

## Mechanisms of reorganization of cell-cell junctions in the testis

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## 1. ABSTRACT

During spermatogenesis, intercellular junctions are dynamically remodeled to facilitate the translocation of developing germ cells from the basal to the adluminal compartment of the seminiferous epithelium. In particular, at stage VIII of the seminiferous epithelial cycle, timely restructuring of cell junctions localized at the blood-testis barrier and apical ectoplasmic specializations are crucial to allow the entry of preleptotene/leptotene spermatocytes to the adluminal compartment for further development and the release of mature spermatids from the epithelium respectively. In this review, the transcriptional, post-transcriptional and post-translational regulations involved in testicular cell junction restructuring are included and specific areas required further attention are also highlighted.

## 2. INTRODUCTION

Spermatogenesis is a process in which diploid spermatogonia differentiate into haploid spermatids in the seminiferous epithelium (for review, see 1). Spermatogenesis can be divided into three phases: (i) mitosis, (ii) meiosis and (iii) spermiogenesis. Primordial germ cells undergo several mitotic divisions either to enter a stem cell renewal pathway or produce diploid resting primary spermatocytes. From there, a diploid primary spermatocyte enters the first prolonged meiotic division to produce two diploid secondary spermatocytes which immediately enter the second meiotic division, resulting in the production of four haploid round spermatids. The round spermatids then go through spermiogenesis in which round spermatids undergo a series of biochemical and

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

Junction type	Location	Protein components
Tight junction	Between Sertoli cells at the BTB	<b>Transmembrane proteins</b> occludin; occludin-1B; claudins (1, 3, 4, 5, 7, 8, 11); JAM-A ; CAR; CLMP
		<b>Peripheral proteins</b> ZO-1, -2; cingulin; $\alpha$ -, F-actin; AF-6; PKC- $\alpha$ ; c-Src; symplekin; AIP4/Itch; rab13
Actin-based anchoring junction		
(i) Adherens junction	Between Sertoli cells and Sertoli-germ cells	<b>Transmembrane proteins</b> nectin-2, -3; N-, E-cadherins; JAM-B, -C; laminin $\alpha$ 3 $\beta$ 3 $\gamma$ 3; $\alpha$ 6 $\beta$ 1 integrin; $\beta$ 2-integrin; CAR
(ii) Ectoplasmic specialization	Between Sertoli cells and Sertoli-germ cells	<b>Peripheral proteins</b> I-afadin; ponsin; $\alpha$ -, $\beta$ -, and $\gamma$ -catenins; p120ctn; vinculin; $\alpha$ -actinin; myosin VIIa; ZO-1; $\alpha$ -, F-actin; zyxin; axin; WASP; c-src; p130cas; rab8B; rhoB, IQGAP1; cdc42; FAK; cofilin; actin; dynamin II; PKC $\alpha$ ;
(iii) Tubulobulbar complex	Between Sertoli cells and Sertoli-germ cells	PKG; ERK; IQGAP1; ROCK; Fer kinase; cofilin; vinculin; Par3; Par6; Pals1, PATJ
Intermediate filament-based anchoring junction		
(i) Desmosome	Between Sertoli cells and Sertoli-germ cells	plakoglobin; vimentin
(ii) Hemidesmosome	Between testicular cells and extracellular matrix	integrin; paxillin
Gap junction	Between Sertoli cells, Sertoli-germ cells and Leydig cells	Connexins (26, 31, 31.1, 32, 33, 36, 37, 40, 43, 45, 46, 50, 57)

This table was prepared based on earlier reviews and reports (5, 8, 9). This table is not intended to be exhaustive. Readers are strongly encouraged to the original articles cited in this review.

morphological changes and give rise to elongated spermatozoa with distinct acrosome and flagellum.

In rodent testes, the development of germ cells requires the timely movement of preleptotene/leptotene spermatocytes across the blood-testis barrier (BTB) at late stage VIII and early stage IX of the epithelial cycle to enter the adluminal compartment for further development (2). When in the adluminal compartment, spermatocytes keep migrating towards the tubular lumen while they differentiate into round and elongating spermatids. Eventually, the mature elongated spermatids detach from the epithelium at the next stage VIII of the epithelial cycle at spermiation. The movement of developing germ cells along the seminiferous epithelium requires extensive junction restructuring between two adjacent Sertoli cells and between Sertoli and germ cells. Disassembly and reassembly of cell junctions must be precisely controlled to allow spatial and temporal opening of junctions ahead of moving germ cells followed by rapid assembly of cell junction behind migrating germ cells.

In this review, we summarize recent findings in the study of junction restructuring in the testis into five areas: (i) structure of cell junctions in the testis, (ii) cross-talk between cell junctions within seminiferous epithelium, (iii) transcriptional regulation, (iv) post-transcriptional regulation, and (v) post-translational regulation of cell junction dynamics.

