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C. Yan Cheng *Editor*

Biology and Regulation of Blood-Tissue Barriers

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BIOLOGY AND REGULATION OF BLOOD-TISSUE BARRIERS
C. Yan Cheng

Biology and Regulation of Blood-Tissue Barriers

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DEDICATION

In memory of my Dad and Mom

PREFACE

I learned about Sertoli cells and germ cells in the testis and the concept of the blood-testis barrier when I was a graduate student in the late 1970s in the laboratory of Professor Barry Boettcher, University of Newcastle, Australia. Thereafter, I joined the Laboratory of Dr. Wayne Bardin in the Population Council's Center for Biomedical Research in New York and began my career to study the biology of the Sertoli cell with Drs. Wayne Bardin, Neal Musto and Glen Gunsalus, in particular how steroid transport proteins (e.g., androgen binding protein) in the testis versus similar functional proteins in the systemic circulation (e.g., testosterone-estradiol-binding globulin also known as sex hormone binding globulin) in regulating testicular function. During this period, I also learned about how the blood-testis barrier near the basement membrane in the seminiferous epithelium regulated protein composition in the tubule lumen versus the blood compartment, besides different surgical techniques in laboratory rats. This earlier exposure had laid the foundation that sparked my interest in this area of science later on in my scientific career. During the last three decades since the completion of my postdoctoral training in the early 1980s, I established my research program at the Population Council and I am fortunate to have an undisrupted period of productive research in reproductive biology, and I remain grateful to Dr. Wayne Bardin for this opportunity and his generosity to nurture the growth of many young scientists at the Council during his tenure. During the past ~17 years since the retirement of Wayne from the Population Council, I shifted my research interest to develop contraceptives for males while working closely with Professor Bruno Silvestrini on the series of compounds he developed in the 1960s and 1970s known as indazole-carboxylic acids. I began to realize the significance of the blood-testis barrier that had considerably limited the bioavailability of many potential contraceptive drugs for men, in particular if they exert their effects on developing germ cells in the compartment behind the blood-testis barrier, such as adjudin, 1-(2,4-dichlorobenzyl)-1H-indazole-3-carbohydrazide, which was developed by Bruno and myself in the late 1990s. As such, I began to learn more about blood-tissue barriers, such as the blood-brain barrier, the blood-retina barrier and the gut barrier, which turned out to be an exceedingly fascinating field of research. It is easily understood that this is a rapidly growing field since many illnesses in the brain

remain difficult to treat therapeutically because of the blood-brain barrier. Thus, the idea of editing a book that encompassed critical discussion and thought-provoking concepts besides summarizing the latest findings in different blood-tissue barriers emerged a few years ago. I am grateful that many outstanding investigators who have spent decades to study different aspects of blood-tissue barrier function share my enthusiasm to prepare such a book for investigators in the field. I am thankful for each of our contributors who have spent numerous hours to work on their book chapters amidst their busy schedules in the laboratory. They have also summarized some of the latest and fascinating development in their fields of research including the blood-brain barrier, the blood-retinal barrier, the gut barrier, the blood-biliary barrier, the blood-follicle barrier, the blood-epididymis barrier, the blood-testis barrier, the tight junction barrier in general as well as barriers in the female reproductive tract. We also have a few chapters that focus on topics that are physiologically applicable to all blood-tissue barriers. Many of these chapters also include information on specific human diseases, such as pathological changes of the gut barrier that cause bowel disorders resulting from inflammation of the epithelial lining in the intestine, and infertility in men as a result of disruption of the blood-epididymal and/or blood-testis barriers; and on new therapeutic approaches (e.g., drug delivery across the blood-brain and the blood-retinal barriers). It is my belief that this book has covered some of the more interesting aspects of research in blood-tissue barriers, and it is my intention as well as the contributors of this book that this work will serve as a helpful and important reference guide to investigators in the field.

I am also indebted to our Publisher, Dr. Ron Landes who entrusted me to edit this book for Landes Bioscience, to Ms. Cynthia Conomos, Ms. Celeste Carlton and their staff at Landes Bioscience, such as Daniel Olasky and Marissa Stewart, who have worked diligently with me and our contributors these past two years while different chapters for this volume were being written, revised and prepared, and also for their extraordinary efforts in copy editing, page layouts and the final production. Without these extraordinary efforts and professionalism, *Biology and Regulation of Blood-Tissue Barriers* would never be published. I hope that our readers will find this volume to be a delightful addition to their personal libraries in their laboratories and offices. It is obvious that this field will undergo rapid expansion in the years to come because of the advances in biochemistry, molecular biology, cell biology and nanotechnology. I also hope that this book will play its role to spark the interest of young investigators to join us to study the biology and regulation of blood-tissue barriers and their significance in diseases and health.

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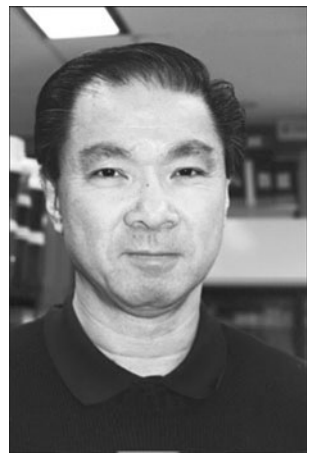

DR. C. YAN CHENG is a native of Hong Kong and a graduate from the Chinese University of Hong Kong in 1977. Shortly after receiving his BSc, he began his graduate training in the laboratory of Professor Barry Boettcher at the University of Newcastle, New South Wales, Australia and received his PhD in 1981. He joined the laboratory of Dr. C. Wayne Bardin as a postdoctoral fellow, working with him and Drs. Neal A. Musto and Glen Gunsalus at the Population Council's Center for Biomedical Research located on the campus of the Rockefeller University in New York City for a little over 4 years. During this period, he had the opportunity to interact with a number of eminent scientists and visiting scientists who were at the Population Council including Drs. Jennie Mather, Vilma Rossi, and Geoffrey Hammond. Dr. Bardin had also created an unprecedented stimulating and competitive environment that shaped his career in life sciences. Through his collaboration with Dr. Vilma Rossi, he met Professor Bruno Silvestrini in the summer of 1985 in New York, and began a scientific collaboration that spans more than two decades to develop new derivatives of indazole-carboxylic acid for male contraception with the development of adjudin, 1-(2,4-dichlorobenzyl)-*1H*-indazole-3-carbohydrazide, as a potential male contraception. He has also used different animal models, such as the adjudin model, the cadmium model, the bisphenol A model and a few genetic models to study different aspects of testicular function in particular the biology and regulation of cell adhesion in the testis. Through this work, he and his colleagues, Drs. Helen Yan, Elissa Wong, Will Lee and Dolores Mruk, have discovered a functional axis that links

the different cellular compartments in the seminiferous epithelium of the testis known as the apical ectoplasmic specialization-blood-testis barrier-hemidesmosome/basement membrane axis that coordinates different cellular events during the seminiferous epithelial cycle of spermatogenesis. They have also identified several putative signaling pathways and nonreceptor protein kinases, such as focal adhesion kinase (FAK) and members of the c-Src kinases, that regulate blood-testis barrier dynamics. Some of these findings are currently under development to design innovative compounds for male contraception. For the past three decades, his laboratory has received supports from the Angelini Research Institute, Rockefeller Foundation, Lupus Foundation of America, CONRAD Program, USAID, and the National Institutes of Health. He has published over 300 research articles and reviews in peer-reviewed journals in the field. He is currently a Senior Scientist and the Head of the Mary M. Wohlford Laboratory for Male Contraceptive Research at the Population Council's Center for Biomedical Research in New York City.

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CONTENTS

CHAPTER 1

REGULATION OF PERMEABILITY ACROSS THE BLOOD-BRAIN BARRIER

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Abstract: The blood-brain barrier refers to the very low permeability across microvessels in the Central Nervous System (CNS), created by the interaction between vascular endothelial cells and surrounding cells of the neurovascular unit. Permeability can be modulated (increased and decreased) by a variety of factors including inflammatory mediators, inflammatory cells such as neutrophils and through alterations in the phenotype of blood vessels during angiogenesis and apoptosis. In this chapter, some of these factors are discussed as well as the challenge of treating harmful increases in permeability that result in brain swelling (vasogenic cerebral edema).

INTRODUCTION

The Blood Brain Barrier (BBB) refers to restrictive properties of vascular endothelial cells in the Central Nervous System (CNS). In early animal experiments, Biedl and Kraus (1898, using bile acids)[1] and Lewandowsky (1900, using sodium ferrocyanide)[2] showed that substances only produced symptoms if introduced into the CNS when injected into Cerebrospinal Fluid (CSF) or by direct intracerebral injection, but had no effect if given intravenously. They correctly attributed this to the exclusion of certain substances from the CNS by the walls of cerebral blood vessels. Erlich, on the other hand (1902)[3] showed that cerebral grey matter was preferentially stained by basic dyes if given intravenously, rather than by acid dyes or dyes containing a sulphate group. He believed that this pointed to a differential affinity of CNS tissue for different dyes, rather than vascular exclusion. Goldman, a pupil of Erlich, also showed (1909)[4] that trypan blue could not penetrate the brain if injected intravenously, but stained the CNS if administered into

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the CSF. He noted that the dye was excluded by small capillaries, but avoided a clear reference to vascular exclusion, perhaps out of deference to Erlich. Stern and Gautier (1921)[5] came closer to the present concept of a "barriere hematoencephalique". Work by Krogh (1946),[6] Davson (1955)[7] and Brodie (1960)[8] established lipid solubility as a key determinant for penetration of the CNS. The role of the vasculature in restricting the passage of lipid insoluble substances between blood and brain was confirmed by Reese, Karnovsky and Brightman (1967,1969)[9,10] who demonstrated that horseradish peroxidase could penetrate freely through the CNS interstitium, but was arrested at the level of the tight junctions between brain endothelial cells. The movement of solutes through the endothelial barrier is restricted therefore by the complex anatomical structure of the tight junctions. Freeze fracture studies showed a greater complexity of junctional strands compared to endothelia outside the CNS.[11] Junctional strands are composed of rows of bead like particles, representing proteins spanning the junction between two endothelial cells. These spanning proteins include claudin 5, occludin and junctional adhesion molecule. The cytoplasmic portion of these transmembrane proteins interact with a submembranous plaque composed of the cytoplasmic accessory proteins zona occludens (ZO)-1, 2 and 3. These accessory proteins connect the tight junction complex to the actin cytoskeleton. Several other accessory proteins have been described including AF6, 7H6 and cingulin. The tight junction is associated with an adherens junction in which cells are bridged by cadherin proteins, including Vascular Endothelial (VE) cadherin, which connect to cytoplasmic catenins and thus to the actin cytoskeleton. The adherens junctions stabilize the connection between endothelial cells. The tight junction is dynamically regulated by a variety of signaling molecules to maintain normal BBB permeability and to trigger permeability increases (reviewed in ref. 12). Brain endothelial cells also have a reduced density of vesicles compared to peripheral endothelial cells and it has been proposed that the shuttling function of vesicles is reduced, which contributes to the low permeability of the barrier.[13] The term 'neurovascular unit' has been substituted for 'blood brain barrier' in recent years to indicate that the endothelium derives its properties from the interaction with many different cell types (Fig. 1). For instance, astrocyte foot processes envelop microvessels in most of the CNS parenchyma. It has been assumed that astrocytes are necessary for a full expression of barrier properties, including restricted permeability. It was shown that permeability across cultured brain endothelial cells increases on exposure to astrocyte conditioned medium[14] and explant experiments showed a role for astrocytes in BBB development.[15] It is interesting that pial microvessels on the brain surface have a low permeability without a physically close interaction with astrocyte foot processes, suggesting that the relationship between astrocytes and BBB permeability properties may not be as straightforward as is often supposed. Although cultured brain endothelial cells are more permeable in the absence of astrocytes, permeability is nevertheless markedly reduced in comparison to peripheral cells and can be markedly increased by non-astrocytic factors such as shear stress[16] or exposure to hydrocortisone in low serum medium.[17] Other cells that may influence the endothelium and its permeability, include pericytes, perivascular macrophages, smooth muscle cells, fibroblasts, mast cells and perivascular nerve fibers. Some of these (e.g., mast cells, nerves) can influence permeability through the generation of inflammatory mediators that interact with receptors on the abluminal surface of the endothelium.

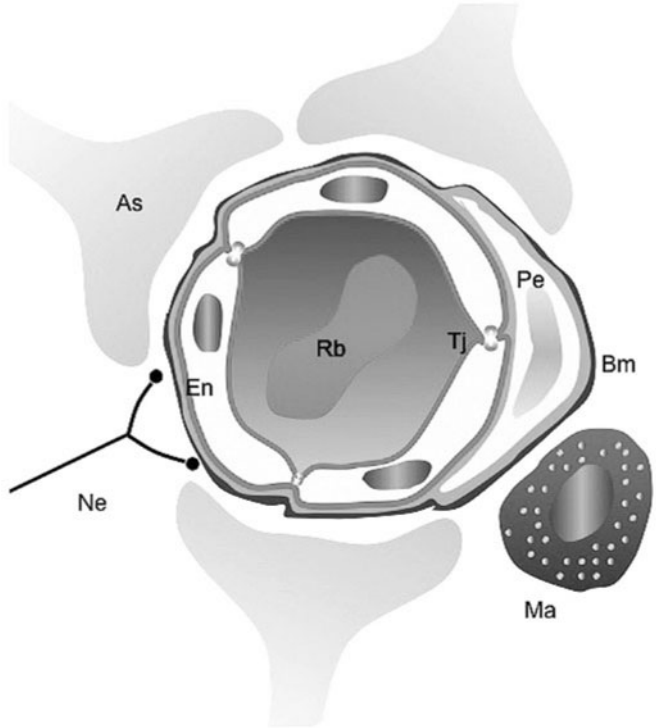

Figure 1. Diagrammatic representation of the blood-brain barrier/neurovascular unit. Symbols: As = astrocyte foot process, Bm = basement membrane, En = endothelial cell, Ma = mast cell, Ne = unmyelinated nerve ending, Pe = pericyte or perivascular macrophage, Rb = red blood cell, Tj = interendothelial tight junction.

PERMEABILITY DEFINED

Permeability is a term that refers to the rate at which a solute moves across a barrier. In general, a solute will move from a solution of higher concentration to one of lower concentration by Brownian motion, by the process of diffusion. The bulk solution containing the solute can itself move if driven by a hydrostatic pressure gradient by the process of convection. The movement of hydrophilic solutes across the endothelial barrier is modeled as occurring through water soluble cylindrical pores. The relative size (radius) of the solute compared to the radius of the pore is what determines the degree of restriction. There are mathematical formulae that describe these relationships. However, in general, to define permeability (P) one needs to know the surface area (S) through which the solute diffuses, the difference in concentration (C) across the interface (δC) and the way this varies over time, t ($\delta C/\delta t$). As long as concentration in the blood exceeds that in the brain, then the solute will accumulate in the brain over time. However, once an equilibrium is reached, then solute will not move from blood to brain, or it may begin to back-diffuse from brain into blood. The delivery of solutes is also influenced by hydrostatic pressure,

but in general this is only important when the radius of the solute begins to approach that of the pore through which it travels, so solutes with a higher molecular weight such as plasma proteins can be affected. Delivery is also dependent on blood flow, but in general this is only rate limiting for lipid soluble substances with a high permeability.

PERMEABILITY MEASURED

Given these theoretical considerations, it is useful to review different methods in use to assess permeability across the BBB. Much work has been carried out in animal models. In general, tracers are infused into the blood and their level of accumulation in brain is assessed as a function of time and in relation to the concentration in blood entering and leaving the brain (Renkin 1959, Crone 1963).[18,19] These tracers vary in their molecular weight and in how they are labeled. For example, dextrans of different molecular weights have been used, labeled with fluorescent markers or molecules are infused attached to radioisotopes. This global approach has several disadvantages as pointed out by Robinson (1990).[20] These include heterogeneity of capillaries (variations in individual capillary blood flow, surface area and length), recruitment of capillaries with changes in blood flow and binding of tracers to plasma proteins such as albumin. Because the surface area of capillaries is not known, data is often expressed as permeability-surface area (PS) products. A technique that we have used recently visualizes the spatial distribution of horseradish peroxidase in brain sections, converting the images obtained by reacting the sections with diaminobenzidine and hydrogen peroxide to obtain a brown reaction product, whose initial rate of formation is proportional to concentration. The resulting permeability maps allow correlations to be made between permeability and histological parameters in the surrounding tissue (Fig. 2).[21]

These approaches have also been supplemented by measurements made in single capillaries on the surface of the brain. Approaches include visualization of fluorescent dyes of known molecular weight, trapped in single vessels with an occluding probe placed at one end of the vessel (Fig. 3).[22] Vessels have also been injected with microelectrodes and the current dissipation between stimulating and recording electrodes used to measure transendothelial electrical resistance (TEER) along the vessel.[23] TEER is also used to measure permeability in endothelial cell culture experiments and is inversely related to permeability, so that a high TEER indicates a low permeability. The use of tissue cultures is of value in recording relative differences in permeability across monolayers of brain endothelial cells. Cultured cells develop a permeability that is several orders of magnitude lower than comparable cultures of peripheral endothelial cells (such as human umbilical vein endothelial cells, HUVEC). Primary bovine cultures in our hands[24] develop a TEER of around 100 $\Omega.cm^2$. By contrast, single pial microvessels[23] in the frog have a TEER of around 2000 $\Omega.cm^2$. This difference in TEER values has prompted many groups to develop methods that increase TEER to in situ values. This has been achieved by coculturing brain endothelial cells with astrocytes or astrocyte conditioned medium, adding agents that increase intracellular cyclic AMP,[25] culturing cells in serum free medium with hydrocortisone[17] and by exposing cells to shear stress (flow conditions).[16] It may be argued that mechanistically, the use of cultured endothelial cells allows the effect of particular factors to be explored in the regulation of BBB permeability, while animal models test the robustness of these simplified observations in the complex in situ situation.

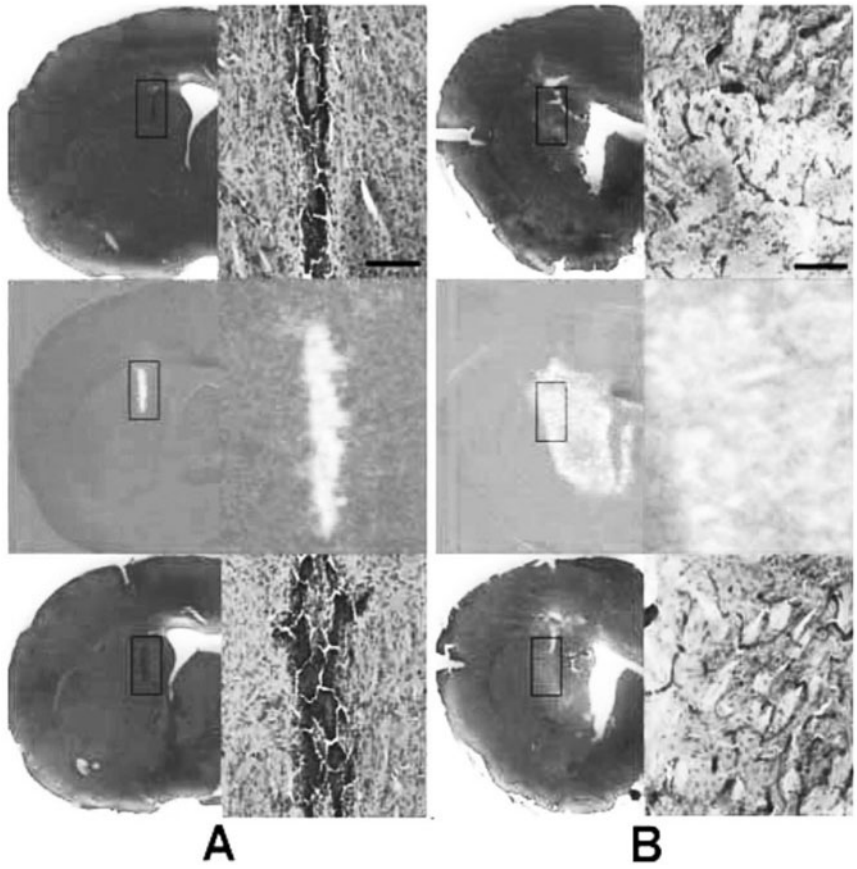

Figure 2. Permeability maps used to measure blood-brain barrier permeability. In this example, neutrophils (panel A) or arachidonic acid (10^{-3} mol/l, panel B) were injected into the striatum of anesthetized rats. Upper and lower panels show H&E stained sections taken anterior or posterior to the middle section. This was reacted to derive an image of the concentration of horseradish peroxidase, from which a map was obtained showing a pixel by pixel measure of permeability-surface area (PS) product. Reproduced from Joice SL et al. Brain Res 2009; 1298:13-23,75 ©2009 with permission from Elsevier.

WHY IS BBB PERMEABILITY SO LOW AND IS IT A STATIC OR DYNAMIC ENTITY?

It is generally assumed that the BBB creates a site of immune privilege in the CNS, by restricting the entry of immune cells. Also, nervous functioning depends on a carefully controlled ionic environment. Thus, the free diffusion of potassium into the CNS to levels seen in blood would result in depolarization and interfere with axonal conduction. The BBB has developed a complex series of energy dependent transporters to drive the movement of essential nutrients such as glucose and amino acids into the CNS. Other transport proteins exist to extrude substances from the CNS, including P-glycoprotein. There is considerable interest in studying these transporters, since these physiological transporters restrict entry of therapeutic drugs into the CNS.

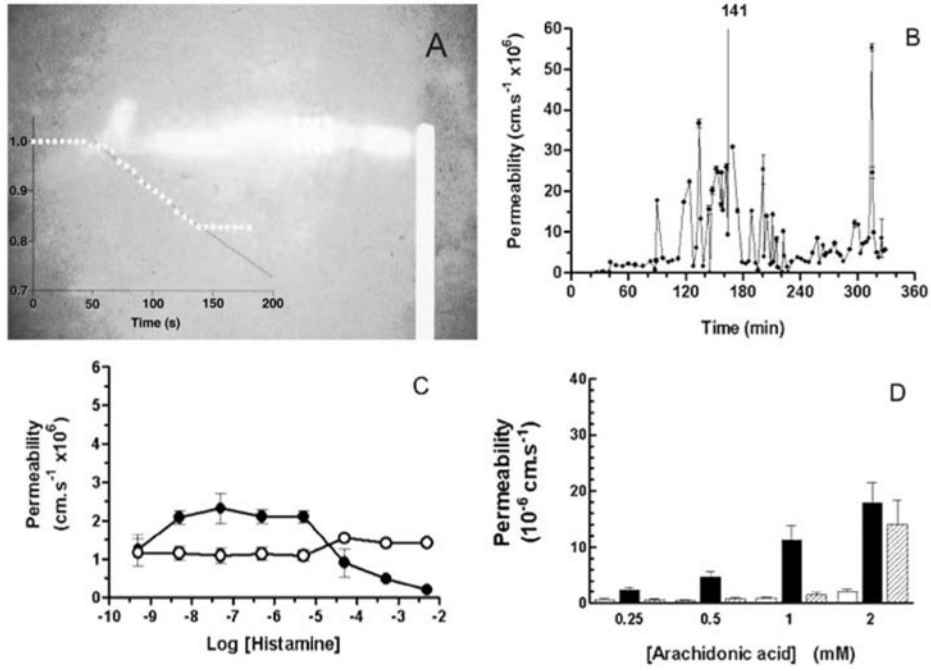

Figure 3. Variation in permeability in single rat pial microvessels. A) Shows an image of a vessel filled with the intravascular fluorescent tracer Lucifer yellow, with an occluding probe placed at one end. The decline in fluorescence in a segment (box) is plotted against time (inset) and a monoexponential fitted to the data to derive permeability values. B) Spontaneous disruption of the barrier was monitored over time in this vessel. Permeability was initially tight, but increased to a steady low level (first phase) followed by much higher fluctuating values (second phase). Adapted with permission from Easton AS et al. J Physiol 1997; 503:613-623,[26] ©1997 John Wiley and Sons. C) The first phase was mimicked by applying histamine. Lower doses (under 10^{-5} mol/l) increased permeability, while higher doses reduced permeability. Adapted with permission from Sarker MH et al. J Physiol 1998; 507:909-918,[43] © 1998 John Wiley and Sons. D) The second phase was mimicked with arachidonic acid. At higher doses, this was not reversible (hatched bars show permeability after application). Adapted with permission from Easton AS, Fraser PA. J Physiol 1998; 507:541-547,[44] ©1998 John Wiley and Sons.

However, there is also an interest in studying factors that regulate increases in BBB permeability. The process of BBB disruption, refers to increases in BBB permeability that cause damage to the CNS. However, there appear to be lower level increases in permeability that may have a homeostatic, essentially physiological role rather than a pathological effect. In work we carried out on single pial microvessels, the permeability after experimental exposure was initially tight, but after 1h or so, it increased to a steady level, approximately 10 times higher than the tight state (Fig. 3). This level of permeability was mimicked with the calcium ionophore A23187 and by applying the inflammatory mediator histamine. Permeability could be reduced by applying substances that increase intracellular cyclic AMP.[26] This small increase in permeability (first phase) appears to be tightly controlled and responsive to external modulation. Possible sources of histamine and other mediators that could generate smaller increases in permeability are the perivascular nerves (unmyelinated histaminergic fibers), mast cells and generation of mediators at the endothelial surface, for example, the generation of bradykinin via

the kallikrien/kinin system. The role of this smaller increase in permeability may be to permit the rapid clearance of substances from brain to blood such as the diffusion of neurotransmitters or locally elevated ions into the blood. If these permeability changes play a role in normal homeostasis of the CNS, then agents that reduce permeability may have detrimental effects if given in the wrong context, as will be discussed in relation to inflammation.

CHANGES IN BBB PERMEABILITY DURING INFLAMMATION

During inflammation, there appears to be a much larger increase in permeability than that just described, defined in single pial microvessels by rapid, transient increases in permeability, to levels that are 100 to 10,000 times greater than that of the tight barrier (Fig. 3).[26] Under these circumstances, it is possible to measure transiently fluctuating increases in permeability to albumin. Permeability to a smaller molecule (Lucifer yellow) remains high while that to albumin fluctuates from high to low within minutes.[27] By mapping the permeability along the length of microvessels, it is possible to visualize a complex and rapidly changing pattern of permeability that varies in both time and space. The clinical importance of albumin leakage across microvessels in the CNS is that albumin (and other plasma proteins) can thereby accumulate in CNS parenchyma and exert osmotic forces that draw and retain water in the CNS that leads to tissue swelling. Albumin clearance is limited, because the CNS lacks a lymphatic system, however albumin can drain along the perivascular spaces, finally draining along the olfactory bulbs into the nasal mucosa where it reaches the peripheral lymphatics.[29] The term 'vasogenic cerebral edema' has been used to describe this important clinical complication of increased BBB permeability during a variety of CNS diseases.[30,31] Vasogenic refers to the role played in its generation by increased blood vessel permeability. Vasogenic edema will only develop if blood flow is delivered to leaky blood vessels, since hydrostatic pressure is required for fluid movement from blood to brain. However, water can also pass through water only pores in the endothelial membrane. In frog pial microvessels with an intact BBB, the hydraulic conductivity was measured and found to be equivalent to the water only pathway in frog skeletal muscle capillaries.[32] The character of these water only pores has been defined in recent years, in studies of the aquaporin family of proteins. These proteins form water channels across the plasma membrane, through which water moves passively, driven by osmotic gradients. In brain and spinal cord there are three aquaporins: Type 1 in choroid plexus epithelial cells, Type 9 which is found in subpial astrocytes and specialized ependymal cells and Type 4, the most abundant form. Type 4 aquaporin is expressed in the foot processes of astrocytes that surround cerebral blood vessels, as well as the subpial astrocytes. It is also expressed by cerebral endothelial cells where it forms water only pores and also by ependymal cells. A recent review summarizes data obtained from aquaporin-4 null mice[33] suggesting that aquaporin-4 plays a major role in the movement of water into cells during cytotoxic edema, as well as a major clearance route for water that accumulates in the CNS during vasogenic edema. Studies were carried out in a model of stroke involving permanent occlusion of the middle cerebral artery. There is swelling of tissue in the region of the infarct, much of which is due to cytotoxic edema. Cytotoxic edema occurs when depletion of metabolic substrates due to vascular occlusion results in failure of membrane ATP dependent pumps. In the case of the sodium pump, this causes sodium to accumulate

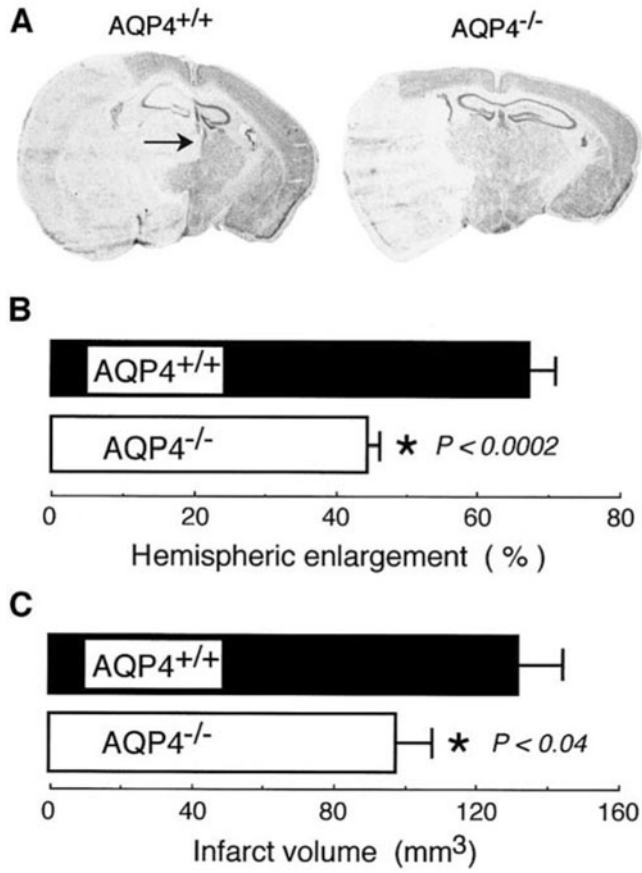

Figure 4. Aquaporin-4 null mice are protected from cytotoxic edema following permanent occlusion of the middle cerebral artery. Panel A shows reduced swelling 24h after occlusion in the null mice compared to wild type mice. Panels B and C quantify this benefit in terms of hemispheric swelling (B) and infarct volume (C). This data implies that water transport into cells as the result of energy failure occurs via aquaporin-4, while null mice fare worse in vasogenic edema, where aquaporin-4 is a major route for water clearance (see text, figure reproduced from Manley GT et al. Neuroscience 2004; 129:983-991, ©2004 with permission from Elsevier).

in the cell, where it drives osmotic movement of water from the extracellular space to the intracellular space. It could be shown that aquaporin-4 mediates most of this water movement, in that aquaporin null mice had markedly reduced edema in this model (Fig. 4).[34] The same paper also showed a similar protective effect from acute water intoxication, in which the reduction in serum osmolarity also drives water from blood to brain, without increases in vascular permeability. Presumably, some of this water accumulates in cerebral endothelial cells. In contrast, the loss of aquaporin-4 was detrimental when isotonic water was infused into the brain, suggesting that it forms a major egress route when extracellular water increases, as will occur in vasogenic cerebral edema, due to increases in vascular permeability. The same detrimental effect was seen in the cold injury model, where vasogenic edema predominates over cytotoxic

edema.[35] This implies that the opening of pores across the endothelium during BBB disruption, provides an additional route for water movement into the CNS, since loss of aquaporin-4 did not prevent vasogenic edema from occurring. These studies raise the intriguing question of the precise dynamics of water flow through the brain. It is not clear to what extent edema fluid moves through aquaporins at the brain-CSF interfaces (glia limitans, ependymal layer) or at the BBB. Also, to what extent other drainage routes predominate during edema, including movement through the perivascular spaces. Water may therefore exit the CNS by drainage into the CSF (reiterating Davson's notion of the CSF 'sink'), into the blood or along the olfactory tracts into the cervical lymphatics. The role of aquaporin-4 in water movement may also provide a clue to the effect that glucocorticoids (dexamethasone) have in the treatment of cerebral edema.[36] Clinically, the edema that develops around brain neoplasms such as high grade gliomas is responsive to glucocorticoids, while the edema developing around brain infarcts (stroke) is unresponsive. When dexamethasone was given to mice implanted with 9L glioma cells, this reduced their production of vascular endothelial growth factor (VEGF) and reduced edema.[37] However, when dexamethasone was given to sheep at doses used in humans, it reduced the expression of aquaporin-4.[38] It also stimulated the expression of the Na-K-Cl cotransporter, which has been implicated in hypoxia.[39] Thus, paradoxically, dexamethasone may remove a key mechanism for water clearance around vasogenic edema by reducing levels of aquaporin-4 and promote water accumulation through effects on sodium transport. Thus, aquaporin expression may represent a therapeutic target for the more effective treatment of stroke related vasogenic edema.

There are two main themes that emerge when considering how the BBB is altered during inflammation. One is the concept of biological redundancy and the other the concept of inflammatory benefit. During CNS diseases a plethora of inflammatory mediators are generated. These include classical inflammatory mediators such as histamine and bradykinin, which have a well established role in changing vascular permeability and have defined endothelial receptors and signal transduction mechanisms. Other mediators play an orchestrating role, including cytokines such as Tumor necrosis factor (TNF) and interleukin (IL)-1 and chemokines. Chemokines stimulate the entry of subsets of leucocytes into the brain, but in recent years their functions have widened to include effects on permeability and angiogenesis.[40] Nitric oxide and arachidonic acid are examples of mediators that do not bind to cell surface receptors, but operate by generating downstream metabolites or by altering the function of signaling proteins. In terms of vasogenic cerebral edema, the large number of different mediators means that blocking the action of one mediator or even a group of mediators is unlikely to be clinically effective. For example, a bradykinin B_2 receptor antagonist (Anatibant) was ineffective in a recent clinical trail in patients with traumatic brain injury.[41] It has been suggested that it may be possible to treat cerebral edema by interfering with common pathways. For instance, the transcription factor nuclear factor kappa B (NF-κB) has been implicated in the generation of multiple inflammatory mediators. Also, cytokines such as TNF may play a central role in the inflammatory response. However, inflammation is not just a detrimental process, because it is part of healing and repair and therefore blockade of common factors could have detrimental effects that outweigh or negate the benefits. Some factors are generated at high concentrations early in disease, when their blockade would be beneficial, while later in the disease, lower doses operate to promote repair and here blockade is detrimental. Careful attempts to delineate when and how particular factors could be blocked to improve outcomes in particular

diseases are needed. Because a large number of mediators are known to increase BBB permeability, this suggests several ways to exploit transient BBB opening, to improve delivery of water soluble drugs to the CNS. One example is the use of the bradykinin agonist labradimil, which has been used to cause transient increases in permeability to deliver chemotherapy to brain tumors.[42] It is beyond the scope of this chapter to describe in detail the extensive work on inflammatory mediators and the BBB. Studies were carried out in pial microvessels by this author and others on several mediators including histamine, bradykinin and arachidonic acid. Histamine is released from several sources during inflammation, including resident mast cells, histaminergic nerve fibers and by invading inflammatory cells such as neutrophils. Histamine has an interesting dual action on permeability, depending on dose. Doses between 5nmol/l to 5μmol/l caused a reversible increase in permeability. This was relatively modest, well under the range defined as first phase during spontaneous BBB disruption. Higher doses of histamine between 50μmol/l and 5mmol/l caused permeability to reduce (Fig. 3). Additional work showed that permeability increases occurred through the histamine H_2 receptor via increases in calcium, while permeability decreases occurred through H_1 receptor stimulation and increased cyclic AMP.[43] This data implies that histamine regulates permeability without causing increases of the magnitude associated with vasogenic edema. This contrasts with the actions of arachidonic acid, which causes permeability to increase into the higher range seen during the later, second phase of spontaneous BBB opening (Fig. 3). The action of millimolar doses of arachidonic acid could be blocked by scavenging oxygen free radicals, but blockers of arachidonic acid metabolism to prostanoids and leukotrienes had no independent effect. However the combination of indomethacin (cyclooxygenase inhibitor) and nordihydroguariaretic acid (lipoxygenase inhibitor) did block the permeability increase, implicating the free radicals being generated during arachidonic acid metabolism.[44] Further work was carried out to delineate some of the complex interactions between bradykinin and other inflammatory mediators and the multiple signaling pathways involved.[45-47] Acute application (ca. 1min) of bradykinin causes modest increases in permeability, that are calcium dependent. These increases are blocked with the same strategies that were effective against arachidonic acid, suggesting that bradykinin can activate phospholipase A_2 to increase arachidonic acid production. The resulting generation of free radicals may punch holes in the plasma membrane that allow calcium entry. Bradykinin also acts through a nitric oxide independent activation of particulate guanylate cyclase and generation of cyclic GMP. Its effects are blocked with zaprinast, which inhibits cyclic GMP breakdown by phosphodiesterase-5 and by leukotriene D_4, which blocks particulate guanylate cyclase. This is in contrast to the histamine H_2 effect, which operates through calcium dependent activation of endothelial NO synthase and NO mediated activation of soluble guanylate cyclase. However, bradykinin and histamine interact, because not only does bradykinin bind directly to B_2 receptors to mediate its effects on permeability through calcium and cyclic GMP, but the bradykinin B_1 receptor agonist [des-arg^9]BK also increases permeability, but is blocked by the histamine H_2 antagonist cimetidine. This implies that bradykinin binds to B_1 receptors, perhaps on perivascular mast cells or nerve fibers, to induce histamine release. While the action of bradykinin is restricted through its removal by enzymes such as angiotensin converting enzyme, continuous application for several minutes results in large permeability increases compatible with vasogenic edema. This also involves the interplay of mediators, since the effect of short applications of bradykinin was potentiated by the cytokine IL1β.

THE RELATIONSHIP BETWEEN BBB PERMEABILITY AND ANGIOGENESIS

Angiogenesis is the formation of new blood vessels from pre-existing vessels. In describing the relationship between increases in BBB permeability and angiogenesis, several principles emerge. First, the process of angiogenesis is regulated by several mediators that also induce increases in BBB permeability. This was briefly summarized in a recent review.[48] The angiopoietins are known to bind to the receptor tyrosine kinase Tie-2. There are four known ligands at Tie-2 including angiopoietin (Ang)-1, Ang-2, Ang-3 and Ang-4. Ang-1 binding to Tie-2 is associated with vessel stability. Ang-2 is an antagonist for Ang-1 and is postulated to initiate angiogenesis by counteracting the stabilizing effect of Ang-1, leading to detachment of pericytes from endothelial cells, allowing them to form sprouts that lead to the formation of new blood vessels. The process of endothelial sprouting is stimulated by various pro-angiogenic factors including Vascular endothelial growth factor, VEGF (the 165 amino acid human isoform, or VEGF-A, through binding to VEGF-receptor 2) and other growth factors. To migrate through the extracellular matrix, sprouting vessels must attach by integrin interactions (including binding of $\alpha_v\beta_3$ to fibronectin) and must digest collagen and other extracellular matrix components by releasing matrix metalloproteinases. Once vessels form patent lumens, there is a downregulation of VEGF, upregulation of Ang-1 and in this context new vessels are stabilized and excess vessels are pruned by induction of vascular endothelial apoptosis, which may be mediated by Ang-2 in the absence of VEGF. VEGF release is induced by hypoxia which activates the transcription factor HIF-1, however it is also induced by other factors including cytokines such as TNF. Many of these mediators have been reported to cause increases in BBB permeability, including TNF, VEGF and the MMPs. Second, the formation of immature capillary type vessels during angiogenesis may be associated with increased permeability, such that angiogenesis bypasses the normal tight phenotype of BBB endothelium and effectively contributes to vasogenic cerebral edema. The precise relationship between angiogenesis during CNS disease and increases in BBB permeability is not clear. Studies in models of stroke have suggested that new vessel formation is not associated with increased BBB leakage. In a model of permanent occlusion of the Middle Cerebral Artery (MCA) in rats, the authors detected an early increase in Evans blue leakage (4h).[49] They also examined the expression of angiogenic factors in the brain at times between 2h and 28 days and related these to the formation of new blood vessels around the stroke. In control animals, there was little expression of the operative VEGF receptor-2, or of Ang-1 and Ang-2, although the operative receptor Tie-2 was detected. However, following stroke, there was an early upregulation of VEGF at 2h that persisted for 28 days and a similar upregulation of VEGF-R2 from 4h-28days. Ang-1 was initially reduced (within hours) but expression increased after 2 days. This coincided with an increased expression of Ang-2 from 1 day and increased Tie-2 expression from 2h. Both angiopoietins had reduced by 28 days. From 2 days, the authors detected vessels with enlarged diameters ('mother vessels') at the margins of the stroke (penumbra) which were still present at 28 days. The authors suggest that early expression of VEGF and downregulation of Ang-1 induces an early increase in BBB permeability. However, other work suggests that BBB permeability reduces between 72-96h[50] and this reduction in permeability may be induced by increased expression of Ang-1 at 2 days. Therefore, the later development of new blood vessels appears to occur after most increases in BBB permeability have taken place. In an earlier study by the same group,[51] the delayed

intravenous administration of VEGF, 48h after permanent MCA occlusion in rats, induced angiogenesis without significant increases in BBB permeability. This was assessed using two methods—gadolinium enhancement on magnetic resonance images and extravasation of Evans blue. In this study, permeability increases were only induced if VEGF was given early (1h) after stroke induction. The implication is that VEGF acts on pre-existing vessels to induce permeability increases early on, but that this response becomes blunted at later times, perhaps through Ang-1 expression. A similar set of results was obtained in an elegant study of angiogenesis occurring in human temporal lobes.[52] Those individuals with temporal lobe epilepsy had an increased density of microvessels that correlated with seizure frequency. Similar to the stroke model, normal controls expressed low levels of VEGF, VEGF-R2 and Tie-2. However, epilepsy tissues showed increased expression of all of these factors. The increased vessel density was associated with perivascular collections of immunoglobulin G, implicating recent increases in BBB permeability. This study implies a continuing stimulus for angiogenesis in temporal lobe epilepsy, perhaps due to repeated cycles of hypoxia. The significance of angiogenesis as a pathological phenomenon is also intriguing. In stroke, angiogenesis is delayed for several days and so cannot rescue cells from necrotic cell death in the earliest stages of injury. However, angiogenesis continues for several weeks after stroke, as suggested by a study in which endothelial proliferation was found 21 days after experimental stroke.[53] The new vessels persisted at 28 days after stroke in the rat[49] and correlated to survival in human material at 92 days.[54] This may be likened to wound repair in general, where angiogenesis occurs to promote recovery, by increasing delivery of phagocytes, by restoring oxygen to hypoxic tissues and by finalizing scar formation. If angiogenesis persists in conditions such as stroke, it may reflect the ongoing stimulus for repair or the 'wound that never heals'. It has been suggested that angiogenesis in stroke promotes recovery by improving cerebral blood flow to surviving brain leading to improved cognitive outcomes and by promoting repair processes such as neurogenesis and synaptogenesis. A number of strategies have been employed to promote angiogenesis in stroke models. This includes direct application of factors such as VEGF or the angiopoietins, infusion of bone marrow derived stem cells and studies on the beneficial effects of exercise and the harmful effects of high cholesterol levels.[48] Acute intravenous administration of VEGF increases infarct size and BBB permeability.[51] However, administration of VEGF by injection into the lateral ventricle had the opposite effect, reducing infarct volume, as well as area of BBB leakage.[55] This parallels a study in which VEGF was applied to the cerebral surface and reduced infarct size without increasing edema.[56] These studies may reflect the ability of VEGF to promote neurogenesis and afford neuronal protection.[57] The smaller infarct that results may generate fewer mediators of increased BBB permeability. Also, it may be that VEGF receptors are polarized between the luminal and abluminal sides of the endothelium, so that VEGF applied to the abluminal surface of blood vessels does not increase BBB permeability. The effect of intravenous VEGF on BBB permeability could also be counteracted by co-administration of an adenoviral vector expressing Ang-1[58] and angiogenesis could be further promoted by co-administration of Ang-2.[59] Another aspect to angiogenesis is the process of endothelial apoptosis. Apoptosis (the process of orchestrated cell death) is a mechanism by which new vessels could be pruned and limited and might conceivably limit increases in permeability associated with angiogenesis. However, apoptosis of pre-existing endothelial cells might have the opposite effect and cause BBB permeability to increase. Studies of apoptosis have been carried out in cultured cerebral endothelial cells, exposed to a variety of stimuli including exposure to bacteria,[60]

to beta-amyloid,[61] oxygen glucose deprivation to mimic stroke like conditions[62] and exposure to mediators such as the TNF family of 'death ligands' known as TNF-related apoptosis inducing ligand (TRAIL) and Fas ligand.[63] In a study of angiopoietin signaling after permanent MCA occlusion in rats, there was a transient increase in endothelial apoptosis at 12h, associated with upregulation of Ang-2.[64] This would coincide with early increases in BBB permeability following stroke and may be limited by subsequent increases in VEGF, since in this context (increasing VEGF/Ang-2 ratio) Ang-2 is said to promote angiogenesis. In a recent study using ionizing radiation, it could be shown that endothelial apoptosis in spinal cord was associated with increases in BBB permeability and that both processes were blocked in mice deficient for acid sphingomyelinase.[65] This suggests that endothelial apoptosis can increase BBB permeability in contexts such as radiation injury and may increase BBB permeability during stroke through transient effects of Ang-2 signaling. It is not clear if its role in vessel regression also limits permeability increases associated with angiogenesis. However, this may be a moot point, since angiogenesis may not be a major factor in harmful increases in BBB permeability.

THE EFFECT OF NEUTROPHILS ON BBB PERMEABILITY DURING INFLAMMATION

One of the inflammatory factors whose role is uncertain is that of neutrophils and BBB permeability. Neutrophils are circulating white blood cells that act as early participants in inflammation. They form part of the innate immune response, which is not antigen specific. The application of inflammatory mediators such as TNF or histamine results in an increased expression of adhesion molecules on the surface of human brain endothelial cells in culture that regulate the adhesion and migration of applied neutrophils.[66,67] These adhesion proteins tether the neutrophil, allowing it to roll along the surface of the cell, firmly adhere and then migrate through the endothelial barrier into the tissues. The proteins involved in these processes including the selectins (P and E selectin on the endothelium, L-selectin on neutrophils) and integrin superfamily members (ICAM-1, ICAM-2, VCAM-1). Selectins induce rolling, while integrins induce firm adhesion and regulate transmigration. Particular subsets of leucocytes are cued to adhere to the endothelium by surface expression of chemokines. In the case of neutrophils these include IL-8 in the human and CXCL1 and CXCL2 in rodents. This basic story has expanded in recent years and now includes migration of neutrophils both between and through endothelial cells, an expanded repertoire of molecular regulators, differences between different vascular beds in vivo, insights into movement through the vascular basement membrane and migration through venules as well as capillaries.[68] Work done by Perry and his colleagues showed a fascinating restriction to the penetration of neutrophils into the CNS. Even when cytokines were injected into the mouse hippocampus and it could be shown that adhesion molecules were expressed, there was little infiltration by neutrophils. These injections were made into control tissues.[69] Later work showed that injection of chemokines or induction of CXCL1 expression by injection of IL1β, was able to override this resistance and induce infiltration by large numbers of neutrophils, accompanied by significant increases in BBB permeability, associated with molecular alterations in the associated microvessels.[70-73] By contrast, when neutrophils are applied to cultured brain endothelial cells both bovine and human, they induce little net changes in permeability. Application of untreated neutrophils to bovine cells initially reduces

permeability and during transmigration induced by placing the chemoattractant fMLP under the cells, the permeability returns to baseline levels (Fig. 5).[74] When neutrophils are activated using leukotriene (Lt)B_4, a similar small increase in permeability occurs, but only back to baseline.[75] When neutrophils were injected into the brain and then activated with LtB_4, a rather similar pattern was seen. Untreated neutrophils reduced permeability around the inflamed injection site, while activated neutrophils did not induce significant permeability increases.[75] In other work, neutrophils were applied to endothelium exposed to oxygen glucose deprivation, to explore their effects in the context of cerebral ischemia.[76] Here, neutrophils induce a profound reduction in permeability that is unresponsive to the application of LtB_4 and only partially responsive to application of PMA. When we induced neutrophil infiltration into a focal stroke by injecting the chemokine CXCL1, there was a reduction in local permeability, rather than the increase noted when CXCL1 was injected into the normal brain, providing evidence that neutrophils reduce permeability in the inflamed brain, but increase permeability when induced to enter an otherwise 'normal' brain (Fig. 5).[77] This likely reflects the balance of pro and anti-inflammatory

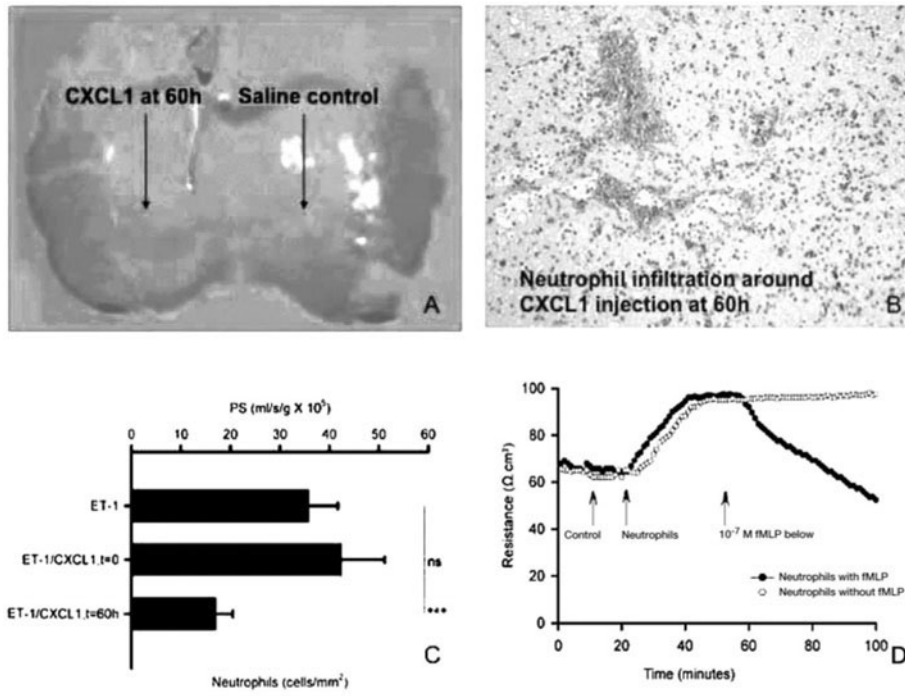

Figure 5. Evidence that neutrophils reduce BBB permeability. Panels A-C show data obtained in a stroke model. Rat striatum was injected with the vasoconstrictor peptide endothelin-1 and co-injected with the neutrophil-specific chemokine CXCL1 60h later. At 72h, the tissue was analyzed. Permeability maps show a reduction on the side of the CXCL1 co-injection (A), with a significant influx of neutrophils (B). The reduction in permeability is summarized in panel C. Panel D shows that neutrophils applied to cultures of primary bovine brain endothelial cells increase transendothelial electrical resistance (reduce permeability, open circles) but this returns to baseline when fMLP is applied to induce neutrophil migration through the cell layer (filled circles). Panel D adapted from Inglis VI et al. Brain Res 2004; 998:218-229,[74] ©2004 with permission from Elsevier.

factors present in the surrounding tissues. Indeed it has been suggested that neutrophils and other factors may operate very differently depending on the size of the lesion. In smaller strokes (hemorrhagic or ischemic) they may promote a reduction in permeability and are generally anti-inflammatory and healing in their effects. In larger strokes, the opposite may apply.[78] It should be noted that there is a dismal lack of effective therapy available to ameliorate stroke. Despite much animal research and clinical trials on many different agents, little seems to have been achieved. One of the classic failures relates to the use of NMDA antagonists. By countering the effect of glutamate, this may cause short term protection, but seems to cause longer term injury, since glutamate at lower concentrations also promotes recovery.[79] The same applies to anti-neutrophil strategies. In clinical trials with enlimomab, a monoclonal antibody that blocks neutrophil infiltration by binding to ICAM-1, the treated group had a worse outcome than the placebo group.[80] Our data suggests that the neutrophil response may be similar to the glutamate response and should not be limited or might even be promoted to reduce injury.

CONCLUSION—WHERE DO WE GO FROM HERE?

