Published in final edited form as:

Cytokine Growth Factor Rev. 2007 ; 18(3-4): 299–311. doi:10.1016/j.cytogfr.2007.04.009.

Regulation of cell junction dynamics by cytokines in the testis – a molecular and biochemical perspective*

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Abstract

Studies in the past decade in the field have demonstrated the significance of cytokines in regulating epithelial and endothelial cell junctions including tight and anchoring junctions in multiple organs including the testis. There are mounting evidences in recent years that cytokines play a crucial role in the restructuring of junctions at the Sertoli-Sertoli and Sertoli-germ cell interface in the seminiferous epithelium during spermatogenesis. These earlier studies, however, were focused on the effects of cytokines in maintaining the steady-state protein levels of integral membrane proteins at the sites of the blood-testis barrier (BTB), and anchoring junctions at the Sertoli-Sertoli and Sertoli-germ cell interface, such as basal and apical ectoplasmic specialization, respectively. The molecular pathway(s) and/or mechanism(s) underlying these effects remain virtually unexplored until very recently. Herein, we summarize and provide some discussions on studies that focused on the role of cytokines in regulating junction restructuring events in epithelia from a molecular and biochemical perspective. Specifically we use the adult rat or mouse testis as a model to highlight the significance of transcriptional and translational regulation. Specific areas of research that require further attentions are also highlighted.

Keywords

Testis; cytokines; blood-testis barrier; Sertoli cells; germ cells; tight junctions; anchoring junctions; adherens junctions; ectoplasmic specialization; tubulobulbar complex; spermatogenesis

Introduction

Spermatogenesis is divided into three distinctive phases during which diploid spermatogonia (2n) differentiate into haploid spermatids (1n) [1]. The primordial germline spermatogonia undergo self-proliferation via mitosis (phase 1) some of which differentiate into primary spermatocytes. These germ cells, in turn, enter into meiosis and become secondary spermatocytes which further differentiate into haploid round spermatids (phase 2). During

*Studies in the our laboratories were supported in part by grants from the National Institutes of Health (U01 HD045908, U54 HD029990 Project 3, to CYC), the CONRAD Program (CICCR CIG 01-72, to CYC), and Hong Kong Research Grant Council (HKU7609/06M to WYL)

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spermiogenesis (phase 3), round spermatids undergo drastic morphological changes (e.g., condensation of chromatin materials and formation of acrosome at the head, elongating of spermatid tail) to become functional and fully developed spermatids. Spermatozoa are then released into the seminiferous tubule lumen at spermiation. In mammalian testes, such as rodents and humans, these changes take place in the seminiferous epithelium and can be categorized into different stages [2,3]. For instance, in the rat, a seminiferous epithelial cycle is divided into 14 stages which are based on the unique cellular association of developing germ cells with Sertoli cells in the epithelium [2,3].

The development of germ cells in the seminiferous tubules requires both structural support and nourishment (e.g., foods, minerals, hormones, cytokines and paracrine factors) supports from the Sertoli cell which, in turn, constitute the seminiferous epithelium (Fig. 1). Sertoli cells extend from the basement membrane (a modified form of extracellular matrix) [4] to the tubule lumen with each Sertoli cell supporting 30–40 developing germ cells at different stages of development [5] (Figs. 1 & 2). The blood-testis barrier (BTB) situated between adjacent Sertoli cells physically divides the seminiferous epithelium into the basal and adluminal (apical) compartment (Figs. 1 & 2). The BTB regulates proteins and/or biomolecules that can enter the adluminal compartment and maintain cell polarity, it also segregates the post-mitotic germ cell development from the systemic circulation to avoid autoimmune response developed against germ cell-specific antigens many of which appear transiently during spermatogenesis [6]. For instance, preleptotene spermatocytes that are differentiated from spermatogonia lie at the periphery of the seminiferous tubules and outside the BTB must traverse this unique barrier at late stage VIII of the epithelial cycle [7], so that further development of post-meiotic germ cells (pachytene spermatocytes and onwards) takes place in the adluminal compartment.

It is conceivable that there are extensive interactions between Sertoli cells and germ cells regardless the localization of germ cells in the epithelium during spermatogenesis (Figs. 1 & 2), and accurate signals directed to Sertoli and germ cells must take place to facilitate the events of cell cycle, mitosis, meiosis, morphogenesis pertinent to germ cell development. There are at least two sources of signals including the external signals such as hormonal factors from the hypothalamic-pituitary axis, and internal signals such as cross-talk between Sertoli and germ cells via paracrine and autocrine regulation. In this review, we first give an update on the junction complexes that are found in the testis. We next summarize and discuss the latest development in the field regarding how cytokines affect junction dynamics via the precise controls on transcriptional, post-transcriptional and post-translation modifications. Recent data regarding the cytokine-mediated (e.g., TGF- β 3 and TNF- α) differential regulations of junction dynamics via distinct signaling pathways is also discussed. This succinct review attempts to serve as a guide for designing functional experiments to probe some unexplored areas of research to decipher the role of cytokines in junction dynamics pertinent to spermatogenesis.