### 3. STRUCTURE OF CELL JUNCTIONS IN THE TESTIS

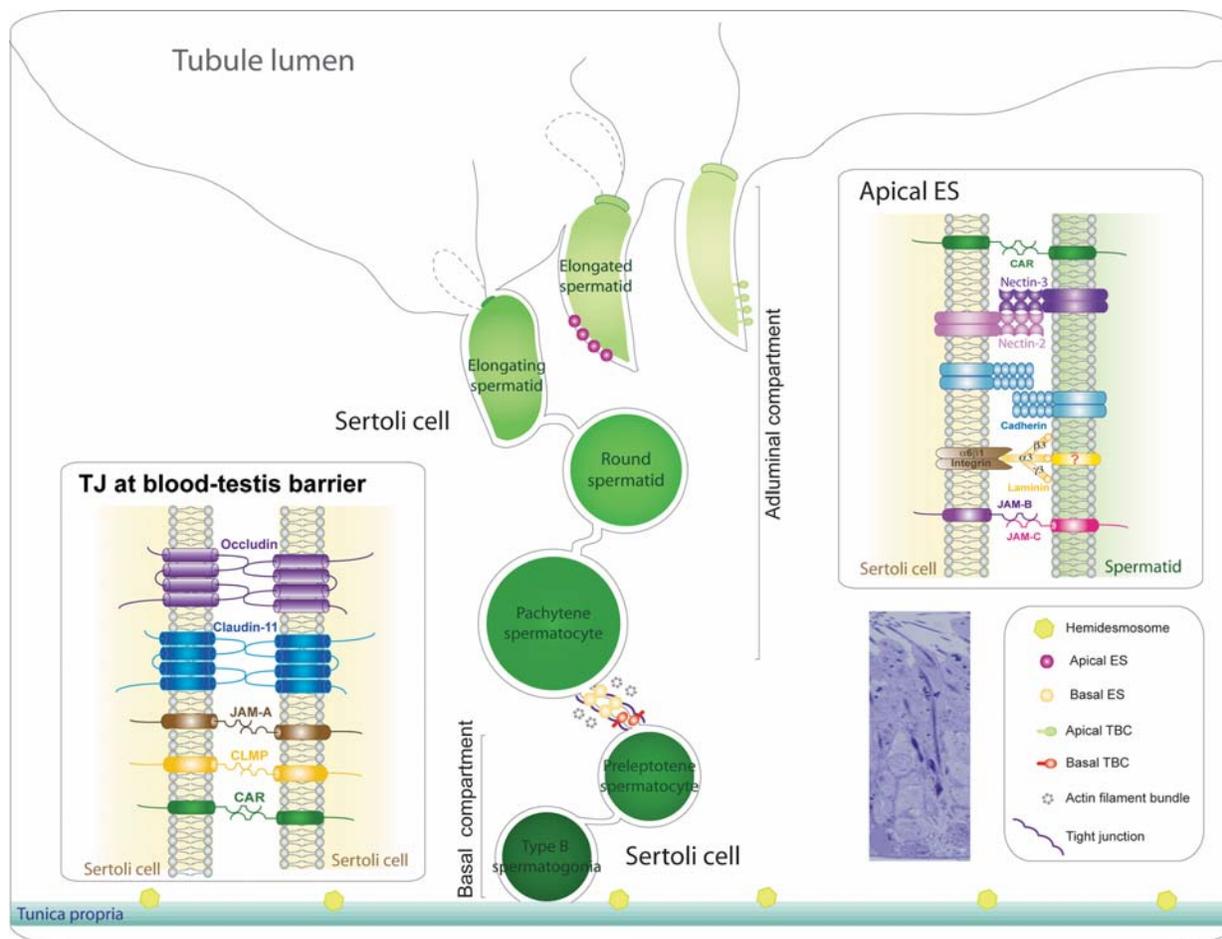
Sertoli cells residing at the basement membrane extend their cytoplasm to the tubule lumen and each Sertoli cell is able to support 30-40 developing germ cells (3, 4). Close interaction between Sertoli and germ cells is crucial

to allow germ cells to gain physical and nourishment supports from Sertoli cells. Attachment of germ cells onto Sertoli cells for communications and structural supports are achieved by gap junctions and anchoring junctions. Gap junctions connect Sertoli and germ cells through end-to-end docking of connexons, formed by a ring of six connexin monomers. Several different connexins of the multigene family have been identified in the testis (Table 1). Anchoring junctions found between Sertoli and germ cells are (i) adherens junctions (AJ) including apical ectoplasmic specializations (ES) and tubulobulbar complexes (TBC) and (ii) desmosome-like junctions (for review, see 5).

Apart from cell junctions formed between Sertoli and germ cells, adjacent Sertoli cells also interact with each other closely to create an immunological barrier and a microenvironment for post-meiotic germ cell development. Sertoli cells interact with each other via tight junctions (TJ) and anchoring junctions. Inter-Sertoli TJ, basal ES and TBC contribute to the formation of the BTB which physically divides the seminiferous epithelium into the basal and adluminal compartments. Spermatogonia, preleptotene and leptotene spermatocytes are located below the BTB (at the basal compartment), while meiotic spermatocytes and post-meiotic germ cells such as elongating and elongated spermatids are found above the BTB (at the adluminal compartment) (Figure 1).