Increases in permeability across the blood-brain barrier are often seen during the complex inflammatory processes associated with CNS disease (Fig. 6). In some cases, the associated cerebral edema is both life threatening and associated with significant morbidity in survivors of an acute episode. In other diseases, like multiple sclerosis, BBB disruption is not associated with clinically important edema unless critical structures such as the optic nerve are affected, but is nevertheless commonly observed and may be functionally important. Attempts to reduce vasogenic edema have met with mixed success. The best therapeutic results are associated with the use of steroids to reduce tumor associated edema. But steroids have little impact on the edema associated with stroke or trauma. In multiple sclerosis, it remains to be seen whether BBB disruption is an epiphenomenon, or whether its amelioration could impact the disease. One approach to this question is to determine whether BBB disruption is related to angiogenesis and therefore amenable to treatment with anti-angiogenic drugs, or by using agents that promote apoptosis of endothelial cells. If BBB disruption is specifically targeted, what impact does this have on the disease itself? One question that remains unanswered is that of the relationship between increases in permeability and the transmigration of immune effector cells into the CNS. If the intercellular route facilitates both processes, then attempts to reduce one process could also mitigate the other. In some cases, such as multiple sclerosis, agents that reduce BBB permeability could therefore have a major impact on the disease, by sealing the main egress route for pathogenic leucocytes into the CNS. On the other hand, BBB permeability may not be easy to manipulate as an independent variable and even if it could be manipulated, there may be little impact on the disease itself. One way to address these questions has been to target the proteins that form the tight junction between cerebral endothelial cells. For instance, it was shown that the severity of experimental allergic encephalomyelitis, a mouse model of multiple sclerosis, was reduced in mice engineered to overexpress tight junctional claudin-1. Claudin-1 overexpression reduced BBB permeability but had no effect on the degree of leucocyte infiltration, showing that permeability alone affects the severity of the disease.[78] This suggests that even when leucocyte infiltration is reduced, the presence of BBB permeability increases is harmful. Perhaps, this relates to simple interference with ionic homeostasis and neuronal

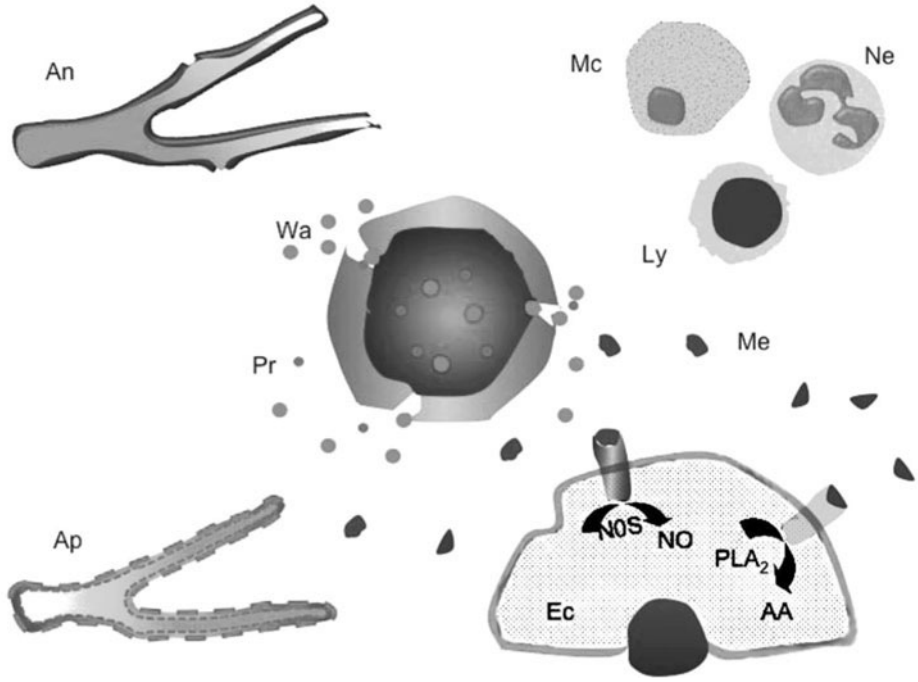

Figure 6. Summary of factors discussed in the chapter that cause increases in BBB permeability. The central picture shows a microvessel with movement of water and protein molecules into the CNS. This is stimulated by inflammatory cells and the release of mediators that act directly, or stimulate production of secondary factors like arachidonic acid or nitric oxide. Permeability may also increase as the result of angiogenesis or endothelial apoptosis. Symbols: AA = arachidonic acid, An = angiogenesis, Ap = apoptosis, Ec = endothelial cell, Ly = lymphocyte, Mc = macrophage, Me = soluble mediators, Ne = neutrophil, NO/NOS = nitric oxide/synthase, PLA_2 = phospholipase A_2, Pr = protein, Wa = water molecules.

functioning, as a significant player in diseases that are otherwise driven by immune cell involvement. The role of permeability in CNS disease and ordinary homeostasis remains an open question. Attempts to reduce permeability in the setting of disease need to be balanced by an understanding of the reparative role that BBB permeability increases might have in disease. It is usually assumed that permeability increases are harmful in the CNS, but this is based on the deleterious consequences of vasogenic cerebral edema. However, in other organs, edema is part of the reparative response to injury, allowing antibodies and compliment to enter injured tissue and to mount an appropriate immune response. So, a greater understanding of permeability changes, particularly at the lower levels unassociated with major protein leakage, and of the impact of modulating these changes, could lead to a more effective approach to therapy in a variety of CNS disorders.

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CHAPTER 2

DRUG TRANSPORTERS AT BRAIN BARRIERS
Expression and Regulation by Neurological Disorders

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Abstract: Drug transport in the central nervous system can be highly regulated by the expression of numerous influx and efflux transport proteins not only at the blood-brain barrier and blood-cerebrospinal fluid barrier but also in brain parenchymal cellular compartments (i.e., astrocytes, microglia, neurons). In particular, members of the ATP-Binding Cassette membrane-associated transporter superfamily and Solute Carrier family are known to be involved in the traffic of several endobiotics and xenobiotics (including drugs) into and out of the brain. These transport proteins have also been implicated in a number of neurological disorders including HIV-encephalitis, Alzheimer's disease, Parkinson's disease and neoplasia. This chapter summarizes recent knowledge on the role of drug transporters in the brain.

INTRODUCTION

Membrane-associated transport proteins are important determinants of drug disposition. In the central nervous system (CNS), tightly regulated influx and efflux transport systems are involved in the traffic of solutes and drugs into and out of the brain.[1] Influx transport systems facilitate the uptake of numerous nutrients (i.e., amino acids, glucose, nucleosides) as well as xenobiotics, whereas, efflux transport systems restrict the entry of a broad spectrum of xenobiotics and extrude endogenous compounds (i.e., neurotoxins, metabolic products) from the brain. In order to characterize the involvement of drug transporters in physiological and pathological events in the CNS, it is crucial to understand their localization and functional expression in several brain cellular compartments. The objectives of this chapter are: (i) to briefly introduce the physiology of the blood-brain barrier, mechanisms

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of transport processes and established methods to study drug transport and (ii) to review the expression, localization and functional activity of drug transporters in different brain cellular compartments under normal physiological and neuropathological conditions.

PHYSIOLOGY OF THE BLOOD-BRAIN BARRIER

The blood-brain barrier (BBB) forms a dynamic physical and biochemical barrier that separates the CNS from systemic circulation. The discovery of the BBB is attributed to the German immunologist Paul Ehrlich who first reported in 1880 that injection of cationic dyes into the systemic circulation stained most of the organs with the exception of the brain and spinal cord (Ehrlich, 1904). Later work from Edwin E. Goldman demonstrated that if the dye is injected in the cerebrospinal fluid (CSF) directly, only the nervous tissue is stained whereas other tissues remain unstained, confirming the existence of a physiological barrier between the systemic circulation and the brain (Goldmann, 1913). It was not until another 70 years when with the use of electron microscopy several researchers observed lack of horseradish peroxidase perfusion in mouse vascular endothelium suggesting the existence of a structural barrier at the blood-brain interface.[2] The identification of tight junction proteins that connect endothelial cells and restrict the paracellular traffic of substrates further confirmed the concept of a tightly regulated physiological barrier surrounding the CNS.[2] To date, the BBB is known as a multi-cellular unit formed of brain microvessel endothelial cells, basal lamina, surrounding pericytes, adjacent astrocytes and neurons (Fig. 1). These cellular compartments and basal lamina are collectively referred to as the "neurovascular unit" (Fig. 1).[3-5]

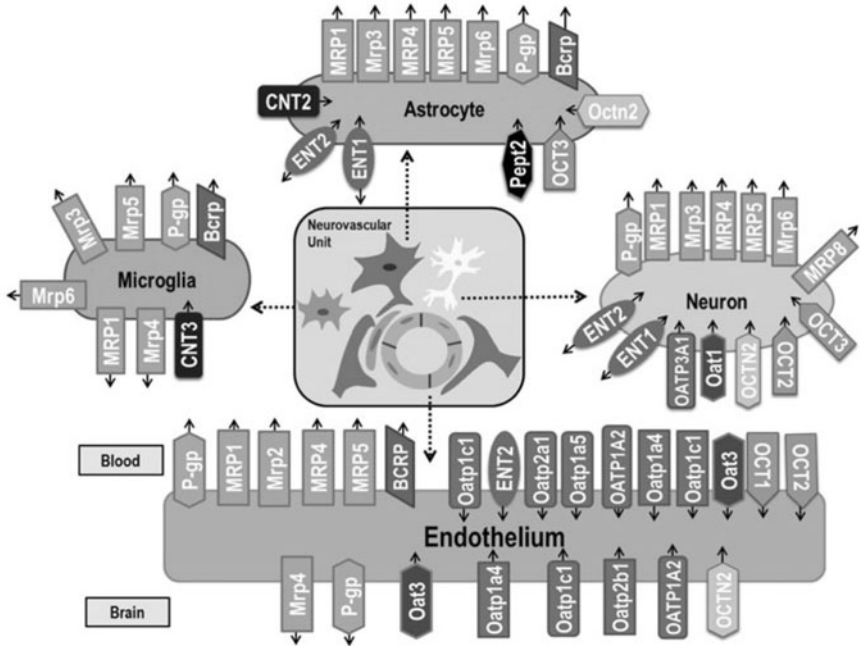

Figure 1. Neurovascular unit and localization of selected ABC and SLC transporters in brain microvessel endothelial cells, astrocytes, microglia and neurons. Arrows indicate the direction of substrate transport.

Neurovascular Unit

Brain Microvascular Endothelial Cells (BMECs)

Brain microvascular endothelial cells (BMECs) have unique structural and functional properties that form a selectively permeable and highly regulated barrier between the CNS and systemic circulation.[2] The intercellular network of tight junctions limits and regulates the traffic of immune surveillance cells (macrophages) and substances from the systemic circulation.[6] Tight junction proteins (i.e., claudins, occludin) and adherent junction proteins (i.e., junctional adhesion molecule 1) expressed in BMECs are responsible for the high transendothelial electrical resistance (1500 to 2000 ohm.cm^2) that restricts the paracellular entry of water and solutes.[7,8] In a functional BBB, BMECs also express a number of polarized receptors, ion channels and active influx and efflux transport proteins.[9] In addition to these systems, endocytosis and transcytosis of hormones and plasma proteins have also been reported in BMECs, which take place in membrane microdomains such as caveolae. The integrity of the BBB in known to be compromised in a number of neuropathological conditions such as stroke, trauma, bacterial or viral infections, multiple sclerosis, Alzheimer's disease, Parkinson's disease, epilepsy, brain tumours and pain.[5,10,11]

Astrocytes

Astrocytes are the most abundant glial cells in the CNS. These cells have a stellate morphology and possess numerous cytoplasmic fibrils that are enriched in glial fibrillary acidic protein.[12] Astrocyte foot processes surround more than 99% surface of the cerebral capillary basement membrane and this close interaction between astrocytes and BMECs has been implicated in the maintenance of tight junction integrity, proliferation and angiogenesis of endothelial cells and regulation of inflammatory responses in the brain.[4,13] Astrocytes also actively participate in maintaining the homeostasis of the CNS by locally removing excess K^+ and excitatory neurotransmitter glutamate released from neurons.[12,14] Astrocytes secrete various neurotrophic factors (i.e., transforming growth factor-beta, glial-derived neurotrophic factor, basic fibroblast growth factors) that are known to promote growth of neurons as well as differentiation and maturation of microglia.[15] Astrocytes also express numerous transporters that mediate the traffic of nutrients, solutes and xenobiotics across the cell membrane.[16,17] These cells are also involved in the regulation of immune and inflammatory events during brain injury and infection. Loss of astrocyte-mediated neuroprotective effects as well as chronic activation of astrocytes have been reported in neurodegenerative diseases, HIV-dementia, hepatic encephalopathy, hyperammonemia and ischemia.[18-20]

Pericytes

Pericytes are perivascular cells within the basal lamina that are important constituents of the BBB.[3] They surround the microvessel endothelial cells and maintain the structural integrity and stability of the BBB.[21] Pericytes are known to regulate many other brain functions such as angiogenesis, capillary flow, immune response as well as hemostasis.[3] Due to their multiple role in maintaining a functional BBB, pericytes have been implicated in the pathogenesis of numerous cerebrovascular, neurodegenerative and neuroimmune

diseases.[22] In addition, pericytes are known to express several drug transport proteins at the gene and/or protein level.[23] However, the role of these transporters at this site is not clear.

Neurons

Neurons are involved in the transmission of nerve signal through axonal processes. The interaction between blood vessels and neurons plays an essential role for the neurovascular network and maintenance of brain homeostasis.[24,25] Neuronal injury or loss has been implicated in many diseases such as Alzheimer's disease, Parkinson's disease, infectious diseases, vascular dementia, stroke and multiple sclerosis.[26] A few studies have demonstrated the expression of several drug transporters at this site.[27]

Other Glial Cells in Brain Parenchyma

Microglia

Microglia, the resident immunocompetent cells in the brain, were first identified by the Spanish neuroanatomist del Rio-Hortega in 1932. Under normal physiological condition, these cells appear in a resting state, characterized by smaller cell body, ramified processes and low expression of surface antigens. In response to injury, ischemia or inflammatory stimuli, microglia become activated and release inflammatory mediators such as pro-inflammatory cytokines, prostaglandins and nitric oxide that further recruit other microglia to the site of injury, trigger the activation of astrocytes and signal recruitment of peripheral monocytes from the systemic circulation. The beneficial role of microglia during CNS injury can be deleterious in situations where they become chronically active. Activated microglia have been implicated in many neuropathological conditions such as Alzheimer's disease, Parkinson's disease, HIV-encephalitis and others.[28] Like BMECs and astrocytes, these cells also express many active transport processes.[29,30] A decade ago, our group demonstrated functional expression of multiple drug efflux transporters in both microglia and astrocytes and proposed that along with astrocytes, microglia constitute a secondary barrier to drug permeability in the brain.[29,31-36]

Oligodendrocytes

Oligodendrocytes are myelin producing cells that are critical for the survival and maintenance of axon structure in the CNS. Therefore, the injury and death of oligodendrocytes are directly related to axonal damage. Diseases such as periventricular leukomalacia, multiple sclerosis, ischemic stroke, traumatic brain or spinal injury, radiation necrosis and leukodystrophies have been associated with oligodendrocyte loss or damage.[37] Although several transporters have been detected at the gene and/or protein level in oligodendrocytes, their expression level is known to vary according to the differentiation state of glial cells and their physical location in the brain.[30]

Blood-Cerebrospinal Fluid Barrier

The blood-cerebrospinal fluid barrier (BCSFB) composed of choroid plexus epithelial cells plays a major role in the permeability of nutrients and xenobiotics. The choroid plexus is a highly vascularised branched structure with numerous villi that project

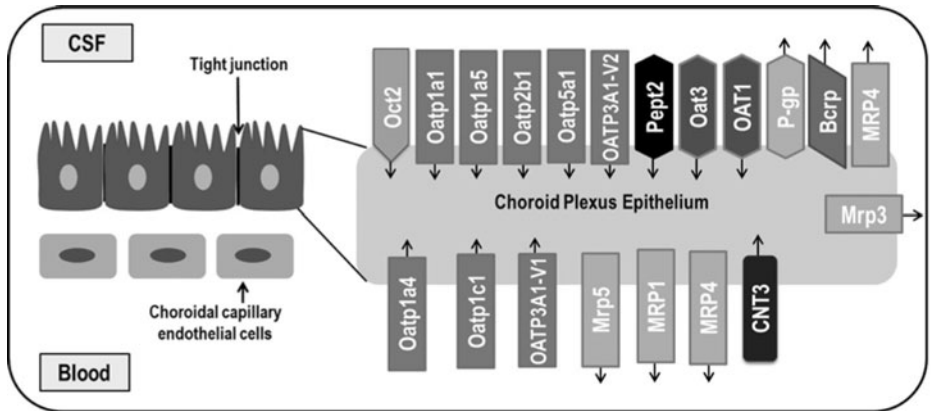

Figure 2. Localization of selected ABC and SLC transporters in choroid plexus epithelial cells. Arrows indicate the direction of substrate transport.

into all four cerebral ventricles.[38] The capillaries of the choroid plexus are fenestrated and provide little resistance to the movement of water and solutes. However, a barrier is formed by a monolayer of polarized epithelial cells surrounding the fenestrated capillaries that are joined together by tight junctional proteins.[39] These tight junctions form a functional barrier that restricts the movement of molecules and ions. The main function of the choroid plexus epithelial cells is to secrete and maintain the homeostatic composition of the CSF. The CSF fills the ventricles of the brain, the spinal canal and subarachnoid space. In humans, the total volume of CSF is approximately 140 ml which is replaced four to five times daily. The CSF also provides a drainage system for the brain, known as the sink effect, into which products of metabolism and other molecules are diluted and subsequently removed.[40] The sink effect is greater for large molecular weight and hydrophilic compounds. At the level of choroid plexus epithelial cells, polarized expression of numerous receptors, ion channels and transporters has been reported (Fig. 2).[38,41,42]

MECHANISMS OF DRUG PERMEABILITY ACROSS THE CNS

Passive Diffusion

Passive diffusion is a spontaneous and concentration gradient dependent process that allows molecules to move across cellular membranes down their electrochemical gradient without the requirement of metabolic energy (Fig. 3). Although passive diffusion of a drug is triggered by concentration differences, several factors can affect the diffusion of a drug across the BBB. For example, lipophilicity or lipid affinity of a drug can be used to predict how readily the drug will partition into the cellular lipid bilayer.[43] Molecular weight can also affect passive transport and is inversely correlated with the partition. For example, lipid soluble molecules with a molecular weight less than 400-600 Da are predicted to rapidly diffuse through the BBB, whereas, water soluble molecules and/or molecules with higher molecular weight are expected to show poor permeation across

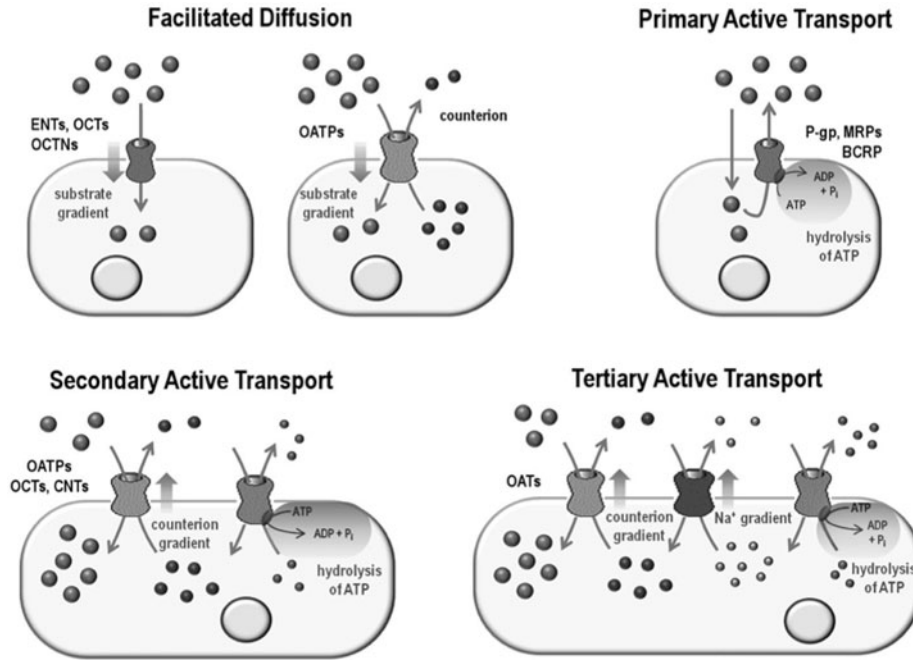

Figure 3. Mechanisms of drug transport.

the barrier.[44,45] However, a large number of drugs with favourable lipophilicity show poor permeability into the brain due to drug efflux transport processes suggesting that other transport mechanisms also influence passive diffusion of a compound.[46]

Carrier-Mediated Transport Processes

Many drugs can permeate into brain cellular compartments via specific carrier proteins or transporters. Uniporters are transporters that facilitate the movement of poorly permeable solutes across cellular membranes down the concentration gradient. Symporters cotransport more than one substrate in the same direction, whereas antiporters move two or more molecules or solutes across the phospholipid bilayer in opposite direction.[47] These carrier-mediated transport processes can further be divided into two categories: (i) facilitated diffusion that does not require metabolic energy and (ii) active transport that requires metabolic energy. Facilitated diffusion involves carrier-mediated passage of solutes across cellular membranes down their concentration gradient (Fig. 3). Transport of substrates via facilitated diffusion is energy-independent and saturable. A number of solute carrier transporters exhibit facilitated diffusion (i.e., glucose transporters with equilibrative nucleoside transporters).[48] Similar to facilitated diffusion, active transport is a carrier-mediated process that allows the passage of poorly permeable solutes across the membrane. However, unlike facilitated diffusion, active transport is energy dependent and uses ATP hydrolysis as an energy source to move molecules against their electrochemical gradient. Primary active transport is directly coupled with the hydrolysis of ATP by ATPase activity. In contrast, secondary and tertiary active transport utilize ion gradients

across the membrane, which are generated by active transport processes (Fig. 3). For example, members of the ATP-Binding Cassette superfamily of transporters are primary active transporters, whereas, members of the Solute Carrier family transporters utilize sodium or proton gradient across the membrane generated by primary active transporters to move solutes against their electrochemical gradient (Fig. 3).[49] In contrast to primary active transporters, secondary and tertiary active transporters do not have ATP-binding sites in their structure.

Endocytosis/Transcytosis

Endocytosis/transcytosis allow the internalization, sorting and trafficking of many plasma macromolecules to their location. Endocytosis is a process where molecules from the circulation are internalized in vesicles and are directed to endosomes or lysosomes within the cells. Transcytosis refers to the transcellular movement of molecules. Caveolae are specialized plasma membrane microdomains that are involved in both endocytosis and transcytosis. These processes can be either adsorptive (nonspecific process) or receptor-mediated.[50] Adsorptive endocytosis/transcytosis facilitates the transport of large peptides such as IgG, histone, albumin, native ferritin, horse radish peroxidase and dextran. Adsorptive processes largely depend upon electrostatic interactions that allow the positively charged moiety of the substrate to bind to the negatively charged cell membrane. Studies suggest that adsorptive-mediated transport efficiency depends on cationic charge and lipophilicity of the peptide, but not peptide size.[51] Receptor-mediated processes are activated by ligand binding to luminal cell surface receptors which lead to internalization of the receptors at the luminal side followed by either endocytosis to endosomes/lysosomes or transcytosis across the membrane to be externalized at the abluminal surface.[50] Unlike adsorptive processes, receptor-mediated endocytosis/transcytosis primarily depends upon the binding of molecules to specific binding sites within clathrin coated vesicles that in turn, directs the molecules to endosomes. Uptake of molecules by these processes is saturable and dependent on the number of available receptors on the cell surface. Peptides such as transferrin, albumin, insulin, insulin growth factor, low-density lipoprotein, ceruloplasmin and others utilize receptor-mediated endocytosis/transcytosis.[52]

MODELS TO STUDY DRUG TRANSPORT AND PERMEABILITY

Over the past several years, numerous techniques have been developed to study drug transport across the BBB as well as into cellular compartments located in brain parenchyma. These techniques can be divided into three main categories: in vivo, in situ and in vitro.

In Vivo

Many key findings involving the physiological and functional role of drug transporters have been identified in in vivo models. Knockout animal systems have provided a convenient method to selectively downregulate a transporter of interest. A number of single, double or even triple knockout mice models are currently available. Comparison of drug accumulation in the brain between wild-type and knockout animals may provide insights

into the functional role or substrate specificity of a transporter; however, compensatory mechanisms within the cells may influence transport properties and data generated from these models must be interpreted cautiously.

In vivo systems available for the study of drug transport across the BBB can be categorized into two classes: single and multi-passage techniques. Single passage techniques measure drug uptake into the CNS after a single passage through the brain following an injection in the blood stream. These techniques include indicator dilution method, brain uptake index and external registration.[53] Single passage techniques do not permit to study time-profile of BBB transport in a single animal and the use of many animals may introduce large data variability. In addition, these methods are not suitable for studying uptake of hydrophilic drugs since compounds with extremely slow uptake have a short exposure time and cannot be accurately measured.[54] Therefore, multi-passage techniques such as intravenous administration and microdialysis are more favourable to study BBB transport of hydrophilic drugs since these techniques allow longer circulation time for the test substance.[55] Intracerebral microdialysis is a technique that allows direct sampling of brain interstitial fluid by implanting a dialysis fibre into the brain. This technique allows the determination of the local concentration of free drug as a function of time in a single animal which reduces the number of animals to be used. However, since only low concentrations of drugs may be present in the dialysate, this method can be limited by the sensitivity of the analytical procedure. Another multi-passage technique that allows the quantification of drug transport from the blood into the CSF is CNS deconvolution technique. This technique is based on serial CSF fluid sampling and numerical deconvolution of data that enables the determination of a complete transport profile of a drug in a single living animal.[56] This technique has the advantage of being model-independent and involves a small number of animals; however, it requires skilled surgical and experimental experience.

Although a number of in vivo models have been established to quantify drug transport across the BBB, most of these models are cost and time-intensive and often require complex mathematical models. Therefore, in situ or in vitro systems are more commonly used.

In Situ

In situ perfusion techniques allow exposure of the brain tissue to the test substance by perfusion with a physiological buffer.[57] The in situ perfusion technique was first introduced by Takasato et al and involves carotid artery perfusion of the brain of an animal (usually rat) and sampling of drug levels.[58] This model was generated to limit the metabolism of the test substance and control conditions such as pH or temperature during transport. However, in situ techniques require fine surgery and experimental set up as well as complex kinetic analysis.

In Vitro

Since cell culture systems provide a broader level of control over environmental factors (i.e., pH, temperature) compared to in vivo systems, they are more commonly used to generate information on the properties of a transporter (i.e., kinetics, specificity). Often substrate specificity and driving force of a transporter are determined using in vitro methods such as reconstituted liposomes, *Xenopus* oocytes and/or transporter

overexpressing cell lines (i.e., MDCK, HeLa, HEK293).[59] To exclusively understand the CNS-specific role of drug transporters, numerous BMEC and glial cell culture systems have been used.[29,60,61]

To date, many researchers have established primary cultures of BMECs from a number of mammalian species (i.e., bovine, porcine, murine, human). In addition, several immortalized rodent and human cell systems are also available (i.e., RBE4, GPNT, b.End3, BB19, NKIM-6, hCMEC/D3).[62] Among these systems, the immortalized hCMEC/D3 cell line generated from primary human BMECs is one of the most established and extensively characterized systems available to study drug transport across the BBB.[63] This cell line has been shown to retain several morphological and functional characteristics of the brain microvessel endothelial cells in vivo. Dauchy et al compared the gene and functional expression of various ABC transporters between hCMEC/D3 and freshly isolated human brain microvessels and reported that P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP) expression are lower in hCMEC/D3 than in human microvessels, but activity remains comparable.[64]

Since the BBB is considered a dynamic multi-compartment unit, advances have been made to establish coculture systems where mixed cultures of cells are grown either together or in separate compartments using transwell systems. A coculture system may allow cell-cell communication and/or generate specific signaling proteins and/or tropic factors necessary for the differentiation and proliferation of neighbouring cells. Therefore, coculture systems with mixed glia or pericytes have been considered a more physiological model of BBB.[65] Several studies have also described dynamic three-dimensional model of the BBB where drug transport properties are investigated in BMECs cocultured with other cellular components of the neurovascular unit under flow conditions. In order to study drug transport in brain parenchymal cells, standard procedures have been established to generate primary cultures of astrocyte or microglia.[31,66] Due to the low yield, a few immortalized microglia cell systems have also been utilized to determine transport properties in vitro.[31,32,36,67,68]

FUNCTIONAL EXPRESSION AND LOCALIZATION OF FAMILIES OF MEMBRANE DRUG TRANSPORTERS IN THE CNS

ATP-Binding Cassette (ABC) Transporters

The ABC superfamily is one of the largest protein families containing numerous functionally diverse membrane-associated transporters that are expressed throughout the human body. To date, 50 ABC proteins have been identified in humans that can be separated in seven subfamilies (A to G) based on the structural organization of these transporters.[69] These membrane-associated proteins are responsible for the movement of a wide range of substrates across the lipid bilayer using ATP hydrolysis as the energy source (Table 1).[73,74] ABC transporters are characterized by the presence of a highly conserved Nucleotide Binding Domain (NBD) containing three distinct motifs, Walker A, Walker B and a signature motif (LSGG).[75] Mutations in a number of ABC genes can lead to diseases such as cystic fibrosis, retinal degeneration, impaired cholesterol/bile transport and others.[76] An increased recognition of these transporters in the absorption, distribution and elimination of a wide spectrum of xenobiotics including several pharmacological agents highlights the significance of these proteins.[77]

Table 1. ABC transporters, localization in the brain and established substrates

Name	Gene	CNS Expression/Localization	Substrates
P-gp (MDR1)	*ABCB1*	Brain microvessel endothelial cells (LM/ALM), choroid plexus epithelium (AP), astrocytes, neurons	*Xenobiotics*: anticancer agents (i.e., doxorubicin, etoposide, vincristine, vinblastine, paclitaxel), antiretrovirals (i.e., darunavir, atazanavir, saquinavir, indinavir, ritonavir, tenofovir, abacavir, raltegravir, maraviroc), steroids (i.e., dexamethasone), immunosuppressant (cyclosporine), antibiotics (i.e., erythromycin), β-blockers (i.e., bunitrolol), calcium channel blockers (i.e., verapamil), cardiac glycosides (i.e., digoxin), analgesic (morphine), anti-histamines (i.e., fexofenadine), dyes (i.e., rhodamine 123), detergents (i.e., triton X-100), ionophores (i.e., gramicidin D) and others (i.e., actinomycin D, loperamide, cimetidine)
P-gp (Mdr1a, Mdr1b)	*Abcb1*	Brain microvessel endothelial cells (LM/ALM), choroid plexus epithelium (AP), astrocytes, microglia, neurons	
BCRP (ABCG2)	*ABCG2*	Brain microvessel endothelial cells (LM), choroid plexus epithelium (AP), astrocytes, microglia, neurons	*Endobiotics*: folic acid, vitamin K3, riboflavin, porphyrins, pheophorbide, estrone-3-sulfate (E3S), estradiol-17β glucuronide ($E_2$17βG), dehydroepiandrosterone sulphate (DHEAS), uric acid *Xenobiotics*: anticancer agents (i.e., mitoxantrone, methotrexate, doxorubicin, daunorubicin, etoposide, topotecan), antiretrovirals (i.e., lamivudine, zidovudine, abacavir), antibiotics (i.e., erythromycin, enrofloxacine, gepafloxacine), calcium channel blockers (i.e., azidopine, dipyridamole), HMG-CoA reductase inhibitors (i.e., atorvastatin, pravastatin, rosuvastatin), fluorescent compounds (i.e., rhodamine123), carcinogens (i.e., aflatoxin B1) and others (i.e., cimetidine)
Bcrp (Abcg2)	*Abcg2*	Brain microvessel endothelial cells (LM), choroid plexus epithelium (AP), astrocytes	

continued on next page

Table 1. Continued

Name	Gene	CNS Expression/Localization	Substrates
MRP1 (ABCC1)	*ABCC1*	Brain microvessel endothelial cells (LM), choroid plexus epithelium (BL), astrocytes, neurons	*Endobiotics*: leukotrienes C_4, E_4 and D_4 (LTC_4, LTE_4 and LTD_4), GSH, GSSG, GSH conjugates (i.e., LTC_4, S-glutathionyl prostaglandin A_2), glucuronide conjugates (i.e., glucuronosylbilirubin), sulphate conjugates (i.e., E3S), folate *Xenobiotics*: antiretrovirals (i.e., atazanavir, saquinavir, ritonavir, indinavir, emtricitabine), anticancer (i.e., doxorubicin, danorubicin, etoposide, vincristine, vinblastine, irinotecan, paclitaxel), antibiotics (i.e., difloxacine), metalloids (i.e., sodium arsenite, sodium arsenate, potassium antimonite), dyes (carboxydichloro fluorescein, calcein and toxins (i.e., methoxychlor)
Mrp1 (Abcc1)	*Abcc1*	Brain microvessel endothelial cells (LM), choroid plexus epithelium (BL), astrocytes, microglia, neurons	
MRP2 (ABCC2)	*ABCC2*		*Endobiotics*: LTC_4, glutathione, bilirubin, steroids (E3S, $E_2$17βG), glucuronides (bisglucuronosyl bilirubin, monoglucuronosyl bilirubin), conjugated bile salts *Xenobiotics*: anticancer agents (doxorubicin, etoposide, methotrexate), antiretrovirals (i.e., atazanavir, ritonavir, saquinavir, indinavir), antibiotics (i.e., ampicillin), dyes (carboxydichloro fluorescein), toxicants and other drugs (i.e., phenobarbital, indomethacin)
Mrp2 (Abcc2)	*Abcc2*	Brain microvessel endothelial cells (LM)	
MRP3 (ABCC3)	*ABCC3*	Unknown	*Endobiotics:* LTC_4, $E_2$17βG, DHEAS, glucuronide conjugates (i.e., bilirubin glucuronide), cholylglycine *Xenobiotics*: anticancer agents (i.e., vincristine, methotrexate, etoposide)
Mrp3 (Abcc3)	*Abcc3*	Brain microvessel endothelial cells, choroid plexus epithelium (tight junction)	

continued on next page

Table 1. Continued

Name	Gene	CNS Expression/Localization	Substrates
MRP4 (ABCC4)	*ABCC4*	Brain microvessel endothelial cells (LM), choroid plexus epithelium (BL), astrocytes	*Endobiotics*: cyclic GMP (cGMP), cyclic AMP (cAMP), LTC_4, GSH conjugates (i.e., cholate, cholylglycine, cholytaurine), prostaglandins E_1, E_2 and $F_{2\alpha}$, thromboxane B_2, DHEAS, $E_2$17βG, folate, urate, ADP *Xenobiotics*: 9-(2-phosphonomethoxyethyl)adenine (PMEA), 6-thioguanine, 6-mercaptopurine, anticancer drugs (i.e., methotrexate, topotecan, irinotecan), antiretrovirals (tenefovir, abacavir, zidovudine)
Mrp4 (Abcc4)	*Abcc4*	Brain microvessel endothelial cells (LM/ALM), choroid plexus epithelium (BL), astrocytes, microglia	
MRP5 (ABCC5)	*ABCC5*	Brain microvessel endothelial cells (LM), astrocytes, neurons	*Endobiotics*: cGMP, cAMP, folate *Xenobiotics*: methotrexate, PMEA, 6-thioguanine, 6-mercaptopurine, heavy metals, antiretrovirals (stavudine)
Mrp5 (Abcc5)	*Abcc5*	Brain microvessel endothelial cells (LM), choroid plexus epithelium (BL), astrocytes, microglia	
MRP6 (ABCC6)	*ABCC6*	Unknown	*Endobiotics*: GSH conjugates (i.e., LTC_4, N-ethymaleimide S-GSH) *Xenobiotics:* anticancer agents (i.e., etoposide, doxorubicin),
Mrp6 (Abcc6)	*Abcc6*	Glial cells, neurons	
MRP7 (ABCC10)	*ABCC10*	Unknown	*Endobiotics*: $E_2$17βG , LTC_4 *Xenobiotics*: anticancer agents (docetaxel, taxane, paclitaxel, vincristine and vinblastine)
Mrp7 (Abcc10)	*Abcc10*	Unknown	
MRP8 (ABCC11)	*ABCC11*	Neurons	*Endobiotics*: cGMP, cAMP, cholylglycine, glycocholate, taurocholate, folate, GSH conjugates (i.e., LTC_4), $E_2$17βG, E3S, DHEAS *Xenobiotics*: PMEA, 5-fluorouracil
MRP9 (ABCC12)	*ABCC12*	Unknown	Unknown
Mrp9 (Abcc12)	*Abcc12*	Unknown	

LM = luminal; ALM = abluminal; AP = apical; BL = basolateral.
Adapted from references: Keppler, 2011; Kis et al., 2010; Zhou, 2008[70]; Deeley and Cole, 2006[71]; Dallas et al., 2006; Mao and Unadkat, 2005; Kim, 2002[72]; Lee et al., 2001.

P-Glycoprotein (P-gp)

P-gp, the first identified ABC transporter, was isolated and characterized by Victor Ling in Toronto, Canada, in a Chinese hamster ovary cell line where this transporter was associated with resistance to colchicine.[78] Since its discovery, overexpression of P-gp has shown resistance to numerous chemotherapeutic drugs, a phenomenon known as "Multidrug resistance (MDR)". P-gp consists of 1276-1280 amino acids with a molecular mass of approximately 170 kDa. It is encoded by the MDR/Mdr gene which has two isoforms in humans (MDR1 and MDR2) and three isoforms in rodents (mdr1a, mdr1b and mdr2).[79,80] MDR2/mdr2 is exclusively expressed in the liver and involved in phosphatidylcholine translocation, whereas, P-gp is encoded by the MDR1 gene in humans. In rodents, both mdr1a and mdr1b are products of the mdr1 gene and demonstrate similar MDR phenotype. The membrane topology of P-gp shows a tandemly duplicated structure with two homologous halves.[81] Each half consists of a highly hydrophobic transmembrane domain (TMD) containing six transmembrane helices and a NBD.[80,82] The two homologous halves of P-gp are connected by a hydrophilic linker region, which is phosphorylated at several sites by protein kinase C, although phosphorylation of this linker region does not appear to affect P-gp function. The N- and C-termini as well as the NBDs are located intracellularly. Both NBDs are critical for P-gp function and ATP binding to both NBDs allows P-gp activity. Using P-gp reconstituted into proteoliposomes, previous studies have shown that P-gp may function as a unidirectional flippase and conformational change induced by ligand binding allows P-gp to move the ligand across the lipid barrier using ATP hydrolysis. Recent advances in structural studies have generated the crystal structure of mouse P-gp which shows an internal cavity with nucleotide-free inward facing conformation.[83] This study also reported two cocrystal structures of P-gp bound to cyclic peptide inhibitor. In the drug-bound conformation, the drug-binding pocket of P-gp is open to the cytoplasm and lipid bilayer.[83] This conformation likely represents a pretransport state of P-gp where drug binding followed by ATP binding causes a dimerization in the NBDs, resulting in the outward facing conformation where the drug is released due to changes in conformation and/or accompanied by ATP hydrolysis.

P-gp can transport many structurally diverse compounds that are weakly amphipathic or relatively hydrophobic such as anticancer, antiretroviral, immunosuppressive, antihypertensive, anti-arrhythmic, anti-depressant, antimicrobial, H2-antagonistic and antiepileptic agents as well as steroids, natural products, fluorescent dyes and others.[57,61,84,85] The majority of P-gp substrates contain an aromatic ring and a positively charged tertiary nitrogen atom in their structure. A list of P-gp substrates can be found in Table 1.

Functional expression of P-gp has been detected at several blood-tissue barriers (i.e., blood-testis, blood-placenta) including the BBB suggesting a predominant role of this transporter in the absorption and elimination of xenobiotics at these sites.[86,87] At the BBB, P-gp localization has been reported in BMECs, adhesive pericytes and adjacent astrocytes.[88] Although P-gp is mainly detected at the luminal membrane in brain endothelium,[89,90] a few studies, including the ones from our group, have reported P-gp localization at the abluminal surface as well as in astrocyte foot processes.[16,88] P-gp functional expression has been characterized in isolated brain capillaries as well as in several primary and immortalized brain cell culture systems including a rat microglia cell line (MLS-9), cultured rat astrocytes, rat brain microvessel endothelial cell line (RBE4) and human brain microvessel cell line.[29,36,60,91,92] At the BCSFB, P-gp has been detected

at the apical side of choroid plexus epithelia.[93] Studies performed in mdr1a/1b knockout mice models further support the protective role of P-gp in the brain.[94,95]

High levels of expression at the BBB and the wide range of substrate specificity of P-gp is considered as one of the major obstacles to drug permeation into the brain. Modulating P-gp expression, therefore, has been extensively studied by many researchers to improve drug delivery into the brain. Numerous physiological and pathological signals have also been implicated in the regulation of P-gp functional expression including pro-inflammatory cytokines, polypeptide hormone endothelin-1, viral proteins, bacterial lipopolysaccharide and β-amyloid.[96,97] Many cellular signaling pathways have been reported to regulate P-gp such as Mitogen-Activated Protein Kinase pathway (MAPK), nuclear factor-kB pathway (NF-κB), nuclear receptors [i.e., pregnane-X-receptor (PXR), peroxisome proliferator-activated receptor (PPAR), constitutive androstane receptor (CAR)], protein kinase C, protein kinase Akt and Rho pathways.[98-103] P-gp has also been implicated in numerous drug-drug interactions. Co-administration of drugs that are substrates, inducers or inhibitors of P-gp can result in altered pharmacokinetic and pharmacodynamic response.[104] Altered P-gp expression at the BBB and in brain parenchymal compartments can affect drug distribution during therapy. The regulation of P-gp in several CNS diseases will be discussed in a later section of this chapter.

Multidrug Resistance-Associated Proteins (MRPs)

Multidrug resistance-associated protein (MRP) family is a group of ABC transporters that primarily confer resistance to a number of organic anions. MRPs are ubiquitously expressed in many tissues including liver, kidney, intestine and brain. The mammalian MRP family (humans, MRP; rodents, Mrp) has 13 members including nine proteins involved in drug transport, MRP1-9 (ABCC1-6 and ABCC 10-12), one ion channel (cystic fibrosis transmembrane regulator gene, CFTR), two receptors (sulfonylurea 1 and 2) and one truncated protein that does not mediate transport (ABCC13).[33,105] With regards to membrane topology, MRP transporters can be classified into two groups (MRP1-3, MRP6-7 and MRP4-5, MRP8-9). MRP1-3, MRP6 and MRP7 have predicted membrane topology of three TMDs showing a topology of 5 + 6 + 6 configuration of transmembrane helices where the first TMD is linked to the core region by a shorter cytoplasmic loop. In the case of MRP1, the linker region is required for function, whereas, the first TMD is not essential for transport activity.[106] In comparison, MRP4, MRP5, MRP8 and MRP9 do not possess the first TMD with five helices at their N-terminus and contain two TMDs each having 6 transmembrane helices and a NBD.[107]

Among the MRP proteins, MRP1 was the first to be identified in a human lung cancer cell line, H69AR, which displayed characteristics of the MDR phenotype in the absence of P-gp expression.[108] MRP1 shows substrate selectivity towards organic anions or neutral organic drugs and unlike P-gp, MRP1 is also capable of transporting glutathione (GSH), glucuronide and sulphate conjugates.[109,110] Substrates of MRP1 include anticancer drugs, inflammatory mediator leukotriene C4 (LTC4), protease inhibitors, oxyanions and several dietary constituents (i.e., bioflavanoids).[33,111] MRP1 function can be distinguished from MRP2 and MRP3 by its high affinity for LTC_4. Mrp1 has been shown to be involved in the transport of a few cationic drugs (i.e., vincristine, etoposide) in the presence of GSH.[112,113] MRP1 is known to be ubiquitously expressed with higher expression in lung, testes and peripheral blood mononuclear cells. In the CNS, MRP1 protein expression has been located in the luminal membrane of BMECs and basolateral membrane of choroid

plexus epithelial cells.[93,114] Studies have also confirmed the localization of this protein in pericytes, astrocytes, microglia, oligodendrocytes and neurons.[23,32,36,115] Functional expression of MRP1 has also been demonstrated in vivo. In Mrp1 knockout mice, vincristine permeation in the knockout animal brain was found to be significantly higher than the wild-type animal.[116] In addition, a few signaling pathways have been identified in MRP1 regulation such as MAPK, NF-κB and Janus Kinase-Signal Transducer and Activator of Transcription (JAK-STAT).[117,118]

MRP1, along with other MRP isoforms (MRP2, MRP4 and MRP5), can also contribute towards cellular defence during oxidative stress by regulating intracellular GSH and glutathione disulfide (GSSG) concentrations. GSH levels within cells are tightly regulated and compromised levels lead to the progression of many disorders including cancer, inflammatory and neurodegenerative diseases. Elevated levels of GSH have been found in tissues from Mrp1 and Mrp2 knockout mice.[119] It is now known that both MRP1 and MRP2 can mediate transport of GSH as a substrate as well as cotransport of GSH in the presence of other compounds. MRP1 mediated transport of GSH has been well characterized in cultured astrocytes and this transporter has also been implicated in the transport of GSSG and other oxidized GSH derivatives.[120] Other MRP isoforms are also known to be involved in GSH transport (MRP4 and MRP5),[121,122,137] although their functional role in brain cellular compartments is not clear. For example, using astrocyte cultures derived from Mrp1 and Mrp5 knockout mice, Minich et al demonstrated that Mrp1, but not Mrp5 is involved in GSH and GSSG transport in cultured astrocytes.[123]

MRP1 and MRP2 share similar substrate specificities, although differences in kinetic properties exist between MRP1 and MRP2 mediated transport. Endogenous substrates of MRP2 include conjugated steroids, bile salts and LTC4. MRP2 also transports many exogenous compounds such as anticancer drugs, antiretrovirals, antibiotics, environmental toxins and metal complexes. MRP2 can also mediate transport of GSH independently or along with other compounds. MRP2 plays an important role in extruding metabolites into bile[124] and mutations in MRP2 gene lead to Dubin-Johnson syndrome. MRP2 has a more limited tissue distribution compared to MRP1. In polarized cells such as hepatocytes and kidney proximal tubule epithelial cells, MRP2 is expressed at the apical surface. At the BBB, Mrp2 has been localized at the luminal side of BMECs and brain capillaries.[91] At the BCSFB, the mRNA expression of Mrp2 has been reported to be negligible.[125] In brain parenchyma, Mrp2 mRNA, but not protein expression in astrocytes isolated from embryonic rats has been detected.[30] Study by Potschka et al demonstrated an increased accumulation of phenytoin, an antiepileptic agent in Mrp2-transport deficient mice suggesting a defensive role of this transporter at the level of the BBB.[126] Although there has been no evidence to date that supports a role of MRP2 in clinical MDR phenotype. In rodent capillaries, Mrp2 has been shown to be regulated by the nuclear receptor, CAR.[102]

MRP3 has a narrower but similar substrate preference as MRP1 and MRP2. However, MRP3 cannot transport GSH and prefers glucuronide conjugates over glutathione conjugates.[127] MRP3 can mediate the transport of monoanionic bile acids. MRP3 is expressed in adrenal gland, kidney, placenta, intestine, pancreas, liver and gallbladder. At the BBB, Mrp3 expression appears negligible and has not been detected in mouse brain microvessels; however, gene expression was reported in hCMEC/D3 cells.[64] Low levels of Mrp3 have also been detected in bovine BMECs whereas expression was not observed in capillary enriched homogenate.[128] In choroid plexus epithelium, Mrp3 staining was observed at the inter-cellular junctions, although the function of this protein at this site is unknown.[129] In brain parenchyma, MRP3/Mrp3 gene expression has been

detected in astrocytes, microglia, oligodendrocytes and neurons.[30,130] Upregulated MRP3 expression has been observed in several gliomas suggesting a role of this transporter in chemotherapeutic resistance.[131]

Unlike MRP1-3, MRP4 has the unique ability of transporting a range of endogenous molecules involved in cellular communication of signaling including cyclic nucleotides (cAMP, cGMP), eicosanoids, urate, conjugated steroid hormones, folate, bile acids, nucleotide and purine analogs.[132] MRP4 substrates also include antiretrovirals, antibiotics, cardiovascular and cytotoxic agents. Cellular localization of MRP4 varies in polarized cells. Basolateral localization has been reported in prostate epithelial cells and hepatocytes, whereas, apical expression has been detected in proximal tubule kidney epithelial cells. Using immunocytochemical analysis, MRP4 expression has been detected at the luminal and abluminal sides of brain capillary endothelial cells and at the basolateral side in the choroid plexus epithelia.[130,133-135] MRP4 is also expressed in astrocytes, microglia, oligodendrocytes and neurons.[136] In our hands, we have also detected MRP4/Mrp4 expression in primary cultures of rat and human astrocytes.[137] In Mrp4 knockout mice model, an enhanced accumulation of the anticancer drug topotecan was observed in brain parenchyma and CSF.[135] Another study reported increased brain penetration of adefovir in Mrp4 knockout mice suggesting a functional role of MRP4 in limiting nucleoside analogue drugs into the brain.[138]

Similar to MRP4, MRP5 can function as a cyclic nucleotide export pump and confers resistance to nucleoside analogs which suggests that these transporters may synergistically contribute to the regulation of tissue levels of cAMP and cGMP. In addition to these cyclic nucleotides, MRP5 substrates include a number of other organic anions such as nucleoside monophosphate analogs, glutathione S-conjugates and fluorescein diacetate. MRP4 and MRP5 do not interact with established substrates of MRP1-3 (i.e., vincristine, daunorubicin, etoposide). GSH is also a substrate for MRP4 and MRP5, although GSH cotransport is not necessary for all the substrates of these transporters. MRP5 is also expressed in brain cellular compartments. In comparison to MRP4 expression, MRP5 is localized at the luminal membrane of BMECs, but is expressed at the basolateral membrane of the choroid plexus.[130,134,139] MRP5 is also highly expressed in pyrimidal neurons and astrocytes.[130] Others have reported Mrp5 gene expression in oligodendrocytes and microglia.[30,130] Using PMEA, a substrate for MRP4 and MRP5, our group has shown that these transporters are functional in a rat microglia cell line (MLS-9).[34]

Functional studies have shown that MRP6 exhibits a weak resistance to chemotherapeutic drugs such as etoposide, doxorubicin and daunorubicin. MRP6 can also transport GSH conjugates (i.e., LTC4, N-ethymaleimide S-glutathione) and cyclic pentapeptide BQ123. Transcripts of Mrp6 have been detected in primary cultures of bovine BMECs, in capillary enriched fraction of bovine brain homogenate and in human brain tissue.[140] Overexpression of MRP6 transcripts was also detected in human BMECs isolated from epileptic patients.[141] Mrp6 expression at the gene and protein level has been detected in glial cells and neurons, but not in pericytes.[23] MRP6 is also known to be expressed in liver and kidney. Loss of MRP6 function causes an autosomal recessive multi-organ disorder known as pseudoxanthoma elasticum, resulting in calcification of elastic fibres.[142]

A few in vitro studies have suggested that MRP7 is a lipophilic anion transporter that confers resistance to several natural product anticancer drugs (i.e., docetaxel, vincristine). MRP7 can also transport physiological substrates such as 17β-glucuronosyl estradiol and LTC4 as shown in membrane vesicles expressing recombinant MRP7. High levels

of MRP7 transcripts have been detected in various tumor specimens including breast, ovary, prostate, lung, colon and pancreas suggesting a potential role of this transporter in the intrinsic sensitivity of tumors.[143] Transcripts of Mrp7 have been detected in brain tissue;[144] however, protein expression as well as the functional role of MRP7 in drug distribution in the brain is currently unknown.

MRP8 has been characterized as a lipophilic anion efflux pump in vitro that shows resistance to PMEA, fluorouracil, steroid sulphates and GSH conjugates such as LTC4. MRP8, similar to MRP4 and 5, confers resistance to several purine and pyrimidine nucleotide derivatives.[145] Gene expression of MRP8 has been detected in liver, placenta, breast, testis, breast cancer samples as well as in several tumor cell lines.[146] Transcripts and protein expression of MRP8 have been detected in human brain samples and gliomas. Using immunofluorescence microscopy, MRP8 protein expression was found to be colocalized with neurofilaments in white matter in human brain suggesting that MRP8 is an axonal protein.[147] The axonal localization of MRP8 together with its substrate affinity towards steroids indicates that this transporter may potentially participate in regulating neurotransmitter receptors by mediating the efflux of neurosteroids from neurons.

High levels of MRP9 transcripts have been detected in brain as well as testes, mammary epithelial and breast cancer cells.[148] However, additional localization and functional studies are required to clarify the role of this transporter in the brain.