Biology and regulation of cell junctions in the seminiferous tubules

Different types of junctions ranging from tight junctions (TJ) to gap junctions (GJ) that are found in the seminiferous epithelium have recently been reviewed [8,9] (see Figs. 1 & 2 and Table 1). Apart from the TJs that are localized and restricted to the BTB, anchoring junctions are also found Sertoli-Sertoli, Sertoli-germ, and Sertoli-basement membrane interface [4] in the seminiferous epithelium. These include: (1) adherens junctions (AJ) including basal and apical ectoplasmic specializations (ES), basal and apical tubulobulbar complex (TBC) (2) desmosome-like junctions and (3) hemidesmosomes. ES is an actin-based testis-specific hybrid AJ type [10,11] which is confined to the region between the plasma membrane of two adjacent Sertoli cells and the cisternae of the endoplasmic reticulum with hexagonally packed actin filaments. Basal ES is limited to the BTB, coexisting with TJ, whereas apical ES is found between Sertoli cells and elongating/elongate spermatids (step 8 and beyond in adult rat testes)

(Fig. 1). They are structurally similar except that the ultrastructural feature pertinent to ES at the apical ES is restricted to the Sertoli cell side and without the TJ (Figs. 1 & 2). TBC is another modified testis-specific AJ. In late stage VIII of the seminiferous epithelial cycle, apical ES begins to disappear which coincides with the appearance of apical TBC that is restricted to the concave side of the elongating spermatid head and the Sertoli cell, and the apical TBC is detectable only for a few days before spermiation. For basal TBC, it is present alongside with TJ, the basal ES, gap junctions, and desmosome-like junctions, which collectively constitute the BTB. Desmosome-like junctions are also found between Sertoli cells and spermatogonia, spermatocytes and round spermatids via intermediate filaments, such as vimentin, as the attachment site. Gap junctions (GJ) found between adjacent Sertoli cells and between Sertoli and germ cells for cell-cell communication.

Unlike other epithelia or endothelia in which AJ forms a continuous belt underneath the TJ, AJ coexisting with TJ at the BTB in seminiferous epithelium (see Fig. 1). Besides the unusual structural features at the BTB, the BTB is different from other blood-tissue barriers in which dynamic restructuring are actively occurring during spermatogenesis to facilitate the entry of preleptotene spermatocytes to the adluminal from the basal compartment for further development. Extensive restructuring of AJs and GJs are also important for germ cell migration across the seminiferous epithelium during spermatogenesis. Furthermore, unlike other blood-tissue barriers, such as the blood-brain and the blood-retina barriers, which are formed by endothelial tight junctions of the specialized microvessels in the brain and the retina, respectively [for reviews, see 6,12], the BTB is created and maintained by adjacent epithelial Sertoli cells in the seminiferous epithelium, located physically away from microvessels which are found in the interstitium (Fig. 1). In short, the endothelial TJ-barriers of the microvessels, unlike other blood-tissue barrier, do not contribute to the BTB integrity in testes.

During the past two decades, an array of constituent proteins of different junction types has been identified (Table 1) and detailed studies have been performed to pin-point the functions of many of these protein components such as knockout and functional studies (Table 2). Due to the page constraints, those findings are summarized in these two Tables and we highly recommend readers seek additional information from recent review on these subject areas.

Regulation of junction dynamics in the testis

It is increasingly clear that multiple signaling pathways are involved in junction restructuring events pertinent to spermatogenesis, such as activation of different protein kinases [for a review, see 13]. Several studies have also implicated the involvement of small GTPases (e.g., Rab and Rho), proteases and protease inhibitors in junction remodeling [for a review, see 13]. However, these are intermediate molecules that serve as the linkers to transduce an upstream signal, leading to significant changes in the status and/or homeostasis of the junction via changes in the steady-state protein levels of integral membrane proteins. There is mounting evidence that cytokines function as the key regulatory molecules to control or initiate signal transduction activation. For instance, binding of cytokines to their receptors triggers a cascade of signaling events. Activation of a specific signaling pathway results in an increase in the expression of junction proteins and their peripheral regulators, or altering the functional properties of junction proteins, which are required for junction remodeling. In this regard, we highlight how cytokines can affect junction dynamics via different regulatory levels including transcriptional, post-transcriptional and post-translational modifications and how these cytokine-mediated signals are fine-tuned so that a specific cytokine can regulate specific integral membrane proteins at a particular site in the seminiferous epithelium, such as the BTB.

Regulation of junction dynamics by cytokine-mediated transcriptional regulation

A spectrum of cytokines, such as tumor necrosis factor α (TNF- α) and transforming growth factor β 3 (TGF- β 3), have been implicated in regulating junction dynamics in the testis via transcriptional regulation (Table 3). Several studies have shown that TNF- α and TGF- β 3 down-regulate the expression of a TJ transmembrane protein - occludin, and impairs TJ permeability barrier in cultured Sertoli cells [27–29]. A number of potential *cis*-acting motifs pertinent to TNF- α -mediated gene transcription, such as nuclear factor- κ B (NF- κ B) and NF-IL6, have been identified within the promoter sequence of occludin [30]. It remains unknown if TNF- α inhibits the transcription of occludin gene via this promoter region in the testes, however, TNF- α was shown to impair TJ barrier function through the suppression of occludin promoter activity in HT-29/B6 cells [30]. In astrocytes, TNF- α was shown to suppress the occludin transcription through TNF- α type I receptor and NF- κ B. NF- κ B might either function as a negative regulator that directly interacted with the occludin promoter or exerted an indirect effect by activating a repressor that acted on the occludin promoter [31]. Thus in studies using astrocytes, the molecular mechanism by which TNF- α regulates junction dynamics has been partially unfolded, which maybe applicable to the testis.