The organization of cell junctions at the BTB is in great difference from those at other blood-tissue barriers. In most blood-tissue barriers, different types of cell junctions are arranged from the apical to the basal and distinctly segregated, with TJ localized at the most apical region and farther away from extracellular matrix (ECM) and followed by AJ and desmosome (for review, see 6). However, TJ at the BTB is localized at the basal region and near to ECM. In addition, it is found to coexist with AJ,

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**Figure 1.** A schematic drawing showing different types of junctions and the relative location in the seminiferous epithelium of adult rat testis. 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 compartments. The molecular architecture of apical ES and tight junction at the BTB in the seminiferous epithelium are also illustrated. CAR, coxsackie and adenovirus receptor; CLMP, CAR-like membrane protein; JAM, junctional adhesion molecule.

basal ES, basal TBC, desmosome-like junction (for review, see 7). Basal ES and basal TBC are testis-specific AJs that are restricted to the Sertoli-Sertoli interface at the BTB.

A myriad of protein components including structural and regulatory proteins in different types of junctions in the seminiferous epithelium has been identified and summarized in Table 1. Due to the page constraint, we recommend readers to seek additional information from recent reviews (for reviews, see 5, 8, 9).

#### 4. EFFECTS OF HORMONES AND CYTOKINES ON CELL RESTRUCTURING IN THE TESTIS

Hormones and cytokines are two major regulators of cell junction restructuring in the testis. Hormones such as testosterone and follicle stimulating hormone (FSH) have been reported to regulate the expression of several junction proteins. For instance, a reduction of endogenous testicular testosterone level by testosterone/estradiol (TE) implants caused sloughing of spermatids (step 8 and

beyond) from the seminiferous epithelium which was resulted from a loss of apical ES function (10, 11) and clearly illustrates the functional significance of testosterone in maintaining spermatid adhesion on the epithelium. Subsequent studies have shown that the protein levels of putative ES regulators such as tyr-phosphorylated focal adhesion kinase (p-FAK) and c-Src are altered in T suppression-induced germ cell loss (12). Recent studies have shown that both testosterone and dihydrotestosterone (DHT) enhance the integrity of the TJ barrier in cultured Sertoli cells as reflected by an increase in transepithelial electrical resistance (TER) (13). Furthermore, there was a significant increase in claudin-11 mRNA in rat Sertoli cells treated with androgens (13). Although androgens showed no effect on overall occludin expression, it promoted the localization of occludin at cell-cell contacts. These results strongly suggested that androgens are potent regulators to modulate TJ formation in the testis. Apart from androgens, FSH is another hormone that regulates the expression of junction proteins in the testicular cells. While Hellani *et al.* has reported that the inhibitory effect of FSH on the level

of claudin-11 (also called OSP) mRNA was observed in cultured mouse Sertoli cells (14); on the contrary, Kaitu'u *et al.* has shown that FSH partially stimulated claudin-11 mRNA in cultured rat Sertoli cells (13). Cytokines such as transforming growth factor- $\beta$  (TGF- $\beta$ ) and tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) are crucial regulators to modulate junction dynamics in the testis. The detailed mechanisms of cytokine-mediated junction restructuring have been recently reviewed. Readers are strongly encouraged to read this other review (8) to gain a more comprehensive view.

## 5. CROSS-TALK BETWEEN CELL JUNCTIONS IN THE TESTIS

Cross-talk between different types of cell junctions enables proper coordination in the event of junction restructuring, which is important in many cellular events such as tissue formation and morphogenesis (15). Many studies have shown that cross-talk between different types of junctions is achieved by differential association of the peripheral and integral junction proteins. For instance, nectin-2 and AF-6 have been shown to interact with ZO-1 to facilitate the recruitment of TJ components such as junctional adhesion molecule (JAM) (16-18). Other than junction assembly, cross-talk between cell junctions also allows a correct sequence of events in junction disassembly. For example, manipulation of AJ function in airway epithelial cells by incubating with E-cadherin antibody subsequently leads to TJ disruption (19).

During spermatogenesis, cross-talk between TJ and AJ is crucial to facilitate the translocation of germ cells across the BTB and their further entry into the adluminal compartment of the seminiferous epithelium for development. Similar to junctions found in other epithelia, TJ and AJ coexisting at the BTB are able to communicate so as to regulate the dynamics of the BTB. Although there is no direct interaction between the TJ transmembrane protein occludin and the AJ transmembrane protein cadherin, these two proteins colocalized at the BTB cross-talk with each other via their peripheral proteins ZO-1 and  $\alpha$ -catenin (20, 21). ZO-1 shuffles between TJ and AJ and links the two junction complexes alternatively to actin cytoskeleton. ZO-1 and catenin indeed are the key molecules to facilitate the cross-talk by differential association with different junction types. TJ and AJ can be structurally associated with each other by the help of these two adaptors, resulting in the reinforcement of BTB integrity at all stages of the epithelial cycle except VIII. Such reinforcement of the BTB must be abolished at stage VIII to allow the translocation of germ cell across the BTB. To achieve this, catenin disengages from ZO-1 and causes a loss of association between cadherin and catenin, which facilitates germ cell movement across the barrier. Such disengagement mechanism prevents unnecessary damage to TJ at the BTB during AJ restructuring (20).

