PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B

Phil. Trans. R. Soc. B (2010) **365**, 1621–1635
doi:10.1098/rstb.2010.0010

Review

Tight junctions in the testis: new perspectives

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In the testis, tight junctions (TJs) are found between adjacent Sertoli cells at the level of the blood–testis barrier (BTB) where they coexist with basal ectoplasmic specializations and desmosome-gap junctions. The BTB physically divides the seminiferous epithelium into two distinct compartments: a basal compartment where spermatogonia and early spermatocytes are found, and an adluminal compartment where more developed germ cells are sequestered from the systemic circulation. In order for germ cells (i.e. preleptotene spermatocytes) to enter the adluminal compartment, they must cross the BTB, a cellular event requiring the participation of several molecules and signalling pathways. Still, it is not completely understood how preleptotene spermatocytes traverse the BTB at stage VIII of the seminiferous epithelial cycle. In this review, we discuss largely how TJ proteins are exploited by viruses and cancer cells to cross endothelial and epithelial cells. We also discuss how this information may apply to future studies investigating the movement of preleptotene spermatocytes across the BTB.

Keywords: testis; blood–testis barrier; tight junction

1. INTRODUCTION

The functional unit of the testis is the seminiferous tubule where spermatozoa are produced from spermatogonia through a step-wise process known as spermatogenesis which spans the entire reproductive cycle of the male (de Kretser & Kerr 1988; Kerr *et al.* 2006). Critical to spermatogenesis is the blood–testis barrier (BTB), a structure situated above migrating preleptotene spermatocytes that is constituted by different types of junctions present between adjacent Sertoli cells (Russell & Peterson 1985; Cheng & Mruk 2002; Mruk & Cheng 2004*a*; Kerr *et al.* 2006) (figure 1). The BTB physically divides the seminiferous epithelium into a basal and an adluminal compartment and provides two unique environments for germ cell development (Dym & Fawcett 1970; Fawcett *et al.* 1970; Setchell & Waites 1975). In the basal compartment, spermatogonia reside in spermatogonial niches (i.e. composed of the basal portion of Sertoli cells and the basement membrane), which are found at the periphery of seminiferous tubules at sites where three different tubules come into proximity, and three different types of spermatogonia have been identified morphologically: (i) single cells (A_s), (ii) proliferating cells (A_{pr} and A_{al}) and (iii) differentiating cells (A_{1-4}, Int and B). In brief, A_s cells divide by mitosis first to replenish and to maintain a constant pool of stem cells within spermatogonial niches, and second to produce A_{pr} spermatogonia which undergo a series of mitotic divisions to form chains of A_{al} spermatogonia that are linked by intercellular bridges. (At present, it is not yet clear if all cells within the A_s pool are true spermatogonial stem cells because recently several investigators have arrived at counts that were significantly lower than the ones originally reported, that is, only ~1/12–1/15 of the A_s pool appears to be composed of true spermatogonial stem cells; Tegelenbosch & de Rooij 1993; Shinonara *et al.* 2000; Yoshida *et al.* 2004; Nakagawa *et al.* 2007.) This is followed by a series of differentiation steps to yield type B spermatogonia which subsequently develop into primary spermatocytes, (i.e. preleptotene spermatocytes) whose fate is to traverse the BTB at late stage VIII of the seminiferous epithelial cycle for entry into the adluminal compartment. Once in the adluminal compartment, spermatocytes undergo two consecutive rounds of meiosis at stage XIV to ultimately yield haploid spermatids (i.e. spermatozoa) that are released from the seminiferous epithelium at spermiation (Setchell 1978; Russell 1993*a*).

As mentioned above, the BTB physically divides the seminiferous epithelium into two compartments (figure 1), and it is constituted by several different types of coexisting junctions: tight junctions (TJs), basal ectoplasmic specializations (ES) and desmosome-gap junctions (D-GJs) (Vogl *et al.* 1993, 2008). Of these, basal ES and D-GJs are unique to the testis; basal ES combine qualities from classic adherens junctions, focal adhesions and TJs to some extent, whereas D-GJs share characteristics of gap junctions and desmosomes (Russell 1993*b*; Cheng & Mruk 2002; Mruk & Cheng 2004*a*; Mruk *et al.* 2008; Vogl *et al.* 2008). As such, both junction types can be defined as hybrid-like in nature. On the other hand, the molecular backbone of TJs in the testis is

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One contribution of 17 to a Theme Issue 'The biology and regulation of spermatogenesis'.

