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Regulation of junction dynamics in the testis—Transcriptional and post-translational regulations of cell junction proteins

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Abstract

Cell junctions are the sites at which cells attach to the neighboring cells. They do not only maintain tissue integrity, their turnover also plays a crucial role in cell development and morphogenesis. In the testis, tight junctions and adherens junctions are dynamically remodeled to allow the movement of post-meiotic germ cells across the seminiferous epithelium and the timely release of spermatids into the tubular lumen. There is growing evidence that this dynamic remodeling of cell junctions is mediated by several mechanisms at the transcriptional and post-translational levels. This review summarizes what is known about the transcriptional regulation, ubiquitination and endocytosis that are involved in modulating junction dynamics in epithelial cells. It also highlights the recent findings on the regulation of junction dynamics in the testis and the specific areas that require further research for a thorough understanding of the role of junction remodeling in spermatogenesis. Understanding the junction dynamics in the seminiferous epithelium may unfold new targets for non-hormonal male contraceptive development.

Keywords: Sertoli cells; Cell junctions; Transcription; Post-translation; Ubiquitination

1. Introduction

In rodent testes, a type A1 spermatogonium (diploid, 2n) gives rise to 256 mature spermatids (haploid, n) in the seminiferous epithelium during spermatogenesis (for reviews, see Cheng and Mruk, 2002; de Kretser and Kerr, 1988). Differentiating germ cells must traverse from the basal to the adluminal compartment of the seminiferous epithelium to complete spermatogenesis. Tight junctions (TJs) between Sertoli cells at the basal compartment form the blood–testis barrier (BTB), which segregates most part of spermatogenesis from the systemic circulation (for reviews, see Dym and Cavicchia, 1977; Dym and Fawcett, 1970; Russell and Peterson, 1985). At the late stage VIII through early stage IX, preleptotene and leptotene spermatocytes must traverse the BTB, where junctions are rapidly broken and subsequently reassemble to allow the spematocytes to pass through (for reviews, see Cheng and Mruk, 2002; Lui et al., 2003d; Russell, 1977).

The translocation of germ cells across the seminiferous epithelium involves not only the dynamic modulation of TJ at the BTB, but also the extensive restructuring of actin-based adherens junctions (AJs) between Sertoli cells as well as between Sertoli and germ cells thereafter, so that differentiating germ cells could move towards the adluminal compartment for further development (for reviews, see Cheng and Mruk, 2002; Lui et al., 2003d; Mruk and Cheng, 2004).

Previous studies in our laboratory have shown that the restructuring of cell junctions in the testis involves an array of biomolecules such as cytokines, proteases, protease inhibitors and extracellular matrix proteins, and signaling molecules including cAMP and cGMP (Lee and Cheng, 2004; Lui et al., 2001, 2003b, 2003f; Siu and Cheng, 2004; Siu et al., 2003a, 2003b; Wong et al., 2004).

Cytokines such as transforming growth factor-β3 (TGF-β3) and tumor necrosis factor-α (TNF-α) have been shown to be involved in regulating junction proteins such as claudin-11, occludin and ZO-1 at their transcriptional levels in Sertoli cells (Lui et al., 2001). Cytokine-mediated transcriptional regulation of those proteins results in altering the permeability of the TJ barrier both in vitro and in vivo (Lui et al., 2001, 2003b, 2003f; Siu and Cheng, 2004; Wong et al., 2004). These results clearly demonstrate that the transcriptional regulation of the junction proteins is one of the major regulatory mechanisms to modulate the junction dynamics in the testis. Apart from transcriptional regulation of junction proteins, our recent studies have demonstrated that junction restructuring is also mediated via post-translational modification of the junction protein. Ubiquitination regulates the bioavailability of targeted junction proteins

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at the site of cell–cell contact, resulting in the opening and closing of cell junctions (Lui and Lee, 2005).

The structure and function of cell junctions in the testis have been reviewed extensively (for reviews, see Cheng and Mruk, 2002; Lui et al., 2003d; Mruk and Cheng, 2004). Here we attempt to highlight specific research areas that deserve attention in future studies. In this review, we shall focus on the (i) transcriptional regulation and (ii) post-translational modification of cell junction proteins at the site of cell–cell contact, which in turn regulate cell junction dynamics.

2. Structure and molecular composition of cell junctions in the testis

Morphologically, TJs form a continuous circumferential seal above the basal lamina of the seminiferous tubules in the testis. The molecular architecture of TJs has been unraveled rapidly in recent years (for reviews, see Fanning et al., 1999; Martin-Padura et al., 1998; Mitic et al., 2000). Three classes of integral membrane proteins have been positively identified in the testis including occludin, claudins and junctional adhesion molecules (JAM) (Martin-Padura et al., 1998; Moroi et al., 1998; Tsukita et al., 2001). Several peripheral membrane proteins, which are linked to the integral membrane proteins have also been identified at the site of TJs in the testis. They include zonula occludens-1 (ZO-1), ZO-2, cingulin and several others (for reviews, see Byers et al., 1991, 1992; Cheng and Mruk, 2002; Jesaitis and Goodenough, 1994; Lui et al., 2003d; Mruk and Cheng, 2004). Some of the peripheral proteins function to connect the integral membrane protein with the actin filament inside the cell.

