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Review

Claudin and occludin expression and function in the seminiferous epithelium

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Integral membrane proteins that contribute to function of the blood–testes barrier (BTB) in mice include claudins 3, 5 and 11 and occludin. Although claudin 11 is expressed throughout all stages of the seminiferous epithelial cycle, claudins 3 and 5 have specific expression at stage VIII. These differences in protein expression suggest that the interactions among, and functions of, these integral membrane proteins may shift over the course of the seminiferous epithelial cycle. Also, differences in expression among rodent species and men may make interpretation of studies across species challenging. This review will discuss the characteristics of claudins and occludin; the expression, regulation and function of these integral membrane proteins in the seminiferous epithelium; and how these properties relate to the unique features of BTB.

Keywords: blood–testis barrier; tight junction; seminiferous epithelial cycle; spermatocyte migration

1. INTRODUCTION

Claudins (Furuse *et al.* 1998*a*,*b*) and occludin (Furuse *et al.* 1993) were the first tight junctional integral membrane proteins identified. Tight junction permeability and epithelial barrier function are primarily mediated by claudins, and the claudin isoforms expressed in a tissue determine the tissue-specific barrier characteristics (for review, see Krause *et al.* 2008). The importance of occludin in mediating epithelial barrier function appears to be tissue dependent (Saitou *et al.* 2000; Schulzke *et al.* 2005). One of those tissues is the seminiferous epithelium. The tight junctions between the Sertoli cells of the seminiferous epithelium, which form the blood–testes barrier (BTB), have several morphological and functional differences when compared with the characteristics of other epithelial barriers. The purpose of this review article is to discuss the expression and function of claudins and occludin in mammalian seminiferous epithelium and how these characteristics relate to the unique properties of the BTB.

2. CHARACTERISTICS OF CLAUDINS AND OCCLUDIN

Claudins and occludin have similar topographical features, including four transmembrane domains, two extracellular loops, a short intracytoplasmic loop and cytoplasmic N- and C-termini (figure 1; Tsukita *et al.* 2001). Both of these proteins interact with scaffolding proteins via their C-termini (figure 2; Tsukita *et al.* 2001). However, there is no sequence homology between the claudin family and occludin (Furuse *et al.* 1998*a*), and the claudins (MW = 20–27 kDa) are also smaller than occludin (MW = 65 kDa; Furuse *et al.* 1993; Van Itallie & Anderson 2006). There are 24 members of the claudin family, and an identifying characteristic is a conserved residue sequence of W-n_x-GLW-n_x-C-n_x-C in the first extracellular domain (Van Itallie & Anderson 2006).

Claudins, occludin and members of the junctional adhesion molecule (JAM) family are the most studied and characterized of the tight junctional integral membrane proteins (Paris *et al.* 2008). Of these proteins, claudins are of primary importance for optimal establishment of tight junctions. JAM proteins are not sufficient for tight junction formation. Numerous primary and established fibroblast cell lines endogenously express JAM-A or JAM-C, yet these cells do not form tight junctions (Morris *et al.* 2006). Fibroblasts that are transfected with occludin can form tight junction strands, albeit small and few in number (Furuse *et al.* 1998*b*). However, occludin is not necessary for the formation or maintenance of tight junctions, as demonstrated by occludin disrupted murine embryonic stem cells (Saitou *et al.* 1998) and occludin knockout mice (Saitou *et al.* 2000; Schulzke *et al.* 2005). Morphologically normal tight junctions are formed in both of these models, albeit with various degrees of physiological dysfunction among epithelia, including the seminiferous epithelium, in occludin knockout mice (Saitou *et al.* 2000).

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

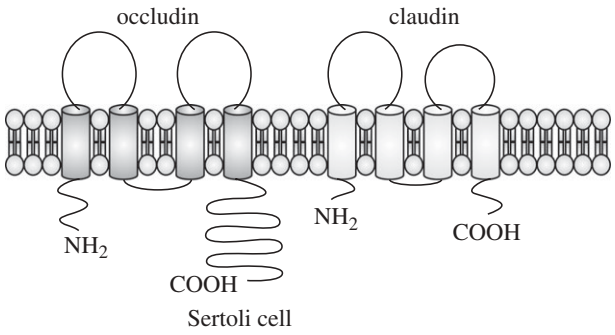

Figure 1. Comparison of occludin and claudin topography. Both occludin and claudins have similar topography, with cytoplasmic N- and C-termini, four transmembrane domains, two extracellular loops, and a short cytoplasmic loop. However, there is no sequence homology between these proteins, and the longer cytoplasmic termini in occludin give it a substantially larger molecular mass than the molecular mass of claudin.

The phenotypes of claudin knockout mice reveal the importance of specific claudins for regulating barrier integrity of distinct epithelial tissues. For example, claudin 1 knockout mice die shortly after birth due to disruption of epidermal barrier function and resultant dehydration (Furuse *et al.* 2002), whereas in claudin 5 knockout mice, the neonatal lethality is attributable to disruption of endothelial barrier integrity in the central nervous system and resultant loss of blood–brain barrier function (Nitta *et al.* 2003). Claudin 11 knockout mice are viable, but have locomotion defects and male sterility due to lack of tight junctions in the myelin sheaths of the central nervous system and between Sertoli cells of the testes, respectively (Gow *et al.* 1999).

The interactions between the claudin proteins within a tissue determine the barrier properties of an epithelial tissue (Krause *et al.* 2008). The interactions can be described by the plane in which they occur (i.e. *cis* within the same cell membrane or *trans* between opposing cell membranes) and by the type of interacting claudins (homophilic between two claudins of the same isoform or heterophilic between two claudins of different isoforms; Krause *et al.* 2008). Paracellular ion pores are formed by *trans*-interactions between the first extracellular loops of certain claudins, whereas tightness against solute diffusion is determined by the respective *trans*-interactions of both extracellular loops (Krause *et al.* 2008). Both *trans*- and *cis*-interactions among claudins are important for formation, morphology and stability of the tight junction strands (Krause *et al.* 2008; Mrsny *et al.* 2008).

The importance of occludin–occludin interactions to epithelial barrier function is uncertain. Disruption of these interactions with peptides that correspond to the first (Tavelin *et al.* 2003; Everett *et al.* 2006) or second (Wong & Gumbiner 1997; Nusrat *et al.* 2005) extracellular loop or a monoclonal antibody against the second extracellular loop (Tokunaga *et al.* 2007) decreases localization of occludin to the cell membrane. Alterations in epithelial barrier function associated with these disruptions in occludin interactions and localization may actually be attributable to altered localization of other tight junctional proteins. Treatment of T84 human intestinal cell cultures with a peptide corresponding to part of the second extracellular loop of occludin alters localization of claudin 1, JAM-A, and the scaffolding protein zona occludens 1 (ZO-1) and increases barrier permeability (Nusrat *et al.* 2005). However, treatment with a monoclonal antibody against that loop does not alter localization of those proteins nor barrier permeability (Tokunaga *et al.* 2007).

In addition to claudin–claudin and occludin–occludin interactions, claudin–occludin interactions may also occur. Occludin can interact with a peptide that corresponds to the first extracellular loop of claudin 1 (Mrsny *et al.* 2008), and claudin 1 and JAM-A can interact with a peptide that corresponds to the second extracellular loop of occludin (Nusrat *et al.* 2005). The relative importance of the various types of interactions among the claudins and occludin to tight junction formation, stability and function remain to be determined.

Claudins and occludin have additional functions in addition to mediating tight junction permeability (table 1). Indeed, prior to being identified as integral membrane proteins of tight junctions, some claudin proteins had been characterized as a marker of androgen withdrawal in rat ventral prostate (claudin 3; Briehl & Miesfeld 1991), as receptors for *Clostridium perfringens* enterotoxin (claudins 3 and 4; Katahira *et al.* 1997*a*,*b*), and as one of several proteins whose genes are deleted in velo–cardio–facial syndrome in humans (claudin 5; Sirotkin *et al.* 1997). In some tissues, the additional functions of occludin may be more important than any contribution occludin may make toward barrier tightness. In gastric epithelia of mice, occludin appears to be necessary for proper differentiation rather than for barrier function (Schulzke *et al.* 2005). Disruption of occludin–occludin interactions in monolayers of a human intestinal epithelial cell line alters cell polarity without affecting barrier permeability (Tokunaga *et al.* 2007).

3. BLOOD–TESTIS BARRIER

The BTB is formed by tight junctions between adjacent Sertoli cells and divides the seminiferous epithelium into basal and adluminal compartments (Mruk & Cheng 2004). Spermatogonia and preleptotene spermatocytes are below in the basal compartment, and leptotene and later spermatocytes and spermatids are above, in the adluminal compartment. This physical separation establishes the distinct microenvironments needed for the different germ cell types (Onoda *et al.* 1990; Mruk & Cheng 2004). The BTB also protects the primary spermatocytes and haploid spermatids from potentially harmful chemicals by limiting the movement of intercellular molecules from the interstitial space into the adluminal compartment (Mruk & Cheng 2004; Su *et al.* 2009). In association with other immunoprotective mechanisms, the BTB protects the haploid spermatids, which express 'foreign' proteins, from immunological attack and helps to establish testicular immune privilege (Fijak & Meinhardt 2006).