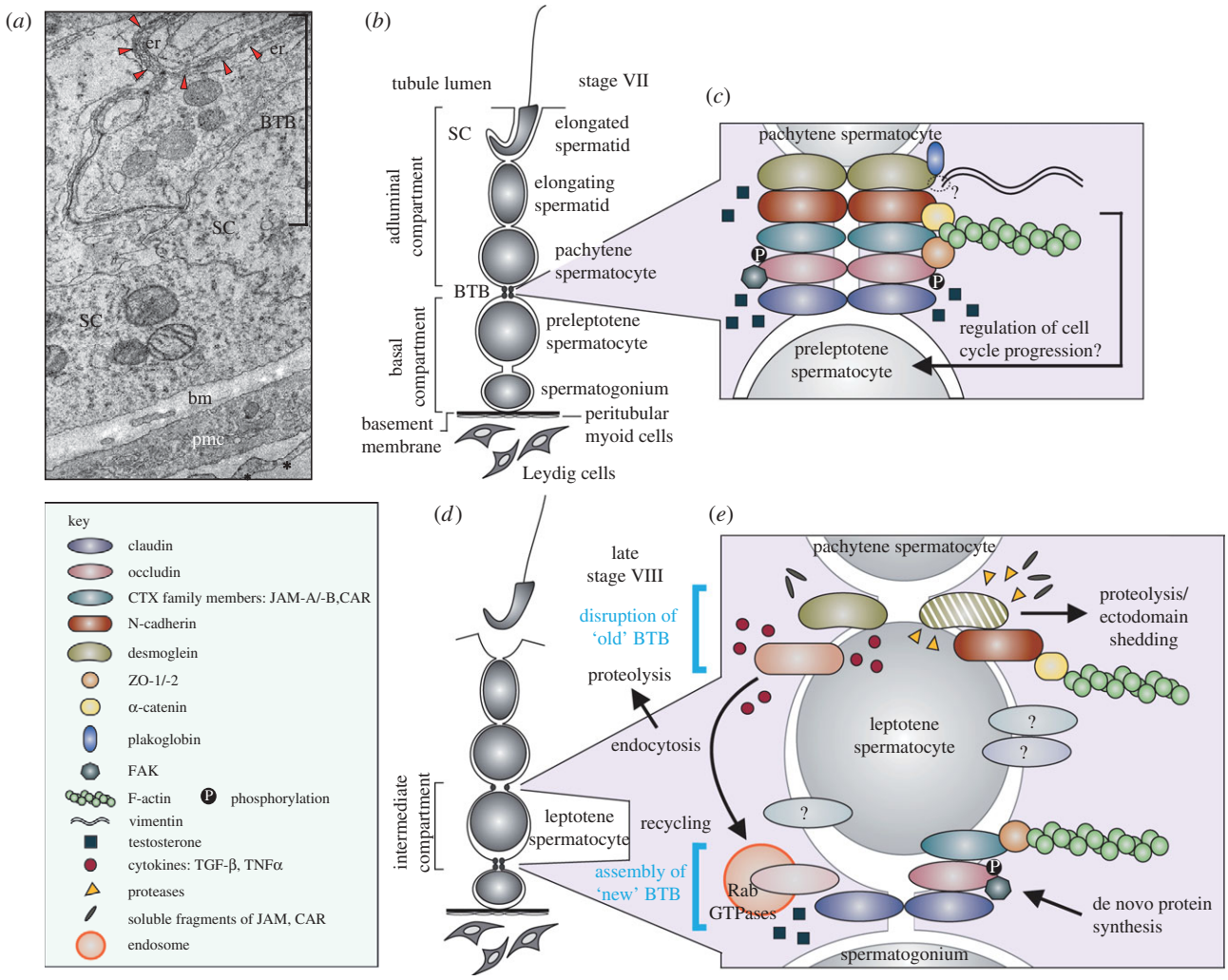

Figure 1. A model describing the movement of preleptotene spermatocytes across the BTB. (*a*) An electron micrograph showing part of the seminiferous epithelium from the adult rat testis. Depicted here are two adjacent Sertoli cells resting on the tunica propria which is composed of the basement membrane (bm), a layer of peritubular myoid cells (pmc) and the lymphatic endothelium (see asterisks). The BTB is typified by coexisting junctions, namely TJs, basal ES and D-GJs. Red arrowheads point to bundles of actin microfilaments sandwiched in between the Sertoli cell plasma membrane and the endoplasmic reticulum (er), and these are characteristic of the basal ES. (*b*) A schematic view of a cross section of the seminiferous epithelium at stage VII of the seminiferous epithelial cycle, showing germ cells (i.e. spermatogonium, preleptotene spermatocyte, pachytene spermatocyte, elongating spermatid and elongated spermatid) at different stages of development. The BTB, which is situated above a migrating preleptotene spermatocyte (see circles), divides the seminiferous epithelium into two compartments: a basal compartment and an adluminal compartment. Sertoli (SC), peritubular myoid and Leydig cells are also depicted. (*c*) A magnified view of the BTB at stage VII of the seminiferous epithelial cycle before a preleptotene spermatocyte transits across the BTB. Different proteins (see key) that constitute the BTB are shown, and these include TJ, basal ES and D-GJ proteins, some of which are phosphorylated. Testosterone synthesized by Leydig cells can upregulate several BTB constituent proteins. Thus, it plays an important role in maintaining barrier function. Desmosome junctions are linked to intermediate filaments through an adaptor such as desmoplakin but as it is not yet known if this protein localizes to the BTB, it is depicted as a dotted line open circle with question mark. As described in the text for viruses and cancer cells, TJ proteins may regulate germ cell cycle progression. (*d*) A schematic view of a cross section of the seminiferous epithelium at late stage VIII of the seminiferous epithelial cycle immediately following spermiation. Shown here is a leptotene spermatocyte within the intermediate compartment. (*e*) A magnified view of the intermediate compartment at late stage VIII of the seminiferous epithelial cycle. During the movement of preleptotene spermatocytes across the BTB, the following cellular events are likely to take place. De novo protein synthesis, as well as testosterone, are likely to facilitate the assembly of a 'new' Sertoli cell barrier below a migrating preleptotene spermatocyte. Protein phosphorylation may also play a critical role in this cellular event. Cytokines released by Sertoli and germ cells regulate BTB constituent proteins by activating proteases, which cleave proteins and/or by initiating endocytosis, which internalizes integral membrane proteins. Endocytosed integral membrane proteins may undergo proteolysis, and soluble fragments of proteins may be produced by ectodomain shedding. These soluble fragments may have functions that are different from their full-length counterparts. Nevertheless, in both cases, the 'old' BTB situated above a leptotene spermatocyte is breached, signalling to preleptotene spermatocytes that they may begin to cross the barrier. Concurrently, proteins that have been endocytosed but not destined for proteolysis may travel to the opposite pole of the leptotene spermatocyte by transcytosis as barrier integrity must be maintained during spermatogenesis. Recycling of endocytosed proteins is probably facilitated by Rab GTPases and other proteins of the endocytic pathway. Before arriving at their final destination (i.e. below a leptotene spermatocyte), it is also possible that endocytosed proteins are transiently inserted back into the Sertoli cell plasma membrane to form homotypic or heterotypic interactions with the migrating germ cell, thereby creating a 'channel.' These events contribute to assembly of a 'new' BTB.