Breast Cancer Resistance Protein (BCRP)

ABCG2 (BCRP) transporter was first identified in a P-gp and MRP1-negative breast cancer cell line that showed high resistance to mitoxantrone, an anthracycline anticancer drug.[149] Unlike P-gp and MRPs, ABCG2 is a half-transporter with six membrane spanning helices and one ATP-binding site.[149] BCRP is predicted to function as a homodimer; however, evidence suggests that BCRP may also function as a monomer. The substrate profile of ABCG2 overlaps with that of P-gp or MRP1 and includes many anticancer drugs (i.e., daunorubicin, doxorubicin, mitoxantrone, etoposide, topotecan, camptothecins, methotrexate), phototoxins (i.e., pheophorbide-A), antiretrovirals (i.e., zidovudine), fluorescent dyes (i.e., rhodamine 123) and many others.[150,151] ABCG2 is expressed in the blood-brain, blood-placenta and blood-testis barriers and is known to colocalize with P-gp at the luminal membrane of BMECs.[152] Several studies have reported the expression of BCRP in primary cultures of human BMECs, mouse brain capillaries and immortalized rat brain endothelial cell line.[153-155] ABCG2 expression has also been detected in pericytes and at the apical membrane of choroid plexus epithelial cells.[156] In brain parenchyma, ABCG2 expression has been detected in astrocytes and microglia. Our group has confirmed Abcg2 protein expression in primary cultures of rat astrocytes and in a microglia cell line, MLS-9.[154] Despite ABCG2 protein expression in multiple brain cellular compartments, functional activity of this transporter in these cell systems is not clear. A few studies have reported ABCG2 mediated transport in cultured human and rodent BMECs, whereas, others have suggested lack of ABCG2 mediated transport in these cells.[152,153] For example, accumulation of mitoxantrone, an established BCRP substrate, did not increase in the presence of ABCG2 inhibitors in primary cultures of human brain endothelial cells as well as in RBE4 cell line. In addition, a lack of Abcg2 mediated transport of mitoxantrone has also been shown in primary cultures of rat astrocytes and in a microglia cell line.[154] In contrast, functionally active ABCG2 has been reported in intact brain capillaries.[157] Several in vivo studies have also supported a functional role of Abcg2 at the BBB. In

Mdr1a knockout mice model, upregulated Abcg2 protein expression was observed in brain microvessels compared to wild type animals suggesting a compensatory role of ABCG2.[158] Recent studies also indicate that ABCG2, in conjunction with P-gp can limit the penetration of tyrosine kinase inhibitors into the brain.[159-161]

Several studies have reported the involvement of nuclear receptors (i.e., CAR, aryl hydrocarbon receptor, PPARγ, PPARα) and other signaling pathways in ABCG2 regulation.[162-164] Using isolated rat and mouse brain capillaries, Wang et al showed CAR mediated upregulation of Abcg2 along with P-gp and Mrp2.[102] A recent report by Hartz et al demonstrated that estrogen can be a modulator of BCRP and treatment with 17-β-estradiol resulted in a decreased Abcg2 functional expression in brain capillaries isolated from rat and mouse.[157] A complex inflammation-mediated regulation of Abcg2 and P-gp has also been reported in porcine BMECs suggesting that ABC transporters at the BBB are susceptible to modulation by pathological conditions.[165]

Solute Carrier (SLC) Transporters

The SLC transporter superfamily includes more than 360 membrane transporters divided into 48 families (SLC1-48) which mediate solute transport across cellular, mitochondrial and vesicular membranes by a variety of transport mechanisms.[166] Protein nomenclature of SLC transporters is based on function (e.g., Organic Cation Transporter family), while gene nomenclature is assigned using the root symbol SLC and a numeral designated to each family (e.g., SLC22) followed by a letter indicating the subfamily (e.g., A) and a number assigned to each isoform within family in the order of discovery.[166] The exception to this rule is the SLCO superfamily (formerly SLC21), which has new nomenclature introduced by Hagenbuch and Meier in 2004, with families numbered 1-6 and subfamilies designated a letter (A, B).[167] Several SLC families such as *SLC15*, *SLC22A*, *SLCO*, *SLC28* and *SLC29* are considered to play the primary role in xenobiotic uptake, and recently *SLC47* family, encoding the multidrug and toxin extrusion (MATE) transporters, was also identified to play a role in drug transport and drug-drug interactions (Table 2).[168]

Organic Anion Transport Systems

Uptake of organic anions is mediated by two families of transporters, the organic anion transporting polypeptides (OATPs, *SLCO* family)[169] and the organic anion transporters (OATs, *SLC22A* family).[170] In general, OATPs transport large amphipathic organic anions with molecular weights greater than 450 Da via sodium-independent transport mechanism.[167] In contrast, OATs transport smaller hydrophilic organic anions eliminated by renal excretion by sodium-dependent mechanism.[171]

Organic Anion Transporting Polypeptides (OATPs). Members of the SLCO superfamily (human: OATPs; rodent: Oatps) play an important physiological role in mediating the hepatobiliary transport and tissue distribution of steroid hormones and their conjugates, thyroid hormones, linear and cyclic peptides and bile salts.[169] Many OATP isoforms are ubiquitously expressed in multiple tissues including brain. Currently, 11 human, 11 rat and 8 mouse OATPs/Oatps have been identified, which are divided into six gene families (i.e., SLCO1-6).[167] Large interspecies variability in OATPs/Oatps makes the correlation between rodent and human data difficult because many rodent Oatps do

not have corresponding human orthologs. For example, in *SLCO1A* subfamily, four mouse and five rat Oatp isoforms (m/rOatp1a1, rOatp1a3, m/rOatp1a4, m/rOatp1a5, and m/rOatp1a6) have one corresponding human OATP1A2 transporter. While most OATP family substrates are amphipathic organic anions, some organic cations and neutral substrates have also been identified.[169]

The OATPs/Oatps are predicted to have 12 helical TMDs, intracellular carboxy- and amino-termini and a conserved OATP "superfamily signature" located between TMD 6 and extracellular loop 3.[169] Most OATPs/Oatps act as anion exchangers, coupling the uptake of organic anions with the efflux of a counterion such as bicarbonate (HCO_3^-), GSH or glutathione-S-conjugates.[172,173] The efflux of intracellular GSH was found to play an important role for Oatp1a1- and Oatp1a4-mediated uptake of organic anions in hepatocytes and choroid plexus epithelial cells, indicating an antiport transport mechanism.[174,175] While OATP-mediated transport is Na^+-independent, substrate/counterion gradient typically acts as a driving force (Fig. 3) and some OATPs/Oatps have been shown to mediate bidirectional transport depending on the direction of the local ion gradient.[172,176] A number of human/rodent OATPs/Oatps also demonstrate pH dependent transport with enhanced substrate uptake at lower extracellular pH.[173,177] In addition, OATP-mediated transport can be regulated by steroid hormones such as androgens (e.g., testosterone), which act as potent inhibitors, or gestagens (e.g., progesterone) that enhance OATP2B1-mediated transport.[178]

Most OATP family members have broad and overlapping spectrum of endogenous substrates (Table 2) including bile acids (e.g., taurocholate, cholate), steroid hormones and their conjugates (e.g., estrone-3-sulphate (E3S), dihydroepiandrosterone (DHEAS), estradiol-17β-glucuronide ($E_2$17βG), cortisol), thyroid hormones, eicosanoids (i.e., prostaglandins), peptides, bilirubin and glucuronide derivatives.[169,179] In addition, many human and rodent OATPs/Oatps transport a diverse spectrum of drugs (Table 2) including the antihistamine, fexofenadine,[180] HMG-CoA reductase inhibitors, i.e., statins (e.g., pravastatin, rosuvastatin),[181,182] endothelin receptor antagonist, BQ-123,[183] opioid receptor agonists, [D-Pen^{2,5}]-enkephalin (DPDPE) and deltrophin II,[184,185] thrombin inhibitor, CRC220, cancer chemotherapeutics (e.g., methotrexate, paclitaxel),[186] HIV protease inhibitors (e.g., saquinavir, lopinavir, darunavir),[187,188] antibiotics (e.g., rifampicin, rifampin)[189] and cardiovascular agents (e.g., digoxin, ouabain) (Table 2).[179]

The OATPs/Oatps are suggested to play an important role in the brain permeability of organic anions. Much of the current knowledge on the role of OATPs in the brain comes from studies in rodents. Gene expression of several rodent Oatps has been detected in the brain, including Oatp1a4, Oatp1a5, Oatp1c1, Oatp2a1, Oatp2b1, Oatp3a1, Oatp4a1 and Oatp5a1.[125,168,190] Furthermore, Oatps have been localized at the BBB (Fig. 1) and the BCSFB (Fig. 2). At the BBB, rat Oatp1a4 and Oatp1c1 are localized at the luminal and abluminal membranes of the brain capillary endothelial cells, Oatp2b1 is localized at the abluminal membrane, and mouse Oatp1a5 is found at the luminal membrane.[134,191] At the choroid plexus, rat Oatp1a1, Oatp1a5 and Oatp2b1 and mouse Oatp5a1 are localized at the apical brush border membrane in contact with CSF,[42,193] while Oatp1a4 and Oatp1c1 are expressed at the basolateral membrane.[168,194] Among these, Oatp1a5 isoform has the highest expression at the rodent choroid plexus, while localization of Oatp1a1 at this site has been controversial because Oatp1a1 antibody also binds Oatp1a5 protein due to the high sequence homology between these isoforms.[42] Protein expression of Oatp3a1 is also detected in various regions of rat brain; however, its localization at the BBB and BCSFB are still unclear.[195] Oatp1c1 expressed at the BBB and BCSFB is believed to play an important

Table 2. SLC transporters, localization in the brain and established substrates

Name	Gene	CNS Expression/Localization	Substrates
ORGANIC ANION TRANSPORT SYSTEMS			
Organic Anion Transporting Polypeptides (OATPs)			
OATP1A2	*SLCO1A2*	Brain microvessle endothelial cells (LM/ALM)	*Endobiotics*: estrone-3-sulfate (E3S), dehydroepiandrosterone sulphate (DHEAS), estradiol-17β- glucuronide ($E_2$17βG), prostaglandin E_2, cholate, glycocholate, taurocholate, tauroursodeoxycholate, chloambuciltaurocholate, thyroxine, triiodothyronine *Xenobiotics*: fexofenadine, ouabain, methotrexate, endothelin receptor antagonist (BQ123), opioid receptor agonists ([D-Pen^{2,5}]-enkephalin (DPDPE) and deltrophin II), statins (i.e., pravastatin, rosuvastatin, pitavastatin), HIV protease inhibitors (i.e., saquinavir, lopinavir, darunavir), rocuronium, levofloxacin, erythromycin, D-penicillamine, unprostone, microcystin-LR, bromosulfophthalein (BSP), N-methyl-quinidine, N-methyl-quinine
OATP1C1	*SLCO1C1*	Brain	*Endobiotics*: $E_2$17βG, E3S, thyroid hormones (e.g., T3, rT3 and T4) *Xenobiotics*: BSP
OATP2B1	*SLCO2B1*	Brain microvessel endothelial cells (LM)	*Endobiotics*: E3S, DHEAS *Xenobiotics*: fexofenadine, glibenclamide, atorvastatin, fluvastatin, pravastatin, benzyl-penicillin, bosentan, BSP
OATP3A1	*SLCO3A1*	Choroid plexus epithelial cells (BL/AP), neurons, glial cells	*Endobiotics*: E3S, thyroxine, prostaglandins E_1 and E_2 *Xenobiotics*: benzylpenicillin, endothelin receptor antagonist BQ-123, vasopressin
rOatp1a1	*Slco1a1*	Choroid plexus epithelial cells (AP)	*Endobiotics*: cholate, glycocholate, taurocholate, deoxycholate derivative (TUDCA), aldosterone, E3S, $E_2$17βG, DHEAS, thyroid hormones T3, rT3, and T4, cortisol *Xenobiotics*: rosuvastatin, fexofenadine, ouabain, α-lactam antibiotics, thrombin inhibitor (CRC220), DPDPE, deltrophin II, dexamethasone, enalapril, BQ123, gadoxetate, ouabain, temocaprilat, pravastatin, rocuronium, BSP, N-methyl-quinidine, N-methyl-quinine, ochratoxin A

continued on next page

Table 2. Continued

Name	Gene	CNS Expression/Localization	Substrates
rOatp1a4	*Slco1a4*	Brain microvessel endothelial cells (LM/ALM), choroid plexus epithelial cells (BL)	*Endobiotics*: bile acids (cholate, glycocholate, taurocholate), E3S, $E_2$17βG, DHEAS, thyroid hormones T3, T4, prostaglandin E2, folate *Xenobiotics*: methotrexate, digoxin, rosuvastatin, fexofenadine, ouabain, β-lactam antibiotics, DPDPE, zidovidine, ochratoxin A, N-methyl-quinidine, N-methyl-quinine
mOatp1a4	*Slco1a4*	Brain microvessel endothelial cells (LM/ALM)	*Endobiotics*: bile acids (cholate, glycocholate, taurocholate), E3S, $E_2$17βG, DHEAS, T3, T4, prostaglandins E_2, folate *Xenobiotics*: rosuvastatin, pravastatin, pitavastatin, digoxin, DPDPE, fexofenadine, BSP, ochratoxin A, N-methyl-quinidine, N-methyl-quinine
rOatp1a5	*Slco1a5*	Choroid plexus epithelial cells (AP)	*Endobiotics*: cholate, glycocholate, taurocholate, deoxycholate derivative (TCDCA, TUDCA), E3S, $E_2$17βG, DHEAS, thyroid hormones T3, T4 *Xenobiotics*: fexofenadine, pravastatin, rosuvastatin, ouabain, BQ123, DPDPE, leu-enkephalin, biotin, digoxin, ruconium, APD-ajmalinium
mOatp1a5	*Slco1a5*	Brain microvessel endothelial cells (LM), choroid plexus epithelial cells (AP)	*Endobiotics*: cholate, glycocholate, taurocholate, deoxycholate derivative (TCDCA, TUDCA), E3S, $E_2$17βG, DHEAS, thyroid hormones T3, T4 *Xenobiotics*: fexofenadine, pravastatin, rosuvastatin, ouabain, BQ123, DPDPE, leu-enkephalin, biotin, digoxin, ruconium, APD-ajmalinium
rOatp1c1	*Slco1c1*	Brain microvessel endothelial cells (LM/ALM), choroid plexus epithelial cells (BL)	*Endobiotics*: thyroid hormones rT3, T4, $E_2$17βG *Xenobiotics*: cerivastatin, troglitazone sulphate
rOatp2b1	*Slco2b1*	Brain microvessel endothelial cells (ALM), choroid plexus epithelial cells (AP)	*Endobiotics*: taurocholate, leukotriene C4 (LTC4), prostaglandins E_1, E_2 and D_2, thromboxane B2, E3S *Xenobiotics*: iloprost

continued on next page

Table 2. Continued

Name	Gene	CNS Expression/Localization	Substrates
Organic Anion Transporters (OATs)			
OAT1	*SLC22A6*	Choroid plexus epithelial cells (AP)	*Endobiotics*: cyclic AMP, cyclic GMP, α-ketoglutarate, prostaglandins E_2 and $F_2\alpha$, urate, indoxyl sulfate, *Xenobiotics*: acetylsalicylate, salicylate, stavudine, lamivudine, didanosine, zidovudine, zalcitabine acyclovir, adefovir, cidofovir, cephaloridine, cimetidine, edaravone sulfate, furosemide, ganciclovir, indomethacin, methotrexate, penicillin G, tetracycline, trifluridine; *Other xenobiotics*: p-aminohippurate (PAH), ochratoxin A
OAT2	*SLC22A7*	Unknown	Endobiotics: DHEAS, E3S, cyclic AMP, glutarate, α-ketoglutarate, prostaglandins E_2 and $F_2\alpha$ *Xenobiotics*: acyclovir, allopurinol, zidovudine, bumetanide, 5-fluo rouracil, methotrexate, paclitaxel, salicylate, tetracycline, valproic acid, L-ascorbic acid, allopurinol, salicylate, *p*-acetylsalicylate, ranitidine, ochratoxin A, PAH
OAT3	*SLC22A8*	Brain microvessel endothelial cells, choroid plexus	Endobiotics: E3S, cyclic AMP, cyclic GMP, DHEAS, prostaglandins E_2 and $F_{2\alpha}$, cortisol, L-carnitine, choline, taurocholate, glycocholate, glutarate, glutathione, urate, indoxyl sulfate *Xenobiotics*: pravastatin, rosuvastatin, tetracycline, valacyclovir, zidovudine, allopurinol, benzylpenicillin, cefazolin, cephaloridine, cimetidine, famotidine, edaravone sulfate, methotrexate, 6-mercaptopurine, 5-fluorouracil, PAH, ochratoxin A
OAT4	*SLC22A9*	Unknown	*Endobiotics*: glutarate, DHEAS, E3S *Xenobiotics:* tetracycline, zidovudine, indoxyl sulphate, PAH, ochratoxin A
m/rOat1	*Slc22a6*	Choroid plexus epithelial cells (AP), ependymal cells (BL)	*Endobiotics*: cyclic AMP, cyclic GMP, urate, indoxyl sulfate *Xenobiotics*: antibiotics, diuretics, nonsteroidal anti-inflammatory drugs (NSAIDs), uricosuric drugs, valproic acid, zidovudine, zalcitabine, PAH
m/rOat2	*Slc22a7*	Choroid plexus	*Endobiotics*: α-ketoglutarate, glutarate, prostaglandins E_2 and $F_{2\alpha}$, propionate *Xenobiotics*: indomethacin, ketoprofen, methotrexate, allopurinol, salicylate, acetylsalicylate, bumetanide, PAH, ochratoxin A

continued on next page

Table 2. Continued

Name	Gene	CNS Expression/Localization	Substrates
rOat3	*Slc22a8*	Brain microvessel endothelial cells (LM/ALM), choroid plexus	*Endobiotics*: E3S, cyclic AMP, cyclic GMP, urate, indoxyl sulfate *Xenobiotics*: antibiotics, diuretics, nonsteroidal anti-inflammatory drugs (NSAIDs), benzylpenicillin, indoxylsulfate, homovanillic acid, uricosuric drugs, PAH
mOat3	*Slc22a8*	Choroid plexus epithelial cells (AP), Brain microvessel endothelial cells (ALM)	*Endobiotics*: E3S, cyclic AMP, cyclic GMP, urate, indoxyl sulfate *Xenobiotics*: antibiotics, diuretics, nonsteroidal anti-inflammatory drugs (NSAIDs), uricosuric drugs, PAH
mUrat1	*Slc22a12*	Brain microvessel endothelial cells, choroid plexus	*Endobiotics*: urate
ORGANIC CATION TRANSPORTER SYSTEMS			
Organic Cation Transporters (OCTs)			
OCT1	*SLC22A1*	Brain microvessel endothelial cells (LM)	*Endobiotics*: choline, acetylcholine, dopamine, serotonin, spermine, spermidine, agmatine *Xenobiotics*: quinidine, quinine, acyclovir, ganciclovir, lamivudine, zalcitabine, cimetidine, metformin, diphenylhydramine, ranitidine, famotidine, atropine, desipramine, 1-Methyl-4-phenylpyridinium (MPP), tetraethylammonium (TEA), tetrapropylammonium, tetrabutylammonium, *N*-methylnicotinamide, N-methylquinine, tetrabutylammonium
OCT2	*SLC22A2*	Brain microvessel endothelial cells (LM), neurons	*Endobiotics*: choline, acetylcholine, dopamine, norepinephrine, epinephrine, serotonin, histamine, agmatine, prostaglandin E_2, prostaglandin F_2 *Xenobiotics*: quinine, famotidine, ranitidine, amantadine, metformin, lamivudine, zalcitabine, cimetidine, cisplatin, memantine, etilefrine, paraquat, MPP, TEA, *N*-methylnicotinamide
OCT3	*SLC22A3*	Neurons, astrocytes	*Endobiotics*: dopamine, norepinephrine, epinephrine, serotonin, histamine *Xenobiotics*: quinine, metformin, atropine, guanidine, etilefrine, MPP, TEA
r/mOct1	*Slc22a1*	Brain microvessel endothelial cells (LM)	*Xenobiotics*: MPP, TEA, picoplatin, metformin

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Table 2. Continued

Name	Gene	CNS Expression/Localization	Substrates
r/mOct2	*Slc22a2*	Brain microvessel endothelial cells (LM), choroid plexus epithelial cells (AP)	*Xenobiotics*: TEA, picoplatin
rOct 3	*Slc22a3*	Glial cells	*Xenobiotics*: TEA, guanidine
Organic Cation/Carnitine Transporters (OCTNs)			
OCTN1	*SLC22A4*	Unknown	*Endobiotics*: L-Carnitine *Xenobiotics*: quinidine, quinine, verapamil ergothioneine, pyrilamine, TEA
OCTN2	*SLC22A5*	Brain microvessel endothelial cells (ALM), neurons	*Endobiotics*: L-Carnitine *Xenobiotics*: quinidine, verapamil, cephaloridine, mildronate, pyrilamine, spironolactone, TEA, valproic acid
mOctn1	*Slc22a4*	Brain (widely distributed)	*Xenobiotics*: ergothioneine
rOctn2	*Slc22a5*	Brain microvessel endothelial cells (ALM), choroid plexus, astrocytes	*Endobiotics*: L-Carnitine *Xenobiotics*: valproic acid
mOctn2	*Slc22a5*	Choroid plexus, astrocytes	*Endobiotics*: L-Carnitine
r/mOctn3	*Slc22a21*	Choroid plexus	*Endobiotics*: L-Carnitine

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Table 2. Continued

Name	Gene	CNS Expression/Localization	Substrates
Multidrug and Toxin Extrusion Transporters (MATE)			
MATE1	*SLC47A1*	Unknown	*Endobiotics*: creatinine, thiamine, E3S *Xenobiotics*: procainamide, cimetidine, acyclovir, ganciclovir, tenofovir, cephalexin, cephradine, metformin, oxaliplatin, paraquat, topotecan, guanidine, MPP, TEA
mMate 1	Slc47a1	Brain	*Xenobiotics:* metformin, cisplatin
NUCLEOSIDE TRANSPORTERS			
Concentrative Nucleoside Transporters (CNTs)			
CNT1	*SLC28A1*	Unknown	*Endobiotics*: adenosine, thymidine, uridine *Xenobiotics*: cladribine, cytarabine, fialuridine, 5-fluorouridine, gemcitabine, stavudine, zalcitabine, zidovudine
CNT2	*SLC28A2*	Brain microvessel endothelial cells, choroid plexus epithelium, astrocytes	*Endobiotics*: adenosine, cytidine, guanosine, inosine, uridine *Xenobiotics*: cladribine, clofarabine, didanosine, fialuridine, 5-fluorouridine, formycin B, ribavirin, tiazofurin
CNT3	*SLC28A3*	Choroid plexus (BL), microglia	*Endobiotics*: adenosine, cytidine, guanosine, inosine, thymidine, uridine *Xenobiotics:* benzamide riboside, cladribine, clofarabine, cytarabine, didanosine, fludarabine, 5-fluorouridine, gemcitabine, 6-mercaptopurine, ribavirin, 6-thioguanine, tiazofurin, zalcitabine, zebularine, zidovudine

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Table 2. Continued

Name	Gene	CNS Expression/Localization	Substrates
Equilibrative Nucleoside Transporters (ENTs)			
ENT1	*SLC29A1*	Brain microvessel endothelial cells, choroid plexus epithelium, astrocytes, neurons	*Endobiotics*: adenosine, guanosine, cytidine, thymidine, uridine *Xenobiotics*: cladribine, clofarabine, fialuridine, gemcitabine, ribavirin, tiazofurin
ENT2	*SLC29A2*	Brain microvessel endothelial cells (LM), choroid plexus epithelium (BL), astrocytes, neurons	*Endobiotics*: adenosine, guanosine, inosine, cytidine, thymidine, uridine *Xenobiotics*: adenine, clofarabine, fialuridine, gemcitabine, guanine, hypoxanthine, tiazofurin,
ENT3	*SLC29A3*	Unknown	*Endobiotics*: adenosine, guanosine, inosine, thymidine, uridine *Xenobiotics*: adenine, cladribine, fludarabine, zebularine, zidovudine
Peptide Transporters			
PEPT1	*SLC15A1*	Unknown	*Endobiotics*: dipeptides, tripeptides, 5-aminolevulinic acid, carnosine *Xenobiotics*: glycylsarcosine, bestatin, β-lactam antibiotics (cefadroxil, ceftibuten, cephradine, cefixime, cephalexin)
PEPT2/ Pept2	*SLC15A2*	Choroid plexus epithelial cells (AP), astrocytes, neurons	*Endobiotics*: dipeptides, tripeptides, 5-aminolevulinic acid, carnosine *Xenobiotics:* glycylsarcosine, bestatin, β-lactam antibiotics (cefadroxil), *l*-kyotorphin

LM = luminal; ALM = abluminal; AP = apical; BL = basolateral.
Adapted from References: Klaassen and Aleksunes, 2010; Konig, 2011; Hagenbuch and Meier, 2003,[192] 2004; Ose et al, 2010; Kusuhara and Sugiyama, 2004, 2005; Koepsell et al, 2007; VanWert, et al, 2010;

physiological role in brain uptake of thyroxine.[196] In humans, gene expression of several OATPs has been detected in the brain, including OATP1A2, OATP1C1 and OATP3A1 which are highly expressed as well as OATP1B1, OATP1B3, OATP2A1, OATP4A1, OATP4C1 and OATP6A1 which are detected at much lower levels.[168] OATP1A2 is ubiquitously expressed in the brain, with high expression in the frontal cortex. This transporter is localized at the luminal and abluminal membranes of the brain capillary endothelial cells, while its expression at the BCSFB has not been reported to date.[42,168] OATP1C1 also demonstrates high protein expression in the CNS and at the BBB; however, its exact localization has not been identified.[197] To date, OATP3A1 is the only isoform detected at the human choroid plexus. Interestingly, two splice variants of OATP3A1 were found to have different localization at the BCSFB (Fig. 2) and in brain parenchyma (Fig. 1).[195] Both of these variants exhibit similar transport activities for prostaglandins E_1 and E_2, thyroid hormone T4, and oligopeptides BQ-123 and vasopressin. Rat Oatp3a1, which is highly homologous to the human v1 variant, demonstrated similar brain localization to both human OATP3A1 variants.[195] Interestingly, expression of OATP1A2 and OATP2B1 is also detected at the blood-tumor barriers of gliomas, suggesting that these OATPs may play a role in tumor distribution of anticancer agents and other drugs and could also control tumor uptake of steroid hormones and their derivatives.[198]

Functional studies in rodents demonstrate that Oatps play an important role in CNS uptake and clearance of organic anions and drugs. Oatp1a4 was found to mediate brain uptake of opioid receptor agonist, DPDPE, across the BBB.[199] This process was inhibited by other Oatp1a4 substrates such as digoxin and $E_2$17βG. Furthermore, brain clearance of several Oatp substrates (i.e., 1-naphtyl 17β-glucuronide, taurocholate, E3S, $E_2$17βG, DHEAS) as well as several drugs (i.e., pitavastatin, rosuvastatin, pravastatin, cyclic peptide RC-160) was demonstrated to involve OATP-mediated transport.[42,181] The clearance of these substrates from the CNS after microinjection was saturable and susceptible to inhibition by probenecid, an OATP/OAT family inhibitor. Moreover, the elimination of $E_2$17βG from the brain was 40% inhibited by digoxin, a high affinity substrate of Oatp1a4, almost completely inhibited by probenecid and taurocholate, while *p*-aminohippurate (PAH), a substrate and inhibitor of OAT family, had a minimal but significant effect.[200] Collectively, these interactions suggest that $E_2$17βG clearance from the brain is primarily mediated by Oatp1a4 and to a lesser degree Oat family transporter (presumably Oat3), and another taurocholate-sensitive transporter also makes a relatively similar contribution.[200,201] Furthermore, since Oatp1a4 and Oatp1a5 have opposite membrane polarization in the choroid plexus epithelial cells and many common substrates (e.g., $E_2$17βG, taurocholate, E3S, DHEAS, digoxin, fexofenadine, ouabain), these isoforms have been suggested to participate in vectorial transport of substrates across the BCSFB resulting in the overall uptake or clearance of these organic anions and drugs from the CNS.[42,201]

The proposed role of OATPs in human brain is mainly based on the data obtained in animal studies. OATP1A2 has the highest homology with rat Oatp1a4 and Oatp1a5, and also demonstrates overlapping substrate specificity and localization with these isoforms. Hence, it has been suggested that human OATP1A2 may also play an important role in the clearance of drugs from the CNS. OATP1A2 transports a diverse spectrum of drug substrates including fexofenadine, ouabain, methotrexate, DPDPE, deltorphin II, GD-B20790 chlorambucil-taurocholate, BQ123, rocuronium, levofloxacin, statins (i.e., pravastatin, rosuvastatin, pitavastatin), HIV-protease inhibitors (i.e., saquinavir, lopinavir, darunavir), erythromycin, D-penicillamine, BSP and unprostone.[169,185,202] The polarized

expression of multiple Oatps/OATPs at the BBB and BCSFB contributes to the net directional movement of multiple endo- and xenobiotics in the CNS. These along with the other ABC and SLC transporter family members contribute to the regulation of CNS drug distribution in humans.

Organic Anion Transporters (OATs). The OATs, which belong to the *SLC22A* gene family, are highly expressed in epithelial cells of the renal tubules, where they mediate active excretion of organic anions into the urine. The first member of the OAT family was identified in 1997 as the PAH transporter in rat, which was later renamed as the OAT1.[203] Members of the Oat/OAT family include Oat/OAT1-4, URAT-1 and more recently identified members such as Oat5-10.[204] Most of the members of this family are located in the proximal tubular epithelial cells of the kidney or in the liver.

The structural features of OATs are similar to the OCTs and OCTNs, which also belong to the major facilitator superfamily (MFS), i.e., the *SLC22A* gene family. OATs have 12 membrane spanning domains with intracellular carboxy- and amino- terminus and have been reviewed in detail by Klaassen and Aleksunes.[168]

Cellular influx of organic anions by OATs is described as Na^+-dependent organic anion exchange. This process is tertiary active since it is energized by the dicarboxylate gradient generated by Na^+/dicarboxylate cotransport system, which is dependent on the Na^+ gradient produced by the primary active Na^+/K^+ ATPase pump (Fig. 3).[204] OATs transport a wide range of endogeneous substrates and xenobiotics including neurotransmitter metabolites (e.g., homovanilic acid), antimetabollites (e.g., methotrexate), xanthine-related compounds, HMG-CoA reductase inhibitors (e.g., pravastatin), NSAIDS, toxins (e.g., ochratoxin), steroid hormones and their metabolites (e.g., estrone-3-sulphate).[171] Some members of the family such as Oat1/OAT1, Oat2, Oat3/OAT3 are expressed in the brain, at the choroid plexus and/or the endothelial cells of the BBB.[125,168,205,206] They play an important role in the permeability of organic anions from the brain or CSF into the blood.

Rodent Oat1 is expressed predominantly in the kidney with lower expression in the brain, where it is localized at the basolateral membrane of the choroid plexus ependymal cells.[207] Human OAT1 is expressed at the apical membrane of the choroid plexus epithelial cells and demonstrates tertiary active transport of organic anion xenobiotics, facilitating their removal from the CSF into the blood.[204] Furthermore, OAT1-mediated uptake of PAH is stimulated by an outwardly-directed dicarboxylate gradient, demonstrating an anion exchange transport mechanism. Rodent Oat2 is detected in epithelial cells of the choroid plexus and this transporter was found to function without a direct sodium or dicarboxylate gradient in in vitro studies.[208] In rodents, Oat3 is also localized at the apical membrane of the choroid plexus and the luminal and abluminal membranes of rat brain endothelial cells.[206] This isoform mediates the extrusion of organic anions from the CSF and brain compartment into blood. Oat3 is mainly involved in the transport of thiopurines such as 6-mercaptopurine and 6-thioguanine used in the treatment of acute lymphoblastic leukemia.[168] Interestingly, Oat3-mediated clearance of thiopurines is thought to be responsible for the relapse of leukemia. The active metabolite of Oseltamivir (i.e., Ro 64-0802) is cleared from the CNS by Oat3. Thus, modulation of Oat3 activity may be implicated in the CNS toxicity of Oseltamivir. Apical localization of the Oats in the choroid plexus and their basolateral localization at the BBB suggest that OATs/Oats may play an important role in the extraction of anionic chemicals from the brain.[42,201]

OATs may also be regulated in neurological disorders associated with neuroinflammation. Pro-inflammatory cytokine-mediated release of matrix metalloproteins from the choroid plexus was found to reduce OAT functional activity.[209] Inflammation near the ventricles in the brain has been reported to reduce the expression of Oat1 and Oat3 at the apical membrane of the choroid plexus epithelial cells and impair the clearance of anionic drugs from the brain.[42]

Organic Cation Transport Systems

Membrane transport of organic cations can be mediated by the *SLC22A* family including organic cation transporters (OCTs), organic cation/carnitine transporters (OCTNs) and the multidrug and toxin extrusion (MATE) transporters encoded by the *SLC47* family. OCTs mediate sodium-independent facilitated diffusion of organic cations and are capable of bidirectional transport driven by the electrochemical gradient of the transported organic cation. OCTNs demonstrate a variety of transport mechanisms, including electroneutral H^+/organic cation exchange, Na^+-independent translocation or Na^+-dependent transport, while MATE transporters mediate active efflux of organic cations, rather than uptake or bidirectional transport, and their mode of transport is electroneutral H^+/organic cation exchange. While these transport families have overlapping substrate specificities for organic cations, OCTNs also serve as carnitine transport systems.

Organic Cation Transporters (OCTs). Members of the OCT/Oct family include human OCT1 (*SLC22A1*), OCT2 (*SLC22A2*) and OCT3 (*SLC22A3*) and their rodent orthologs Oct1, Oct2 and Oct3. While the OCT1 and OCT2 have highest expression in the liver and kidney, respectively, they are also expressed in the brain. OCT3 (the extraneuronal monoamine transporter) has a wider tissue distribution and is expressed in glial cells, heart, skeletal muscles, blood vessels and liver.[210]

Both OCTs and OCTNs share similar structure characteristics of the MFS, with 12 α-helical transmembrane domains, an intracellular amino- and carboxy- terminus, as described previously.[210]

Members of the OCT/Oct family share similar transport mechanism, facilitating diffusion of small organic cations down their electrochemical gradient by Na^+-independent and electrogenic mechanisms. Electrogenecity of OCT-mediated transport of organic cations was demonstrated for rat rOct1, rOct2 and rOct3 as well as human transporters hOCT1 and hOCT2. While most OCT substrates are weak organic bases with net positive charge at physiological pH, some substrates are not charged. Most OCTs transport a broad spectrum of endogenous substrates such as tetraethyl ammonium (TEA), 1-methyl-4-phenylpyridinium (MPP), choline, acetylcholine, prostaglandins and monoamine neurotransmitters (i.e., dopamine, epinephrine, norepinephrine, histamine), as well as a large spectrum of xenobiotics including antidiabetic drugs (metformin), antiviral agents (e.g., acyclovir, lamivudine, zalcitabine), acid-lowering drugs (e.g., famotidine, ranitidine), antineoplastic agents (e.g., cisplatin), quinidine and quinine.[168,210] The OCT1 and OCT2 have overlapping substrate specificities. They mediate the transport of a large number of chemically unrelated small organic cations like MPP, TEA, quinine and metformin. The OCT2 also transports choline, several neurotransmitters (i.e., dopamine, norepinephrine, epinephrine), glutamate receptor antagonists (i.e., amantidine and memantine) and histamine receptor antagonists

such as cimetidine and ranitidine. The extraneuronal monoamine transporter, OCT3, has overlapping spectrum of endogenous substrates with OCT2 including many neurotransmitters and also transports several pharmacological agents (i.e., quinine, metformin, atropine, guanidine, etilefrine).[210]

OCT3 is the most predominantly expressed OCT in the brain (neurons and glial cells). It is highly expressed in hippocampus, cerebellum, and cerebral cortex where it participates in serotonin uptake.[211] Treatment of mice with methamphetamine causes increased locomotor activity. Reduced protein expression of Oct3 in mice was associated with substantially enhanced locomotor activity induced by methamphetamine. This suggests that OCT3/Oct3 plays an important role in the clearance of monoaminergic neurotransmitters in the brain.[212] Furthermore, two *SLC22A3* (OCT3) polymorphisms were identified in subjects with methamphetamine dependence, which were associated with the development of substance abuse in these subjects, indicating that human OCT3 plays a role in the transport of neurotransmitters in the brain.[213] OCT1/Oct1 and OCT2/Oct2 are expressed at the luminal side of the brain microvessel endothelial cells isolated from brain cortex of humans, rats and mice and human OCT2 has also been localized in neurons.[201,210] Luminal localization of OCTs at the BBB suggests that these isoforms mediate the uptake of substrates across the BBB into the brain. In rodents, Oct2 is also expressed apically in the choroid plexus epithelial cells.[206] However, the role of OCT transporters in neurons and glial cells is yet to be elucidated at the functional level.

Organic Cation/Carnitine Transporters (OCTNs). Members of the OCTN/Octn family, including human OCTN1 (SLC22A4), OCTN2 (SLC22A5), and OCTN3 (SLC22A21) and rodent Octn1, Octn2, and Octn3, mediate facilitated diffusion of organic cations, but are also capable of transporting carnitine.[210] OCTN1/Octn1 can act as organic cation uniporter or H^+/organic cation antiporter capable of bidirectional transport depending on the direction of the local ionic gradients. OCTN2/Octn2 can serve as sodium-independent organic cation uniporter or sodium-dependent organic cation transporter.[214] Mouse Octn1 and Octn2 mediate sodium-dependent carnitine transport, while Octn3 transports carnitine in a sodium-independent manner. These rodent isoforms appear to be more specific for carnitine transport compared to human orthologs, which also transport a number of organic cations.[215]

The OCTNs are predicted to have structural characteristics of the MFS, as described for OATs and OCTs.[168,210] Interestingly, OCTNs can function by a variety of transport mechanisms. OCTN1 transport mechanism is dependent on the electroneutral H^+/organic cation exchange. OCTN2 can function as Na^+-dependent transporter, using the Na^+/organic cation gradient as the driving force, or Na^+-independent carrier, as observed in the translocation of cationic substrates, TEA, choline and verapamil. OCTN3 mediates Na^+-independent transport of carnitine.[210]

OCTN1 transports a diverse spectrum of substrates including L-carnitine, zwitterions, TEA, quinidine, pyrilamine, verapamil, stachydrine and antioxidant ergothionine and is implicated in their renal clearance and intestinal absorption in humans. Octn1 protein expression is also detected in different regions of mouse brain.[168] Similarly, OCTN2 is a high affinity sodium-dependent transporter for L-carnitine but also transports many zwitterions and cations in a sodium-independent manner.[210] Other substrates of OCTN2 include acetyl L-carnitine, β-lactam antibiotic, cephaloridine, valproic acid, spironolactone, and cationic substrates, TEA, choline, verapamil and pyrilamine.[168,210]

OCTN2 has been shown to localize at the abluminal membrane of the brain capillary endothelial cells where it may facilitate the transport of organic cations/carnitine across the BBB.[216] The expression of rodent Octn2 has also been demonstrated in glial cells such as astrocytes as well as in different regions of mouse and rat brain, such as choroid plexus, cortex, cerebellum, spinal cord, hippocampus, and hypothalamus [217] OCTN3 functions primarily as a sodium-independent carnitine transporter[215] and is predominantly expressed in testes, but mouse Octn3 expression has also been detected in choroid plexus, brain cortex, cerebellum, olfactory bulb and nerve, hippocampus, and hypothalamus.[168]

Multidrug and Toxin Extrusion (MATE) Transporters. Although MATE transporter family (SLC47) belongs to the solute carrier superfamily, these carriers act as active efflux transporters for organic cations, rather than influx or bidirectional transporters.[168] Human MATE transporters include rodent and human MATE1 (*SLC47A1*), human MATE2-K (*SLC47A2*), and rodent Mate 2 which are not orthologous to the human isoform.[210,218,219] In the kidney, MATE transporters mediate electroneutral H^+/organic cation exchange, using the inwardly directed proton gradient as a driving force for the efflux of organic cations, including typical OCT substrates (e.g., TEA, MPP, N1-methylnicotinamide) and a number of cationic drugs (e.g., cimetidine, metformin, procainamide, quinidine, cephalexin, verapamil).[210] The expression of rodent Mate1 has been reported in mouse brain; however, its localization in specific cell types is still unclear. The expression of other MATE/Mate transporters in various brain compartments has not been reported to date.[210]

Nucleoside Transport Systems

Nucleoside transporters are highly preserved between species due to their important role in tissue distribution of nucleosides (i.e., cytidine, uridine, adenosine, guanosine, thymidine, and inosine) used as precursors for the synthesis of RNA/DNA nucleotides necessary for cell growth and body homeostasis. Some of these nucleosides also act as neurotransmitters, explaining why nucleoside transporters are widely expressed in various brain compartments, in particular, the BBB and the BCSFB.[220,221] Since many antiviral drugs and cancer chemotherapeutics are nucleoside analogs, ENTs and CNTs play an important role in drug pharmacokinetics, tissue distribution and CNS toxicity. The major difference between the CNTs and the ENTs is their mode of transport. While CNTs mediate an active (concentrative) transport of nucleosides and other substrates against their concentration gradient, the ENTs mediate facilitated diffusion (equilibrative) transport of nucleosides down their concentration gradient.[222]

Concentrative Nucleoside Transporters (CNTs). The CNTs, encoded by the *SLC28A* gene family, are high-affinity transport systems for nucleosides, with pyrimidine nucleosides preferentially transported by hCNT1, purine nucleosides by hCNT2, and both purine and pyrimidine nucleosides by hCNT3. These transporters, found primarily in specialized epithelial tissues, constitute secondary active nucleoside uptake systems capable of accumulating their substrates in specific cellular compartments. In addition to the diverse spectrum of endogenous substrates, CNTs also play a role in membrane transport of several drugs such as nucleoside reverse transcriptase inhibitors (e.g., antivirals).[222]

The predicted structure of CNT1 and CNT2 contains 13 transmembrane helices with intracellular amino terminus and extracellular carboxyl terminus; their exact crystal structure has not been determined.[223,224] CNTs utilize Na^+ gradient as a driving force for the influx of nucleosides and nucleoside analog compounds against their concentration gradient.

Pyrimidine and purine nucleosides have important functions in the brain, and since they are hydrophilic molecules, they need to be transported across cell membranes by specialized nucleoside transporters. Furthermore, the transport of nucleoside analog drugs may have important pharmacological implications. The expression of CNTs in brain compartments appears to be lower compared to ENTs. In rodents, gene expression of Cnt1 and Cnt2 has been detected at low levels in the brain,[225] while at the choroid plexus, high levels are reported.[125,220] However, CNT3 is the only concentrative nucleoside transporter detected at the human choroid plexus. Functional studies in freshly isolated choroid plexus slices demonstrated sodium-dependent inosine transport which was likely mediated by hCNT3. When expressed in *Xenopus laevis* oocytes, hCNT3 could mediate transport of several nucleoside derivatives, such as the antiviral agents: zidovudine, 2′3′-dideoxycytidine (zalcitabine) and 2′3′-dideoxyinosine (didanosine); however, its affinity for these derivatives was lower compared to the transport of endogenous substrates. In glial cells, our group has also demonstrated the activity of a sodium dependent thymidine transporter.[66]

Equilibrative Nucleoside Transporters (ENTs). The ENTs belong to the *SLC29A* family and represent a bidirectional facilitated transport system for nucleosides/nucleoside analogs, mediating membrane translocation of nucleoside substrates down their concentration gradient. The direction of transport depends on the local substrate gradient. ENTs are also expressed within the mitochondrial membrane where they facilitate mitochondrial uptake of nucleoside precursors, and may also be implicated in the drug-induced mitochondrial toxicity. Although ENTs and CNTs have overlapping substrate-specificities, the affinity of common substrates for the ENTs is generally much lower compared to their affinity for the CNTs.[221]

A topology of hENT1 has been confirmed experimentally, with eleven membrane-spanning helical domains, intracellular amino terminus and extracellular carboxy terminus. Similar topology is predicted for other human and rodent ENTs/Ents. While hENT1 and hENT2 have similar broad specificities for purine and pyrimidine nucleosides, hENT2 also mediates transport of nucleobases.[226] hENT3 has a broad specificity for nucleosides and nucleobases and is expressed not only at the cell surface but also in intracellular organelles such as lysosomes.[227] In contrast, hENT4 is selective for adenosine, but can also transport a diverse spectrum of organic cations. In regards, to transport characteristics, hENT3 and hENT4 are pH sensitive, with optimal activity demonstrated under acidic conditions.

Human choroid plexus expresses three equilibrative nucleoside transporter family members, hENTs 1-3; hENT2 is expressed at higher levels and involved in the transport of inosine, along with hCNT3.[220,228] In addition to many physiological functions in the brain, such as nucleoside and nucleobase uptake and neurotransmission, ENTs also play a role in brain permeability of drug that are synthetic nucleosides such as agents used for the treatment of cancers and viral diseases. Human ENT2, expressed in *Xenopus* oocytes, mediates transport of zidovudine, zalcitabine, and didanosine.[229] Basolateral to apical permeability of zidovudine across the choroid plexus epithelium is significantly higher compared to the permeability of sucrose, a paracellular flux marker, demonstrating

a carrier-mediated process.[230] Didanosine transport across the BBB in guinea pigs was found to involve nucleoside transporters, while OCT-like transport system was implicated in didanosine transport from blood into the CSF across choroid plexus epithelium.[231] Human ENT3 is also capable of transporting zidovudine, zalcitabine, and didanosine.[232]

Peptide Transporters

Peptide transporters belong to the proton-coupled oligopeptide transporter (POT) family (*SLC15A*), which consists of four members (i.e., PEPT1, PEPT2, PHT1, PHT2). While PHT1 and PHT2 are primarily involved in the transport of histidine, PEPT1 and PEPT2 mediate the transport of di- and tri-peptides and can also transport many drugs (e.g., β-lactam antibiotics). PEPT1 demonstrates high capacity and low affinity for di- and tri-peptides, whereas PEPT2 has low capacity and high affinity for the peptides.[233]

The mammalian peptide transporters PEPT1 and PEPT2 have twelve transmembrane α-helical domains, intracellular carboxyl and amino termini and a large extracellular loop between TMDs 9 and 10. In addition, transmembrane domains 1-4 and 7-9 were found to contribute to affinity and substrate binding characteristics.[234] These transporters use an inwardly directed proton gradient as a driving force for the translocation of their substrates, such as peptides, across the membranes against their concentration gradient. Peptide transport by PEPT1 and -2 is coupled with the inward translocation of protons leading to electrogenic transport. Typical substrates for peptide transporters are di- or tri-peptides, with free amino and carboxy termini, another free amino group in the α or β position and many also contain hydrophobic side chains and stereoselectivity for L-amino acid peptides.[235]

Peptide transporters have a very broad and overlapping substrate spectrum including many di-peptides, tri-peptides and peptidomimetic compounds (e.g., drugs), but not amino acids or tetrapeptides.[233] PEPT1 and PEPT2 have different affinities for their common substrates, with PEPT1 described as low affinity/high capacity systems and PEPT2 as high affinity/low capacity transporter. Glycylsarcosine (GlySar) is a prototypical substrate for Pept/PEPT1 and -2 transporters.[236] A number of pharmacological agents are substrates of Pept/PEPTs, including β-lactam antibiotics of cephalosporin and penicillin classes (e.g., cefadroxil, cefixime, ceftibuten), angiotensin converting enzyme inhibitors, renin inhibitors, dopamine receptor antagonists, the photosensitizing agent 5-aminolevulinic acid, antitumour agents, amino-acid ester prodrugs and bestatin, used as ancillary anticancer treatment.[233]

Tissue localization of PEPT1 and PEPT2 differs significantly. While PEPT1 is primarily localized in the small intestine, functioning as a nutrient transporter, PEPT2 demonstrates highest expression in the kidney, where it mediates re-absorption of peptides and other substrates, but is also expressed in other tissues including the brain. In rat, Pept2 mRNA is detected in different cell types, including astrocytes, subependymal cells, ependymal cells and the epithelial cells of choroid plexus.[237,238] At the BCSFB, Pept2 is localized at the brush-border membrane of the choroid plexus epithelial cells, suggesting it may be involved in the clearance of peptide fragments and neuropeptides from the CSF.[238] Smith et al demonstrated using Pept2-knockout mouse model that Pept2 plays a critical role in the brain distribution and clearance of peptides such as GlySar, peptidomimetics (i.e., 5-aminolevulinic acid), neuropeptide carnosine and peptide-like drugs (i.e., cefadroxil) and is the main transport system responsible for their uptake by the choroid plexus epithelial cells as well as astrocytes.[239,240]

REGULATION OF MEMBRANE DRUG TRANSPORTERS BY NEUROLOGICAL DISORDERS

HIV-1 Associated Encephalopathy

Human immunodeficiency virus 1 (HIV-1), an enveloped retrovirus, can significantly compromise the immune system by infecting immune cells and ultimately results in acquired immunodeficiency syndrome (AIDS) in humans.[241-243] HIV-1 can penetrate the CNS early in the course of the infection and infect glial cells, primarily microglia and to a lesser extent astrocytes.[19,244] HIV-1 infection may result in a neuropathological condition known as HIV-encephalitis (HIVE). HIVE is characterized by elevated monocyte/macrophage infiltration into the brain, myelin pallor, multinucleated giant cells, activated microglia, reactive astrocytosis (proliferation and activation of astrocytes) and presence of microglial nodules.[245] The chronic neuroinflammation associated with HIV-1 infection often leads to the development of neurocognitive disorders and ultimately, can result in memory loss, apathy and a poor quality of life in infected individuals. Despite the availability of highly active antiretroviral therapy, neurological disorders are highly prevalent and on the rise as patients live longer with HIV-1.[246] Poor penetration of antiretroviral drugs across the BBB and subsequently into parenchymal cells remains a major obstacle to the suppression of HIV-1 infection in the brain.[35] The inadequate permeability of different antiretrovirals, in particular HIV-1 protease inhibitors and nucleoside-reverse transcriptase inhibitors, into different brain cellular compartments is primarily attributed to the functional expression of ABC transporters.

Evidence in the literature suggests that functional expression of ABC transporters in the brain is altered during HIV-1 infection. Increased P-gp immunoreactivity in glial cells has been reported in brain autopsy tissues from patients with HIVE.[247] Furthermore, shed viral proteins (i.e., gp120, Tat) and secreted pro-inflammatory cytokines during HIV-1 infection are also known to alter expression of ABC transporters. Hayashi et al have shown HIV-1 viral protein Tat-induced upregulation of P-gp expression in both murine BMECs and astrocytes.[248] Zhong et al have further reported the involvement of intact lipid rafts and Rho signaling pathways in Tat-mediated P-gp upregulation.[99] Our group has extensively investigated P-gp regulation in an inflammatory model of primary cultures of rat astrocytes triggered by HIV-1 gp120. Our work in rodent astrocytes has demonstrated that HIV-1 gp120 can mediate secretion of pro-inflammatory cytokines (IL-6, IL1-β and TNF-α) by interacting with chemokine receptors and significantly downregulate the functional expression of P-gp. We have further delineated the effect of different cytokines on P-gp expression and demonstrated that IL-6 can profoundly decrease P-gp expression in primary cultures of rat astrocytes, whereas, TNF-α or IL-1β exposure results in an enhancement in P-gp expression.[96] Recently, an IL-6 mediated decrease and TNF-α mediated increase in P-gp expression has been reported in cultured human BMECs, further supporting that P-gp expression can be altered by cytokines.[249] We have recently confirmed similar findings in primary cultures of human fetal astrocytes and demonstrated that HIV-1 has a down-regulatory effect on P-gp functional expression suggesting altered antiretroviral drug accumulation within brain parenchyma during HIV-1 infection.[250]

Altered MRP1 expression has also been reported in the context of HIV-1 infection. Hayashi et al demonstrated Tat induced upregulation of Mrp1 at the mRNA and protein level in cultured astrocytes with an involvement of the MAPK pathway.[117] In primary

cultures of rat astrocytes, we have also observed an increase in Mrp1 functional expression in response to HIV-1 gp120 treatment which correlated with an enhanced efflux of GSH and GSSG, suggesting a potential role of MRP1 in regulating oxidative stress in glial cells.[137] We have further shown that NF-κB and JNK pathways are involved in the gp120-mediated upregulation of Mrp1 in these cells. Our data suggest that JNKs are involved in the direct regulation of Mrp1, whereas, NF-κB mediates Mrp1 regulation via the release of TNF-α, illustrating the complexity of signaling pathways interactions involved in transporter regulation.[118]

Antiretroviral therapy itself can also modulate the expression of ABC transporters.[251-253] Nuclear receptors (i.e., PXR, CAR) have been implicated in the xenobiotic-mediated regulation of these transporters. Using a human endothelial cell line system (hCMEC/D3), our group has demonstrated that in the presence of atazanavir or ritonavir, two HIV-protease inhibitors, a PXR dependent induction in P-gp functional expression occurs.[253] Using primary cultures of human BMECs, Bousquet et al showed atazanavir induced increase in P-gp functional expression in vitro.[254] In addition, data from a recent study have suggested that other classes of antiretroviral drugs such as chemokine receptor antagonists (maraviroc, vicriviroc) and HIV-1 integrase inhibitor (elvitegravir) may have the potential to modulate P-gp and/or BCRP expression.[255] Taken together, these observations provide evidence that ABC transporters are highly regulated during HIV-associated neuroinflammation which in turn may result in altered pharmacotherapy of brain HIV-infection. Although a few SLC transporters (i.e., nucleoside transporter, hCNT1) have been implicated in the uptake of antiretrovirals into the brain, the regulation of these transporters in the context of brain HIV-1 infection is not yet characterized.