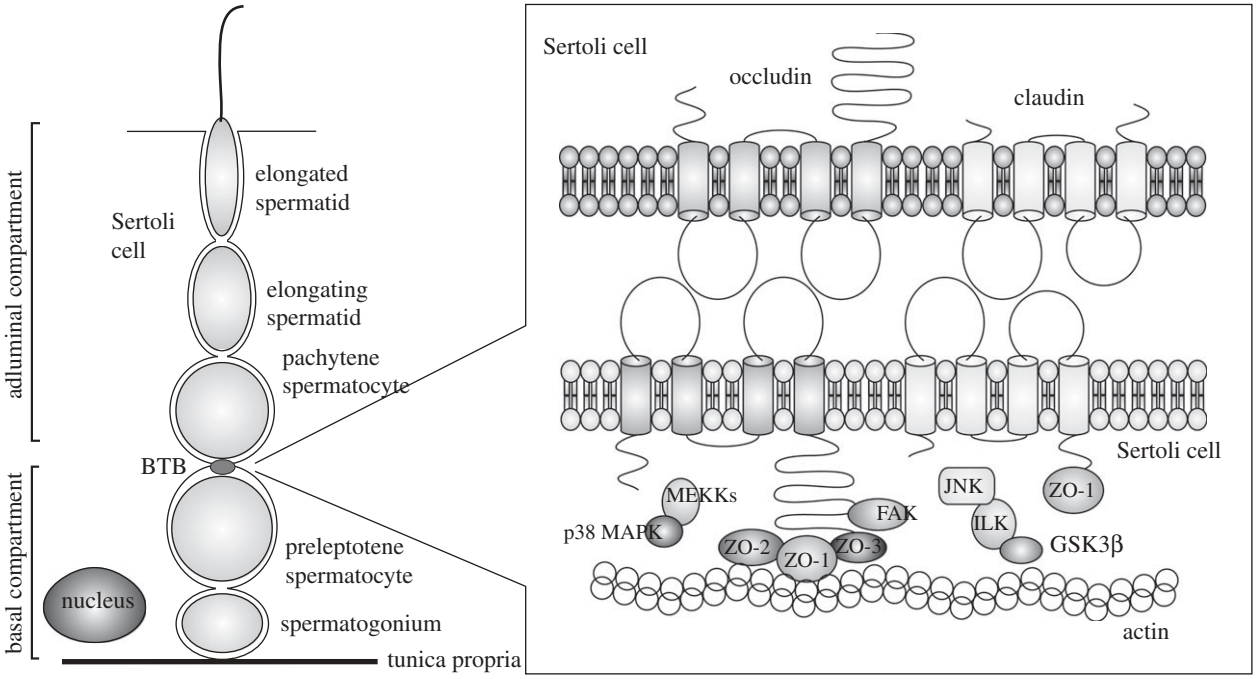

Figure 2. Location of occludin and claudin in the seminiferous epithelium. Occludin- and claudin-based tight junctions are components of the BTB, which is located apical to preleptotene spermatocytes and basal to pachytene spermatocytes in the seminiferous epithelium. Both occludin and claudin interact with scaffolding proteins (ZO-1, -2, and -3) and occludin interacts with the signalling molecule FAK. These interactions with scaffolding proteins connect occludin and claudin to the actin cytoskeleton and allow localization of cell signalling molecules to tight junctions. Through these various interactions, tight junctions can influence cell cycle pathways.

Compared with the tight junctions of other polarized epithelial cells, the tight junctions between Sertoli cells have several unique features. In other polarized epithelia, tight junctions are located in a circumferential band towards the apical membrane; whereas in Sertoli cells, the tight junctions are located in a circumferential band toward the basement membrane (figure 3; Gilula *et al.* 1976; Wong *et al.* 2008). In other polarized epithelia, the adherens junction band and then the desmosome band are located just basal to the tight junction band, and gap junctions are located throughout the lateral cell membrane. Although the bands of tight junctions, adherens junctions, and desmosomes support each other functionally through interactions with scaffolding proteins and the cytoskeleton, these different types of cell–cell contact are physically separate from each other in most polarized epithelia (Wong *et al.* 2008). However, at the BTB in Sertoli cells, the tight junctions and specialized adherens junctions (basal ectoplasmic specialization and basal tubulobulbar complex), as well as the basal gap junctions and desmosome-like junctions are all intermingled with each other (Russell & Peterson 1985; Parreira *et al.* 2002; Wong *et al.* 2008). In addition to the tight junctions, the basal ectoplasmic specialization (Yan & Cheng 2005; Lie *et al.* 2006; Yan *et al.* 2007) and desmosome-like junctions (Li *et al.* 2009) appear to be integral, functional components of the BTB. Another feature of the tight junctions and other components of the BTB is the need to allow for the cyclic passage of spermatocyte cohorts while maintaining barrier integrity (Mruk & Cheng 2004).

4. CLAUDIN AND OCCLUDIN CONTRIBUTIONS TO BLOOD–TESTIS BARRIER INTEGRITY

Claudins 3 (Meng *et al.* 2005), 5 (Morrow *et al.* 2009), and 11 (Gow *et al.* 1999) and occludin (Saitou *et al.* 2000; Chung *et al.* 2001) contribute to BTB integrity. The contributions of claudins 3 and 5 were determined in mice via deletion of genes for transcription factors that are upstream regulators of the claudins (androgen receptor (AR) for claudin 3; Meng *et al.* 2005) and ets variant 5 (ETV5) for claudin 5; Morrow *et al.* 2009). Mice that are gene-deleted for AR in the Sertoli cells and for ETV5 in all cells have deficient BTBs as demonstrated by leakage of interstitially injected tracers into the seminiferous tubule lumens. Males in both of these lines are infertile. However, the infertility is not primarily attributable to the barrier deficiency. Mice with AR specifically deleted from Sertoli cells are infertile due to the inability of the Sertoli cells to nurture the germ cells past the round spermatid stage (Holdcraft & Braun 2004). The transcription factor ETV5 is needed in Sertoli cells (Chen *et al.* 2005) and germ cells (Tyagi *et al.* 2009) for proper regulation of the spermatogonial stem cell niche and renewal of spermatogonial stem cells.

The contribution of claudin 11 and occludin to BTB integrity was determined by gene-deletion in mice (Gow *et al.* 1999; Mazaud-Guittot *et al.* 2010). Tight junction strands between adjacent Sertoli cells from testes of claudin 11 knockout mice are not detected on ultrastructural examination (Gow *et al.* 1999). At the light microscopic level, morphological differences between the testes of wild-type and claudin

Table 1. Roles of occludin and claudins in addition to mediating barrier permeability.

function	protein
coactivator for soluble matrix metalloproteinases	claudins 1, 2, 3, 4 and 5 (Miyamori *et al.* 2001; Takehara *et al.* 2009)
coreceptor for hepatitis C virus	claudins 1, 6 and 9 (Evans *et al.* 2007; Zheng *et al.* 2007); occludin (Ploss *et al.* 2009)
mediate cell adhesion (weak)	claudins 1, 2 and 3 (Kubota *et al.* 1999)
mediate cell cycle regulation	claudin 11 (Mazaud-Guittot *et al.* 2010)
mediate cell differentiation	occludin (Schulzke *et al.* 2005)
mediate cell polarity	occludin (Tokunaga *et al.* 2007)
modulate cell migration and invasion	claudins 2, 3, 4 and 11 (Michl *et al.* 2003; Agarwal *et al.* 2005, 2009; Mima *et al.* 2008)
receptor for *Clostridium perfringens* enterotoxin	claudins 3 and 4 (Katahira *et al.* 1997*a*,*b*)

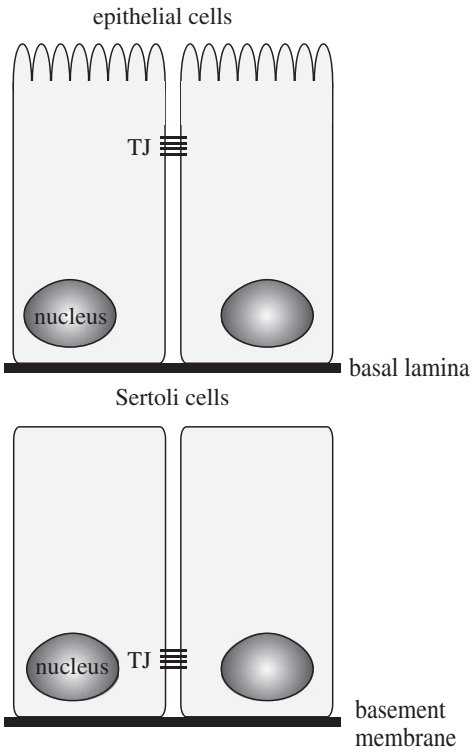

Figure 3. Comparison of distribution of tight junctional complexes between the seminiferous epithelium and other epithelia. Unlike other epithelia in which tight junctions are located apically, in the seminiferous epithelium tight junctions are located basally.

