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·*Review*·

Orchestration of occludins, claudins, catenins and cadherins as players involved in maintenance of the blood-epididymal barrier in animals and humans

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Abstract

Although spermatozoa are formed during spermatogenesis in the testis, testicular spermatozoa are immature and cannot swim or fertilize. These critical spermatozoal functions are acquired in the epididymis where a specific luminal environment is created by the blood-epididymal barrier; proteins secreted by epididymal principal cells bind to maturing spermatozoa and regulate the maturational process of the spermatozoa. In the epididymis, epithelial cell-cell interactions are mediated by adhering junctions, necessary for cell adhesion, and by tight junctions, which form the blood–epididymal barrier. The regulation of these cellular junctions is thought to represent a key determinant in the process of sperm maturation within the epididymis. Tight junctions between adjacent principal cells permit the formation of a specific microenvironment in the lumen of the epididymis that is essential for sperm maturation. Although we have made significant progress in understanding epididymal function and the blood-epididymal barrier, using animal models, there is limited information on the human epididymis. If we are to understand the normal and pathological conditions attributable to human epididymal function, we must clearly establish the physiological, cellular and molecular regulation of the human epididymis, develop tools to characterize these functions and develop clinical strategies that will use epididymal functions to improve treatment of infertility. *(Asian J Androl 2007 July; 9: 463–475)*

Keywords: claudins; cadherins; catenins; human; rat; mouse; tight junction; adherens junction

1 Introduction

The formation of apical tight junctions between adjacent epithelial cells of the epididymis and the creation of the blood-epididymal barrier represents a key element of epididymal physiology. The formation of this cellular barrier is critical not only for the protection of spermatozoa from the immune system but, in cooperation with the secretory and endocytic functions of the epithelial cells, regulates the composition of the luminal environment of the epididymis that is responsible for sperm maturation. Much of our knowledge on the regulation and the cellular and molecular makeup of the blood-epididymal barrier has been acquired from animal models, which provide the basis of our understanding of this barrier. Although animal model studies are critical for understanding human function in many systems, including male reproduction, there are limitations regarding the applicability of observations made using animal models to studies on normal functions and pathologies of the human epididymis leading to reduced fertility. The objectives of this review are to examine what we have learned about the blood-epididymal barrier in animal models and to compare it with recent data on the human epididymis by assessing the similarities and differ-

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ences between species.

2 The epididymal junctional complex

Tight junctions between epithelial principal cells are responsible for the formation of the blood-epididymal barrier [1, 2]. The ultrastructural anatomy of the blood-epididymal barrier was first described by Friend and Gilula [3] who reported the presence of a series of tight junctions between adjacent principal cells of the epididymis. Freeze fracture studies by Suzuki and Nagano [4] provided further evidence of a blood-epididymal barrier by showing the presence of tight junction mesh networks between principal cells. They also demonstrated that these junctions varied in number and complexity along the epididymis, being more extensive in the caput epididymidis and least extensive in the cauda epididymidis. These observations were substantiated by routine EM analyses, which showed that in the initial segment, the junctional complex between adjacent principal cells is composed of an extensive tight junction that spans a considerable length of the adjacent plasma membranes, but contains relatively few desmosomes, whereas in the other regions of the epididymis the extent of the tight junction is reduced and there are more desmosomes [5]. We postulated that because the epididymal lumen in the initial segment is smaller than in the other regions of the epididymis, the pressure on the epithelium might be greater and this might account for the need for a more extensive tight junction [5].

Morphological evidence for the integrity of the barrier was provided by Hoffer and Hinton [6], who demonstrated that although both lanthanum and inulin can cross the blood vessels and basement membrane of the epididymis, these markers cannot cross the tight junctions between the epithelial principal cells to enter the lumen of the tubule. Using lanthanum exclusion experiments, Agarwal and Hoffer [7] showed that the blood-epididymal barrier begins to form in the rat caput epididymidis by 18 days of age and is complete throughout the epididymis by 21 days. Guan *et al*. [8] reported that in the Wistar rat the blood–epididymal barrier is already fully formed by 7 days of age. Pelletier [9] reported that the blood-epididymal barrier is functional during embryonic development in the mink. In the murine model, we have shown that occludin is localized to tight junctions by embryonic day 13.5 and that an epididymal lumen is already beginning to form at this age. Although this observation does not preclude the possibility that the murine blood-epididymal barrier is leaky, it does suggest that a barrier is already in place by that age. These studies point to the necessity for re-examining the timing of the formation of the blood-epididymal barrier at this window in time to identify the factors regulating the formation of the blood-epididymal barrier. Horseradish peroxidase perfusion studies have demonstrated the presence of the blood-epididymal barrier in primates, including in humans [10].

Intercellular junctions are dynamic interactions. Portions of the lateral plasma membrane of adjacent principal cells are internalized along the entire epididymis, forming annular junctions [5]. In Sertoli cells of the testis, tight and gap junctions are also disposed through annular junctions [9]. In these cells, it has been postulated that the turnover of junctions is associated with the movement of proliferating spermatocytes from the basal compartment to the adluminal compartment. In the epididymis, only blood cells, monocytes and lymphocytes appear between adjacent principal cells, but junctions between these two cell types are not present, nor do they penetrate the junctional complex under normal circumstances [11, 12]. Turnover of plasma membranes in the epididymis suggests a mechanism whereby junctional proteins may be renewed.

3 Tight junctions and the blood-epididymal barrier

Although the data described above provide physical evidence for a blood-epididymal barrier, the evidence of a functional blood-tissue barrier has been suggested by the large concentration differences between ions, inorganic and organic molecules within the lumen of the convoluted tubule and the extracellular fluid [13]. Using direct micropuncture of the tubule following perfusion with labeled organic molecules, the epididymis can concentrate certain organic molecules such as carnitine and inositol from 10-fold to 100-fold, while other compounds such as inulin, glucose and serum albumin can be effectively excluded [14]. This indicates that the barrier is selective and transports certain molecules against a concentration gradient into the epididymal lumen.

