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SIGNALING PATHWAYS REGULATING THE BLOOD-TESTIS BARRIER

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Abstract

Throughout mammalian spermatogenesis, preleptotene/leptotene spermatocytes traverse the blood-testis barrier during stages VIII–XI of the seminiferous epithelial cycle while trapped within a dynamic intermediate compartment that is sealed at north and south poles by tight junctions, basal ectoplasmic specializations, desmosomes and gap junctions. In order for spermatocytes to gain entry into the adluminal compartment of the seminiferous epithelium for continued development, ‘old’ junctions present above migrating spermatocytes disassemble, while ‘new’ junctions assemble simultaneously below these germ cells. In this way, the integrity of the blood-testis barrier and the homeostasis of the seminiferous epithelium can remain intact during spermatogenesis. Previous studies have shown an array of cellular events, including protein internalization and cytoskeletal remodeling, to underline blood-testis barrier restructuring, whereas other studies have reported BTB dysfunction to associate with activation of the p38 mitogen-activated protein kinase pathway. Herein, we discuss the signaling pathways and mechanisms involved in blood-testis barrier restructuring in the mammalian testis.

Keywords

blood-testis barrier; Sertoli cell; testis; p38 mitogen-activated protein kinase pathway; testosterone

1. Introduction

Throughout spermatogenesis in the seminiferous epithelium of the mammalian testis, germ cells develop into spermatozoa through a series of cell divisions and morphological changes after which they are released from the epithelium (i.e., spermiation) (O’Donnell et al., 2011, de Kretser and Kerr, 1988, Kerr et al., 2006). During this time, developing germ cells are anchored to and supported by Sertoli cells, ‘nurse-like’ epithelial cells that give rise to the basic structure of the seminiferous epithelium. One crucial function of Sertoli cells is the formation of the blood-testis barrier (BTB), which separates the seminiferous epithelium into two distinct compartments, adluminal and basal. The BTB is constituted by different junction types, including tight junctions (TJs), basal ectoplasmic specializations (ESs; a testis-specific anchoring junction), desmosomes and gap junctions (GJs) (Mruk and Cheng, 2004, Cheng and Mruk, 2002). Unlike other mammalian blood-tissue barriers, the BTB is unique because junctions are not organized into discrete domains; instead, they are

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intermixed, co-existing and co-functioning. This unparalleled organization of junctions allows for strict coordination across the different structures and makes the BTB one of the tightest blood-tissue barriers. While the BTB is known to protect post-meiotic germ cells from the systemic circulation (i.e., immune system), it also restructures cyclically to allow preleptotene/leptotene spermatocytes entry into the adluminal compartment for further development. In rats, for instance, spermatocytes traverse the BTB during stages VII-XI of the seminiferous epithelial cycle, which is comprised of 14 stages in total (Russell et al., 1990, Hess and Renato de Franca, 2008, Clermont and Perey, 1957). Previous studies have demonstrated that 'old' junctions situated above migrating spermatocytes disassemble, while 'new' junctions assemble simultaneously below these germ cells (Figure 1). This creates a brief scenario where a migrating spermatocyte can be microscopically observed as trapped in between two barriers, thereby creating an intermediate compartment. This highly coordinated process is mediated by an elaborate signaling network that involves the participation of an array of molecules from the extracellular milieu. Among these, autocrine and paracrine factors (e.g., cytokines and testosterone) secreted locally by testicular cells under the regulation of the hypothalamus and the pituitary gland are the best studied regulators of BTB function (Xia et al., 2005, Li et al., 2009b). In other studies, we have shown that biologically-active laminin fragments generated during spermiation, which occurs at late stage VIII of the seminiferous epithelial cycle, also facilitate BTB restructuring via a local autocrine axis (Yan and Cheng, 2006, Yan et al., 2008b, Su et al., 2012) (Figure 1). Herein, we briefly discuss our current understanding of how extracellular factors activate common signaling pathways within Sertoli cells to ultimately bring about BTB restructuring, a prerequisite for germ cell movement.