The molecular mechanism by which TGF- β 3 down-regulates the expression of occludin in the testis has not been studied. Studies of epithelial-mesenchymal transition have shown that two Snail superfamily of zinc-finger transcription factors, Snail and Slug, are found to repress the occludin transcription in multiple epithelial cells including human breast cancer cells MCF7 and mammary epithelial cells Eph4 [32,33]. The proximal Snail promoter could be activated by TGF- β through several signaling pathways including MAPK, PI3 kinase/Akt and Smad proteins [34–36]. These studies thus illustrate that cytokine-mediated alteration of the levels of transcription factors can be an indirect but major mechanism to modulate the availability of junction proteins. Knockout studies have demonstrated that Slug plays a role in spermatogenesis as testicular atrophy have been detected in adult Slug^{-/-} male mice [37]. It is worthy to elucidate whether the transcriptional repression machinery of occludin in testes is also mediated via the regulation of Snail family of repressors.

Claudin-11 is a highly expressed TJ-integral membrane protein in the testis, it is also a major building block that constitutes TJ strands between Sertoli cells at the BTB [16]. TGF- β 3 and TNF- α both exert negative effects on claudin-11 expression in the testis [27,28,38]. Our recent studies of claudin-11 transcription have shown that Smad proteins, downstream mediators of TGF- β signaling pathway, could suppress GATA-1 and CREB transactivation of claudin-11 gene via an overlapping GATA/NF-Y motif within the core promoter region [39]. The activation of Smad proteins assists the recruitment of histone deacetylase and its co-repressor mSin3A to the promoter region and facilitates the down-regulation of claudin-11 gene transcription [39].

Besides TJ proteins, gap junction proteins in the testis have recently been shown to be regulated by cytokines via transcription control. Connexin33 (Cx33) and Cx43 display stage-specific expression in the seminiferous epithelium coinciding with the event of residual body phagocytosis by Sertoli cells [40]. During phagocytosis of residual bodies, Cx43 is weakly expressed within the seminiferous epithelium, in contrast, Cx33 displays an enhanced expression. Such differential expression pattern is under the precise control of IL-1 α and ERK activation since the exposure of Sertoli cells to IL-1 receptor antagonist was shown to reverse this expression pattern [40].

For adherens junctions (AJ), no study has been performed thus far to investigate how cytokines regulate the transcription of AJ proteins in the testis except our on-going study of JAM-B

transcription regulation in Sertoli cells, which was shown to be mediated by the combined action of TNF α and IFN- γ . It was shown that exposure of Sertoli cells to TNF- α and IFN- γ reduced the steady-state JAM-B protein level as a result of down-regulation of JAM-B transcription. The promoter region responsible for the TNF- α /IFN- γ -mediated down-regulation consists of two IFN- γ -activated site (GAS) elements. Work is now in progress to investigate whether these two GAS motifs are involved in TNF- α /IFN- γ -mediated regulation (Leung & Lui, unpublished observations).

Apart from cytokine-mediated direct regulation of gene transcription of junction proteins, cytokines could alter the transcription of proteins that contributed to junction dynamics. For instance, studies have shown that TNF- α , other than exerting its effect to down-regulate occludin transcription, could promote the transcription of matrix metalloproteinase-9 (MMP-9) [29]. An increase in MMP-9 facilitates the cleavage of the collagen network in the basement membrane, thereby perturbing the TJ-barrier in Sertoli cells [29]. The mechanism by which TNF- α up-regulates MMP-9 transcription in Sertoli cells remains to be elucidated, however, similar up-regulation of MMP-9 transcription via TNF- α -mediated PKC- ζ -NF κ B signaling pathway has been reported in C6 glioma cells [41,42]. Besides, TNF- α was shown to enhance the intestinal Caco-2 epithelial TJ-barrier permeability by facilitating NF- κ B p50/p65 binding and activation of the myosin light chain kinase (MLCK) promoter. NF- κ B p50/p65 activation of the MLCK promoter results in enhanced MLCK transcription, expression and activity, leading to MLCK-mediated opening of the intestinal TJ-barrier [43].

In short, much work is needed in this area to clearly define the role of transcriptional regulation of junction dynamics in the seminiferous epithelium via cytokines. It is also not known if different cytokines (e.g., TGF- β 3 and TNF- α) exert their effects on junction dynamics via a few physiologically related transcription factors to regulate the homeostasis and steady-state levels of integral membrane proteins and their peripheral adaptors, kinases and phosphatases at the BTB and cell adhesion at the Sertoli-Sertoli and Sertoli-germ cell interface. Nonetheless, current research in this area strongly suggests that local production of cytokines by Sertoli and germ cells into the microenvironment of the BTB, such as at stage VIII of the seminiferous epithelial cycle, can exert their effects on adjacent Sertoli cells at the BTB since receptors for TGF- β 3 and TNF α are restricted to Sertoli cells rather than germ cells [for reviews, see 13, 44], inducing transient “opening” of the BTB to accommodate preleptotene spermatocyte migration across the BTB as illustrated in a recent functional study [45]. For instance, it was shown that TNF α administered locally to the testes at a dose range comparable to its endogenous level could effectively and transiently “open” the BTB, permitting diffusion of a fluorescent dye (e.g., FITC) into the seminiferous epithelium beyond the BTB [45].