4 Creation of a specific epididymal luminal environment

Tight junctions are essential structures for functional epithelial physiology. They serve several functions, including a "barrier" role between cells and the paracellular space [15, 16], as well as a boundary between the apical and basolateral plasma membranes, which generates and maintains cell polarity, the so-called "fence" function [17, 18]. Both barrier and fence functions of tight junctions, in addition to vectorial transport across the epithelium, permit the development of a specific luminal microenvironment. In the epididymis, it has long been thought

that the luminal environment is responsible for sperm maturation. Although the structural components of adhering and tight junctions are necessary for the formation and maintenance of the blood-epididymal barrier, the selective transport of ions and solutes across the epithelium as well as proteins secreted and endocytosed by principal cells, all contribute to form this specific luminal environment.

The movement of solutes, ions and water across epithelia occurs through both transcellular and paracellular routes. The total transepithelial transport across an epithelium is the sum of the two distinct components: transcellular and paracellular transport. Transcellular transport results from the regulated movement of ions, solutes and water across the apical and basolateral membranes by specific protein pumps, channels and transporter proteins. In this way, these systems maintain the proper acidic pH of the lumen, cell volume, intracellular pH and rapid movement of water across the epithelium. Transcellular transport has a very high degree of molecular specificity, is tightly regulated, and is variable among different epithelia. This type of transport is active, dependent either on hydrolysis of ATP or on the electro-osmotic gradient generated by basolaterally positioned Na^+/K^+-ATPase. Maintenance of these gradients is dependent on limiting back diffusion between cells through the paracellular pathway. In this way, the tightness of the paracellular barrier and its molecular selectivity in the epididymis contribute significantly to overall epithelial transport characteristics and maintenance of the epididymal lumen to perform its function of sperm maturation [19, 20].

5 Adhering junctions and cadherins

The formation of intercellular junctions involves the interactions of cell adhesion molecules between adjacent cells, followed by the addition of junctional proteins that assemble into tight and gap junctions [21, 22]. The process of cell adhesion is a fundamental cornerstone to further intercellular junction formation. Adhering junctions are formed by cadherins, a large family of calcium-dependent cell adhesion molecules, which mediate calcium-dependent homotypic interactions between adjacent cells (Figure 1). Over 100 different cadherins have now been identified [23]. Cadherins are single-pass transmembrane glycoproteins that are anchored to the actin-based cortical cytoplasm via catenins [24, 25]. Cadherins have been subdivided into several subgroups: classical/Type I cadherins; atypical/Type II cadherins; desmosomal cadherins; protocadherins; and cadherin-related proteins [26]. We will focus exclusively on classical or Type I cadherins in this review, because they are the only cadherins that have thus far been identified in the epididymis. The primary sequence of classical cadherins consists of a long extracellular region composed of ectodomains, which contain the cadherin recognition and binding site as well as calcium-binding sites. The first ectodomain contains an HAV amino acid sequence necessary for cadherin-cadherin binding [27, 28]. The amino acids adjacent to the HAV sequence are necessary for the recognition specificity of cadherins. Calcium binding sites are present on each subdomain of the extracellular region and the presence of calcium is essential for cadherin-mediated cell adhesion. The cytoplasmic domain of cadherins forms a tight complex with several proteins which either link cadherins to the cytoskeleton or are involved in signal transduction pathways. These include catenins, actinin, vinculin and tight junctional protein 1 (also known as zonula occludens-1; TJP1). The cadherin-catenin complex is essential for cadherin-mediated cell adhesion. In a variety of cell types, the levels of cadherins appear to regulate the process of

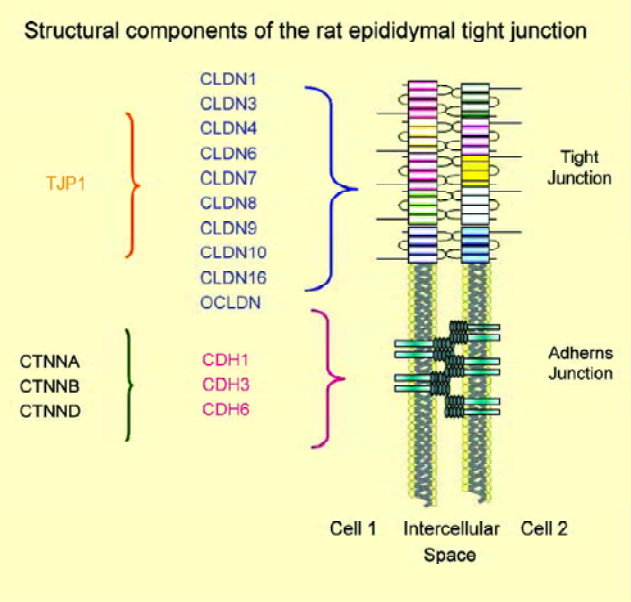

Figure 1. Schematic representation of the epididymal tight and adherens junctional complex. Several transmembrane tight junctional proteins named claudins (CLDNS) have been identified in epididymal tight junctions as well as tight junctional protein 1 (TJP1), which binds to the cytoplasmic tail of CLDNS and acts as both an anchor to the cytoskeleton and a signal transduction protein. Several cadherins (CDH) have also been identified in the rat epididymis as well as several members of the catenin (CTNN) family that bind to the cytoplasmic terminal of cadherins and are implicated in linking cadherins to the cytoskeleton and in signal transduction.

adhesion, suggesting that the cytoplasmic binding proteins are present in excess [29]. Hence, one of the functions of cadherins might be to sequester β-catenin from the cytoplasm into adhering junctions. This might indirectly alter gene expression, because cytoplasmic β-catenin can be stabilized by signaling pathways and may then be translocated to the nucleus where it interacts with the transcription factor LEF-1 to regulate the expression of a variety of genes [30].