2. Functions

Cytokines are secretory proteins capable of eliciting a signal transduction cascade upon binding to cell surface receptors, resulting in pleiotropic effects. Several cytokines are known to be produced by Sertoli and/or germ cells, including tumor necrosis factor (TNF)- α , members of the transforming growth factor (TGF) superfamily (e.g., TGF- β 2 and - β 3, activins and inhibins), interleukins (IL; e.g., IL-1 α and IL-6) and interferons (Li et al., 2009b, Xia et al., 2005). On the other hand, testosterone, a steroid synthesized by Leydig cells residing in the interstitium, is critical for spermatogenesis (Walker, 2011, Verhoeven et al., 2010). Because testosterone is produced locally, its level is approximately 100-fold higher in the testis than in the systemic circulation (O'Donnell et al., 2006). In general, BTB function is disrupted by cytokines, but enhanced by testosterone. It is also worth noting that many of the reported effects of cytokines and testosterone on BTB function are derived from Sertoli cells cultured at high density on MatrigelTM-coated substrata. This allows Sertoli cells to polarize and to assemble junctions that structurally and functionally mimic the BTB *in vivo* (Mruk and Cheng, 2004).

Transcriptional regulation

The binding of cytokines to their receptors results in the recruitment of adaptor proteins to the ligand/receptor complex, which in turn activates a cascade of signaling events that ultimately affect gene transcription and cell function. For instance, TGF- β s are best known to regulate transcription via SMAD-mediated pathways (Kang et al., 2009), whereas TNF- α and IL-1 α are both capable of activating nuclear factor- κ B and promoting the transcription of proinflammatory molecules (Rickert et al., 2011, O'Neill, 2008). Likewise, testosterone can elicit genomic effects via classical testosterone signaling upon binding to androgen receptors in Sertoli cells (Walker, 2010, Walker, 2011). However, these classical genomic pathways fail to account for some of the effects of cytokines and testosterone in Sertoli cells. For example, TNF- α is known to perturb Sertoli cell TJ integrity, resulting from a down-regulation in occludin mRNA and protein levels (Siu et al., 2003, Lydka et al., 2012) (Figure

1). Nevertheless, BTB function is controlled in part through transcriptional regulation, which is triggered by cytokines and testosterone to elicit changes in the steady-state levels of BTB constituent proteins (Lui and Cheng, 2007). In the following sections, we discuss recent studies that point to non-genomic effects which are equally important in the regulation of BTB dynamics during spermatogenesis.

Endocytosis, recycling and degradation

Biochemical studies have shown the kinetics of internalization of integral membrane proteins (e.g., occludin and N-cadherin) in Sertoli cells to be accelerated by both testosterone and cytokines, including TNF- α , TGF- β 2, - β 3 and IL-1 α (Xia et al., 2009, Yan et al., 2008a, Lie et al., 2011) (Figure 1). Under the influence of testosterone or cytokines, these proteins are internalized within early endosomes via a clathrin-mediated mechanism, resulting in junction restructuring (Xia et al., 2009). These findings are also supported by an increase in the association of occludin with caveolin-1 and Rab-11, regulators of endocytosis/transcytosis and recycling, respectively (Su et al., 2010), suggesting that a caveolin-mediated mechanism may be involved in BTB restructuring. Furthermore, testosterone facilitates recycling of integral membrane proteins back to the plasma membrane, thus supporting assembly of the 'new' BTB below migrating spermatocytes (Yan et al., 2008b) (Figure 1). In contrast, TGF- β 2 and - β 3 are known to perturb TJ integrity, which is the result of protein degradation as shown by an increase in occludin association with the late endosome marker Rab9 as well as by an induction in the level of the ubiquitin-conjugating enzyme E2 J1 (Su et al., 2010). Unlike TGF- β , IL-1 α decelerates the kinetics of occludin degradation (Lie et al., 2011), possibly promoting subsequent recycling when induced by other factors such as testosterone. In light of the opposed effects of testosterone and cytokines on BTB function, the events of protein endocytosis, recycling and degradation require strict coordination to facilitate junction restructuring during germ cell movement.