remarkably similar to those found in other epithelia, but their regulation is believed to be unique because TJs in the testis must open transiently to allow passage of preleptotene spermatocytes into the adluminal compartment while still maintaining the homeostasis of the seminiferous epithelium. In other words, the BTB is programmed to restructure cyclically throughout spermatogenesis. Thus far, our understanding of these cellular events is limited because it is difficult to design functional experiments to investigate *how* primary spermatocytes traverse the BTB *in vivo*. Nevertheless, many reports have successfully used primary Sertoli cells cultured *in vitro* to identify the molecules and signalling pathways involved in junction restructuring as an *in vitro* model of germ cell movement is unavailable. The goal of this article is to provide a selective review of the current status of research relating to TJs with emphasis on the testis, focusing on key challenges that should be addressed in future functional studies. For general background information on TJ biology and regulation, readers are asked to refer to the following excellent articles: Anderson & van Itallie (1995, 2008), Cereijido *et al.* (1998), Cereijido & Anderson (2001), Tsukita *et al.* (2001), Matter & Balda (2003), Shin *et al.* (2006), Balda & Matter (2009), Feigin & Muthuswamy (2009) and Findley & Koval (2009).

2. TJ STRUCTURE AND FUNCTION: BACKGROUND

Ultrastructurally, TJs appear as close contact points or 'kisses' between the plasma membranes of adjacent epithelial and endothelial cells, and they have two main functions: TJs function (i) as a 'barrier' to prevent free passage of solutes, ions and water and (ii) as a 'fence' to separate the plasma membrane into apical and basolateral regions, thereby conferring cell polarity (Matter & Balda 1999; Cereijido & Anderson 2001; Tsukita *et al.* 2001; Shin *et al.* 2006; Anderson & van Itallie 2008; Furuse 2010). Thus far, the molecular architecture of the TJ has been largely elucidated, and more than a 100 TJ-associated proteins have been described, including the multi-pass membrane proteins occludin (including MarvelD3, a novel member of the occludin family), claudin and tricellulin (Tsukita *et al.* 1999; Balda & Matter 2000*a*; Heiskala *et al.* 2001; Ikenouchi *et al.* 2005; Furuse & Tsukita 2006; van Itallie & Anderson 2006; Steed *et al.* 2009); single membrane-spanning proteins such as CAR (coxsackie and adenovirus receptor) and JAM (junctional adhesion molecule) that function in cell adhesion and cell movement (Bazzoni 2003; Ebnet *et al.* 2004; Coyne & Bergelson 2005); cytoplasmic scaffolding proteins such as ZO-1 (zonula occludens-1), MAGI-1 (membrane-associated guanylate kinase) and MUPP1 (multi-PDZ (post-synaptic density protein, *Drosophila* disc large tumour suppressor and zona occludens-1 protein) domain protein 1) (Tsukita *et al.* 1999; Gonzalez-Mariscal *et al.* 2000); signalling molecules such as cingulin, PKC-α (protein kinase C-α), PALS1 (protein-associated with lin-7), FAK (focal adhesion kinase) and c-YES (Mitic & Anderson 1998; Clarke *et al.* 2000; Chen *et al.* 2002; Siu *et al.* 2009*a*); and several transcription factors such as Jun, Fos and C/EBP (CCAAT/enhancer binding protein) (Betanzos *et al.* 2004). In addition to these proteins which contribute to barrier and fence, TJ function requires a perijunctional ring of F-actin and myosin, a motor protein that moves along actin while hydrolysing ATP (Hartsock & Nelson 2007; Ivanov 2008; Miyoshi & Takai 2008).

More recently, TJs have also been reported to be involved in other cellular events such as in the regulation of cell proliferation and cell motility (Kavanagh *et al.* 2006). For instance, overexpression of ZO-2 in MDCK cells was shown to downregulate and promote the degradation of cyclin D1 (Gonzalez-Mariscal *et al.* 2009; Tapia *et al.* 2009) (a cell cycle regulator whose activity is required for G1–S phase cell cycle progression; Fu *et al.* 2004; Klein & Assoian 2008; Wolgemuth 2008), thereby inhibiting proliferation (Tapia *et al.* 2009). These results reveal that ZO-2 also functions as a tumour suppressor. Interestingly, cyclin D1 is a target gene of ZONAB/DbpA (Sourisseau *et al.* 2006), a Y-box transcription factor which can also be recruited to the TJ by binding to ZO-1 (Balda & Matter 2000*b*), thereby sequestering ZONAB in the cytoplasm and suppressing cell proliferation (Balda *et al.* 2003). Indeed, TJs are known to sequester many transcriptional regulators (e.g. ZONAB) that transit to the nucleus upon junction disassembly to activate genes that control cell division (Perez-Moreno & Fuchs 2006; Matter & Balda 2007). Another TJ-associated protein that mediates G1–S phase cycle progression is GEF-H1 (Benais-Pont *et al.* 2003; Aijaz *et al.* 2005), a RhoA exchange factor (Birkenfeld *et al.* 2008) and ZONAB interacting protein, whose transient overexpression in MDCK cells was reported to stimulate cyclin D1 expression (Nie *et al.* 2009) possibly leading to aberrant cell proliferation. As another example, ubinuclein (a nuclear and adhesion complex (NACos) member; Balda & Matter 2009) was shown to be restricted to TJs in confluent cells, co-localizing with occludin, claudin 1 and ZO proteins, but was found in the nucleus in non-confluent cells (Aho *et al.* 2009). A role in cell proliferation has been proposed for ubinuclein because its overexpression in MDCK cells prevented cells from entering cytokinesis (Aho *et al.* 2009). Changes (including both up- and downregulation) in the expression of claudin, an integral membrane protein, have also been reported in tumour cells as compared with normal cells (Gonzalez-Mariscal *et al.* 2007). For example, the motility and invasiveness of gastric epithelial cells was shown to increase following siRNA-mediated knockdown of claudin-11 (Agarwal *et al.* 2010). Moreover, certain types of bacterial infections (e.g. *Helicobacter pylori*) and inflammation which disrupt TJs have also been shown to induce cell proliferation in epithelial cells (Amieva *et al.* 2003). TJ proteins have also been shown to be targets of oncogenic viruses for proteasome-mediated degradation (Javier 2008). For instance, MUPP1 (multi-PDZ domain protein 1, a TJ protein known to bind to claudin-1 and JAM; Hamazaki *et al.* 2002), MAGI-1 and PATJ (PALS-associated TJ protein, a protein functioning in the establishment of apico-basal cell polarity and cell