Alzheimer's Disease

Alzheimer's disease (AD) is the most prevalent neurodegenerative disorder worldwide. It is characterized by the decline of cognitive performance including memory and mental processing in affected individuals. Pathological features of AD include neurofibrillary tangles and formation of amyloid plaques primarily characterized by the expression of amyloid-β (Aβ) peptides (40 or 42 amino acids in length) that are known to play a central role in the pathogenesis of AD.[256] Impaired clearance of Aβ across the BBB has been implicated in the progression of AD.[257] The main receptor for Aβ transport from brain to blood is low-density lipoprotein receptor related protein-1 (LRP1), whereas, blood to brain transport of Aβ is primarily mediated by receptor for advanced glycation end products (RAGE). Evidence also suggests a potential role of drug efflux transporter, P-gp, in Aβ transport from brain to blood. Using P-gp enriched plasma membrane vesicles, Lam and colleagues have shown ATP-dependent transport of Aβ.[258] P-gp mediated active transport of Aβ has been further demonstrated in overexpressing P-gp cell line.[259] Fluorescent labelled accumulation of Aβ in the lumen of isolated mouse brain capillaries has been shown to be reduced by P-gp specific inhibitors.[260] In addition, increased accumulation of Aβ (1-40) was observed in a human brain microvessel endothelial cell line (hCMEC/D3) in the presence of tariquidar and vinblastine suggesting a role of P-gp in Aβ transport in these cells.[261]

Several in vivo studies have also attempted to identify the role of P-gp in Aβ peptide transport. A study by Cirrito et al reported suppression of Aβ (1-40; 1-42) elimination from the brain in mdr1a/1b double knockout mice model.[262] However, this study also reported a downregulation in the expression of LRP1 in cerebral vessels of the knockout animals

which may also contribute to the decreased Aβ elimination.[262] Due to the compensatory mechanisms involved in knockout animal models, it is overall difficult to conclude to what extent P-gp contributes towards Aβ clearance. Therefore, in another study, Ito et al injected rodent brains with radiolabelled Aβ (1-40) and measured Aβ elimination from the brain in the presence of P-gp inhibitors, quinidine or verapamil. But none of the inhibitors significantly reduced Aβ (1-40) elimination suggesting that P-gp may not play a major role in Aβ clearance.[263] However, these data should be interpreted with caution since wild type rodents do not exhibit age-dependent accumulation of Aβ. It is anticipated that transgenic mice or aged rodent models represent a more appropriate system to determine Aβ clearance. Interestingly, using aging animals or transgenic models of AD, several studies have confirmed that P-gp is involved in the clearance of Aβ in vivo. A recent study reported an age dependent loss of brain capillary P-gp expression as well as increased accumulation of Aβ in aging rats suggesting a role of this transporter in trafficking Aβ across the BBB.[264] In addition, Hartz et al reported reduced functional expression of P-gp at the brain capillary level in a transgenic mouse model of AD suggesting P-gp-mediated transport of Aβ across the BBB.[260]

Alteration of other ABC transporters has been reported in AD. Upregulation of ABCG2 at gene and protein level has been reported in brain tissues obtained from patients with cerebral amyloid angiopathy.[265] In Abcg2 knockout mice, a higher brain accumulation of Aβ was observed compared to wild-type mice after intravenous administration of Aβ suggesting that this transporter may act as a gatekeeper at the BBB by preventing blood-borne Aβ peptides from entering the brain.[265] A study by Shen et al has demonstrated that overexpression of ABCG2 is associated with reduced reactive oxygen species (ROS)-mediated toxicity and inflammatory response in cultured cell systems.[266] This study further suggests that upregulation of ABCG2 may partly be responsible for inhibiting ROS-mediated NF-kB activation.[266] Based on their findings, the authors have proposed that upregulation of ABCG2 in AD brain may be an adaptive response to protect the brain tissue against ROS and inflammatory damage.[266]

Among MRP isoforms, an increase in MRP1 protein expression was observed in hippocampal samples from Alzheimer's patients.[267] Although a correlation between P-gp and MRP1 expression was not established in these tissue samples, the upregulation of MRP1 could be a compensatory mechanism for the loss of P-gp efflux activity. Further studies need to elucidate the complex role of drug transporters in the context of AD.

Parkinson's Disease

Parkinson's disease (PD) is the second most common neurodegenerative disorder worldwide that is pathologically characterized by a loss of nigral dopaminergic neurons and formation of lewy bodies. The progression of this disease results in bradykinesia, akinesia, muscle rigidity and tremor.[268] PD may result from familial or sporadic etiology.[269,270] In sporadic PD, several environmental neurotoxins (i.e., pesticides, drug contaminants) as well as lack of brain detoxification have been implicated.[270] Since neurotoxicity in PD could potentially occur by accumulation of xenobiotics, impaired function of drug transporters may be associated with the accumulation of neurotoxins. Therefore, genetic polymorphism in the MDR1 gene has been implicated as a risk factor for PD. Although correlation between MDR1 polymorphism and PD remains controversial, several studies support that MDR1 polymorphism may increase the risk of PD by making individuals susceptible to environmental factors.[271,272]

Alteration of P-gp expression has been reported in PD. Using autopsy samples, Westerlund et al reported a reduction in MDR1 mRNA in postmortem human Parkinsonian subject endothelial cells compared to control samples.[273] An increase in intracerebral uptake of radiolabelled verapamil, a substrate of P-gp, has also been reported in the midbrain of patients with PD suggesting an impaired function of this efflux transporter.[274] Another study reported an increase in radiolabelled verapamil brain distribution in patients with advanced PD compared to healthy volunteers indicating a downregulation of P-gp, whereas, an upregulation of P-gp was observed in patients with less advanced PD suggesting that different stages of disease may have different effect on the transporter.[275] Furthermore, P-gp has been shown to interact with several antiparkinsonian drugs such as levodopa and bromocriptine in brain capillary endothelial cells.[276,277] Bromocriptine is known to be a substrate as well as an inhibitor of P-gp in vitro,[278] however, data from mdr1a knockout mice suggest that bromocriptine is not a P-gp inhibitor in vivo.[279] Other anti-parkinsonian drugs such as budipine, pramipexole and pergolide are also known to be P-gp substrates.[277,280] To date, the interaction of antiparkinsonian drugs with other members of the ABC family are not known.

Epilepsy

Epilepsy is a neurological disorder characterized by recurrent seizures. Chronic administration of antiepileptic drugs (AEDs) is used to treat epilepsy. However, in about 40% of the patients, AEDs fail to suppress recurrent seizures resulting in pharmacoresistance or refractory epilepsy.[281] Enhanced efflux of AED at the BBB is considered one of the major mechanisms behind refractory epilepsy and several studies have confirmed upregulation of drug efflux transporters in brain tissue obtained from both patients with refractory epilepsy or animal model of epilepsy.[282,283] Tishler et al first reported the involvement of drug transporters in the development of pharmacoresistant epilepsy where high expression of P-gp was detected in brain capillary endothelial cells isolated from tissues collected from patients with pharmacoresistant epilepsy.[284] Since then, enhanced expression of P-gp as well as other efflux transporters has been reported by many others. For example, upregulation of P-gp has been observed in capillary endothelium as well as in reactive astrocytes in epileptogenic tissue.[285] Upregulation of Abcg2 protein expression has also been observed in a rat model of status epilepticus.[286] Among the members of the MRP family, MRP1, MRP2 and MRP5 are known to be upregulated in the brain tissue of pharmacoresistant patients.[287,288] In addition, polymorphism in the MRP2 gene has been found to be associated with adverse neurological drug reactions to carbamazepine.[289]

Numerous in vitro and in vivo studies have further demonstrated that several AEDs (i.e., phenytoin, phenobarbital, levetiracetam, topiramate) are substrates of P-gp or MRPs (i.e., phenobarbital, levetiracetam).[290] Several studies have further demonstrated that upregulation of ABC transporters is associated with reduced brain penetration of antiepileptic drugs. For example, using brain microdialysis in rats, brain extracellular levels of phenytoin and carbamazepine were significantly increased by P-gp and MRP inhibitors suggesting a role of ABC transporters in limiting antiepileptic drug penetration in the brain.[283] In contrast, in Mdr1 knockout mice, brain penetration of phenytoin and carbamazepine was not different than wild-type animals, although significant increase in other AEDs was observed (i.e., topiramate, lamotrigine and gabapentin).[291,292] This variability may be due to compensatory mechanisms in the knockout animals. Although MRPs have been implicated in AED transport, Luna-Tortos et al reported that common

AEDs (i.e., carbamazepine, valproate, levetiracetam, phenytoin, lamotrigine and Phenobarbital) are not substrates for human MRP1, MRP2 or MRP5 in vitro[293] suggesting the need for further investigation in vivo. Although the severity of the disease state seems to affect the expression of the transporters, AEDs themselves may not be involved in regulating the expression of these transporters.[294]

Brain Neoplasia

Gliomas are the most common tumors in human brain. Combination of chemotherapy, radiation and surgery are used to treat and eradicate these tumors. However, one of the major obstacles against successful chemotherapeutic outcome is development of MDR phenotype in cancer cells. Numerous in vitro and in vivo studies have demonstrated that a broad spectrum of chemotherapeutic agents are substrates of ABC transporters and many members of this family have been implicated in the development of MDR phenotype. For example, using single (mdr1a/1b –/–, Bcrp–/–) and triple (mdr1a/1b –/–Bcrp–/–) knockout mice models, de Vries et al demonstrated that both P-gp and Bcrp can limit the brain penetration of erlotinib, a chemotherapeutic agent recommended for glioblastoma.[295] Similarly, others have demonstrated inadequate penetration of other chemotherapeutic agents (i.e., gefitinib, sorafenib) due to active efflux transport processes such as P-gp and BCRP.[161,296]

Upregulation of P-gp expression has been reported in tumor microvasculature in both gliomas and glioblastomas.[297] Upregulation of P-gp and ABCG2 has been reported in a number of neuroblastomas, astrocytomas and glioblastomas suggesting their contribution in generating resistance towards a number of therapeutic substrates.[131] In addition, overexpression of MRP1 has been strongly associated with MDR phenotype in many human brain tumors including astrocytomas, glioblastomas, meningiomas, neuroblastomas and oligodendrogliomas.[298] In particular, high expression of MRP1 in neuroblastoma has been found to be associated with poor treatment outcome.[299] Studies have shown enhanced sensitivity towards anticancer drugs in cells with downregulated MRP1 expression either due to administration of SiRNA or MRP specific inhibitors. For example, Burkhart et al have identified a potent inhibitor of MRP1, reversan, which increased tumour sensitivity to anticancer agents in a mouse model of neuroblastoma.[300] Using malignant human glioma T98G cells, another study demonstrated that targeting HIF-1α using siRNA resulted in reduced MRP1 transcript levels and increased cellular susceptibility to chemotherapeutic agents.[301] Recently, the tumor-promoting hedgehog signaling pathway inhibitor, HhAntag691, has been identified as a potent inhibitor of both P-gp and ABCG2 and a mild inhibitor of MRP1. This compound is now investigated in a clinical trial against various tumors due to its potent role in inhibiting hedgehog signaling as well as transporters involved in MDR phenotype.[302]

Among other MRP isoforms, increased expression of MRP3 has also been implicated in MDR phenotype in gliomas. MRP3 is expressed at relatively low levels in normal brain, however, overexpressed MRP3 transcript and protein expression have been observed in glioblastoma multiforme, the most aggressive astrocytic tumors.[303] MRP4 and MRP5 have also been detected in glioma cell lines.[131] Additional studies are needed to further understand the role of these transporters in gliomas since the expression of these transporters may differ between different phases (i.e., metastatic, malignant) or types of tumor. Among SLC transporters, transcripts of various OATP isoforms have been detected in human gliomas; however, the functional expression of these transporters in drug distribution during brain neoplasia is not yet characterized.

CONCLUSION

Limited drug penetration in the brain remains a major obstacle to brain pharmacotherapy for many neurological diseases. Although the localization and functional characterization of many influx and efflux transport processes have been investigated in vitro and/or in vivo in the CNS, modulating the expression of these transporters to improve brain pharmacotherapy still remains elusive. Moreover, functional relevance of several SLC and ABC transporters in brain cellular compartments is yet to be discovered. In addition, wide spectrum of overlapping substrate specificities of these drug transporters may lead to unexpected drug-drug interactions leading to failure in brain pharmacotherapy. In order to identify novel mechanisms to overcome poor drug penetration in the brain and effective drug targeting to the CNS, a more comprehensive understanding of human drug transporters and their regulatory mechanisms in the context of neuropathological processes is required.

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CHAPTER 3

THE BLOOD-RETINA BARRIER
Tight Junctions and Barrier Modulation

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Abstract: The blood-retina barrier (BRB) is composed of both an inner and an outer barrier. The outer BRB refers to the barrier formed at the retinal pigment epithelial (RPE) cell layer and functions, in part, to regulate the movement of solutes and nutrients from the choroid to the sub-retinal space. In contrast, the inner BRB, similar to the blood brain barrier (BBB) is located in the inner retinal microvasculature and comprises the microvascular endothelium which line these vessels. The tight junctions located between these cells mediate highly selective diffusion of molecules from the blood to the retina and the barrier is essential in maintaining retinal homeostasis. In this chapter, we summarize the key differences between the iBRB and oBRB and outline the molecular constituents of the tight junctions associated with the iBRB. We also describe a process for modulation of the iBRB to enhance systemic delivery of therapeutics to the retina, a technology which may pave the way for safer and more effective therapies for retinal diseases.

INTRODUCTION: GENERAL ANATOMY OF THE HUMAN EYE

The eye is one of the most specialized organs of the human body. It allows for light from the environment, mediated by specialized cells in the retina, to be converted into specific signals required for the process of vision. The retina, located at the back of the eyeball, contains photoreceptor cells (rods and cones) which receive the light, and the resulting neural signals subsequently undergo complex processing by other neurons of the retina, and are transformed into action potentials in retinal ganglion cells whose axons form the optic nerve. The optic nerve forms at the point of the retina where ganglion cell

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axons converge at the optic disk, and allows for the transmission of information to the brain, to be processed for the function of vision.

The retina is made up of a distinct group of neurons, including photoreceptor cells, bipolar cells, ganglion cells, Müller glial cells and various forms of interneurons. In general, the retina can be divided histologically into ten distinct layers, with each layer formed by certain cell types. The human retina has the highest oxygen consumption per weight of any tissue in the body. The high metabolic rate of the neural retina underlines the need for a distinct and regulated blood supply, and this is mediated via the Blood Retina Barrier (BRB), which will be discussed later in this chapter.

There are two sources of blood supply to the retina; the central retinal artery arises adjacent to the optic nerve, passing forward into the neural retina in the center of the optic nerve accompanied by the central retinal vein, and the choroid, which functions to nourish the outer layers of the retina. The choroid however is thin and loose compared with the highly regulated barrier properties of the vasculature associated with the inner retina, and is situated at the posterior portion of the eye, with the choriocapillaris providing nutritional requirements for outer retinal cells and the photoreceptors. At the inner retina, retinal capillaries arising from the central artery permeate the retina as far as the outer plexiform layer (OPL), with the outer segments of the retina remaining avascular.[1]

THE BLOOD-RETINA BARRIER (BRB)

Similar in structure and function to the Blood Brain Barrier (BBB), the Blood Retina Barrier (BRB) in the eye allows for the maintenance of neural tissue environments through the regulation of ion concentrations, water permeability, delivery of amino acids and sugars, and by preventing the exposure of the neural tissue to circulatory factors such as antibodies and immune cells.[2] In contrast to the BBB, however, the BRB consists of both an inner blood retina barrier (iBRB) and an outer blood retina barrier (oBRB). The iBRB comprises retinal endothelial cells, which line the micro-vessels allowing for the maintenance of blood vessel integrity and preserving the vessel's homeostasis. The oBRB is made up of Retinal Pigment Epithelial (RPE) cells and Bruch membrane, and it acts as a filter to restrict the passage of macromolecules to the outer segments of the photoreceptors. The RPE cells that comprise the oBRB allow for supporting functions essential to photoreceptor survival. These include phagocytosis of photoreceptor outer segments and transport of nutrients from the choroid to the sub-retinal space. The RPE also allows for the transport and processing of Vitamin A, general adhesion of the retina, and the absorption of scattered and out of focus light.[3] The apical surface of the RPE interacts with the photoreceptor outer segments in the outer nuclear layer of the retina, while the basolateral side interacts with the choroid, acting as a barrier to the highly perfused and permeable choriocapillaris.[4]

Both the iBRB and oBRB contain tight junctions that confer highly selective properties on barrier function. Tight junctions contain a unique assembly of proteins which constitute a highly selective barrier. Proteins making up the tight junction include Occludin and the Claudins, which span the plasma membrane and interact homotypically, while a series of peripheral cytoplasmic proteins function to anchor the transmembrane proteins to the actin cytoskeleton. Among others, these peripheral proteins include Zonula occludens-1 (ZO-1), Zonula occludens (ZO-2), and Zonula occludens-3 (ZO-3), which act through multiple protein-protein interaction domains, and are crucial for the distinct organization and initial formation of tight junctions.[5-8] Although the principle of the tight junctions is

inherently similar at the iBRB and the oBRB, i.e., the restriction of paracellular diffusion of molecules into the neural environment, the molecular composition of these junctions differs considerably.

TIGHT JUNCTIONS

Tight junctions were first identified in 1963 in epithelial cells, using electron microscopy.[9] Since then, our understanding of the tight junction and its composition and localization has increased dramatically. Tight junctions are formed at the apical periphery of endothelial cells of the iBRB and RPE cells of the oBRB. They perform the dual role of creating a primary barrier to the diffusion of solutes through the paracellular pathway, while also maintaining cell polarity as a boundary between the apical and basolateral plasma membrane domains.[10] Tight junctions are complex structures, which are composed of a series of integral and peripheral membrane proteins. The transmembrane proteins of the tight junction include occludin, Junctional Adhesion Molecule (JAM) and claudins, and they extend into the paracellular space, creating the seal characteristic of the tight junction.[11] Of the known integral membrane proteins of the tight junction, the proteins ZO-1, -2, -3 and cingulin have been shown to play an integral role in the scaffolding of transmembrane proteins, while also creating a link to the perijunctional actin cytoskeleton.[12] The 220 kDa phosphoprotein ZO-1 in particular has three PDZ (PSD-95, DLG, ZO-1) domains that could potentially bind to a wide variety of protein partners and allow for the control of tight junction assembly.[13,14] The PDZ domains associated with ZO-1 will be discussed in more detail later in this chapter.

At the ultra-structural level in freeze-fracture replicas, tight junctions have been shown to appear as an intricate network of fibrils encircling the apical end of the lateral membrane in cells expressing tight junctions. These fibrils have previously been identified as transmembrane proteins.[15,16]

Breakdown of the iBRB is a hallmark of many degenerative retinal diseases, including diabetic retinopathy, sickle-cell disease, and cystoid macular edema.[17-21]

TIGHT JUNCTION PROTEINS

Occludin

Occludin is an integral membrane protein located at the tight junction. It has been described as a phosphoprotein which can exist in many phosphorylated states, and normally migrates on SDS-PAGE between the molecular weights 60–100 kDa.[22]

Occludin has four transmembrane domains with both the N- and C-terminal located cytoplasmically. It contains two extracellular loops, which are involved in interactions between adjacent cells, regulating permeability and certain selectivity functions. The first loop contains approximately 60% Tyrosine and Glycine residues, which may possibly play an important role in cell-cell coupling through homotypic interactions with other extracellular occludin loops.[23]

The second loop has been implicated in the formation of the paracellular barrier characteristic of the tight junction. The C-terminal end of occludin interacts with ZO-1 and ZO-2 and the last 150 amino acids of the C-terminal interact with F-actin.[24-27]

It has been shown previously that overexpression of chicken occludin in Madin-Darby Canine Kidney cells (MDCK) increases the trans-epithelial/endothelial electrical resistance (TER), which correlates with decreases in paracellular permeability. However, the COOH-terminal truncated occludin in MDCK cells increases paracellular flux of small tracer molecules from the apical to basolateral domain. This increase in paracellular flux upon transfection of the COOH-terminal cytosolic domain of occludin suggests that this domain may be involved in regulating paracellular permeability.[28]

It has been suggested that occludin associates with ZO-1 at the tight junction upon recruitment by JAM. This conclusion was drawn after co-transfection of Chinese Hamster Ovary (CHO) cells with both JAM and occludin, increased the localization of occludin to the tight junction.[5]

With regard to occludin's association with the tight junction protein ZO-1, it has been reported that this interaction may play a fundamental role in modulating the function of occludin at the tight junction. It was reported that at low to normal levels of occludin, an optimum amount of ZO-1 would be present to interact with it, thus mediating an increase in TER and a decrease in paracellular flux. However, upon overexpression of occludin, the binding sites on ZO-1 may become saturated; leading to a surplus of occludin that may mediate the formation of pores as opposed to sealing the paracellular space.[29] This study only further highlighted the dynamics of this transmembrane protein's function at the tight junction, and its involvement in the maintenance of tight junction integrity.

Phosphorylation of Occludin as a Regulatory Mechanism

In Retinal Microvascular Endothelial cells (RMECs), occludin exists in multiple phosphorylation states.[30-32] In RMECs, occludin has been shown to migrate at molecular weights of 60 and 62 kDa on SDS-PAGE, with higher molecular weight bands frequently appearing at between 75 and 100 kDa. Higher molecular weight forms of occludin have previously been proposed to be richly phosphorylated on serine residues while also exhibiting phosphorylation on threonine residues.[10]

These post-translational modifications to occludin may have a significant bearing on the overall function of the tight junction protein and the overall integrity of the tight junction itself. When occludin was immunoprecipitated and treated with a phosphatase, the higher molecular weight bands disappeared. Also, the addition of a peptide constituting an extracellular domain of occludin decreased the TER of MDCK cells along with a decrease in the higher molecular weight forms of occludin.[33] This strongly suggests that hyper-phosphorylated occludin is essential in maintaining tight junction integrity.

The phosphorylation of occludin on tyrosine residues can increase paracellular permeability in endothelial and epithelial cells, further highlighting the importance of specific residue phosphorylation status in determining occludin localization at the tight junction of cells.[34] Interestingly, at its C-terminal tail, the tyrosine phosphorylation status of occludin is essential in mediating its binding properties to ZO-1, ZO-2 and ZO-3. It has also been shown that the binding properties of ZO-1,-2 and -3 to the C-terminal tail of occludin were significantly decreased compared with non-phosphorylated occludin.[35] Tyrosine phosphorylation of occludin has also been directly postulated as a mediator in disrupting the association of occludin and ZO-1, thus leading to an increase in paracellular permeability.[36] Shear stress has also been shown to decrease occludin content while increasing phosphorylation, allowing for increases in Bovine Aortic Endothelial cell (BAEC) permeability.

It is clear however that the post-translational phosphorylation status of occludin is of fundamental importance in mediating its cellular localization and the regulation of paracellular permeability, with threonine and serine phosphorylation occurring in tandem with maintenance of tight junction integrity, while tyrosine phosphorylation appears to occur concomitant with increases in paracellular permeability.

It is not sufficient however to say that occludin alone can determine tight junction integrity, as it has been shown that differentiated embryonic bodies isolated from embryonic stem cells in which the occludin gene was knocked out, still developed a normal network of tight junction fibrils between adjacent epithelial cells.[37] It is however possible that in cells with an established tight junction, occludin will mediate important and highly significant regulations of the junction via interactions with integral membrane proteins and adjacent tight junctions associated with neighboring cells.

Zonula Occludens-1 (ZO-1)

ZO-1 is a member of the Membrane Associated Guanylate Kinase (MAGUK) family of proteins. It is a tight junction phosphoprotein with a molecular weight of approximately 220 kDa, and it was among the first tight junction associated proteins to be identified.[38]

ZO-1 contains several PDZ domains. These domains are regions of sequence homology found in a diverse range of signaling proteins. The name "PDZ" derives from the first three proteins in which these domains were identified: PSD-95, a protein involved in signaling at the post-synaptic density; DLG, the Drosophila Discs Large protein; and ZO-1, the zonula occludens-1 protein. PDZ domains are also sometimes called DH domains or GLGF repeats.[39]

By recruiting downstream proteins in a signaling pathway, PDZ domains mediate assembly of specific multi-protein complexes, including those complexes necessary for tight junction formation. Proteins that contain PDZ domains play important roles in many key signaling pathways, including the maintenance of epithelial cell polarity and morphology, organizing the postsynaptic density in neuronal cells, and regulating the activity and trafficking of membrane proteins.

As well as the PDZ domains, ZO-1 also contains a Src homology (SH3) domain which mediates intracellular protein-protein interactions through the recognition of proline-rich sequence motifs on cellular proteins. A guanylate kinase (GK) domain adjacent to the SH3 domain facilitates interaction of ZO-1 with occludin.[40]

ZO-1 also contains a long carboxyl terminal region with an acidic module, a proline rich domain and several alternative-splicing sites, (Fig. 1). Due to this alternative splicing, ZO-1 can be found in two different isoforms ZO-1α^+ and ZO-1α^-. The two isoforms differ by an internal 80 amino acid domain.[41]

ZO-1 is a peripheral membrane protein enriched at tight junctions, however, cells with decreased cell-cell contact reportedly display a strong presence of ZO-1 staining in the nuclei, and therefore ZO-1 may mediate certain signaling mechanisms un-related to the tight junction.[42]

ZO-1 is found to associate initially with adherens junction components prior to final localization at the tight junction, and it has been shown to bind to the adherens junction protein α-Catenin and the gap junction associated protein connexin-43.[43-46] Only in certain conditions will it localize to the nucleus.[38] It has been proposed that in the nucleus, ZO-1 interacts via its SH3 domain with a novel transcription factor, namely, ZO-1 associated nucleic acid-binding protein (ZONAB), and regulates gene expression of components involved in epithelial cell proliferation and cell density,including ErbB-2.[47]

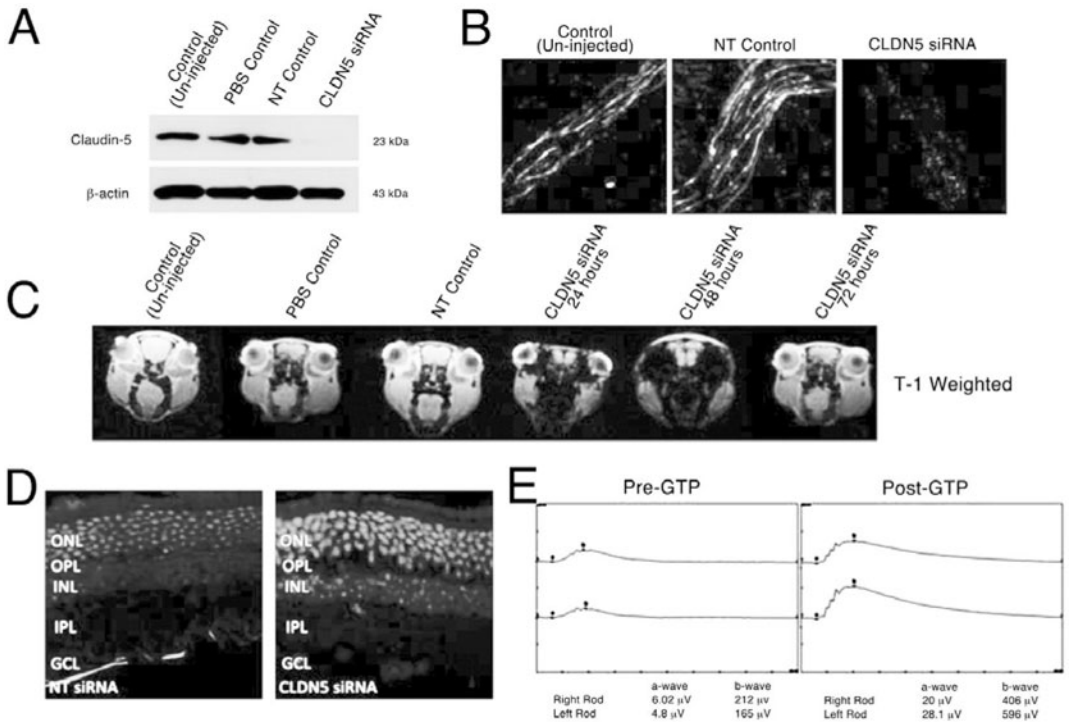

Figure 1. A) Systemic injection of siRNA targeting claudin-5 caused a transient decrease in expression of this tight junction protein in the brain and retina 48 h post injection. B) This suppression was manifested by a change in localization of claudin-5 from the microvascular endothelial cell tight junctions. C) Following injection of the contrast agent Gd-DTPA (742Da) however, intense contrasting was observed both 24 and 48 h post injection of claudin-5 siRNA when observed with T-1 weighted imaging. This was manifested as intense dark contrasting in the ocular region of the mouse. D) Immunohistochemical analysis of claudin-5 levels (red staining) in retinal cryosections, it was clear that levels were decreased in all retinal layers. Sections were counter-stained with DAPI (blue staining). E) Rod isolated ERG tracings in IMPDH1−/− mice pre- and post- injection of GTP with NT siRNA showed no distinct changes in waveform, however, upon analysis of ERG tracings pre- and post- GTP injection with claudin-5 siRNA, well formed a- and b- waves were observed in the retinas of mice. Reproduced from Campbell M et al. Proc Natl Acad Sci USA 2009; 106:17817-22;[73] A color version of this figure is available online at http://www.landesbioscience.com/curie.

The overall molecular structure of ZO-1 facilitates a capacity for multiple protein-protein interactions. At the tight junction, ZO-1 binds to the actin cytoskeleton through its carboxyl terminal end, and forms a bridge between the C-terminal sequences of occludin and β-actin. ZO-1 interacts with ZO-2 and ZO-3 through its second PDZ domain, while as previously discussed, associating with adherens junction proteins α-Catenin and gap junction proteins Connexins -43 and -45 at this domain.[48]

In a certain percentage of breast cancers, the putative expression of ZO-1 has been shown to be significantly decreased.[49] Therefore, as well as playing an important role in the regulation of the tight junction, ZO-1 and its respective family members may play a distinct and important role in the regulation of cell growth and differentiation.

ZO-1 Localization in the Mammalian Retina

Unlike the tight junction protein occludin, ZO-1 has been shown to localize to numerous regions and cell types of the mammalian retina, again highlighting its importance in mediating more cellular functions than solely barrier properties. ZO-1 has been shown to localize to retinal blood vessels and the RPE of the mammalian retina, however, it is also present at the Outer Limiting Membrane (OLM) mediating important interactions between the junctions of Müller cells and photoreceptor cells. ZO-1 is expressed in the blood vessels of the choroid and the core stroma of the ciliary process, however, as these vessels are highly permeable to solutes, it has been proposed that ZO-1 does not feature predominantly as a barrier related protein, but rather mediates important cell-cell interactions in these vessels.[50-52] ZO-1 is expressed at the borders of the anterior and the posterior epithelial cell layers in the iris and also in the epithelial layer of the ciliary body.[53] ZO-1 appears to be a dynamic protein whose functions are more diverse and complex than previously thought, and its wide distribution in the mammalian retina may be fundamental in the overall health and integrity of the eye.

Zonula Occludens-2 and -3 (ZO-2 and -3)

ZO-2 is a 160 kDa phosphoprotein located peripherally on the cytoplasmic side of the tight junction. It was originally identified through co-immunoprecipitation experiments with ZO-1. ZO-2 has 51% homology to ZO-1 most of which occurs in the MAGUK domain. ZO-1 and ZO-2 associate with each other through their second PDZ domains.[54] ZO-2 can interact with claudins through its first PDZ domain,[45] with occludin through its GK region and also with cingulin and catenin.[38,55]

The proline rich domain at the C-Terminal of ZO-2 binds to actin. It has also been proposed that ZO-2 interacts with transcription factors such as Fos, Jun and C/EBP (CCAAT/enhancer-binding protein),[56] and it has previously been shown to localize to the nucleus of epithelial cells.[57] This may imply that ZO-2 has a role to play in cell signaling and tight junction formation.

Our knowledge to date of the expression and localization of the tight junction protein ZO-3 is still at a very early stage, due mainly to a lack of antibodies that can specifically distinguish ZO-3 from the proteins ZO-1 and ZO-2. ZO-3 is a 130 kDa phosphoprotein that was initially identified as a protein that immunoprecipitated with ZO-and it has been shown to interact with both ZO-1 and occludin, but not ZO-2.[58] Interestingly, ZO-3 expression has not been detected at the OLM of the mammalian retina and appears to localize predominantly at the RPE.[59]

Both ZO-1 and ZO-2 have previously been reported to localize to the nucleus in dividing cells, which suggests a role for these proteins in distinct and specific signals necessary for tight junction formation.[60] As it is proposed that ZO-1 may interact with a transcription factor called Zonula-Occludens Associated Nucleic Acid-Binding protein (ZONAB) to regulate specific gene expression including that of cell division kinase (CDK) 4.

Claudins

Like occludin, the claudin family of proteins are transmembrane proteins that allow for the maintenance of tight junction integrity. Claudins, like occludin, span the membrane four times and interact with ZO-1 via their C-terminal domain, which all contain a tyrosine and a valine as the last two amino acids, allowing for interaction with ZO-1.[61] It has been shown that co-expression of occludin and claudin-1 in fibroblast cells causes co-localization of both proteins at the periphery of the cells in tight junction-like strands.[62] This suggests that, like occludin, claudins may play a role in regulating barrier function and the overall development of the tight junction.

Claudin-1 overexpression in Madin-Darby Canine Kidney (MDCK) cells has previously been shown to increase Trans-Epithelial Electrical Resistance (TER) and reduce Flourescien Isothiocyanate (FITC)-dextran flux across the monolayer, again suggesting it plays a role in regulating barrier function.[63] In 2003, claudin-5 knockout mice were first reported.[64] Although these mice died within hours of birth for reasons that have yet to be elucidated, they formed intact tight junctions at the blood-brain barrier (BBB). Following a series of tracer molecule experiments however, it was observed that the mice had a size-selective compromise to the BBB, where molecules below approximately 800 Da were able to freely diffuse form the blood to the brain while larger molecules were restricted to the blood. These findings offered clues to the function of claudin-5 at the neuronal barrier and it was proposed that claudin-5 played a fundamental role in the formation of paracellular pores at the tight junctions. Not only was this an important finding relating to the molecular biology of the neurovascular unit, but it also raised the possibility that levels of claudin-5 could be modulated to allow for the diffusion of low molecular weight drugs from the blood to the brain or retina.

A NOVEL THERAPEUTIC STRATEGY FOR RETINAL DISEASES

Globally, an estimated 161 million people are visually handicapped.[65] The most common causes of visual handicap are cataract and glaucoma, however in the developed world, age-related macular degeneration (AMD) is the most prevalent cause of registered blindness in the older population, closely followed by diabetic retinopathy. In the US, 1.75 million people are estimated to have advanced AMD defined as geographic atrophy or neovascularisation in at least one eye, with 7.3 million people at risk of developing the disease owing to the presence of drusen.[66] With regard to Diabetic retinopathy, in the US, for example, 4.1 million people over the age of 40 have the condition with almost 1 million people having sight-threatening disease.[67] Given that, in the overall, only limited therapies are available for these diseases, their negative social and economic impact is immense. The cost of AMD, involving diagnosis, monitoring, visual aids, habitation, accident treatment, rehabilitation, treatment of associated depression and anxiety, as well as direct treatment of the disease itself has been estimated to amount to approximately

€200,000 per patient in any five year period.[67] Costs associated with the management of diabetic retinopathy are also high.

In regard to therapeutic intervention in these major causes of visual handicap, there is currently no means of prevention of the dry form of AMD, apart from dietary supplementation and attempting to reduce the impact of environmental risk factors such as cigarette smoking. In regard to some wet cases of the disease, monoclonal antibodies targeting VEGF, Lucentis® and Avastin®, are now being used with increasing frequency, although the latter is licensed only for anti-tumor use and is therefore currently being used 'off label' for AMD.[68] These antibodies are introduced into the vitreous of the eye by direct intraocular injection at intervals of approximately one month to six weeks. While improvement in vision has been reported in numerous trials, each intraocular injection can result in the development of severe endophthalmitis, retinal hemorrhage, retinal detachment and cataract in up to 0.1% of patients. Other estimates in relation to the general intra-ocular injection of material into the eye put this figure at closer to 2%. The proliferative retinopathy associated with diabetes is treatable by scatter laser surgery, in which up to 2,000 burns are placed in areas of the retina away from the macula.[69] In addition, in cases of macular edema, focal laser surgery may be used to introduce burns around the macula. While treatments are generally useful, they do to an obvious extent, compromise retinal function and do not cure the disease and in addition, neovascular vessel leakage can occur post laser surgery in a significant proportion of cases.

Highly relevant to the advancement of preventive therapies for AMD and diabetic retinopathy, however, is the fact that an estimated 98% of drugs with established anti-neovascular, neuroprotective, anti-inflammatory or anti-apoptotic potential, do not easily cross the BBB or iBRB, rendering systemic delivery of such compounds either impractical or highly inefficient.[70] The endothelial cells of the microvasculature supplying the brain and inner retina contain tight junctions, composed of a variety of interacting proteins which form a tight seal, constituting the BBB and iBRB respectively.[71] Permanent and un-controlled opening of these barriers to large molecules such as anaphylatoxins, antibodies, other soluble proteins or pathogens would be disastrous for neuronal viability. However, controlled and transient modulation of these barriers for short periods of time to allow passive diffusion of very low molecular weight compounds could have substantial therapeutic potential, avoiding the necessity for regular invasive delivery to ocular tissues.

RNAi MEDIATED BARRIER MODULATION

In 2008, Campbell et al[72] reported for the first time on the use of barrier modulation technology to suppress levels of the tight junction protein claudin-5 at the BBB and iBRB. In this study, they employed the hydrodynamic delivery technique to introduce siRNA's to the microvasculature associated with the brain and retina. It was observed that 48 h post injection of the siRNA, the BBB and iBRB became transiently permeable to low molecular weight molecules similar to the phenotype observed on the claudin-5 knockout mouse. Molecules lower than approximately 1 kDa were able to freely passively diffuse across the BBB and iBRB, while molecules with molecular weights greater than this were restricted to the blood. This initial study was followed by a report in 2009 that barrier modulation could be used to enhance delivery of drugs with potential to prevent oxidative stress induced damage to the murine retina. Following modulation of the iBRB by suppression of claudin-5, Campbell et al,[73] demonstrated that the calpain inhibitor

ALLM could prevent light induced retinal degeneration in albino mice. Moreover, they also showed that mice lacking the enzyme IMPDH1, the rate limiting enzyme in the d novo synthesis of GTP, could be supplemented with systemically administered GTP and gain periods of functional retinal electrophysiology (Fig. 1). These studies provided the platform for the experimental use of barrier modulation as a means to enhance systemic drug delivery to neuronal tissues, however for chronic conditions such as those observed on retinal disease, repeated systemic injection of siRNA would not be a feasible approach and therefore alternative strategies were needed.

ADENO-ASSOCIATED VIRUS (AAV)-MEDIATED BARRIER MODULATION

In developing barrier modulation technology for general drug delivery in patients, it was necessary to develop a process for inducible, periodic and reversible modulation either of the BBB or the iBRB exclusively (i.e., modulating one, or other, but not both). In this regard, barrier-modulating machinery was incorporated into an AAV2/9 vector such that when sub-retinally introduced into the retina the virus accessed the vasculature, and reversible barrier modulation can periodically be induced by systemic treatment with a well tolerated inducing agent, doxycycline. It is of note that AAV infection of retinal tissues is long lasting or may even be permanent following a single sub-retinal inoculation and hence repeated ocular injections of this inducible barrier-modulating agent would not be required. Moreover, it is of note that AAV delivery systems have now been approved for use in a growing number of gene therapy trials, including those recently initiated for the congenital retinopathy, Leber congenital amaurosis (LCA).[74-76]

The objective was to develop a minimally invasive system, requiring only a once-off sub-retinal injection for periodic reversible modulation of the iBRB in mice, which could subsequently be used in human subjects. While AAV systems have been used extensively for the delivery of cDNAs and shRNAs to retinal tissues in mice a limitation of this form of delivery was that until recently, no viral serotype was available to allow efficient infection of retinal vascular endothelia. However, Foust et al[77] reported that AAV-2/9 preferentially targets neonatal neurons and adult astrocytes, and in so doing, traverses the blood-brain barrier. While no data were presented on transduction of ocular tissues, the similarities between brain and retinal vascular endothelia strongly suggested that this particular AAV serotype could also transfect the latter. This opened up the possibility of introducing an inducible barrier opening system into the genome of an AAV virus such that it could be delivered specifically to the retina. The iBRB could then be periodically and selectively opened by systemic administration of the inducing agent, doxycycline, which is required to traverse only a single lipid bilayer, that of the endothelial cells of the inner retinal vessels, in order to activate claudin-5 targeted shRNA (Fig. 2).

In 2011, it was reported for the first time that this AAV could allow for the enhanced systemic delivery of VEGF receptor antagonists systemically to prevent the development of laser induced choroidal neovascularisation (CNV), the hallmark pathology associated with wet AMD. The commercially available low molecular weight drugs Sunitinib malate (Sutent) and 17-AAG were used in his study and they were both shown to be highly efficacious in treating CNV development. Establishment of enhanced delivery of anti-neovascular compounds following reversible modulation of the iBRB provides a direct proof of principle for further development of an AAV-mediated system for

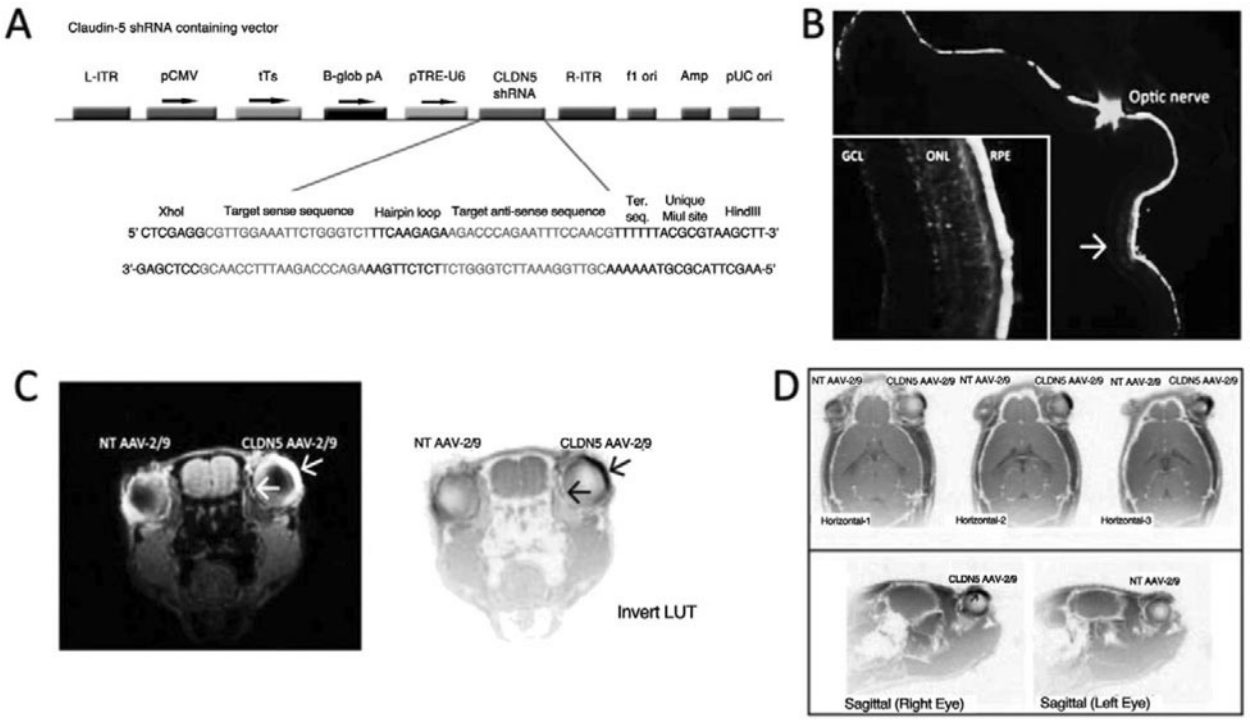

Figure 2. A) The plasmid incorporating the inducible system with claudin-5 shRNA or a non-targeting (NT) luciferase shRNA was cloned into the plasmid pAAV-MCS, such as to incorporate left and right AAV inverted terminal repeats. (L-ITR and R-ITR). AAV2/9 was then generated using a triple transfection system in a stably transfected HEK-293 cell line for the generation of high-titer viruses. B) An adeno-associated virus (AAV-2/9) with an eGFP reporter gene was injected sub-retinally in an adult C57/Bl6 mouse and 3 weeks post-injection, a retinal whole-mount showed the pattern of transduction to be widespread (green). C,D) Contrast enhanced magnetic resonance imaging (MRI) showed extravasation of the MRI contrasting agent Gd-DTPA (MW 742 Da) in mice injected in the right eye with CLDN5 AAV-2/9, but not in the left eye, injected with NT AAV-2/9 when animals were supplemented with the inducing agent doxycycline (2 mg/ml with 5% sucrose in their drinking water) for 3 weeks post-injection. Reproduced from Campbell M et al. EMBO Mol Med 2011; 3:235-45;[78] ©2011 with permission of John Wiley and Sons. A color version of this figure is available online at http://www.landesbioscience.com/curie.

inducible barrier opening, extending the use of this novel drug delivery system to an extremely important condition. Inducible suppression of claudin-5 in humans may pave the way for the controlled delivery of low molecular weight therapeutics currently deemed useless as they do not cross the iBRB.[78] In this regard, barrier modulation technology may have significant implications for the enhancement of drug delivery in general for a wide range of neurodegenerative conditions. In a clinical setting, a patient would require a "once-off" inoculation of the inducible AAV either by sub-retinal or intra-vitreal injection. Following this procedure, the iBRB could be induced to modulate by the administration of doxycycline via an oral tablet. Claudin-5 levels would then be sufficiently reduced in the retina 2–3 d post administration of doxycycline, at which time the active component could be taken by systemic or oral routes. This dosing regimen could be used in chronic conditions such as AMD or Retinitis pigmentosa and would negate the need for regular, risky and in many patients, stressful intra-ocular injection of monoclonal antibodies.

CONCLUSION

Barrier modulating technologies are now being tested for a range of neuronal conditions, not just limited to retina diseases. However a range of safety/toxicology studies are on-going so that AAV-mediated barrier modulation can be used in future human clinical trials associated with enhanced systemic delivery of therapeutics to the retina for AMD and Retinitis pigmentosa treatments. These non-invasive methods for chronic drug delivery should pave the way for a safer, more patent friendly option for treating the most common debilitating forms of blindness.

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CHAPTER 4

THE INNER BLOOD-RETINAL BARRIER
Molecular Structure and Transport Biology

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Abstract: The inner blood-retinal barrier (inner BRB) is created by complex tight junctions of retinal capillary endothelial cells. Although this barrier prevents the free diffusion of substances between the circulating blood and the neural retina, the inner BRB efficiently supplies nutrients to the retina and removes endobiotics and xenobiotics from the retina to maintain a constant milieu in the neural retina. We review herein the molecular structure and transport mechanism at the inner BRB.

INTRODUCTION

In mammals, the retina is a thin tissue (~0.5 mm in thickness) which lines the interior of the posterior globe of the eye, is a highly organized ultrastructure composed of about ten cell layers and plays a key role in vision. Retinal neuronal cells are the most sensitive and critical cells in the eye analogous to neurons in the brain. Therefore, it is necessary to have developed a specialized structure to isolate neuronal cells from blood to maintain a stable ionic environment and to ensure appropriate activities of neuronal cells in a specialized microenvironment. The blood-retinal barrier (BRB), which is created by complex tight junctions of retinal capillary endothelial cells (inner BRB) and retinal pigment epithelial cells (outer BRB), restricts nonspecific transport between the neural retina and the circulating blood (Fig. 1).[1,2] The inner two-thirds of the human retina is nourished by the inner BRB and the remainder is covered by choriocapillaris via the outer BRB.[2]

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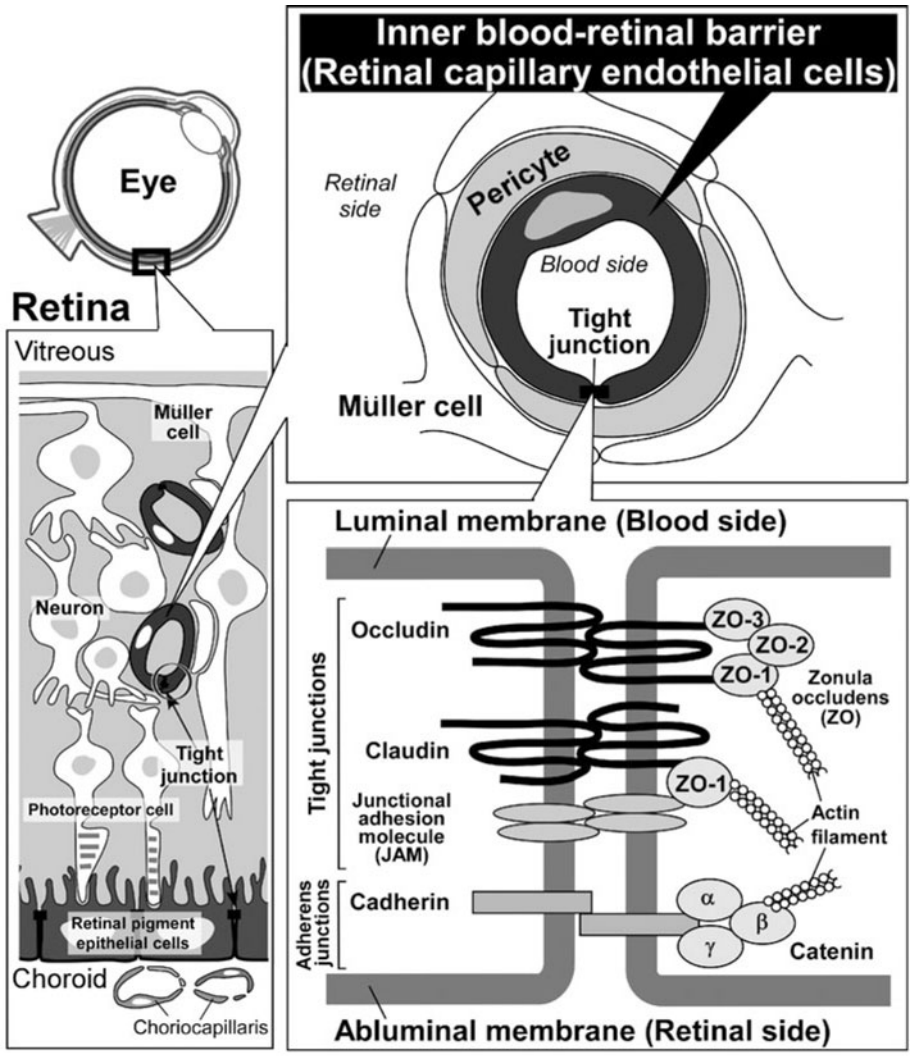

Figure 1. Schematic diagram of the inner blood-retinal barrier. The inner blood-retinal barrier consists of tight junctions of the retinal endothelial cells which are covered with pericytes and Müller cells. The tight junction complex includes two classes of transmembrane molecules such as occludin and claudins, which interact with transmembrane proteins on adjacent endothelial cells. The cytoplasmic tails of the occludin and claudin complex are linked to the actin cytoskeleton via a number of accessory proteins, including members of the zonula occludens family, ZO-1, ZO-2 and ZO-3. Vascular endothelial cadherin and catenins help to seal between endothelial cells as adherens junctions.

The concept of the BRB was first proposed by Schnaudigel in 1913 based on the similarity to the blood-brain barrier (BBB).[3] He found that dyes injected into vein did not stain the retina as well as brain even though peripheral tissues were stained.[3] The concept was then formulated in greater detail during the 20th century with the advance of technology of an electron microscopy.[1] Like the BBB, the inner BRB is composed of

tight junctions of the retinal endothelial cells which are covered with pericytes and glial cells. Müller cells are the most prominent glial cells of the retina, although astrocytes are predominantly present in the brain. They are also present in species having a retinal circulation. In these species, astrocytes are restricted largely to the nerve fiber layer at the inner boundary of the retina. In contrast, outer BRB is formed by the retinal pigment epithelium, where tight junctions between epithelial cells create the barrier, but the choriocapillaris are fenestrated (Fig. 1). The inner BRB has been proposed to be a glio-vascular unit since endothelial barrier constructed by an extensive network of endothelial cells, Müller cells/astrocytes and neurons to regulate retinal capillaries by paracrine interactions.[4]

CELLULAR FACTORS IN GLIO-VASCULAR INTERFACE

The inner BRB properties are not intrinsic to endothelial cells but are inducible by the surrounding pericytes and glial cells. Several lines of evidences suggest that glial cells such as Müller cells and astrocytes play this role in the retina as astrocytes do in the brain. Astrocytes induce BBB properties in endothelial cells.[5] Indeed, Type I astrocytes injected into the anterior eye chamber of the rat are able to induce a host-derived angiogenesis and some barrier properties in endothelial cells of nonneural origin.[6] When bovine retinal endothelial cells were cocultured with astrocytes from rat brain, astrocytes increase barrier properties in retinal endothelial cells.[7] These observations suggest that soluble factor(s) from astrocytes modulate barrier properties of endothelial cells. Müller cells can enhance the barrier properties of retinal blood vessels by production of factors that contribute to tight junction formation.[8] Conditionally immortalized rat Müller cell line (TR-MUL cells) can up-regulate barrier-related marker enzyme activities such as alkaline phosphatase in conditionally immortalized rat retinal capillary endothelial cell line (TR-iBRB cells) by production of transforming growth factor-β (TGF-β).[9] Moreover, glia cell-derived neurotropic growth factor in the TGF-β family, interleukin-6 and basic fibroblast growth factor (bFGF) are also involved in barrier regulation.[10] Pericytes also induce barrier function of endothelial cells in a manner similar to astrocytes by production of angiopoietin-1.[11] Pericytes communicate with endothelial cells via gap junctions between both cells and considered contractile cells that help to regulate blood flow.[12] Pericytes contract when exposed to endothelin-1, angiotensin II,[13] hypoxia and ATP and relax on exposure to CO_2,[14] nitric oxide (NO) and adenosine.[15]

MOLECULAR STRUCTURE OF THE INNER BRB

The inner BRB is composed of a tightly sealed monolayer of retinal endothelial cells, which normally prevent passive diffusion via paracellular transport of solute across endothelial cells between the circulating blood and the neural retina. Interestingly, blood-to-retina influx permeability rate of D-mannitol, which is a nonpermeable paracellular marker, is more than 300 times less than that of D-glucose and amino acids, which are transported via a carrier-mediated transport mechanism (Table 1), suggesting that the inner BRB acts as a 'selective' barrier. Retinal endothelial cells are normally connected via a junctional complex composed of tight junctions and adherens junctions (Fig. 1).