## 6. TRANSCRIPTIONAL REGULATION OF CELL JUNCTION DYNAMICS

Transcription is among the most highly regulated biological processes. Transcriptional regulation is the

primary determinant of developmental and homeostatic cellular functions of no exception in spermatogenesis (22). Temporal and spatial expression of a gene in testicular cells is achieved by the interplay of general transcription factors (GTF) and gene-specific transcription factors. To allow stringent gene expression in testicular cells, differential expressions of GTFs and gene-specific transcription factors in cell type-specific or stage-specific manner are crucial to ensure proper and efficient transcriptions to occur throughout spermatogenesis.

### 6.1. Expression of general transcription factors in testicular cells

As far as the expression of GTFs is concerned, three proteins that are important for pol II transcription machinery, namely TFIIB, TATA-binding protein (TBP) and RNA polymerase II, are abundantly expressed in haploid round spermatids than in somatic cells. In fact, the level of nuclear TBP in testicular cells, on average, is 8- and 11-fold higher than those in liver and spleen cells, respectively (23). These findings suggest that high level of the pol II transcription apparatus might be a general property of this stage of spermatogenesis. Apart from exceptional high expression level of several GTFs in particular type of germ cells, several GTFs such as TFIIA $\alpha$ / $\beta$ -like factor (ALF) and TFIIA $\tau$  are expressed almost exclusively in the testis. It is believed that they might be uniquely important to testis biology (24, 25). However, it is not known whether such differential expression of GTFs do regulate the temporal and spatial transcriptional activation of certain cell junction proteins such as nectin-3 and JAM-C in haploid elongating spermatids that are localized at the apical ES before spermiation.

### 6.2. Expression of gene-specific transcription factors in testicular cells

Recent studies have been more focused on identifying the role of individual transcription factors in transcriptional regulation of junction proteins in testicular cells. In this section, several transcription factors pertinent to the junction protein expression have been selected for review. Their expression and mechanism of action in regulating the transcription of junction protein genes are discussed.

#### 6.2.1. CREB protein

cAMP response element binding protein (CREB) is capable of forming homodimers or heterodimers with other family members such as activating transcription factor-1 (ATF-1) upon cAMP stimulation (26, 27). The dimers recognize cAMP responsive element (CRE) with a consensus sequence, TGACGTCA (27). In adult rodent testes, CREB is expressed predominantly in pachytene spermatocytes, round spermatids and Sertoli cells (28, 29). In situ hybridization analyses have shown that there is a cyclic change in CREB mRNA in Sertoli cells along with the seminiferous epithelial cycle. CREB mRNA is found to be present in Sertoli cells at stages I-VIII and reach its maximum level at stages VII-VIII, followed by a significant decrease to an undetectable level at stages IX-XIV (28, 29).

### 6.2.2. Smad proteins

Smad heteromeric complex comprising R-Smads and Co-Smad activates transcription via physical interaction with Smad-binding element (SBE) and functional co-operation with other transcription factors, co-activators and co-repressors. The minimal SBE contains an AGAC sequence or its complementary sequence, GTCT (30, 31). Some reports have revealed that BMP-regulated R-Smads preferentially bind to the GC-rich sequences, GCCGNC or GRCGNC (32, 33).

In situ hybridization and immunohistochemistry studies have shown that Sertoli cells, interstitial cells and meiotic germ cells, from preleptotene to pachytene spermatocytes, express Smad2 mRNA and protein (34). Smad2 mRNA and its protein are detected in the seminiferous epithelium throughout the spermatogenic cycle. The strongest signal is associated with pachytene spermatocytes at stages I-VII, while expression significantly reduces at stages VIII-X (34). Smad3 has been detected in the cytoplasm of Sertoli cells throughout the spermatogenic cycle (35) whereby a shift in the localization of Smad3 from cytoplasm to the nucleus occurs at stages VII-VIII (35), suggesting that nuclear Smad3 might involve in mediating stage-specific gene expression.

### 6.2.3. Sp1/KLF family

The Sp1/KLF multigene family is sub-divided into two groups namely Sp subgroup (Sp1-6) and KLF subgroup (KLF1-17) (for review, see 36). Because of this highly conserved DNA binding domain, Sp and KLF are able to recognize and bind to the same DNA sequence, but with different affinities (for review, see 37). A major difference at the N-terminal region helps these two subgroups to recruit and facilitate interaction with different co-factors such as CtBP and mSin3A required for the transcription machinery (for review, see 38).