movement; Shin *et al.* 2006, 2007) were all shown to bind adenovirus 9 (Ad9) E4-ORF1 and high-risk papillomavirus (HPV) type 18 E6 oncoproteins (Lee *et al.* 2000; Glaunsinger *et al.* 2001; Latorre *et al.* 2005). Other examples include MAGI-2 and -3 which were also shown to be targets of HPV type 18 E6 (Thomas *et al.* 2002), ZO-2 which interacted with Ad9 E4-ORF1 (Glaunsinger *et al.* 2001) and MAGI-3 which associated with HTLV (human T-lymphotrophic virus)-Tax1 (Ohashi *et al.* 2004), the causative agent of T-cell leukaemia (Poiesz *et al.* 1980; Hinuma *et al.* 1981). In the study involving ZO-2, interaction with Ad9 E4-ORF1 resulted in the aberrant sequestration of ZO-2 within the cytoplasm, but it is not known if this affected TJ barrier function (Glaunsinger *et al.* 2001). In essence, these data illustrate that TJs are sophisticated structures with pivotal roles in intercellular adhesion and cell motility that also function as hubs for signal transduction and transcriptional regulation. In the context of the testis, these observations may be of importance because they imply that restructuring of Sertoli cell TJs during spermatogenesis may be linked directly or indirectly to germ cell cycle progression, as well as to germ cell movement across the BTB (figure 1). Thus, this postulate may be worthwhile to investigate in future studies.

3. TJ PROTEINS: MODERATORS OF VIRUS AND CANCER CELL MIGRATION ACROSS BARRIERS

In this section, we focus our discussion on several examples of TJ proteins that play roles in virus and cancer cell migration across endothelial and epithelial barriers under pathological conditions. This information is likely to provide useful insights on how germ cells cross the Sertoli cell barrier, thereby laying the foundation for future investigation.

(a) *Claudins*

Claudins are transmembrane proteins responsible for creating charge-selective pores within TJs (note: TJs are not absolute barriers), and they are essential for TJ formation. Thus far, 24 distinct claudins have been identified in most mammals (except humans and chimpanzees which have 23 claudins) and found to be expressed by several tissues including the testis (Chiba *et al.* 2008; Krause *et al.* 2008; Furuse 2009) (figure 1). Interestingly, several *in vitro* and *in vivo* studies are available to support the participation of cytokines, as well as androgens, in claudin regulation (Hellani *et al.* 2000; Florin *et al.* 2005; Meng *et al.* 2005; van Itallie & Anderson 2006; Kaitu'u-Lino *et al.* 2007; Capaldo & Nusrat 2008) (figure 1). Claudin levels are also regulated by proteolysis, ubiquitylation, palmitoylation, phosphorylation and endocytosis (Angelow *et al.* 2008; Findley & Koval 2009; Lal-Nag & Morin 2009). For instance, claudin internalization was recently reported to occur via two unique mechanisms. In the first well-studied model, tightly apposed plasma membranes (i.e. 'kissing points') detach, and each claudin molecule is endocytosed into its respective cell as two distinct vesicles. In the second model, however, tightly apposed plasma membranes never detach. Instead, both claudin (but not occludin) molecules are co-endocytosed into one cell as a vesicle that was shown to be immunoreactive for Rab7, a late endosome marker (Matsuda *et al.* 2004), illustrating that claudins may be destined for lysosomal degradation when plasma membranes fail to disassociate. At this point, additional studies are needed to better understand this new mechanism of protein endocytosis and degradation, and to determine whether Sertoli cells use a similar mechanism.

As discussed briefly above, claudins are known to have functions outside of the TJ. For instance, claudins 1–5, 7 and 11 have all been reported to associate with and to possibly be regulated by tetraspanin family members such as tetraspanins 24 (CD151), 28 (CD81) and 29 (CD9) (Tiwari-Woodruff *et al.* 2001; Kovalenko *et al.* 2007; Kuhn *et al.* 2007; Harris *et al.* 2008). Tetraspanins (also known as the transmembrane 4 superfamily, TM4SF) are transmembrane proteins that participate in cell proliferation, differentiation and migration; tumour suppression; signal transduction; protein trafficking, virus entry into host cells and fertilization (Berditchevski & Odintsova 2007; Hemler 2008; Charrin *et al.* 2009; Zoller 2009). Interestingly, tetraspanins did not localize to the TJ, and they were shown to stabilize non-junction-associated claudins (Kovalenko *et al.* 2007), suggesting that tetraspanins may maintain this separate pool of claudins to facilitate junction disassembly and cell movement. Indeed, others have reported claudin localization at the basolateral membrane (Gregory *et al.* 2001; Rahner *et al.* 2001; Kiuchi-Saishin *et al.* 2002). This is also in line with a co-immunoprecipitation and confocal microscopy study in oligodendrocytes which demonstrated OAP-1 (OSP (oligodendrocyte-specific protein)/claudin 11-associated protein 1), a tetraspanin that was found to be expressed by the testis, to interact with claudin 11, as well as with β1 integrin (Tiwari-Woodruff *et al.* 2001). These findings are interesting because they seemingly support the existence of another mechanism of junction disassembly, one in which neighbour proteins (i.e. tetraspanins) can temporarily 'kidnap' and dislocate or remove TJ proteins (i.e. claudins) from the junctional complex, thereby leading to junction disassembly and cell migration during tumourigenesis. However, additional functional studies would be needed to confirm and expand these observations, as well as to determine if this mechanism precedes or is an extension of other mechanisms of junction restructuring (i.e. proteolysis, ubiquitylation, phosphorylation and/or endocytosis). On a final note, claudin 11 was recently shown to be critical for maintaining Sertoli cell quiescence because Sertoli cells from claudin 11-null mice displayed a loss in cell polarity, detached from the basement membrane, underwent a change in cell shape and proliferated (Mazaud-Guittot *et al.* 2010), illustrating yet again that TJs function outside of barrier and fence roles.