Regulation of cell junction dynamics via cytokine-mediated post-transcriptional modification

Post-transcriptional events, operating at the level of transcript stability, play a significant role in regulating the localization and abundance of proteins [for reviews, see 54,55]. It is increasingly clear that mRNA degradation is dependent on both *cis*-elements in the RNA and *trans*-acting factors in the nucleus and cytoplasm [for review, see 56].

Many mRNA species contain highly conserved AU-rich elements (AREs) within the 3'-untranslated region (UTR). The ARE appears to be an important determinant in regulating mRNA stability [57]. Mutagenic analyses of the ARE sequences have demonstrated that the functional ARE motif could be UUAUUA(U/A)(U/A) or UUAUUUAUU [58,59] in which binding of specific proteins to these sites could alter mRNA stability, and the binding of *trans*-acting factors into ARE at the 3'-UTR also alters mRNA stability. In the testis,

tristetraprolin (TTP), AUF1 and HuR are three RNA-binding proteins that have recently been identified [60].

The steady-state levels of junction-associated proteins have recently been shown to be modulated by direct alteration of their mRNA levels. For example, the AREs in β -catenin mRNA transcripts are implicated in the regulation of β -catenin mRNA stability in HeLa cells. Deletion of the AREs from 3'-UTR destabilizes the β -catenin mRNA, suggesting that AREs contribute to stabilization of the β -catenin mRNA transcripts [61]. It was also found that HuR, one of well-known RNA-binding proteins, could contribute to the stabilization of β -catenin transcripts in a colon cell line [62]. And knockdown of HuR led to a reduction in both β -catenin mRNA and protein levels [62]. These studies have clearly illustrated that the AREs at the 3'-UTR of the transcript and RNA-binding proteins are equally important in regulating the cellular junction protein levels.

It is well-documented that cytokines regulate the expression of junction proteins via changes in their mRNA levels, resulting in alteration of cell adhesion [47,53] (Table 3). For instance, granulocyte macrophage-colony stimulating factor (GM-CSF) is capable of altering β_3 -integrin mRNA and integrin $\alpha_v\beta_3$ in immature osteoclast precursors [53]. mRNA stability studies have demonstrated that the increase in β_3 -integrin mRNA upon GM-CSF treatment is the result of an increase in the stability of β_3 -integrin mRNA. GM-CSF can increase β_3 -integrin mRNA's half-life from 6.5 to 38 h [53]. IFN- γ is known to increase the expression of intercellular adhesion molecule-1 (ICAM-1) in many cell types including primary human fibroblasts and chondrosarcoma [48]. The increase in ICAM-1 gene expression in murine monocytic cell line, P388D1, is mediated, at least in part, by stabilizing the ICAM-1 mRNA [47]. Unlike other cytokines, IFN- γ enhances the level of ICAM-1 mRNA by stabilizing itself even in the absence of the conserved AUUUA pentanucleotide sequences in the 3'-UTR [47].

Studies from our laboratory have unraveled the crucial role of TNF- α in the regulation of junction dynamics in the testis. TNF- α reciprocally regulates the expression of several junction proteins via different distinct mechanisms. In a recent study using TM4, a mouse Sertoli cell line, we have shown that TNF- α down-regulates a newly-identified TJ integral membrane protein, coxsackie-and adenovirus receptor-like membrane protein (CLMP), at both mRNA and protein levels. The 3'-UTR of CLMP transcript containing ARE was found to respond to TNF- α treatment, which destabilized in CLMP mRNA transcripts via JNK1 activation [Sze and Lui, unpublished observations].

Based on these limited studies, it is apparent that junction dynamics in the testis are regulated, at least in part, by cytokine-mediated post-transcriptional modifications of junction proteins. However, this area of research deserves much attention in future studies since post-transcriptional regulation of gene expression can ensure rapid disappearance of mRNA of a target protein, such as a TJ protein, in an epithelium or endothelium once the transcription is turned off [56], which can be an effective approach to achieve stage-specific expression of junction proteins during the seminiferous epithelial cycle.

Cytokines regulate junction dynamics via post-translational modification

Integral membrane protein endocytosis and recycling

It is no doubt that cytokine-mediated transcriptional and post-transcriptional regulations of the junction proteins are two major mechanisms to control the expression of junction proteins [63–66]. However, for the junction proteins that are already present at the cell-cell interface, how can cells respond to the rapid junction remodeling during epithelial morphogenesis in which some cellular events last for <1 hour [67] and perhaps minutes when the half-lives of many junction proteins are much longer, perhaps up to several hours, such as 12 h for occludin

[68]? It is apparent that rapid junction restructuring could not be solely achieved by transcriptional and post-transcriptional controls, the alteration of protein stability and/or its bioavailability at the cell-cell interface are equally important in modulating junction dynamics in response to morphogenesis or changes in the physiological microenvironment. Current research has shown that endocytosis and ubiquitination are two efficient mechanisms that can effectively and rapidly remove a junction protein from the cell-cell interface, resulting in rapid junction restructuring, such as at different stages of the seminiferous epithelial cycle, when junctions are required to disassemble and reassemble to facilitate germ cell translocation and morphogenesis [69–75] (Fig. 3). Much of the current research have been focused on how cytokines induce or trigger these protein trafficking processes in kidney and intestinal epithelial cells, leading to changes in the homeostasis of integral membrane proteins at the cell-cell interface. Yet, these two important areas of research are presently missing in the field of testicular physiology. The information summarized below based on recent studies in other epithelia, which may serve as a blueprint for investigators in the field to explore the regulation of junction restructuring in the testis.