Sp1 and KLF4 (also known as gut-enriched KLF) are the best characterized Sp/KLF family members in the testis. Immunohistochemistry and in situ hybridization analyses have shown that Sp1 exhibits cell type-specific expression (39). Sp1 is predominantly expressed in preleptotene, leptotene, pachytene and zygotene spermatocytes, whereas spermatogonia and spermatids express a relatively low level of Sp1 (39, 40). Unlike Sp1, KLF4 shows tissue-specific expression pattern and is abundantly expressed in colon followed by testis, lung and small intestine (41). In the testis, KLF4 mRNA level increases after postnatal day 18 and remains at high level afterward (42). KLF4 is expressed in Sertoli cells and is more predominant in round spermatids (42). In situ hybridization analyses have revealed that KLF4 shows a stage-dependent expression pattern with the highest expression at stage VII of the spermatogenic cycle (42).

Sp1 and KLF4 are known transcription factors that are important in regulating the expression of several adhesion molecules such as laminin  $\gamma$ 1 and laminin  $\alpha$ 3A in epithelial cells. For instance, Sp1 and KLF4 exert synergistic effects in activating rat laminin  $\gamma$ 1 chain gene transcription (43, 44).

### 6.2.4. GATA family

All GATA family members contain a highly conserved DNA-binding domain composing of two zinc-fingers. GATAs bind to DNA consensus sequence, WGATAR, via the zinc-finger located at the C-terminus adjacent to the basic region (for review, see 45).

Of the six members, GATA-1, -4 and -6 are expressed in the testis (46-48). Northern blot analysis has shown that GATA-1 mRNA level in the mouse testis peaks at 2-week of age and reduces steadily thereafter. GATA-1 expression is stage-specific and is exclusively found at stages VII-IX of the spermatogenic cycle (46). For GATA-4, its expression remains steadily throughout testicular development (47, 48). Sertoli cells and Leydig cells are found to express GATA-4; however, the presence of GATA-4 in germ cell remains controversial (47-49). In rodent testis, the expression of GATA-6 is restricted to Sertoli cells and overlapped with that of GATA-4.

Similar to Sp1/KLF family, GATA proteins are involved in regulating the expression of junction proteins. In cardiac mesodermal cells, GATA-4 is a key transcription factor that activates N-cadherin gene transcription via direct binding to the consensus AGATAA motif of the N-cadherin promoter (50). Other than N-cadherin, GATA-4 and GATA-6 have been shown to bind to  $\alpha$ T-catenin promoter in cardiac HL-1 cells (51).

### 6.3. Role of transcription factors in pertinent to the expression of junction proteins in testicular cells

Some of the above-mentioned transcription factors such as Sp1/KLF and GATA family have been reported to regulate the expression of junction proteins in other epithelial cells (52, 53). Indeed, these four groups of transcription factors including CREB and the Smad proteins are key molecules in regulating transcription of junction proteins in testicular cells. For instance, the basal transcription of two junction proteins nectin-2 and claudin-11 in Sertoli cells have been found to be regulated via binding of CREB to the CRE and GATA/NF-Y motifs, respectively (54, 55). Nectin-2, an integral membrane protein, is expressed by Sertoli cells and localized at apical ES, while claudin-11 is a TJ transmembrane protein found at the BTB. The cyclic expression pattern of CREB is believed to be crucial to allow the cyclic events of junction restructuring to be taken place in the seminiferous epithelium (54). For instance, a significant reduction in CREB at early stage IX is of particular importance in facilitating the disassembly of apical ES at Sertoli-spermatid interface via repression of CREB-mediated nectin-2 gene activation, resulting in the timely release of mature spermatids at late stage VIII and early stage IX. In fact, analysis of the staged tubules by RT-PCR has confirmed that the cyclic expression of CREB and nectin-2 coincide with the event of apical ES restructuring between Sertoli and germ cells (54). It is apparent that CREB is one of the crucial regulators to mediate timely transcription of junction proteins in the testis. Apart from CREB, our studies have revealed that c-Jun and Sp1 are two major transcription factors that are involved in nectin-2 gene transcription. The results suggest that transcription of

junction protein is controlled by an array of transcription factors which can work in either separate or co-operative manner. This observation also provides an explanation why mice having CREB knockout (knockout of CREB  $\alpha$  and  $\delta$ ) remain fertile (56). It is possible that other transcription factors such as c-Jun and Sp1 supersede CREB in mediating transcriptional activation of junction proteins in the knockout animals.