The identification of catenins binding to the cytoplasmic domain of cadherins has been shown to be important not only for linking cadherins to the cell cytoskeleton but also for intracellular signaling [31–34]. Both β-catenin and γ-catenin, or plakoglobulin, have a high degree of homology with the Armadillo family of proteins, which encodes components of a signal transduction pathway in *Drosophila* [35].

There has been considerable interest in signal transduction pathways and cadherins. Tyrosine kinases have been identified in adherens junctions and their phosphotyrosine activity can be induced [36]. Increased tyrosine phosphorylation by transfection of cells with the *Src* and *Ras* oncogenes is correlated with a loss of intercellular adhesion [36]. Tyrosine phosphatase inhibitors have been reported to increase intercellular adhesion. Proteins such as p100/p120 are also members of the Armadillo family and have been localized to adhering junctions. Activation of protein kinase C (PKC), a serine/threonine kinase, results in a dephosphorylation of p100/p120 by perturbing the phosphorylation cycle, leading to increased permeability across epithelial cell monolayers [37]. Hence, the regulation of cadherins, either directly or indirectly, is an important factor contributing to the regulation of intercellular junctions [38]. Small GTPases (Rac, Rho and Cdc42), have also been implicated in cadherin-mediated adhesion [39]. These proteins appear to be involved in regulating actin-membrane interactions. Although the physiological role of these proteins is unclear, they appear to be involved primarily in the assembly and/or disassembly of adherens junctions [33, 39]. Nectins and integrins have also been implicated in regulation of cadherins [40].

E-Cadherin (CDH1) and P-Cadherin (CDH3) are present in the rat epididymis [41, 42]. CDH3 mRNA levels are maximally expressed in the first 7 days postnatally, whereas CDH1 mRNA peaks at the time of puberty. Immunogold labeling at the electron microscope level indicates that CDH1 is localized between the lateral plasma membranes of adjacent principal cells at the level of apically located junctional complexes in the adult rat epididymis, as well as in the deeper underlying regions of the extracellular space between the lateral plasma membranes [43, 44]. Similar observations were also noted in the human and murine epididymis [45-47].

As tissues differentiate, they switch from one cadherin to another to form new cell contacts. This essential characteristic of cell adhesion molecules in developing tissues leads to the conclusion that they must be regulated either by endocrine, autocrine and/or paracrine factors, which allow cells to coordinate the expression of their cell adhesion molecules to establish new contacts with each other. In the caput-corpus epididymidis, age-dependent changes in CDH1 mRNA concentrations during postnatal development increase to peak at 42 days of age [43]. These changes in CDH1 mRNA levels in the caput-corpus epididymidis are correlated with developmental changes in 5α-reductase activity [48]. This enzyme catalyzes the conversion of testosterone to its active metabolite dihydrotestosterone. Interestingly, CDH1 mRNA concentrations in the cauda epididymidis are not regulated in a similar fashion [43]; rather, CDH1 mRNA levels do not increase with rising serum levels of androgens that occur during postnatal development. However, in adult orchidectomized rats whose testosterone levels are maintained, Cyr *et al*. [42] demonstrate that in all segments of the epididymis, including the cauda, CDH1 mRNA levels are androgen-dependent. Hence, the regulation of CDH1 in the cauda epididymidis might reflect multifactorial regulation. CDH1 immunostaining in the corpus epididymidis decreased from an intense immunoreaction in 3-month-old rats to below the levels of detection in 24-month-old aging Brown-Norway rats. The blood-epididymal barrier in these older rats was compromised and immunostaining for both occludin and TJP1 was absent. In contrast, in the initial segment, CDH1 immunostaining increased as a function of age, and in this region the blood-epididymal barrier remained functional [44].

Alpha-Catenin (CTNNA) was localized along the lateral plasma membranes of adjacent principal cells as well as between principal and both clear and basal cells. Immunostaining was more intense in the distal corpus and cauda regions of the epididymis compared with other regions. A postnatal developmental analysis of CTNNA distribution revealed that in the epididymis, immunostaining was already intense by day 7 between the adjacent epithelial cells in all regions except the cauda, and by day 28 staining was present throughout the epididymis. Because androgen levels are still low at these ages, the data suggest that CTNNA is not regulated by androgens [49]. Similar observations have also been made for β-catenin [49]. Furthermore, $p120^{ctn}$, a catenin that binds cadherins and can alter cell adhesion, has also been identified in the epididymis by immunostaining [49].

Interestingly, in orchidectomized rats, there is an increase in cytoplasmic staining for both α-catenin and β-catenin immunostaining. This suggests that in the absence of androgens there might be a degradation or disruption of the adhering junction [49]. These data indicate that the cadherin-catenin complex in the epididymis resembles that of other epithelial cells, but appears to be regulated, at least in part, by testicular androgens.

6 Occludin

Occludin was the first transmembrane protein identified in tight junctional strands. It is a phosphoprotein of approximately 65 kDa containing four membrane spanning domains and two extracellular loops [50]. It has a long extracellular carboxy-terminal and a short intracellular amino terminal and two extracellular loops. One occludin binds to an occludin molecule from an adjacent cell and has been shown to be a functional component of tight junctions. In *Xenopus*, the expression of truncated occludin increases paracellular leakage, whereas in cultured epithelial cells, synthetic peptides corresponding to the second extracellular loop of occludin decrease transepithelial resistance [51]. Occludin has been identified in a variety of tissues and is associated with the cytoskeleton through a direct interaction with various proteins [50, 52].