Adherens junctions link cytoskeletal elements from one cell (e.g. sertoli cell) to the same type or another type of cell (e.g. germ cell) or to the extracellular matrix, creating a network that maintains the architecture of the testis. More importantly, the dynamic nature of AJs in the testis permits the translocation of developing germ cells across the seminiferous epithelium during spermatogenesis. There are three classes of interlocking protein complexes identified at the AJs. They are the cadherin–catenin complex, nectin–afadin complex and integrin–laminin complex (Bouchard et al., 2000; Chapin et al., 2001; Chung et al., 1999; Lee et al., 2003; Ozaki-Kuroda et al., 2002; Siu et al., 2003b; Wine and Chapin, 1999). The regulatory mechanisms of the formation and disruption of TJs and AJs have been examined extensively (for reviews, see Cheng and Mruk, 2002; Lui et al., 2003d, 2003e; Mruk and Cheng, 2004), and the promoter analyses of occludin, claudins and cadherin have also begun (Batlle et al., 2000; Bolos et al., 2003; Chen et al., 2000; Comijn et al., 2001; Li and Mrsny, 2000; Mankertz et al., 2000).

3. Regulation of TJ dynamics by transcriptional regulation of tight junction proteins

Previous studies from our laboratory and others have revealed that cytokines down-regulate the transcription of the junction proteins in primary Sertoli cells and the human intestinal cell line, HT-29/B6 (Hellani et al., 2000; Lui et al., 2001; Mankertz et al., 2000; Siu et al., 2003a). This cytokine-mediated transcriptional repression resulted in the reduced expression of junction proteins, concomitant with an increase in barrier permeability.

Although major advances have been made in the past 20 years in identifying the components of TJs as well as the signaling pathways involved in the regulation of TJ dynamics (for reviews, see Cheng and Mruk, 2002; Lui et al., 2003d, 2003e; Mruk and Cheng, 2004), the transcriptional regulation of TJ proteins involved in modulating TJ junction dynamics is limited to the transmembrane proteins such as occludin and claudins (Ikenouchi et al., 2003; Luk et al., 2004; Mankertz et al., 2000; Sakaguchi et al., 2002; Wachtel et al., 2001). It is obvious that studies of transcriptional regulation of other TJ protein members, including the peripheral membrane proteins, should be expanded to further our understanding of the precise regulatory mechanism(s) by which TJ dynamics are regulated at the transcriptional level.

3.1. *Occludin*

Occludin, a 64 kDa protein, was the first TJ integral membrane protein identified in many epithelia including the rat seminiferous epithelium (Furuse et al., 1993). Numerous studies have implicated that cytokines such as TNFα and interferon γ (IFNγ) exert negative regulatory effects on the expression of occludin in epithelial cells along with the disruption of TJ barrier monitored by transepithelial resistance (TER) measurement (Hellani et al., 2000; Lui et al., 2001; Mankertz et al., 2000; Siu et al., 2003a; Wachtel et al., 2001). Studies from Mankertz et al. (2000) have identified the promoter sequence essential for the regulation of occludin expression and TJ formation in HT-29/B6 cells. The 208 bp DNA fragment upstream from the putative transcription start site of occludin gene was shown to be necessary and sufficient in mediating the basal promoter activity. It was also shown that TNFα impaired TJ barrier function by lowering the expression of occludin in HT-29/B6 cells through the suppression of the promoter activity (Mankertz et al., 2000). Although a number of potential *cis*-acting motifs pertinent to TNFα-mediated gene transcription, such as NF-IL6 and NF-κB (GGGAGGAGGC, at position 1753), were identified within the human occludin promoter sequences, the detailed intracellular pathway that mediates TNFα-dependent occludin gene repression remains to be elucidated.

Other studies by Wachtel et al. (2001) have shown a similar negative effect of TNFα on occludin gene transcription in astrocytes, but not in brain endothelial cells and Madin–Darby canine kidney cells (MDCK). It was found that TNFα suppressed occludin mRNA level, but not ZO-1 expression in astrocytes and the removal of TNFα from astrocytes could restore basal expression of occludin. The effect of TNFα on occludin expression in astrocytes is mediated through TNFα type-1 receptor and NF-κB. It was suggested that NF-κB might either function as a negative regulator through a direct interaction with the *cis*-acting motif located on occludin promoter or exert an indirect effect by activating a repressor that acted on the occludin promoter (Wachtel et al., 2001).

TNFα could decrease occludin expression via the transcriptional repression in HT-29/B6 cells and astrocytes, but it showed

no effect on brain endothelial cells and MDCK cells. These studies have demonstrated that TNFα represses the occludin promoter in a cell-specific manner. Recent findings in our laboratory have demonstrated that TNFα and TGF-β3 play a crucial role in regulating TJ dynamics in the testis. They have been shown to exert a negative effect on occludin transcription, which in turn reduces the occludin protein level thus leading to an increase in the testicular barrier permeability (Lui et al., 2001, 2003c; Siu et al., 2003a). The effect of TGF-β3 on occludin expression has been confirmed recently in in vivo studies (Lui et al., 2003f); however, the molecular mechanism on how TNFα and TGF-β3 down-regulate the occludin promoter activity in Sertoli cells is not clearly known. Work remains to be done to elucidate the transcriptional repression machinery and the significance of the transcriptional repression of occludin in modulating TJ dynamics in the testis.

Recent studies have demonstrated that the Snail superfamily of repressors encoding transcription factors of the zinc-finger type are involved in transcriptional regulation of occludin (Ikenouchi et al., 2003). The two members of this superfamily, Snail and Slug, were found to repress the occludin transcription in many epithelial cells including the human breast cancer cell, MCF7 and the mammary epithelial cell, Eph4 (Ikenouchi et al., 2003; Ohkubo and Ozawa, 2004). Most of the studies of Snail and Slug have been focused on their role in the epithelium-mesenchyme transition (for reviews, see Nieto, 2002; Thiery, 2002). The functional importance of Snail superfamily in spermatogenesis has not been investigated. In this regard, it is noteworthy to examine whether Snail or Slug could elicit a stage-specific expression of junction proteins in the seminiferous epithelial cycle. Would Snail and Slug be involved in the complete shutdown of occludin expression at the stages VIII-IX of the epithelial cycle, when the BTB is disassembled to allow the translocation of preleptotene and leptotene spermatocytes towards the adluminal compartment of the seminiferous epithelium? The molecular function of Snail superfamily in the testis has yet to be defined.