Cytoskeletal remodeling

At the BTB, TJs, basal ESs and GJs link to filamentous (F-) actin. The basal ES is most notably characterized by hexagonally-packed actin bundles sandwiched in between the plasma membrane and the endoplasmic reticulum (Mruk and Cheng, 2004, Vogl et al., 2008). While this unique arrangement of F-actin stabilizes ES-mediated adhesion, the cyclic restructuring of the BTB is thought to be facilitated by the remodeling of these actin bundles into a highly branched and less organized network (Figure 1). These changes in F-actin organization are mediated by key actin-regulatory proteins under the control of cytokines as shown by recent studies. For instance, IL-1 α induces the mislocalization of epidermal growth factor receptor pathway substrate 8 (Eps8; an actin-capping and actin bundling protein) away from the Sertoli cell surface, thereby destabilizing actin bundles, cell junctions and BTB integrity (Lie et al., 2011, Lie et al., 2009). Concomitantly, IL-1 α also increases the steady-state level of actin-related protein (Arp) 3 (Lie et al., 2011), a component of the Arp2/3 complex, which is an actin nucleation machinery capable of forming nascent branches on actin filaments (Goley and Welch, 2006). The effects of cytokines on F-actin dynamics are not restricted to IL-1 α ; the association between Arp3 and drebrin E, an actin-binding protein, is induced by TNF- α and TGF- β 3, suggesting that an increase in Arp2/3 recruitment by drebrin E may facilitate actin branching (Li et al., 2011). Furthermore, TGF- β 3 also increases the active GTP-bound form of Cdc42 (Wong et al., 2010), a Rho GTPase capable of activating Wiskott-Aldrich syndrome protein (WASP), which in turn is required for the activation of the Arp2/3 complex (Goley and Welch, 2006). Collectively, these cytokines elicit similar effects in favoring the formation of a branched actin network, which facilitates the internalization of integral membrane proteins and the restructuring of the BTB.

3. Cascades

Despite our growing knowledge on the multitude of effects elicited by different autocrine and paracrine factors, it is still poorly understood how signals are propagated and how they are coordinated within Sertoli cells to bring about BTB restructuring. One of the best-studied intracellular signaling pathways to associate with BTB disassembly is the p38 mitogen-activated protein kinase (MAPK) cascade (Cheng et al., 2011, Wong and Cheng, 2005) (Figure 2). On the other hand, signaling pathways that associate with BTB assembly are much more difficult to study because a multitude of cellular events are known to take place during spermatogenesis. At the very least, classical and non-classical testosterone signaling pathways are involved in BTB assembly and integrity (Walker, 2010, Walker, 2011, Verhoeven et al., 2010).