11 knockout mice are first detectable at postnatal day 20 (Mazaud-Guittot *et al.* 2010). In both prepubertal and adult claudin 11 knockout mice, the lumens of the seminiferous tubules are narrowed and often filled with aggregates of Sertoli cells (Gow *et al.* 1999; Mazaud-Guittot *et al.* 2010). Round spermatids are the most mature germ cell type detected, and there is evidence of increased germ cell apoptosis (Mazaud-Guittot *et al.* 2010). As expected with this level of testicular phenotype severity, claudin 11 knockout mice are sterile (Gow *et al.* 1999). In occludin knockout mice, the testes appear histologically normal at six weeks of age (Saitou *et al.* 2000). However, at 40 to 60 weeks of age the seminiferous tubules are atrophied and have a Sertoli-cell only phenotype. Despite the apparently normal spermatogenesis in young occludin knockout mice, these male mice are sterile.

An adult model of occludin disruption in the testes of rats also demonstrates the importance of occludin to BTB integrity (Chung *et al.* 2001). In this model, rat testes are injected with a synthetic peptide that corresponds to a portion of the second extracellular loop of occludin and disrupts occludin–occludin interactions. Loss of elongate spermatids can be detected by post-injection day 8, with loss of spermatocytes and spermatids in all tubules by day 27, reappearance of spermatocytes by day 47, and complete recovery by day 68. The time points with disrupted spermatogenesis correspond with time points in which there are increases in tracer leakage across the BTB in treated testes.

The integral membrane proteins JAM-A and JAM-B are also found at the BTB (Mruk & Cheng 2004). However, the contribution of these JAMs to BTB integrity is uncertain. Mice gene-deleted for JAM-B have normal fertility (Sakaguchi *et al.* 2006), and mice gene-deleted for JAM-A have normal testes morphology, with the subfertility in the male mice attributable to the role of JAM-A in regulating spermatozoal motility (Shao *et al.* 2008). Whether this reflects redundancy between JAM-A and JAM-B in regulating the BTB (Shao *et al.* 2008) or that JAMs have a minimal contribution to BTB integrity remains to be determined.

5. CLAUDIN AND OCCLUDIN EXPRESSION IN THE TESTES

Claudin mRNAs that have been identified in rodent testis via Northern blot analyses include claudins 1, 2, 3, 4, 5, 7, 8 and 11 (Furuse *et al.* 1998*a*; Morita *et al.* 1999*a*,*b*). Additionally, claudins 10, 12 and 23 have been detected by microarray analysis (Singh *et al.* 2009). Of those, the mRNA and protein expression patterns of claudins 1, 3, 5 and 11 have been further characterized.

Claudin 1 mRNA and protein are detected in mouse testes at postnatal day 3, are increased at postnatal day 10, but are decreased on postnatal days 16 and 30, compared with day 3 mRNA and protein expression levels (Gye 2003*b*). These data were generated from whole testes extracts and were not normalized for the number the Sertoli cells. Thus, the decrease in claudin 1 expression in testes from 16- and 30-day-old mice probably represents a dilutional effect as rapid germ cell proliferation causes a relative increase in the numbers of germ cells and a relative decrease in the numbers of Sertoli cells

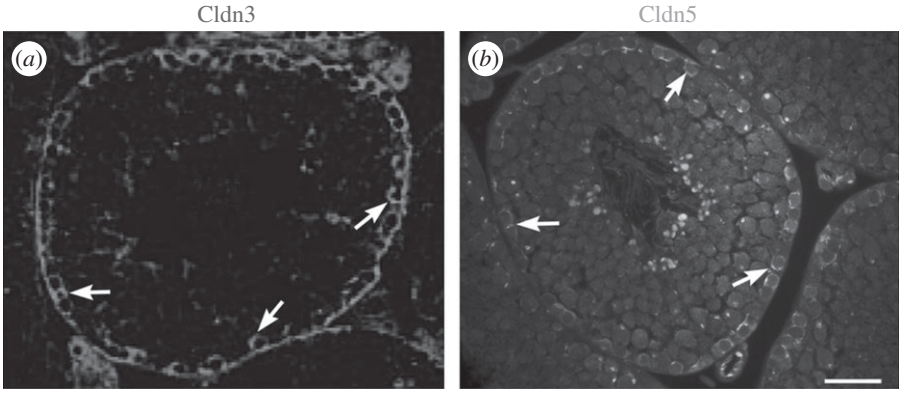

Figure 4. Claudin 3 (red) and 5 (green) protein expressions in mouse testes at stage VIII. Expressions of both claudins peak during Stage VIII of the seminiferous epithelial cycle (arrows), with expressions not detectable to minimal at other stages. (*a*) Claudin 3 image reprinted from Meng *et al.* (2005) (Copyright © 2005, National Academy of Sciences, USA). (*b*) Claudin 5 image reprinted from Morrow *et al.* (2009). Scale bar, 40 μm.

(Gye 2003*b*). In rats, expression of claudin 1 protein adjusted for testes weight increases from postnatal day 16–35 and then plateaus in adulthood (Yan *et al.* 2008*a*). Although claudin 1 protein expression was determined by Western blot analyses in mice and rats, determination of the cell-specific expression pattern via immunohistochemical analyses was not performed. Whether claudin 1 is a functional component of the BTB or not remains to be determined.

Claudin 3 mRNA is first detected in mouse testes at postnatal day 15, has an apparent peak at day 20, and then decreases to levels comparable to day 15 during puberty and adulthood (Meng *et al.* 2005). In primary cultures of Sertoli cells from 19- to 21-day-old rats, claudin 3 mRNA expression was not detected (Kaitu'u-Lino *et al.* 2007). On Western blot analyses, claudin 3 protein in mouse testes is first detected at day 15, has a peak at day 25, and then gradually decreases to levels comparable to day 20 during puberty and adulthood (Meng *et al.* 2005), whereas in rat testes the peak is detected at postnatal day 35 (Yan *et al.* 2008*a*). The data for the Western blots and the mouse qPCR analyses were generated from whole testes extracts and were not normalized for the number of Sertoli cells.

Immunofluorescence analyses of claudin 3 expression in mouse testes revealed interesting patterns. Claudin 3 is first detected by this method at postnatal day 15 and is seen at all levels of the seminiferous tubules (Meng *et al.* 2005). At day 20, the claudin 3 immunosignal is localized to the area of the BTB (Meng *et al.* 2005). In adult mice, claudin 3 expression is stage-specific and is observed during the stages when the preleptotene and leptotene spermatocytes migrate across the BTB (Meng *et al.* 2005). During stage VII, claudin 3 is detected just apical to the preleptotene spermatocytes, whereas during stage VIII and early stage IX, the immunosignal is seen both apically and basally to the preleptotene and leptotene spermatocytes, and during late stage IX and early stage X, claudin 3 is localized basal to the leptotene spermatocytes (Komljenovic *et al.* 2009). This expression pattern led to the conclusion that claudin 3 could be used as a marker to delineate the intermediate compartment that was hypothesized by Russell (1977). Claudin 3 immunosignal was not detected at the BTB at other seminiferous epithelial stages in mice (Meng *et al.* 2005), and was not detected in the seminiferous epithelium at all in rats (Kaitu'u-Lino *et al.* 2007). In Djungarian hamsters (*Phodopus sungorus*), claudin 3 is transiently expressed at the BTB during simulated transitioning from short-day to long-day photoperiods (Tarulli *et al.* 2008); Djungarian hamsters are seasonal breeders in which active spermatogenesis is induced by long-day (16 h of light) photoperiods and subsequent FSH secretion. Claudin 3 in testes is also associated with interstitial cells and spermatids in mice (Meng *et al.* 2005) and Djungarian hamsters (Tarulli *et al.* 2008) and interstitial cells in rats (Kaitu'u-Lino *et al.* 2007).

The differences in claudin 3 protein expression in the seminiferous epithelium among mice, rats and hamsters suggest that the role of claudin 3 in the BTB may be species-specific. However, fixation technique is important for detection of claudin 5 in the seminiferous epithelium (Morrow *et al.* 2009), and if this is a factor for the inability to detect claudin 3 in rat seminiferous epithelium is unknown. The inability to detect claudin 3 mRNA in primary cultures of rat Sertoli cells (Kaitu'u-Lino *et al.* 2007) suggests that rats do not express claudin 3 in their seminiferous epithelium. However, claudin 3 protein is detected on Western blot analysis of whole testes extracts from rats (Yan *et al.* 2008*a*). Whether these differences between these rat studies reflect contributions from the interstitial compartment for claudin 3 expression on Western blot analysis or *in vivo* versus *in vitro* differences in claudin 3 expression in rat Sertoli cells remains to be determined.