(b) *Occludin*

Occludin is another well-studied TJ protein presumed to have a regulatory role as it does not appear to be an integral component of TJ fibrils (figure 1), that is, barrier function was unaffected in occludin −/− mice (Saitou *et al.* 2000). Occludin is a highly

phosphorylated protein, and TJ disruption generally brings about dephosphorylation on Ser and Thr residues but phosphorylation on Tyr residues (Sakakibara *et al.* 1997; Feldman *et al.* 2005; Gonzalez-Mariscal *et al.* 2008). In support of this, occludin has been shown to interact with several protein kinases and phosphatases such as Src (Basuroy *et al.* 2003), c-YES (Chen *et al.* 2002), PKCη (Suzuki *et al.* 2009), FAK (Siu *et al.* 2009*a*), ERK1/2 (Basuroy *et al.* 2006), PP2A and PP1 (Sakakibara *et al.* 1997; Seth *et al.* 2007). In a recent study, PKCη phosphorylated occludin on Thr 403 and 404 which was required for the insertion of occludin into functional TJs (Suzuki *et al.* 2009). Moreover, PKCη knockdown by shRNA in MDCK cells was shown to compromise barrier function (Suzuki *et al.* 2009). In another recent study by Siu *et al.* (2009*a*,*b*), barrier integrity was also shown to be regulated by FAK, a non-receptor protein tyrosine kinase that was demonstrated to associate with occludin and ZO-1. Here, FAK knockdown by siRNA in Sertoli cells *in vitro* also perturbed barrier function (Siu *et al.* 2009*a*). These results suggest that FAK silencing may have rendered occludin non- or less phosphorylated and removed it from the site of the TJ possibly via endocytosis as highly phosphorylated occludin is known to concentrate at TJs (Sakakibara *et al.* 1997; Wong 1997) (figure 1). It is also important to note that FAK binds Src (Cox *et al.* 2006; Mitra & Schlaepfer 2006), and Tyr phosphorylation of occludin by Src was shown to perturb its interaction with ZO-1 (Kale *et al.* 2003), a scaffolding protein (Furuse *et al.* 1994; Fanning *et al.* 1998). As FAK is an important regulator of cell movement (Broussard *et al.* 2008; Tomar & Schlaepfer 2009), we believe that it may have a critical role in spermatocyte movement across the BTB. At this point, further studies are needed in the testis to investigate the possible connection between protein phosphorylation and endocytosis as both cellular processes involve changes in the localization of TJ proteins. For instance, cytokines such as TGF (transforming growth factor)-β and TNF (tumour necrosis factor) α have been demonstrated to accelerate the kinetics of occludin endocytosis in Sertoli cells (Yan *et al.* 2008; Xia *et al.* 2009) (figure 1). Thus, the next logical step would be to investigate whether protein kinases play a role in cytokine-mediated protein internalization. In other words, can knockdown of FAK or Src by siRNA in control Sertoli cells (i.e. untreated with TGF-β and TNF α because these cytokines are known to affect barrier function; Lui *et al.* 2001; Li *et al.* 2006; Xia *et al.* 2006, 2009) affect the kinetics of TJ protein endocytosis, thereby perturbing barrier function (Siu *et al.* 2009*a*). Interestingly, studies have shown both caveolin and clathrin (both function in the formation of endocytic vesicles; Kirchhausen 2000; Razani *et al.* 2002) to be phosphorylated by Src (Martin-Perez *et al.* 1989; Li *et al.* 1996), revealing that protein kinases have an important role in endocytosis.

While the precise function of occludin within the junctional complex is not yet clear, entry of viruses such as hepatitis C virus (HCV) into hepatocytes has been shown to require occludin; as well as claudins 1, 6 and 9; and CD81 which function as 'receptors' (Pileri *et al.* 1998; Evans *et al.* 2007; Zheng *et al.* 2007; Meertens *et al.* 2008; Liu *et al.* 2009; Ploss *et al.* 2009). Interestingly, siRNA knockdown of occludin impaired the infection of Hep3B cells by HCVpp (pseudoviral particles), as well as inhibited the infection of Huh-7.5 cells by HCVpp and HCVcc (cell-culture-derived virus) (Benedicto *et al.* 2009; Liu *et al.* 2009; Ploss *et al.* 2009), demonstrating that occludin is indispensable for HCV infection. Likewise, infection of primary hepatocytes with HCV could be perturbed by anti-CD81 antibodies or siRNAs directed against CD81 mRNA (Molina *et al.* 2008). Moreover, an intact microtubule network was also required for HCV entry because microtubule-affecting drugs (i.e. vinblastine, nocodazol and paclitaxel) markedly inhibited HCV infection, and α- and β-tubulins were identified as binding partners of the HCV core protein which forms the capsid shell (Roohvand *et al.* 2009). An association between HCV proteins and actin microfilaments has also been reported (Lai *et al.* 2008; Coller *et al.* 2009). In the latter cited study which employed single particle tracking of HCV infection, HCV was shown to be internalized via endocytosis, and this involved the participation of several proteins including clathrin, epsin (an endocytic accessory protein; Horvath *et al.* 2007), cofilin, CDC42 and ROCK2 (Coller *et al.* 2009). The mechanism described above used by *viruses* is intriguing because it is somewhat similar to the mechanism used by *cells* (e.g. leucocytes and germ cells) to cross physiological barriers (e.g. endothelial and Sertoli cell barriers). For instance, transit of preleptotene spermatocytes across the BTB requires disassembly of junctions (i.e. TJs, basal ES and D-GJs) present above these germ cells, and this involves proteolysis and endocytosis of proteins (figure 1). At the same time, transit of preleptotene spermatocytes requires assembly of new junctions below these germ cells so that the immunological barrier and spermatogenesis can be maintained, and this involves de novo synthesis and recycling of proteins (Mruk & Cheng 2004*a*; Yan *et al.* 2008; Xia *et al.* 2009). While it is not known if preleptotene spermatocytes use integral membrane proteins (e.g. occludin and claudin) as 'receptors' to seal the BTB during germ cell movement (figure 1), this hypothesis is worthwhile to investigate in future studies.