Endocytosis and recycling of integral membrane proteins at cell junctions are the effective mechanism used by epithelial cells that undergo rapid changes in morphology upon extracellular stimuli [70,71,74,75]. Integral membrane proteins can be internalized via different endocytic structures including caveolin-coated vesicle, clathrin-coated vesicle and actin-coated vacuole [76] (Fig. 3). TNF- α and IFN- γ are two known cytokines that regulate the barrier function in different epithelia via different mechanisms. These two cytokines not only exert their effects on junction proteins at transcriptional and post-transcriptional levels [30,47,77], they also rapidly alter the distribution of junction proteins in response to changes in physiological environment via endocytosis [78–80]. Exposure of the human intestinal epithelial cell line, T84, to IFN- γ was shown to induce the internalization of occludin, junctional adhesion molecule-A (JAM-A), claudin-1, and claudin-4, leading to TJ disassembly without obvious changes in the overall cellular contents of these proteins [79]. Subsequent studies have shown that those internalized TJ-integral membrane proteins are temporarily stored in a recycling endosomal compartment from where they can rapidly be recycled back to the plasma membrane upon removal of IFN- γ [81]. More recent studies have shown that IFN- γ mediates macropinocytic process through reorganization of the cortical actin cytoskeleton [82]. The reorganization of actin cytoskeleton is achieved by IFN- γ -induced expression of Rho-associated kinase (ROCK), which then activates RhoA, leading to increased phosphorylation of myosin II regulatory light chain (MLC) and activation of myosin II [82]. Not unlike IFN- γ , TNF- α was shown to increase Caco-2 TJ-barrier permeability by down-regulating the redistribution of ZO-1 protein [83].

AJ dynamics can also be regulated via integral membrane protein endocytosis. Using cell surface biotinylation and recycling assays, E-cadherin was shown to be actively internalized from cell surface in Ca²⁺-depleted environment through the clathrin-coated endocytic pathway, which can be rapidly recycled back to the plasma membrane in Ca²⁺-repleted medium [71]. If such endocytic activities are present in the seminiferous epithelium, this may present new targets for intervention in cytokine-mediated junction remodeling, such as development novel contraceptives for men. Indeed, a recent study has demonstrated C-type natriuretic peptide (CNP), its over-expression in Sertoli cells can disrupt the TJ-barrier, is capable of accelerating the endocytosis of TJ-integral membrane protein (e.g., occludin) in Sertoli cells cultured *in vitro* with functional TJ and BTB [94].

Protein ubiquitination

Ubiquitination is another cellular trafficking process that can efficiently regulate the steady-state levels of integral membrane proteins in testes, such as at the BTB. This cellular event

requires the actions of several enzymes including ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin ligase (E3). Ubiquitin is first activated by E1 and forms an E1-ubiquitin intermediate coupled with a high-energy thioester bond. Activated E1 then transfers a thiol group onto E2. E2 can then transfer activated ubiquitin to a target protein recognized by E3. A target protein tagged with polyubiquitin chain is recognized and degraded by the 26S proteasome with the release of ubiquitin and short peptides [85–88]. This degradation process can be reversed and prevented with the help of a deubiquitinating enzyme. It is apparent that the bioavailability of a protein, such as integral membrane proteins at cell junctions, is tightly regulated by the action of ubiquitinating and deubiquitinating enzymes [89]. Several ubiquitinating and deubiquitinating enzymes specifically targeted to junction proteins have been identified. Hakai and Itch are two E3 ligases that specifically recognize E-cadherin and occludin and facilitate their degradation, respectively. Studies from our laboratory have shown that cAMP is one of the signal mediators that triggers the expression of Itch and UBC4 (an ubiquitin-conjugating enzyme) in primary Sertoli cells and promotes the occludin protein degradation by the 26S proteasome, leading to an increase in TJ-barrier permeability [75]. Although the upstream biological molecule and/or factor involved in cAMP-mediated occludin degradation via the action of Itch has yet to be identified, it is possible that cytokines, such as TNF- α and interleukin, may be involved as it is well-documented that these cytokines can activate cAMP-mediated signaling function [for a review, see 90].