3.2. Claudins

Similar to occludin, claudins are also integral membrane proteins (∼23 kDa) found at the sites of TJs in both epithelia and endothelia (for reviews, see Heiskala et al., 2001; Morita et al., 1999a; Tsukita and Furuse, 1999). They are from large gene family with more than 20 members (for reviews, see Morita et al., 1999a; Tepass, 2003). Different mammalian tissues have their own combination of claudin members (Morita et al., 1999a). For instance, several claudin members have been found in the testis and they are claudin-1, -3, -4, -5, -7, -8, and -11 (Tsukita et al., 2001). Among them, claudin-11 is highly expressed in the testis and is an important TJ building block that constitutes TJ strands between Sertoli cells in the testis (Gow et al., 1999; Morita et al., 1999b).

Studies from Ikenouchi et al. and Ohkubo et al. have unraveled the importance of Snail in the transcriptional regulation of claudins (Ikenouchi et al., 2003; Ohkubo and Ozawa, 2004). The promoters of mouse claudin-3, -4 and -7 were characterized and each promoter has at least six E-boxes (Ikenouchi et al., 2003). The E-box motif, with sequence [CA(G/C)(G/C)TG], is identical to the binding sites for Snail (Ikenouchi et al., 2003). When Snail expression vector and claudin reporter constructs were co-transfected into Eph4 cells, the promoters of claudin-3, -4 and -7 were repressed remarkably. While co-transfection of mutant Snail lacking the N-terminal SNAG domain, which is essential for the repressor activity, the repression effect of Snail on claudin-7 promoter was impaired (Ikenouchi et al., 2003). These studies clearly illustrated that the transcription of claudins was directly regulated by Snail through modulating their promoter activities. However, studies from Ohkubo and Ozawa (2004) have shown that Snail is involved in direct transcriptional repression of occludin, but not claudin-1. It was found that overexpression of Snail in MDCK cells could only decrease the protein synthesis of claudin, suggesting that Snail down-regulated claudin-1 through the control of post-transcriptional events (Ohkubo and Ozawa, 2004). Such discrepancy in the role of Snail on transcriptional regulation of claudin genes as revealed by the two studies might be related to the use of different cell lines as well as the members of claudin gene chosen for study.

In view of the physiological significance of Snail superfamily in transcriptional regulation of TJ components, it is important that Snail and Slug should be studied more vigorously in the testis for the purpose of elucidating their role in the migration of preleptotene/leptotene spermatocytes across the BTB during the late stage VIII and early stage IX of the epithelial cycle.

Apart from Snail, several transcription factors such as the β-catenin/Tcf complex, Cdx homeodomain proteins/hepatocyte nuclear factor-1α and Sp1 were reported to bind directly to claudin-1, -2 and -19 promoters, respectively (Luk et al., 2004; Miwa et al., 2000; Sakaguchi et al., 2002). For instance, β-catenin/Tcf complex binds the two putative Tcf4 binding elements in the 5′ flanking region of claudin-1 to activate transcription (Miwa et al., 2000). HNF-1α enhances Cdx2-mediated activation of claudin-2 promoter in Caco-2 cells and HNF-1α is an organ-specific regulator of claudin-2 expression in the liver (Sakaguchi et al., 2002). These findings indicate that expressions of different claudin members in different tissues are under unique and sophisticated regulatory control.

Previous studies from our laboratory have shown that TGF-β3 can down-regulate the expression of claudin-11 in cultured Sertoli cells at the time of TJ assembly, which in turn perturbs the TJ permeability barrier (Lui et al., 2001). These results thus suggest claudin-11 plays a vital role in the formation and maintenance of TJ barrier in the testis. Recent studies have also revealed that follicle stimulating hormone (FSH) and TNFα exert a negative effect on claudin-11 transcription in mouse Sertoli cells (Hellani et al., 2000). It was also found that the FSH-driven transcriptional repression of claudin-11 gene is mediated through the cAMP/protein kinase A pathway (Hellani et al., 2000). It is believed that a unique transcriptional mechanism might exist to confine the tissue-specific expression of claudin-11 in the testis and brain. Such a postulate is supported by the observation that organ-specific transcription factor such as HNF-1α is involved

in regulating the expression of claudin-2 in the liver, but not in other tissues (Sakaguchi et al., 2002).

Several *cis*-acting motifs including NY-F and GATA motifs have been identified to be involved in the activation of mouse claudin-11 transcription. Gel-shift analysis and co-immunoprecipitation studies have shown that NY-F, GATA-1 and CREB form a transcriptional complex and bind to the same GATA/NY-F overlapping motif on mouse claudin-11 promoter, and overexpression of these transcription factors significantly increased the claudin-11 promoter activity (Lui et al., unpublished observation). These findings clearly suggest that the transcriptional machinery of claudin-11 gene is different from the other claudin members. In the proximal claudin-11 promoter, some testis-specific *cis*-acting motifs such as SRY were identified by sequence analysis. It is noteworthy to determine how SRY plays a role in regulating the expression of claudin-11 gene in the adult testis as it has been suggested that during the testicular development in the fetus, the expression of claudin-11 gene is probably under the control of SRY (Hellani et al., 2000).