(c) *JAM and CAR*

The JAM family of immunoglobulin-like proteins is composed of five members (i.e. JAM-A, -B, -C, -4 and JAM-like) that localize to sites of intercellular contact in epithelial and endothelial cells (figure 1). JAMs are capable of mediating homophilic and heterophilic interactions, and they are known to be involved in the regulation of cell proliferation, migration and invasion; cell polarity, platelet aggregation and junction assembly (Bazzoni 2003; Mandell & Parkos 2005; Severson & Parkos 2009*a*,*b*). JAM-A also functions as a receptor for reovirus, a family of viruses that infects the gastrointestinal and respiratory systems in humans (Barton *et al.* 2001; Guglielmi *et al.* 2007).

JAMs began to receive special attention when their extracellular domains were found to interact with integrins (Ostermann *et al.* 2002; Santoso *et al.* 2002; Naik *et al.* 2003; Mandell *et al.* 2005), transmembrane receptors that connect to proteins in the extracellular matrix (ECM), thereby mediating adhesion between cells and the basement membrane via focal contacts (Delon & Brown 2007; Caswell *et al.* 2009; Hynes 2009). In essence, JAM-A was illustrated to be indispensable for the internalization of integrins, a pre-requisite for cell movement (Cera *et al.* 2009). Interestingly, knockdown of JAM-A was shown to dramatically affect cell morphology, diminish the level of cell-surface-associated β1-integrin, inhibit cell–ECM interactions and reduce cell migration; and these cellular events were mediated by an increase in the small GTPase Rap1 (Mandell *et al.* 2005; Severson *et al.* 2009). In yet another study, overexpression of dimerization-defective JAM-A mutants or treatment of 293T cells with a dimerization inhibiting antibody decreased the level of β1-integrin and inhibited cell spreading and migration on fibronectin (Severson *et al.* 2008). In addition to JAM's role in cell migration, loss of JAM-A in HepG2 cells also resulted in the mislocalization of several TJ proteins (i.e. occludin, claudin 1 and ZO-1), and in the disruption of cell polarity and junction assembly (Konopka *et al.* 2007). This is in agreement with an investigation by Laukoetter *et al.* (2007) which demonstrated an increase in barrier permeability in SK-CO15 cells transfected with JAM-A siRNA. These findings, if taken collectively, are interesting because they clearly illustrate the active role of JAMs in TJ assembly and cell migration, as well as demonstrating an unexpected interaction between a TJ and a focal contact protein. As a second example, ZO-1 (also a JAM-binding partner; Bazzoni *et al.* 2000; Ebnet *et al.* 2003) was found to interact with α5β1-integrin at lamellae and to support migration of cancer cells, an interaction that was dependent on PKC-ε (Tuomi *et al.* 2009). In essence, the subcellular localization of ZO-1 was regulated via phosphorylation by PKC-ε which allowed ZO-1 to move away from TJs and to translocate to lamellae. Again, these findings illustrate that proteins from distinct junction types (i.e. TJs and focal contacts) can interact to mutually control cell function (i.e. cell migration). On a final note, JAM-A can be proteolytically cleaved by ADAMs (a distintegrin and metalloproteinase domain, a family of membrane-bound proteins that function in cell adhesion by interacting with integrins; Wolfsberg *et al.* 1995; Black & White 1998) to release a soluble fragment (sJAM) in a process known as ectodomain shedding which can drastically change the functional properties of the soluble protein. Thus far, more than 50 proteins have been reported to undergo ectodomain shedding (Arribas & Borroto 2002; Huovila *et al.* 2005; Garton *et al.* 2006; de Wever *et al.* 2007; Reiss & Saftig 2009). Shedding was associated with a downregulation of cell-surface JAM, and it is upregulated by pro-inflammatory cytokines such as INF-γ and TNF α (Koenen *et al.* 2009) (figure 1). Interestingly, sJAM appeared to block migration in a model using endothelial cells (Koenen *et al.* 2009).

