the BTB.⁶ Collectively, these findings illustrate the myoid cell layer in the tunica propria contributes to the BTB integrity, at least in rodent testes.

The BTB, which physically divides the seminiferous epithelium into the basal and the adluminal (apical) compartments, segregating virtually the entire events of post-meiotic germ cell development and maturation from the systemic circulation, is located closely to the basement membrane (a modified form of extracellular matrix, ECM) (Fig. 1).⁷ This morphological layout is in sharp contrast to other epithelia where TJ is located at the apical portion of the cell epithelium, to be followed by the adherens belt (composed of AJ) and desmosomes. Such physical intimacy between the BTB and the basement membrane thus illustrates the possible role of ECM on junction dynamics at the BTB in the testis.^{2,3,8,9} Indeed, it was reported that infertile patients with aspermatogenesis were shown to have abnormal basement membrane structures.^{10,11} Recent studies have also demonstrated the crucial role of ECM components, such as collagens and laminins, in junction dynamics since these proteins were shown to work in concert with proteases, protease inhibitors, cytokines (e.g., TNF α), and focal adhesion (FA) components found at the ES to regulate the steady-state levels of integral membrane proteins at the cell-cell interface.¹²⁻¹⁶ In this chapter, we intend to highlight the recent advances of how ECM proteins and their partners regulate junction dynamics in the testis.

Unique Features of Extracellular Matrix (ECM) in the Testis

ECM, largely composed of glycoproteins and polysaccharides, fills the extracellular space at the cell-cell contact sites.⁷ In rodent testes, a specialized form of ECM, constituted largely by type IV collagen and laminins, along with heparan sulfate proteoglycan¹⁷ and entactin,¹⁸ forms the basement membrane (~0.15 μ m thick), which encloses each seminiferous tubule and is in contact with the base of Sertoli cells and spermatogonia (Fig. 1). One interesting feature of the basement membrane is that it is adjacent to the blood-testis barrier (BTB),^{2,3} where tight junctions (TJ) coexist with adherens junctions (AJ) (Fig. 1), such as basal ectoplasmic specialization (ES) and basal tubulobulbar complex (TBC),¹⁹⁻²¹ and desmosome-like junctions (DS);²² unlike other blood-tissue barriers (e.g., blood-brain

barrier and blood-retina barrier) where TJs are furthest away from the ECM, and are localized to the apical portion of the epithelium/ endothelium, to be followed by AJ, desmosomes and gap junctions.²³

Functions of the Blood-Testis Barrier (BTB)

The BTB divides the seminiferous epithelium into the basal and adluminal compartments and thus creates a unique microenvironment for spermatogenesis (Fig. 1). It maintains an immunological barrier by sequestering post-meiotic germ cell development from the systemic circulation, regulates the passage of molecules into the adluminal compartment or vice versa and confers cell polarity.^{2,3,24} As such, developing germ cells depend exclusively on Sertoli cells for structural, anchoring and nutrient supports. Although BTB confers one of the tightest blood-tissue barriers in mammalian body, it is highly dynamic in nature since it must 'open' (or 'disassemble') at stage VIII of the epithelial cycle in adult rat testes on the apical portion of the migrating preleptotene and leptotene spermatocytes and then 'close' (or 'reassemble') at the basal portion of the cell to facilitate cell migration while maintaining the barrier integrity.²⁴ Without this, spermatogenesis cannot complete. At present, the mechanism(s) governing this timely BTB restructuring is not entirely clear. However, recent studies have shown that ECM components, such as collagen IV, are working in concert with proteases, protease inhibitors and cytokines (e.g., TNF α) to regulate TJ dynamics in the testis.^{8,9,14}