4. Regulation of AJ dynamics by transcriptional regulation of adherens junction proteins

Ectoplasmic specializations (ES) are specialized actin-based cell–cell AJs unique to the testis. Cadherin-catenin, nectin/afadin and integrin/laminin complexes are the interlocking protein complexes that can be found at the ES in the testis (for reviews, see Cheng and Mruk, 2002; Lui et al., 2003e; Mruk and Cheng, 2004). They can be found between Sertoli cells at the basal region of the seminiferous epithelium (basal ES) as well as at the apical region of the seminiferous epithelium where developing and mature spermatids attach onto Sertoli cells (apical ES). The turnover of ES permits the movement of spermatocytes across the epithelium and allows the release of mature spermatids from the seminiferous epithelium (for reviews, see Cheng and Mruk, 2002; Mruk and Cheng, 2004). Previous studies in our laboratory have identified biomolecules such as Rho GTPases and signaling pathways such as integrin-linked kinase that are involved in AJ disassembly and reassembly in the testis (Lui et al., 2003a; Siu et al., 2003b).

Studies from this and other laboratories have demonstrated that the transcriptional regulation of AJ components also plays a critical role in modulating the expression of AJ proteins on epithelial cells, which results in the destruction of cell junctions (Batlle et al., 2000; Cano et al., 2000). Understanding the transcriptional mechanism by which transcription factors act on the promoters of AJ components in other epithelial cells will provide a useful guideline on similar studies in the testis and the modulation of AJ dynamics in the testis.

4.1. Cadherin

Previous studies have shown that the loss of cadherin expression is responsible for the breakdown of intercellular adhesion, suggesting that the regulation of cadherin gene transcription is one of the predominant mechanisms to control the AJ dynamics in epithelial cells (Perl et al., 1998).

The transcriptional regulation of cadherin was extensively studied in the field of cancer biology since the down-regulation of E-cadherin expression is highly pertinent to the development of tumors and their progression (Birchmeier and Behrens, 1994; Takeichi, 1993). Recent studies have identified several transcriptional factors including bHLH factor E12/E47, the two-handed zinc factors ZEB-1 (δEF1) and ZEB-2 (SIP-1) that are involved in transcriptional repression of the cadherin gene (Comijn et al., 2001; Grooteclaes and Frisch, 2000; Perez-Moreno et al., 2001). Interestingly, all these transcriptional repressors act through the interaction with specific E-boxes on the proximal promoter of cadherin, resulting in down-regulation of cadherin expression (Comijn et al., 2001; Grooteclaes and Frisch, 2000; Perez-Moreno et al., 2001).

There is emerging evidence showing that the Snail superfamily of transcriptional factors are involved in the regulation of cadherin gene transcription (for reviews, see Nieto, 2002; Thiery, 2002). Studies from several laboratories have demonstrated that Snail and Slug zinc-finger proteins repressed the endogenous E-cadherin expression in a panel of epithelial tumor cell lines of different origins, ranging from bladder carcinoma to breast carcinoma (Batlle et al., 2000; Hajra et al., 2002; Kurrey and Bapat, 2005). The Snail- and Slug-mediated repression pathways act through the putative E-box motifs on the E-cadherin proximal promoter (Batlle et al., 2000; Bolos et al., 2003; Ikenouchi et al., 2003). This specific E-cadherin repression mechanism has been unraveled by Peinado et al. It was found that Snail mediated E-cadherin repression by the recruitment of the Sin3A/histone deactylase 1 (HDAC1)/HDAC2 complex at the E-cadherin promoter (Peinado et al., 2004). At there, HDAC1/HDAC2 deacetylated the histone H3 and H4 proteins. This suggests that Snail mediates chromatin remodeling and histone modifications to repress the cadherin expression (Peinado et al., 2004).

4.2. Nectin

Nectin is a newly identified AJ integral membrane protein. Much of the works performed previously focused on the identification of its interacting partners and localization. Until recently, the gene knockout studies have illustrated that nectin-2 is a major component of the ES in the testis and plays a crucial role in spermatogenesis (Bouchard et al., 2000). Loss of nectin-2 in male mice results in infertility. Proper formation and destruction of nectin-2-based AJs between Sertoli cells and germ cells allow the movement of developing germ cells (Bouchard et al., 2000; Ozaki-Kuroda et al., 2002).

In our laboratory, we have recently isolated the mouse nectin-2 promoter for detailed characterization. It was found that Sp1 and cAMP response element (CRE) motifs at the proximal promoter played a crucial role in regulating gene transcription. Interestingly, gel-shift assays, overexpression analysis and chromatin immunoprecipitation assays have unequivocally shown that not only CREB protein interacts with the CRE *cis*-acting motif, c-Jun, but not c-fos, also acts through the CRE motif to up-regulate the nectin-2 transcription in Sertoli cells (Lui et al, unpublished observation). This transcriptional regulation is functionally significant to the testicular physiology as cyclic