Table 1. Influx transporters/transport processes at the inner blood-retinal barrier

Transporter/ Transport Process	Substrate	Influx Permeability Rate [μL/(min · g retina)]	Plasma Concentration (μM)	Km (μM)	Refs.
GLUT1	D-Glucose	544[b]	~12,500	5,000	41
	DHA	2,440	10	93	42
ENT2	Adenosine	25.8	0.09	29	46
CAT1	L-Arginine	118	170	11	49
TAUT	Taurine	259	100-300	22	50,52
LAT1 (System L)	L-Leucine	203	180	14	50,54
xCT (System Xc^-)	L-Cystine	286[c]	100-200	10	60,61
GlyT1	Glycine	8.6	200	55	62
OCTN2	Acetyl-L-carnitine	2.3	18	26	63
CRT	Creatine	10.7	140-600	15	64
SMVT	Biotin	5.6	0.006	146	68
Choline transport	Choline	271	10	6-100	69
RFC1	MTF	N.D.	~0.05	5.1	71
MCT1	L-Lactic acid	N.D.	10,000	1,600	72-74
SR-BI	α-Tocopherol/HDL	N.D.	-	-	75
Passive diffusion	D-Mannitol[a]	0.6	-	-	63

The influx permeability rate was determined by integration plot analysis after intravenous injection of radiolabeled compound. [a]D-Mannitol is a nonpermeable paracellular marker (passive diffusion). [b]Influx permeability rate of D-glucose [544 μL/(min · g retina)] is calculated from the influx rate of D-glucose (6.8 μmol/(min · g retina))/normal D-glucose concentration in rat plasma (12.5 mM).[41] [c]indicates μL/(min · g eye). N.D.: not determined. DHA: dehydroascorbic acid, MTF: methyltetrahydrofolate.

Tight junctions are composed of multiple transmembrane, scaffolding and signaling proteins. There are at least three different types of transmembrane proteins within tight junctions: occludin, claudin and junctional adhesion molecule (JAM).[16-18] Transcript levels of tight junction proteins in isolated rat and mouse retinal endothelial cells were quantified. Caludin-5, occludin and JAM-1 mRNAs are predominantly expressed in rat and mouse retinal capillary endothelial cells compared with nonretinal endothelial cells.[19,20] The cytoplasmic tails of the occludin and claudin complex are linked to the actin cytoskeleton via a number of accessory proteins, including members of the zonula occludens family, ZO-1, ZO-2 and ZO-3.[21,22] Vascular endothelial cadherin (VE-cadherin) and catenins help to seal between endothelial cells as adherens junctions.[23] The tight junctions act not only as a physical barrier, but also as a segregator of the luminal (blood) and abluminal (retina) domains in the endothelium[24] (Fig. 1).

Reduction of occludin by the anti-sense oligonucleotides increases permeability.[25] Thus, occludin likely regulates barrier function.[25] The endothelial expression of occludin in the retina is reduced in experimental diabetes.[26] In addition, vascular endothelial growth factor (VEGF) significantly reduced occludin in the retinal endothelial cell culture and increased permeability across the endothelial cell monolayers.[27] VEGF promotes leakage of plasma proteins from blood vessels.[28] It has been shown to induce an increase in vascular permeability via synthesis or release of NO.[28] Increased

VEGF and NO production was observed in the rat retina in response to hypoxia.[29] Generation of reactive oxygen species (ROS), resulting in oxidative stress, occurs in many tissues in hypoxia-ischemia. In ocular pathologies, ROS have been correlated with neovascularisation in diabetic eyes in humans[30] and in eyes with retinopathy of prematurity.[31] Enhanced production of VEGF, NO and ROS underlies disruption and the increased permeability of the inner BRB. Inhibition of these factors is beneficial for treating retinal diseases such as diabetic retinopathy, ischemic central retinal vein occlusion and retinal hypoxia.[32]

INFLUX TRANSPORTERS AT THE INNER BLOOD-RETINAL BARRIER

The tight junctions of retinal capillary endothelium control paracellular impermeability for hydrophilic compounds. Since neural cells, including photoreceptor cells in the retina require a large amount of metabolic energy for phototransduction and neurotransduction, metabolic substrates, such as D-glucose, amino acids and their metabolites must cross the endothelial wall transcellularly to reach their neural targets or leave the retina.[33,34] In general, transcellular transport across the inner BRB can be classified into three main categories: passive diffusion (lipid-mediated transport), carrier-mediated transport and receptor-mediated transport. The carrier-mediated transport systems at the inner BRB consist of facilitated transport, secondary active influx and efflux transport and primary active efflux transport (Fig. 2).

The inner BRB transporters play an essential role in the blood-to-retina transport of hydrophilic nutrients such as D-glucose, amino acids, vitamins and nucleosides.[35] The in vivo blood-to-retina transport of a test compound across the BRB is evaluated by integration plot analysis after intravenous injection of the radiolabeled compound in rats.[36] This approach allows determination the influx permeability rate of text compound. The

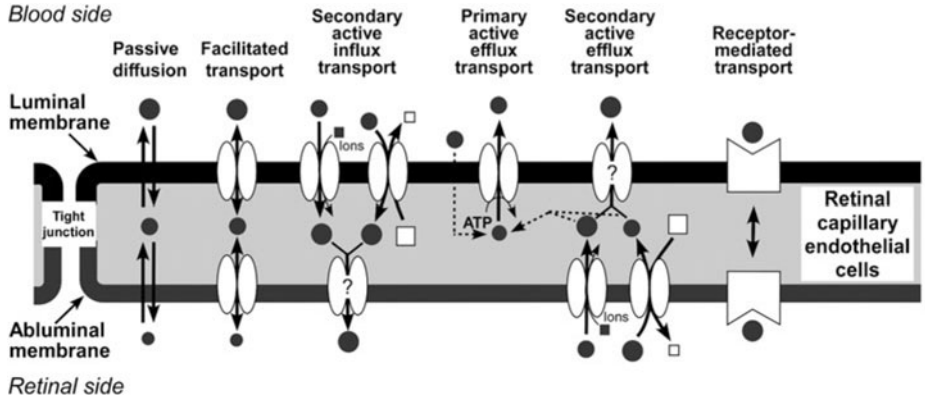

Figure 2. Schematic representation of transport systems at the inner blood-retinal barrier. The transport systems at the inner blood-retinal barrier can be classified into three main categories; (i) passive diffusion (lipid-mediated transport), (ii) carrier-mediated transport and (iii) receptor-mediated transport. The carrier-mediated transport systems at the inner BRB consist of facilitated transport, secondary active influx and efflux transport and primary active efflux transport.

influx permeability rates differ for transporter-mediated transport and passive diffusion (Table 1). The molecular identify of the transporters at the inner BRB has been established using TR-iBRB cells as an in vitro model of inner BRB.[2,37]

GLUT1

D-Glucose, which is the main energy souse for the retina, is transported from the circulating blood to the retina via facilitative glucose transporter 1 (GLUT1/SLC2A1). GLUT1 is a member of a gene family of Na^+-independent glucose transporters, which is expressed exclusively at the inner BRB. GLUT1 transports hexoses and dehydroascorbic acid (DHA), which is an oxidized form of vitamin C.[38] The expression of GLUT1 on the abluminal membrane of the inner BRB is 2- and 3-fold greater than that on the luminal membrane in human and rats, respectively.[39,40] The asymmetrical distribution of GLUT1 at the inner BRB provides a homeostatic control for D-glucose influx into the retina by preventing D-glucose accumulation in the interstitial fluid of the retina at levels higher than those in the blood. The influx permeability rate of D-glucose is 544 μL/(min · g retina)[41] (Table 1). DHA transport is mediated via GLUT1 at the inner and outer BRB and DHA has the influx permeability rate with 2,440 μL/(min · g retina)[42] (Table 1). Even though GLUT1 is not a concentrative transporter, DHA is rapidly reduced to ascorbic acid and thus is trapped within the retina,[42] most likely by Müller cells.[43] The Michaelis-Menten constant (Km) of GLUT1 for D-glucose is ~8 mM and normal plasma D-glucose concentration in most mammals is ~5 mM[44] while the Km of GLUT1 for DHA is 93 μM and DHA is present in the circulation at much lower level (~10 μM).[42] Therefore, DHA transport via GLUT1 across the BRB does not exhibit complete inhibition under physiologic conditions. On the other hand, DHA transport from the blood to the retina decreases with increasing blood D-glucose concentration (~20 mM) under diabetic conditions.[45] Diabetic patients may experience enhanced oxidative stress in the retina because of reduced influx of DHA.

ENT2

Adenosine, which is a purine nucleoside, plays a number of roles in retinal neurotransmission, blood flow, vascular development and response to ischemia through cell-surface adenosine receptors. Although the influx permeability rate of adenosine is 25.8 μL/(min · g retina) which is about 10-fold lower than that of amino acids (Table 1), the blood-to-retina transport of [^{3}H]adenosine is inhibited by unlabeled adenosine and thymidine but not by cytidine.[46] Similar features are evident for adenosine uptake by TR-iBRB cells which express equilibrative nucleoside transporter 2 (ENT2/SLC29A2) mRNA.[46] The Km of ENT2 for adenosine is ~30 μM, which is much higher than adenosine concentration in plasma (~0.1 μM), suggesting that ENT2 at the inner BRB is not saturated by the plasma adenosine under physiologic conditions. Since the nucleotide drugs, such as 3′-azido-3′-deoxythymidine (AZT), 2′ 3′-dideoxycytidine (ddC), 2′3′-dideoxyinosine (ddI), cladribine, cytarabine, fludarabine, gemcitabine and capecitabine, are substrates for ENT2, this transporter is potentially involved in the delivery of such drugs into the retina.[47,48]

CAT1

Unlike the anionic amino acids such as L-glutamic acid and L-aspartic acid, essential and semi-essential cationic amino acids such as L-lysine and L-arginine cannot be synthesized by the retina at adequate rates to meet the needs for retina metabolism and protein synthesis. Thus, the retina requires a steady and balanced supply of these cationic amino acids from the circulating blood. L-arginine is the precursor for generation of NO via nitric oxide synthases. NO not only regulates vascular tone and blood flow but also is a critical component in a variety of cell signaling pathways. It is also an important determinant in the progression of retinal pathology in diseases such as diabetic retinopathy and glaucoma. L-arginine uptake by TR-iBRB cells is mediated by the Na^+-independent cationic amino acid transporter 1 (CAT1/SLC7A1).[49] CAT1 is expressed in retinal capillary endothelial cells. The influx permeability rate of L-arginine is 118 μL/(min · g retina)[49] (Table 1) and the blood-to-retina transport of [^{3}H]L-arginine is inhibited by unlabeled L-arginine and L-lysine.[50] Since NO synthesis depends on extracellular L-arginine, the function of CAT1 in the delivery of L-arginine into the retina across the inner BRB may represent a rate-limiting step in NO production in the retina. Since CAT1 also transports a variety of arginine- and lysine-based inhibitors of nitric oxide synthases, this transporter at the inner BRB can be exploited for delivery of such compounds into the retina in the treatment of specific retinal diseases associated with overproduction of NO (e.g., inflammation).

TAUT

Taurine is a non-essential amino acid in humans, but is considered to be an essential amino acid during fetal growth and lactation. In the retina, taurine exerts a number of neuroprotective functions as an osmolyte and antioxidant and accounts for more than 50% of the free amino acid content. The activity of cysteine sulfinic acid decarboxylase, a rate-limiting enzyme for taurine biosynthesis from L-cysteine, in the rat retina is low in comparison with the abundance of retinal taurine,[51] leading the hypothesis that the blood-to-retina transport system(s) of taurine play a key role in maintaining the taurine concentration in the retina. The influx permeability rate of taurine is 259 μL/(min · g retina)[52] (Table 1) and the blood-to-retina transport of [^{3}H]taurine is inhibited by substrates of taurine transporter (TAUT/SLC6A6).[50] Taurine uptake by TR-iBRB cells is Na^+-, Cl^-- and concentration-dependent with a Km of 22.2 μM and inhibited by substrates of TAUT.[52] TAUT is expressed in TR-iBRB and primary cultured human retinal endothelial cells.[52] We found that TAUT transports γ-aminobutyric acid (GABA) as a substrate with a lower affinity than taurine.[53] Although the physiological role of TAUT in GABA transport in the retina is not fully understood, TAUT most likely mediates taurine transport from the circulating blood to the retina across the inner BRB.

LAT1

The retina requires a steady stream of essential large neutral amino acids (e.g., L-leucine, L-phenylalanine and L-tryptophan) to maintain metabolic function and protein synthesis. The system L consists of L (leucine-referring)-type amino acid transporter 1 (LAT1/SLC7A5)

and an additional subunit protein of the heavy chain of 4F2 cell surface antigen (4F2hc/SLC3A2) and is involved in the transport of branched-chain and aromatic amino acids. LAT1 appears to operate principally via an Na^+-independent, substrate-coupled antiport, although it can mediate net influx. An immunohistochemical study showed that LAT1 is expressed at the inner BRB.[54] The influx permeability rate of L-leucine is 203 μL/(min · g retina)[54] (Table 1) and the blood-to-retina transport of [^{14}C]L-phenylalanine is inhibited by substrates of LAT1.[50] L-Dopa is transported across the BBB by system L and is readily biotransformed to dopamine in the brain.[55] Many patients with Parkinson's disease have blurred vision or other visual disturbances, which are reflected in the reduced retinal dopamine concentration and delayed visual evoked potentials.[56] L-Dopa administration has been reported to reduce these delayed visual evoked potentials in Parkinson's disease.[57] Indeed, we recently found using retinal uptake index (RUI) method that the blood-to-retina transport of [^{3}H]L-Dopa is 8-fold greater than that of the [^{3}H]D-mannitol and inhibited by unlabeled L-Dopa.[58] LAT1 is also potentially important for drug delivery into the retina.[59]

xCT

The system Xc^- consists of xCT(SLC7A11) and 4F2hc and is involved in transport of L-cystine and L-glutamic acid. The system Xc^- appears to operate principally via an Na^+-independent, substrate-coupled antiport, although it can mediate net influx of L-cystine. To protect the retina against light-induced oxidative stress and maintain intracellular antioxidants, such as gluthathione (GSH) and vitamin C, at an appropriate level, transport of L-cyst(e)ine (one of the constitutent amino acid for GSH) and vitamin C into the retina is critical for the health of the retina. xCT is expressed at the inner BRB and transports L-cystine,[60] which is also one of the rate-limiting precursor of GSH synthesis. The influx permeability rate of L-cystine is 286 μL/(min · g eye) (Table 1).[61] An in vivo intravenous administration study has shown that L-cystine uptake by the eye is activated by pretreatment with diethyl maleate (DEM), a reagent used to deplete intracellular GSH in order to induce oxidative stress.[61] This uptake is inhibited in the presence of L-glutamic acid and L-α-aminoadipic acid, selective substrates of system Xc^-.[61] TR-iBRB cells express xCT and 4F2hc mRNA and L-cystine uptake by TR-iBRB cells takes place in an Na^+-independent and concentration-dependent manner with a Km of 9.2 μM and is inhibited by substrates and inhibitors of system Xc^-.[60] DEM treatment causes significant induction of xCT mRNA, L-cystine uptake and the GSH concentration in TR-iBRB cells.[60] These results suggest that L-cystine influx transport at the inner BRB is mediated by system Xc^- and induced under oxidative stress by enhanced transcription of the xCT gene.

GlyT1

Glycine plays a pivotal role in neurotransmission and in the biosynthesis of creatine and GSH in the retina. It is also a co-agonist for the *N*-methyl-D-aspartate (NMDA) receptor. The influx permeability rate of glycine is 8.6 μL/(min · g retina) (Table 1) and the blood-to-retina transport of [^{14}C]glycine is inhibited by unlabeled glycine.[62] Glycine uptake by TR-iBRB cells is Na^+-, Cl^-- and concentration-dependent with a Km of 55.4 μM and inhibited by substrates and inhibitors of Na^+ and Cl^--dependent glycine transporter 1 (GlyT1/SLC6A9).[62] GlyT1 most likely mediates the blood-to-retina transport of glycine across the inner BRB.

OCTN2

L-Carnitine has multiple roles in mammalian cells, even though its primary and most recognized function is to promote fatty acid oxidation for energy production. Acetyl-L-carnitine is effective in improving visual function in patients with early age-related macular degeneration. Uptake of L-carnitine and acetyl-L-carnitine in TR-iBRB cells occurs via the Na^+-dependent organic cation/carnitine transporter 2 (OCTN2/SLC22A5).[63] The influx permeability rate of acetyl-L-carnitine is 2.33 μL/(min · g retina) (Table 1) and the blood-to-retina transport of [^{3}H]acetyl-L-carnitine is inhibited by unlabeled L-carnitine and acetyl-L-carnitine.[63] OCTN2 is expressed in isolated rat retinal capillary endothelial cells. The Km value for the transport of L-carnitine and acetyl-L-carnitine via OCTN2 in TR-iBRB cells is ~30 μM, a value similar to the physiological levels of these compounds in plasma (L-carnitine, ~50 μM; acetyl-L-carnitine, ~20 μM).[63] Exogenous administration of L-carnitine and acetyl-L-carnitine would therefore be able to increase the retinal levels of these protective compounds through OCTN2-mediated transfer across the inner BRB.

CRT

Creatine plays a vital role in the storage and transmission of phosphate-bound energy in the retina. The Na^+- and Cl^--dependent creatine transporter (CRT/SLA6A8) plays a role in the influx of creatine into the retina at the inner BRB.[64] CRT is localized on both the luminal and abluminal membranes of rat retinal capillary endothelial cells.[64] CRT expressed on the luminal membrane would mediate creatine supply to the retina, but the function of CRT on the abluminal membrane is not yet known. The influx permeability rate of creatine is 10.7 μL/(min · g retina) (Table 1).[64] Creatine supplementation into the retina is a potentially promising treatment for gyrate atrophy of the choroid and retina with hyperornithinemia. However, CRT at the inner BRB is almost saturated by plasma creatine (140-600 μM in mice and rats), since the Km for creatine uptake in TR-iBRB cells (~15 μM) is much lower than these plasma concentrations.[64] Although creatine is biosynthesized in Müller cells from L-arginine and glycine,[65,66] which are transported from blood across the inner BRB as above mentioned,[49,62] CRT at the inner BRB constantly supplies creatine to the retina.

SMVT

Biotin, a water-soluble vitamin, acts as a cofactor for five carboxylases catalyzing essential steps in fatty acid biosynthesis, glyconeogenesis and catabolism of several branched-chain amino acids and odd-chain fatty acids. Recently, Arunchaipong et al reported that biotin is involved in glutamine synthetase activity and glutamate decarboxylase activity as a 42 kDa biotin-coupled protein in the chick retina.[67] Biotin uptake by TR-iBRB cells is Na^+- and concentration-dependent with a Km of 146 μM and inhibited by substrates and inhibitors of Na^+-dependent multivitamin transporter (SMVT/SLC5A6).[68] The influx permeability rate of biotin is 5.6 μL/(min · g retina) (Table 1) and the blood-to-retina transport of [^{3}H]biotin is inhibited by substrates of SMVT.[68] SMVT is expressed in isolated rat retinal capillary endothelial cells.[68] SMVT plays a role in the influx of biotin into the retina at the inner BRB.

OTHER TRANSPORTERS

Several other transporters and transport processes have been identified as the blood-to-retina influx transport of nutrients. Choline is an important cell membrane constituent in the form of phosphatidylcholine and sphingomyelin. It is also a precursor of neurotransmitter acetylcholine. Choline uptake by TR-iBRB cells is Na^+-independent, potential- and concentration-dependent with Km of 6.4 and 99.7 μM.[69] The influx permeability rate of choline is 271 μL/(min · g retina)[69] (Table 1) and the blood-to-retina transport of [^{3}H]choline is inhibited by unlabeled choline and hemicholinium-3.[70] Even though it is clear that a specific choline transport system is responsible for transfer of choline into the retina across the inner BRB, the molecular identify of the transporter remains to be established.

Folates play an essential role as cofactors for one-carbon metabolism in cells and, consequently, they are required for the de novo synthesis of purines, pyrimidines, some amino acids and for the conversion of homocysteine to methionine. Folate deficiency in the retina has been associated with increased risk of nutritional amblyopia and methanol-induced retinal toxicity. Much of the folate in the plasma of most mammals is in the reduced form, methyltetrahydrofolate (MTF) and its concentration is 5-50 nM. The MTF uptake by TR-iBRB cells is Na^+- and Cl^--independent and concentration-dependent with a Km of 5.1 μM.[71] This process is inhibited competitively by reduced folate carrier 1 (RFC1/SLC19A1) substrates, such as methotrexate and formyltetrahydrofolate.[71] RFC1 mRNA, compared with other folate transporters, such as H^+-coupled folate transporter (RCFT/SLC46A1), is predominantly expressed in TR-iBRB cells and isolated rat retinal capillary endothelial cells.[71] RFC1 plays a role in the influx of MTF into the retina at the inner BRB.

The retina produces more L-lactic acid aerobically than any other tissue. Moreover, L-lactic acid appears to be required as an energy source, in addition to D-glucose, in photoreceptors. Alm and Törnquist were the first to use the RUI method to show that L-lactic acid transport across the rat BRB exhibits saturability, pH-dependence and is inhibited by pyruvate and 3-hydroxybutyrate.[72] In addition to this functional evidence, Gerhart et al used immunoelectron microscopy to provide morphological evidence that monocalboxylate transporter 1 (MCT1/SLC16A1) is localized on both the luminal and abluminal membranes of the inner BRB.[73] TR-iBRB cells express MCT1 mRNA and L-lactic acid uptake by TR-iBRB cells was shown to be a temperature-, H^+- and concentration-dependent process with a Km of 1.7 mM.[74] L-Lactic acid uptake was inhibited by a protonophore, MCT inhibitors and a number of other monocarboxylates.[74] MCT1 plays a role in the influx of monocarboxylates into the retina at the inner BRB.

SR-BI

Vitamin E has preventive and therapeutic effects in human retinopathy. Among the member of the vitamin E family, α-tocopherol has the highest biological activity and is exclusively associated with high-density lipoprotein (HDL), in the circulating blood. HDL-associated [^{14}C]α-tocopherol ([^{14}C]α-tocopherol-HDL) uptake by TR-iBRB cells exhibited a time-dependent increase and a temperature-dependence.[75] The uptake of [^{14}C] α-tocopherol-HDL was inhibited by in the presence of block lipid transport-1, a specific inhibitor of the scavenger receptor class B, Type I (SR-BI)-mediated lipid transfer

between HDL and cells.[75] The expression of SR-BI protein was detected in TR-iBRB cells and immunostaining of SR-BI was observed along the rat retinal capillaries.[75] The inhibition of SR-BI protein expression by RNAi using SR-BI-specific siRNA duplexes resulted in a reduction in [^{14}C]α-tocopherol-HDL uptake.[75] SR-BI plays a role in uptake of HDL-associated α-tocopherol by the retinal capillary and may transfer α-tocopherol into the retina.

S1P AND LPA RECEPTOR

Sphingosine 1-phosphate (S1P) and lysophosphatidic acid (LPA) are simple bioactive lysophospholipids which exhibit an effect on blood vessels via their G protein-coupled receptors.[76] Hyponatremia is a frequently encountered clinical disorder, in which the Na^+ concentration and osmolarity in the plasma are significantly reduced and causes neural cell swelling in the retina and contributes to the development of edema.[77] Retinal capillary endothelial cells first detect the reduction in plasma osmolarity and should rapidly respond to hypoosmotic stress. Otherwise, the breakdown of the inner BRB would lead to further damage of retinal function. Under hypoosmotic stress, although the cell volume is regulated by releasing inorganic ions, such as K^+, Cl^- and organic osmolytes, such as taurine,[78] taurine seems to be an ideal organic osmolyte because of its metabolic inertness and abundance in the inner BRB.[79] S1P and LPA significantly enhanced the taurine release from TR-iBRB cells under hypotonic conditions in a time- and concentration-dependent manner, whereas S1P and LPA had no significant effect under isotonic conditions.[80] S1P receptor, $S1P_1$ and $S1P_4$ and LPA receptor LPA_4 mRNAs are predominantly expressed in isolated rat retinal capillary endothelial cells.[80] The S1P-enhanced taurine release under hypotonic conditions was significantly inhibited by $S1P_1$ receptor antagonists.[80] Since S1P and LPA are present in the serum, they play a novel role in the regulation of osmolyte efflux from the inner BRB in response to hypoosmotic stress via the activation of their specific receptors.

EFFLUX TRANSPORTERS AT THE INNER BLOOD-RETINAL BARRIER

Since the retina produces various metabolites and neurotoxic compounds and their accumulation is believed to one of the causes of neurodegenerative diseases, the efflux transport systems at the inner BRB are required to eliminate such compounds as a means to protect the retina from unwanted harmful effects. There are two distinct mechanisms in the elimination process. The endobiotics and xenobiotics including drugs are taken-up from the circulating blood into the retinal capillary endothelial cells via either passive diffusion or some influx transporters and subsequently undergo efflux into the circulating blood via a primary active efflux transport system. ATP-binding cassette (ABC) transporters as an efflux transport system are mainly present on the luminal membrane of the inner BRB (Fig. 2). Of ABC transporter family, ABC transporter A (ABCA), B (ABCB), C (ABCC) and G (ABCG) play a role in transporting endobiotics and xenobiotics in the plasma membrane. The second mechanism involves transcellular transport of endobiotics and xenobiotics from subretinal space to the circulating blood. The first step is the uptake of these compounds by secondary active transporters on the abluminal membrane and subsequently these compounds undergo efflux to the circulating

blood via primary/secondary active transporters on the luminal membrane of the inner BRB. Of secondary active transporters on the abluminal membrane, organic anion transporting polypeptide (OATP/SLCO/SLC21A) and organic anion transporter (OAT/SLC22A) family take part in the transport of organic anions (Fig. 2).

Oatp1a4

Many clinically important drugs including antibiotics, anti-tumor drugs, anti-HIV drugs and anti-inflammatory agents are organic anions. The limited distribution of β-lactam antibiotics in the vitreous humor/retina after systemic administration is problematic, resulting in reduced efficacy in the treatment of bacterial endophalmitis. Some β-lactam antibiotics are substrates for organic anion transporting polypeptide (oatp) 1a4 (Slco1a4; oatp2).[81]

We used microdialysis to carry out an in vivo evaluation of vitreous/retina-to-blood efflux transport in rats and to determine the efflux transport of organic anions across the BRB.[36,82] Estradiol 17-β glucuronide (E17βG) was injected with D-mannitol, a bulk flow marker, into vitreous humor of the rat eye and a microdialysis probe was placed in the vitreous humor.[82] E17βG and D-mannitol were bi-exponentially eliminated from the vitreous humor after vitreous bolus injection.[82] The elimination rate constant of E17βG during the terminal phase was 2-fold greater than that of D-mannitol and it was significantly inhibited by organic anions including digoxin,[82] a specific substrate of oatp1a4.[83] In addition to functional evidence, oatp1a4 is expressed in rat retinal capillary endothelial cells.[84] Moreover, oatp1a4 and 1c1 (Slco1c1/oatp14) mRNA are predominantly expressed in isolated rat retinal capillary endothelial cells.[19] Oatp1c1 transports E17βG as is the case with oatp1a4 whereas oatp1c1 does not have high affinity for digoxin.[85] This suggests that oatp1c1 and oatp1a4 play distinctive roles in the retina-to-blood efflux transport in terms of the specificity of the drugs and xenobiotics. Further studies are needed to clarify the individual contribution of oatp1c1 and oatp1a4 to the efflux of specific anionic drugs across the inner BRB.

OAT3

Some β-lactam antibiotics, such as benzylpenicillin (PCG) are substrates for organic anion transporter (OAT) 3 (SLC22A8).[86] 6-Mercaptopurine (6-MP) is frequently used for cancer chemotherapy in patients with childhood acute lymphoblastic leukemia. Relapse of childhood acute lymphoblastic leukemia involving eye is a rare but challenging problem.[87] This is probably due to the restricted distribution of 6-MP in the eye. One possible factor in the restricted drug distribution in the retina/eye is the retina-to-blood efflux transport of such anionic drugs across the BRB.

Betz and Goldstein demonstrated that *p*-aminohippuric acid (PAH) uptake by isolated retinal capillaries was slightly greater than that of the extracellular marker, sucrose and inhibited by fluorescein and penicillin.[88] Oatp does not transport PAH, but OAT family transporters prefer PAH as a substrate. Therefore, in addition to oatp, OAT is also involved in the organic anion transport at the inner BRB.

PAH, PCG and 6-MP were bi-exponentially eliminated from the vitreous humor after bolus injection into vitreous of the rat eye.[89] The elimination rate constant of PAH, PCG

and 6-MP during the terminal phase was about 2-fold greater than that of D-mannitol.[89] This efflux transport was reduced in the retina in the presence of probenecid, PAH and PCG, relatively specific substrates of OAT3,[86] but not in the presence of digoxin. OAT3 is localized on the abluminal membrane of retinal capillary endothelial cells.[89] Thus, OAT3 is involved in the uptake of PAH, PCG and 6-MP across the abluminal membrane of retinal capillary endothelial cells and contributes to the efflux transport of PAH, PCG and 6-MP from vitreous humor/retina into blood across the inner BRB.

SYSTEM A (ATA2)

As mentioned above, LAT1,[54] xCT,[60] TAUT,[52] GlyT1[62] and CAT1[49] have been identified as an amino acid transporter at the inner BRB. They play an essential role in supplying L-leucine, L-cystine, taurine, glycine and L-arginine to the retina. However, byproducts from the biosynthesis pathway of amino acids in the retina need to excrete from retina to the circulating blood. It has been suggested that small neutral amino acid transporter, system A is present on the abluminal membrane of the inner BRB because α-methylaminoisobutyric acid (MeAIB), which is a substrate for system A is taken up from the retinal side using isolated bovine retinal capillaries and this process is ouabain-sensitive.[88]

MeAIB uptake by TR-iBRB cells is Na^+- and concentration-dependent with a Km of 234 μM and this process is strongly inhibited by substrates of system A, such as L-alanine, L-proline, glycine, L-serine and L-methionine.[62] On the other hand, L-proline uptake by TR-iBRB cells is Na^+- and concentration-dependent with a Km of 392 μM and this process is strongly inhibited by substrates of system A.[90] System A isoform, ATA2 mRNA (SLC38A2/SNAT2/SAT2) is predominantly expressed in TR-iBRB cells and isolated rat retinal capillary endothelial cells.[90] L-Proline and D-mannitol are bi-exponentially eliminated from the vitreous humor after vitreous bolus injection. The elimination rate constant of L-proline during the terminal phase was 2-fold greater than that of D-mannitol and it was significantly inhibited by MeAIB.[90] We found an evidence using RUI method that [^{14}C]MeAIB undergoes limited blood-to-retina transport across the BRB.[62] Thus system A is most likely localized on the abluminal membrane of the inner BRB. L-Proline is biosynthetically derived from L-glutamic acid in the retina and can act as an agonist or antagonist of excitatory L-glutamic acid.[91] L-Proline, L-alanine and other amino acids concentrations in the vitreous humor of rat are increased after chronic intraocular pressure elevation.[92] System A, most likely ATA2 is responsible for the retina-to-blood transport of L-proline across the inner BRB and plays a role in maintaining the concentration of small neutral amino acids in the retina.

ABCB1 (P-GLYCOPROTEIN)

P-Glycoprotein (P-gp/ABCB1), which is an ATP-dependent 170 kDa membrane glycoprotein, exhibits a protective role by restricting the entry of several classes of drugs, including antibiotics, anti-tumor drugs, anti-HIV drugs, steroids and immunosuppressants into the eye. P-gp is localized on the luminal membrane of the inner BRB[2] and the expression of mdr1a mRNA is predominantly exhibited in isolated rat[19] and mouse[20] retinal capillary endothelial cells. P-gp is expressed in TR-iBRB[37] and primary cultured

bovine retinal endothelial cells[93] and accumulation of rhodamine 123[94] and paclitaxel[93] is enhanced in the presence of inhibitors of P-gp in TR-iBRB and and primary cultured bovine retinal endothelial cells, respectively. The active efflux transport function of P-gp at the inner BRB could lower the blood-to-retina transport of its substrates. Cyclosporin A, which is a substrate of P-gp, was not detected in the intraocular tissues of cyclosporin A-treated rabbits,[95] rats[96] and human,[97] although significant level of cyclosporin A can detected in the plasma. These pieces of evidence indicate that P-gp at the inner BRB plays an important role in protecting the retina from xenobiotics and, therefore, hinders the retinal transfer of therapeutic drugs.

ABCC4 (MRP4)

The multidrug resistance-associated protein (MRP/ABCC) family plays a role in transporting anionic compounds, such as glucuronic acid conjugates and glutathione conjugates.[98] The transcript levels of ABCC transporters at the inner BRB have been quantified in isolated mouse[20] and rat retinal capillary endothelial cells.[35] MRP4 (ABCC4) was shown to be most highly expressed at the transcript level and MRP3 (ABCC3) (mouse)[20] and MRP6 (ABCC6) (mouse and rat)[20,35] are also expressed at lower levels. Consistent with these transcript data, it was recently shown that MRP4 protein is localized on the luminal membrane of the inner BRB in mice.[99] MRP4 are known to transport PAH, 6-MP and β-lactam antibiotics as substrates.[100,101] These findings suggest that MRP4 is involved in excreting of PAH, PCG and 6-MP from cell to blood across the luminal membrane of retinal capillary endothelial cells and contributes to the efflux transport of PAH, PCG and 6-MP from vitreous humor/retina into blood across the inner BRB.

ABCG2 (BCRP)

Breast cancer resistance protein (BCRP/MXR/ABCP/ABCG2) is localized on the luminal membrane of the inner BRB in rats.[102] ABCG2 prefers not only drugs (e.g., mitoxantrone and doxorubicin) but also photosensitive toxins, including pheophorbide a, a chlorophyll-derived dietary phototoxin related to porphyrin.[103] The retina is subject to high levels of cumulative irradiation and, therefore, vulnerable to light-induced damage caused by a variety of phototoxic compounds including porphyrins. TR-iBRB cells express ABCG2 protein and Ko143, an ABCG2 inhibitor, inhibits the excretion of pheophorbide a from TR-iBRB cells.[102] ABCG2 at the inner BRB also plays a role in protecting the retina from xenobiotics.

ABCAs

Although the transport function and the precise cellular localization of ABCA family at the inner BRB is not fully understood, ABCA1 plays a role in efflux of sterols.[104] ABCA3 and ABCA9 mRNA are highly expressed in isolated mouse retinal capillary endothelial cells.[20] Transport function and localization of ABCA3 and ABCA9 at the inner BRB remains to be established.

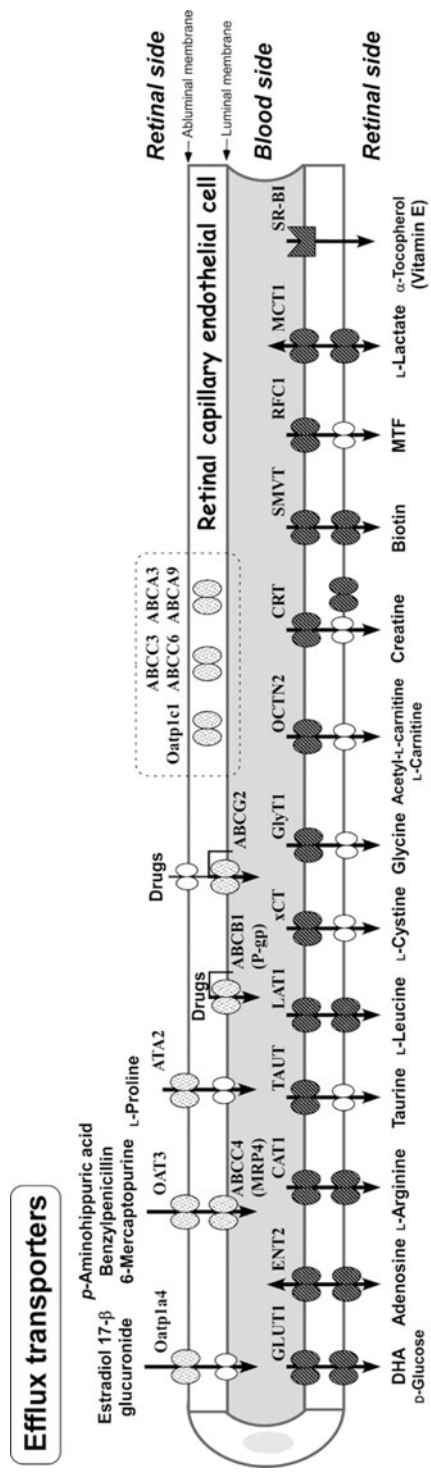

Figure 3. Hypothetical localization and physiological function of the inner blood-retinal barrier transporters. Inner blood-retinal barrier transporters play an essential role in supplying nutrients to the retina and carrying out the efflux transport of endobiotics and xenobiotics. DHA: dehydroascorbic acid; MTF: methyltetrahydrofolate.

CONCLUSION

It has been almost 100 years since the physiological concept of the inner BRB was initially proposed.[3] Recent progress in the inner BRB research has revealed that retinal capillary endothelial cells interact with glial cells and pericytes and are sealed by junctional barrier proteins. The inner BRB expresses a wide variety of transporters essential for the blood-to-retinal influx of nutrients. There are also several transporters that contribute to the protective function of the inner BRB by mediating the retina-to-blood efflux of toxins and drugs (Fig. 3). Thus, the inner BRB acts as dynamic interface between the neural retina and the circulating blood.[105] Several retinal acting drugs have been discovered, but not yet successful in treating the diseases in retina because the inner BRB acts not just the structural barrier but also efflux pump. Better understanding of the influx and efflux transport mechanisms at the inner BRB will help in the design of optimal drug candidates as well as the prediction of drug penetration.

FUTURE CHALLENGES

Numerous challenges remain. At the inner BRB level, for instance, a number of carrier-mediated transporters, such as choline, peptides and vitamins, several active efflux transporters and receptor-mediated transport processes need to be identified. The role and regulation of the junctional barrier proteins and transporters at the inner BRB in the pathogenesis of diabetic retinopathy, age-related macular degeneration and retinal vein occlusion remains to be explored. Developing an absolute quantification method for membrane proteins using liquid chromatography-tandem mass spectrometer (LC/MS/MS)[106] will enable us to construct a quantitative atlas of transporters and junctional barrier proteins at the inner BRB and to compare protein expression profiles with other barriers such as blood-brain and blood-placental barriers. If the inner BRB transporters and junctional barrier proteins contribute to progression of retinal disease such as via a breakdown of the inner BRB, manipulation of transport systems and barrier functions may offer innovative neuroprotective and treatment strategies. The inner BRB specific transporters and receptors could also be potentially utilized as portals of entry for retinal drug targeting systems.

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CHAPTER 5

ENDOTHELIAL AND EPITHELIAL BARRIERS IN GRAFT-VERSUS-HOST DISEASE

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Abstract: Endothelial and epithelial cells form selectively permeable barriers that separate tissue compartments. These cells coordinate movement between the lumen and tissue via the transcellular and paracellular pathways. The primary determinant of paracellular permeability is the tight junction, which forms an apical belt-like structure around endothelial and epithelial cells. This chapter discusses endothelial and epithelial barriers in graft-versus-host disease after allogeneic bone marrow transplantation, with a focus on the tight junction and its role in regulating paracellular permeability. Recent studies suggest that in graft-versus-host disease, pathological increases in paracellular permeability, or barrier dysfunction, are initiated by pretransplant conditioning and sustained by alloreactive cells and the proinflammatory milieu. The intestinal epithelium is a significant focus, as it is a target organ of graft-versus-host disease, and the mechanisms of barrier regulation in intestinal epithelium have been well characterized. Finally, we propose a model that incorporates endothelial and epithelial barrier dysfunction in graft-versus-host disease and discuss modulating barrier properties as a therapeutic approach.

INTRODUCTION

A key function of endothelia and epithelia is to form selectively permeable barriers that separate distinct tissue compartments. Transport across these barriers is accomplished by coordinating the specific, but saturable, transcellular pathway with the nonspecific, nonsaturable paracellular pathway. The paracellular space must be at least partially sealed in order to maintain the concentration gradient established by active transcellular transport. Such trans-epithelial and -endothelial gradients also direct passive paracellular transport. The tight junction, a component of the apical junctional

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complex, is the primary determinant of paracellular permeability, and can be regulated in response to physiological and pathological stimuli.

Tight Junction Structure and Barrier Function

A large number of transmembrane and cytosolic proteins, most notably the claudin family, the tight junction-associated MARVEL protein (TAMP) family, which includes occludin, and the zonula occludens proteins ZO-1, ZO-2 and ZO-3, form the tight junction, which creates a belt-like structure at contact sites between adjacent epithelial or endothelial cells.[1,2] The interaction between tight junction transmembrane proteins at these sites creates a selectively permeable seal. While endothelial and epithelial cells express similar tight junction proteins, the structure is more complex and highly developed in epithelium,[1] where tight junctions are clearly separated from the more basal adherens junction. In contrast, it can be difficult to differentiate the endothelial tight junction and adherens junctions, and these structures are collectively referred to as the interendothelial junction.[3]

Transepithelial, or transendothelial, electrical resistance measures the properties of the seal that limits movement of ions across the paracellular pathway under most experimental conditions,[4] and is generally higher in epithelia than endothelia.[5-9] Although most endothelia are leaky, some do form tight seals, particularly at specialized sites. For example, endothelia that form the blood-brain barrier have extremely well developed tight junction structures and are some of the least permeable in the body,[10] contrasting sharply with tight junctions within postcapillary venules. This difference in endothelial paracellular permeability depending on site illustrates the reduced permeability that is generally associated with a greater need to prevent mixing of separate compartments. While the vascular space is typically sterile, many epithelia are in direct contact with the external environment, including the airspaces and gut lumen. This requires a tighter paracellular barrier to prevent contamination of the tissues and subsequent inflammatory responses. In contrast, the leaky endothelial barrier supports free exchange of nutrients and waste products. In addition, although inflammatory cells can cross epithelia, primarily in the setting of disease, immune cells regularly traffic across vascular and lymphatic endothelium. This is particularly true in postcapillary venules, which readily allow immune cell extravasation.[9]

Just as there are barrier differences between endothelia at separate sites, barrier function also varies between epithelia.[11] For example, barrier function increases progressively from proximal to distal along the renal tubule.[12-14] This reflects the need to establish a steep electrochemical gradient in order to concentrate or dilute urine in the distal tubule and collecting duct. It follows that bladder epithelium forms one of the least leaky mucosal barriers in the body.[11,15,16] Although small intestinal and colonic epithelia are much leakier than those of the distal nephron and bladder, a qualitatively similar pattern of decreasing paracellular permeability occurs along the length of the intestine and correlates with the need to extract water and ions from lumen. Further, there is a gradient of barrier function along the small intestinal crypt-villus axis.[17] Villus tight junctions are structurally well developed and have been characterized as preventing paracellular flux of molecules with radii greater than ~6 Å.[18] In contrast, crypt tight junctions accommodate molecules up to 50 Å radius.[18] This correlates with greater numbers and complexity of tight junction strands in villus, relative to crypt, epithelium[19] (Fig. 1). As with the proximal to distal permeability gradient of the intestines and renal tubule, this crypt to villus gradient also reflects function. The crypt is primarily secretory, and the permeable tight junctions at that site support the paracellular water flow that follows transcellular Cl^- secretion.

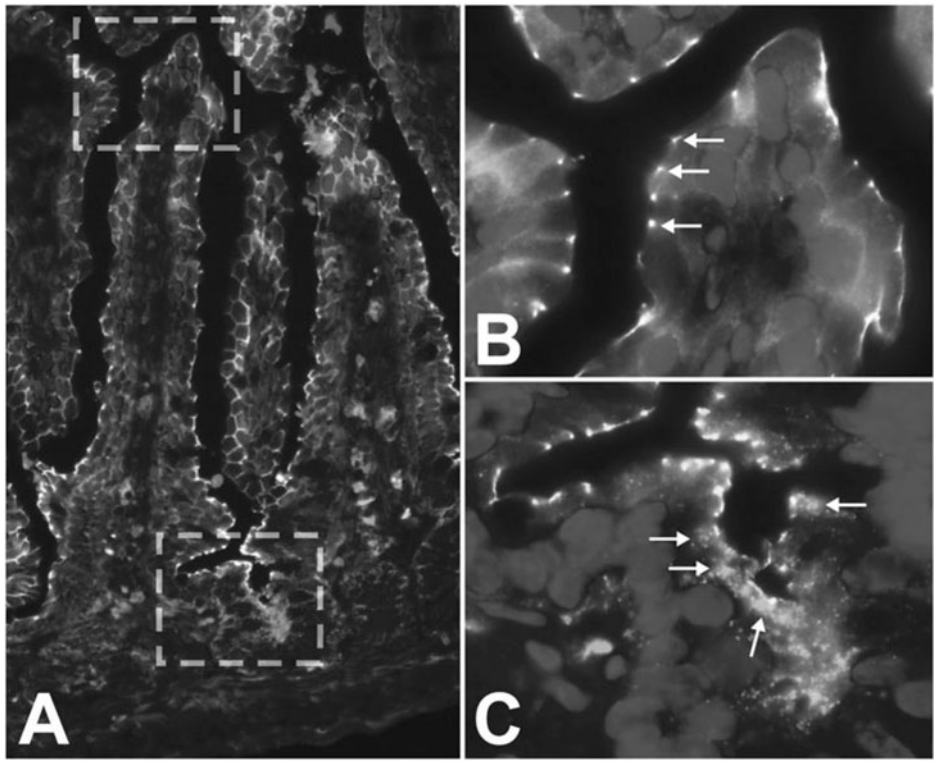

Figure 1. Normal mouse small intestine was stained for occludin (red), ZO-1 (green) and nuclei (blue). Note the difference in subcellular occludin localization in villous versus crypt epithelium, which corresponds with more and less complex tight junction structure, respectively. A) Low magnification. B) High magnification of villus epithelium, which shows almost all occludin is localized at the tight junction (arrows). C) High magnification of crypt epithelium, where there is a vesicular pool of occludin (arrows). A color version of this figure is available at www.landesbioscience.com/curie.

This flow into the gut lumen creates a protected, relatively sterile crypt lumen, which minimizes the potential hazards of a leaky gut barrier. In contrast, the villous epithelium must establish a relatively tight barrier to prevent backflow of absorbed nutrients and tissue contamination by luminal microbes and their products.

REGULATION OF THE PARACELLULAR BARRIER

The paracellular permeability of endothelial and epithelial cells is not static. In addition to factors that govern baseline barrier function, there are separate mechanisms that regulate tight junction permeability in response to physiological and pathological mediators. For example, permeability of small intestinal epithelial villous tight junctions increases after activation of Na^+-nutrient, e.g., Na^+-glucose, cotransport.[20-22] This allows the transepithelial osmotic gradient established by active transcellular transport to drive passive paracellular nutrient absorption.[23,24] In pathological conditions, such as graft-versus-host disease that develops following allogeneic bone marrow transplantation, increases in paracellular

permeability of both endothelial and epithelial tight junctions occurs in many organs.[25-28] Similar increases in epithelial and endothelial tight junction permeability are found in inflammatory bowel disease, celiac disease, ischemia reperfusion injury, and acute lung injury, where they are thought to enhance disease pathogenesis.[29-35] As discussed in this chapter, efforts to re-establish and maintain intestinal barrier function may be a viable therapeutic approach in graft-versus-host disease. Using this hypothesis as a framework, we will address the mechanisms that lead to increased paracellular permeability in endothelia and epithelia, how barrier dysfunction contributes to disease, and how modulating barrier function may alter the course of disease. Overall, the intestine is the focus, as it is one of the target organs of graft-versus-host disease, and the mechanisms of barrier regulation in intestinal epithelium have been well characterized in other diseases.

Endothelium

Endothelial paracellular permeability can be increased by interendothelial junction disassembly, cell contraction, and cell retraction.[36] Interendothelial junction disassembly is primarily accomplished by redistribution of interendothelial junction scaffolding proteins, which destabilizes overall structure. In addition, endocytosis removes transmembrane proteins, including claudins, occludin, and junctional adhesion molecules (JAMs), from the junctions in response to inflammatory or ischemic insults.[5,37-39] This redistribution alters the localization of other tight junction proteins, creating an additive effect on interendothelial junction disassembly and barrier dysfunction.[40]

Proximal signaling events for interendothelial junction disassembly are mostly regulated through phosphorylation of interendothelial junction components.[41] For example, thrombin induces disassembly of the interendothelial junction and increases permeability by stimulating PKCα activation and translocation to the interendothelial junction, where it phosphorylates residues on the adherens junction proteins VE-cadherin and p120.[42-44] These phosphorylation events cause both target proteins to be removed from the interendothelial junction, which destabilizes structural integrity and increases paracellular permeability. Vascular endothelial growth factor (VEGF) increases permeability by a similar mechanism that involves phosphorylation and endocytosis of VE-cadherin, junction disassembly, and barrier dysfunction.[45] In this case, VEGF uses p21-activated kinase (PAK), not PKCα, as an intermediate to induce VE-cadherin phosphorylation. Similar processes drive internalization of the tight junction protein occludin in response to VEGF.[5] While the specific kinase(s) involved in VEGF-induced occludin phosphorylation are not known, Rho-associated kinase (ROCK), PKCβ and PKCδ have all been implicated in VEGF-dependent endothelial barrier regulation.[41,46]

Endothelial cell contraction causes barrier loss that is less pronounced than interendothelial junction disassembly. Nevertheless, it leads to development of small interendothelial gaps. These gaps are formed by an actin driven process that changes endothelial cell shape, re-organizes intercellular junctions, and increases paracellular permeability. This process requires myosin II regulatory light chain (MLC) phosphorylation, by myosin light chain kinase (MLCK) or ROCK, to drive actin-myosin cross-bridge cycling.[47,48] ROCK can also potentiate actomyosin contraction by phosphorylating and inhibiting MLC phosphatase.[49-51] Upstream events that activate MLCK include and Ca^{2+}/calmodulin signaling.[6,52]

Further mechanisms that allow flux of cells and nutrients from the blood stream to the interstitium are cell retraction and transcellular passage. These mechanisms are unique to

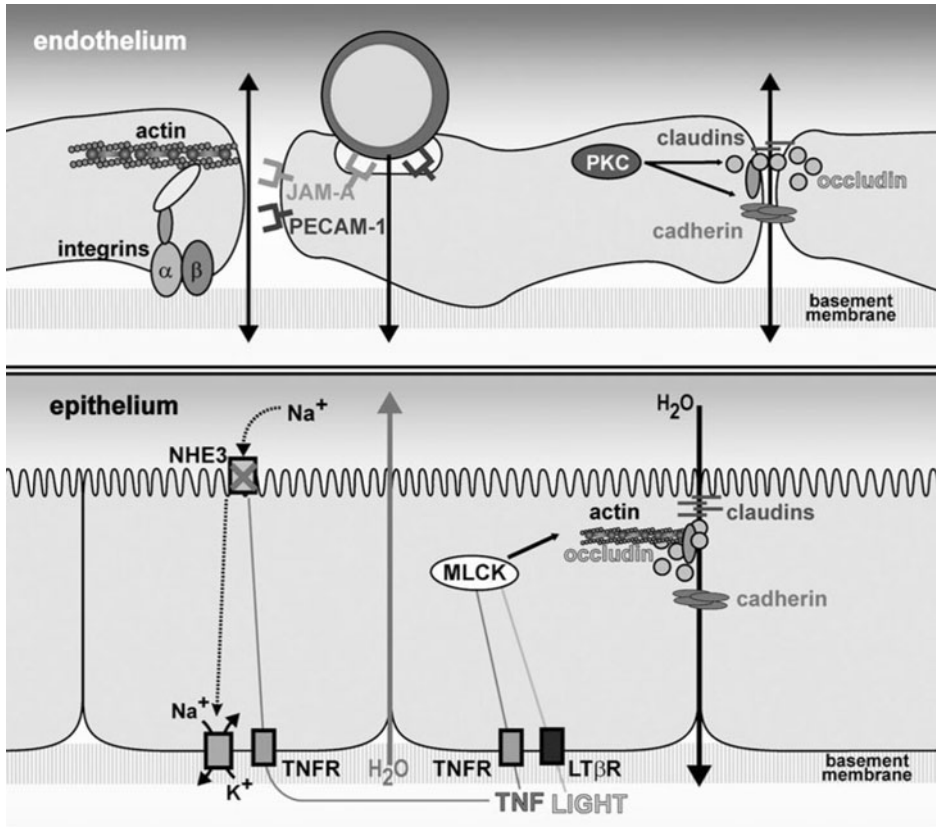

Figure 2. Mechanisms of permeability in endothelium (top) and epithelium (bottom). Cell retraction (far left) and transcellular migration are unique to endothelium, while junctional disassembly and actomyosin-mediated barrier regulation occur in both endothelium and epithelium. In absorptive epithelium, water movement from the lumen follows Na^+ absorption. The proinflammatory cytokines TNF and LIGHT lead to increased MLCK activity and actomyosin-mediated barrier regulation, thereby increasing paracellular permeability to water and small solutes. In addition, TNF triggers endocytosis of the Na^+-H^+ exchanger NHE3 from the apical membrane, which reduces Na^+ absorption and results in net water flux into the lumen. A color version of this figure is available at www.landesbioscience.com/curie.

endothelium and have not been reported in epithelium (Fig. 2). Although endothelial cell retraction does generate interendothelial gaps that increase paracellular permeability, it is distinct from cell contraction in that retraction is neither driven by actin nor associated with cell shape changes. Rather, retraction is thought to be mediated by phosphorylation of integrin-linked proteins such as vinculin and talin, which are critical for maintaining cell-cell and cell-matrix contacts.[36]

Transcellular movement refers to the passage of cells and solutes from the lumen to the interstitium directly through endothelial cells. This was clearly demonstrated by ultrastructural examination of serial sections of inflamed guinea pig skin.[53] Mechanistic analysis has revealed that transcellular movement utilizes components of the endothelial lateral border recycling compartment, termed the LBRC,[54] and suggest that the LBRC is recruited to the luminal surface where it surrounds the transmigrating leukocyte and

creates a transcellular pore that mimics paracellular transit.[55] Thus, transcellular movement relies on many of the same molecules required for paracellular cell migration, including platelet/endothelial adhesion molecule (PECAM)-1, JAM-A and CD99.[55] While epithelia transport nutrients, immunoglobulins, viral proteins, bacterial products, and even whole bacteria transcellularly,[56-59] there have been no reports of transcellular leukocyte migration. Rather, morphological and physiological data suggest that leukocytes traverse epithelial cells only at intercellular junctions. This is not surprising, as transport of unwanted luminal materials through the transcellular pore may be acceptable in the case of endothelium, which interfaces with the sterile vascular space, but not, for example, in intestinal epithelium, which protects the internal milieu from gut microbiota.