Cytokine-mediated differential regulation of junction dynamics via distinct signaling pathways in the testis

It is of interest to note that cytokines, such as TGF- β 3, can exert distinctive yet differential effects on junction restructuring events in the testis (Fig. 4). For instance, TGF- β 3 can concurrently disrupt the BTB between adjacent Sertoli cells and AJs between Sertoli and germ cells in the seminiferous epithelium upon the activation of p38 MAPK [91]. And TGF- β 3-mediated activation of ERK1/2 can lead to AJ disruption at the Sertoli-germ cell interface only in particular apical ES without any obvious disruptive effect on the BTB integrity [92]. Using CdCl₂-treated (TJ disruption) and Adjudin-treated (AJ disruption) animal models, our laboratory have shown that by preventing the activation of MAPKs in the testes using specific kinase inhibitors [such as inhibiting p38 MAPK by SB202190 and MEK (upstream of ERK1/2) by U0126] can protect the seminiferous epithelium from the disruptive effects of CdCl₂ and Adjudin on the BTB and AJs, respectively [28,91,93]. Recent studies have identified the mechanism by which the TGF- β 3-T β R1 protein complex interacts with different adaptors, which, in turn, differentially activates different MAPK pathways, leading to disruption of either “AJ alone” or “BTB and AJ” in the seminiferous epithelium [92] (Fig. 4). By recruiting adaptors CD2AP and TAB1 to T β R1 at stage VIII of the seminiferous epithelial cycle, this thus activates p38 MAPK and ERK signaling pathways, leading to the reduction on the steady-state protein levels of occludin, ZO-1 and cadherins at the BTB and cadherins (and possibly other proteins) at ES, resulting in the disruption of BTB and apical ES in the seminiferous epithelium [92]. Such activations thus facilitate both spermiation at the apical compartment and the migration of preleptotene spermatocyte across the BTB at the basal compartment, since both events occur concurrently at stage VIII of the epithelial cycle. While at other stages, CD2AP, but not TAB1, is predominantly recruited to the TGF- β 3-T β R1 complex, leading to the activation of the ERK pathway. The activation of ERK pathway results in the disruption of Sertoli-germ cell adhesion alone without compromising the BTB integrity [92]. These studies have clearly illustrated that CD2AP and TAB1 function as molecular switches to selectively turn “on” either the TGF- β 3-mediated p38 or the TGF- β 3-mediated ERK pathways, so that the opening (or disassembly) of specific types of junctions could be facilitated at different stages of the seminiferous epithelial cycle.

Furthermore, BTB disruption also occurs when TNF- α is administered intra-testicularly into rat testes at levels similar to that present in the microenvironment. This loss of BTB integrity is accompanied by a transient activation of both p38 and ERK signaling pathways concomitant with a reduction of steady-state protein levels of occludin, ZO-1 and N-cadherin [45]. These data suggest that TNF- α , perhaps is working in concert with TGF- β 3, to facilitate the restructuring of BTB and anchoring junctions in the seminiferous epithelium to facilitate germ cell movement across the epithelium during spermatogenesis. It will be of interest to examine whether TNF- α can have dual regulatory effects on junction remodeling via binding of different signaling adaptors.

Concluding remarks and future perspectives

It is not entirely unexpected that these two cytokines, namely TGF- β 3 and TNF- α , may share the common transcription factors to regulate the steady-state levels of either the integral membrane proteins, their peripheral adaptors, protein/lipid kinases, protein/lipid phosphatases, or a combination of these proteins at the Sertoli-Sertoli and Sertoli-germ cell interface, which, in turn, determines the “opening” or the “closing” status of the cell junctions, such as those at the BTB. Recent studies using genome wide gene profiling techniques and an *in vivo* animal model in which rats were treated with Adjuvant to induce junction restructuring mimicking similar events that occur during spermatogenesis have identified several transcription factors, namely Egr-1 & -2, Bhlhb2, Jun, Nupr1, Pawr, c-fos, Atf3, Irf1, Myc, and Stat3, that are pertinent to these events [84]. The information obtained from such gene profiling and bioinformatics approach should be helpful to expand this area of research, unraveling the cytokine-mediated biochemical and molecular regulation of junction dynamics. Needless to say, much of the efforts in the field thus far investigating transcriptional regulation of spermatogenesis are the results of scattered efforts targeting a small subset of genes from different laboratories instead of a concerted effort among investigators to study these transcription factors systematically. It is likely that the use of bioinformatics will be our next step to delineate the coordinated regulatory roles of different transcription factors on specific cellular events during spermatogenesis, such as cell cycle regulation, spermatogonial stem cell mitotic proliferation/renewal, spermiogenesis, spermiation, acrosome formation, and the like.

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Fig. 1. Ultrastructural features of the major junction types in the adult rat testis