p38 MAPKs are a class of serine/threonine protein kinases that are activated in response to a wide range of extracellular stimuli, especially those generated under stress and inflammatory conditions (Kumar et al., 2003). Under the three-tiered kinase cascade followed by MAPK pathways in general, p38 MAPKs are phosphorylated and activated by several MAPK kinases (MKKs; e.g., MKK3, 4 and 6), which in turn are activated by MAPK kinase kinases (MEKKs; e.g., MEKK1-4 and TGF- β -activated kinase 1) (Cuadrado and Nebreda, 2010) (Figure 2). Upstream of this cascade, signals from the cell surface are relayed by activators such as small G proteins, including Rac and Cdc42 (Keshet and Seger, 2010). In the testis, the p38 MAPK pathway is clearly implicated in BTB disassembly. The loss of barrier function induced by TGF- β 3 and TNF- α , as well as by the environmental toxicant cadmium, associates with an increase in p38 phosphorylation (Lui et al., 2003a, Li et al., 2006, Lui et al., 2003b). Bisphenol A, another environmental toxicant, also triggers the phosphorylation of extracellular signal-regulated kinases (ERK) 1/2 in Sertoli cells (Li et al., 2009c, Li et al., 2009a). Equally important, TGF- β 3-induced TJ disruption is blocked by a specific p38 inhibitor SB202190 (Lui et al., 2003a). Within this signaling pathway, MEKK2 likely functions as a p38 activator given its increase in mRNA level after TGF- β 3 administration (Lui et al., 2003a). Further upstream, activation of the p38 MAPK pathway in Sertoli cells is Cdc42-dependent since p38 phosphorylation is induced by overexpression of Cdc42, but not overexpression of its dominant negative mutant T17N (Wong et al., 2010). Collectively, these results illustrate that BTB disassembly induced by TGF- β 3 and TNF- α is mediated via the activation of the p38 MAPK pathway. While small GTPases that activate the p38 pathway are generally not considered to be part of this cascade, they are likely to promote BTB disruption through additional effectors. For instance, both Cdc42 and Rac are activators of nucleation promoting factors, namely WASP and WASP family verprolin-homologous protein (WAVE), respectively, which in turn promote Arp2/3-mediated actin nucleation during junction restructuring (Goley and Welch, 2006).

4. Key molecules

Cytokines, testosterone and receptors

Cytokines such as TNF- α , TGF- β s and IL-1 α are synthesized as precursor proteins of ~30 kDa in Sertoli and/or germ cells, which are subsequently cleaved into mature forms of ~17 kDa. Despite numerous studies illustrating their effects on BTB dynamics, these cytokines are largely dispensable for spermatogenesis because knockout models are either perinatally lethal or viable and fertile (Xia et al., 2005). On the other hand, testosterone is indispensable for male fertility; in the absence of testosterone or functional androgen receptors, spermatogenesis cannot go to completion as a result of (i) compromised BTB integrity, (ii) inability of germ cells to develop beyond meiosis, and (iii) spermiation failure (Verhoeven et al., 2010, Walker, 2010, Walker, 2011). Since germ cells do not express androgen receptors, Sertoli cells are the main transducers of testosterone signals within the

seminiferous epithelium that are essential for both germ cell development and BTB integrity. This is illustrated by the up-regulation in Sertoli cell androgen receptor expression during stages VI-VIII of the seminiferous epithelial cycle, which coincides with BTB restructuring and spermiation. As discussed previously, testosterone signaling is known to be mediated via both classical and non-classical pathways (Walker, 2010, Walker, 2011). In the classical pathway, testosterone binds to androgen receptors present within the Sertoli cell cytoplasm. Upon ligand binding, heat shock proteins release androgen receptors so that they may enter the nucleus to initiate transcriptional regulation. More recent studies in Sertoli cells have described the existence of at least two non-classical pathways, which involve the influx of Ca^{2+} ions and the stimulation of epidermal growth factor receptor via Src activation (Walker, 2010). As described previously, testosterone-induced endocytosis and recycling of BTB proteins is likely mediated via a non-classical pathway as these effects are rapidly manifested (Yan et al., 2008b), but additional studies are needed to better understand the mechanisms at play.