Tight junctions are formed in the murine epididymis as early as embryonic day 12, as shown by freeze fracture electron microscopy [54]. At this age, a mesh network of tight junctions surrounds the entire circumference of the epithelial epididymal cells at the juxtaluminal position [54]. Occludin is expressed in the murine epididymis as early as embryonic day 13.5, although at this age there is a strong cytoplasmic reaction, suggesting that the formation of tight junctions at this age might still be incomplete. Although it has been reported that tight junctions increase in the epididymis during embryonic development [54], we could not assess by immunofluorescence whether or not occludin levels were also increasing [52]. The expression of occludin in the area of the tight junctions in the developing epididymis (embryonic day 18.5) occurs much earlier than noted in the seminiferous epithelium (postnatal day 14). Occludin is localized to the apical cell surface of the mouse epididymis by embryonic day 18.5, which coincides with peak androgen levels, suggesting that androgens might be important in the regulation of embryonic epididymal tight junctions. Clearly, the results of these experiments suggest that the regulation of occludin expression in testicular tight junctions differs from that in epididymal tight junctions.

In the adult, occludin has been noted apically between adjacent principal cells except in the proximal initial segment. In this region, occludin has been seen only in association with narrow cells, this despite the fact that there is an extensive tight junction in the initial segment. These data suggest that other tight junctional proteins must be present between principal cells in this region, as tight junctions have been shown to exist between these cells [5].

7 Claudins (CLDNS)

The discovery of a second family of transmembrane tight junctional proteins named claudins (CLDNS) demonstrates the complexity of tight junctions. To date, 20 claudins have been identified, and their tissue distribution is widespread and variable. CLDNS can co-localize with occludin; however, in the absence of occludin, claudins are still recruited to tight junctions, suggesting a crucial role in tight junction formation [53, 55].

We reported that *Cldn1* mRNA transcripts and protein are present in all epididymal segments in rat, as early as postnatal day 7 [56]. Additionally, immunohistochemical studies show that CLDN1 is not exclusively localized to tight junctions, but is also located along the lateral plasma membranes of epithelial principal cells, and between principal and basal cells, in all regions of the epididymis. This suggests that although CLDN1 might be involved in the formation and maintenance of epididymal tight junctions, it might also be involved in other cell–cell interactions. It has been suggested that CLDNS along the lateral plasma membrane might represent reserves of CLDNS prior to their incorporation into the tight junctions.

Tight junctions are comprised of multiple CLDNS. Reports in the rat epididymis indicate that tight junctions are comprised of CLDN1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11. However, CLDN5 was localized only to the endothelial cells and although *Cldn2* transcripts are present in the epididymis, these are not translated into proteins. The significance of such a large number of proteins that comprise epididymal tight junctions is unknown, as is the exact nature of the relationship between these proteins in the regulation of the blood-epididymal barrier. In MDCK cells, certain CLDNS, such as CLDN2, might increase paracellular permeability without altering the number of tight junctional strands [57]. Turksen and Troy [58] reported epidermal barrier dysfunction in transgenic mice that overexpressed CLDN6. These animals lived only a short time after birth, putatively as a consequence of increased water loss due to a defective epidermal barrier. The authors suggested that CLDN6 was involved in both epithelial differentiation as well as development of the

epidermal barrier. These findings are similar to those reported by Furuse *et al*. [59], who observed rapid water loss due to lack of an intact epidermal barrier, and death shortly thereafter, in *Cldn1* deficient (knockout) mice. Both studies were among the first to demonstrate the importance of CLDNS in tight junctions of stratified epithelia (such as epidermis), as well as in simple epithelia. It appears that certain CLDNS might play specific roles in regulating paracellular permeability within the tight junctional complex.

The paracellular component of transport is a passive process, resulting from paracellular dissipation of the electro-osmotic gradients established by transcellular transport. It also lacks the vast functional diversity and molecular discrimination of the transcellular component. Paracellular transport has two basic characteristics: (i) permeability, the magnitude of the barrier; and (ii) selectivity, the ability to discriminate molecular size and ionic charge. In the kidney, CLDN16 has been shown to be important for Mg^{2+} transport between cells [60]. Numerous claudins have been implicated in regulation of paracellular barriers in a variety of epithelia [15].

Although these functions have been demonstrated in the epididymis, changes in ionic composition along the epididymis suggest that paracellular transport is likely to represent a critical component in the regulation of luminal ionic homeostasis. This might explain why there are differences in the expression of CLDNS along the epididymis.

8 Other tight junction proteins

Tight junctions are also composed of integral transmembrane proteins that are linked to membrane-associated proteins, such as junction-associated membrane proteins and peripheral membrane proteins, including the membrane-associated guanylate kinase (MAGUK homologues, such as TJP proteins) family and non-MAGUK proteins; namely, symplekin, cingulin and 7H6 antigen.

Byers *et al*. [46] reported the presence of both TJP1 and cingulin both *in vivo* and in cultured epithelial principal cells. In the adult rat epididymis, TJP1 is localized apically between adjacent principal cells [44, 49]. Immunoprecipitation studies suggest that TJP1 associates with components of the adhering junction [49]. Because TJP1 is a signaling protein, it is possible that it is involved in signaling pathways between adhering and tight junctions. To determine if β-catenin is associated with TJP1, proteins were isolated from 7, 18 and 91-day-old rats and immunoprecipitated with either β-catenin or TJP1 to compare differences in protein binding. These studies showed that TJP1 immunoprecipitates contain β-catenin at all ages but that they are associated in greater quantities in younger rats as compared to adults. These findings suggest that TJP1 and β-catenin interact extensively during the formation of the epididymal tight junctional strands, and much less so after the formation of the tight junctional complex appears to stabilize (Figure 2) [49].