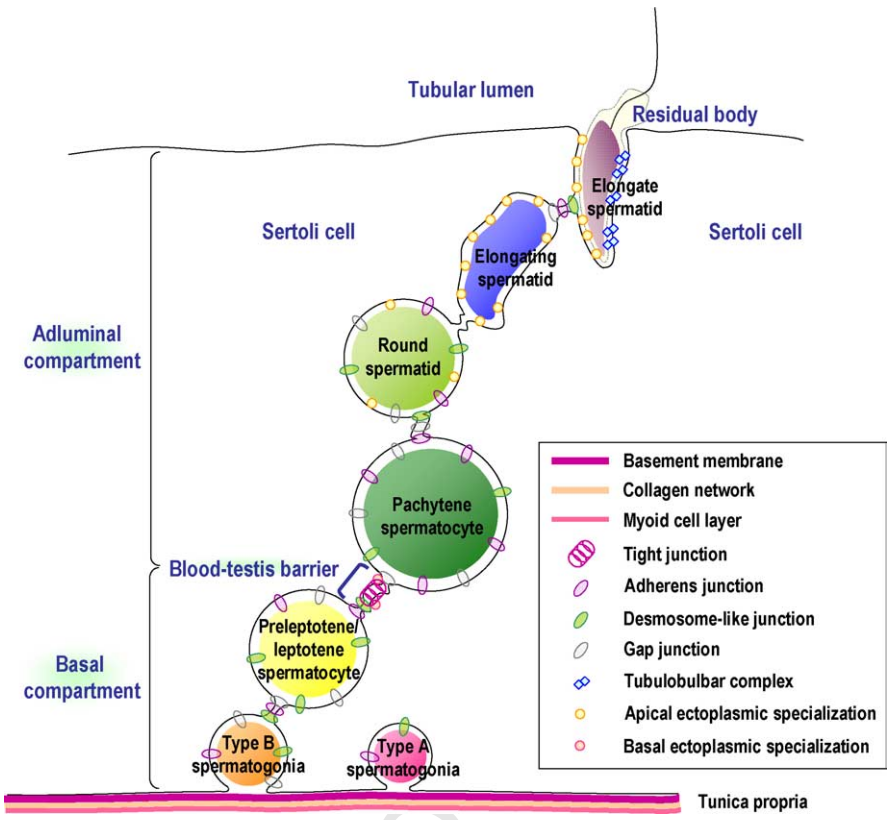

Fig. 1. A schematic diagram illustrating the location of the cell junctions between Sertoli cells and different types of germ cells in the seminiferous epithelium. The blood–testis barrier, which is constituted by tight junctions, divides the epithelium into the basal and adluminal compartment. Anchoring junctions that are present in the seminiferous epithelium including (i) cell–cell actin-based adherens junctions such as ectoplasmic specialization and the tubulobulbar complex, (ii) cell–cell intermediate filament-based desmosome-like junctions. The dynamics rearrangement of cell junctions is essential to allow the movement of germ cells from the basal compartment to the adluminal compartment to complete spermatogenesis.

expression of CREB in the seminiferous epithelium concomitant with the expression of nectin-2 gene was observed when isolated staged seminiferous tubules were analysed (Don and Stelzer, 2002; Waeber et al., 1991).

5. Regulation of junction dynamics via post-translational modification of junction protein at the site of cell–cell contact

Cell junctions between Sertoli–Sertoli and Sertoli–germ cells are dynamically modulated and such changes can result in the translocation of differentiating germ cells from the basal to the adluminal compartment of the seminiferous epithelium for spermiation. Undoubtedly, transcriptional regulation of the junction components is an important mechanism to control the expression of the junction proteins (Ivanov et al., 2005; Nieto, 2002; Thiery, 2002). Still, the fate of the existing junction proteins at the site of cell–cell contact is a major determinant factor on junction dynamics. For instance, remodeling of junction proteins during epithelial morphogenesis occurs in 1 h (Schock and Perrimon, 2002), whereas the half-lives of the junction proteins are much longer, up to 12 h for occludin (Wong and Gumbiner, 1997). The disparity between the stability of junction proteins and rapid junction remodeling apparently suggests that the transcriptional regulation of the junction protein is not the sole mechanism to achieve rapid remodeling of the cell junctions.

Ubiquitination and endocytosis are recognized as the two essential mechanisms of targeted protein degradation whereby the targeted proteins are removed by the proteasome and the lysosomal system, respectively (for reviews, see Le Roy and Wrana, 2005; Takei et al., 2005; Wilkinson, 2000). There is growing evidence that ubiquitination and endocytosis of junction proteins at the site of cell–cell contact are effective mechanisms for the remodeling of cell junctions in dynamic situations, where junctions must be rapidly broken and reassembled (Fujita et al., 2002; Kamei et al., 1999; Le et al., 1999; Lui and Lee, 2005; Paterson et al., 2003; Taya et al., 1998; Traweger et al., 2002).

Herein, we attempt to review (i) the mechanism of protein ubiquitination and deubiquitination, (ii) the covalent attachment of ubiquitin to the targeted junction proteins or removing ubiquitin to rescue degradation, and (iii) endocytosis of cell junction proteins (Fig. 1).

6. Ubiquitination

The ubiquitin conjugation system is composed of ubiquitin and three enzymes namely ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin-ligase (E3) (for reviews, see Hershko and Ciechanover, 1998; Hicke, 2001).