Collagen IV

Its Expression and Localization in the Testis

Type IV collagen and laminins are the building blocks of the basement membrane in the testis.^{7,23,25} Type IV collagen network is formed by the association of monomer, which is a triple helical structure composed of three α chains.^{25,26} Each monomer is characterized by an N-terminus noncollagenous 7S domain (~15 amino acid residues), a middle collagenous domain (~1400 residues of Gly-Xaa-Yaa repeats) and a carboxyl terminal noncollagenous (NC1) domain (~230 residues). There are six genetically distinct α chains including ubiquitous $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ chains and more restricted $\alpha 3(\text{IV})$ - $\alpha 6(\text{IV})$ chains.^{26,27} $\alpha 1(\text{IV})$ and $\alpha 5(\text{IV})$ chains are present in rodent testes.²⁸⁻³⁰ Moreover, $\alpha 3(\text{IV})$ and $\alpha 4(\text{IV})$ chains are the major (~80%) collagen chains found in the basement membrane of bovine testes,³¹ implicating the unique structural and/or functional role of $\alpha 3(\text{IV})$ and $\alpha 4(\text{IV})$ chains in the seminiferous tubule basement membrane in the testis. Collagen $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ chains are products of Sertoli and myoid cells,^{28,32,33} whereas $\alpha 3(\text{IV})$ is a product of Sertoli and germ cells in the rat.³⁴

Roles in TJ Dynamics

There is mounting evidence that collagen functions perhaps not just as a scaffolding protein.²³ For instance, recent studies have shown that Sertoli cell TJ-barrier assembly in vitro was associated with a transient but significant increase in collagen $\alpha 3(\text{IV})$ indicating de novo synthesis of collagens is associated with TJ assembly, suggesting the involvement of collagen $\alpha 3(\text{IV})$ in TJ dynamics.¹⁴ Furthermore, the presence of an anti-collagen antibody in Sertoli cell cultures during TJ-barrier assembly reversibly disrupted the TJ-

barrier, further supporting that the interference of an ECM function affects TJ dynamics. Although the underlying mechanism is presently unclear, subsequent studies have shown that these effects were mediated, at least in part, by cytokines, such as TNF- α , which regulate ECM homeostasis via proteolysis.¹⁴

TNF α

TNF α and its Receptor and Testicular Function

ECM harbors a pool of cytokines, such as TNF α , which can be released when ECM proteins, such as collagens, are degraded.⁷ TNF α (a ~50 kDa trimeric protein, consisting of three identical subunits of 17 kDa each) is produced mainly by activated monocytes and macrophages in the systemic circulation, and is crucial to inflammation, cell proliferation and apoptosis.³⁵ In the testis, TNF α is a product of germ cells (e.g., round and elongating spermatids), macrophages, and Sertoli cells.¹⁴ Its receptors, p55 and p75, are two structurally related, but functionally distinct receptors found in epithelial cells, including Sertoli cells;³⁶ however, the p55 TNF α receptor (TNFR p55) in Sertoli cells is the main receptor for TNF signaling.^{12,37} In the testis, TNF α plays a crucial role in regulating germ cell apoptosis,³⁸ Leydig cell steroidogenesis³⁹ as well as junction dynamics (Fig. 1). For instance, it is now known that in adult rat testes, the number of Sertoli cells, at ~40 million cells, remain relatively stable throughout the adulthood^{40,41} since by day 15 post-partum, Sertoli cells cease to divide.⁴⁰ As such, these limited number of Sertoli cells cannot support an unlimited number of developing germ cells. Indeed, it has been shown that each Sertoli cells support ~40-50 developing germ cells in adult rodent testes,⁴² and that as much as 75% of the developing germ cells undergo apoptosis and/ or spontaneous degeneration,^{43,44} failing to become mature spermatozoa, which is the mechanism being used in the seminiferous epithelium to regulate the precise number of developing germ cells.⁴⁵ Interesting, TNF α was shown to reduce germ cell spontaneous degeneration in rat and human seminiferous tubules cultured in vitro,^{38,46} illustrating its germ cell survival promoting effect.

Roles in TJ Dynamics

The role of TNF α on BTB dynamics has been elucidated by both recent in vitro and in vivo studies and a summary of the results on these studies are depicted in Figure 1. In a recent in vitro study, the presence of recombinant TNF α was shown to perturb the Sertoli cell TJ-permeability barrier dose-dependently and specifically since the disrupted TJ-barrier can be resealed upon its removal.¹⁴ This in vitro effect of TNF α on the Sertoli cell TJ-barrier was also confirmed by an in vivo study.¹² In this study, transient and reversible BTB disruption was shown when adult rats were treated with 2 μ g recombinant TNF α per testis, which is comparable to its endogenous intratesticular level (~0.5 μ g per testis when estimated by a solid-phase immunoblot assay), via an intratesticular injection and assessed by electron microscopy, fluorescent microscopy and a functional assay that monitors the diffusion of a fluorescent dye (fluorescein thioisocyanate, FITC, Mr 389) from the systemic circulation to the seminiferous epithelium behind the BTB.¹² These in vitro and in vivo studies, along with the observations that the expression of TNF α is stage-specific, being highest at stages VII-VIII,¹⁴ coinciding with the events of preleptotene and leptotene spermatocyte migration