9 Regulation of epididymal tight junctions

The factors regulating the formation and maintenance of the blood-epididymal barrier are unknown. Suzuki and Nagano [4] have reported that orchidectomy of adult mice results in a decrease in the number of tight junctional strands in the epididymis. This suggests that maintenance of epididymal tight junctions is regulated by one or more testicular factors. In the mink, a seasonal breeder, Pelletier [9] has reported that the blood-testis

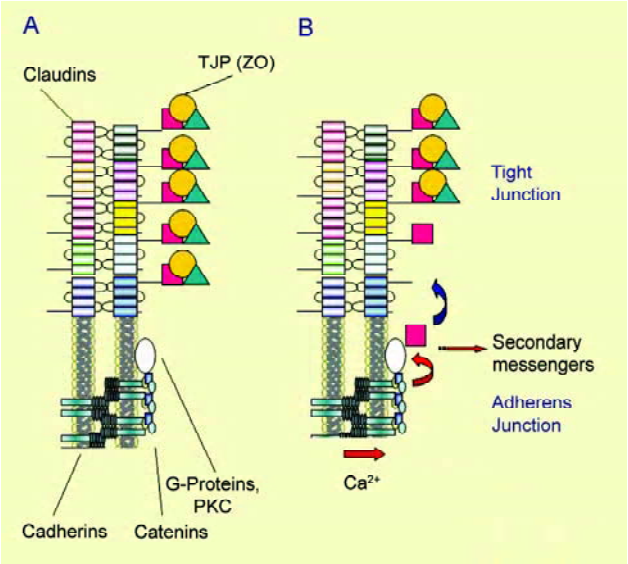

Figure 2. Schematic representation of the assembly of tight junctions in the epididymis. (A): Tight junctions are composed of occludin, transmembrane tight junctional proteins named claudins (CLDNS) and tight junctional protein (TJP) as well as several other cytoplasmic proteins (not shown). Adherens junctions are composed of cadherins and catenins. It has been proposed in the calcium switch model, in which calcium induces the formation of tight junctions, that cadherin-cadherin interaction (B) activates several small G-proteins, such as Rho. The activation of G-proteins and the breakdown of membrane phospholipids, such as phosphoinositol triphosphate and diacylglycerol, stimulate Ca^{2+} uptake. Protein kinase C (PKC) is activated and phosphorylates TJP1. In the epididymis, TJP1 associates with catenin of the adhering junction during the formation of epididymal tight junctions. This association is thought to be important to localize TJP1 to the apical region of the cell. TJP1 is then available to associate with CLDNS and occludin in formation of tight junctions.

barrier is compromised at the end of the reproductive season when serum testosterone levels decrease. However, unlike the blood-testis barrier, the blood-epididymal barrier remains intact throughout the year, suggesting that it is regulated differently from the blood-testis barrier. Based on the ultrastructure of the blood-epididymal barrier during embryonic and postnatal development, it would appear that at least two factors regulate epididymal tight junctions. The first occurs during embryonic development, when tight junctions are formed and the second, postnatally, when the number of junctional strands increases to form the barrier.

The regulation of epididymal tight junctions and the blood–epididymal barrier is poorly understood. In the adult, the integrity of the barrier is unaltered by experimental treatments with either estradiol or vasectomy. The only evidence for manipulation of the barrier is the morphological observation that the tight junctions between principal cells 3 days following orchidectomy appeared leaky [4], suggesting that testosterone or other testicular factors might regulate the integrity of the blood-epididymal barrier. Such an effect might be a result of a dedifferentiation of cells, which might occur following the removal of androgens [4].

We have shown that orchidectomy in rats resulted in changes to the staining pattern of CLDN1 in the initial segment of the epididymis. In this region of the epididymis, there was an absence of CLDN1 immunostaining in the area of the epididymal tight junction 14 days after orchidectomy. Testosterone replacement maintained expression apically between the lateral plasma membranes of adjacent principal cells. No changes to CLDN1 expression were noted in any other regions of the epididymis. Therefore, androgens appear to regulate CLDN1 expression in epididymal tight junctions in a segment-specific manner. Postnatal developmental studies indicate that factors other than androgens regulate CLDN1 expression in the initial segment and other epididymal regions, because CLDN1 is present in the epithelium as early as day 7, at a time when androgen levels are below detection [48].

Thyroid hormones also appear to play a role in the regulation of epididymal CLDN1. In studies in which rats were made hypothyroid during pubertal development by the administration of n-propylthiouracil (PTU), CLDN1 expression does not occur in the initial segment of the epididymis (Figure 3). CLDN1 expression was unaltered in other regions of the epididymis.

The recent development of epididymal cell lines in different species, including the rat, which express CLDN1, has provided long awaited tools for studying epididymal gene expression [61–64]. We investigated the transcriptional regulation of the *Cldn1* gene in the rat epididymis. A 1.8-kb sequence of the 5' flanking region of the rat *Cldn1* gene was cloned. The transcriptional start site was identified as an adenine located at the –198 position relative to the first codon and 26 bp downstream of the putative TATA box. It is the only start site for the *Cldn1* gene transcription in the rat epididymis. The *Cldn1* promoter was inserted into a luciferase gene expression vector and transfected into a rat caput epididymidal cell line (RCE-1) [64]. Sequential deletion analysis revealed that minimal promoter activity was achieved with the construct containing –61 bp to +164 bp of the promoter. This sequence contained a TATA box and two consensus SP1 binding sites. Electrophoretic mobility shift and supershift assays confirmed that SP1 and SP3 were present in RCE-1 cell and epididymal nuclear extracts, and that they bind to the 5′ SP1 binding motif of the promoter. Site directed mutagenesis of the 5′ SP1 binding site resulted in a 4-fold decrease in transactivation of the minimal promoter sequence [64]. These findings indicate that SP1 binds to the *Cldn1* promoter region and that this interaction influences the expression of *Cldn1*