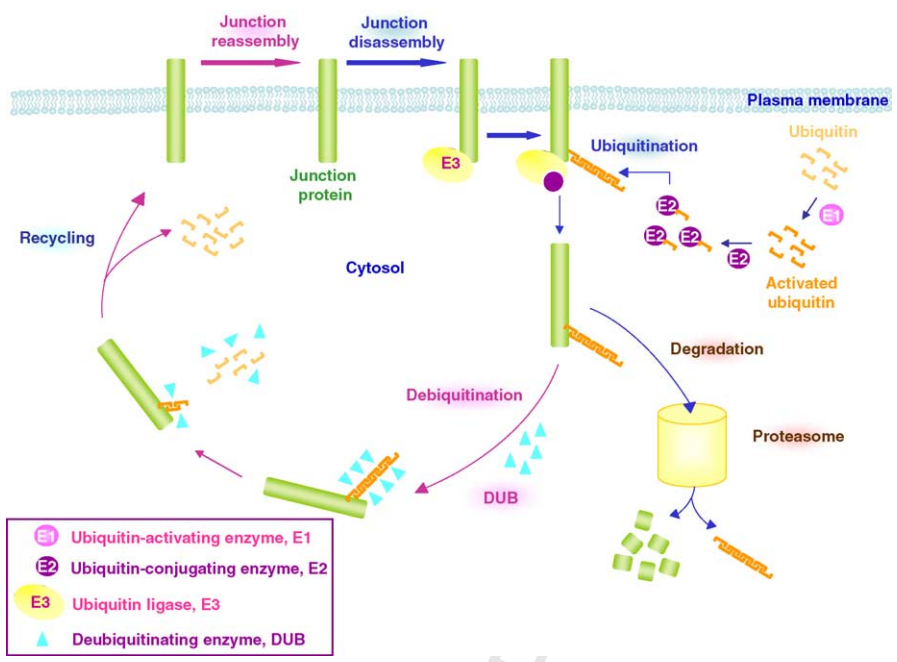

Fig. 2. A schematic drawing illustrating the dynamic regulation of junction proteins through ubiquitination and deubiquitination. At steady-state, junction proteins are organized at the site of cell–cell contacts. Under certain conditions such as cAMP-mediated junction disassembly, E3 ligase recognizes and binds to the specific junction protein substrate. Meanwhile, E1 activates ubiquitin and the activated ubiquitin is then conjugated with E2. With the aid of E2, E3 ligase functions to mediate the transfer of ubiquitin chains to the junction protein. The ubiquitinated protein may either be degraded into short peptides by proteasome with the release of ubiquitin chain, or undergo deubiquitination by deubiquitinating enzymes (DUBs). DUBs act on the ubiquitin chain and cleave the ubiquitin chain from that protein, resulting in rescuing the protein from degradation. The junction protein can be recycled back to the cell surface, which in turn facilitates the reassembly of cell junctions.

Ubiquitin, an evolutionary conserved protein with 76 amino acid residues, is first activated through the glycine residue in its C-terminus by E1. An E1-ubiquitin intermediate is formed with a high-energy thioester bond. The activated ubiquitin then passes a thiol group onto one of the E2. The E2 when bound to an E3 ligase transfers activated ubiquitin to the target protein (Fig. 2). Most organisms contain one or two E1s since E1 is able to carry out the activation of ubiquitin for all modifications, whereas E2 and E3 have their substrate specificities. Each E2 transfers ubiquitin specifically to a single or several E3 ligases, whilst each E3 ligase can associate with unique protein substrates through recognizing similar motifs (for review, see Pickart, 2001).

The protein substrate tagged with a polyubiquitin chain is then recognized and degraded into short peptides by the 26S proteasome complex. The ubiquitin is then released and recycled (for reviews, see Hicke, 2001; Pickart, 2001) (Fig. 2).

7. Deubiquitination

Ubiquitination can be reversed by members of a large family of enzymes known as isopeptidases or deubiquitinating enzymes (for reviews, see Kim et al., 2003; Wilkinson, 2000; Wing, 2003). Generally, the deubiquitinating enzymes can be divided into two main types (for review, see Wing, 2003). The first group functions to regenerate ubiquitin from proteolytic remnants produced by the proteasomes, thereby to speed up the proteasome-dependent proteolysis. The second group of the deubiquitinating enzymes is responsible for the reverse process of ubiquitin conjugation. In the process of deubiquitination, the enzymes cleave the isopeptide bond of poly-ubiquitin chain proximal to the target proteins or hydrolyze the poly-ubiquitin chains into ubiquitin monomers, and thereby prevent the degradation of protein by the proteasome (Hochstrasser, 1995). By the action of the deubiquitinating enzymes, the ubiquitinated protein which is originally destinated to the degradation process can then be rescued (Fig. 2).

8. Attachment of ubiquitin to the targeted proteins for degradation by proteasome

Using yeast two-hybrid screening, an E3 ubiquitin ligase Itch was identified to bind specifically to the NH_2-terminal portion of occludin (Traweger et al., 2002). This novel interaction between Itch and occludin is involved in the ubiquitination of occludin in MDCK cells, and the degradation of occludin is sensitive to proteasome inhibition (Traweger et al., 2002). Such interaction can also be found in the Sertoli cells by which TJs are dynamically rearranged to allow the movement of germ cells in the seminiferous epithelium (Lui and Lee, 2005). In addition, a novel interaction between Itch and UBC4 (an ubiquitin-conjugating enzyme) on occludin was detected by co-immunoprecipitation. Using the cAMP-mediated TJ disruption model, we have shown that an increase in protein levels of Itch and UBC4 along with a significant reduction in endogenous occludin was detected when TJs were disrupted by dibutyryl-cAMP (db-cAMP). Addition of MG-132 (a proteasome inhibitor) could prevent db-cAMP-induced TJ disruption by altering the rate of occludin degradation (Lui and Lee, 2005). These studies support the notion that

the ubiquitination of TJ protein is one of the regulatory mechanisms in modulating the junction dynamics in the testis.

Recently, a specific E3 ubiquitin ligase targeted to E-cadherin was identified by yeast two-hybrid system (Fujita et al., 2002). This E3, known as Hakai, contains SH2, RING, zinc-finger and proline-rich domains. Hakai interacts with E-cadherin in a tyrosine phosphorylation-dependent manner and induces ubiquitination of the E-cadherin complex (Fujita et al., 2002). The activation by tyrosine kinases induces the tyrosine phosphorylation of the E-cadherin, which in turn attracts Hakai. Such interaction triggers the ubiquitination and degradation of the E-cadherin complex (Fujita et al., 2002; Pece and Gutkind, 2002).

Apart from the identification of E3 ubiquitin ligases specific to the integral protein of cell junctions, it has been reported that F-box/WD40-repeat protein functions as an E3 ubiquitin ligase to regulate the ubiquitination of β-catenin, a peripheral protein associated with cadherin at the AJ (Hart et al., 1999; Jiang and Struhl, 1998; Latres et al., 1999; Marikawa and Elinson, 1998). The ubiquitinated β-catenin is recognized by the proteasome and rapidly degraded. Several studies have illustrated the function of F-box/WD40-repeat protein on the degradation of β-catenin, however, all studies focused on its effect on growth and development via the Wingless-Wnt signaling pathway (Hart et al., 1999; Jiang and Struhl, 1998; Latres et al., 1999; Marikawa and Elinson, 1998). Therefore, the effect of E3-ligase-mediated β-catenin degradation on cell adhesion has not been fully characterized.