CAR was initially characterized as a cell surface protein (i.e. receptor) required for the entry of coxsackie B and adenoviruses into cells (Coyne & Bergelson 2005), but later reported to be a component of the TJ complex and a regulator of TJ assembly when it was shown to co-localize with occludin and immunoprecipitate ZO-1 (Cohen *et al.* 2001; Coyne *et al.* 2004; Excoffon *et al.* 2004; Mirza *et al.* 2005; Raschperger *et al.* 2006) (figure 1). While homophilic interactions are known to underlie CAR function, there are at least two reports illustrating a heterophilic interaction with JAML, a protein which is known to mediate monocyte migration across TNF α-treated endothelial monolayers (Zen *et al.* 2005; Luissint *et al.* 2008). CAR is also highly homologous to JAM. Generally speaking, loss of CAR expression resulted in weakened cell adhesion, thereby promoting migration of cancer cells (Okegawa *et al.* 2001*a*; Bruning & Runnebaum 2004; Matsumoto *et al.* 2005; Anders *et al.* 2009). While these findings are opposite to those of JAM discussed above (i.e. knockdown of JAM-inhibited cell migration), they demonstrate that CAR is also important in cell adhesion. Indeed, CAR immunoprecipitated β-catenin from epithelial cell lysates (Walters *et al.* 2002). Moreover, CAR is likely to have additional functions such as a role in cell proliferation and differentiation (Okegawa *et al.* 2001*a*,*b*; Kim *et al.* 2003; Excoffon *et al.* 2004). For instance, in bladder carcinoma cells, CAR expression resulted in upregulation of $p21^{CIP}$, an inhibitor of cyclin-dependent kinases (Okegawa *et al.* 2001*b*). This suggests that CAR may interrupt the cell cycle and halt uncontrolled proliferation of cancer cells, resulting in their death. In a recent study using DLD-1 and IEC-6 cells, knockdown of CAR by siRNA downregulated α-catenin (Stecker *et al.* 2009), a cytoplasmic protein that connects cadherin to the actin cytoskeleton (Nelson 2008), leaving the authors to speculate that CAR interacts structurally with α-catenin as well (Stecker *et al.* 2009) (figure 1). As CAR is known to associate with actin (Huang *et al.* 2007), it is believed that CAR regulates actin dynamics and mediates junction restructuring, thereby resulting in the movement of viruses (e.g. adenovirus) and cells (e.g. leucocytes and preleptotene spermatocytes) across epithelial and endothelial barriers. Moreover, a redistribution of endothelial CAR from TJs has been reported during virus infection (Walters *et al.* 2002). CAR overexpression, on the other hand, upregulated p44/p42 MAPK (also known as ERK1/2; Sturgill 2008) which activated β1 and β3 integrins, facilitated adenovirus type 5 (Ad5) attachment to CAR and initiated infection (Farmer *et al.* 2009). It may be that p44/p42 phosphorylates CAR, possibly changing its cellular localization, because in Caco-2 cells p-ERK and ERK were shown to bind another TJ protein occludin, and to prevent oxidative TJ disassembly via EGF (Basuroy *et al.* 2006). While it is interesting that both CAR and JAM activate integrins, suggesting that they may have a similar function and share a common mechanism in the regulation of cell movement, CAR did not co-immunoprecipitate JAM (Walters *et al.* 2002). Similar to JAM-A, CAR can be

cleaved to produce several soluble fragments (i.e. sCAR 4/7, sCAR 3/7 and sCAR 2/7) which can bind to full-length CAR, thereby weakening cell adhesion and facilitating cell migration (Dorner *et al.* 2004; Reimer *et al.* 2007) (figure 1). It is critical that sCAR be characterized in the testis and that its role in junction restructuring at the Sertoli cell barrier and at the apical ES be investigated. As a starting point, it would be interesting to see if sCAR can modulate TJ dynamics by downregulating the levels of other proteins, thereby perturbing BTB function.

4. GERM CELL MOVEMENT ACROSS THE BTB: INSIGHTS FROM VIRUS AND CANCER CELL MIGRATION MODELS

In the previous section, we focused on several integral membrane proteins, including occludin, claudin, JAM and CAR, that not only contribute to the barrier and fence function of TJs, and to briefly summarize, each one of these proteins played a pivotal role in virus or cancer cell movement. If we appreciate the mechanisms defining virus or cancer cell movement across endothelia and epithelia, we will probably gain important and much-needed insight on the mechanism(s) used by preleptotene spermatocytes to cross the BTB at stage VIII of the seminiferous epithelial cycle (figure 1). All of the integral membrane proteins discussed in the previous section are expressed by the testis (Cheng & Mruk 2002; Mruk & Cheng 2004*a*). For example, JAM-A and -B were found in Sertoli cells, localizing specifically at the BTB (Gliki *et al.* (2004) (figure 1), whereas JAM-A, -B and -C were present at the site of the apical ES (Gliki *et al.* 2004; Sakaguchi *et al.* 2006; Shao *et al.* 2008), a hybrid type of junction found between Sertoli cells and elongating/elongated spermatids (Mruk & Cheng 2004*b*). Of these, JAM-A also localized to the head and flagellum of sperm (Shao *et al.* 2008), whereas JAM-C was essential for the polarization of round spermatids during spermiogenesis and for fertility (Gliki *et al.* 2004), intriguing results given that JAMs are proteins that localize to TJs in other endothelia and epithelia. CAR is another TJ protein expressed by germ cells (i.e. round spermatids and spermatozoa) that was shown to interact with JAM-C when testis lysates were used for co-immunoprecipitation (Mirza *et al.* 2006, 2007; Wang *et al.* 2007). It is worth noting that CAR is also expressed by Sertoli cells, localizing to the BTB and apical ES (Wang *et al.* 2007). Thus, we ask why germ cells would express putative TJ proteins if they do not assemble functional TJs *in vivo*. It is possible that JAM and/or CAR have a role in moving round spermatids away from the BTB and towards the tubule lumen in anticipation of spermiation as JAM is known to associate with polarity proteins (Ebnet *et al.* 2001, 2003) (figure 1). If this is the case, then cross-talk may exist between the two pools of JAM/CAR found at the apical ES and the BTB. While the molecules that coordinate this probable cross-talk are presently unknown, we believe cell polarity and cytoskeletal proteins to be key players. A further understanding of this regulation may provide insight on how BTB restructuring and spermiation are coordinated at stage VIII of the seminiferous epithelial cycle, two critical events that occur at opposite ends of the seminiferous epithelium (figure 1). A second example rests with CAR which was reported to participate in germ cell movement because migrating preleptotene spermatocytes at the BTB were strongly immunoreactive for CAR (Mirza *et al.* 2007). This led the authors to speculate that CAR contributes to the formation of a 'tunnel' that surrounds germ cells as they traverse the BTB (Mirza *et al.* 2007) (figure 1). This mechanism, which is somewhat similar to the one used by leucocytes to cross the endothelium (defined as diapedesis), would make clear why germ cells express putative TJ proteins, as well as explain how barrier integrity is maintained during periods of restructuring. In addition, this mechanism would complement Russell's morphological studies which showed that preleptotene/leptotene spermatocytes become trapped within a transient intermediate compartment, thereby sealing these cells off from the rest of the seminiferous epithelium via TJs and basal ES (Russell 1977, 1993*b*). In this context, it is worth noting that as leucocytes cross the endothelium, they become surrounded by the lateral border recycling compartment (LBRC), an interconnected reticulum of membrane that functions as a 'channel.' This channel is lined with several proteins, namely PECAM (platelet endothelial cell adhesion molecule), CD99 (cluster of differentiation 99) and JAM-A but not VE-cadherin which appeared to be endocytosed (Xiao *et al.* 2005; Gavard & Gutkind 2006), that are needed for leucocytes to cross the endothelium (Mamdouh *et al.* 2003, 2008, 2009). Interestingly, depolymerization of microtubules blocked the accumulation of the LBRC around leucocytes and transmigration (Mamdouh *et al.* 2009). At this point, additional studies are needed to investigate if preleptotene spermatocytes can also express other TJ proteins. Indeed, Morrow *et al.* (2009) recently demonstrated that spermatogonia and preleptotene spermatocytes express claudin 5.