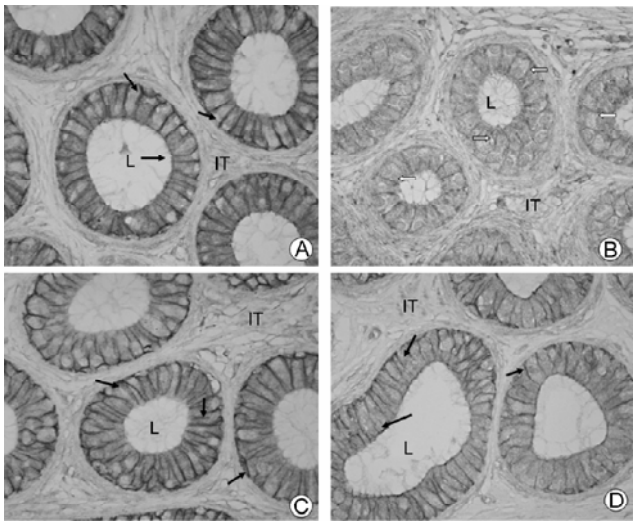

Figure 3. Thyroid hormones regulate the expression of transmembrane tight junctional proteins named claudin-1 (CLDN1) in the initial segment of the epididymis. Rats were treated with the antithyroid drug n-propylthiouracil (0.01%; PTU) in their drinking water from days 0 to 28. Controls received water alone. At day 30, CLDN1 (arrows) is localized along the lateral plasma membrane and at the region of the tight junction in the initial segment (A) and caput (C) of the epididymis. A similar pattern of immunostaining was observed in the corpus and cauda epididymidis (not shown). In PTU-treated rats, CLDN1 immunostaining was absent from the initial segment (B) but its expression in the caput epididymidis (D) and other regions of the epididymis was unaltered (not shown). (400 ×). L, Lumen; IT, interstitium.

in the rat epididymis. Protein binding to the 5′ regulatory region of the *Cldn1* gene was also demonstrated, although the binding factors have yet to be identified.

10 The human epididymis

The human epididymal epithelium is a pseudostratified columnar epithelium, which presents different cell types including principal and basal and clear cells. Principal cells, the predominant secretory cells of the epididymis, are characterized by the presence of a secretory apparatus (endoplasmic reticulum [ER], Golgi apparatus, secretory granules) and an endocytic apparatus (coated pits, endosomes, multivesicular bodies and lysosomes). Basal cells, which are hemispherical in appearance, adhere to the basement membrane and do not have direct access to the lumen of the duct [49]. Different secretory and endocytic organelles could be observed in the cytoplasm of the principal cells as well as apical tight junctional complexes between adjacent principal cells (Figure 4). As in the rodent model, the human tight junctional complex is localized between adjacent epithelial cells that surround the lumen of the epididymis. The ultrastructure of the tight junction appears similar between the different regions of the epididymis.

11 Human pathologies and the blood-epididymal barrier

Almost 12% of couples experience fertility problems and male infertility accounts for approximately half of these cases [65]. Male infertility can be classified into three major categories: pre-testicular dysfunction (endocrinological and genetic disorders), intra-testicular disorders (e.g. Sertoli cell only syndrome, spermatogenic defects, gonadotoxin exposure, impaired sperm DNA integrity and cryptorchidism), and post-testicular disorders (obstruction, infection, immune, ejaculatory and coital disorders). In patients with normal or high sperm numbers and sperm motility who are diagnosed with idiopathic infertility, it is generally thought that post-testicular factors might contribute to their infertility, as the problem may be associated with the process of sperm maturation [66, 67]. Although male gametes are formed in the seminiferous epithelium of the testis through spermatogenesis, testicular spermatozoa do not have the ability to fertilize or swim. These functions are acquired in the epididymis [12]. Therefore, post-testicular infertility might be the result of pathological dysfunction in the epididymis, which results in incomplete or dysfunctional sperm maturation. There are several lines of evidence for a direct role of epididymal dysfunction in male infertility. Boué *et al.* [68] identify a protein, P34H, secreted by the epididymis and which binds to spermatozoa and is required for binding of the sperm to the zona pellucida of the egg. This protein accumulates on the sperm during epididymal transit and has been proposed as a marker of sperm maturation. In fertile patients, P34H is detectable in all patients by western blot analyses. However, in infertile patients, 40% of patients have undetectable P34H levels, suggesting that epididymal sperm maturation was incomplete [68].

A second line of evidence for epididymal dysfunction in infertile patients is related to the presence of sperm-reactive antibodies. These antibodies in seminal fluid or bound to spermatozoa have been reported in 6–11% of infertile patients and are usually absent or present in low titers in men with no fertility problems [69, 70]. These antibodies are usually not present until the time of puberty. Sperm are antigenic and are protected from the immune system in the testis during the first wave of spermatogenesis by the formation of tight junctions between Sertoli cells [71]. These tight junctions are necessary for spermatogenesis and, therefore, because these patients have