across the BTB, further support the hypothesis that TNF α secreted by Sertoli and germ cells into the microenvironment at the BTB at stage VIII contributes to the transient BTB “opening” to assist preleptotene spermatocyte migration. This effect of TNF α in “opening” the BTB perhaps is working in concert with its germ cell survival promoting ability so that the migrating preleptotene spermatocytes that likely to take place in “clones” would not undergo spontaneous degeneration. For instance, it is known that germ cell maturation and development occur in “clones” via inter-cellular bridges as they traverse the seminiferous epithelium.^{47,48} Perhaps it is important in future studies to design functional experiments to assess if the level of TNF α at the BTB microenvironment is sufficient to induce BTB restructuring while promoting germ cell survival.

Regulation of TJ Dynamics: Effects on TJ-Proteins and ECM Proteins

Furthermore, TNF α apparently exerts its effects on the Sertoli cell TJ-permeability barrier function by regulating the expression of TJ-proteins (e.g., occludin) as illustrated by both in vitro and in vivo studies,^{12,14} thereby determining the steady-state protein levels of the integral membrane proteins at the BTB. Besides regulating TJ-proteins, TNF α was shown to induce Sertoli cell collagen $\alpha 3(\text{IV})$, matrix metalloprotease (MMP)-9 and tissue-inhibitor of metalloproteases (TIMP)-1, and to promote the activation of pro-MMP-9 to proteolytically active MMP-9.¹⁴ MMPs and TIMPs are proteases and protease inhibitors respectively, that work synergistically to regulate ECM remodeling.⁴⁹ As such, these results suggest that the activated MMP-9 induced by TNF- α may be used to breakdown the existing collagen network by cleaving collagen IV, separating the middle collagenous domain from the N-terminal 7S and the COOH-terminal NC1 domains in the ECM. Such cleavage process possibly affects the scaffolding function of ECM,⁴⁹ thus inducing a loss of other basement membrane proteins (e.g., laminins) and cytokines (e.g., TNF α and TGF- β), which, in turn, contributes to TJ disruption and BTB restructuring because Sertoli cells can no longer attach to an intact ECM. Furthermore, the released biologically active fragments, the NC1 domains, can bind to the middle collagenous domain, inhibit the assembly of intact collagen IV network.⁵⁰ Also, these biologically active fragments can have a negative feedback effect that inhibits collagenase production and thus affecting collagen degradation.⁵¹ As such, the induced collagen $\alpha 3(\text{IV})$ and TIMP-1 by TNF α may be a negative feedback mediated by the biologically active fragments, so as to replenish the collagen network in the disrupted TJ-barrier and limit the activity of MMP-9. Obviously, this hypothesis must be vigorously examined in future studies. In short, the following question must be address. First, can the collagen $\alpha 3(\text{IV})$ NC1 synthetic peptides regulate Sertoli cell MMP-9 and TIMP-1 production and/or their activation using Sertoli cells cultured in vitro? If they can, can they also regulate Sertoli cell TJ-permeability barrier when administered in vitro, or perhaps in vivo? Second, can these in vitro studies be reproduced in vivo that an administration of the NC1 domain peptides intratesticularly that leads to a disruption of the BTB function by disrupting the levels of MMPs and TIMPs in the basement membrane?

After reviewing the involvement of collagen in TJ dynamics, the following section introduces the crucial role of laminin, another ECM component, in ES dynamics.

Ectoplasmic Specialization (ES)

ES is a testis-specific, actin-based adherens junction residing in the basal (defined as basal ES) and apical (defined as apical ES) compartment of the seminiferous epithelium.^{3,52,53} Both basal and apical ES consist of a layer of hexagonally packed actin bundles sandwiched between the plasma membrane of the Sertoli cell and the cisternae of endoplasmic reticulum. Basal ES is localized at the Sertoli-Sertoli cell interface at the BTB, present side-by-side with TJ, desmosome-like junctions and gap junctions. Apical ES is found between the heads of developing elongating/elongate spermatids (step 8 and beyond in rat and mouse testes) and Sertoli cells which persists until replacing by apical tubulobulbar complex (apical TBC) restricted to the concave side of the elongated spermatid heads just a few hours before spermiation that occurs at late stage VIII of the seminiferous epithelial cycle in adult rat testes.^{20,52}