Another interesting observation gained from virus and cancer cell model systems is that viruses have an inherent mechanism to breach endothelial and epithelial barriers, thereby exposing receptors such as occludin, JAM and CAR which under normal physiological conditions are not readily accessible to viruses. For instance, MAV-1 (mouse adenovirus type 1), which causes fatal encephalitis in mice, is capable of breaking down the blood–brain barrier (BBB). In addition, there are several other examples of pathogenic viruses that can break down barriers as well, including HIV (human immunodeficiency virus), MHV (mouse hepatitis virus) and WNV (West Nile virus), the latter of which can also infect the testis and cause orchitis (Zhou *et al.* 2003; Smith *et al.* 2004; Toborek *et al.* 2005; Armah *et al.* 2007; Ivey *et al.* 2009; Medigeshi *et al.* 2009). While the identity of the mechanism(s) underlying TJ disruption remains unknown, it may involve protein transduction domains, sort stretches of sequence found within certain proteins (e.g. TAT (transactivator of transcription) and HSC-1 VP22 (herpes simplex virus type-1 VP22)) (Gump & Dowdy 2007). These

findings raise many important questions regarding the possible role of preleptotene spermatocytes in BTB remodelling at stage VIII of the seminiferous epithelial cycle (figure 1). For example, how do Sertoli cells know when the BTB must be restructured? What molecules, besides those already reported in the literature (e.g. cytokines, proteases and protein kinases), can trigger disassembly of the Sertoli cell barrier? Are these molecules produced specifically by preleptotene spermatocytes? What signalling pathways are involved? Finally, if future studies demonstrate preleptotene spermatocytes to express TJ proteins, how will this shape our understanding of BTB dynamics? Moreover, viruses have also been shown to infect cells using endocytic mechanisms which may be separate from the mechanism described above (Wang *et al.* 1998; Meier & Greber 2004; Gruenberg 2009). It is not yet clear, however, if endocytosis of viruses requires endocytosis of integral membrane proteins. While preleptotene spermatocytes do not appear to cross the BTB enveloped in an endocytic vesicle, endocytosis was recently shown to be responsible for moving TJ and basal ES proteins away from the plasma membrane of Sertoli cells treated with different cytokines (Yan *et al.* 2008; Xia *et al.* 2009). Endocytosis of integral membrane proteins would explain in part how TJs and basal ES situated above a migrating preleptotene spermatocyte are disassembled, thereby allowing germ cells to enter the adluminal compartment for further development. It is conceived that endocytosed structural proteins journey from above preleptotene spermatocytes to below them and that these proteins are inserted back into the Sertoli cell plasma membrane, thereby creating the intermediate compartment. It is also possible that some of these endocytosed proteins become inserted back into the Sertoli cell plasma membrane while *en route* to below a migrating spermatocyte, and this may create a 'channel' lined with proteins similar to the one described for leucocytes (i.e. LBRC) (figure 1). At this point, additional studies and new models are needed to test these hypotheses. For instance, there is no *in vitro* model to study preleptotene spermatocyte movement across the Sertoli cell barrier, whereas *in vivo* the intermediate compartment is a transient structure observed in a small percentage of seminiferous tubules, making it difficult to study how preleptotene spermatocytes can cross the BTB. Culturing Sertoli cells and preleptotene spermatocytes in a three-dimensional environment using commercially available bioscaffolds, which would mimic more closely the behaviour of these cells in the testis, may alleviate some of these technical difficulties.

5. FUTURE PERSPECTIVES

In this review, we have highlighted how TJ proteins participate in virus and cancer cell migration across endothelial and epithelial barriers. It is hoped that this information can provide new and important insights on germ cell migration across the BTB which is critical for spermatogenesis and fertility. First and foremost, an *in vitro* model that more closely mimics the behaviour of Sertoli and germ cells *in vivo* is needed to study these cellular events. In this respect, three-dimensional culture models, which have shown promise in the field of cancer cell biology, should be explored. Other useful but more simple studies may focus on the role of germ cells in cell junction dynamics. For instance, can germ cells (i.e. preleptotene spermatocytes) affect the assembly and/or maintenance of the Sertoli cell barrier *in vitro*? Alternatively, can routine Sertoli cell–preleptotene spermatocyte co-cultures provide important clues on cell–cell interactions? Finally, the study of BTB dynamics should be expanded to include other non-TJ proteins such as ICAMs (intercellular adhesion molecules) which are known to have critical roles in leucocyte transmigration across endothelial barriers (Lawson & Wolf 2009; Wittchen 2009). While several years of research are needed before we completely understand how germ cells cross the BTB, it will certainly be an exciting time filled with unexpected and interesting discoveries.

Research supported by NICHD, NIH (R03HD061401 to D.D.M.; R01HD056034 and U54HD029990, Project 5 to C.Y.C.).

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