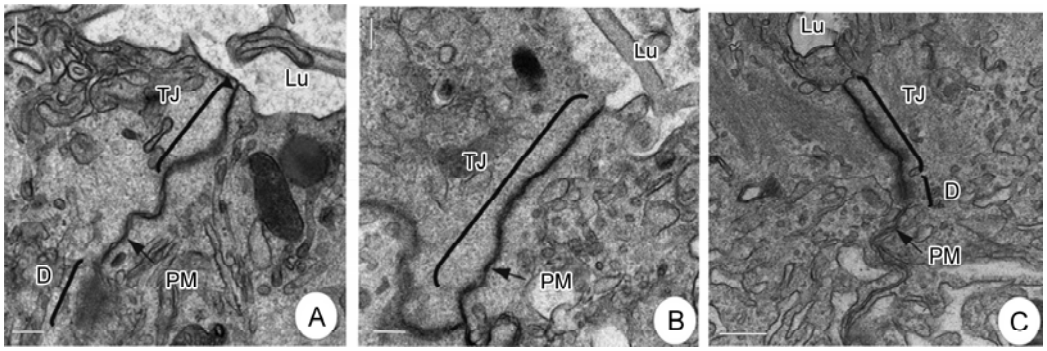

Figure 4. Electron micrograph of apical tight junctions in the human epididymis. Apical tight junctions in the caput (A), the corpus (B) and cauda (C) epididymidis. D, desmosome; Lu. Lumen; PM, lateral plasma membrane; TJ, tight junction. Scale bar = 0.5 μm.

normal sperm counts, sperm-reactive antibodies are unlikely to arise as a result of testicular dysfunction. In the epididymis, tight junctions between the epithelial cells that line the lumen of the tubule are responsible for forming the blood-epididymal barrier [1]. One of the roles of this barrier is to protect sperm from the immune system. Diekman *et al.* [72] analyze a range of potential antigens associated with sperm-reactive antibodies. They report that these antibodies are directed against an epididymal glycoprotein that shares homology with a CD52 lymphocyte surface molecule, suggesting that the blood-epididymal barrier is compromised and that the immune system had been activated. In addition there are many other causes associated with the development of sperm-reactive antibodies, including obstructive epididymal azoospermia, acute epididymitis, infections, scrotal/testicular trauma and post-vasectomy [73, 74]. Because inflammatory cytokines are recognized as repressors of tight junctional proteins, it is conceivable that these inflammatory processes are associated with the disruption of the blood-epididymal barrier.

12 Proteins implicated in human epididymal tight junction

There is no information on the proteins that comprise human epididymal intercellular junctions. To understand the regulation and functions of epididymal tight junctions, based on our previous studies in the rat, it is essential to establish the components of both adherens and tight junctions that are expressed in the epididymis. To this end, we used a genomics approach to study the expression of epididymal genes. Epididymides from four fertile patients undergoing radical orchidectomy for testicular cancer confined within the testicular tunica albuginea were obtained and subdivided into the caput, corpus and cauda epididymidis. All patients had normal hormone levels, showed intact normal gross anatomy of their epididymides, and by light and electron microscopy the ultrastructural features of the epithelium had a normal morphological appearance.

Using oligonucleotide microarrays, we identified 2 980 genes that were differentially expressed by at least 2-fold throughout the epididymis. Genes that were expressed included several encoding for adhesion proteins (cadherins and catenins) and tight junctional proteins (claudins and tight junction proteins). The human epididymis expresses a large number of both cadherins and protocadherins (Tables 1 and 2). CDH1 was among the highest expressed cadherin, which is also the case for the rat epididymis [42]. Interestingly, CDH22 (also known as PB-cadherin) and CDH24 are also highly expressed in the human epididymis. It has been reported in the published literature that both CDH22 and CDH24 have two transcripts. CDH22 has been reported to be expressed in gonocytes of the testis of young rats [75]. All three of these cadherins are expressed at similar levels throughout the epididymis. Only one cadherin, CDH16 (also known as Ksp-cadherin), showed large differences in its expression pattern along the epididymis, with high expression in the corpus and cauda epididymidis. CDH16 was first identified in the kidney and has been shown to be expressed in the murine epididymis during embryonic development [76]. This cadherin does not have a characteristic HAV sequence in its binding domain and lacks an extensive cytoplasmic region that is present in classical cadherins [76]. Several catenins were also expressed in the epididymis, including three members of the α-catenin family (CTNNA1, CTNNA2 and CTNNA3), β-catenin (CTNNB1) and two p120 catenins (CTNND1

Table 1. Relative expression of cadherins in the human epididymis. The results were obtained by microarrays (n = 4). –, not expressed; +, very weakly expressed (0–30); ++, weakly expressed (30–100); +++, moderately expressed (100–200); ++++, highly expressed (200 and more).

Gene name	Relative intensity		
	Caput	Corpus	Cauda
CDH1	++++	++++	++++
CDH2	+++	++	+
CDH3	+++	+++	+++
CDH4	++	++	++
CDH5	+	–	+
CDH6	++	++	++
CDH7	+ (n = 1)	–	+ (n = 1)
CDH8	+	+ (n = 1)	+
CDH9	+	+	+
CDH10	+	–	+
CDH11	++	++	++
CDH12	++	++	+
CDH13	++	++	++
CDH15	++	++	++
CDH16	++	++++	++++
CDH17	–	–	–
CDH18	+ (n = 1)	–	+ (n = 1)
CDH19	–	–	–
CDH20	–	–	–
CDH22	++++	++++	++++
CDH23	++	++	+
CDH24	++++	++++	++++
CDH26	+ (n = 1)	–	+ (n = 1)

and CTNND2 [Table 3]). Interestingly, the epididymis contained high expression levels of CTNNAL1 (also known as α-catulin). This member of the catenin family is involved in the signal transduction pathway of small G-proteins, such as Rho [77]. The expression of high levels of Rho in the human epididymis suggests that the Rho signaling pathways might play an important role in epididymal function and the regulation of cadherin mediated intracellular signaling.