For claudin-11, the timely repression of claudin-11 in facilitating BTB disassembly at late stages VIII-IX can be achieved not only by a significant reduction of CREB at early stage IX, but also by a shift in the localization of a negative regulator, Smad3, from cytoplasm to nucleus at stages VII-VIII of the spermatogenic cycle (35, 55), which alters the ratio of positive/negative regulators and might favor gene repression. In fact, Smad3 is able to inhibit CREB-mediated claudin-11 transactivation by competitive binding at the GATA/NF-Y motif of the claudin-11 promoter (55). This binding of Smad3 assists in the recruitment of HDAC1 and mSin3A to the GATA/NF-Y motif and alters the acetylation status of histones, resulting in effective repression of claudin-11 gene (55). Apart from claudin-11, our recent studies have shown that Smad3 and Smad4 are also involved in modulating the expression of JAM-A and JAM-B in Sertoli cells (Lie *et al.*, Wang *et al.*, unpublished observations). Overexpression of Smad3 and Smad4 could significantly reduce JAM-A and JAM-B promoter activities. Electrophoretic mobility shift assays have shown that Smad3 and Smad4 bind onto the TGIF motif at the JAM-B promoter region (Wang *et al.*, unpublished observations). Taken collectively, Smad proteins are important negative regulators in controlling the transcription of junction proteins in the testis.

Apart from CREB and Smad proteins, the Sp1/KLF protein family indeed plays a crucial role in controlling the expression of AJ and TJ integral membrane proteins. Sp1 and Sp3 are capable of up-regulating the expression of two AJ integral membrane proteins nectin-2 and JAM-B in Sertoli cells (54). Overexpression studies and chromatin immunoprecipitation analyses have shown that Sp1 and Sp3 significantly increase the promoter activities and bind onto the minimal promoter regions of the corresponding genes (54); Wang *et al.*, unpublished results). Sp1 and KLF4 also work but reciprocally on CLMP gene transcription (57). CLMP is a newly identified TJ integral membrane protein that is co-localized with ZO-1 at the BTB (57). Within the Sp1/KLF family, KLF4 exerts a positive regulatory effect on CLMP gene transcription. Unlike the effects of Sp1 on nectin-2 and JAM-B gene activation, Sp1 and Sp3 function as negative regulators on CLMP gene transcription. In addition, co-transfection of KLF4 and Sp1 proteins has shown that Sp1, but not Sp3, significantly abolishes KLF4-mediated transactivation possibly by effective competition of the binding to the promoter as well as to the interacting partners (57). Studies of the transcriptional regulations of nectin-2, JAM-B and CLMP genes have illustrated that transcription factors belonging to the same family are able

to exert opposite effects in modulating gene transcription of junction proteins.

Studies have shown that the involvement of GATA proteins in basal transcription of claudin-11 and CLMP genes. While overexpression of GATA proteins alone significantly up-regulate the promoter activity of claudin-11 gene (~7-fold increase), suggesting GATA proteins are strong positive regulators in claudin-11 gene transcription (55), their role in regulating transcription of CLMP gene is different. Although the basal transcription of CLMP requires the presence of GATA proteins as GATA proteins are components of a ternary protein complex needed for the basal transcription machinery (57), overexpression of GATA proteins alone shows no significant increase in CLMP promoter activity (57). It has been suggested that GATA proteins are required to work in concert with another transcription factor in the ternary protein complex, such as KLF4, to mediate their regulatory effect.

Based on our studies on the nectin-2 and CLMP gene transcription, the minimal promoter regions of the nectin-2 and CLMP genes in different cell types such as TM4 (Sertoli cells), GC-2Spd(ts) (germ cells), and other non-gonadal cell types such as NIH3T3 and Caco-2 cells are the same, but with different magnitude of fold changes (54, 57). However, the cis-acting motifs and trans-acting factors involved in transcription activation are different. For instance, SRY motif is found to be a crucial cis-acting motif in driving the CLMP gene transcription in TM4 cells, but not in NIH3T3 and Caco-2 (57). These results suggest that the transcriptional regulation of some junction proteins is unique to testicular cells. Since most of the promoter analyses have been performed in testicular cell lines, it is worthy to select some of the important pathways and re-examined them using testicular cells freshly isolated from testes.

Apart from TJ and AJ, the expression of gap junction proteins such as connexin33 and connexin43 in the seminiferous epithelium have been found and modulated via transcriptional regulation in response to IL-1 $\alpha$ -mediated ERK activation (58). However, the transcription factors involved in this event have not yet been identified.

Taken collectively, transcriptional regulations of junction proteins in the testicular cells require functional cooperation of different transcription factors. Stage-specific expression of particular transcription factors indeed provides a mean to control the timely expression of a particular junction protein.

## 7. POST-TRANSCRIPTIONAL REGULATION OF CELL JUNCTION DYNAMICS

Besides transcription, there are accumulating evidences showing that the modulation of mRNA stability is also an effective mechanism to regulate the level of steady-state mRNA. The regulation can be in effect at a relatively short period of time upon external stimuli and will ultimately alter the protein abundance (for reviews, see

59, 60). Factors that control the rate of mRNA decay are the cis-acting elements on the mRNA and trans-acting factors in the cytosol (61). One of the best characterized cis-acting elements controlling mRNA degradation is the adenylate- and uridylylate-rich (AU-rich) element (ARE) with sequence of UUAUUA(U/A)(U/A) or UUAUUUAUU at the 3'-untranslated region (UTR) (62-64). Binding of specific RNA-binding proteins to the ARE alters mRNA stability. Three RNA-binding proteins including tristetraproline (TTP), AUF1 and HuR have been identified in the testis (65).