Epithelium

In contrast to the endothelium, which regulates paracellular permeability by gaps that involve interendothelial junction disassembly, the epithelial tight junction does not generally undergo such drastic structural changes. While there is biochemical and morphological evidence of actomyosin contraction, gross cell separation, as seen in endothelia, does not occur. Instead, smaller changes to tight junction protein-protein interactions and trafficking, some of which are mediated by actin and myosin, are thought to account for increases in epithelial paracellular permeability. As discussed above, formation of endothelium-like gaps in epithelia would be dangerous given that most epithelia separate sterile and nonsterile compartments.

MLCK is an important mediator of epithelial barrier regulation under physiological and pathological conditions. This has been most carefully demonstrated in intestinal epithelial cell lines and in vivo models of intestinal barrier dysfunction.[60-66] The first direct evidence for MLCK-mediated increases in paracellular permeability in response to physiological stimuli was obtained using an in vitro model of Na^+-glucose cotransport in intestinal epithelial cells.[20] It was later shown that the same mechanisms of MLCK-driven barrier dysfunction are activated in proinflammatory conditions, both in vitro,[63,67,68] and in vivo.[29] These studies also showed that the inflammatory cytokine tumor necrosis factor (TNF) was an important signaling molecule that enhanced MLCK expression and activity in the intestinal epithelium. The related cytokine LIGHT, (lymphotoxin-like inducible protein that competes with glycoprotein D for HVEM on T cells) a TNF core family member, as well as IL-1β also upregulate MLCK expression and activity to cause barrier dysfunction.[69-72] Importantly, TNF, LIGHT, and IL-1β signaling have all been implicated in graft-versus-host disease,[73-78] and the potential of TNF neutralization in graft-versus-host disease therapy is under active investigation.[79-83] This confluence suggests that it may also be appropriate to assess the role of MLCK activation and barrier dysfunction in graft-versus-host disease[84] (Fig. 3).

MLCK-MEDIATED BARRIER DYSFUNCTION

The basis for our understanding of MLCK-mediated barrier dysfunction has been established in vitro using cell lines and in vivo using mouse models of acute immune-mediated diarrhea. TNF treatment of intestinal epithelium is commonly used as a method to induce barrier loss in vitro.[67,68,70] This is pathophysiologically relevant, as TNF is a central mediator in human diseases involving intestinal epithelial barrier

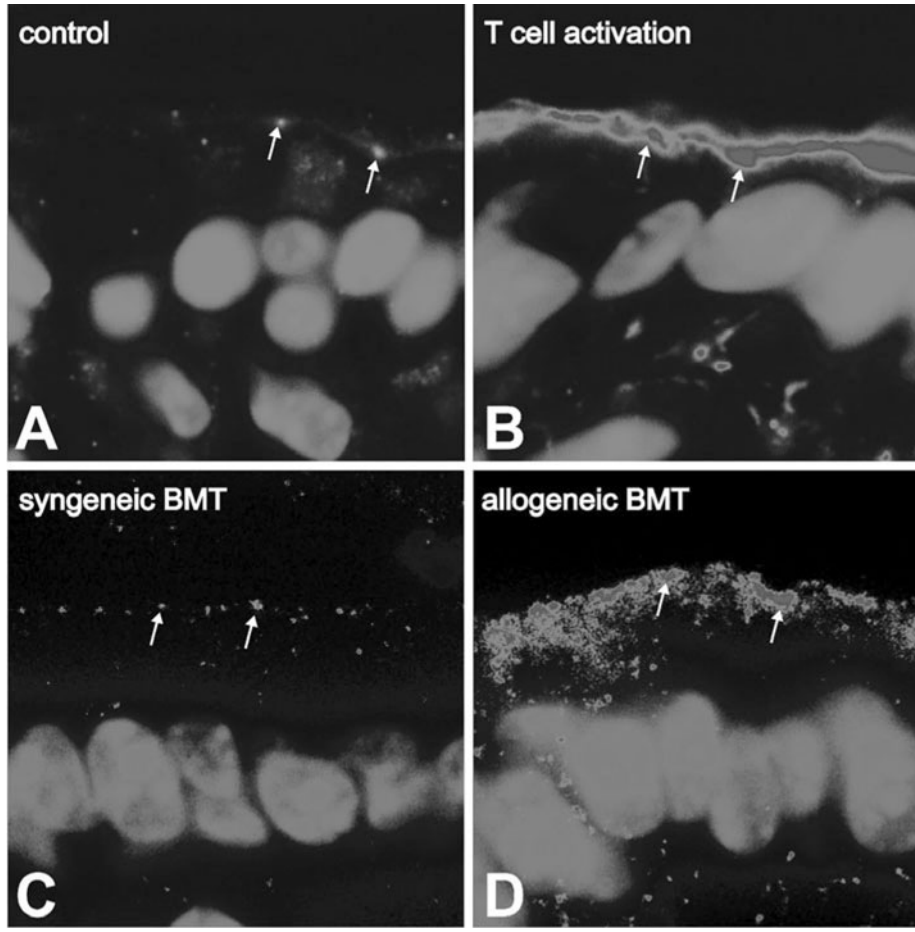

Figure 3. MLC phosphorylation is increased in acute intestinal barrier dysfunction (T-cell activation) and experimental graft-versus-host disease. Mouse small intestinal sections were stained for phosphorylated MLC (red) and nuclei (blue), 3 hours after acute T-cell activation (B) or 14 days after bone marrow transplantation from syngeneic (C) or allogeneic minor mismatch donors (D). These similarities suggest that both disease models involve MLCK-dependent intestinal epithelial barrier dysfunction. Panels A and B adapted from Clayburgh DR et al. J Clin Invest 2005; 115:2702-15;[29] with permission from the American Society for Clinical Investigation. A color version of this figure is available at www.landesbioscience.com/curie/.

dysfunction, including Crohn's disease, ischemic intestinal damage, and graft-versus-host disease.[85] Early studies suggested that TNF-induced apoptosis could be at least partly responsible for barrier loss in cultured epithelial monolayers,[86] however, subsequent work has shown that TNF-induced barrier dysfunction can also occur without significant apoptosis.[63,68,87,88] Instead, the bulk of TNF-mediated barrier loss is the result of MLCK activation.[29,67,70,72,89] MLCK has likewise been demonstrated to play a key role in driving TNF-induced endothelial barrier dysfunction in vitro.[90,91] In these studies, MLCK activation has been linked to increased permeability due to both cell contraction and caspase-dependent apoptotic signaling.[92,93]

Myosin Light Chain Kinases: Expression and Subcellular Localization

Three separate proteins are produced from the mammalian *MLCK* gene.[94,95] "Short" MLCK is a ~120 kD protein expressed in smooth muscle that is required for muscle contraction.[96] Short MLCK knockout results in perinatal death,[97] while inducible knockout in adult animals causes hypotension, bladder dysfunction, and severe disruption of peristalsis.[96] Telokin is a ~20 kD protein expressed from a intronic promoter within the distal portion of the MLCK gene. Telokin contains only carboxy terminal domains distal to the enzymatic and regulatory domains and has not been studied extensively.[98] The third gene product, "long" MLCK, is a ~225 kD protein expressed in epithelium, endothelium, and most cultured cell lines.[95,99-102] At least five long MLCK splice variants are expressed in endothelia,[94] but only two of these, MLCK1 and MLCK2, are expressed in intestinal epithelium.[99] These intestinal epithelial MLCK splice variants differ by only a single 69 amino acid region, encoded by a single exon, that is present in MLCK1 but not MLCK2. This region contains Src kinase phosphorylation sites and a potential SH2-binding domain,[94,103] and one study suggest that Src-mediated phosphorylation of this domain enhances MLCK1 activity.[103] In human intestinal epithelium, MLCK1 expression is restricted to well-differentiated villus enterocytes, where it localizes to the perijunctional actomyosin ring.[99] In contrast, MLCK2 is expressed throughout the crypt-villus axis and is present in multiple subcellular locations.[99] Selective knockdown of MLCK1 increases intestinal epithelial barrier function in vitro.[99] Moreover, in patients with inflammatory bowel disease, MLCK1 expression correlates with disease activity.[104]

Acute MLCK Activation

Systemic T-cell activation induced by anti-CD3 administration or direct injection of recombinant TNF both cause acute intestinal epithelial barrier dysfunction and diarrhea in mice.[29,105-108] Both stimuli trigger long MLCK activation and MLC phosphorylation within the intestinal epithelium.[29,108] To further asses the role of MLCK in these models, investigators turned to long *MLCK*$^{-/-}$ mice.[30] These mice are viable, fertile, and grow and gain weight normally.[30] However, *MLCK*$^{-/-}$ mice are protected from acute barrier dysfunction and diarrhea induced by either systemic T-cell activation or TNF administration.[29,108] Pharmacological MLCK inhibition also prevented acute barrier dysfunction induced by these stimuli.[108] However, while both genetic and pharmacological MLCK inhibition prevented net intestinal water secretion, absorption was still diminished.[108] Further study showed that the effects of TNF and LIGHT on barrier dysfunction are similar and additive,[108] but that TNF induces water secretion while LIGHT increases absorption.[108] This striking difference is explained by TNF-, but not LIGHT-, induced inhibition of Na^+ absorption, due to endocytic removal of the apical Na^+-H^+ exchanger NHE3.[108] Thus, the direction of paracellular water movement can be defined by the status of active transepithelial ion transport. These experiments illustrate the passive nature of paracellular transport and how transcellular and paracellular pathways can be coordinated.[85]

Systemic T-cell activation and TNF or LIGHT administration induce endocytosis of the tight junction protein occludin.[29,108] This occludin endocytosis requires MLCK, as it does not occur in *MLCK*$^{-/-}$ mice or mice treated with a pharmacological MLCK inhibitor.[29,85] In vivo analysis using transgenic mice expressing fluorescent tight junction proteins showed that TNF-induced endocytosis is preceded by focal concentration of occludin at the tight junction.[109] This endocytosis requires cholesterol-enriched membrane

microdomains, dynamin II, and caveolin-1, consistent with caveolar endocytosis.[109] Further, each of the treatments that blocked occludin endocytosis also prevented TNF-induced barrier loss and diarrhea.[109] While this could suggest that occludin endocytosis is merely a marker of barrier loss, mice that overexpress occludin within the intestinal epithelium are partially protected from TNF-induced barrier loss.[109] Thus, acute occludin contributes removal from the tight junction functionally to the observed increases in permeability. Similar conclusions have recently been reported on the basis of in vitro analyses.[109,110]

The role of MLCK has also been evaluated in acute in vivo models of pathological increases in endothelial permeability as well.[30,111-114] *MLCK*$^{-/-}$ mice or mice treated with a pharmacological MLCK inhibitor have significantly reduced pulmonary inflammatory injury after lipopolysaccharide (LPS) lavage or mechanical ventilator stress, and in the heart after ischemia-reperfusion. Mechanistically, a recent study in the LPS model showed that MLCK inhibition decreased both lung leukocyte infiltration and serum cytokine signaling.[114] These studies have established a role for MLCK as a viable target to improve outcome after various forms of acute injury.[30,112,114,115]

Chronic MLCK Activation

Although intestinal epithelial MLCK activation is a requirement for acute TNF-induced barrier dysfunction and diarrhea, chronic MLCK activation is not sufficient to cause overt disease. Mice with intestinal epithelial-specific expression of constitutively active MLCK (CA-MLCK) grow and gain weight normally, despite persistent increases in epithelial myosin light chain phosphorylation and intestinal paracellular permeability.[35] However, detailed analysis revealed subclinical mucosal immune activation, as demonstrated by elevated colonic mucosal transcription of IFNγ, TNF, IL-13 and IL-10.[35,116] Similarly, another study has shown that transient intestinal barrier defects induce an immunoregulatory response that can protect mice from subsequent insults.[117] A similar phenomenon may also occur in humans, where intestinal barrier defects are associated with disease, but are, in and of themselves, insufficient to cause disease. This is demonstrated by the fact that as many as 10% of first-degree relatives of Crohn's disease patients have elevated intestinal permeability despite being healthy.[32,118] Although these relatives tend to carry a specific mutation in the Crohn's disease susceptibility gene *NOD2*,[119] it is not known if they have an increased risk of developing disease. However, there are some data in human subjects that suggest an association between increased intestinal permeability and disease onset. For example, during remission, increased intestinal permeability is associated with an increased risk of relapse to active Crohn's disease.[34] Together, these observations suggest that intestinal permeability defects may contribute to Crohn's disease pathogenesis. Moreover, a single episode of infectious gastroenteritis caused by *Salmonella* or *Campylobacter*, which is associated with increased intestinal permeability,[120] confers a significant two-to-three-fold increase in risk of developing inflammatory bowel disease.[121]

To directly assess the contribution of barrier dysfunction to pathogenesis of intestinal disease, colitis was induced in CA-MLCK transgenic mice using the $CD4^{+}CD45RB^{hi}$ adoptive transfer model.[35] In this model, immunodeficient mice receive $CD4^{+}CD45RB^{hi}$ effector T cells.[122] The colitis that develops requires the presence of the gut microbiota[123] and induces changes in cytokine, chemokine, and chemokine receptor expression that are similar to human inflammatory bowel disease.[124] The CA-MLCK transgenic mice lost weight and developed clinical and biochemical features of disease more rapidly than their nontransgenic littermates.[35] Moreover, the transgenic mice developed more

severe disease and had reduced survival.[35] Thus, while increased intestinal permeability is insufficient to cause disease, it can accelerate disease progression and increase severity. Ongoing studies suggest that the converse may also be true, as disease onset is delayed and severity is decreased after $CD4^{+}CD45RB^{hi}$ adoptive transfer in *MLCK*$^{-/-}$ mice.[64] The clinical implications of these data are significant, as they suggest that MLCK inhibition may be useful in treatment of active disease and, perhaps, as maintenance therapy to prevent relapse from clinical remission. The role of chronic MLCK activation in endothelia, and its potential as a therapeutic target, has not been investigated in inflammatory bowel disease.

INFLAMMATORY BOWEL DISEASE AND GRAFT-VERSUS-HOST DISEASE

Shared Disease Mechanisms

Inflammatory bowel disease and graft-versus-host disease have many characteristics in common (Table 1). The first is a dependence on the gut microbiota. Neither $CD4^{+}CD45RB^{hi}$ adoptive transfer colitis nor the spontaneous colitis observed in IL-$10^{-/-}$ mice develops in germ-free conditions.[125-128] Moreover, the broad spectrum antibiotic metronidazole can prevent colitis in IL-$10^{-/-}$ mice[129] and has been used to maintain remission in Crohn's disease.[130,131] Similarly, severity of experimental graft-versus-host disease is reduced in both germ-free and antibiotic-treated mice,[132-134] and antibiotics are associated with reduced incidence and severity of graft-versus-host disease in human patients.[135-139] While the specific bacterial components responsible for these features are not clear, it is notable that antagonism of lipopolysacchride (LPS) signaling can also reduce graft-versus-host disease in mice.[140,141]

A second feature shared by inflammatory bowel disease and graft-versus-host disease is the central role of TNF signaling. TNF is elevated in many acute and chronic experimental colitis models, including $CD4^{+}CD45RB^{hi}$ adoptive transfer colitis and IL-$10^{-/-}$ mice,[35,142-145] and TNF neutralization reduces disease severity.[122,146,147] Moreover, TNF neutralizing antibodies have been extraordinarily potent therapeutic agents in Crohn's disease and some ulcerative colitis patients,[148-150] where they dampen immune responses and restore intestinal barrier function.[149,151,152] In graft-versus-host disease, serum TNF concentrations correlate with disease severity,[28,77] and TNF neutralization reduces target organ injury.[27,74] Moreover, although not the standard of care, clinical trials of TNF neutralizing antibodies are ongoing in graft-versus-host disease.[79,80,83]

A third mechanistic link between inflammatory bowel disease and graft-versus-host disease is compromised barrier function. As discussed above, the association of barrier dysfunction with inflammatory bowel disease is extensively documented in both patients and experimental models. Similarly, barrier function is compromised in human and experimental graft-versus-host disease,[26,27] and the extent of barrier loss correlates with severity of graft-versus-host disease.[26] Experimental graft-versus-host disease is also associated with increases in serum LPS concentration and bacterial translocation.[28,153-156] The barrier dysfunction that occurs in graft-versus-host disease may be related to TNF production, as LPS stimulation can cause both donor and host cells and host to produce proinflammatory cytokines that may contribute to barrier loss.[157,158]

TLRs in Experimental Inflammatory Bowel Disease and Graft-Versus-Host Disease

In addition to the shared mechanisms above, inflammatory bowel disease and graft-versus-host disease are both associated with inherited single nucleotide polymorphisms (SNPs) in pattern recognition receptors (PRRs) that recognize conserved microbe-specific molecular patterns.[159] These include the extracellular toll-like receptors (TLRs) and intracellular nucleotide-binding oligimerization domain (NOD) receptors, such as NOD2. Given the aforementioned contributions of the intestinal microbiota to inflammatory bowel disease and graft-versus-host disease, the role of microbial sensing via both TLRs and NODs has been investigated.

TLRs are expressed on both hematopoietic and nonhematopoietic cell types and recognize a variety of microbial molecules such as lipopolysaccharide (TLR4), flagellin (TLR5), and unmethylated cytosine phosphorothioate-guanine (CpG) DNA that is present in viruses and bacteria (TLR9). Support for the role of TLRs in colitis models is provided by studies showing that MyD88, the intracellular signal transducer for all TLRs except TLR3, is required for donor cells to cause disease in $CD4^{+}CD45RB^{hi}$ colitis[160,161] as well as colitogenesis in IL-$10^{-/-}$ mice.[162] T-cell responses to self and luminal antigens can drive colitis,[163,164] and, in the case of $CD4^{+}CD45RB^{hi}$ colitis, disease can be cured by adoptive transfer of regulatory T cells.[165] While incompletely understood, TLR-mediated signals are thought to trigger secretion of proinflammatory cytokines and inhibit the function of T regulatory cells.[166-168]

In graft-versus-host disease models, host conditioning leads to epithelial damage, systemic exposure to microbial products, and TLR activation.[169,170] Consistent with this, *TLR9*$^{-/-}$ mice have decreased graft-versus-host disease and improved survival after allogeneic bone marrow transplantation.[155,171] Bone marrow chimera studies suggest that TLR9 signaling in nonhematopoietic cells (i.e., epithelial cells) is responsible for decreased alloreactivity of donor T cells.[171] LPS-mediated stimulation of TLR4 has also been shown to play an important role in experimental graft-versus-host disease.[172] Donor cells resistant to LPS cause less graft-versus-host disease, and antagonism of LPS with a lipid-A analog immediately after bone marrow transplant decreased graft-versus-host disease.[76,140] However, TLR signaling may also moderate disease, as suggested by the observation that host TLR4 inhibition exacerbates experimental graft-versus-host disease.[173] The importance of homeostatic TLR sensing has been noted in other experimental models. For example, acute colitis induced by oral administration of dextran sulfate sodium, which causes diffuse colonic damage, is far more severe in MyD88$^{-/-}$ mice or in mice pretreated with antibiotics for four weeks prior to induction of colitis.[174] In addition, spontaneous colitis develops in a subset of TLR5$^{-/-}$ mice.[175] However, MyD88 deficiency in host antigen-presenting cells does not appear to affect graft-versus-host disease severity.[176] The explanation for this unexpected result is not certain, although it has been proposed that there may be non-TLR-mediated activation pathways that are sufficient to drive graft-versus-host disease.[177] Alternatively, because it is necessary for signaling by most TLRs, MyD88 knockout may have limited both pathogenic and homeostatic effects of TLR activation and thereby negated any protective effects of TLR inhibition.

Table 1. Shared features of active inflammatory bowel disease and graft-versus-host disease

	Active Inflammatory Bowel Disease	Graft-Versus-Host Disease
Intestinal histopathology	Crypt architectural distortion Crypt abscesses Epithelial regeneration Ulceration and fissures Dense lamina propria neutrophilic and lymphoid infiltrates Granulomas (Crohn's disease) Transmural disease, strictures (Crohn's disease) Serositis (Crohn's disease)	Crypt dropout (atrophy) Crypt cell apoptosis Ulceration is uncommon Sparse lamina propria Lymphoid infiltrates
Clinical symptoms	Abdominal pain Nausea Malabsorption Weight loss Bloody or mucoid diarrhea Obstruction Extraintestinal disease can involve skin, eyes, joints, and liver	Abdominal pain Nausea Malabsorption Weight loss Watery diarrhea Extraintestinal disease can involve skin, lungs, and liver
Treatments for human disease	Immunosuppressants (e.g., corticosteroids) Antiinflammatory agents (e.g., 5-amino-salicylates) Immunomodulators (e.g., methotrexate) Biologics (e.g., infliximab) Calcineurin inhibitors (e.g., tacrolimus) Antibiotics (to maintain remission) Surgery	Immunosuppressants (e.g., corticosteroids) Calcineurin inhibitors (e.g., tacrolimus) Antibiotics Biologics (e.g., infliximab, in clinical trials)
Role of microbiota in experimental disease	Required	Required
TNF	Tissue TNF elevated in patients Serum TNF elevated in experimental disease Anti-TNF treats clinical and experimental disease	Serum TNF correlates with severity of clinical and experimental disease Anti-TNF treats experimental disease
Barrier dysfunction	Present in patients with active disease Present before overt disease in experimental models May predict relapse in patients during remission Present in some healthy first-degree relatives Improves after anti-TNF therapy in patients and experimental models	Correlates with disease severity in patients Present in experimental models Improves after anti-TNF therapy in experimental models

continued on next page

Table 1. Continued

	Active Inflammatory Bowel Disease	Graft-Versus-Host Disease
Toll-like receptors (TLRs)	Polymorphisms of TLR1, TLR2, TLR4, TLR 5, TLR6 and TLR9 are associated with disease	Recipient TLR4 and TLR9 polymorphisms associated with reduced graft-versus-host disease severity and improved survival, respectively
NOD2	First inflammatory bowel disease-associated gene identified	Donor and recipient mutations are associated with more severe graft-versus-host disease

NOD2 in Inflammatory Bowel Disease and Graft-Versus-Host Disease

NOD2 is an intracellular sensor for microbial molecules that has been linked to the pathogenesis of both inflammatory bowel disease and graft-versus-host disease. NOD2 detects muramyl dipeptide, a product of the synthesis and breakdown of peptidoglycan, which is a cell wall component of most bacteria.[177] Experimental graft-versus-host disease was exacerbated in *NOD2*$^{-/-}$ recipients receiving wild-type bone marrow, but *NOD2*$^{-/-}$ bone marrow did not alter disease severity in wild-type recipients.[177] This may be because *NOD2*$^{-/-}$ antigen presenting cells stimulate allogeneic T cells more effectively than wild-type antigen presenting cells.[177] This hypothesis is consistent with data indicating a role for NOD2 regulation of antigen presenting cell function in experimental colitis.[178-180]

Despite these data, the mechanisms by which *NOD2* mutations affect inflammatory bowel disease and graft-versus-host disease are a matter of debate.[181,182] One proposed effect of *NOD2* mutations is impaired production of antimicrobial molecules by the specialized intestinal epithelial Paneth cells.[183-185] This may allow bacterial overgrowth and thus stimulate an inflammatory response. Alternatively, others have suggested that *NOD2* mutations result in dysregulated TLR signaling. The most direct evidence for this hypothesis is the tolerogenic effect of NOD2 activation in antigen presenting cells.[178-180,186]

Genetic Links in Human Inflammatory Bowel Disease and Graft-Versus-Host Disease

Although the genetics of inflammatory bowel disease are complex, the mutations most strongly associated with inflammatory bowel disease are polymorphisms in the *NOD2* locus.[187,188] In the context of allogeneic bone marrow transplantation, several studies have shown a correlation between donor and recipient *NOD2* polymorphisms and greater severity of graft-versus-host disease.[189-192] Consistent with the importance of pattern recognition receptors, TLR mutations have also been linked to inflammatory bowel disease.[193-197] Although fewer studies have analyzed TLR mutations in graft-versus-host disease, recent reports suggest that recipients with *TLR9* gene variants associated with reduced TLR9 expression had a significantly lower treatment-related mortality and relapse

rate.[198,199]. Although these results are not universal,[200,201] this may be due to reduced impact of TLR mutations as a consequence of antibiotic use during the peritransplant period.[177]

Features of Disease in Human Inflammatory Bowel Disease and Graft-Versus-Host Disease

Histologically, the most characteristic feature of intestinal graft-versus-host disease is crypt cell apoptosis.[202] When severe, there may be villous blunting and crypt loss. Inflammatory bowel disease is less characterized by apoptosis, but, like graft-versus-host disease, crypts are destroyed by infiltrating neutrophils and other inflammatory cells. Clinically, patients with inflammatory bowel disease and graft-versus-host disease can both present with abdominal pain, nausea, diarrhea, nutrient malabsorption and weight loss. However, diarrhea is typically watery in graft-versus-host disease, in contrast to the bloody or mucoid diarrhea of inflammatory bowel disease. Thus, although clear distinctions exist, there can be significant clinical and histological overlap between graft-versus-host disease and inflammatory bowel disease.

In addition to shared mechanisms and clinical features, treatments for inflammatory bowel disease and graft-versus-host disease can be similar. Immunosuppressants, including corticosteroids, tacrolimus and cyclosporine A, and methotrexate are often used for prophylaxis against and treatment of graft-versus-host disease.[83] Corticosteroids are also a mainstay of inflammatory bowel disease therapy, and tacrolimus can be useful in management of Crohn's disease.[203-207] As noted previously, anti-TNF antibodies are tremendously effective in inflammatory bowel disease and have also shown promise in early studies of graft-versus-host disease.[79,81-83,208-211] Moreover, growth factors, including keratinocyte growth factor (KGF), have been used in experimental animals and patients after bone marrow transplantation to protect the intestine from the pretransplant conditioning and limit graft-versus-host disease,[154,212-214] and to accelerate mucosal healing and re-establish barrier function in inflammatory bowel disease.[215,216]

The role of the intestinal microbiota in inflammatory bowel disease is established, and multiple studies have shown that antibiotics reduce graft-versus-host disease in patients,[135-139] particularly when administered during the interval immediately after bone marrow transplantation. Although antibiotics do not sterilize the gut, they do alter microbial composition. Thus, one mechanism of their efficacy may be to correct the loss of bacterial diversity and outgrowth of *Enterobacteriaceae* in graft-versus-host disease[217] and inflammatory bowel disease.[217-220] Although side-effects can limit the utility of chronic antibiotic use in inflammatory bowel disease, prophylatic antibiotics are a standard part of bone marrow transplantation protocols at some institutions.[221,222]

A final clinical feature linking inflammatory bowel disease and graft-versus-host disease is the involvement of specific extraintestinal target organs in each disease. Graft-versus-host disease is a systemic disease that preferentially targets the intestine, liver, skin and lungs.[74,172] Extraintestinal disease manifestations in inflammatory bowel disease also involve liver and skin, although lungs are less commonly affected.[223-227] The reason for the constellations of target organs in inflammatory bowel disease and graft-versus-host disease is not clear, although ongoing research suggests that it is likely a combination of the unique ability of intestinal damage to propagate systemic inflammation and the function of these target organs as epithelial barriers that interface with immune-stimulating antigens.[84]

ENDOTHELIAL AND EPITHELIAL BARRIER DYSFUNCTION IN GRAFT-VERSUS-HOST DISEASE

Given the role of barrier dysfunction in inflammatory bowel disease, and the similarities between inflammatory bowel disease and graft-versus-host disease, it is possible that barrier dysfunction is a crucial regulator of graft-versus-host disease severity. The role of intestinal epithelial barrier dysfunction in inflammatory bowel disease has been modeled previously.[85,89,228] We propose a similar model to describe the role of endothelial and epithelial barrier dysfunction in graft-versus-host disease (Fig. 4). This model has

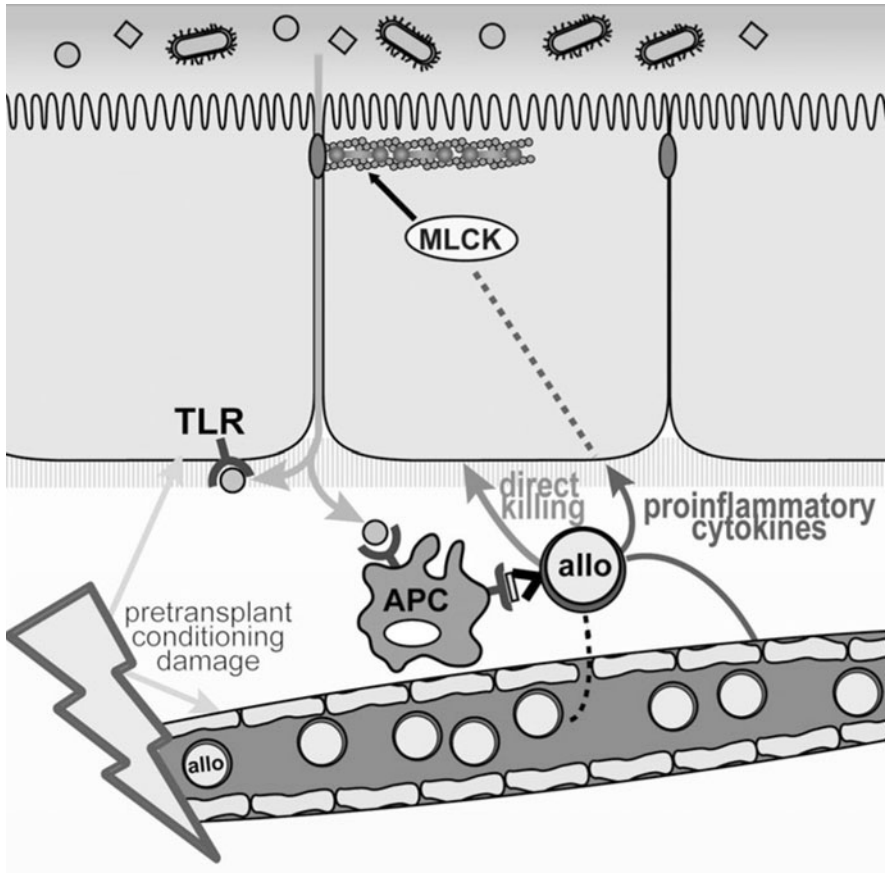

Figure 4. Modeling endothelial and epithelial barrier dysfunction in graft-versus-host disease. Pretransplant conditioning with irradiation and/or chemotherapy damages the endothelial and epithelial barriers. This leads to increased extravasation of alloreactive donor T cells from the endothelium into target tissue. In the intestinal epithelium, conditioning-induced barrier loss leads to translocation of luminal contents and activation of the underlying antigen presenting cells (APCs) through TLRs. This enhances activation and proliferation of alloreactive donor cells, which secrete proinflammatory cytokines and directly kill target tissue. This results in further proinflammatory cytokine production, which acts on endothelium and epithelium to sustain barrier dysfunction and donor T-cell infiltration and activation, leading to graft-versus-host disease. A color version of this figure is available at www.landesbioscience.com/curie.

implications for graft-versus-host disease treatment, and suggests that restoration of barrier dysfunction might limit the inflammatory environment that drives graft-versus-host disease, thereby reducing the need for immunosuppressants.

The Host Inflammatory Environment in Graft-Versus-Host Disease

The target of nearly all immunosuppressive treatments is donor T cells, which have been shown to be the main mediators of graft-versus-host disease.[172,229] However, experimental graft-versus-host disease models have shown that donor T cells are highly dependent on the host inflammatory environment to become fully activated and cause graft-versus-host disease.[230-232] Much like a cognate T-cell response, donor cells rely on "danger signals" to become licensed, enter target tissue, and cause graft-versus-host disease. Thus, altering the inflammatory environment could be a potential means to decrease T-cell stimulation and reduce graft-versus-host disease pathogenesis.

Endothelial Barrier Dysfunction in Graft-Versus-Host Disease

It is well recognized that endothelial damage is a feature of graft-versus-host disease.[233,234] Endothelial damage post-bone marrow transplantation can contribute to transplant-associated microangiopathy, veno-occlusive disease, accelerated arteriosclerosis, and graft-versus-host disease.[234] Studies on the origin of the endothelial damage in graft-versus-host disease point to pretransplant conditioning with either irradiation or chemotherapy.[235-238] Consistent with this, the extent of endothelial damage correlates with radiation dose.[236,237] The resulting barrier dysfunction[25] is likely to enhance donor cell infiltration into target tissues. It is currently unclear whether restoration of endothelial barrier function, by preventing endothelial cell contraction for example, might be beneficial in graft-versus-host disease outcome.

Epithelial Barrier Dysfunction in Graft-Versus-Host Disease

A role for intestinal epithelial barrier function in graft-versus-host disease has been implicated in several experimental models. The data suggest that intestinal epithelial barrier dysfunction caused by the pretransplant conditioning is propagated by alloreactive donor cells and the inflammatory milieu. Consistent with this, both barrier dysfunction and intestinal damage were blocked by TNF antagonism in experimental graft-versus-host disease.[27] Along with TNF, other proinflammatory cytokines such as LIGHT and IL-1β are implicated in graft-versus-host disease.[27,73,77,239-241] As described above, each of these has been shown to reduce intestinal epithelial barrier function by enhancing MLCK expression and activity.[63,68-72] Our recent studies have focused on MLCK-mediated barrier dysfunction in graft-versus-host disease after minor antigen mismatch bone marrow transplantation. Analysis of intestinal permeability over the course of graft-versus-host disease reveals that both syngeneic and allogeneic recipients have increased permeability in the peritransplant period, likely due to irradiation conditioning, but that barrier dysfunction persists only in allogeneic recipients. Moreover, *MLCK*$^{-/-}$ mice have significantly reduced graft-versus-host disease severity.[84]

Modeling Barrier Dysfunction in Graft-Versus-Host Disease and Therapeutic Approaches

We propose that endothelial and epithelial barrier dysfunction due to pretransplant conditioning with irradiation and/or chemotherapy initiates damage to endothelial and epithelial barriers. In the case of the endothelium, this leads to activation, upregulation of leukocyte homing molecules, and increased infiltration of alloreactive donor T cells into target tissue. In the intestinal epithelium, conditioning-induced barrier dysfunction leads to translocation of luminal contents and activation of the underlying lamina propria cells, such as macrophages and other antigen presenting cells. This enhances activation and proliferation of alloreactive donor cells, which return to target tissue and cause further tissue damage. This results in greater inflammatory cytokine production and increased barrier dysfunction, donor T-cell infiltration, and graft-versus-host disease in a self-amplifying cycle. This model explains the observations that reducing the intensity of pretransplant conditioning and use of prophylactic antibiotics limits graft-versus-host disease in both experimental models and patients.[28,133-135,137,242-244]

Approaches to reduce tissue damage from pretransplant conditioning have been used clinically, including intestinal protection with KGF.[245] However, this particular study only evaluated protection after autologous bone marrow transplantation. Other studies have investigated the use of KGF in allogeneic bone marrow transplantation and observed a decrease in oral mucositis, but not a reduction in intestinal damage or overall graft-versus-host disease.[241,246,247] This may indicate that while KGF reduces epithelial damage from pretransplant conditioning, it is insufficient to counter a strong allogeneic reaction. Recently, several studies have focused on R-spondin1, a Wnt agonist that has been shown to specifically stimulate intestinal stem cells and protect from epithelial damage induced by chemotherapy or irradiation.[248,249] Importantly, pretransplant R-spondin1 treatment reduced systemic graft-versus-host disease in mice,[249] consistent with the hypothesis that the intestine is an initiation site for systemic graft-versus-host disease.[84]

In addition to reducing tissue damage and bacterial translocation induced by pretransplant conditioning, restoring barrier function during ongoing graft-versus-host disease could have beneficial effects. To date, no studies have investigated the direct effect of reducing endothelial barrier dysfunction during graft-versus-host disease. Endothelial MLCK and ROCK are both potential therapeutic targets. Endothelial MLCK has already been successfully targeted in other in vivo models of endothelial barrier dysfunction,[30,112,114] and ROCK inhibitors have been used to reduce endothelial barrier loss.[250-252] Further, given emerging experimental data, epithelial MLCK inhibitors could be a viable approach to limit intestinal barrier dysfunction in graft-versus-host disease.

CONCLUSION AND FUTURE PERSPECTIVE

Endothelial and epithelial barriers serve to regulate water, solute and cell movement between tissue compartments. When these barriers are disrupted, such as from pretransplant conditioning prior to bone marrow transplantation, they can initiate and perpetuate an inflammatory environment that leads to graft-versus-host disease. Further studies on

the role of the endothelial and epithelial barriers in graft-versus-host disease will allow development of additional therapeutic approaches aimed at maintaining or re-establishing the barrier, thereby reducing the inflammatory environment and disease development. As the severity of graft-versus-host disease has a significant reliance on the extent of antigen mismatch, it is likely that multiple approaches targeting the endothelial and epithelial barriers and other nonhematopoietic aspects will have to be used in concert, along with reduced immunosuppression, to exert a productive and reproducible effect in patients.

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CHAPTER 6

STRUCTURE AND REGULATION OF INTESTINAL EPITHELIAL TIGHT JUNCTIONS

Current Concepts and Unanswered Questions

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Abstract: Intestinal epithelium serves as a key interface between internal body compartments and the gut lumen. The epithelial layer forms a physical barrier that protects the body from the harmful environment of the lumen and also mediates vectorial fluxes of fluids, nutrients and waste. Increased permeability of the epithelial barrier is a common manifestation of different gastrointestinal diseases that enhances body exposure to external pathogens thereby exaggerating mucosal inflammation. Barrier properties of the intestinal epithelium are regulated by specialized adhesive plasma membrane structures known as tight junctions (TJs). It is generally believed that disease-related increase in intestinal permeability is caused by defects in TJ structure and functions. This chapter describes the molecular composition of intestinal epithelial TJs, basic mechanisms that regulate TJ functions in healthy gut mucosa as well as molecular events that contribute to increased mucosal permeability during intestinal inflammation. The chapter outlines our current understanding of TJ structure and dynamics and highlights several unresolved questions regarding regulation of this junctional complex under normal conditions and in gastroenterological diseases.

INTRODUCTION

Epithelial lining of the gut plays a number of vital roles including regulation of water, nutrient and waste fluxes and establishment of the protective barrier between the body interior and noxious content of the gut lumen.[1-3] Differentiated intestinal

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epithelium represents a monolayer of columnar-shaped polarized cells with a free apical pole facing the gut lumen, a basal pole attached to the basement membrane and extended lateral surfaces that form adhesive contacts with adjacent cells. Such architecture provides a physical basis for establishment of the paracellular barrier and regulation of the vectorial transcellular transport of solutes and macromolecules.[1-3]

Integrity and barrier properties of the intestinal epithelium are determined by several types of adhesive structures located along the lateral plasma membrane that are called junctions.[2,4] The most apical tight junctions (TJs) play a key role in formation of the paracellular barrier and establishment of the apico-basal cell polarity. TJs have been initially visualized by transmission electron microscopy (EM) as areas of very close intercellular contacts sealing the paracellular space.[5] Subsequent high resolution freeze-fracture EM revealed an elaborated architecture of this sealing zone that appeared as a honeycomb network of interconnecting strands or fibrils physically linking two opposing plasma membranes.[6] This fibrillar network encircles the entire cell and its complexity (number of strands) is thought to correlate positively with the tightness of the paracellular barrier.[7,8]

The TJ barrier has two major functional properties, permeability and permselectivity, that can be determined experimentally.[2,9] Permeability is measured by transepithelial electrical resistance (TEER), whereas permselectivity is a qualitative characteristic that indicates barrier preferences for either cations or anions and within the particular ion series. Depending on their barrier properties, gastrointestinal epithelia have been classified into three categories: Leaky, with TEER below 200 Ω cm^2, moderately leaky, with TEER in the range of 300-1000 Ω cm^2 and tight with TEER higher than 1,000 Ω cm.[2,9,10] Mammals have leaky epithelium in the small intestine, moderately leaky in the colon and tight epithelial barrier in the gastric fundus and esophagus.[9,10] Likewise, human colonic carcinoma-derived epithelial cell lines that are frequently used to study TJ regulation in vitro, create either moderately leaky (Caco-2 cells) or tight (T84, SK-CO15) barriers.[11-14] Despite the differences in the permeability, leaky and tight intestinal epithelial TJs have similar permselectivity. They are cation selective and show preference for K^+ and Na^+ over Cl anions.[9,10] Such cation selectivity is important for epithelia with apical chloride secretion such as in the small intestine where preferential paracellular passage of Na^+ and limited back diffusion of Cl^- is important for excretion of NaCl and water.

Recent studies provided the first semi-quantitative model of the TJ barrier in simple mammalian epithelia.[15,16] A key feature of this model is the existence of two distinct paracellular pathways. The major pathway that carries most of the ionic fluxes has been described as the pore pathway that is permeable for small solutes with a molecular radius below 4 Å. An additional nonpore pathway is thought to represent temporary breaks in TJ contacts that are permeable for larger than 4 Å molecules. These two pathways have been examined in cultured intestinal and renal epithelial cell monolayers as well as in small intestinal epithelium ex vivo.[10,15,16] The pore and nonpore pathways have different regulatory mechanisms and may play different roles in normal epithelial permeability and during barrier breakdown in diseases.[10,15,17,18] It is noteworthy that such a two-pathway model is based exclusively on the results of permeability profiling experiments and ultrastructural studies are needed to visualize molecular architecture of the paracellular barrier within epithelial TJs.

MOLECULAR COMPOSITION OF INTESTINAL EPITHELIAL TJs

It is generally accepted that TJ fibrils are composed by large complexes of integral and peripheral membrane proteins.[10,19-21] The integral membrane proteins directly mediate cell-cell adhesions and create the paracellular barrier, whereas peripheral membrane components that assemble a so called 'cytosolic plaque' play key roles in regulating TJ stability and remodeling.[10,19-21] The adhesive properties of TJs are determined by three major types of integral proteins that include members of the claudin family, tight junction-associated MARVEL proteins (TAMP) family and immunoglobulin-like proteins such as junctional adhesion molecule (JAM)-A and coxsackievirus and adenovirus receptor (CAR).[10,19-21] The cytosolic plaque of TJ contains a large number of molecular constituents including *Zonula occludens* (ZO)-1 proteins, cingulin and afadin.[20-22] Although numerous studies from different laboratories have examined the contribution of individual TJ proteins in the integrity and functional properties of epithelial barriers, the exact role of many of these junctional components remain elusive and controversial.

Claudins

Claudins consist of a large protein family with approximately 24 members in mammalian epithelia.[20,23] They are small, four transmembrane domain (tetraspan) proteins possessing two extremely hydrophobic extracellular loops that mediate various adhesive interactions at the opposing plasma membranes. Expression of claudins in fibroblastic L cells was shown to generate TJ-like plasma membrane fibrils,[24] whereas genetic or pharmacological removal of claudins from the plasma membrane resulted in TJ disassembly in various model epithelia.[25-27] These experiments highlighted claudins as key structural components of TJ strands. Different types of epithelial cells simultaneously express several claudins and therefore it is not surprising that these proteins can be engaged in homotypical and heterotypical adhesive interactions.[20,23] Interestingly, reconstruction experiments using claudin-expressing L cells revealed certain specificity of such heterotypical interactions. For example TJ strands were formed by mixing claudin-1 and 3 or claudin 2 and 3 expressing cells but not cells bearing claudin 1 and 2.[10,20,23] However, it remains poorly understood how different claudins interact within native TJs in the intestinal epithelium.

Several studies employing overexpression of different claudin isoforms firmly established that claudins control permeability and permselectivity of the paracellular pore pathway.[10,20,23] Based on their functional effects, these proteins can be divided into two groups: Tight claudins, expression of which increases TEER and leaky claudins that decrease barrier properties of model epithelial monolayers. The majority of claudins tested so far (claudin-1, 4, 5, 7, 8, 11, 14, 15, 16, 18, and 19) belong to the tight group whereas only claudins 2 and 10 are leaky.[10,20,23] Expression of 19 claudin isoforms in the intestinal mucosa of small rodents has been examined in several studies. They detected mRNA and protein expression for the majority of claudins except claudin 6, 16 and 19.[28,29] It is noteworthy that different claudin isoforms had distinct localization patterns within the gut or even within the same gut segments. For example, claudins 8 and 13 were predominantly expressed in colon, whereas claudins 12 and 15 had the strongest expression in ileum and jejunum respectively.[28] Furthermore, even co-expressed claudins can be spatially separated along the crypto-villous axis. For example, colonic expression of claudins 2 and 15 was limited to the crypt epithelium, whereas claudin 4 was found exclusively

at the surface.[28,29] Such mosaic expression of different claudins in the gut is likely to determine differences in paracellular ionic fluxes in each segments of the intestinal tract.

Recent pharmacological and genetic studies demonstrated the roles of several claudins in regulating TJ morphology and barrier integrity in cultured colonic epithelial cells and gastrointestinal mucosa of experimental animals (Table 1). For example, synthetic peptides that mimic the first extracellular loop of claudin-1 were shown to induce the decrease in TEER and TJ disassembly in T84 cells and to increase permeability of gastric epithelium in vivo.[26] Furthermore, *Clostridium perfringens* enterotoxin that is known to selectively displace claudins-3 and 4 from TJs[27] increased permeability of Caco-2 monolayers[30] and enhanced absorption of macromolecules in rat intestine.[25] Abnormal development of the intestinal tract has been detected in claudin-15 deficient mice that were characterized by a dramatic expansion of small intestine (duodenum and jejunum) resulting in a megaintestine.[31] This abnormal phenotype was not due to altered epithelial cell-cell adhesions and was associated with increased proliferation of intestinal epithelial cells. Similarly, claudin-15 knockout in zebrafish resulted in abnormal formation of multiple gut lumens which was not accompanied by noticeable changes in intestinal TJ structure and permeability.[32] Claudin-1 and claudin-5 deficient mice died just after birth and morphology and functions of their intestinal epithelium have not been investigated.[33]

TAMP Family

The TAMP family of transmembrane TJ proteins is composed of occludin, tricellulin and marvelD3.[36,39] These tetraspan proteins possess structural domains that mediate cell-cell adhesion as well as intracellular trafficking and protein targeting to membrane rafts.[36] In cultured intestinal epithelial cells and tissue sections of intestinal mucosa, occludin and marvelD3 were uniformly localized in all TJs whereas tricellulin selectively accumulated at tricellular junctions.[36,48] Despite the fact that TAMP represent the first identified transmembrane components of TJs, their exact physiological roles remain unclear. For example, a peptide or a monoclonal antibody that inhibit interactions with the second extracellular loop of occludin were shown to attenuate TJ re-assembly and barrier recovery in calcium-switched T84 cells,[34,35] whereas siRNA-mediated knockdown of occludin delayed development of the paracellular barrier in Caco-2 monolayers.[36] On the other hand, overexpression of occludin in L cells did not lead to assembly of TJ-like fibrils,[49] while occludin-knockout mice did not show obvious abnormalities in intestinal epithelial barrier architecture and permeability[38] and did not develop spontaneous gut diseases.[37] Similarly, siRNA-mediated downregulation of other members of the TAMP family failed to prevent establishment of the paracellular barrier in Caco-2 cells.[36,39] Overall, this apparent dispensability of individual TAMP for proper functioning of the intestinal epithelial barrier can be explained by either high redundancy of these homologous proteins or that they play other cellular roles, which are unrelated to regulation of epithelial cell-cell adhesions.

JAM-A and CAR

Immunoglobulin-like proteins JAM-A and CAR represent another type of integral membrane constituents of intestinal epithelial TJs. These proteins have a single transmembrane domain and two extracellular Ig-like domains which can be engaged in either

Table 1. Effects of individual TJ protein inhibition on structure and permeability of the intestinal epithelial barrier

TJ protein	Method of Inhibition	Effect on the Barrier	Effect on TJ Structure	Reference
Claudin-1	Inhibitory peptides	Blocked TEER recovery after calcium switch	Disassembled mature TJs	26
Claudins 3 & 4	*Clostridium perfringens* enterotoxin	Increased steady-state permeability of cultured cell monolayers and rat jejunum		25,30
Claudin 15	(a) Claudin-15 null mice	(a) Decreased ion permeability of jejunum	(a) Decreased complexity of TJ strands	(a) 31
	(b) Zebrafish mutant lacking claudin-15		(b) Normal TJs but multiple lumens in the gut	(b) 32
Occludin	(a) Inhibitory peptide	(a) Attenuated TEER recovery after calcium switch	(a) Attenuated TJ re-assembly	(a) 34
	(b) Inhibitory antibody	(b) Impaired fence function of TJ and apico-basal cell polarity	(b) Attenuated TJ re-assembly	(b) 35
	(c) siRNA mediated gene knockdown	(c) Attenuated TEER recovery after calcium switch		(c) 36
	(d) Occludin null mice	(d) Did not affect intestinal permeability	(d) Did not affect structure of intestinal TJs	(d) 37,38
Tricellulin	siRNA mediated gene knockdown	Attenuates TEER recovery after calcium switch		36
MarvelD3	siRNA mediated gene knockdown	Attenuates TEER recovery after calcium switch	Did not affect TJ architecture and assembly	36,39
ZO-1	(a) siRNA-mediated gene knockdown	(a) Attenuated TEER recovery after calcium switch	(a) Attenuated TJ re-assembly	(a) 13
	(b) *Zonula occludens* toxin	(b) Increased permeability of rabbit ileum		(b) 40
	(c) ZO-1 null mice	(c) Embryonic lethality		(c) 41
ZO-2	ZO-2 null mice	Embryonic lethality		42
ZO-3	ZO-3 null mice		Did not affect structure of intestinal TJs	42
JAM-A	(a) Inhibitory antibody or siRNA knockdown in vitro	(a) Attenuated TEER recovery after calcium switch	(a) Attenuated TJ re-assembly	(a) 43,44
	(b) JAM-A null mice	(b) Increased permeability of healthy gut	(b) Did not alter TJ structure in healthy gut	(b) 45,46
CAR	Extracellular CAR-Fc protein	(a) Attenuated TEER recovery after calcium switch		47

homotypical or heterotypical interactions.[19,21,50] Strong evidence implicated JAM-A and CAR in regulation of TJ structure and functions. For example, treatment with anti-JAM-A antibodies or siRNA-mediated knock-down of JAM-A increased permeability of cultured intestinal epithelial cell monolayers and attenuated TJ re-assembly.[43,44] Furthermore, JAM-A knockout mice were characterized by increased baseline permeability of the gut and by exaggerated intestinal inflammation during experimental colitis.[45,46] Likewise, exposure of T84 cells to soluble extracellular fragments of CAR attenuated development of the paracellular barrier whereas overexpression of CAR resulted in the barrier enhancement.[47] On the other hand, knockdown of CAR in mice or zebrafish caused cardiac and renal abnormalities without disrupting TJs in these organs.[51,52] Effects of CAR deletion on TJ structure and integrity of the gut epithelial barrier in vivo remain to be investigated.