Claudin 5 protein expression in mouse testes has several interesting patterns (Morrow *et al.* 2009). In testes from 8-day-old pups, it is seen at all levels of the seminiferous epithelium surrounding all of the germ cells. In adult testes, claudin 5 protein localizes to the BTB with stage-specific expression at stages VIII and IX, similar to claudin 3 expression (Meng *et al.* 2005; figure 4). Claudin 5 protein is also

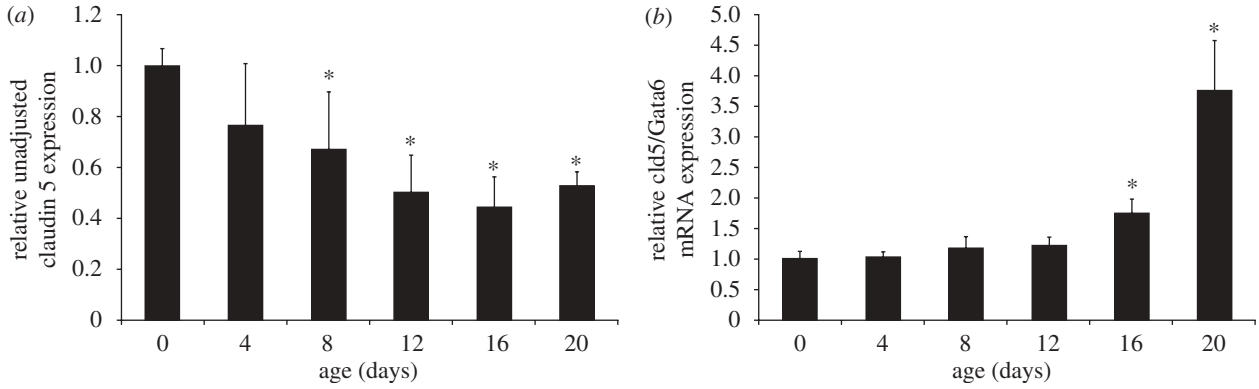

Figure 5. Developmental claudin 5 mRNA expression in mouse testes ($n = 2–4$ mice/group). (*a*) Unadjusted and (*b*) adjusted claudin 5 expression in whole testes extracts relative to expression values on day 0. To adjust for a germ cell dilution effect that occurs with age, claudin 5 expression was determined relative to Gata 6, a gene expressed in Sertoli cells but not germ cells. The adjusted claudin 5 value in panel b is thus normalized for the number of seminiferous tubules. * $p < 0.05$ compared with day 0 value. Modified from Morrow *et al.* (2009).

associated with spermatogonial cell membranes and cytoplasm and an unknown perinuclear organelle in spermatocytes, cells that do not have tight junctions. Immunocytochemistry on enriched populations of germ cells and Sertoli cells isolated from 8-day-old pups revealed claudin 5 expression in spermatogonia, spermatocytes and Sertoli cells. The specificity of the immunostain was confirmed by use of preincubation of the claudin 5 antibody with the immunogenic peptide. Claudin 5 protein is also expressed in testicular vascular endothelium and rete testis epithelium.

Because claudin 5 protein is expressed in multiple cell types in the testes, interpreting developmental claudin 5 mRNA expression is challenging. In whole testes extracts, claudin 5 mRNA has its peak expression on postnatal day 0 and declines to a plateau of about half the day 0 level at day 12 and in older mice (figure 5). However, when these claudin 5 mRNA data are normalized for the number of seminiferous tubules, claudin 5 mRNA expression remains constant in 0-day-old through 12-day-old mice and then increases at day 16 to reach a plateau of about 3 to 3.5 times the day 0 level in 20-day-old and adult mouse testes (Morrow *et al.* 2009). Because germ cells also express claudin 5, normalization by a Sertoli cell marker adjusts claudin 5 mRNA to the level of the seminiferous tubule and not the Sertoli cell (Morrow *et al.* 2009); thus the increase in claudin 5 expression mRNA may also have contributions from germ cells. The normalization also assumes that relative claudin 5 mRNA contributions from the seminiferous epithelial and vascular endothelial compartments remain consistent throughout development.

Claudin 11 mRNA expression is first detected prenatally in mouse testes at postcoitum day 12, at about the same time as the formation of the testes cords (Hellani *et al.* 2000). Postnatally, unadjusted claudin 11 mRNA expression peaks between days 6 and 15 and then declines through puberty to reach a low plateau in adulthood (Hellani *et al.* 2000). When developmental claudin 11 mRNA expression is normalized for the number of Sertoli cells, expression increases from postnatal day 0 through adulthood (Johnston *et al.* 2004; Willems *et al.* 2009).

Claudin 11 protein is first detectable in mouse testes by use of immunohistochemical staining at postnatal day 13 (Mazaud-Guittot *et al.* 2010). At this age, the immunosignal is throughout the entire seminiferous epithelium, from the basement membrane to the lumen. By day 20 and in adult mice, claudin 11 staining is primarily localized to the area of the BTB (Morita *et al.* 1999*b*; Hellani *et al.* 2000; Mazaud-Guittot *et al.* 2010), with fainter staining observed between Sertoli cells at the middle and apical regions in 1 report (Hellani *et al.* 2000). In Djungarian hamsters exposed to long-day photoperiods and undergoing active spermatogenesis, claudin 11 localization at the BTB is basal to the germ cells at the basement membrane during stages I–III and XI–XII, apical to those germ cells at stages VII–VIII, and intermediate to those germ cells at stages IV–VI and IX–X (Tarulli *et al.* 2008). Claudin 11 protein expression can be detected at all stages of the seminiferous epithelium cycle in mice (Hellani *et al.* 2000; Meng *et al.* 2005; Mazaud-Guittot *et al.* 2010), men (Fink *et al.* 2009) and Djungarian hamsters during active spermatogenesis (Tarulli *et al.* 2008). At the ultrastructural level, claudin 11 immunogold labelling is associated with the tight junction strands between Sertoli cells in mice (Morita *et al.* 1999*b*). In all of these reports, claudin 11 protein was only detected in Sertoli cells.

Occludin protein is detected via immunofluorescence in the testes cords of mice at embryonic day 13.5 (observation of vaginal plug = embryonic day 0.5; Cyr *et al.* 1999). At this time point and also at embryonic day 16.5, occludin immunostain is diffuse in the gonocyte cytoplasm. However, at embryonic day 18.5, the immunostain appears as a filiform-like network in the Sertoli cell cytoplasm with no signal associated with the gonocytes. The filiform staining pattern continues to be observed in neonatal, prepubertal, and adult mice, albeit with decreasing intensity as the mice age (Cyr *et al.* 1999). By postnatal day 14, occludin immunostain is detected as focal, wavy bands toward the base of tubules that contain more advanced germ cells. By postnatal day 23 and in adult mice, these bands are present in all tubules

at all stages of the seminiferous epithelial cycle. Occludin is also expressed in testicular vascular endothelium and rete testes epithelium in mice (Cyr *et al.* 1999). On Western blot analysis of whole testes extracts from rats, unadjusted occludin expression increases from postnatal days 18–35, and then plateaus in adulthood (Yan *et al.* 2008*a*).

Similar to mice, occludin immunostain is detected at all stages of the seminiferous epithelial cycle in dogs (Gye 2004) and Korean wild rabbits (*Lepus sinensis coreanus*; Yoon *et al.* 2009). However, in rats, occludin expression is stage-specific, with immunostain not detectable in Stage VIII tubules but strongly expressed at all other stages (Li *et al.* 2006). Occludin expression is also stage-specific in Djungarian hamsters exposed to long-day photoperiods; the stage distribution was not reported (Tarulli *et al.* 2008). Interestingly, occludin is not expressed in the seminiferous tubules of guinea pigs (*Cavia porcellus*) and men (Moroi *et al.* 1998).

These differences in occludin expression among species imply that the importance of occludin in regulating BTB integrity may be species-specific. In species and stages in which occludin is not detected, the functions of occludin would be superseded by claudins or JAMs. Alternatively, the lack of occludin detection in men and guinea pigs may be attributable to occludin splice variants in the seminiferous epithelium that are not recognized by antibodies against the C-terminus of wild-type occludin (Moroi *et al.* 1998). Splice variants with altered C-termini have been identified from various human cell lines (Mankertz *et al.* 2002; Gu *et al.* 2008); however, the distribution of these variants in the testes has not been evaluated. Differences in the expression patterns among the rodent species may also be attributable to differences in antibody specificity among the antibodies used in the various studies (Ramos-Vara 2005).

6. REGULATION OF CLAUDIN AND OCCLUDIN EXPRESSION IN THE TESTES

There are myriad factors that regulate claudin and occludin transcription, translation and cellular localization in the testes (see Mruk & Cheng 2004; Yan *et al.* 2008*b*; Lie *et al.* 2009 for reviews on regulation and dynamic restructuring of the BTB). This review will focus on the transcription factors, reproductive hormones and cellular associations that are important for claudin and occludin expression in the testes.

(a) *Testosterone and androgen receptor*

Testosterone is important for maintaining the integrity of the BTB (Janecki *et al.* 1991). Indeed, testosterone supplementation is able to counteract the disruptive effects of cadmium in primary cultures of rat Sertoli cells (Chung & Cheng 2001). The effects of testosterone and AR signalling on mRNA expression, protein expression, and protein localization of tight junctional proteins, including claudins 1, 3 and 11 and occludin, have been the focus of numerous studies (table 2).