Basal ES

Cadherins⁵⁴ and nectin-2⁵⁵ are two AJ transmembrane proteins that are currently found at the basal ES. Recent study has shown that there is an engagement/disengagement mechanism between basal ES and TJ proteins via their corresponding peripheral adaptors, catenins and ZO-1,⁵⁶ perhaps being used to reinforce the BTB conferring its barrier function, making the BTB as one of the “tightest” blood-tissue barriers in the mammalian body. Such mechanism was suggested to facilitate preleptotene/leptotene spermatocyte migrate across the BTB at stage VIII of the epithelial cycle that the TJ and basal ES proteins become “disengaged” during BTB restructuring to facilitate germ cell migration across the barrier. However, much study is needed to elucidate the intriguing cross-talk mechanism(s) between basal and apical ES since the “opening” (or restructuring) of the BTB near the basement membrane and the disruption of the apical TBC at spermiation at the luminal edge of the epithelium take place almost simultaneously and since both ultrastructures are present at the opposite ends of the Sertoli cell epithelium, it is not entirely unexpected these events are intimately regulated in the Sertoli cell. Indeed, recent studies have shown that FAK (focal adhesion kinase) is restricted to the basal ES at the BTB where its activated and phosphorylated form, pFAK, is restricted to the apical ES,¹⁵ suggesting that protein kinases that are found at the apical and basal ES are likely play a crucial role to coordinate cross-talk between different cellular events that occur at the opposing ends of the Sertoli cell epithelium.

Apical ES: A Hybrid Cell-Matrix-Cell Junction Type

Besides cadherin-catenin, nectin-afadin protein complexes that are found at both basal and apical ES, apical ES also consists of integrin-laminin complex,^{13,15,16,57} which is usually restricted to the focal contact in cell-matrix interface in other epithelia.⁵⁸ Such hybrid cell-matrix-cell junction type is suggested to be involved in the rapid junction remodeling facilitating the orientation and movement of spermatids at spermiation.^{8,9} While most of the previous studies on ES were largely focus on its morphology, recent studies have shifted the focus to identify the putative components in the ES in order to explore the mechanisms that regulate ES restructuring during the epithelial cycle.^{8,9,13,15,16,57,59,60} These findings will be summarized and discussed herein; in particular, recent data regarding the involvement of

laminin-integrin complex and its downstream effectors in facilitating germ cell migration are discussed.

Integrin: The First FA Component Found at the Apical ES

Integrin, a heterodimeric transmembrane receptor composed of α and β subunits, is the first integral membrane protein positively identified at the ES.⁶¹ To date, there are 18 α subunits and 8 β subunits present in mammals. Among them, $\alpha 1$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 6$, $\alpha 9$, $\beta 1$, and $\beta 3$ integrins have been identified in testes.^{8,9,61-65} $\alpha 1$, $\alpha 3$, $\alpha 9$, $\beta 1$ subunits are detected in the basement membrane of the seminiferous epithelium, whereas $\alpha 1$, $\alpha 4$, $\alpha 5$, $\alpha 6$ and $\beta 1$ subunits are found at the apical ES. When $\beta 1$ integrin subunit was first detected at the ES in 1992,⁶¹ this study first demonstrated the presence of a ECM-associated protein at the nonbasement membrane site namely the ES, since studies from other epithelia have shown that integrins are largely restricted to focal contacts and hemidesmosomes at the sites of cell-matrix anchoring junctions.^{8,9,66} For instance, $\alpha 6\beta 1$ integrin is a known receptor for a wide variety of ECM including collagens, fibronectin and laminin in other epithelia.⁶⁷ However, there is no ECM protein present in nonbasement membrane at the time when integrin was first reported at ES. Until recently, more than 10 years after integrin being detected at ES, laminin $\alpha 3\beta 3\gamma 3$ ^{13,56} and other FAC component proteins^{15,16,56,60} were detected and structurally linked to integrin at the apical ES. Such findings, along with the presence of proteases activity at the apical ES site,¹³ illustrating that apical ES is utilizing the most efficient migration device usually restricted to cell-matrix anchoring junctions to facilitate germ cell movement across the epithelium during spermatogenesis.