So far, there is only one report in the literature demonstrating the importance of mRNA stability in the regulation of junction dynamics in the testis. This study has demonstrated that TNF $\alpha$ , a cytokine known to induce junction disassembly, is capable of destabilizing CLMP mRNA transcript, which leads to a significant reduction in CLMP protein level (66). In fact, TNF $\alpha$  induces the expression of a RNA-binding protein, TTP, via JNK activation and promotes the binding of TTP to the ARE of the CLMP transcript (66). It is likely that this post-transcriptional modification of mRNA mediated by cytokine provides a means to regulate cell junction dynamics in the testis.

In fact, several studies performed in other epithelial cells have demonstrated that alteration of mRNA stability affects the expression of junction proteins. RNA-binding proteins interacting with the ARE can either stabilize or destabilize mRNA transcripts encoding junction proteins. For example, studies performed in colon cell line have shown that binding of HuR onto ARE of the  $\beta$ -catenin mRNA stabilizes the transcripts and a knockdown of HuR results in significant reduction of  $\beta$ -catenin mRNA and protein levels (67). Besides  $\beta$ -catenin, the expression of  $\beta$ 3-integrin is upregulated via stabilizing its mRNA in response to granulocyte macrophage-colony stimulating factor (GM-CSF) (68). Upon GM-CSF stimulation, the half-life of  $\beta$ 3-integrin mRNA extends from 6.5h to 38h (68). Apart from mRNA stabilization, it has reported that lipopolysaccharide (LPS)-induced inflammation causes a significant reduction in connexin32 in the liver via a mechanism by which the connexin32 transcripts are destabilized (69).

It is apparent that post-transcriptional modification is an effective mechanism to control the steady-state mRNA level of a particular gene available for translation. The functional significance of this modification regarding to junction restructuring is well-established in many epithelia. However, this area of research in the field of male reproduction is just at the beginning and attention is highly deserved to elucidate its significance in spermatogenesis.

## 8. POST-TRANSLATIONAL REGULATION OF CELL JUNCTION DYNAMICS

Based on the fact that the half-lives of most junction proteins are much longer than the time required for junction remodeling in many physiological events (e.g. the half-life of occludin is 12h vs the duration of epithelial

morphogenesis is <1h) (70, 71), it is apparent that apart from transcriptional and post-transcriptional approaches, post-translational modification of junction proteins at the site of cell-cell contact should provide more effective mechanism in controlling the bioavailability of junction proteins for a particular cellular event. For instance, synthetic occludin peptides either injected intratesticularly or carried by FSH vehicle block the existing occludin at the site of cell-cell contact and thus induce premature release of spermatids from the seminiferous epithelium (72, 73). These studies have clearly shown that alteration of bioavailability of junction proteins results in male infertility. Ubiquitination and endocytosis are two known mechanisms that control protein bioavailability via targeted protein degradation.

Ubiquitination is a process of conjugating a chain of ubiquitin (Ub) onto a target protein. An ubiquitin-activating enzyme (E1) is responsible for the activation of Ub to form an E1-Ub intermediate with a thiol group. Activated Ub is then conjugated onto the target protein recognized by E3 ligase with the help of ubiquitin-conjugating enzyme (E2). Protein tagged with a polyubiquitin chain is recognized by proteasome and degraded into short peptides. Ub is eventually released and recycled (for reviews, see 74, 75). In fact, ubiquitin conjugation can be reversed by deubiquitinating enzymes. Deubiquitinating enzymes hydrolyze the polyubiquitin chain into ubiquitin monomer and rescue the protein from proteasome-mediated degradation (for reviews, see 76, 77). Ubiquitination and deubiquitination work in coordination to control the bioavailability of proteins required for a particular cellular event.

Studies have shown that testis is the organ that exhibits the highest rate of ubiquitination (78). High rate of ubiquitination in the adult testis correlates with massive cellular events taken place during germ cell development (79, 80). For instance, studies have unraveled the mechanism on how ubiquitination of occludin modulates the BTB function in the testis (81). It has been shown that Itch, an E3 ligase, and UBC4, an E2 enzyme, are up-regulated in cultured Sertoli cells in response to cAMP stimulation. High level of Itch promotes the recognition of occludin. With the help of UBC4, poly-ubiquitin chain is conjugated onto occludin and this triggers proteasome-sensitive occludin degradation, resulting in TJ disruption (81). Although limited studies have been performed so far to unravel the significance of ubiquitination in regulating junction restructuring in the testis, studies performed in other epithelia may serve as a blueprint for researchers in the field seeking further investigation.

Endocytosis is another mechanism that allows rapid junction remodeling by internalization of integral membrane proteins at the site of cell-cell contact and results in junction restructuring. (82-84). Proteins are internalized into different endocytic structures including actin-coated vacuoles, clathrin- or caveolin-coated vesicles (85, 86). Internalized proteins are then delivered into the early endosomes in which proteins are either channelled back to cell surface through recycling endosomes or

delivered to lysosome for degradation via late endosomes (85, 86).