9. Deconjugating ubiquitin from the multiubiquitinated substrate to avoid protein degradation

Protein ubiquitination is a reversible process, and it has become increasingly obvious that ubiquitin deconjugation plays an important role in rescuing the ubiquitinated proteins from proteolysis (for reviews, see Kim et al., 2003; Wilkinson, 2000; Wing, 2003).

In the ubiquitin-proteasome pathway, the ubiquitinated substrates either undergo degradation by the proteasome or stabilization through the action of the deubiquitinating enzyme, suggesting that the bioavailability of proteins is tightly regulated by the action of the ubiquitinating and deubiquitinating enzymes (for review, see Wilkinson, 2000). As a result, the disassembly and reassembly of cell junctions could be manipulated by the precise regulation on the junction protein levels at the site of cell–cell contacts through the specific pathways of ubiquitination and deubiquitination (Fig. 2).

Studies by Taya et al. (1998) have shown that one of the deubiquitinating enzymes, Fam, is capable of regulating the degradation of peripheral component of nectin-based cell–cell adhesion. Immunofluorescence staining has shown that Fam is specifically localized at the sites of cell–cell contact in confluent MDCKII cells. Fam not only co-localizes with AF-6 (afadin), but also interacts with AF-6 in vitro and in vivo. More importantly, Fam can exert its deubiquitinating activity in vivo to release ubiquitin from the ubiquinated AF-6, suggesting that the degradation of AF-6 at the site of cell–cell adhesion is regulated by Fam (Taya et al., 1998).

Apart from deubiquitinating the ubiquitinated AF-6, other studies have demonstrated that Fam also interacts with another AJ peripheral components, β-catenin. It was found that Fam could stabilize β-catenin by inhibiting its degradation, thereby prolonging the half-life of β-catenin (Taya et al., 1999). Although the detailed mechanism underlying the stabilization of β-catenin by Fam has not been fully elucidated, it is presumably through the deubiquitination of β-catenin.

It is obvious that the degradation of peripheral membrane proteins AF-6 and β-catenin are regulated through the FAM-mediated deubiquitination. However, virtually no deubiquitinating enzyme specific to the integral membrane protein of AJs and TJs has been identified. Work should be expanded to identify the candidates involved in deubiquitination of the junction proteins as well as to understand the precise regulatory mechanism(s) by which junction dynamics are regulated utilizing these deubiquitinating enzymes.

Although the exact mechanisms of ubiquitination and deubiquitination are not fully understood, the junction proteins at the site of cell–cell contacts are in an ubiquitination-deubiquitination equilibrium. Thus, the activity and the expression of the E3 ligases and the deubiquitinating enzymes at the site of cell–cell contacts play a crucial role in affecting this equilibrium, which in turn modulates the junction dynamics (Fujita et al., 2002).

10. Endocytosis of junction proteins

The level of junction proteins at the site of cell–cell contacts could be modulated by transcriptional regulation and/or protein degradation through the ubiquitin-proteasome pathway (Thiery, 2002). The endocytosis and recycling of junction proteins have recently emerged as an alternative mechanism allowing cells to undergo rapid changes in morphology in response to extracellular stimuli (Kamei et al., 1999; Le et al., 1999; Paterson et al., 2003) (Fig. 3).

Studies from Ivanov et al. have demonstrated that internalization of TJ proteins such as occludin, JAM-1, claudins-1 and -4 were observed in T84 epithelial cells by proteinase protection assay and immunocytochemistry (Ivanov et al., 2004). Addition of pharmacological inhibitors of clathrin-mediated endocytosis blocked the process, suggesting the endocytosis is clathrin-dependent. However, those TJ proteins are targeted neither for recycling nor for degradation in lysosomes. It was proposed that the endocytosed TJ proteins were shuffled into a unique storage compartment, hence providing a new mechanism to disrupt the TJ barrier (Ivanov et al., 2004).

Matsuda et al. have shown that the dynamic remodeling of TJs involves the elongation and shortening of individual TJs between two adjacent cells during intercellular motility (Matsuda et al., 2004). During the shortening of the individual TJs, vesicular structures containing claudin-3 were found in the cytoplasm. Interestingly, occludin, ZO-1 and JAM, which are the major building blocks of TJs, were not detected in the claudin-containing vesicles, suggesting that claudin-3, but not other TJ components were selectively segregated during TJ internalization (Matsuda et al., 2004). These results suggest that distinctive