Integrins, laminins and focal adhesion proteins

Integrins are cell surface receptors comprised of transmembrane heterodimers that mediate cell-cell and cell-matrix adhesion by binding ligands such as laminins. Besides having roles in adhesion, integrins are also important signal transducers between cells and the external environment via outside-in, as well as inside-out signaling (Margadant et al., 2011). In the seminiferous epithelium of the mammalian testis, the integrin/laminin complex localizes to two adhesion sites, namely the (i) apical ES at the Sertoli cell-elongating/elongated spermatid interface and the (ii) hemidesmosome at the Sertoli cell-basement membrane interface (Yan and Cheng, 2006, Yan et al., 2008b) (Figure 1). Recent studies show that integrins and laminins mediate crosstalk among the apical ES, hemidesmosome and BTB, forming a local autocrine axis to facilitate barrier restructuring. Prior to spermiation at late stage VIII, laminin at the apical ES is proteolytically cleaved to generate biologically-active fragments, which are capable of disrupting BTB integrity by decreasing the steady-state level of occludin (Yan et al., 2008a, Su et al., 2012). Furthermore, these laminin fragments also down-regulate the level of $\beta 1$ -integrin at the hemidesmosome, which also indirectly affects BTB integrity (Yan et al., 2008a) (Figure 1). These data support the contention that BTB restructuring and spermiation, which occur at opposite ends of the seminiferous epithelium, are strictly coordinated. At this point, the precise biological actions of laminin fragments are not completely understood, and additional studies are needed.

5. Associated pathologies and concluding remarks

Herein, we have summarized the current state of knowledge as it relates to the regulation of the blood-testis barrier in mammals. Since BTB integrity is critical for spermatogenesis and fertility, additional studies are needed to better understand how this unique ultrastructure is regulated. This is important because this information may help to identify new targets for non-hormonal contraception in the future. BTB dysfunction may also associate with unexplained cases of infertility, and additional studies along these lines are needed as well.

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Signaling network facts

- Blood-testis barrier (BTB) function is regulated by the combined effects of autocrine and paracrine factors such as cytokines, steroids and biologically-active laminin fragments.
- BTB restructuring, which is essential for germ cell movement, is the net result of multiple events including, but not limited to, transcriptional regulation, protein trafficking and cytoskeletal remodeling.
- The p38 mitogen-activated protein kinase pathway associates with BTB dysfunction.

Figure 1.

BTB function in the seminiferous epithelium of the mammalian testis is regulated by several factors and signaling cascades. This illustration depicts two polarized Sertoli cells sitting atop the basement membrane. Sandwiched in between these Sertoli cells is a migrating preleptotene/leptotene spermatocyte trapped within a dynamic intermediate compartment that is sealed at north and south poles by TJs, basal ESs, desmosomes and GJs. As this spermatocyte moves upwards, 'old' junctions situated above this germ cell disassemble, while 'new' junctions assemble simultaneously below it. This cellular event is facilitated by transcriptional regulation, protein trafficking (e.g., endocytosis, recycling and degradation) and cytoskeletal remodeling (e.g., actin branching), which collectively result in junction disassembly. For instance, junction disassembly is facilitated by internalization of integral

membrane proteins, which is triggered by the binding of cytokines (e.g., TGF- β s, TNF- α and IL-1 α) to their receptors as well as by the binding of testosterone to its receptor. It is also worth noting that testosterone facilitates recycling of integral membrane proteins back to the plasma membrane, thus supporting assembly of the 'new' BTB below migrating spermatocytes. Recent studies also show that integrins and laminins mediate crosstalk among the apical ES, hemidesmosome and BTB, forming a local autocrine axis to facilitate barrier restructuring. Specifically, biologically-active laminin fragments generated during the release of elongated spermatids from the seminiferous epithelium are capable of disrupting BTB integrity by decreasing the steady-state level of occludin. These laminin fragments also down-regulate the level of β 1-integrin at the hemidesmosome, which also indirectly affects BTB integrity.

Figure 2.

BTB restructuring is mediated in part by serine/threonine kinases belonging to the MAPK pathway, which transduces signals received from external stimuli (e.g., cytokines and environmental toxicants). In general, the MAPK pathway branches into four cascades, leading to the activation of p38, ERK1/2, c-Jun N-terminal kinase (JNK) or ERK5. Here, MAPKs are phosphorylated and activated by MAPKKs, which in turn are phosphorylated and activated by MAPKKs.