In primary cultures of mouse Sertoli cells, testosterone supplementation or coculturing with Leydig cells has negligible effects on the expression of claudin 1 mRNA (Gye 2003*a*,*b*). Treatment of adult rats with the AR antagonist flutamide for 6 days did not significantly affect the levels of claudin 1 mRNA in whole testes extracts (Gye & Ohsako 2003). AR signalling does not appear to alter claudin 1 mRNA expression.

Claudin 3 dependence on AR signalling was detected in one line of Sertoli cell-specific AR knockout mice, in which claudin 3 mRNA was reduced by 10-fold and claudin 3 protein was not detected by use of immunofluorescence in testes from 8-week-old mice (Meng *et al.* 2005). Because of altered splicing of the floxed AR allele, the parental AR-flox mice have reduced pancellular AR signalling; this means that in addition to absent AR signalling in the Sertoli cells, the other cells of this line of Sertoli cell-specific AR knockout mice have reduced AR signalling (Holdcraft & Braun 2004). Interestingly, although claudin 3 mRNA is downregulated in the testes of Sertoli cell-specific AR knockout mice, it is not downregulated in the testes of parental AR-flox mice (Eacker *et al.* 2007). The parental AR-flox mice have greatly increased levels of serum testosterone, compared with wild-type mice (Holdcraft & Braun 2004). Assuming this reflects increased testosterone concentration in the testes, this may provide adequate stimulation for claudin 3 transcription in the Sertoli cells of the parental AR-flox mice. Testosterone supplementation to TM4 Sertoli cells transiently transfected with an AR expressing plasmid increased claudin 3 mRNA expression after 48 h, compared with values in non-supplemented cells (Meng *et al.* 2005). Although numerous potential AR response elements in the promoter region of mouse claudin 3 suggest that AR may directly mediate claudin 3 transcription (Eacker *et al.* 2007), this has yet to be confirmed. Also, results of microarray analysis of the testes of two other lines of Sertoli cell-specific AR knockout mice did not reveal changes in claudin 3 mRNA expression (Tan *et al.* 2005; Wang *et al.* 2006).

Claudin 11 responsiveness to AR signalling was detected by numerous *in vitro* models, in which primary cultures of mouse or rat Sertoli cells, receiving testosterone supplementation, had increased claudin 11 mRNA and protein expression, compared with expression values in cells that did not receive testosterone (Gye 2003*a*; Florin *et al.* 2005; Kaitu'u-Lino *et al.* 2007). There was also increased localization of claudin 11 protein to the cell membrane in cultured rat Sertoli cells that received testosterone (Kaitu'u-Lino *et al.* 2007). Addition of the AR antagonist flutamide and removal of testosterone to cultured rat Sertoli cells that had been previously supplemented with testosterone caused claudin 11 mRNA expression to decrease to levels comparable to cells grown in non-supplemented media (Kaitu'u-Lino *et al.* 2007). Interestingly, when the flutamide was removed and testosterone resupplemented to the cells, claudin 11 mRNA expression was not different compared with levels during flutamide treatment and to levels in non-supplemented control cells at the same time point (Kaitu'u-Lino *et al.* 2007). Flutamide treatment had no effect on basal levels of claudin 11 mRNA expression in cells that did not receive testosterone (Kaitu'u-Lino *et al.* 2007).

Table 2. Effects of testosterone and AR signalling on expression of BTB integral membrane mRNA and proteins. AR, androgen receptor; SCARKO, Sertoli cell-specific androgen receptor knockout.

protein	endpoint	treatment	effect[a]	reference
claudin 1	mRNA expression	flutamide treatment of adult rats	—	Gye & Ohsako (2003)
		testosterone supplementation to primary cultures of mouse Sertoli cells	day 2: ↑; day 4: —	Gye (2003*b*)
		bicompartmental coculture of mouse Sertoli and Leydig cells	day 2: —	Gye (2003*a*)
claudin 3	mRNA expression	mice with hypomorphic AR allele	—	Eacker *et al.* (2007)
		SCARKO mice (Braun line)	↓	Meng *et al.* (2005)
		testosterone supplementation of TM4 Sertoli cells	48 h: ↑	Meng *et al.* (2005)
	protein expression	SCARKO mice (Braun line)	↓	Meng *et al.* (2005)
	protein localization	SCARKO mice (Braun line)	not visualized	Meng *et al.* (2005)
claudin 11	mRNA expression	mice with loss of AR signalling in all cells	—	Johnston *et al.* (2004), Tan *et al.* (2005)
		SCARKO mice (Verhoeven line; Chang line)	↓	Tan *et al.* (2005), Wang *et al.* (2006)
		flutatmide treatment of adult rats	—	Gye & Ohsako (2003)
		bicompartmental coculture of mouse Sertoli and Leydig cells	day 2: ↑	Gye (2003*b*)
		testosterone supplementation to primary cultures of rat Sertoli cells	4–24 h: —; 48–96 h: ↑ day 0–3: —; day 4–7: ↑; day 9: ↑; day 13: ↑	Florin *et al.* (2005) Kaitu'u-Lino *et al.* (2007)
		primary rat Sertoli cells treated with testosterone on days 1–5, followed by flutatmide treatment on days 6–9, then testosterone on days 10–13 of culture	compared with media control cells: day 9: —; day 13: — compared with continuous testosterone supplementation: day 9: ↓; day 13: ↓	Kaitu'u-Lino *et al.* (2007)
		flutamide treatment of primary cultures of rat Sertoli cells on days 6–9 of culture	day 9: —; day 13: —	Kaitu'u-Lino *et al.* (2007)
	protein localization	testosterone supplementation to primary cultures of rat Sertoli cells	increased localization to the cell membrane at days 5, 9 and 13	Kaitu'u-Lino *et al.* (2007)
		primary rat Sertoli cells treated with testosterone on days 1–5, followed by flutatmide treatment on days 6–9, then testosterone on days 10–13 of culture	compared with continuous testosterone supplementation: decreased localization to the cell membrane on day 9, similar localization on day 13	Kaitu'u-Lino *et al.* (2007)
		flutamide treatment of primary cultures of rat Sertoli cells on days 6–9 of culture	decreased localization to the cell membrane on day 9	Kaitu'u-Lino *et al.* (2007)
occludin	mRNA expression	flutatmide treatment of adult rats	↓	Gye & Ohsako (2003)
		SCARKO mice (Chang line)	↓	Wang *et al.* (2006)
		testosterone supplementation to primary cultures of rat Sertoli cells	10–24 h: ↑ day 1–7: —	Chung & Cheng (2001) Kaitu'u-Lino *et al.* (2007)
	protein expression	testosterone supplementation to primary cultures of rat Sertoli cells	day 1: ↑; day 5: —	Yan *et al.* (2008*a*)

(*Continued.*)

Table 2. (*Continued.*)

protein	endpoint	treatment	effect[a]	reference
	protein localization	testosterone supplementation to primary cultures of rat Sertoli cells	increased endocytosis at 15, 30 and 60 min; no change in endocytosis at 180 min	Yan *et al.* (2008*a*)
			increased recycling from endosomes to cell membrane at 5 and 15 min	(Yan *et al.* 2008*a*)
			increased localization to the cell membrane at day 7	(Kaitu'u-Lino *et al.* 2007)
		primary rat Sertoli cells treated with testosterone on days 1–5, followed by flutatmide treatment on days 6–9, then testosterone on days 10–13 of culture	compared with continuous testosterone supplementation: decreased localization to the cell membrane on day 9, similar localization on day 13	(Kaitu'u-Lino *et al.* 2007)

[a]↑: increased expression; ↓, decreased expression; —, no change in expression.

The role of AR in mediating claudin 11 expression *in vivo* is less clear. In mice in which AR signalling is absent in all cells of the body, through either a natural mutation in AR (*Tfm* mice; Johnston *et al.* 2004) or through experimental gene deletion (Tan *et al.* 2005), there was no difference in the amount of claudin 11 mRNA per Sertoli cell, compared with values in wild-type mice. Also, treatment of adult rats with flutamide for 6 days did not change testicular claudin 11 mRNA expression (Gye & Ohsako 2003). However, in two of three different Sertoli cell-specific AR knockout mouse lines, claudin 11 mRNA expression is decreased in the testes (Tan *et al.* 2005; Wang *et al.* 2006); in the third line, claudin 11 mRNA expression was not reported to be changed on microarray analysis (Eacker *et al.* 2007). The effect of suppressed AR signalling on claudin 11 protein expression and localization in the aforementioned knockout mice and flutamide-treated rat models was not evaluated.