As in the rat model, the human epididymis also expressed a large number of CLDNS (Table 4). CLDN4, CLDN5 and CLDN7 appeared to be expressed at highest levels in the epididymis. We have shown in the rat that CLDN4 is expressed at high levels and CLDN5 was localized to endothelial cells. CLDN7 has been reported to be localized in the basolateral region of the distal nephron of the

Table 2. Relative expression of protocadherins in the human epididymis. The results were obtained by microarrays (n = 4). –, not expressed; +, very weakly expressed (0–30); ++, weakly expressed (30–100); +++, moderately expressed (100–200); ++++, highly expressed (200 and more).

Gene name	Relative intensity		
	Caput	Corpus	Cauda
PCDH1	++	++	++
PCDH7	+ (n = 1)	–	+ (n = 1)
PCDH8	+	+	+
PCDH10	–	–	–
PCDH12	++	+	++
PCDH15	–	–	–
PCDH17	++	++	++
PCDH18	+++	+++	+++
PCDH19	–	–	–
PCDH20	–	–	–
PCDH21	+	–	+
PCDHB1	+ (n = 1)	–	+ (n = 1)
PCDHB3	–	–	–
PCDHB4	++	++	++
PCDHB5	++	++	++
PCDHB6	++ (n = 1)	–	++ (n = 1)
PCDHB7	++	++	++
PCDHB8			
PCDHB9	+	–	+
PCDHB10	+ (n = 1)	–	+ (n = 1)
PCDHB11	++	++	++
PCDHB12	++	++	++ (n = 1)
PCDHB13	+++	+++	+++
PCDHB14	++	++	+
PCDHB15	+ (n = 1)	–	+ (n = 1)
PCDHB16	++	++	++
PCDHAC2	+++ (n = 1)	–	+++ (n = 1)
PCDHGA8	++	++	++
PCDH11Y	–	–	–
PC-LKC	++	++	++

Table 3. Relative expression of catenins in the human epididymis. The results were obtained by microarrays (n = 4). –, not expressed; +, very weakly expressed (0–30); ++, weakly expressed (30–100); +++, moderately expressed (100–200); ++++, highly expressed (200 and more).

Gene name	Relative intensity		
	Caput	Corpus	Cauda
CTNNA1	++++	++++	++++
CTNNA2	+++	++	+++
CTNNA3	++	++	+
CTNNAL1	++++	++++	++++
CTNNB1	++	++	++
CTNND1	++	++	++
CTNND2	++	++	++

Table 4. Relative expression of transmembrane tight junctional proteins named claudins (CLDNS) in the human epididymis. The results were obtained by microarrays (n = 4). –, not expressed; +, very weakly expressed (0–50); ++, weakly expressed (50–200); +++, moderately expressed (200–600); ++++, highly expressed (600 and more).

Gene name	Relative intensity		
	Caput	Corpus	Cauda
CLDN1	++	++	++
CLDN2	++	+++	++++
CLDN3	++	++	++
CLDN4	++++	++++	++++
CLDN5	+++	++++	++++
CLDN6	+	+	+
CLDN7	++++	++++	++++
CLDN8	++	+	+
CLDN9	++	++	++
CLDN10	++	++++	++++
CLDN11	++	++	++
CLDN12	++	++	++
CLDN14	+	+	+
CLDN15	+	+	+
CLDN16	++	–	++
CLDN17	+	+	+
CLDN18	+	+	+
CLDN19	++	++	++
CLDN22	–	–	–
CLDN23	++	+	+

kidney and along with CLDN8 have been reported be part of the cation barrier of the nephron [78]. The expression of most CLDNS was equivalent along the epididymis. However, both CLDN8 and CLDN10 showed segment-specific expression levels that were opposite from one another. CLDN8 was more highly expressed in the caput and less so in the corpus and cauda, which is consistent with a role as part of the cation barrier. CLDN10 was more strongly expressed in the corpus and cauda epididymidis and is also considered part of the cation barrier.

Immunolocalization of CLDN1, 3, 4, 8 and 10 revealed that the localization of CLDNS also differed along the epididymis. CLDN1, 3 and 4 were localized to tight junctions, along the lateral margins of principal cells and between basal and principal cells in all three segments of the human epididymis. CLDN8, in the caput, was localized to the lateral margins of principal cells and to tight junctions whereas, in the corpus, CLDN8 was localized to tight junctions and between principal and basal cells. In the cauda, CLDN8 was exclusively localized to tight junctions. CLDN10, TJP1 and occludin were exclusively localized to tight junctions in all three segments of the epididymis. CLDN10 immunoreaction was much more intense in the cauda epididymidis than in other regions of the epididymis. This is in contrast with studies on the rat in which CLDN10 was expressed primarily in the initial segment [8].

These data suggest that the proteins that comprise epididymal adherens and tight junctions appear to be conserved between the rodent animal models and human. However, there are differences in either expression pattern or localization of proteins that comprise these intercellular junctions. At the moment, it is unclear why these differences exist or if there are redundancies in the expression of certain proteins that might functionally compensate for differences. Clearly, however, as we begin to attempt to understand the regulation of human epididymal protein to assess there function in fertility, new tools will need to be developed that will permit more extensive studies on the regulation of human epididymal proteins implicated in the formation and maintenance of the human blood–epididymal barrier.

13 Conclusion

Cellular interactions in the epididymis involve complex interactions between large multimember families of proteins. In the epididymis, these interactions are further complicated by the influence of testicular factors that regulate the expression of epididymal genes and cellular targeting of proteins. Although these influences increase the complexity of cell-cell interactions, they also provide a unique model in which it is possible to modulate cellular interactions *in vivo*. Demonstrating how the various cellular interactions in the epididymis function with respect to one another and their significance to epididymal physiology and sperm maturation remains a major but crucial challenge for our understanding of the epididymis.

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