Cytosolic Plaque TJ Proteins

The cytosolic plaque of epithelial TJs contains a number of scaffolding, signaling, polarity, and cytoskeletal proteins. These proteins are responsible for correct TJ assembly and remodeling and they act by clustering transmembrane junctional components and by regulating their trafficking and association with the cytoskeleton.[20,22,53] Members of ZO protein (ZO-1, 2, and 3) are prototypical constituents of the cytosolic TJ plaque. They possess key protein-protein interacting PDZ and SH3 domains and are able to associate with claudins, occludin, JAM-A and CAR.[20,22,53] Experiments with expressional downregulation of different ZO isoforms in cultured epithelial cells demonstrated distinct roles of these scaffolds in the regulation of the epithelial barrier. For example, knock-down of ZO-2 or ZO-3 did not affect TJ formation,[33,42] whereas depletion of ZO-1 delayed TJ re-assembly and establishment of the paracellular barrier in renal and intestinal epithelial cells.[13,54] A simultaneous knock-down of all three ZO isoforms resulted in a complete loss of TJs and a severe impairment of barrier properties in mammary epithelial cell monolayers.[55] These experiments established a key role of ZO scaffolds in formation of TJs and indicated some functional redundancy of their isoforms. Little is known about functions of mammalian ZO proteins in vivo since ZO-1 or ZO-2 null animals exhibited embryonic lethality whereas ZO-3 null mice did not show any adverse phenotype.[41,42] Interestingly, a *Vibrio cholerae* protein toxin that is known to specifically interact with ZO-1 increased permeability and disrupted TJ both in Caco-2 monolayers and in rabbit ileum, thereby supporting roles of ZO-1 in regulating the gut barrier in vitro and in vivo.[40,56] Another abundant scaffold at cytosolic TJ plaque is a myosin II- and Rho-A interacting protein, cingulin.[22] However, its importance for intercellular junctions remains unclear since knockdown of cingulin in renal epithelial cell monolayers did not affect TJ structure and epithelial permeability.[57]

DYNAMICS OF EPITHELIAL TJs IN NORMAL CONDITIONS AND DISEASES

Epithelial TJs are characterized by an intrinsic plasticity, which is manifested as the ability to partially or completely remodel (disassemble and re-assemble) their structure. For example, live imaging of cells expressing fluorescently-labeled claudins showed a rapid break-down and re-assembly of TJ strands,[58] as well as a continuous internalization of claudin-containing vesicles from intact TJs in confluent cell monolayers.[59] Furthermore, a

recent study involving time-resolved microscopy of different TJ proteins has revealed their extensive intramembrane mobility even after incorporation into mature TJs.[60] At physiological conditions, the dynamics of apical junctions is likely to be essential for fine modulation of the paracellular barrier by various physiological stimuli, including nutrients and hormones.[1,61,62] Additionally, a steady-state junctional plasticity is essential for re-organization of cell-cell contacts during tissue morphogenesis and normal rejuvenation of epithelial layers.[63,64] However in disease conditions, the accelerated junctional dynamics results in TJ disassembly and leakiness of epithelial barriers.[62,65] Indeed, increased epithelial permeability is a known consequence of mucosal inflammation that contributes to the pathophysiology of different gastroenteropathies and particularly to inflammatory bowel disease (IBD) that includes Crohn's disease (CD) and ulcerative colitis (UC).[62,66,67] This notion is based on clinical data demonstrating that the decline in barrier function of the intestinal epithelium positively correlates with the degree of mucosal inflammation in CD and UC patients,[68] and that the increased epithelial permeability can precede clinical relapse of CD.[69]

Dysfunction of the epithelial barrier during intestinal inflammation is likely to be mediated by perturbations of normal TJ structure. This conclusion is supported by extensive immunocytochemical studies that documented the loss of the characteristic labeling pattern for different TJ proteins after exposure of model epithelial monolayers to pro-inflammatory agents such as cytokines, free radicals and microbial products.[62,70-72] In cultured epithelia, proinflammatory mediators are known to disrupt barrier integrity by various mechanisms involving expressional down-regulation or post-translational modification of TJ proteins, endocytosis of apical junctions and remodeling of the perijunctional cytoskeleton.[8,62,65,66,73,74]

Defects in the organization of intestinal epithelial junctions have also been observed in animal models of inflammation and tissue biopsies from IBD patients. For example, internalization of occludin and JAM-A from TJs has been shown to occur in the small intestine of mice with experimental T-cell dependent intestinal inflammation,[75] whereas loss of junctional localization of ZO-1 was detected in the colonic epithelium of mice with dextran sulfate sodium-induced colitis.[76] Furthermore, lipopolysaccharide-dependent sepsis in rats was found to induce rapid disorganizations of TJs in colonic epithelium.[77] The animal model data are in good agreement with several clinical studies, which demonstrated substantial loss of occludin, ZO-1, JAM-A, and claudin-1 from TJs in intestinal mucosa of CD and UC patients.[78,79] Such a redistribution of junctional proteins in the intestinal epithelium of IBD patients is consistent with the decreased complexity of TJ strands as identified by freeze-fracture EM.[80] Together, these data strongly suggest that TJ disassembly represents a key mechanism of epithelial barrier dysfunction observed in inflamed intestinal mucosa in vivo.

Studies in knockout animals have provided a strong causal link between disruption of the epithelial barrier and exaggerated gut inflammation. For example, two recent studies revealed increased colonic epithelial permeability in JAM-A knockout mice[45,46] that was accompanied by signs of chronic intestinal mucosal inflammation.[45] Additionally, JAM-A-null animals demonstrated dramatically exaggerated inflammatory response and higher mortality during experimental colitis compared to wild-type controls.[45,46] Finally, a recent report has demonstrated that pharmacological enhancement of the intestinal epithelial barrier function significantly ameliorated mucosal inflammation in spontaneous colitis-prone mice.[81] Together, these studies provided the first direct evidence that specific defects in epithelial junctional structure are sufficient to disrupt the intestinal epithelial barrier and accelerate mucosal inflammation in vivo.

UNANSWERED QUESTIONS ABOUT REGULATION OF INTESTINAL EPITHELIAL TJs

Despite the enormous progress achieved during last two decades in understanding the organization and functioning of epithelial barriers, our knowledge of structure and regulation of epithelial TJs remain very fragmented and incomplete. One can still compose an endless list of questions to address unknown molecular architecture of TJs, exact physiological roles of their protein constituents or the hierarchy and interplay of intracellular signaling cascades that regulate junctional dynamics. Below, I outline a handful of questions or controversial points that may provide food for thought for the future studies of these fascinating cellular structures in intestinal epithelium.

Question 1: What do we know about the diversity and regulation of TJs in the gastrointestinal tract in vivo?

The majority of our knowledge about structure and functions of TJs in the gastrointestinal tract has been generated by in vitro studies that used intestinal epithelial cell lines. The most popular cell lines represent transformed cells originating from colorectal tumors, which display morphological characteristics of either human enterocytes (Caco-2BBE cells) or colonocytes (T84, SK-CO15, HT29-Cl.19A cells). These cultured epithelial cells form morphologically distinct TJs and develop a measurable paracellular barrier, however it remains unclear how well their TJs resemble analogous adhesive structures formed by human intestinal epithelial cells in vivo. Extensive morphological and biochemical studies indicate a close similarity in the ultrastructure and molecular composition of TJs in cultured cell monolayers and in the gut.[82,83] However the regulation of TJs in model cell lines and in the mammalian gut is likely to be different. Evidence suggests that compared to normal intestinal mucosa transformed intestinal epithelial cells form more stable TJs and much tighter paracellular barrier, which can be resistant to modulation by a number of physiological and pathophysiological stimuli. For example, well-studied T84 and SK-CO15 colonic epithelial cell monolayers develop TEER values in the range of 1,500-3,000 $\Omega \times cm^2$,[11-13] which is significantly higher than 350-730 $\Omega \times cm^2$ of TEER reported for rodent colon.[9] Such a tight barrier can be difficult to disrupt. Indeed, T84 and SK-CO15 cells did not respond to TNFα with junctional disassembly even after prolonged (72 h) cytokine treatment.[13,84] By contrast, TNFα administration induced rapid (within 1 h) and massive disruption of TJs in mouse colon.[85] The hyporesponsiveness of transformed epithelial cells is likely to be explained by their frequent chromosomal deletions or genetic mutations that may inactivate important intracellular signaling pathways. A comparative analysis of TJ regulation in transformed and nontransformed primary intestinal epithelial cell lines is required to fully understand physiological implications of the results obtained in commonly used intestinal epithelial cell lines.

There is another reason to ask if our current knowledge about TJs in cultured enterocytes or colonocytes is sufficient enough to understand the complexity of the intestinal epithelial barrier in vivo. Although absorptive epithelial cells are the most abundant cell-type in the intestinal mucosa, this tissue also contains exocrine goblet and Paneth cells.[86,87] Since exocrine and absorptive cells have clearly different morphological features, one can suggest that these cell types may also have differences in structure and regulation of TJs. This suggestion is supported by a study that used freeze-fracture

EM to directly compare enterocyte and goblet cell junctions in the rat ileum.[88] While absorptive epithelial cells showed complex TJs with uniform number and depth of junctional strands, goblet cells TJs appeared to be variable and somewhat abnormal. Such abnormalities included consisting of few strands junctions, strands fragmentation and poor cross-linking.[88] Furthermore, lanthanum and barium tracers easily penetrated goblet cell TJs in contrast to absorptive enterocyte junctions.[88] Secretory activity of goblet and Paneth cells may explain structural and functional peculiarities of their TJs. Indeed, extensive remodeling of the apical surface that accompanies granule secretion in exocrine cells is incompatible with formation of a rigid apical actin cytoskeleton that is essential for stabilization of TJs and enhancements of barrier properties in absorptive enterocytes.[61,65,74] Furthermore, TJs appear to be a 'hot spots' for docking and fusion of exocytic vesicles with the plasma membrane.[89,90] Even apically-targeted proteins can be initially delivered to perijunctional areas of the lateral plasma membrane from which they are transcytosed to the final destination at the apical surface.[91] Whether or not similar events happen in intestinal goblet and Paneth cells remains to be investigated, but it is tempting to speculate that intensive perijunctional vesicle trafficking may destabilize TJ structure and weaken the paracellular barrier in exocrine epithelial cells.

Question 2: What mechanisms regulate integrity and remodeling of the TJ-associated actomyosin cytoskeleton?

Association with the apical actin cytoskeleton plays key roles in the integrity and remodeling of epithelial TJs.[61,65,74] This conclusion is based on EM studies of absorptive intestinal epithelia that showed a close association of TJs with a meshwork of actin filaments lining the interior part of the plasma membrane.[92,93] Furthermore, many studies have shown that specific actin-depolymerizing drugs disrupted integrity of the epithelial barrier and impaired TJ structure and remodeling.[12,94-96] TJ-associated actin filaments are enriched in nonmuscle myosin II (NM II), a molecular motor that converts chemical energy of ATP hydrolysis into mechanical forces thereby mediating tension and contractility of the actin cytoskeleton.[97] This motor protein works as a molecular ensemble of two heavy chains, two essential, and two regulatory myosin light chains (RMLC).[97] The NM II heavy chain has a globular head, which binds to actin filaments and hydrolyzes ATP, and an extended tail that coils together with another heavy chain tail to form a rigid rod-like structure. The tails of multiple NM II molecules readily undergo a side-by side self-association, creating myosin filaments. Such a high-order organization of NM II is critical for two major functions of this protein. One function is the sliding of actin filaments against each other, which mediates the myosin II-dependent contractility, whereas the other is the cross-linking (bundling) of actin filaments thereby producing thick actomyosin fibrils.[97] Intestinal epithelial cells express three different NM II heavy chains, IIA, IIB and IIC that have different tissue distribution and may play unique roles in regulating cell shape, cell-cell and cell-matrix adhesions and cell motility.[11,98]

Several recent studies that used either pharmacological or siRNA approaches to block NM II activity have demonstrated a critical role of this actin motor in regulating epithelial TJs. For example, inhibition of NM II with blebbistatin prevented TJ disassembly caused by IFNγ in T84 cells and by protein kinase C-activating tumor promoters in HPAF II pancreatic epithelial cells.[99,100] On the other hand, blebbistatin treatment dramatically attenuated calcium-dependent reformation of TJs in vitro.[95] Similarly, genetic depletion

of NM II motor was shown to diminish barrier functions of mature TJs and to attenuate their disassembly and re-assembly triggered by various external stimuli.[11,99] Despite strong evidence supporting a key role of NM II in junctional dynamics, mechanisms that regulate activity of this motor at TJs remain poorly understood. There is a common believe that NM II-dependent remodeling of epithelial junctions is driven by the increased phosphorylation of RMLC, which is mediated by either Rho-associated kinase (ROCK) or myosin light chain kinase (MLCK).[53,62,65,101] This concept is based on two lines on evidence. First, RMLC phosphorylation is a classical activation mechanism that enhances the ATPase activity and promotes the self-assembly of myosin II heavy chains.[97,102] Second, many studies have demonstrated that either pharmacological or genetic inhibition of ROCK and MLCK activities disrupted the integrity of epithelial barriers and impaired junctional structure/remodeling.[53,62,65,103] However, recent data suggest that RMLC phosphorylation may not be essential for the activity of NM II motor, which is selectively associated with epithelial TJs. Indeed, effects of phospho-RMLC on NM II functions appeared to be heavy chain isoform specific and limited to NM IIB, while the assembly and activity of NM IIA was found to be independent on the level of RMLC phosphorylation.[104,105] On the other hand, we have recently identified NM IIA isoform is a unique regulator of TJ structure and dynamics in well-differentiated epithelia. For example, NM IIA comprised a majority (65-85%) of all NM II heavy chains in high-resistance T84 and HPAF II epithelial cell monolayers,[98] where expression of NM IIB protein was undetectable.[11,98] Furthermore, NM IIA is abundantly expressed in well-differentiated surface epithelium of normal human colon whereas NM IIB expression appears to be restricted to the less-differentiated crypt epithelium (A.I. Ivanov, unpublished observation). Finally, selective downregulation of NM-IIA was shown to attenuate remodeling (disassembly and re-assembly) of TJs in SK-CO15 cells whereas depletion of NM IIB did not affect such a TJ dynamics.[11] Since NM IIA functioning is poorly sensitive to the level of RMLC phosphorylation, alternative mechanisms should regulate self-assembly and motor activity of the TJ-associated NM IIA in epithelial cells. These mechanisms should specifically target NM IIA heavy chains and may involve either heavy chain phosphorylation or their binding to various accessory proteins such as Mts1, septins and Shroom. An important remaining question is how to explain effects of ROCK and MLCK inhibition of TJ remodeling if such remodeling is independent of RMLC phosphorylation? It is likely that TJ can be regulated by alternative molecular targets of these kinases. For example, ROCK is known to mediate F-actin turnover by controlling cofilin-dependent filament disassembly,[106] and F-actin turnover is essential for TJ dynamics.[12,94] On the other hand, MLCK was recently shown to regulate integrin functions,[107] and thereby may have indirect integrin-mediated effects on apical junctions and the paracellular barrier.[108] Further studies will clarify the signaling pathways that link NM II, ROCK, MLCK activities and remodeling of epithelial TJs.

Question 3: Which endocytic pathways mediate TJ disassembly and how these pathways become activated in disease conditions?

Endocytosis is an emerging mechanism that mediates rapid disassembly of epithelial TJs under physiological conditions and in diseases.[62,64,73,74] This process is not only essential for reversible opening of the paracellular barrier but it also contributes to the loss of epithelial cell phenotype during epithelial to mesenchymal transition in invasive tumors.[73] Generally, plasma membrane components can be internalized *via*

multiple endocytosis pathways, and at least three such pathways have been implicated in TJ internalization. Examples include clathrin-dependent endocytosis of TJ proteins in calcium-depleted[109] or transforming growth factor-treated[110] epithelial cells, as well as lipid raft/caveolae-mediated endocytosis[96] or macropinocytosis[111] of TJs in cells treated with a F-actin-depolymerizing drug and IFNγ respectively. Additionally, caveolar-mediated endocytosis was shown to mediate TNFα-induced disruption of the intestinal epithelial barrier in vivo.[85] Such a multiplicity of endocytic pathways involved in TJ disassembly can be explained by the diversity of external stimuli that trigger junctional internalization as well as by a predominance of the particular endocytosis machinery in different types of epithelia. Despite of the fact that virtually all stimuli that trigger a sustained junctional disassembly result in internalization of TJ proteins, a little is known about mechanisms that activate this process. Two possible scenarios can be envisioned. First, it is known that the perijunctional actin cytoskeleton stabilizes TJs and antagonizes their internalization in stationary, well-differentiated epithelia.[96] On the other hand, many stimuli that induce junctional disassembly also trigger cytoskeletal re-arrangements, and thereby may simply relieve the cytoskeletal inhibition of TJ endocytosis.[61,65] An alternative mechanism involves specific signaling that stimulates interactions between various endocytosis regulators and TJ proteins that triggers junctional internalization. This mechanism has been recently highlighted by the findings of increased associations between occludin and clathrin adaptors epsin-1 and Eps15 during vascular growth factor-induced disassembly of endothelial junctions.[112] Post-translational modification of TJ proteins is likely to be important for the junctional recruitment of endocytic adaptors. For example, occludin and claudin-1 can be ubiquitinated by different ubiquitin ligases and such modification is sufficient to promote TJ disassembly and internalization.[112-114] Virtually nothing is known about signals/mechanisms that stimulate lipid raft/caveolae-mediated endocytosis of TJs. Since this pathway is regulated by intracellular level of caveolin-1,[115] the acute modulation of caveolin-1 expression may dramatically affect stability and internalization of epithelial junctions. Additionally, tyrosine phosphorylation of caveolin-1 by Src kinases that promotes caveolae formation[116] may accelerate TJ endocytosis especially in growth-factor treated epithelial cells. Occludin appears to link epithelial TJs to lipid raft/caveolae domains. Indeed, occludin and caveolin-1 were found to colocalize and physically interact at normal and internalized TJs.[115,117] Furthermore, occludin was shown to regulate caveolin-1 partitioning in lipid rafts,[17] whereas caveolin-1 knockdown inhibited cytokine-stimulated occludin endocytosis.[85]

Question 4: Does programmed cell death play a role in disruption of epithelial TJs during intestinal inflammation?

Intestinal epithelium is a very dynamic tissue that undergoes a continuous self-renovation. This process is characterized by a constant appearance of new enterocytes from stem cells that are located in gut crypts with their subsequent differentiation and migration up the crypt axis.[87] In the small intestine, terminally-differentiated enterocytes reach tips of the villi where they undergo apoptosis and are shed into the lumen. In the large intestine, which is devoid of villi, apoptotic enterocytes are shed from the flat colonic surface. Shedding of epithelial cells is an intensive process with estimated normal loss of approximately 1400 cells from each villus every 24 h.[118] Despite the fact that extrusion of apoptotic cells from epithelial monolayers breaks the continuity

of their TJs, cell shading in normal gut does not compromise integrity of the mucosal barrier.[119] This can be explained by a so called 'purse-string' mechanism of apoptotic cell extrusion that involves formation and rapid contraction of actomyosin rings around apoptotic cells by their neighbors.[120] Contraction of such rings results not only in forceful squeezing out of shedding cells from the monolayer but also leads to a rapid resealing of the focal TJ breaks.

Apoptosis is known to be dramatically exaggerated in model epithelial monolayers treated with various proinflammatory agents[121] as well as in intestinal mucosa of IBD patients.[8] Since excessive apoptosis in vitro was shown to disrupt the epithelial barrier[122] it has been proposed that enhanced cell death and shedding can be responsible for increased TJ disassembly and barrier leakiness in the inflamed intestinal mucosa. However, this mechanism remains controversial and dependent on employed experimental models. For example, apoptosis appeared to be a major mediator of increased epithelial permeability caused by IL-13,[123] whereas this mechanism has been repeatedly dismissed by studies of IFNγ-induced disruptions of the mucosal barrier.[84,124] Conflicted results were obtained for the role of apoptosis in TJ disassembly and barrier breakdown caused by another important proinflammatory cytokine TNFα.[8,125] It is noteworthy, that dismissal of cell death as a barrier-disruptive mechanism is often based on the failure of caspase inhibitors to prevent cytokine-induced disruption of TJs and increase in epithelial permeability.[84,124] However, caspase inhibitors, while blocking apoptosis, can induce alternative modes of cell death such as necrosis or autophagy.[126] Furthermore, autophagy itself is likely to be an abundant alternative cell death mechanism in inflamed intestinal mucosa where epithelial cells are exposed to pro-auhophagic conditions such as nutrient deprivation and oxidative stress. Additional studies are needed to explore a diversity of epithelial cell death pathways and their contribution to disruption of the mucosal barrier during intestinal inflammation.

CONCLUSION

Tight junctions represent key cellular structures that control the architecture and functions of epithelial layers. These structures are especially important in the gut where TJs mediate formation of the protective barrier that dramatically limits body exposure to pathogens and their toxins. Recent studies convincingly demonstrated that defects in TJ structure exaggerate mucosal inflammation, thereby highlighting intestinal epithelium as an active player in the mucosal immune responses. Two major features of epithelial TJs became obvious in recent years: Their enormous complexity and their dynamic nature. Future research in the field will be focusing on these features aiming to reconstruct a precise molecular organization of TJs and to understand mechanisms of junctional plasticity. Such knowledge is critical for the development of new therapeutic strategies that will prevent disruption of the intestinal epithelial in various diseases.

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CHAPTER 7

POLARITY PROTEIN COMPLEX SCRIBBLE/Lgl/Dlg AND EPITHELIAL CELL BARRIERS

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Abstract: The Scribble polarity complex or module is one of the three polarity modules that regulate cell polarity in multiple epithelia including blood-tissue barriers. This protein complex is composed of Scribble, Lethal giant larvae (Lgl) and Discs large (Dlg), which are well conserved across species from fruitflies and worms to mammals. Originally identified in *Drosophila* and *C. elegans* where the Scribble complex was found to work with the Par-based and Crumbs-based polarity modules to regulate apicobasal polarity and asymmetry in cells and tissues during embryogenesis, their mammalian homologs have all been identified in recent years. Components of the Scribble complex are known to regulate multiple cellular functions besides cell polarity, which include cell proliferation, assembly and maintenance of adherens junction (AJ) and tight junction (TJ), and they are also tumor suppressors. Herein, we provide an update on the Scribble polarity complex and how this protein complex modulates cell adhesion with some emphasis on its role in Sertoli cell blood-testis barrier (BTB) function. It should be noted that this is a rapidly developing field, in particular the role of this protein module in blood-tissue barriers, and this short chapter attempts to provide the information necessary for investigators studying reproductive biology and blood-tissue barriers to design future studies. We also include results of recent studies from flies and worms since this information will be helpful in planning experiments for future functional studies in the testis to understand how Scribble-based proteins regulate BTB dynamics and spermatogenesis.

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INTRODUCTION

In invertebrates and vertebrates, epithelial tissues are arranged as sheets of cells that segregate and protect an organism from changes in its environment. In short, epithelial cells are arranged side-by-side with the apical region of their plasma membranes facing the environment, while the basal area is anchored to the basal lamina (i.e., extracellular matrix, also known as basement membrane in the testis[1,2]), and the lateral area in contact with each other. This feature is achieved via the development of "polarity" during embryogenesis. Thus, epithelial/endothelial cells of an epithelium/endothelium can be divided into three discrete domains: the apical, the lateral, and the basal domains. Furthermore, at the cell-cell and the cell-extracellular matrix interface, there exist a series of junction complexes. It is noted that an important function of the epithelial barrier is to limit paracellular diffusion from the outside (e.g., environment, blood) to the inside, and this is conferred by tight junctions (TJs) in vertebrates or septate junctions (SJs) in invertebrates. Adhesion between neighboring epithelial cells is conferred by adherens junctions (AJs) lying immediately behind TJs.[3,4] Therefore, AJs maintain the epithelial sheet whereas TJs act as a "gate" that seals the intercellular space and permits selective exchange of substances (e.g., ions, sugars, amino acids, electrolytes) across an epithelium. The selective permeability of epithelial cells also relies on the polarized distribution of specialized channel proteins on their cell membranes.

Establishment of epithelial cell polarity, including the blood-testis barrier (BTB) in the seminiferous epithelium of mammalian testes, is critical for maintaining cell morphology and function. This requires targeting of membrane and peripheral proteins precisely to either apical or basolateral membrane domains.[5] The asymmetric distribution of proteins in different parts of membranes is an important feature of epithelial cell polarity. Although the signaling network that controls cell polarity has not been elucidated, a number of membrane proteins that regulate cell polarity have been identified based on genetic and biochemical studies in *Caenorhabditis elegans*, *Drosophila melanogaster*, and also in mammals during these past several decades. These polarity proteins are organized into several functional complexes that distribute at different membrane domains, some of which are mutually exclusive, facilitating the formation of the apicobasal axis and the assembly and maintenance of epithelial cell junctions.[6] The best studied polarity complex thus far is the partitioning-defective (Par) protein complex, which is located at the apical domain and is composed of Par6, Par3 (also known as *Drosophila* Bazooka) and atypical protein kinase C (aPKC).[7,8] This tripartite complex also associates with the small GTPase, Cdc42.[9,10] In mammals, Par6 serves as an adaptor, recruiting other proteins to this complex to facilitate TJ assembly (Fig. 1).[11,12] The other apical polarity module is composed of the transmembrane protein Crumbs (Crb), the cytoplasmic protein Pals1 [protein-associated with Lin-7, also known as *Drosophila* Stardust, an adaptor and a member of the membrane-associated guanylate kinase (MAGUK) protein family] and Patj (Pals1 associated tight junction protein) (Figs. 1, 2).[13] Recent studies have shown that Crb complex proteins are tumor suppressors besides serving as polarity proteins.[6,14] In *Drosophila*, there is also a basolateral polarity protein complex known as the Yrt/Coracle group, which is composed of FERM proteins Yurt (Yrt), Coracle (Cora); and the membrane proteins Neurexin IV (Nrx-IV) and Na^+, K^+-ATPase.[15] The Yrt/Cora complex promotes basolateral membrane stability and it also displays negative regulatory interactions with the apical Crb protein module.[15]

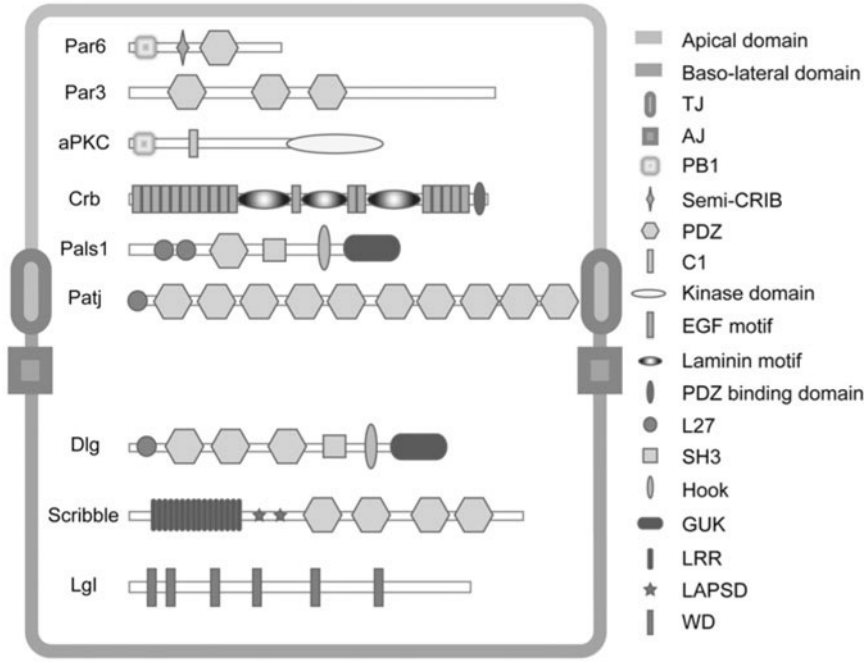

Figure 1. Schematic drawing that illustrates the different functional domains of Scribble complex components in the baso-lateral region versus other apical polarity proteins in the apical region of mammalian epithelial cells. This drawing is also applicable to the testis except that the BTB in the testis lies adjacent to the basement membrane (a modified form of extracellular matrix also known as basal lamina in other epithelia) in the seminiferous epithelium where components of the Scribble protein complex are localized (see Figs. 4 and 5), whereas apical polarity proteins (e.g., Par6) are found both at the Sertoli cell-spermatid interface in the apical region of the seminiferous epithelium and at the BTB.[72] Abbreviations used: TJ, tight junction; AJ, adherens junction; PB1, Phox and Bem1p domain; semi-CRIB, semi-Cdc42/Rac interactive binding motif; PDZ, PSD-95/Discs-large/ZO-1 conserved domain; C1, protein kinase C conserved region 1; EGF motif, epidermal growth factor motif; L27, Lin2 and Lin7 binding domain; SH3, Src homology 3 domain; GUK, guanylate kinase homologs conserved domain; LRR, leucine-rich repeats; LAPSD, LAP specific domain; WD, WD-40 repeats.

In the basolateral domain, there is a protein complex composed of three tumor suppressors: Scribble (Scrib), Lethal giant larvae (Lgl) and Discs large (Dlg), which function cooperatively to regulate cell polarity, junction formation and cell growth in epithelial cells (Figs. 1, 2). This is mediated via mutually exclusive interactions between the Scribble- and the Par-based protein complexes (Fig. 2). Although there is no evidence of physical interaction between the three protein components in the Scribble complex except in *Drosophila* neuronal synapses, where Scribble associates with Dlg via a protein named GUK holder (Fig. 1),[16] the three genes show strong genetic interaction (e.g., the knockout of either Scrib, Lgl or Dlg leads to similar phenotypic changes in mutants versus the wild-type) and these proteins appear to utilize a common pathway to regulate cell architecture and cell proliferation.[17] Mutations of these three genes also display similar phenotypes in different epithelia, suggesting that they are components of a fundamental machinery that creates the distinctive architecture of

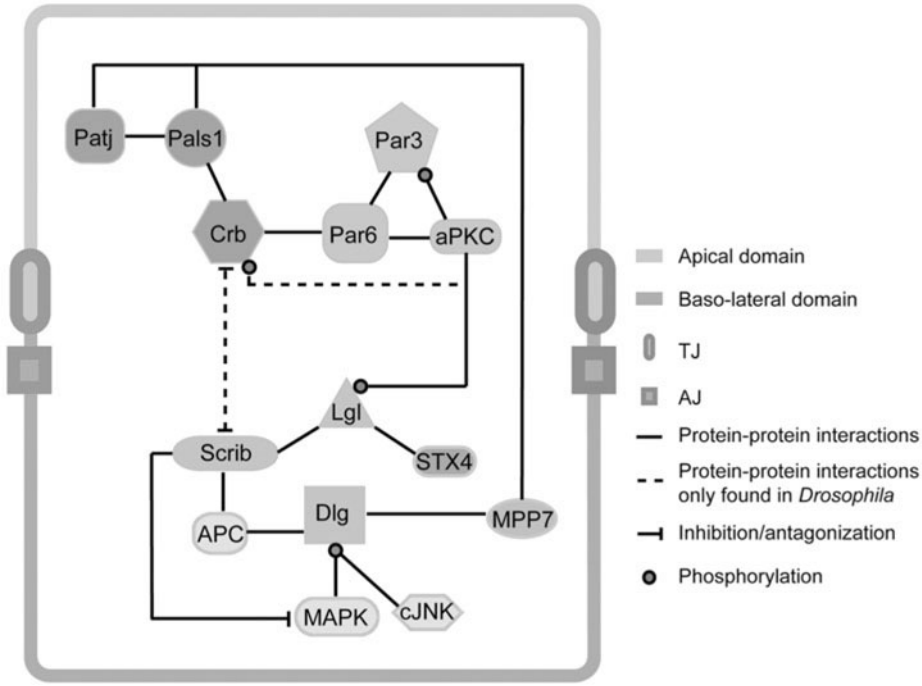

Figure 2. Interactions of Scribble complex components with other polarity or signaling proteins in polarized epithelial cells. The information depicted here is applicable to the seminiferous epithelium of mammalian testes where many components of these proteins have recently been identified. Abbreviations used: Patj, Pals1 associated tight junction protein; Pals1, protein-associated with Lin-7; Crb, Crumbs; Par, partitioning-defective proteins; aPKC, atypical protein kinase C; Scrib, Scribble; Dlg, Discs-large; Lgl, lethal giant larvae; APC, adenomatous polyposis coli; MAPK, mitogen-activated protein kinase; cJNK, c-Jun N-terminal kinase; STX4, Syntaxin 4; MPP7, membrane-palmitoylated protein 7.

epithelial cells and tissues. In this chapter, we first summarize biochemical features of Scribble complex components. We next focus on their cooperative but distinct roles in regulating epithelial cell junctions and polarity in blood-tissue barriers. We also discuss recent findings on this complex in regulating epithelial barrier functions in different tissues in particularly the BTB in the testis.

IDENTIFICATION AND MOLECULAR STRUCTURE OF SCRIBBLE COMPLEX COMPONENTS

Members of Scribble complex contain multiple protein–protein interaction domains, in particular PDZ, SH3, and guanylate kinase domains, indicating they can recruit a complex network of proteins to regulate polarity and other cellular functions (Fig. 1). Herein, we focus on the molecular structural features of Scribble, Lgl and Dlg and their functions.

Dlg

The *Drosophila* discs large tumor suppressor gene, *dlg* (lethal discs-large), was first discovered in 1972.[18] Mutations of this gene were found to display a phenotype similar to that of the *lgl* mutation.[18,19] *dlg* mutants were found to have imaginal discs fused with each other and to the larval ventral ganglion as they overgrew versus the wild type.[20] *dlg* was shown to be crucial for normal epithelial structure and growth in the embryo,[21] indicating the function of this gene is not limited to imaginal discs. Dlg was identified as a member of the membrane-associated guanylate kinase homolog (MAGUK) family, which often locates at cell junctions and contains distinct peptide domains namely PDZ1-3, SH3, HOOK, and GUK (Fig. 1).[22,23] Studies using different Dlg derivatives have shown that the subcellular targeting of Dlg to the plasma membrane requires PDZ2 and HOOK domains, leading to the precise localization of Dlg at SJs.[21]

To date, five mammalian homologs of *Drosophila* Dlg have been identified (Table 1), with Dlg1 being the best studied Dlg member. The human homolog of *Drosophila* discs large called *hdlg1* was found to contain a C-terminal GUK domain, an SH3 domain, a HOOK domain and three PDZ domains.[24] In addition, Dlg1 contains a L-27 domain at its N terminus which can bind to membrane-palmitoylated proteins (MPPs), Lin2/CASK, and Lin7.[25-28] In vitro study has shown that PDZ1-2 or HOOK domains of hDlg can function independently to localize exogenous hDlg to the basolateral membrane of cells.[29]

The three homology repeats (PDZ domains) in Dlg were first reported in PSD-95 protein from rat forebrain,[30] which was also found in the mammalian TJ protein ZO-1.[31] Thus, the name PDZ domain (PSD-95, Discs-large, ZO-1) was used to reflect the origin and distribution of this domain.[32] In *Drosophila*, Dlg colocalizes with Scribble via a protein named GUK holder, which can interact with both GUK domain of Dlg and the PDZ2 domain of Scribble (Fig. 1).[16]

Table 1. Components of the Scribble complex in different species

Drosophila		*C. elegans*		Mammals		
	Mr (kDa)		Mr (kDa)		Mr (kDa)	Gene Symbol
Scribble	190	LET-413	75	Scrb1/Crib1/ Vartul/hScrib	250*/175	*SCRB1*
Lgl	127	–		Lgl1	115	*LLGL1*
				Lgl2	113	*LLGL2*
				Lgl3/syntaxin-binding protein5	127	*LLGL3*
				Lgl4/syntaxin-binding protein5-like	130	*LLGL4*
Dlg	107	Dlg1	107	SAP97/hDlg1	130*/100	*DLG1*
				PSD93/ Chapsyn110	65	*DLG2*
				SAP102/NE-Dlg	90	*DLG3*
				SAP90/PSD95	85	*DLG4*
				LP-Dlg	202	*DLG5*

*This apparent Mr was obtained by immunoblot analysis based on SDS-PAGE and the corresponding specific antibodies performed in our laboratory using rat testes as lysates.

Scribble

Scribble was initially identified in *Drosophila* where it was found to be a crucial regulator of morphogenesis.[33] There is only one *Drosophila* homolog of Scribble in mammals known as Scrib or Vartul (Table 1).[34] Scribble is a LAP [LRR (leucine-rich repeats) and PDZ (PSD-95/Discs-large/ZO-1) domain] protein containing 16 LRRs and 4 PDZ domains (Fig. 1). The first LAP protein, Densin 180, which contains a set of LRRs as well as a PDZ domain, was first isolated in rat brain.[35] "LAP" is a collective name[33] referring to proteins that contain the LRR and PDZ domains found in fruitflies, worms, rodents and humans.[33,36,37] The LAP family has three subfamilies according to the number of PDZ domains, namely LAP0, LAP1 and LAP4.[38] The LRRs and PDZ domains of LAP proteins are implicated in mediating protein-protein interactions. Due to their unique polarized localization within the cell membrane, LAP proteins play a key role in regulating subcellular protein distribution.[33]

LAP family members are strikingly conserved from *Drosophila* to humans, with respect to their primary amino acid sequence, subcellular localization (basolateral in epithelial cells or postsynaptic in neurons) and protein architecture, suggesting common biological functions.[33] A structure-function correlation study on *Caenorhabditis elegans* LET-413, human Erbin and human Scribble (hScrib) revealed that the LRR domain and the LAPSD (LAP specific domain, a 38-amino acid LRR-like domain located between LRR and PDZ domain) are crucial for anchoring members of this protein family to the plasma membrane.[39] In short, LRR domains are necessary and sufficient to attach Scribble to the plasma membrane, but it is necessary to have the LAPSD domain to organize a polarized epithelium.[40]

PDZ domains are composed of 80-90 amino acids, folded into two α helixes and six β strands that interact with C-terminal sequences of other proteins.[41] Because PDZ domains are frequently found in proteins that regulate cell polarity, it is expected that these motifs are involved in epithelial polarization. However, domain analysis in Scribble has shown that PDZ domains are not indispensable for forming an epithelium, yet they enhance the ability of the LRR to localize proteins to SJs, and to regulate cell proliferation.[40]

Lgl

The *Drosophila* tumor suppressor gene, lethal (2) giant larvae (*l(2)gl*), was first discovered in 1930,[42,43] and *lgl* was cloned and sequenced in the 1980s.[44-46] Mutation of *lgl* in *Drosophila* caused the imaginal discs to overgrow, lose their epithelial structure and the ability to differentiate, forming solid tumors that continued to grow until the death of larvae.[18] *lgl* encodes a 130-kDa protein that contains stretches of sequences that are similar to cell adhesion proteins.[45] Homologs of Lgl have been identified in a variety of organisms, all of which share conserved primary sequences.[47] It has been demonstrated that mammalian Lgl proteins (Lgl 1-4, Table 1) contain at least 4-5 putative WD-40 repeat motifs[48] (Fig. 1). WD-repeats, each of which comprises a four-stranded anti-parallel β sheet,[49,50] are known to contain minimally conserved domains of ~40-60 amino acids that begin with a glycine-histidine (GH) dipeptide 11 to 24 residues from the N terminus and end with a tryptophan-aspartic acid (WD) dipeptide at the C terminus.[51] WD-repeat proteins often function as coordinators of macromolecular protein complexes, such as for protein-protein interaction, receptor-ligand interaction in signal transduction,[52-54] pre-mRNA processing,[55,56] cell cycle regulation,[57] and cytoskeleton

assembly.[47] Recent studies have illustrated the cellular function of WD-40 repeat motif in Lgl family members. For instance, WD-40 repeats are implicated in promoting the binding of Lgl with the LRR domain of Scribble.[58]

RELATIONSHIP BETWEEN CELL JUNCTIONS AND CELL POLARITY IN EPITHELIAL CELLS

Epithelial cell polarity refers to the asymmetric distribution of cellular organelles of different structures and/or functions between the apical and the basolateral domains. This apical-basolateral polarization is essential for epithelial cell function, including cell division (mitosis and meiosis), cell migration, and molecular transport,[59-61] and it is intimately related to TJ and AJ dynamics at the cell-cell interface.

TJs that create the limit of the apical and the basolateral domains are composed of three types of proteins: occludins, claudins and junctional adhesion molecules (JAMs). These TJ proteins all bind to the N-terminal PDZ domains of zonula occludens (ZO-1, -2 and -3), members of the MAGUK (membrane-associated guanylate kinase-like homology) family, via their cytoplasmic tails. Since TJs prevent diffusion of membrane proteins between the apical and the basolateral regions in a cell epithelium, it serves as a "fence" or a "barrier" that leads to epithelial cell polarity.

AJs are located immediately underneath TJ in virtually all epithelial barriers.[4,62,63] AJs are formed by homophilic interactions between extracellular domain of cadherins and nectins. The cytoplasmic tails of cadherins and nectins associate with a series of intracellular peripheral proteins (e.g., catenins and afadins, which are adaptors), which take part in different biological events, including cytoskeleton organization[64-66] and regulation of gene expression.[67,68] The interactions between functional polarity complexes, the apical Par- and the Crumb-based modules, and the basolateral Scribble module, generating zones of mutual exclusion around AJs that define the apicobasal axis of epithelial polarity.[6]

The actin-based cytoskeletal remodeling at the AJ is regulated by Rho, Rac and Cdc42 GTPases.[69] However, interactions between AJs and Rho GTPases are bidirectional since AJs also modulate the intrinsic activity of Rho GTPases to adjust cell structure and polarity. Cdc42 is a regulator of actin cytoskeleton and is associated with Par3-Par6-aPKC polarity complex.[11] Cdc42, together with Par proteins, were found to regulate AJ stability via their effects on protein trafficking, such as protein endocytosis, in *Drosophila melanogaster* neuroectodermal epithelium, illustrating functional interactions between cell polarity proteins and endocytosis that are critical to stabilize basolateral AJs.[70] Recent studies in the testis have shown that Cdc42 is a component of the Par6-based polarity complex.[71,72] Cdc42 is crucial to BTB remodeling during spermatogenesis by regulating endocytic vesicle-mediated protein trafficking,[71] since overexpression of a dominant negative mutant of Cdc42 in Sertoli cells that blocks the Cdc42 function can lead to a loss of the ability of TGF-β3 to accelerate protein endocytosis at the Sertoli cell BTB,[71] thereby promoting the Sertoli cell TJ-barrier integrity.[73,74] Earlier studies have shown that cytokines (e.g., TGF-β2, TGF-β3) are potent regulators of BTB dynamics by perturbing TJ-barrier function, which is mediated via their effects in accelerating the kinetics of clathrin-mediated endocytosis of integral membrane proteins (e.g., N-cadherin, occludin) at the Sertoli cell BTB,[75,76] as well as their subsequent endosome-/ubiquitin-mediated degradation.[76,77]

SCRIBBLE COMPLEX IN REGULATING CELL JUNCTION DYNAMICS AND CELL POLARITY

Mutations in *scribble* were found to cause aberrant cell shapes and loss of epithelial organization in *Drosophila* embryos.[33] Interestingly, mutation in either Lgl or Dlg led to similar phenotypic changes in epithelia *versus* Scribble mutant.[17] Furthermore, Scribble and Dlg colocalized and overlapped with Lgl in embryonic epidermal epithelium, and *scribble*, *dlg* and *lgl* displayed strong genetic interactions.[17] These findings thus indicate that these three tumor suppressors share a common role in regulating epithelial polarity and cell growth.

Dlg in Regulation of Cell Junctions and Cell Polarity

The product of the *Drosophila* tumor suppressor gene *dlg* is a member of the MAGUK family, which is located at the cytoplasmic face of SJs and is necessary for the formation of cell junctions and the maintenance of apico-basal polarity.[22,78,79] In *Drosophila*, the complete loss-of-function of *dlg* allele (dlg^{m52}) was shown to induce: (i) rearrangement of the cytoskeletal components (e.g., actin and tubulin) and proteins having a polarized distribution (e.g., Ex, Cor), (ii) mis-localization of transmembrane proteins (e.g., fasciclin III, neuroglian), (iii) loss of SJs and growth control, and (iv) a loss of ability to differentiate.[79]

In mammals, hDlg1 (human homolog of Disc large) colocalizes with E-cadherin at the cell-cell contact site in intestinal and renal epithelial cells.[80] A knockdown of hDlg in intestinal epithelial cells by siRNA severely alters AJ integrity and also prevents the recruitment of p85/PI3K to E-cadherin-based cell-cell contact,[80] which is required for the association of AJ components with the cytoskeleton and also for AJ assembly.[81] This is consistent with findings in *C. elegans* which illustrated a disrupted AJ associated with a mis-localization of AJM-1 (apical junction molecule, an AJ marker) in epidermis and intestinal epithelial cells after Dlg1 knockdown.[82] In short, hDlg1 is a crucial regulator of AJ and TJ assembly in mammalian epithelia, even though the underlying regulatory mechanism(s) remains known.

Scribble in Regulation of Cell Junctions and Cell Polarity

Mutations in *Drosophila scribble* led to embryos displaying corrugated cuticular surface which was riddled with holes, while the wild type embryonic cuticle formed a smooth, continuous sheet, and hence the name *scribble* was used.[33] Scribble is located basal to Armadillo (Arm, the homolog of vertebrate β-catenin) in the epithelial SJs, and codistributes with Coracle (a SJ marker belongs to protein 4.1 family).[33] Thus, Scribble localizes to epithelial SJs in *Drosophila*, and restricted to the boundary of the apical and basolateral cell surfaces. In vertebrates, however, Scribble is distributed along the entire basal and lateral domains of epithelial cells (Figs. 1 and 2). In embryonic epithelial cells of *C. elegans*, the homolog of Scribble, Let-413, also localizes in the basolateral region and it is involved in locating AJ components properly at a discrete subapical position.[37,83] For instance, a loss of *scribble* (or *let-413* in *C. elegans*) rendered a disorganization of epithelia, leading to a mis-distribution of apical proteins (e.g., Arm, Sas, Dlt, and Crb) and AJ proteins (e.g., E-cadherin in *Drosophila*, and HMP-1, JAM-1 in *C. elegans* embryos) with these proteins moved to the basolateral cell surface, but the localization

of basolateral proteins was found to be unaffected.[33,83] This preferential loss of apical protein restriction that leads to polarity defects in *scrib* mutant epithelial cells is the result of mis-distribution of apical proteins.

Although Scribble, together with Dlg,[17] are localized to SJs in *Drosophila*, no protein-protein interaction between either one of these proteins with SJ components was found. During development, Scribble and Dlg are enriched in the membrane before the appearance of SJs,[84] so it was assumed that Scribble might predetermine the site of future SJs by recruiting other SJ components to the site for its assembly.[40]

The biological roles of mammalian Scribble (Scrb1, hScrib) are largely unknown. However, hScrib was localized to cell junctions of MCF10-2A, Caco-2 and MDCK cells, colocalizing with β-catenin.[85] hScrib-depleted MCF10A or MDCK cells did not lead to cell polarity defects as seen in *Drosophila*.[86,87] However, hScrib knockdown in MCF10A cells exhibited defects in wound closure and chemotatic movement in vitro. For instance, hScrib silenced cells at the wound edge failed to polarize and to recruit Cdc42 and Rac1 to the leading edge, which is required for the formation of polarized lamellipodia and to facilitate subsequent migration.[86] Also, hScrib mutant mice were found to have defect in epidermal wound healing.[86] A recent study in mammary epithelial cells demonstrated that depletion of hScrib led to a disrupted 3D acinar morphogenesis by inhibiting the establishment of apicobasal polarity; and, most interestingly, cell apoptosis triggered by activation of *c-myc* oncogene was blocked in these cells.[88] These results thus illustrate the crucial role of hScrib in regulating cell polarity and perhaps cell apoptosis.

In contrast to flies and worms, mammalian Scribble was found to interact with cell junction proteins. For example, co-expression of hScrib and ZO-2, a TJ adaptor protein and a tumor suppressor,[89,90] in COS-7 cells has demonstrated a direct interaction between these two proteins, which is mediated by the PDZ3 and PDZ4 domains of hScrib (Fig. 1) and the carboxyl-terminus of ZO-2.[91] In MDCK cells, a knockdown of Scribble was found to disrupt E-cadherin-mediated cell adhesion, with these cells acquiring a mesenchymal appearance, migrating more rapidly but with a loss of directionality.[87] Interestingly, this adhesion disruption could be partially rescued by overexpressing an E-cadherin-α-catenin fusion protein but not E-cadherin alone. This result thus suggests that Scribble, as a tumor suppressor, it stabilizes the coupling between E-cadherins and catenins, regulating epithelial cell adhesion and migration.[87]

In addition to epithelial cells, mammalian Scribble is likely involved in planar cell polarity (PCP) in mammals (note: PCP originally refers to the directional polarity of hairs on the wings of fruitflies,[92] analogous to the directional alignment of elongating/elongated spermatids with their heads pointing toward the basement membrane in the seminiferous epithelium during spermatogenesis). The study of planar polarization in mammals has demonstrated the role of Scribble in PCP pathway.[93] Mutation of *Scribble* gene in mouse led to defects in the polarization of stereociliary bundles in cochlea, and similar but more severe defects were observed in animals with mutation in *Vangle2*, a mammalian homolog of the *Drosophila* PCP gene *Strabismus*/*Van Gogh*.[93] Moreover, polarization defects in animals heterozygous for Vangl2 and Scrb1 were comparable to Vangl2 homozygotes, demonstrating genetic interactions between these two genes in regulating PCP in mammals.[93] A subsequent study in mammalian cells has shown that Scrb1 and Vangl2 interacted with each other through the PDZ domains of Scribble.[58] These results illustrate that Scribble is involved in regulating PCP in *Drosophila* and mammals. However, the involvement of Scribble in spermatid alignment in the seminiferous epithelium during spermiogenesis with their heads pointing to the basement membrane but their tails to the seminiferous tubule

lumen, which appears to be a form of planar cell polarity, remains to be established. It is likely that the spermatid PCP in the seminiferous epithelium requires the concomitant contribution of PAR-, Crumbs-, and the Scribble-based modules. This possibility must be carefully evaluated in future studies.

Mutual Inhibition of Lgl and Apical aPKC-Par6 in Regulating Epithelial Cell Polarity

Similar to *dlg*, *lgl* was initially identified as a tumor suppressor, because mutation in this gene resulted in tissue-specific tumors in larvae that led to their eventual death.[94,95] Studies in *Drosophila* embryos have shown that Lgl is localized at cell membranes or intercellular matrix of embryonic cells and it is likely involved in cell-cell interactions.[45,96] In plasma membrane, Lgl was found to form large aggregates which were resistant to solubilization by nonionic detergents, indicating its involvement in cytoskeletal matrix.[97] Lgl also forms homo-oligomers and directly interacts with nonmuscle myosin II.[98] Furthermore, Lgl was found to tightly associate with aPKC in the Par protein complex.[99] The phosphorylation of Lgl by aPKC leads to a release of Lgl from the plasma membrane, dissociating from nonmuscle myosin II without affecting Lgl homo-oligomerization.[100] Additionally, genetic and phenotypic analyses in mutant *Drosophila* embryos suggested that the *lgl* product was required in different types of epithelial cells to control cell shape during development in vivo.[101] Collectively, these studies illustrate the involvement of Lgl in the formation of cytoskeletal network, and its interaction with cell membrane is mediated via its phosphorylation by aPKC.

The mutual inhibition of Lgl and aPKC has been reported in *Drosophila*. When Lgl is phosphorylated on three conserved Ser residues by aPKC, Lgl dissociates from the cytoskeleton and becomes inactivated, which, in turn, disrupts proper protein localization to the apical cell cortex.[99] Also, phosphorylation of Lgl causes an auto-inhibitory interaction of its N-terminus with its C-terminus, which prevents the binding of its C-terminus to the cytoskeleton.[102] On the other hand, aPKC mutants were found to display a phenotype opposite to that of the *lgl* mutants, i.e., a reduced aPKC level suppressed both cell polarity and cell proliferation in *lgl* mutants.[103] In *Drosophila*, the maintenance of Par6 localization to the apical side of epithelial cells requires Lgl, which is active on the basolateral side and excludes Par6 from the cell cortex. While on the apical side, Lgl is inactivated and excluded by aPKC phosphorylation.[104] These results suggest that mutual inhibition and exclusion of aPKC and Lgl on opposite sides of an epithelial cell participate in the formation of complementary corticle domains and correct epithelial structure.

In vertebrates, the Lgl homologs, Lgl1 and Lgl2, display similar antagonistic interactions with aPKC-Par3-Par6 complex in regulating epithelial cell polarity and proliferation. In contact-naive MDCK cells caused by the absence of Ca^{2+} in spent medium, cell adhesion and TJ formation were blocked with Lgl accumulated in punctate cytoplasmic structures. After the addition of Ca^{2+} to the medium, Lgl was found to associate with the lateral surface following E-cadherin appearance and TJ formation.[105] This indicates that the engagement of Lgl to the lateral membrane correlates with the development of a polarized structure in mammalian epithelial cells. This lateral domain restriction of mammalian Lgl (mLgl) is dependent on its phosphorylation within a highly conserved region, because a phosphorylation-resistant recombinant is observed to distribute in a nonpolarized manner.[105] In MDCK cells, mLgl competes with Par3 for its interaction with aPKC-Par6 to form a protein complex.[106] During epithelial polarization, mLgl temporally colocalizes with aPKC-Par6 at the cell-cell interface and inhibits the

formation of TJs, while another complex, Par3-aPKC-Par6, promotes TJ formation. Importantly, the phosphorylation of mLgl by aPKC is involved in its dissociation with aPKC-Par6 during polarization of epithelial cells.[106] These findings indicate that aPKC/Par6 selectively associates with either Par3 or Lgl, depending on the intrinsic activity of aPKC, to exerts its regulatory function on epithelial cell polarity.

Using siRNA knockdown, it was shown that mLgl regulated the assembly of apical membrane domains and cell polarization in MDCK cells.[107] Utilizing the Ca^{2+}-switch model, MDCK cells cultured in Ca^{2+}-depleted medium were found to have reduced levels of peripheral apical proteins and F-actin due to their redistribution from the cell surface to cell cytosol. However, in the mLgl-knockdown cells, the redistribution of these proteins was significantly suppressed and most of the mLgl-knockdown cells retained the apical proteins and F-actin at the cell periphery. Importantly, overexpression of dominant-negative aPKCλ was found to antagonize the effect of the mLgl knockdown on the redistribution of apical proteins.[107] This indicates that in mammalian epithelial cells, aPKC activity also contributes to suppressing the disassembly of apical proteins during cell depolarization, and mLgl counterbalances this aPKC activity to facilitate apical protein disassembly. This study also revealed that the suppression of apical Par3-aPKC-Par6 complex activity by mLgl is mediated through the suppression of an interaction of the aPKC-Par6 complex with Par3 or Cdc42.[107] p32, a mLg1 binding protein, was found to form a complex with mLgl and aPKC in regulating cell polarity.[108] Overexpression of p32 could rescue the reduced apical actin filaments caused by aPKC inhibitor whereas its knockdown induced defects in cell polarity.[108] This study thus suggests that p32 regulates cell polarization by binding to mLgl and aPKC and enhancing phosphorylation of mLgl via aPKC. These results thus illustrates that the mutual suppression between mLgl and aPKC-Par6 also plays an important role in the establishment of mammalian epithelial cell polarity.