In vitro results reveal that testosterone and AR signalling can increase testicular claudin 11 mRNA expression. However, *in vivo* results suggest that multiple factors, in addition to AR signalling, control basal expression of claudin 11 mRNA. It is probable that in some mouse lines or treatment models, these factors can maintain basal claudin 11 mRNA expression in the absence of AR signalling. Indeed, results of an analysis of the core region of the claudin 11 promoter revealed 2 GATA/NF-Y binding sites and did not reveal any AR response elements (Lui *et al.* 2007). Both GATA/NF-Y binding sites are needed for optional transcription activity (Lui *et al.* 2007). It remains to be determined whether AR can bind and mediate claudin 11 transcription at non-core regions of the promoter or whether AR mediation of claudin 11 transcription is indirect.

Testicular occludin mRNA expression is decreased in adult rats treated with the AR antagonist flutamide for 6 days, compared with values in untreated rats (Gye & Ohsako 2003). Testicular occludin mRNA expression is also decreased at postnatal day 10.5 in a line of Sertoli cell-specific AR knockout mice (Wang *et al.* 2006). In primary cultures of Sertoli cells from 20-day-old rats, testosterone supplementation stimulated a mild increase in occludin mRNA expression in one experiment (Chung & Cheng 2001) and had no effect in another experiment (Kaitu'u-Lino *et al.* 2007). Whether AR-mediated signalling of occludin transcription is direct or indirect, and its relative contribution to basal occludin levels, remain to be determined. In primary Sertoli cell cultures from 20-day-old rats, testosterone stimulates a transient increase in occludin protein expression (Yan *et al.* 2008*a*) and regulates protein recycling and localization to the cell membrane (Kaitu'u-Lino *et al.* 2007; Yan *et al.* 2008*a*).

Protein localization appears to be the primary mechanism through which testosterone increases BTB integrity (table 2). In primary cultures of rat Sertoli cells, which were supplemented with testosterone on days 1–5, then treated with flutamide on days 6–9, and resupplemented with testosterone on days 10–13, mRNA expression of claudin 11 remained suppressed on day 13, whereas claudin 11 protein had re-localized to the cell membrane (Kaitu'u-Lino *et al.* 2007). In contrast, occludin protein was transiently increased in primary cultures of rat Sertoli cells after testosterone supplementation, with high levels of occludin endocytosis and recycling to the cell membrane (Yan *et al.* 2008*a*). What effect disrupted testosterone and AR signalling have on protein localization of claudin 11 and occludin *in vivo* remains to be determined.

(b) *Other transcription factors*

The transcription factor SOX8 regulates claudin 3 expression in an age-dependent manner (Singh *et al.* 2009). Male mice gene-deleted for SOX8 have defects in spermiation, dysregulation of the seminiferous epithelial cycle, sloughing of spermatocytes and round spermatids and testicular atrophy, all of which worsen as the mice age (O'Bryan *et al.* 2008). In testes from 10- and 20-day-old mice, claudin 3 mRNA expression is greater in SOX8 knockout mice than in heterozygote control mice. However, after establishment of the BTB, this expression pattern

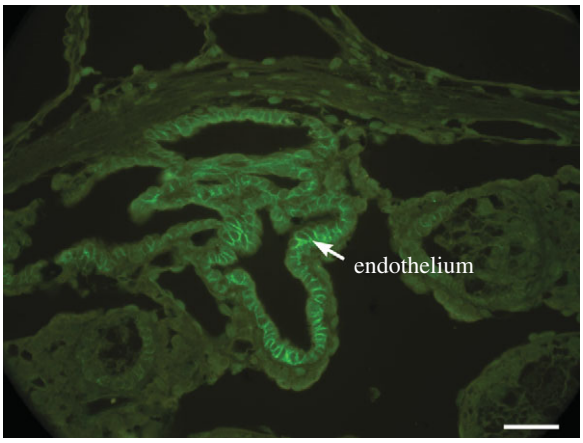

Figure 6. Claudin 5 expression in ETV5 knockout mice. Claudin 5 immunostain (green) is detectable in the rete testes epithelium of ETV5 knockout mice but is not detectable in the seminiferous epithelium (lower right). Claudin 5 is also expressed in testicular vascular endothelium (not shown). Reprinted from Morrow *et al.* (2009). Scale bar, 50 μm.

changes such that testicular claudin 3 mRNA expression is greater in heterozygote controls than in SOX8 knockout mice during puberty and adulthood (Singh *et al.* 2009). The mechanisms that are involved in the switch of SOX8 regulation of claudin 3 transcription after establishment of the BTB remain to be determined, as is a determination of whether SOX8 regulation is direct or indirect.

In mice gene-deleted for the transcription factor ETV5, claudin 5 protein is not expressed in the seminiferous epithelium but is expressed in vascular endothelium and rete testes epithelium (figure 6; Morrow *et al.* 2009). Thus, claudin 5 expression is ETV5-dependent in the seminiferous epithelium but ETV5-independent in vascular endothelium and rete testes epithelium. Analyses of the claudin 5 promoter in various endothelial cell models reveal a variety of binding sites and transcription factors that can regulate claudin 5 expression (Fontijn *et al.* 2008; Burek & Forster 2009). What role these other transcription factors have in mediating ETV5-independent expression of claudin 5 in testicular vascular endothelium and rete testis epithelium remains to be determined. In wild-type mice, claudin 5 protein is expressed in both Sertoli cells and germ cells (Morrow *et al.* 2009). It is not known if ETV5 is required in both of these cell types for each to express claudin 5, or if ETV5 expression in 1 cell type with subsequent intercellular signals to the other is sufficient for claudin 5 expression in the seminiferous epithelium. It also needs to be determined if ETV5 regulation of claudin 5 transcription within a Sertoli or germ cell is direct or indirect.

Claudin 11 expression in Sertoli cells is influenced by numerous transcription factors and coregulators (Lui *et al.* 2007). Specifically, the transcription factors GATA, NF-YA, and CREB form a complex that binds to the GATA/NF-Y regions of the claudin 11 promoter and induce transcription of claudin 11 mRNA. Negative control of claudin 11 transcription is mediated by Smad proteins, which bind to the GATA/NF-Y site that is furthest upstream from the origin. Smad proteins recruit the histone deacetylase 1 (HDAC1)/mSin3A complex, thus inducing histone deacetylation with the subsequent increase in DNA winding and hindrance for other transcription factors to bind. Analyses of the reported expression patterns of these transcription factors in the testes suggest that the ratio of the positive to the negative regulatory factors is important for maintaining claudin 11 expression throughout the stages of the seminiferous epithelial cycle (Lui *et al.* 2007). In gastric epithelial cells, hypermethylation of the claudin 11 promoter is associated with decreased expression of claudin 11 mRNA and protein (Agarwal *et al.* 2009); whether this is also true for Sertoli cells remains to be determined.

Promoter analysis of occludin in Sertoli cells has not been reported. In vascular endothelial cells, promoter analyses revealed that glucocorticoid receptor is important for occludin expression (Felinski *et al.* 2008; Harke *et al.* 2008). Activation by the transcription factor Sp3 and repression by YY1 are important for differential expression of occludin in various endothelial beds (Sade *et al.* 2009).

(c) *Follicle stimulating hormone*

The effects of FSH on the mRNA and protein expression of claudins 3 and 11 and occludin have been extensively studied in the Djungarian hamster (table 3), a seasonal breeding rodent (Tarulli *et al.* 2006, 2008). Increased light exposures, during the long-day photoperiods of summer, stimulate FSH secretion and result in active spermatogenesis. Conversely, FSH secretion is inhibited and spermatogenesis is quiescent during the short-day photoperiods of winter. When exogenous FSH is administered to hamsters exposed to short-day (8 h of light) photoperiods, claudins 3 and 11 and occludin proteins all undergo dramatic relocalization to the BTB. Whereas the claudin 3 protein expression at the BTB is transient, claudin 11 and occludin protein remain localized at the BTB in hamsters exposed to long-day (16 h of light) photoperiods. Through the relocalization of these and other proteins to the BTB, exposure to FSH changes the permeability of the BTB from leaky to tight. Interestingly, FSH suppresses the mRNA expression of claudins 3 and 11 and occludin in these hamsters, in that the mRNA expression values are lower in hamsters exposed to long-day photoperiods compared with values in those exposed to short-day photoperiods. Thus in Djungarian hamsters, increased mRNA expressions of claudins 3 and 11 and occludin do not indicate increased BTB functionality. It was noted that in Djungarian hamsters, the responses of testicular claudins 3 and 11 and occludin to FSH stimulation occur during a time period when intratesticular testosterone levels are low (Tarulli *et al.* 2008). This was interpreted as a difference between Djungarian hamsters and other rodent species regarding the importance of testosterone in establishing and maintaining the BTB.