Laminin 333 and $\alpha 6\beta 1$ Integrin form a Bona Fide Complex at the Apical ES

Laminins are heterotrimers composed of one each of the α , β and γ chains. To date, 5 α -subunits, 4 β -subunits, and 3 γ -subunits have been found in mammalian tissues, which can give rise to at least 16 different functional laminins.^{68,69} By binding to their transmembrane receptors, integrins, at the cell/matrix anchoring junctions, also known as focal contacts constituted by focal adhesion complexes (FAC), laminins and integrins provide not only adhesion between epithelial cells and basal lamina, they also mediate signaling through the downstream effectors, the FAC, leading to cell migration during normal and in pathological conditions, such as tumor invasion.⁷⁰⁻⁷² Laminin $\gamma 3$ was the first laminin subunit found at the apical ES by immunofluorescent microscopy and was shown to form a bona fide complex with $\beta 1$ integrin by coimmunoprecipitation studies.^{13,73} Subsequently, this [α 3] $\beta 3$ chain was found to form a functional laminin protein complex with the $\alpha 3$ and $\beta 3$ chains, known as laminin 333, which are restricted to the elongating/elongated spermatids and also interact with $\beta 1$ integrin at the apical ES.⁵⁷ Perhaps the most important of all, the pivotal role of laminin 333 at the apical ES was demonstrated by the perturbed adhesion between Sertoli and germ cells (mostly spermatids), leading to germ cell loss from the epithelium following treatment of adult rat testes with the laminin blocking antibodies, including anti-laminin $\alpha 3$ or $\gamma 3$ IgG.⁵⁷

Proteolysis of Laminin by MMP-2 and MT1-MMP Regulate Apical ES Dynamics

At apical ES, not unlike the cell-matrix interface, proteolysis may also present to regulate its restructuring. For instance, the remodeling of laminin can occur via the effects of proteases since MMP-2, MT1-MMP (a membrane anchored metalloprotease that can activate MMP-2) and TIMP-2 were found to colocalize with laminin $\gamma 3$ and $\beta 1$ integrin at the apical ES in adult rat testes.^{13,74} Furthermore, MMP-2 and MT1-MMP were shown to be activated when germ cells, especially spermatids, were detached from the epithelium *in vivo* after treating rats with Adjudin, formerly called AF-2364 [1-(2,4-dichlorobenzyl)-1H-indazole-3-carbohydrazide], which is a potential male contraceptive derived from indazole-3-carboxylic acid and has the capability to selectively disrupt adherens junction between Sertoli cells and germ cells.⁷⁵⁻⁷⁷ Perhaps the most important of all, the use of a specific MMP-2 and MMP-9 inhibitor, (2R)-2-[(4-biphenylsulfonyl)amino]-3-phenylpropionic acid, could effectively delay the loss of spermatids from the epithelium induced by Adjudin, indicating the potential role of proteolysis in apical ES disassembly.¹³ Such proteolysis of laminin by MMPs leading to the production of laminin fragments at apical ES may be essential for spermatid movement and spermiation since laminin-5 fragments has been shown to affect migration in both breast epithelial cells and prostate cancer cells (see Fig. 1).⁷⁸⁻⁸¹ This possibility should be vigorously tested in future studies.

FA Complexes at the Apical ES

The laminin integrin complex at the apical ES confers its cell-matrix FA property. Such property is further confirmed by the discovery of numerous FA components (see Table 1), including $\beta \alpha$ -integrin, vinculin, c-Src, Csk, ILK, phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂), phospholipase C (PLC)- γ , Fyn and Keap1 in the ES site.^{2,53,64,82-86} Recent findings in our laboratories further explore another vital FA component, phosphorylated focal adhesion kinase (pFAK), and its downstream effectors, the p85 subunit of phosphatidylinositol 3-kinase (PI3K), protein kinase B (PKB), p21 activated kinases (PAKs) and Crk-associated substrate (CAS), at the apical ES.^{15,16} FAK is a nonreceptor protein tyrosine kinase (PTK) that may be a crucial linker for $\beta 1$ integrin, recruiting ES components to apical ES.¹⁵ When FAK interacts with $\beta 1$ integrin, FAK undergoes autophosphorylation at Tyr-397, creating high-affinity-binding site for multiple molecules, including (i) SH2-domain-containing molecules, such as Src family protein kinases, (ii) effector proteins, such as PI3K and PLC- γ , and (iii) adaptor proteins, such as growth-factor-receptor-bound protein (Grb)7 and Nck-2.⁸⁷⁻⁸⁹ Furthermore, the newly recruited Src-family kinases at the apical ES can further enhance FAK catalytic activity by inducing phosphorylation of FAK at Tyr-576 and Tyr-577 in the kinase domain activation loop. Two other FAK-associated proteins, CAS and paxillin, can also be phosphorylated by Src-family kinases, leading to Rho family GTPase-mediated cell motility.⁸⁷⁻⁹⁵