(A) This is the cross-section of a stage V seminiferous tubule from adult rat testes, showing the intimate relationship between the seminiferous epithelium, composed of Sertoli and developing germ cells (spermatogonia, spermatocytes, round spermatids, and elongating spermatids), resting on the tunica propria. The seminiferous epithelium is physically divided into the basal and adluminal compartment by the blood-testis barrier. SC, Sertoli cell Sg, spermatogonium Ps, pachytene spermatocyte RS, round spermatid Es, elongating spermatid. (B) This is an enlarged image in the seminiferous epithelium showing an early elongating spermatid (both the condensed nucleus and the early acrosome, Ac, are visible) attached to Sertoli cells via the apical ectoplasmic specialization (ES) (a testis-specific adherens junction type) characterized by the presence of actin filament bundles (white arrowheads) sandwiched between the endoplasmic reticulum (ER) and the Sertoli cell plasma membrane. The two apposing white arrowheads illustrate the relative location of the two apposing Sertoli and germ cell plasma membranes. The ultrastructural features of apical ES (namely actin filament bundles sandwiched between ER and plasma membrane) are limited to the Sertoli cell side. In contrast, the basal ES at the blood-testis barrier (BTB) (the boxed area in C illustrates the typical features of the BTB in adult rat testes) (C) found between two adjacent Sertoli cells (white arrowheads represent the two apposing Sertoli cell plasma membranes) near the basement membrane displaying these ultrastructural ES features, namely, actin filament bundles sandwiched between ER and plasma membrane of the Sertoli cell, are present on both sides of the two Sertoli cells, which also co-exist with tight junctions (TJ, see black arrowheads with white tails). Also clearly visible at the BTB is the desmosome-like junctions (Des) typified by

the presence of electron-dense substances but without the basal ES. The tunica propria shown in A was also magnified in C which is composed of two acellular zones (namely the basement membrane, see white asterisks, and the collagen fibril network, c) and two cellular zones (namely the peritubular myoid cell layer, and the lymphatic vessel underneath). Bar in A, B and C is 10 μm , 0.2 μm , and 1 μm , respectively.

Fig. 2. A schematic drawing showing different junction types and their relative location in the seminiferous epithelium of adult rat testes

The intimate relationship between developing germ and Sertoli cells and the relative location of different junction types in the epithelium at the Sertoli-Sertoli and Sertoli-germ cell interface are shown (see left panel). Differentiating preleptotene spermatocytes must traverse the BTB at stage VIII of the epithelial cycle, and the BTB has also physically divided the seminiferous epithelium into the basal and adluminal compartment. The molecular architecture of apical ES (middle right panel) and tight junction at the BTB (bottom right panel) in the seminiferous epithelium are also illustrated.

Fig. 3. A schematic drawing illustrating the dynamic regulation of integral membrane proteins through ubiquitination and endocytosis

At steady-state, junction proteins are organized themselves at cell-cell contact sites. Upon certain stimulation, target proteins will be recognized and underwent ubiquitination (top panel) or endocytosis (bottom panel) for degradation. For ubiquitination, E3 ligase first recognizes and binds to the target protein. Meanwhile, E1 activates ubiquitin and activated ubiquitin is then conjugated to the target protein with the help of E2, resulting in the formation of polyubiquitin tail onto the target protein. The polyubiquitinated protein may either be degraded by 26S proteasome or undergo deubiquitination by deubiquitinating enzymes (DUBs). The action of DUBs helps cleaving the poly-ubiquitin chain from the target protein, resulting in

rescuing the protein from degradation. Protein can then be recycled back to the membrane surface and facilitate the reassembly of junctions. For endocytosis, junction proteins are internalized via various endocytic structures including clathrin-coated vesicle, caveolin-coated vesicle and actin-coated vacuolae. Internalized proteins are delivered into early endosomes. They are then delivered to either late endosomes in which targets will be degraded by lysosomes or recycling endosomes for channeling back to the cell membrane.

Fig. 4. A schematic drawing illustrating the differential interaction of TGF-β3 and its receptor with different adaptors in Sertoli cells can lead to the activation of different signaling pathways, which selectively perturbs the BTB and/or anchoring junction dynamics

The interactions of TAB1 and CD2AP with the TGF-β3/TGF-β-receptor complex can activate both the p38 or the ERK signaling pathways, which, in turn, causes the disruption of both BTB and AJs (right panel). When the TGF-β3/TGF-β-receptor complex associates only with CD2AP, but not TAB1, this selectively activates only the ERK signaling pathway, resulting in AJ disruption without compromising the BTB (left panel). Without such selective interaction between the TGF-β3/receptor complex and any of the adaptors, both the BTB and anchoring junction remains intact (middle panel). Keys to the symbols in this figure can be found in Fig. 2.

Table 1
Constituent proteins of different junction types in the seminiferous epithelium*