Experiments evaluating the effects of FSH on claudin 11 mRNA expression in mice and rats have had variable results (table 4). In cultured mouse Sertoli cells, FSH supplementation decreases claudin 11

Table 3. Effects of FSH on expression of claudins 3 and 11 and occludin in Djungarian hamsters (Tarulli *et al.* 2006, 2008).

	effect[a,b]					
	claudin 3		claudin 11		occludin	
treatment	mRNA expression	protein localization	mRNA expression	protein localization	mRNA expression	protein localization
exposure of Djungarian hamsters to 16 h light per day for 12 weeks to stimulate endogenous FSH release	↓	associated with elongate spermatids	↓	expression at BTB	↓	stage-specific BTB expression
exposure of Djungarian hamsters to 8 h light per day for 12 weeks to suppress endogenous FSH release	↑	expression at apical regions of Sertoli cells	↑	cytoplasmic expression	↑	cytoplasmic expression
exogenous FSH given to Djungarian hamsters exposed to 8 h light per day for 12 weeks		day 2: expression at apical regions of Sertoli cells and at BTB; day 4: diminished apical expression, strong BTB expression day 10: variable expression at BTB		days 2 and 4: relocalization from cytoplasm to BTB day 10: expression at BTB similar to that of long-day photoperiod hamsters		days 2 and 4: expressed at apical regions of Sertoli cells and at BTB day 10: diminished apical expression, strong BTB expression

[a]↑: increased expression; ↓, decreased expression; —, no change in expression.
[b]mRNA expression is on a Sertoli cell basis.

mRNA expression during the first 24 h, with values returning to baseline by 48 h (Hellani *et al.* 2000). In cultured rat Sertoli cells supplemented with FSH for 7 days, claudin 11 mRNA expression is similar to unsupplemented cells for the first 2 days, increases on day 3, and remains elevated through day 7 (Kaitu'u-Lino *et al.* 2007). Differences between these *in vitro* studies were attributed to differing culture conditions (Kaitu'u-Lino *et al.* 2007). *In vivo*, FSH suppresses testicular claudin 11 mRNA expression in Djungarian hamsters but stimulates claudin 11 expression in mice, as claudin 11 mRNA expression per Sertoli cell is decreased in mice gene-deleted for the β-subunit of FSH or for the FSH receptor, compared with values in adult wild-type mice (Johnston *et al.* 2004). Differences in FSH signalling among various lines of AR knockout mice, attributable to different levels of FSH secretion or Sertoli cell FSH receptors, were considered a potential reason for different expression levels of claudin 11 mRNA per Sertoli cell among those lines (Tan *et al.* 2005). The effects of FSH on claudin 11 protein localization in mice and rat Sertoli cells has not been reported. Regarding occludin, in primary cultures of rat Sertoli cells, supplementation with FSH for up to 7 days has no effect on its mRNA expression (Kaitu'u-Lino *et al.* 2007).

(d) *Germ cells*

Germ cells are necessary for expression of claudin 5 in murine seminiferous epithelium. In the testes of W/W^v mice that received spermatogonial stem cell transplants, there is a mixture of aspermic tubules and tubules in which spermatogenesis has been successfully established. Claudin 5 protein is not detected in aspermic tubules but it is detected in tubules with spermatogenesis (figure 7; Morrow *et al.* 2009). Similar to wild-type mice, both Sertoli cells and germ cells appear to express claudin 5 protein. The germ cell stimulatory signal that induces claudin 5 expression in Sertoli cells remains to be determined.

Postmeiotic germ cells have an inhibitory effect on claudin 11 expression in rat seminiferous epithelium, whereas spermatogonia and premeiotic spermatocytes have no effect on claudin 11 expression (Florin *et al.* 2005). There is no difference in claudin 11 mRNA and protein expression per Sertoli cell between irradiated testes of rats killed 10 days after local X-ray irradiation and non-irradiated testes of control rats;

Table 4. Effects of FSH on mRNA expression of claudin 11 and occludin in mice and rats.

mRNA	treatment	effect[a]	reference
claudin 11	FSH supplementation of primary cultures of mouse Sertoli cells	6–24 h: ↓; 1 h, 48 h: —	Hellani *et al.* (2000)
	FSH supplementation of primary cultures of rat Sertoli cells	day 1 and 2: —; days 3–7: ↑	Kaitu'u-Lino *et al.* (2007)
	gene deletion of the β-subunit of FSH in mice	↑	Johnston *et al.* (2004)
	gene deletion of the FSH receptor	↑	Johnston *et al.* (2004)
occludin	FSH supplementation of primary cultures of rat Sertoli cells	days 1–7: —	Kaitu'u-Lino *et al.* (2007)

[a]↑: increased expression; ↓, decreased expression; —, no change in expression.

at this time point spermatogonia are absent but spermatocytes and spermatids are present (Florin *et al.* 2005). In rats killed 45 days after testes irradiation, claudin 11 mRNA and protein expression per Sertoli cell are increased, compared with values in non-irradiated testes; the irradiated testes have spermatogonia and spermatocytes by not postmeiotic spermatids at this time point (Florin *et al.* 2005). In cocultures of Sertoli cells and either spermatocytes or spermatids, claudin 11 mRNA expression is not different in spermatocyte cocultures, whereas it is decreased in spermatid cocultures, compared with values in Sertoli cell-only cultures (Florin *et al.* 2005). Among the proteins produced by round spermatids, growth differentiation factor 9 (GDF9) is a candidate for the claudin 11 inhibitory factor. Addition of GDF9 to primary cultures of rat Sertoli cells decreases claudin 11 protein expression and localization to the cell membrane; localization of occludin and ZO-1 were also affected (Nicholls *et al.* 2009).

In men, the neoplastic germ cells of testicular intraepithelial neoplasia (TIN) lesions also affect Sertoli cell claudin 11 expression and localization. Compared with the expression in tubules with normal spermatogenesis, claudin 11 mRNA and protein are both increased in tubules with TIN (Fink *et al.* 2009). However, claudin 11 protein localization in tubules with TIN is substantially decreased at the BTB and increased in the cytoplasm (Fink *et al.* 2009). This altered claudin 11 localization is associated with similar alterations in ZO-1 and -2 localization and leakage of tracer across the BTB (Fink *et al.* 2006).

7. CLAUDIN FUNCTIONS IN THE TESTES

In addition to contributing to BTB integrity, claudin 3 has been proposed as a marker for the hypothetical intermediate compartment (Komljenovic *et al.* 2009). This transient compartment was described by Lonnie Russell, who observed ultrastructurally normal tight junctions, both apically and basally to the preleptotene and leptotene spermatocytes, during Stages VIII–IX in rats (Russell 1977). In addition to claudin 3, these flanking tight junctions also contain occludin (Mirza *et al.* 2007). However, there is no evidence that both of the flanking tight junctions are functional at the same time. In a study in which tracers were injected intratubularly, intravascularly, or both, leptotene spermatocytes in Stages IX–X were surrounded by the intravascular tracer that was stopped just above these cells, whereas those in Stage X were surrounded by the intratubular tracer that was stopped just below these cells; the presence of two competent barriers associated with leptotene spermatocytes was not detected (Cavicchia & Sacerdote 1988).

The increase in expression of claudins 3 and 5 during Stage VIII and the known interactions between these claudin isoforms in other epithelia (Coyne *et al.* 2003; Daugherty *et al.* 2007) suggest that interactions between claudins 3 and 5 in Sertoli cells may be important for BTB dynamics during migration of preleptotene and leptotene spermatocytes (Morrow *et al.* 2009). One possible contribution of claudin 3–claudin 5 interactions may be to provide structural integrity during a period when other integral membrane proteins are being rapidly recycled. However, in other cell types, the barrier characteristics produced by claudin 3–claudin 5 interactions is highly dependent on cell type, expression levels of claudin 3 or claudin 5, and expression levels of other claudins (such as claudin 1; Coyne *et al.* 2003). It cannot be predicted whether the interactions between the extracellular loops of claudins 3 and 5 would induce a 'leakier' or 'tighter' barrier phenotype between Sertoli cells at Stage VIII versus the barrier phenotype at stages when these proteins are not expressed. Also, current models of BTB dynamic restructuring during Stage VIII suggest that induced expression of basal ectoplasmic specialization proteins may provide a 'patch' to maintain barrier integrity while the tight junctions are opened to allow spermatocyte passage (Yan *et al.* 2008*b*). In a model in which barrier integrity is maintained by adherens junctional proteins, structural integrity provided by homophilic and heterophilic interactions between claudins 3 and 5 would be minimal.

It is also possible that claudins 3 and 5 expression during Stage VII may contribute to cell signalling pathways rather than providing only direct structural support. The intracellular framework among tight junctional integral membrane and scaffolding proteins acts as a platform for numerous signalling molecules (Zahraoui *et al.* 2000; Siu *et al.* 2009). Indeed, claudin 11-based tight junctions are necessary for cell cycle regulation of Sertoli cells, implying a cell signalling function for claudin 11 (Mazaud-Guittot *et al.* 2010).