Recent studies using both *in vitro* and *in vivo* models, including Adjudin and androgen suppression models, to study ES dynamics have illustrated the involvement of several signaling pathways which are initiated by $\beta 1$ integrin/pFAK during apical ES restructuring. These pathways include (i) the integrin/pFAK/c-Src/pERK,¹⁵ (ii) the integrin/pFAK/PI3K/

pPKB/PAK/ pERK¹⁶ and (iii) the integrin/pFAK/c-Src/Cas/Crk/Dock180 (Siu and Cheng, unpublished observations) (see Fig. 1). All these three pathways have the ability to modulate cell adhesion, migration, tissue remodeling and development, and tumor cell metastasis as shown in studies of other epithelia.⁸⁷⁻⁹⁵ These signaling pathways were shown to be triggered within a few hours after treating adult male rats with a single or multiple doses of Adjudin (40-50 mg/kg b.w.) either via i.p. or by gavage, which also matched quite nicely with the subsequent germ cell depletion events, especially spermatids at the apical ES, at 6-8 h after treatment.^{15,16} Perhaps the most important of all, pretreatment of rats with anti- α 1 integrin antibody,¹⁶ PP1 (a c-Src inhibitor),⁵⁹ wortmannin (a PI3K inhibitor),¹⁶ or U0126 (an ERK inhibitor)⁹⁶ via intratesticular injection were shown to delay the Adjudin-mediated spermatid loss from the epithelium, further confirming the involvement of these signaling pathways in the regulation of apical ES restructuring. Furthermore, the integrin/pFAK/c-Src/pERK pathway has recently been validated and expanded by another in vivo model, the androgen suppression model, in which rats were treated with androgen and estrogen implants to suppress the intratesticular androgen level, leading to the alteration of the Sertoli-germ cell apical ES function and the subsequent germ cell sloughing.⁶⁰ All of these findings thus illustrate that the cell-cell anchoring junction in the testis is indeed a hybrid cell-cell and cell-matrix junction type.

Furthermore, recent studies have also demonstrated the presence of TJ component proteins at the apical ES, which include the coxsackie and adenovirus receptor (CAR)⁹⁷⁻⁹⁹ and JAM-C (junctional adhesion molecule-C).¹⁰⁰ These results thus illustrate that the apical ES is also having the structural and perhaps the functional properties of the TJ. While the precise physiology underlying these observations is not entirely clear, it is increasingly clear that the apical ES is adopting some of the best features found in AJ, focal contacts and TJ to regulate the rapid events of germ cell migration and orientation essential to facilitate the rapid junction restructuring event pertinent to spermatogenesis.

Concluding Remarks and Future Perspectives

As briefly reviewed herein, there are mounting evidence illustrating the pivotal role of the basement membrane, a modified form of ECM, on the junction restructuring events that occur at the Sertoli-Sertoli and/or Sertoli-germ cell interface at the BTB and ES, many of which are mediated via the effects of cytokines (e.g., TNF α and TGF- β 3) on the steady-state levels of the integral membrane proteins at these sites. Interestingly, some of these effects are likely mediated via the homeostasis of the proteases and their endogenous inhibitors, which in turn, affects the structural and physico-chemical properties of the basement membrane and/or protein levels at the cell-cell interface. It is obvious that much new information will be added in the years to come and some of the postulates put forth here and depicted in Figure 1 schematically will be updated and/or rewritten. Perhaps it is also important that future studies should include a detailed analysis on the peritubular myoid cells and their role on the BTB function, spermatogonial stem cell renewal, and perhaps ES restructuring such as the use of Sertoli-myoid cell and Sertoli-germ-myoid cell cocultures. The recent deployment of molecular, biochemical and cellular techniques to study junction dynamics in the testis has yielded some unprecedented opportunities for investigators to

identify new leads to develop male contraceptives. They also offer exciting opportunities to understand the impact of environmental toxicants on male reproductive physiology.