Junction type	Protein components
Occluding junction	
<i>Tight junction</i> between Sertoli cells at the BTB	<p>Transmembrane proteins : <u>occludin</u>; <u>occludin-1B</u>; <u>claudins</u> (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12–24); <u>JAM-A</u>, <u>-B</u> and <u>-C</u>; <u>CAR</u>; <u>CLMP</u>; <u>CRB1</u></p> <p>Peripheral proteins : <u>ZO-1</u>, <u>-2</u>, <u>-3</u>; <u>cingulin α</u>- and <u>F-actin</u>; <u>AF-6</u>; <u>PTEN</u>; <u>JEAP</u>; <u>ZA-1 TJ</u>; <u>7H6 antigen</u>; <u>fodrin</u></p> <p>Signaling proteins : <u>PKC-α</u>; <u>c-src</u>; <u>sympleskin</u>; <u>AIP4/Itch</u>; <u>ASIP/Par3</u>; <u>PKC-ζ</u>; <u>ZAK</u>; <u>ZONAB</u>; <u>c-yes</u>; <u>Gα 19B1</u></p> <p>Trafficking proteins : <u>rab13</u>; <u>rab3b</u>; <u>cdc42</u>; <u>Sec6/8</u>; <u>VAP-33</u></p>
Anchoring junction with actin as attachment site	
<i>Adherens junction</i> between Sertoli cells and Sertoli-germ cells	<p>Transmembrane proteins : <u>nectin-1</u>, <u>-2</u>, <u>-3</u> and <u>-4</u>; <u>N-</u>, <u>E-</u>, and <u>P-cadherins</u>; <u>vezatin</u></p> <p>Peripheral proteins : <u>I-afadin</u>; <u>ponsin</u>; <u>α-</u>, <u>β-</u>, and <u>γ-catenins</u>; <u>p120^{cas}</u>; <u>vinculin</u>; <u>α-actinin</u>; <u>myosin VIIa</u>; <u>ZO-1</u>; <u>α-</u> and <u>F-actin</u>; <u>LIN-7</u>; <u>zyxin</u>; <u>axin</u>; <u>WASP</u>; <u>c-Src</u>; <u>p130 Cas</u>; <u>rab8B</u>; <u>rhoB</u></p>
<i>Ectoplasmic specialization</i> between Sertoli cells and Sertoli-germ cells	<u>IQGAP1</u> ; <u>cdc42</u> ; <u>FAK</u>
<i>Tubulobulbar complex</i> between Sertoli cells and Sertoli-germ cells	<u>MN7</u> ; <u>cofilin</u> ; <u>actin</u> ; <u>dynamamin III</u> ; <u>PKCα</u>
Anchoring junction with intermediate filament as attachment site	
<i>Desmosome</i> between Sertoli cells and Sertoli-germ cells	<u>plakoglobin</u> ; <u>plakophilin</u> ; <u>vimentin</u> ; <u>desmoglein-1</u> , <u>-2</u> , and <u>-3</u> ; <u>desmocollin-1</u> , <u>-2</u> , and <u>-3</u> ; <u>desmoplakins</u>
<i>Hemidesmosome</i> between testicular cells and the extracellular matrix	<u>integrin</u> ; <u>paxillin</u> ; <u>desmoplakin-like protein</u>
Communicating junction	
<i>Gap junction</i> between Sertoli cells, Sertoli-germ cells and Leydig cells	<u>connexins</u> (26, 31, 31.1, 32, 33, 36, 37, 40, 43, 45, 46, 50, 57)

* This table was prepared based on earlier reviews and reports [9–11,13,14]. Proteins localized to junctional sites in the testis are underlined. This table is not intended to be exhaustive. Readers are strongly encouraged to read some of the original articles cited in this and earlier reviews.

Table 2

Phenotype in male mice following deletion of target transmembrane junction proteins

Junction protein	Reproductive function in male KO mice	Major phenotype changes	Reference
Occludin	Infertile	Devoid of germ cells only Sertoli cells retained in the epithelium	[15]
Claudin-11	Infertile	Tubules filled with aggregates of nucleated cells	[16]
CAR	Not applicable ⁺	Not applicable	[17]
JAM-A	Fertile	No major change	[18]
JAM-B	Fertile	No major change	[19]
JAM-C	Infertile	Half of the size of normal testis; lack of differentiated elongated spermatids; azoospermia	[20]
Nectin-2	Infertile	Lack of F-actin covered elongated spermatids; mishapen nuclei found in steps 11–16 spermatids	[21]
N-cadherin	Not applicable	Not applicable	[22]
E-cadherin	Not applicable	Not applicable	[23,24]
Connexin43	Not applicable	Not applicable	[25]
Connexin43 [*]	Infertile	Contained only Sertoli cells and actively proliferating early spermatogonia	[26])

⁺ Not applicable, these knock out (KO) mice either die at embryonic stage or shortly after birth, thus the effects of these deletions on fertility and/or spermatogenesis are not known;

^{*}, Sertoli cell-specific Cx43 knockout.

Table 3 Cytokine-mediated changes in the steady-state mRNA levels of junction component proteins in different epithelia

Cytokine	Mr	Subunit structure	Target junction protein	Cis-acting factors and signaling pathway involved	Reference
TNF- α	50 kDa	Trimer, 3 identical subunits of 17kDa each	Occludin (-), claudin-11 (-), CLMP(-)	NF- κ B (p50/p65), JNK1	[30,31] Size and Lui, unpublished observations
TGF- β 3	24 kDa	Dimer, 2 identical subunits of 12 kDa each	Occludin (-), claudin-11 (-)	Snail, Slug, mSin3A, MAPK, PI3K/Akt, Smad	[32-36,39]
IFN- γ	34 kDa	Dimer, 2 identical subunits of 17 kDa each	ICAM (+), ZO-1 (+)	STAT1	[46-50]
HGF	103 kDa	Dimer, a 69 kDa α -chain and a 34 kDa β -chain	Occludin (-), JAM-A (-)	-	[51,52]
VEGF	46 kDa	Dimer, 2 identical subunits of 23 kDa each	Occludin (-)	-	[46]
IL-1 α	18 kDa	Monomer	Connexin33 (+), connexin43 (-)	ERK	[40]
GM-CSF	15.8 kDa	Glycoprotein monomer	β 3-integrin (+)	-	[53]
TNF- α + IFN- γ			JAM-B (-)	JAK2	Leung and Lui, unpublished observations

(-), inhibition; (+), stimulation.