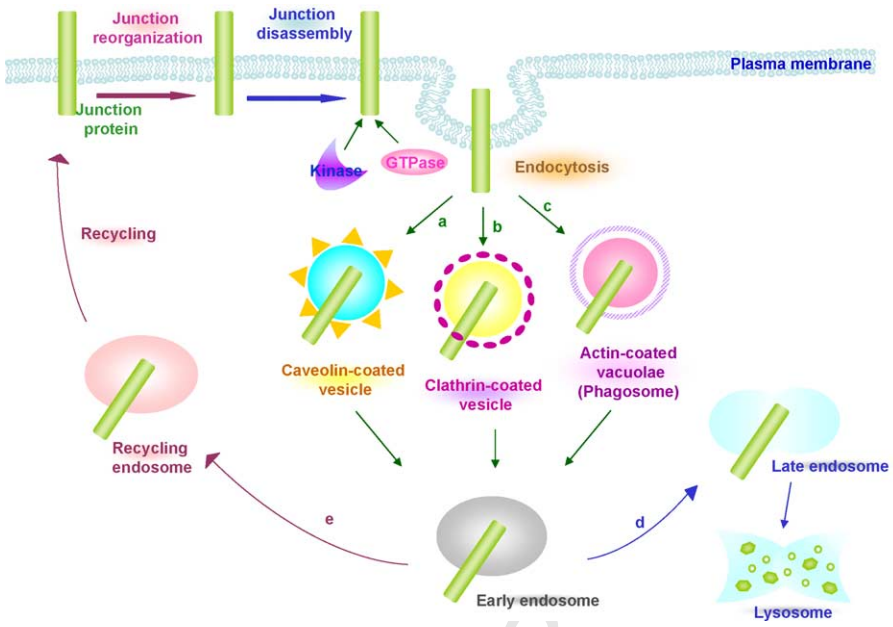

Fig. 3. Disassembly and reassembly of cell junctions are mediated by endocytosis and recycling of cell junction proteins. Junction proteins are internalized via different endocytic structures including caveolin-coated vesicle (a), clathrin-coated vesicle (b) and actin-coated vacuolae (phagosome) (c). Internalized proteins are delivered into early endosomes. Subsequently they are delivered to either late endosome which targets for degradation in lysosome (d) or recycling endosome for channeling back to the cell surface (e).

internalization of claudins plays a crucial role in the remodeling of TJs and the selective regulation of claudin endocytosis is important in intercellular motility (Matsuda et al., 2004).

Endocytosis not only plays a role in TJ dynamics, its effect on the regulation of AJ dynamics is well-documented (Kamei et al., 1999; Le et al., 1999; Palacios et al., 2001, 2002). For instance, using surface biotinylation and recycling assays, Le et al. have shown that some of the E-cadherin at the cell surface are actively internalized and then recycled back to the plasma membrane through the classic clathrin-mediated endocytosis pathway (Le et al., 1999). When cells were cultured in Ca^{2+}-depleted medium, a significant increase in the endocytosis and recycling cell surface E-cadherin was observed. The reassembly of AJs by Ca^{2+}-repletion was inhibited by bafilomycin-mediated disruption of the endocytosed E-cadherin recycling (Le et al., 1999). These results support the notion that endocytosis and the recycling of the junction proteins are involved in the regulation of junction dynamics.

Subsequent studies have also identified the signaling cascades and biomolecules involved in the internalization of AJ proteins (Kamei et al., 1999; Palacios et al., 2001, 2002; Paterson et al., 2003). The diversity of the internalization mechanisms for junction protein has already been covered in an excellent recent review (Ivanov et al., 2005). Readers are strongly encouraged to seek additional information on these subject areas from this article.

Previous studies in our laboratory have demonstrated that RhoB GTPase are also involved in regulating Sertoli–germ AJ dynamics (Lui et al., 2003a). Several small GTPases such as Rho and Rab family members are involved directly and indirectly in the endocytosis and recycling of E-cadherin in several epithelial cells (Kamei et al., 1999; Palacios et al., 2001, 2002; Paterson et al., 2003). For instance, the activation of ARF6 in MDCK cells promotes the clathrin-dependent internalization of E-cadherin, resulting in the disassembly of AJs without the remodeling of actin filament (Palacios et al., 2001). Recently, the molecular mechanism of ARF6 on AJ disassembly has been identified. It was found that ARF6-GTP interacted with and recruited Nm23-H1, a nucleoside diphosphate kinase, to facilitate dynamin-mediated endocytosis during AJ disassembly (Palacios et al., 2002). All these studies clearly suggest small GTPases play an important role in regulating the endocytosis of junction proteins, resulting in junction remodeling.

11. Concluding remarks

In this review, we have summarized some of the recent findings in the study of junction dynamics in epithelial cells, some of the potentially important regulators such as E3 ligase, and regulatory pathways of junction dynamics recently identified in the testis. It is obvious that many questions remain to be addressed. For instance, the precise transcriptional regulation in controlling the testis-specific expression of junction proteins and transcriptional repression of TJ proteins at the stages VIII and IX, by which preleptotene and leptotene spermatocytes traverse the BTB, remain unknown.

Studies of the effect of ubiquitination on junction dynamics in the seminiferous epithelium are very limited, apart from a recent study assessing the role of Itch (E3 ligase) on the Sertoli TJ barrier in vitro. This apparently is a priority area that needs to be further investigated in the near future. As such, the identification of specific E3 ligases targeted to different junction proteins and the elucidation of the precise regulatory mechanisms are needed to be addressed.

Another interesting topic that deserves further investigation is whether endocytosis plays a role in modulating the junction dynamics in the testis. Many studies have demonstrated that endocytosis of junction protein is a rapid and effective way in reorganizing cell junctions. This information from the studies of other epithelial cells provides a blueprint for us to examine the role of endocytosis on junction dynamics in the seminiferous epithelium.

A thorough understanding of germ cell migration during spermatogenesis, in particular, the cell junction dynamics in the seminiferous epithelium, would allow the identification of new targets for non-hormonal male contraceptive development. For instance, selective repression of the junction protein expression at the apical ES, such as nectin-2, might induce premature release of spermatids into the tubular lumen via the transcriptional regulation. Alternatively, if prolonged expression of junction proteins could be procured, germ cells might be trapped in the seminiferous epithelium for an extended period leading to apoptosis. This post-meiotic approach of male contraception can be achieved by the identification of the testis-specific transcription factors that are involved in regulating junction protein expression in the seminiferous epithelium.

The precise control of junction protein turnover in the seminiferous epithelium provides another line of potential for male contraceptive development. For example, alteration of ubiquitination or endocytosis of junction proteins will interfere the dynamic control of junction disassembly and reassembly in the seminiferous epithelium and thus may cause the loss of fertility in the male.

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