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Figure 1.

A schematic drawing illustrating the latest model on the regulation of junction dynamics in adult rat testes, including the blood-testis barrier (BTB) and the ectoplasmic specialization (ES). For instance, junction restructuring events that occur at the blood-testis barrier (BTB) and the apical ectoplasmic specialization (apical ES) in the seminiferous epithelium during spermatogenesis apparently are regulated via intriguing interactions between cytokines (e.g., TNFα), proteases (MMP-2, MMP-9, MT1-MMP), protease inhibitors (TIMP-1, TIMP-2), collagens, laminins, adaptors, kinases and phosphatases. Legend continues on following page.

As described in the text, TNF α regulates the homeostasis of the proteases and protease inhibitors in the basement membrane, which in turn affects the collagen ultrastructural network, perhaps forming biologically active fragments that regulate BTB and/or ES dynamics. However, it remains to be shown if NC1 domain of collagen $\alpha 3(\text{IV})$ is indeed responsible for the transient “opening” of the BTB to accommodate preleptotene spermatocyte migration across the barrier that occurs at stage VIII of the seminiferous epithelial cycle, which should be vigorously examined in future studies. Recent studies have shown that similar mechanism(s) is also operating at the apical ES to regulate the transient opening and/or closing of the apical ES to facilitate spermatid movement during spermatogenesis and perhaps also the cellular events that occur at spermiation at late stage VIII of the epithelial cycle, which also involve the participation of proteases and protease inhibitors. This figure was prepared based on recent findings in the field as described in the text.

Table 1

ECM-related proteins that are found in the ectoplasmic specialization (ES) in adult rat/mouse testes; their interacting partners, functions, properties, and phenotypes after their knock-outs in mice *

Proteins	Mr (kDa)	Binding Partners	Functions/ Properties	Phenotypes in Knock-Out Mice
ECM proteins				
Laminin $\gamma 3$	146	$\beta 1$ Integrin, pFAK ³⁹⁷ , c-Src, MMP-2, MT1-MMP	Adhesion, signaling	n.k.
Laminin $\alpha 3$	165	Laminin $\beta 3$, Laminin $\gamma 3$	Adhesion, signaling	Neonatal lethality
Laminin $\beta 3$	140	Laminin $\alpha 3$, Laminin $\gamma 3$	Adhesion, signaling	n.k.
Transmembrane proteins				
$\beta 1$ Integrin	140	Laminin $\gamma 3$, $\alpha 6$ Integrin, pFAK ³⁹⁷ , c-Src, PI3K, p130 Cas, paxillin, vinculin, ILK, N-cadherin, β catenin, actin	Adhesion, signaling	Embryonic lethality on E5.5
$\alpha 6$ Integrin	118	$\beta 1$ Integrin, paxillin, actin	Adhesion, signaling	Neonatal lethality
Signaling proteins				
pFAK ³⁹⁷	125	$\beta 1$ Integrin, PI3K, c-Src, p130 Cas, paxillin, vinculin, gelsolin	PTK	Embryonic lethality on E8.5
c-Src	60	$\beta 1$ Integrin, pFAK ³⁹⁷ , PAKs 1/2, p130 Cas, Csk, ERK2, N-cadherin, Fer kinase, zyxin, axin, WASP, MTMR2, CAR, actin	PTK	Postnatal lethality
PI3K	80	$\beta 1$ Integrin, p-FAK ³⁹⁷ , p130 Cas, paxillin, vinculin, gelsolin, CAR	PTK	Perinatal lethality (in mice lacking all isoforms of PI3K p85 α)
PTEN	55	Actin, α tubulin, vimentin	Lipid phosphatase	Early embryonic lethality
PKB / pPKB	60	PAK1, actin, α tubulin	Ser/Thr protein kinase	Die shortly after birth
PAKs 1/2	62-65	c-Src (both PAK 1/2), PDK1 (PAK 1 only), PKB (PAK 1 only)	Ser/Thr protein kinase	n.k.
Csk	50	c-Src	PTK	Embryonic lethality on E10
Fyn	59	Actin	PTK	Viable, fertile
ERK1/2 / pERK1/2	44/42	c-Src, vinculin	MAP kinase	Viable, fertile (in ERK1 -/- mice) Early embryonic lethality after the implantation stage (in ERK2 -/- mice)
ILK	59	$\beta 1$ Integrin, vinculin, N-cadherin, β catenin	Ser/Thr protein kinase	Die at the peri-implantation stage
Adaptors				
p130 Cas	130	$\beta 1$ Integrin, pFAK ³⁹⁷ , c-Src, PI3K, Crk, Dock180, paxillin, vinculin, gelsolin	Adaptor	Died in utero
Paxillin	68	$\beta 1$ Integrin, $\alpha 6$ integrin, FAK, p130 Cas, vinculin, p120 ^{cas} , actin, tubulin	Adaptor, signaling	Viable, fertile
Vinculin	130	$\beta 1$ Integrin, pFAK ³⁹⁷ , p130 Cas, ERK2, paxillin, ILK, p120 ^{cas} , espin, CAR, actin	Adaptor	Embryonic lethality on E10