The function of claudin 5 in germ cells is unknown. It has been suggested that interactions between claudin 5 and membrane-type matrix metalloproteinases (MT-MMPs) on the germ cell membrane may enable activation of soluble MMPs in the basal compartment (Morrow *et al.* 2009). Although this interaction between claudins, MT-MMPs and soluble MMPs

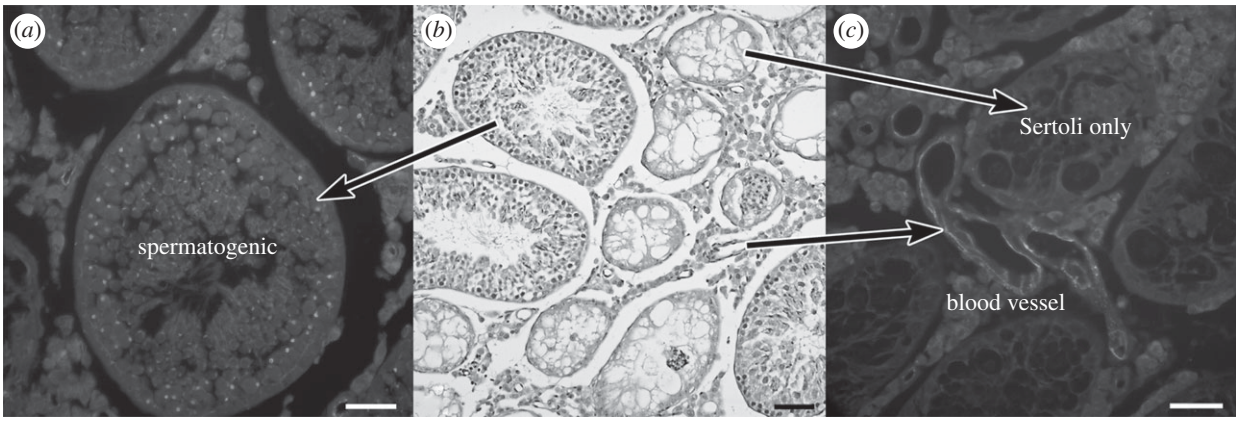

Figure 7. Effect of germ cells on claudin 5 expression. Representative images from W/W^v mice that received spermatogonial stem cell transplants from wild-type donors showing (*a*) expression of claudin 5 in tubules in which spermatogenesis was established; scale bar, 50 μm; (*b*) mixture of spermatogenic and aspermic tubules (PAS/hematoxylin); scale bar, 100 μm; (*c*) absence of claudin 5 in aspermic tubules; scale bar, 75 μm. Claudin 5 is also expressed in the vascular endothelium. (Note: the images are from different mice; modified from Morrow *et al.* (2009).)

has been demonstrated *in vitro* in other epithelial cells, there is no *in vivo* or *in vitro* experimental evidence for this model in germ cells.

Claudin 11 is necessary for the morphology of the tight junction strands in the testes. Tight junction strands between Sertoli cells are mostly parallel to each other with few branch points, whereas in other epithelia the tight junction strands have numerous anastomoses (Gilula *et al.* 1976). Fibroblasts transfected with claudin 11 develop parallel tight junction strands, similar to the strands in Sertoli cells (Morita *et al.* 1999*b*). Interestingly, Sertoli cells in claudin 11 knockout mice that are detached from the basement membrane lose polarity and acquire a shape similar to fibroblasts (Mazaud-Guittot *et al.* 2010).

Claudin 11 tight junctions between Sertoli cells have a role in cell cycle regulation. Sertoli cells in adult claudin 11 knockout mice are able to proliferate *in vivo* (Mazaud-Guittot *et al.* 2010). This proliferation is not due to retention of an immature state or to dedifferentiation, as these Sertoli cells express numerous differentiation markers. It was hypothesized that claudin 11 tight junctions enable contact inhibition between Sertoli cells, thereby maintaining the Sertoli cells in a quiescent state (Mazaud-Guittot *et al.* 2010). Regulation of the seminiferous tubule microenvironment by claudin 11 tight junctions is also important for attachment of the Sertoli cells to the basement membrane, proper epithelial organization, and nurturing of germ cells (Mazaud-Guittot *et al.* 2010).

8. FUTURE DIRECTIONS

Species comparisons of the claudins and occludin are important, especially in the field of pharmaceutical development. Much information about the BTB, particularly the structures and proteins involved in dynamic restructuring during preleptotene spermatocyte migration, has been discovered from the use of mice and rats (Mruk & Cheng 2004; Lie *et al.* 2009; Cheng *et al.* 2010). However, comparison among rodent species reveals differences in the expression of claudin 3 (Meng *et al.* 2005; Kaitu'u-Lino *et al.* 2007; Tarulli *et al.* 2008), and comparison among rodent species and men reveals differences in occludin expression (Moroi *et al.* 1998; Cyr *et al.* 1999; Li *et al.* 2006). Updated characterization of occludin and claudin 11 protein expression and initial characterization of claudins 3 and 5 protein expression in the seminiferous epithelium of men will aid in determining the applicability of discoveries made in rodent models to humans.

Results of investigations of claudin–claudin interactions in the extracellular space have revealed that the types of claudin–claudin interactions and resultant barrier characteristics are a function of the claudin isoforms expressed in the cells (Morita *et al.* 1999*b*; Hoevel *et al.* 2002; Coyne *et al.* 2003; Van Itallie *et al.* 2003; Wen *et al.* 2004). Specifically, claudins 3, 5, and 11 have homophilic interactions with their respective isoforms, claudins 3 and 5 have heterophilic interactions with each other, and the barrier characteristics (neutral molecule flux, transelectrical resistance) are different among cell cultures that express claudin 3 only, claudin 5 only, or both claudins 3 and 5 (Coyne *et al.* 2003; Morita *et al.* 1999*b*). However, it is unknown how the heterophilic and homophilic interactions of claudins 3 and 5 are altered when claudin 11 is also expressed. Investigation of the barrier characteristics of cells expressing various combinations of claudins 3, 5, and 11 would provide insight into the barrier functions of the seminiferous epithelium during the various cycle stages, particularly during preleptotene spermatocyte migration at Stage VIII when expression of claudins 3 and 5 are highest. Cell signalling molecules known to be involved in BTB dynamic restructuring should also be evaluated in addition to the barrier characteristics. This would be helpful in determining the relative structural versus cell signalling roles of claudins 3 and 5 in Sertoli cells and provide for integration of these claudins into current models BTB dynamic restructuring (Yan *et al.* 2008*b*).

Germ cells are needed to stimulate expression of claudin 5 by Sertoli cell *in vivo* (Morrow *et al.* 2009). It can be presumed that coculture with germ cells or supplementation with the unidentified germ cell-derived stimulant would be necessary for Sertoli cells to express claudin 5 *in vitro*. Alternatively, until the germ cell-derived stimulant is identified, transfection of Sertoli cells with vectors containing claudin 5 DNA may be necessary to induce claudin 5 expression in Sertoli cell cultures.

Expression of claudin 5 in germ cells, cells that do not have tight junctions, suggests that additional roles for this protein may be found (Morrow *et al.* 2009). Ultrastructural examination and immunogold analysis to discover the germ cell organelles with which claudin 5 is associated is an initial step towards discovering those roles. Also, in other cell types, the membrane proteins MMP14 (Miyamori *et al.* 2001) and connexin 43 (Nagasawa *et al.* 2006) are associated with claudin 5. Interactions between claudin 5 and MMP14 on germ cell membranes to activate pro-MMP2 has been suggested as a germ cell function of claudin 5 (Morrow *et al.* 2009), and gap junctions derived from connexin 43 are a source of cell–cell communication from Sertoli cells to spermatogonia and spermatocytes (Decrouy *et al.* 2004). Therefore MMP14 and connexin 43 are recommended as initial candidates for evaluation of colocalization with claudin 5 on germ cell membranes.

The expression of claudin 5 in germ cells along with the expression of claudins 3 and 5 in Sertoli cells during Stage VIII suggests that claudin–claudin interactions between germ cells and Sertoli cells may be a means of guidance during preleptotene spermatocyte migration. However, if claudin–claudin interactions were a guide for germ cell migration, these *trans* interactions would be different than the interactions proposed by current models. The *trans* claudin–claudin interactions described by current models result in formation of tight junctions and obliteration of the intercellular space, which are not observed between germ cells and Sertoli cells.

Modalities used to evaluate integral membrane proteins of the BTB include mRNA quantification by use of Northern blot or quantitative PCR analyses, protein quantification via Western blot analysis, and protein localization by use of immunohistochemical analysis. It is interesting that some conditions associated with increased (or decreased) mRNA expression were also associated with decreased (or increased) protein localization to the cell membrane (Tarulli *et al.* 2006, 2008; Kaitu'u-Lino *et al.* 2007; Fink *et al.* 2009). Future studies should be cautious in making statements regarding integral membrane protein functionality that are based solely on mRNA quantification. Ultimately, protein localization analyses should be performed as a more definitive aid in determining protein functionality in the BTB.

9. CONCLUSION

The recent discovery of claudins 3 and 5 as components of the BTB have increased the complexity involved in studying the function of this unique epithelial barrier. The similar expression patterns of claudins 3 and 5 in murine seminiferous epithelium suggest that interactions between these proteins may be important for mediating BTB dynamics during preleptotene germ cell migration. Future studies should be directed towards understanding the interactions between integral membrane proteins of the BTB and identifying species differences in protein expression and function among the rodent species and men.

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