Studying the effects of endocytosis in modulation of testicular junction restructuring has begun in recent years. The results obtained by far have clearly demonstrated the physiological significance of this mechanism in regulating junction dynamics and thus facilitating the timely germ cell movement during spermatogenesis. Studies have shown that C-type natriuretic peptide (CNP) secreted by Sertoli and germ cells is able to regulate the BTB dynamics by disrupting the integrity of TJ-barrier *in vitro* and *in vivo*. Addition of synthetic CNP peptide into cultured Sertoli cells results in the disappearance of TJ and AJ proteins including JAM-A, occludin and N-cadherin) and leads to TJ disruption. This disappearance of junction proteins at the cell-cell interface is in fact associated with an acceleration of junction protein internalization (87). A more recent study has reported that testosterone and transforming growth factor  $\beta$ 2 (TGF- $\beta$ 2) regulate the BTB dynamics via their differential effects on the kinetics of protein endocytosis and recycling (88). Both testosterone and TGF- $\beta$ 2 have been found to promote the internalization of the BTB integral membrane proteins on Sertoli cells via clathrin-mediated endocytic pathway. However, testosterone and TGF- $\beta$ 2 exert different effects on the internalized proteins. Testosterone enhances the kinetics of recycling of internalized BTB proteins and channels them back to the cell surface; whereas TGF- $\beta$ 2 targets the internalized proteins in early endosomes to late endosome in which lysosome-mediated degradation is taken place (88). It is postulated that during germ cell migration across the BTB, TGF- $\beta$ 2 perturbs the apical region of the BTB by targeting junction proteins to degradation whereas testosterone enhances junction protein recycling back to the Sertoli cell surface via transcytosis to reassemble the BTB at the basal region. This coordinated action by TGF- $\beta$ 2 and testosterone facilitates the transit of germ cells across the BTB and at the same time maintains the immunological barrier in the seminiferous epithelium. These two recent studies indeed provide strong evidence that endocytosis and recycling of proteins are crucial in modulating junction dynamics in the testis (87, 88).

## 9. CONCLUDING REMARKS

It is apparent that cell junction reorganization in the testis is via an array of regulatory mechanisms. Whereas transcriptional and post-transcriptional regulations control the amount of mRNA transcripts available for protein translation, post-translational modification controls the bioavailability of junction proteins at the site of cell-cell contact. Efforts have been made in the last decade by various laboratories to identify the biomolecules and signaling pathways involved in this event. Along the line, delineation of the coordinated roles of TGF- $\beta$  and TNF $\alpha$  in regulating the dynamics of adherens and tight junctions has provided a model to explain how germ cells migrate along the basal and the adluminal compartments of the seminiferous epithelium (8). Delineation of the differential effects of testosterone and TGF- $\beta$ 2 has also provided a work model to explain how preleptotene spermatocytes

traverse across the blood-testis barrier without affecting the barrier integrity (88). However, concerted efforts among researchers in the field have to make to delineate the molecular mechanisms on regulating the stability of mRNA transcripts encoding junction proteins as it is increasingly recognized as a crucial means of controlling gene expression (66, 89). It is also important to further investigate the importance of Smad proteins in transcription of junction proteins. It is apparent that Smad proteins downregulate the transcription of several junction proteins. In tumor models, the transcription factor Snail, has been identified as a master transcription factor that suppresses the transcription of junction proteins (90). It is worthy to examine whether Smad proteins can, serve as a master transcription factor, function like Snail, to ubiquitously control the expression of junction proteins in the testis.

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**Abbreviations:** BTB: blood-testis barrier; ES: ectoplasmic specializations; TBC: tubulobulbar complex; TJ: tight junctions; ECM: extracellular matrix; AJ: adherens junctions; FSH: follicle stimulating hormone; TE: testosterone plus estradiol; TGF- $\beta$ : transforming growth factor- $\beta$ ; TNF $\alpha$ : tumor necrosis factor  $\alpha$ ; ZO-1: zonula occludens-1; JAM: junctional adhesion molecule; GTF: general transcription factors; TBP: TATA-binding protein; ALF: TFIIA $\alpha$ / $\beta$ -like factor; CREB: cAMP response

element binding protein; ATF-1: activating transcription factor-1; CRE: cAMP responsive element; SBE: Smad-binding element; IL-1 $\alpha$ : interleukin-1 $\alpha$ ; ARE: adenylate- and uridylylate-rich element; UTR: untranslated region; TTP: tris-trapoline; GM-CSF: granulocyte macrophage-colony stimulating factor; LPS: lipopolysaccharide; Ub: ubiquitin; E1: ubiquitin-activating enzyme; E2: ubiquitin-conjugating enzyme; CNP: C-type natriuretic peptide

**Key Words:** Testis, Spermatogenesis, Cell Junction, Transcription, Post-Transcription, Post-Translation, Regulation, Review

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