Proteins	Mr (kDa)	Binding Partners	Functions/ Properties	Phenotypes in Knock-Out Mice
Proteins pertinent to actin or microtubule remodeling				
PI(4,5)P ₂	0.971	actin	Gelsolin Inhibitor	n.k.
PLC-γ1	148	actin	Hydrolyzes PI(4,5)P ₂	Embryonic lethality on E9
Keap1	68	Myosin VIIa	Nrf2 regulator	Postnatal lethality
Proteins pertinent to matrix remodeling				
MMP-2	64-68	MT1-MMP, TIMP-2, laminin γ3, β1 integrin	Degrades ECM proteins	Viable, fertile
MT1-MMP	45,60-63	MMP-2, TIMP-2, laminin γ3, β1 integrin	Involves in MMP-2 activation, degrades ECM proteins	Early postnatal lethality
TIMP-2	22	MMP-2, MT1-MMP	Involves in MMP-2 activation, inhibits MMPs	Viable, fertile

Abbreviations used: n.k., not known; CAR, coxsackie- and adenovirus receptor; Crk, an oncogene identified in a chicken sarcoma called chicken tumor virus number 10, encoding an activator of PTK; Csk, carboxyl-terminal Src kinase, a PTK that phosphorylates a Tyr residue in src family kinases; Dock180, CED-5 (cell death abnormal-5)/180 kDa protein downstream of chicken tumor virus number 10 (Crk); ERK2, externally regulated kinase-2, a mitogen activated protein (MAP) kinase; FAK, focal adhesion kinase; Fer kinase; the Fujinami sarcoma/feline sarcoma (*fps/fes*) proto-oncogene encoding a 94 kDa nonreceptor PTK called *Fps/Fes* kinase; ILK, integrin-linked kinase; MMP-2, matrix metalloproteinase-2; MT1-MMP, membrane-type 1-matrix metalloproteinase; MTMR2, myotubularin related protein-2; PI(4,5)P₂, also called PtdIns(4,5)P₂, phosphatidylinositol 4,5-bisphosphate; PTK, protein tyrosine kinase; P13K, phosphoinositide (or phosphatidyl inositol) 3-kinase; p130 Cas, Crk-associated protein encoded by the *Crkas* gene; PKB, protein kinase B, also known as Akt, a Ser/Thr protein kinase, a product of the normal gene homolog of *v-akt*, the transforming oncogene of AKT8virus; PLC-γ1, phospholipase C-γ1; TIMP-2, tissue inhibitor of metalloproteinases-2; PAK, p21-activated kinase, a Ser/Thr protein kinase;; PDK1, 3-phosphoinositide-dependent protein kinase 1; PTEN, phosphatase and tensin homolog deleted on chromosome 10, a protein tyrosine phosphatase that shares homology with tensin, and a tumor-suppressor gene located on chromosome 10q23; p120^{ctn}, p120 catenin; c-Src, a nonreceptor PTK of the transforming gene of Rous sarcoma virus; WASP, Wiskott-Aldrich Syndrome protein.

* This table was prepared based on the following articles and/or reviews (1-3, 7-9, 13, 15, 16, 57, 58, 68, 69, 87). Due to the page limit, many original articles were not cited, however, these references can be found in the cited reviews and/or articles listed herein.