Interactions between Scribble Complex and Other Apical Polarity Complexes in Epithelia

As briefly described above, several protein complexes that are involved in epithelial apicobasal polarity have been identified. Using genetic and biochemical approaches, details of the protein-protein interactions that connect these complexes are increasingly clear (Fig. 2). For example, the spatial relationship between Crb complex and Par complex is supported by findings in *Drosophila* and mammalian cells.[109-113] Co-immunoprecipitation in MDCK cells has illustrated direct interaction between Crb3, one of the Crumbs homolog in mammals, and Par6, which is required for the establishment of mature junction structure in epithelial cells.[109] In *Drosophila*, the cytoplasmic tail of Crb that contains two conserved Thr was shown to bind directly to aPKC. Moreover, Dpatj of the Crb complex can regulate the phosphorylation of Crb by aPKC, which induces Dpatj and Scribble to localize to the proper cell membrane domain.[110]

In addition to the antagonistic interaction between Par3-aPKC-Par6 complex and Lgl, another complex that belongs to apical domain of epithelial cells, the Crb complex, has also been found by genetic screening to have functional interactions with components of Scribble complex in *Drosophila*.[114,115] Crb is an integral apical protein that plays a role in specifying the apical domain of epithelial cells by forming a complex with Stardust (Sdt) and Dpatj in *Drosophila*.[116,117] Overexpression of Crb results in the expansion of the apical domain and a decrease in the basolateral domain, which is consistent with the phenotype caused by impaired activity of basolateral components of Scribble

complex.[118,119] Interestingly, genetic study revealed that the epithelial defects caused by *crb* mutation were markedly suppressed by *scribble* mutation.[114,115] Thus, it is likely that the Scribble complex antagonizes Crb in setting the limits of the apicobasolateral domains and in determining the relative distribution of cell junction proteins in these domains.

While no intrinsic catalytic activity is detected in components of the Scribble complex, several protein-protein interaction domains, such as PDZ, SH3 and GUK domains, are found in their primary sequences, illustrating the likely interactions between Scribble/Lgl/Dlg complex and other proteins, which can affect epithelial polarity and cell proliferation. Using biochemical approaches in human epithelial cells, MPP7 (membrane-palmitoylated protein 7), another member of MAGUK family was identified to interact with hDlg1 via its N-terminal L27 domain, and also with Pals1 (MPP5) and Patj, which are components of the Crb complex, via its SH3-HOOK domain.[25,120] In Caco2 cells, both hDlg1 and MPP7 displayed partial colocalization with occludin and ZO-1, and shRNA-mediated knockdown of either one of these proteins compromised TJ assembly, indicating the interactions between these polarity complexes affect cell junction function.[25] In migrating astrocytes, there is evidence that membrane-associated Dlg1 interacts with microtubule-bound APC (adenomatous polyposis coli, another tumor suppressor) through its PDZ domains to polarize the microtubule cytoskeleton during cell migration, which is required for polarization of astrocytes.[121] Furthermore, PKCζ-Par6 complex activated by Cdc42 was shown to control microtubule recruitment of APC and cortical recruitment of Dlg1.[121] This suggests a hierarchical biochemical connection exists between the two distinct polarity complexes.

These observations suggest that components of Scribble complex co-operate and compete with the apical polarity proteins to define the apico- and baso-lateral domains by recruiting respective junction proteins to the sites during cell polarity establishment.

SCRIBBLE COMPLEX PARTICIPATES IN CELL SIGNALING CASCADES

As summarized above, the Scribble complex and its homologs regulate both cell polarity and cell proliferation. Yet little is known about the underlying mechanism(s) particularly in mammals. However, recent findings have shed lights on the complex signaling network through which Scribble/Dlg/Lgl affects cell polarity and proliferation (Fig. 2).

SAPK3/p38γ is a one of the p38 MAPKs (p38 mitogen-activated protein kinases) which is activated in mammalian cells in response to hyperosmotic stress.[122,123] It has a C-terminal sequence that can dock with the PDZ domains of different proteins, one of which is hDlg. Studies by immunofluorescence and co-immunoprecipitation in Hela, PC12 and SH5-SY5Y cells have detected interaction between SAPK3/p38γ and hDlg, revealing specific phosphorylation of hDlg by SAPK3/p38γ in vivo in response to cellular stress.[124] In addition, this phosphorylation of hDlg was found to induce its dissociation from guanylate kinase-associated protein (GKAP), which is necessary for its targeting to cytoskeleton.[124,125] c-Jun N-terminal kinase (JNK) was also found to induce phosphorylation of hDlg during osmotic stress that contributed to its accumulation within the cell membrane at cell-cell contact site, which was coupled with an increase in E6-induced degradation of hDlg.[126] These data thus suggest that the cellular localization, function and proteasome-mediated degradation of hDlg are phosphorylation-dependent events.

Furthermore, the involvement of mammalian Scribble in oncogenic transformation was shown to be mediated via a suppression of MAPK signaling.[127] For instance, simultaneous knockdown of Scribble by shRNA and an activation of Ras-signaling in nontransformed

human MCF10A mammary epithelial cells were shown to promote cell invasion.[127] Moreover, a two-fold increase in the phosphorylation of ERK (extracellular-regulated kinase, an MAPK effector) and an acute hyperphosphorylation of ERK in response to EGF, were also detected in Scribble-depleted cells.[127] In Scribble-depleted cells, an activation of Raf-MEK-ERK signaling was found to enhance the defects of Scribble loss. Furthermore, Scribble-overexpressed cells were found to suppress the development of invasive protrusions of Ras-activated cells, and Scribble overexpression also facilitated the restoration of cell polarity in Ras-activated cells.[127] These data thus illustrate that Scribble acts as a tumor suppressor by limiting cell invasion, which is mediated downstream of Ras but upstream of MAPK in the signaling pathway.

A recent study has revealed the likely mechanism by which Scribble suppresses the Ras-Raf-MEK-ERK pathway.[128] Two ERK binding sites (kinase interaction motif, KIM) were identified on hScrib, and hScrib was found to regulate the ERK signaling pathway in human keratinocytes by inhibiting the phosphorylation and nuclear translocation of ERK.[128] In vitro study also revealed the two phospho-acceptor sites of hScrib were putative substrates for PKA and ERK1.[128] These results thus support the notion that a loss of Scribble can affect mitotic proliferation in tumors by promoting MAPK phosphorylation and nuclear translocation of ERK.

In mammalian tissue barriers, there is also evidence that Scribble complex components participate in endocytic vesicle-mediated trafficking and membrane fusion events. For example, membrane-associated mLgl from MDCK cells was found to directly interact with Syntaxin 4 (STX4), a component of the exocytic pathway at the basolateral membrane.[105] Recently, a study in HaCaT cells has shown that three components of the Scribble complex, hScrib, mLgl and hDlg all display significant degree of colocalization with STX4 at cell membrane.[129]

THE SCRIBBLE MODULE AND MAMMALIAN EPITHELIAL BARRIER FUNCTION

Epithelial monolayers that display apico-basal cell polarity serve as tissue barriers in multiple organisms. Cell polarity and impermeability are two important attributes of these tissue barriers. The polarity protein complexes described above cooperate with each other to confer differential distribution of functional proteins on plasma membrane. On the other hand, junction complexes formed at correct cortical positions help to maintain distinctive cell membrane domains and resist material exchanges between two sides of the epithelial monolayer. Therefore, the development of cell polarity plays an important role in epithelial barrier establishment and function. In this section, we focus on the role of Scribble polarity complex on the formation and function of several mammalian epithelia and the likely underlying mechanism(s).

Scribble Regulates Barrier Function of Intestinal Epithelium

In the small intestine, the gut barrier conferred exclusively by TJs of the intestinal epithelium protects the host animal from invasion of pathogens during food digestion and absorption. Although the causal relationship between the establishment of TJs and cell polarity remains unclear, the role of TJs in sealing the paracellular space that creates the gut barrier has been established.[130,131]

The role of Scribble in TJ assembly was first demonstrated in T84 and SK-CO15 intestinal epithelial monolayers in which Scribble was found to colocalize with TJ proteins occludin and ZO-1.[132] This is in contrast to other mammalian epithelia in which Scribble was restricted to the basolateral plasma membrane.[91,133] Moreover, similar to the findings in Scribble-depleted MDCK cells,[87] a knockdown of Scribble by RNAi was found to significantly delay gut barrier assembly, and to perturb the TJ-barrier function of Scribble-deficient cells.[132] Interestingly, Scribble knockdown in SK-CO15 cells did not affect the TJ ultrastructure, nor impeded the development of apicobasal cell polarity.[132] These data illustrate Scribble is crucial to the gut barrier function in vitro.

In the intestinal epithelium, Scribble was found to interact directly with ZO-1[132] and ZO-2,[91] which are cytosolic scaffolds that stabilize integral membrane TJ proteins (e.g., occludins, claudins, JAMs). A study to monitor the subcellular distribution of Scribble in mouse small intestine epithelium showed that Scribble was localized to the basolateral membrane. In addition, Scribble was also detected at the site of apical junction complex.[134] Moreover, a distinctive colocalization of Scribble and E-cadherin was detected at the apical junction site, illustrating an apical and basolateral localization of Scribble in mouse intestinal epithelium.[134]

Although there is little evidence of physical interaction between components of Scribble complex in mammalian epithelia, a recent report in the gut barrier has revealed selective binding between Scribble and Lgl-1, but not Dlg-1, during TJ reassembly.[132] More important, a knockdown of Dlg-1, but not Lgl-1, was found to attenuate the gut barrier function.[132] Furthermore, interferon (IFN)-γ, a pro-inflammatory cytokine, was found to induce down-regulation of junctional Scribble during TJ disassembly caused by the IFN-γ-induced intestinal inflammation in vivo.[132] These findings seemingly suggest the absence of a trimeric Scribble/Lgl/Dlg complex in mammalian intestinal epithelium, and Scribble is likely to play a unique role in gut barrier function.

Scribble Complex Controls Renal Epithelial Polarity

In the kidney, tubular epithelium is composed of polarized epithelial cells with the apical membrane domain facing the tubular lumen, and cell junctions are either restricted to the cell-cell interface or to the cell-basal lamina interface at the basolateral domain. In the kidney, cell polarity and cell junctions in the epithelium of convoluted tubules are necessary to fluid/electrolyte re-absorption and secretion. Studies by confocal microscopy showed that hScrib was located at the cell-cell interface of MDCK (Madin Darby canine kidney) cells, colocalizing with β-catenin. Calcium switch assay also demonstrated hScrib was retained to cell junction upon engagement of E-cadherin by its LRR region in MDCK cells.[85] Binding of hScrib to E-cadherin at the lateral side is important for normal adhesion between polarized renal epithelial cells, since knockdown of hScrib was found to increase cell motility and reduce adhesion.[87] mLgl was localized to basolateral membranes and it interacted with basolateral exocytic machinery in MDCK cells.[105] Further studies in renal epithelium also revealed the interaction between mLgl and aPKC-Par6 complex.[106] In hDlg1-deficient mice, abnormalities were found in the renal and urogenital organs. For instance, the kidneys and ureters of hDlg1-deficient mice became hypoplastic due to reduced cell proliferation in the uretic or renal epithelium.[135] These findings thus illustrate the involvement of Scribble complex in the development, cell proliferation, and maintenance of renal epithelial polarity and integrity.

Scribble Complex and the BTB

Based on studies in the field as briefly reviewed and summarized above, it is increasingly clear that the Scribble/Dlg/Lgl polarity protein module, whose components are tumor suppressor genes, is crucial to cell junction dynamics in mammalian epithelial cells. Furthermore, each of the components in this complex can recruit numerous partner proteins via their protein interacting domains (e.g., PDZ domain). As such, a complex network of proteins can be recruited to specific cellular sites/domains and that this complex can regulate a wide range of cellular functions besides cell polarity. Interestingly, even though components of the Scribble protein complex, namely Scribble, Dlg, and Lgl, were discovered ~3 to 4 decades ago in *Drosophila*, and their mammalian homologs were since identified and shown to possess similar physiological functions, there is no report in the literature that examined their function in the testis, such as the BTB during spermatogenesis. As shown in Figure 3, Scribble, Dlg1 and Lgl2 were shown to be expressed in the testis, with these proteins expressed by both Sertoli and germ cells. Using an antibody specific to Scribble, its localization in the seminiferous epithelium of the rat testis has also been examined by immunohistochemistry (Fig. 4), illustrating

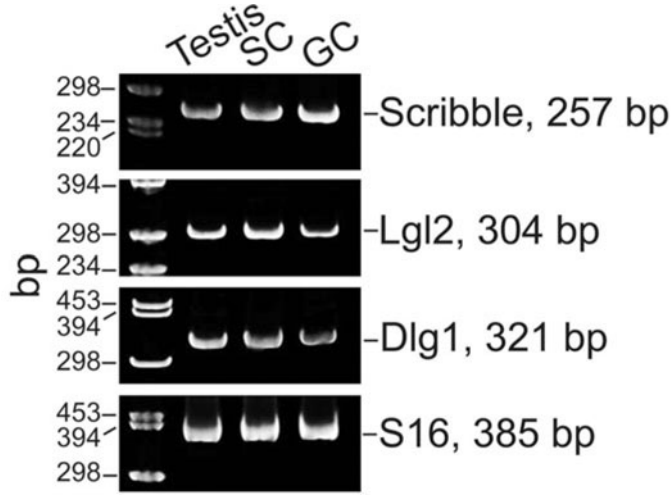

Figure 3. Components of the Scribble protein complex in the testis. Scribble, Lgl2 and Dlg1 were detected in the testis, as well as in Sertoli (isolated from 20-day-old rat testes after 4 days in culture with negligible germ cell contamination) and germ cells (isolated from 90-day-old rats after ~12-hr in culture) by RT-PCR. Primers used in PCR were as follows: (i) Scribble: sense, 5'-CTGGCACTGCTCACAGATCT-3 (nucleotides 724-743); antisense, 5'-AGCACCTCAAGATGATTCCG (nucleotides 961-980) (GenBank Accession #:XM_002726943.1); (ii) Lgl2: sense, 5'-TCCACCATCTCGAACACTCG (nucleotides 442-461); antisense, 5'-TGCTGGATGACAACAGCCTG (nucleotides 726-745) (GenBank Accession #:NM_001127549.1); (iii) Dlg1: sense, 5'-GTTGACCTCAGAGCTGCAAG (nucleotides 2000-2019); antisense, 5'-CCACCACTCGTCATCAGAAG (nucleotides 2301-2320) (GenBank Accession #:NM_012788.1); which were co-amplified with (iv) S16: sense, 5'-TCCGCTGCAGTCCGTTCAAGTCTT (nucleotides 177-200); antisense, 5'-GCCAAACTTCTTGGATTCGCAGCG (nucleotides 538-561) (GenBank Accession #:XM_001078234). RT-PCR was performed essentially as earlier described.[138,139] The cycling parameters for PCR reaction were: denaturation at 94 °C for 1 min, annealing at 57 °C for 2 min, and extension at 72 °C for 3 min, for a total of 21-25 cycles, which were followed by an extension period of 15 min at 72 °C. The identity of the PCR product was confirmed by direct nucleotide sequencing at Genewiz. SC, Sertoli cells; GC, germ cells, bp, base-pair.

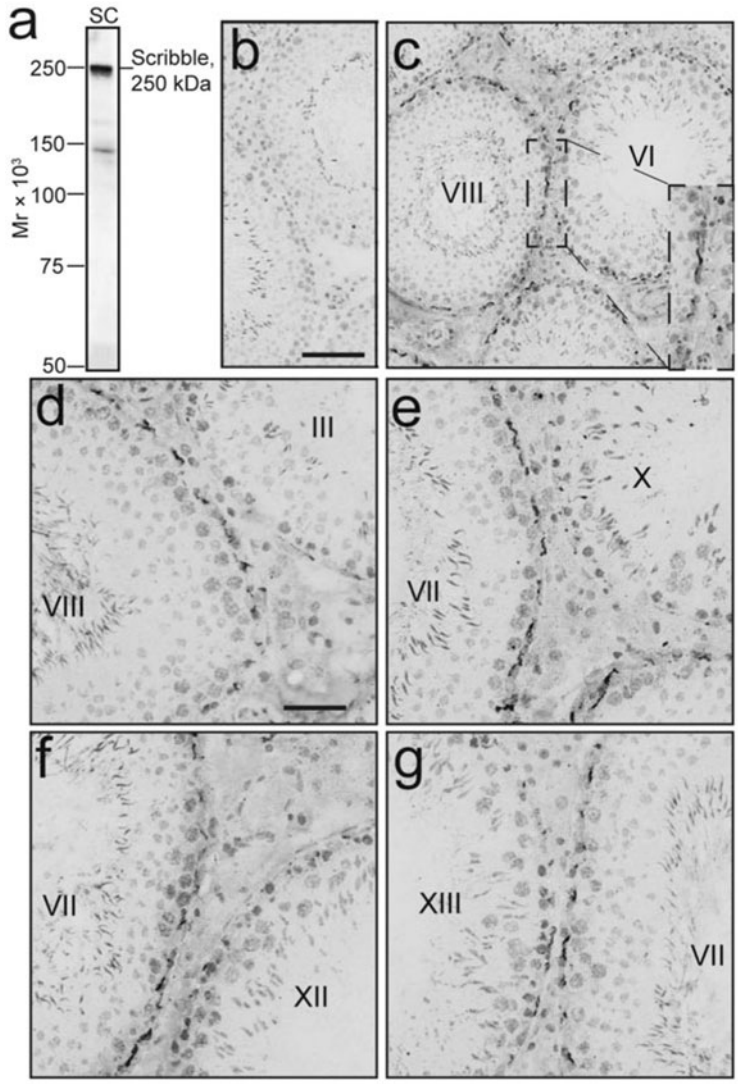

Figure 4. Stage-specific expression of Scribble in the seminiferous epithelium of adult rat testis during the seminiferous epithelial cycle of spermatogenesis. Immunohistochemistry was performed using frozen cross-sections of testes from adult rats and a goat anti-Scribble antibody (Santa Cruz, Cat. # sc-11048) which cross-reacted with Scribble in rats. Using immunoblot analysis and lysates of rat Sertoli cells (SC), this anti-Scribble antibody (at 1:750 dilution for immunoblotting) was found to be specific to Scribble (a). This antibody was used for immunohistochemistry (at 1:50 dilution) (c-g) versus normal goat serum which served as the negative control and shown in (b). Immunoreactive Scribble appears as reddish-brown precipitates (c-g). It is noted that Scribble was localized almost exclusively near the basement membrane in the seminiferous epithelium, consistent with its localization at the BTB (note: this pattern of localization also illustrates its polarized localization) in tubules, most prominently at Stages IV-VIII but it diminished rapidly thereafter to an almost nondetectable level at Stages X-XII but gradually re-appeared from Stage XIII. Bar in b = 80 μm, which applies to c; bar in d = 40 μm, which applies to e-g. Mr, molecular weight. A color version of this figure is available online at www.landesbioscience.com/curie.

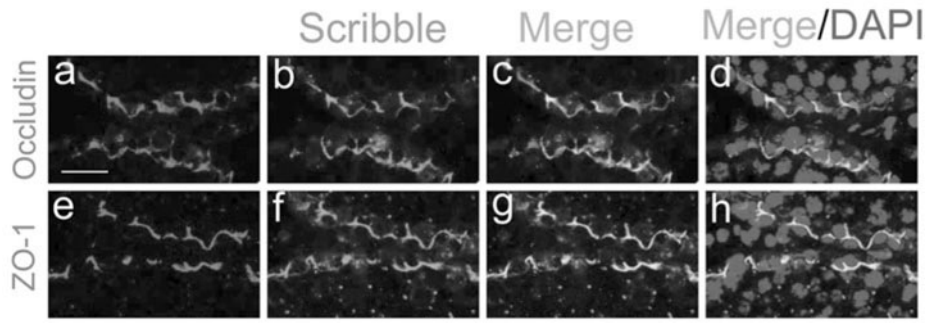

Figure 5. A study by dual-labeled immunofluorescence analysis to assess the colocalization of Scribble with TJ-proteins occludin and ZO-1 at the BTB. In order to further confirm the observations shown in Figure 4 that Scribble is indeed an integrated component of the BTB, dual-labeled immunofluorescence analysis was performed in which Scribble (green fluorescence in b, f) was found to colocalize with either occludin (a TJ-integral membrane protein at the BTB, red fluorescence in a) or ZO-1 (a TJ-associated adaptor protein at the BTB, red fluorescence in e) with the merged images shown in c, d, and g, h, which appear as orange-yellow fluorescence. Cell nuclei were visualized by DAPI staining (d, h). Bar in a = 40 μm, which applies to b-h. A color version of this figure is available online at www.landesbioscience.com/curie.

Scribble is localized at the basal compartment of the seminiferous epithelium near the basement membrane, consistent with its localization at the BTB (Fig. 4). More important, the localization and expression of Scribble at the BTB appears to be stage-specific since Scribble is abundantly found at the BTB at Stages VII to VIII of the seminiferous epithelial cycle at the time of BTB restructuring to accommodate the transit of preleptotene spermatocytes at the site (Fig. 4). The localization of Scribble at the BTB has also been confirmed by dual-labeled immunofluorescence analysis, in which Scribble was found to colocalize with putative integral membrane protein occludin and TJ adaptor protein ZO-1 at the BTB (Fig. 5). These findings thus demonstrate unequivocally that Scribble is an integrated component of the BTB. These results also illustrate that functional studies can now be performed to probe the physiological role of the Scribble/Dlg/Lgl at the BTB. Furthermore, it will be important in future studies to examine how these proteins interact with the Par-based polarity proteins, such as Par 6 and 14-3-3, which were shown to regulate spermatid orientation and cell adhesion at the BTB, via their effects on the endocytic vesicle-mediated protein trafficking.[71,136,137]

CONCLUSION AND FUTURE PERSPECTIVES

The Scribble/Dlg/Lgl complex has been shown to regulate cell polarity, TJ and AJ dynamics, and cell proliferation in species ranging from fruitflies, worms and mammals (e.g., the gut barrier, the tubular barrier in the kidney) as summarized above. It is anticipated that this polarity complex is a multifaceted functional complex at other blood-tissue barriers, such as the BTB, where components of this protein module were found. Also, it will be important in future studies to assess its functional relationship with the PAR- and the Crumb-based complexes since PAR-6,[136] 14-3-3,[137] and Cdc42[71] were shown to be integrated components of the Sertoli cell that regulated BTB dynamics,

and how these three polarity modules fine tune the timely restructuring of the BTB during the seminiferous epithelial cycle of spermatogenesis. For instance, it remains to be examined if the Scribble complex regulates endocytic vesicle-mediated protein trafficking at the BTB; and if it does, how this complex works with the PAR-based protein complex to modulate protein endocytosis, transcytosis, recycling, and endosome-/ubiquitin-mediated degradation.

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CHAPTER 8

THE BLOOD-BILIARY BARRIER, TIGHT JUNCTIONS AND HUMAN LIVER DISEASES

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Abstract: Tight junction (TJ) composes of an intriguing class of cell junction molecules, for which these molecules share similar organizations and structure features among different organs. Four types of transmembrane molecules namely occludins, claudins, junctional adhesion molecules and coxsackievirus and adenovirus receptors act as core units and each link directly and indirectly with a panel of peripheral molecules and underlying cytoskeletons to constitute the functional protein complexes at TJs. Individual TJ complex alone or in co-operation with other complexes via cross-talk mediated by peripheral molecules activate signaling pathways pertinent to various physiological and pathological processes in livers. In human livers, TJs are located at two regions in association with hepatocytes and cholangiocytes and perform major roles in controlling bile flow and metabolism. Apart from this physiological function, the other functions of TJs relating to liver diseases of hepatitis and liver cancer are gradually uncovered. The understanding of how TJs are involved in these clinical conditions hint for the development of new treatments at the molecular level.

INTRODUCTION

Cell junctions constitute a unique structural feature lining the cell-cell border. Because of this specific cellular localization, junctions were discovered initially to maintain cell-cell adhesion and interactions, contributing a main role in cell adhesion and communication. These functions are exemplified clearly in intestinal epithelium, such that adjacent epithelial cells adjoin each other via cell junctions and that in turn leads to the formation of epithelial cell sheet. The adherence of nearby epithelial cells also facilitates cell-to-cell communications. Establishment of selective permeable barrier is an additional function associated with the formation of intestinal epithelium, thereby

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guarding the unwanted entry of micro-organisms and pathogens while at the same time facilitating the absorption of the luminal contents.[1-3] However, it is not possible to describe all the properties associated with various junctions in different parts of the body and readers should refer to other chapters in this book for more details. Apart from the physiological functions of different junctions, research is also devoted to investigate the cellular mechanism related to cell junctions. In a broad sense, cell junctions are involved actively in several signaling pathways during physiological and pathological processes, such as growth, differentiation, disease progression and degeneration.[4-6] Cell junction-related Wnt/β-catenin pathway is one such pathway that is often activated when cancers progress.[7,8] Therefore, cell junctions are key players in a series of cellular processes. In view of the significance of cell junctions in this aspect, they remain targets for biomedical research in particular for those involving infectious diseases and cancers.

In eukaryotes, junctions exist in various locations in different organs and tissues, conferring structural integrity and maintaining functional property. Mammalian cell junctions are classified into various types based largely on the anatomical features and structural compositions. Five major junction types are found, which are known as tight junction (TJ), adherens junction, gap junction, desmosomes, and hemidesmosomes.[9-11] These junctions situate precisely at their respective locations. Taking epithelium as the example, TJ is located at the most proximal position sealing the gap between neighboring epithelial cells, whereas adherens junctions, desmosomes and gap junctions are frequently found at the lateral faces of these cells. Hemidesmosomes are those junctions anchoring cells with the extracellular matrix.[3,9,12] It is therefore obvious that different junctions serve intrinsic roles in adjoining, communicating, situating and positioning cells to assemble a functional system. Among the many types of junctions, TJ is exceptional to certain extent as TJs are sometimes named after an organ based on their specific locations. For instance, blood-brain barrier is found in brain and blood-testis barrier is located in the testis.[13,14] Since liver is the theme of this chapter, TJs found in the liver will be further described and discussed.

ANATOMY AND PHYSIOLOGICAL FUNCTIONS OF TJs IN THE LIVER

Liver is one of the most complicated organs in the body, carrying out numerous metabolic activities-like glucose metabolism, detoxification, bile secretion, urea metabolism and others. Basically, it is composed of heterogeneous cell types, such as hepatocytes, cholangiocytes/bile duct cells, hepatic stellate cells, blood cells, and others. Among these, hepatocytes contribute most of the tissue mass in the mammalian livers.[15,16] Similar to other epithelia and tissues, cells in the livers are also connected and communicated to each other via the establishment of different junctions.[17-19] For the interest of this chapter, we focus only on TJs. TJs in the livers are found associating with two cell types, namely hepatocytes and cholangiocytes.[17,20] Both of these cells are polarized epithelial cells with apical and basolateral surfaces that are enriched with various junctions to maintain hepatic anatomy and to sustain physiological functions of the livers. They perform similar functions as TJs in other epithelia by modulating or restricting the passage of small molecules and ions across the cell monolayers. In particular, TJ found lining the apical face of hepatocytes surrounding the bile canaliculi is referred specifically as blood-biliary barrier. From its name, this barrier functions mainly to enable collection of bile acids and bile salts inside the bile canaliculi away from blood circulation and prevent the backflow

of bile to liver parenchyma. In addition, it helps to maintain the polarity of the hepatocytes by segregating the apical surface from the basolateral surface, therefore enabling the formation of the liver cell plate.[16,20,21] The function of cholangiocyte-associated TJ is largely similar to the hepatocyte-associated TJ by regulating the bile flow and secretion. Other specific functions inherited by cholangiocytes include their abilities in modifying the composition of bile during bile transit in the bile ducts.[22,23] Therefore, it is obvious that TJ molecules of these two cell types perform similar but different functions, which can be glimpsed based on their distinct, yet overlapping, molecular compositions.

MOLECULAR COMPONENTS OF TJ IN THE MAMMALIAN LIVER

As in other tissues, TJ in the liver is also assembled by an array of TJ complexes, which are composed of integral membrane molecules linked to the underlying cytoskeletons via peripheral adaptor molecules. Integral membrane molecules are core elements of the TJ complexes. The cytoplasmic domains of these molecules are usually associated with peripheral adaptor molecules, which act as a bridge between integral molecules with cytoskeletal network.[24-27] With all these components, this structural architecture constitutes the functional unit of TJ complexes (Fig. 1). Though largely similar in their molecular compositions, the two types of TJ in liver demonstrate their uniqueness by possessing their own distinct molecules (Table 1). For instance, it is known that coxsackievirus and adenovirus receptor-1 (CAR-1) is expressed exclusively in the cholangiocyte-associated TJ, but not found associating with hepatocytes, in mouse livers.[28] This phenomenon highlights the similar, yet distinct, functions of TJs locating at different positions in the livers. One postulation for such differences might be the different strength of adhesiveness at the TJ between cholangiocytes, such that both CAR-1 and CAR-2 are residing there to perform their roles in bile ducts. Similar observation was found for claudin-7, for which it is found associating with biliary epithelial cells, but not hepatocytes, in canine liver tissues.[29] Additional research is needed to understand the physiological basis of such differences.

Integral Membrane Molecules

Until now, four classes of integral membrane molecule have been identified in mammalian livers, which are occludins, claudins, junctional adhesion molecules (JAMs) and CARs (Table 1). Among them, occludins and claudins share similar molecular structure by having four transmembrane domains, cytoplasmic amino- and carboxyl-domains, two extracellular loops and two intracellular tails at each end.[24,30,31] JAMs and CARs distinguish themselves from occludins and claudins by having a single pass transmembrane region with extracellular amino- and intracellular carboxyl-terminus. At the amino-terminus, two loops are found for both JAMs and CARs.[27,31] Regardless of their differences in molecular architecture, all of them perform cell adhesion function via interacting with adjacent molecules utilizing the extracellular loop regions.

Peripheral Molecules

Peripheral molecules are those attaching to the cytoplasmic domain of integral membrane molecules in a direct or indirect manner. The nature of these molecules varies and they can be adaptors, protein kinases or phosphatases, cytoskeleton-binding proteins

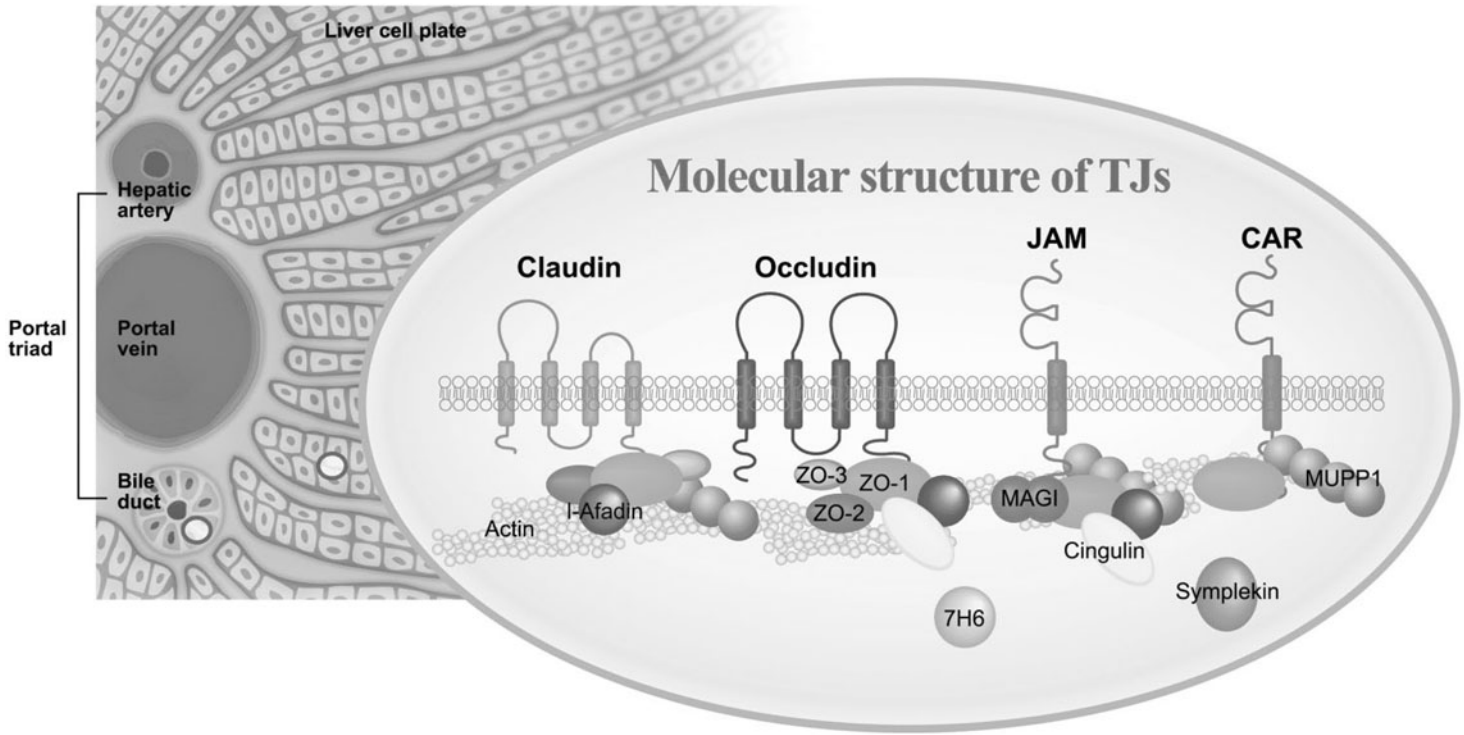

Figure 1. Molecular structure of TJ in mammalian liver. TJs in the livers are found associating with hepatocytes in the liver cell plate and cholangiocytes in the portal triad (as indicated by circles), for which the former is located around the bile canaliculus and the latter is found in the bile duct. These two types of TJs share similar but distinct structural components. Both of them are comprised members of the claudins, occludins, JAMs and CARs as the basic structural elements, for which these molecules are linked indirectly to underlying actin cytoskeleton via a panel of peripheral molecules-like ZO proteins. Therefore, these TJ complexes confer structural anatomy and execute physiological functions in livers. Abbreviations: CAR, coxsackievirus and adenovirus receptor; JAM, junctional adhesion molecule; MAGI, membrane-associated guanylate kinase inverted; MUPP1, multiple PDZ domain protein-1; TJ, tight junction; ZO, zonula occludens.

Table 1. TJ molecules in mammalian livers

TJ Molecules	Species	Liver	Hepatocyte-Associated TJ	Cholangiocyte-Associated TJ	Selected References
Integral membrane proteins					
CAR	H	+	+	+	94
CAR-1	M	+	-	+	28
CAR-2	M	+	+	+	28
Claudin-1	H, M	+	+	+	90,95
Claudin-2	H, M, R	+	+	-	95-97
Claudin-3	M, R	+	+	+	97-99
Claudin-5	M, R	+	-	-	97,98
Claudin-6	H, M	+	n.k.	n.k.	56,100
Claudin-7	C, M	+	-	+	29,100
Claudin-8	M	+	n.k.	n.k.	98
Claudin-9	H	+	n.k.	n.k.	56
Claudin-10	H	+	n.k.	n.k.	91
Claudin-12	M	+	n.k.	n.k.	100
Claudin-13	M	+	n.k.	n.k.	99
Claudin-18	H, M	+	n.k.	n.k.	101
JAM-1	M	+	n.k.	n.k.	102
JAM-2	M	+	n.k.	n.k.	102
JAM-3	M	+	n.k.	n.k.	102
Occludin	H, M	+	+	n.k.	103,104
Occludin 1B	M	+	n.k.	n.k.	104
Peripheral proteins					
7H6	R	+	+	n.k.	105
Cingulin	H	+	n.k.	n.k.	106
l-Afadin	R	+	n.k.	n.k.	107
MAGI-1	M	+	+	+	108,109
MAGI-3	H	+	n.k.	n.k.	110
MUPP1	H	+	n.k.	n.k.	111
Symplekin	H, M	+	+	+	17,112
ZO-1	H, M, R	+	+	n.k.	97,103,108
ZO-2	C	+	+	n.k.	113
ZO-3	M	+	n.k.	n.k.	114

+, presence; -, absence; C, canine; CAR, coxsackievirus and adenovirus receptor; H, human; JAM, junctional adhesion molecule; M, mouse; MAGI, membrane-associated guanylate kinase inverted; MUPP1, multiple PDZ domain protein-1; n.k., not known; R, rat; TJ, tight junction; ZO, zonula occludens.

and others. The best studied peripheral molecules at TJ belong to the zonula occludens (ZO) family members. ZO-1 is the first known adaptor in this class with its ability to bind actin cytoskeleton directly or indirectly via clustering with other actin-binding molecules. Since then, other members named ZO-2 and ZO-3 are identified for their abilities in bridging integral molecules with the cytoskeletal network.[32,33] Apart from ZO family members, a number of peripheral molecules are found in this TJ complex in mammalian livers and these include cingulin, symplekin and others (Table 1). In

addition to the structural function of these peripheral molecules, some of them are involved actively in other cellular mechanisms and this is particularly true for ZO proteins. ZO proteins are signature molecules of this class for their ability to shuttle into the nucleus.[32] However, the exact roles of these nuclear ZO proteins remain to be elucidated. Most studies reveal the existence of these peripheral molecules in mammalian livers using biochemical methods-like western blot and polymerase chain reaction. Not all these studies employed immunological techniques, such as immunofluorescence and immunoprecipitation, to prove the direct interaction or clustering among these peripheral molecules with the integral molecules. Based on the molecular and biochemical studies performed in other systems, we still believe that the architecture of these peripheral molecules in association with their integral molecules is in resemblance to other epithelia (Fig. 1).

Cytoskeleton

Cytoskeleton allows the anchorage of TJ-associated protein adhesion complexes including integral membrane molecules, peripheral protein kinases and phosphatases via the interaction with adaptor molecules. Different categories of cytoskeletal elements are found associating with various junctions such as intermediate filament at desmosomes and microtubule at adherens junctions.[34,35] Among them, actin is the only known element constituting the actin filament underlying various TJ complexes.[36] The roles of actin filament seem to be universal because of its presence in various junction complexes at TJ, offering structural integrity.[25] Other than this function, actin filament is known to be involved in a series of signaling transduction events.[37,38]

JUNCTION-TO-JUNCTION INTERACTION AT TJ IN THE LIVER

Even though TJ alone seems to be a working unit in modulating paracellular passage and maintaining cellular polarity, the functions of TJ are indeed extended to the molecular level. Studies in other epithelia and endothelia reveal TJ also acts as a central unit in an array of signaling networks originated from other junctions. It is not difficult to imagine TJ to play a central commanding role among cell junctions because of its close proximity with them in the plasma membrane. Liver is not the sole organ employing such mechanism for attaining functional integrity. Of note, such kind of junction interaction is commonly observed in the testis, which is another organ with intriguing junction complexity. In testes, most junctions locate in the same way as in other epithelia with the exception of TJ. Unlike other TJs at the most apical region of the epithelial layers, testicular TJs situate near the basement membrane, co-existing with gap junction and desmosome, and they are dynamic ultrastructures, enabling the transit of preleptotene spermatocytes for further differentiation and maturation into sperms.[14,39] In view of this unusual arrangement of TJs in the testes, testicular TJs intermingle with other junctions and can "cross-talk" with other junctions via associating molecules. Testes achieve this inter-junction communications via the mobilization of associated peripheral molecules-like ZO-1 between different junction complexes.[40,41] This results in either selective enhancement or disruption of the biological functions of a specific junction-type without perturbing the others.

TJs in the liver also mediate cross-talk with other junctions and this notion has been exemplified clearly from several studies. In studies using connexin 32-deficient mouse hepatocytes, ectopic expression of a gap junction molecule connexin 32 in these cells induced the expression of TJ molecules including occludin, claudin-1, ZO-1 and membrane-associated guanylate kinase inverted-1 (MAGI-1), thereby enhancing cell polarity.[18,42,43] These studies suggest the impact of manipulating gap junction component on TJ integrity. On the other hand, TJ also possesses abilities to modulate adherens junction, such that depletion of JAM-1 in human hepatoma cells enhances the level of E-cadherin, an adherens junction molecule.[44] Until now, the molecular mechanism(s) underlying these observations remain unknown. It is hypothesized that such cross-talk is the result of shuttling abilities of the peripheral molecules of different junctions and the properties of these adaptors as versatile molecules among different junctions. In short, peripheral molecules from one junction-type can exert their effects in another junction-type via signaling transduction pathways downstream so that signaling events among different junctions can be connected in response to changes in environment or during pathogenesis. Among a panel of TJ-related peripheral molecules, ZO family members seem to be a plausible candidate in mediating these processes because of these adaptors are having a variety of binding partners in different junctions.[27]

INVOLVEMENT OF TJ MOLECULES IN LIVER DISEASES

Liver diseases range from chronic inflammation, hepatitis, fibrosis, cirrhosis and cancers. Most of these diseases are not drug treatable, except for hepatitis infection in which antiviral drugs can be used to suppress the body viral loads and prevent further damage to the livers.[45-47] In fact, many of these liver diseases belong to a series of consequential events when liver cancer develops in the background of inflammation, fibrosis and/or cirrhosis.[48,49] Liver cancer ranks fifth and seventh as the most prevalent cancer in men and women, respectively, worldwide with more than half a million new cases annually. Among all liver cancer cases, hepatocellular carcinoma (HCC) is the major-type with high incidence in Asia and Africa and a recent rise in Western countries.[50] Due to late symptom presentation, aggressive phenotype and limited treatment option, HCC is a deadly disease with nearly equal number of occurrence and death.[50] For any diseases, early detection and effective treatment remain gold standards for leading to survival benefits. Therefore, understanding the cellular mechanism associated with different diseases hints for identification of specific biomarkers and development of potential therapy. For disease biomarkers, these molecules should possess certain features. For instance, these molecules should have elevated level during the early phase of pathogenesis in order to differentiate patients from normal subjects having low or negligible level of them. At the same time, these molecules should be found in body fluids, enabling the development of non-invasive screening assays for quantification of their level. For treatment, delineation of the cellular mechanism hints for the recognition of key disease-related molecules and hence postulating the development of targeted therapy. Targeted therapy functions differently from other general medicine with a broad spectrum of actions, instead it works specifically by targeting specific molecules. The advantage of this mode of therapy is the potential minimization of adverse side-effects and brings less harmful side-effects to patients. Among all liver diseases, only hepatitis C virus (HCV) infection and HCC have proven correlation with deregulation of TJ and its associated complexes.

HCV Infection

Hepatitis virus infection is the most common etiological factor leading to development of liver cancers. At least seven types of hepatitis viruses have been discovered todays and new species are emerging.[51] Among them, hepatitis B virus (HBV) and HCV being most relevant to HCC development and have attracted most attention in clinical research.[52] HBV- and HCV-infected patients demonstrate a distinct while overlapping geographical distribution around the globe. The underlying reason is not totally deciphered. HCV is an RNA virus and its infection is mediated via body fluids, such as blood.[53] Due to the lack of effective vaccines, HCV has no preventive measure except for avoiding direct contact with contaminated materials. This also poses clinical challenges in treating HCV infection. Currently, a combination treatment of pegylated interferon and ribavirin becomes a standard treatment for all HCV genotypes. In some cases, early treatment using these drugs leads to stabilization of clinical conditions.[53,54]

TJ molecules in co-operation with other adjuvant molecules are key molecules in the course of HCV infection. During viral infection, HCV binds to claudin-1, for which this molecule acts as coreceptor for viral entry.[55] Besides claudin-1, claudin-6 and claudin-9 also function similarly in the same process.[56] In addition, occludin is also shown to be an essential molecule in the postbinding step of HCV entry.[57] Indeed, this virus-junction protein interaction to mediate the initial viral entry is not solely reserved for hepatitis virus since several other viruses-like adenovirus and coxsackievirus also employ similar mechanism to initiate infection.[58] Apart from these TJ molecules, other studies have also demonstrated the involvement of CD81 and human scavenger receptor class B member 1 (SR-BI) as coreceptors for the entry of HCV across the TJ-barrier.[59,60] In addition of having occludin and claudins in the initial entry step of HCV, other TJ molecules also involve in this process substantially. Incorporation of the genomic replicons of HCV in HCC cells was found to induce disarrangement of TJ molecules and at the same time led to retention of occludins in the endoplasmic reticulum that failed to assemble to the TJ-fibrils.[61] In line with these studies, several studies have further focused on the investigation of claudin-1 in HCV infection. HCV internalization stimulates the production of claudin-1, but not CD81, in HCC cells.[62] This study implicates for the capability of HCV in facilitating further viral entry via its effect on stimulating the production of viral receptor, such as claudin-1. This notion is further supported by another study showing an impaired viral susceptibility of HCC cells when claudin-1 expression was re-organized due to inactivation of protein kinase A.[63] With these data, it is increasingly clear regarding the role of TJ molecules in particular claudin-1 and occludin in the process of HCV infection in the livers and how a better understanding of these molecules can contribute to the better treatment of HCV infection. It is envisioned that interference of TJ molecules having a prominent role in HCV entry might give hints for developing better therapeutic approach, such as by blocking viral entry into the livers. In order for this to become a treatment option, care should be taken since these TJ molecules are necessary for the normal functioning of the livers. Thus, much research is needed to explore the possibility of targeting TJ molecules as a therapeutic target for HCV treatment.

HCC

Development of HCC mostly results from hepatitis virus infection and other etiologies include alcohol abuse, aflatoxin ingestion, non-alcoholic steatohepatitis and several metabolic diseases.[64] HCC is a deadly disease with no definite cure and the prognosis

remains poor. Until now, surgical resection remains the first line treatment for HCC patients, however this method does not suitable for late-stage patients or those with poor liver functions.[65] As HCC is asymptomatic, patients are usually in the advanced stage when it is first diagnosed and this thus excludes them for surgical treatment. Liver transplantation is another surgical approach for treating HCC and its use is limited by the shortage of liver grafts.[65,66] Other treatments include percutaneous ethanol injection, radiofrequency ablation and transcatheter arterial chemoembolization are in clinical practice, but none of them associate with desirable response rate and with limitation in patient selection.[67,68] No effective chemotherapeutic drug is available or developed for treating HCC because of its strong drug resistance nature. In practice, only Sorafenib, a multi-kinase inhibitor, has gone through the clinical trials and is the only approved drug for treating late-stage HCC patients. Despite that, this drug treatment does not prolong the survival significantly and with marginal benefits in extending the lifespan of patients.[65,69,70] Thus, the clinical condition of HCC is dismal and only early treatment would lead to better prognosis. Early treatment means early detection and this is hindered greatly because of the late presentation of symptoms of HCC. Clinical procedure for detecting HCC comprises ultrasonography and detection of serum alpha-fetoprotein (AFP) level. Most of these tests are performed in high-risk populations and those with hepatitis and/or cirrhosis. While ultrasonography for tumor detection is operator-dependent, quantification of serum AFP level sometimes associates with false-negative or false-positive results.[71-73] Until now, no reliable detection method is devoted for early HCC detection. These clinical limitations of HCC undesirably trigger high death rate in patients. In view of this clinical limitation in HCC detection, many studies are devoted to find alternative biomarkers for early detection using differential profiling.[74-77] Among the many HCC-related molecules, some of them like cancer-testis antigens and microRNAs are known to have potential applications in clinics.[78-80] However, further development of this research idea is needed to put the concept into practice.

During hepatocarcinogenesis, livers undergo several consequential events from preneoplasia, dysplasis to neoplasia.[49] In each step of progression, tumor masses possess their unique expression profiles of molecules representing the dynamic alteration in the expression of a panel of tumor suppressors or oncogenic molecules.[81,82] These expression changes can be readily revealed using various profiling techniques-like gene and proteomic profiling by comparing with the adjacent nontumor and normal tissues from the same origin.[83,84] In addition, detecting the expression of specific molecules in these categories of tissues can be accomplished by measuring the gene or protein level using molecular methodologies-like polymerase chain reaction, western blot and immunohistochemistry. Using these platforms, identification of HCC-related molecules can be accomplished when the changes in the expression of these molecules in tumorous conditions are accounted.

Decades of research work have identified numerous HCC-related molecules belonging to various families, which compose of at least heat shock proteins, cell adhesion molecules and a panel of oncofetal molecules of different natures.[7,85-87] Amid these molecules, TJ molecules also relate to liver tumorigenesis in certain ways. Some of them can be treated as cancer biomarkers because of their deregulated expression in cancerous tissues, such that measuring their expression indicates the advent of tumors. In addition, deregulated expression of these molecules sometimes denotes clinical features of the disease and gives information on the cancer status in the aspects of tumor pathology (e.g., tumor differentiation, size of tumor and tumor stage) and prognosis of patients (such as tendency to develop tumor recurrence after treatment, and disease-free survival and overall survival).

Table 2. Expression of TJ molecules in human HCC

TJ Molecules	Expression in Cultured HCC cells	Expression in HCC Tissues	Selected References
CAR	-	↓	94
Claudin-1	+	↓	90,115
Claudin-3	+	-	115
Claudin-4	+	-	115
Claudin-5	-	↓	116
Claudin-10	+	↑	88,91,92
Occludin	-	↓	103
Symplekin	-	↓	17
ZO-1	-	↓	103

*, abbreviations used: ↑, induced expression; ↓, reduced expression; +, positive expression in certain HCC cells; -, not studied; CAR, coxsackievirus and adenovirus receptor; HCC, hepatocellular carcinoma; TJ, tight junction; ZO, zonula occludens.

Most TJ molecules including CAR, claudin-1, occludin, symplekin and ZO-1 have reduced expression in HCC tissues and clinical analyses have correlated their reduction to poor tumor differentiation, enhanced tumor invasiveness and poor survival (Table 2). Interestingly, not all of the TJ molecules exhibit down-regulation in HCC and claudin-10 is one such exceptional example that shows an up-regulation. Rather than exhibiting a loss of expression in HCC, claudin-10 seems to involve in tumorigenesis and its elevated level indicates high risk for patients to have post-operative tumor recurrence in a cohort of HCC subjects.[88] For these molecules to be used as biomarkers for cancer surveillance, they should be found with deregulated expressions in body fluids-like serum and urine for their quantification in a non-invasive manner. None of these TJ molecules have been screened regarding their levels in body fluids in HCC patients. More work should be dedicated to investigate whether there is a positive correlation between the levels of these molecules in tumor tissues and body fluids.

TJ molecules belong to a family of cell adhesion molecules with prominent functions in liver physiology by maintaining the dynamics and integrity of junction barriers. It is not difficult to understand the presence of these molecules in healthy liver environment. During HCC development, hepatocytes undergo series of changes in cellular properties and acquire tumorigenic phenotypes. This process frequently associates with loss of TJ functions and diminution of TJ molecules with physiological functions. As mentioned above, many TJ molecules including CAR, claudin-1, occludin, symplekin and ZO-1 have reduced expressions in HCC tissues suggestive of their roles in fundamental TJ integrity. These molecules sustain TJ functions in livers partially by conferring cell anatomy via their interactions with the underlying actin-based cytoskeleton and preventing these cells from uncontrolled proliferation and migration. Claudin-1 is known to possess anti-proliferative feature as supported by two published reports. Claudin-1 was found to have a preferential expression in fetal cell-type of human hepatoblastoma, but not in the highly proliferating embryonal cell-type.[89] In addition, a loss of claudin-1 expression is positively correlated with tumor aggressiveness in human HCC.[90] Other studies show reduced expression of TJ molecules in HCC (Table 2), supporting the concept of TJ molecules in liver physiology and that HCC occurs in the background with the loss of

TJ functions. Due to the large number of TJ molecules, queries might exist speculating whether all TJ molecules act as tumor suppressors. While most TJ molecules function as tumor suppressors, others might be tumorigenic in nature. Claudin-10 is one such molecule involving in liver tumorigenesis. High expression of claudin-10 is observed in HCC clinical samples and a reliable predictor for the recurrence of tumors after hepatic resection and poor prognosis.[88,91] When the expression of claudin-10 is manipulated in HCC cells, ectopic expression stimulates presentation of tumor phenotypes and vice versa for expression silencing.[92] Apart from claudin-10, claudin-7 is implicated to have a role in liver tumorigenesis as demonstrated in a study using epidermal growth factor to induce HCC in mice and discovered an induced expression of claudin-7 in small HCCs.[93] However, the expression of claudin-7 has yet to be examined in human cases of HCC. Altogether, these studies uncover the tumorigenic properties of claudin-7 and claudin-10 in HCC, suggesting that a targeted suppression of their levels may offer potential treatments for HCC.

CONCLUSION AND FUTURE PERSPECTIVES

Hepatic TJs associate with hepatocytes and cholangiocytes in the livers that perform unique physiological functions in relation to bile secretion, flow and metabolism. As in other epithelia and endothelia, TJs in the livers also adopt a similar architecture by having integral membrane molecules link to the underlying cytoskeletons via a panel of peripheral molecules. At least four integral molecules, namely occludins, claudins, JAMs and CARs, are found in mammalian livers and these TJ complexes co-ordinate with each other downstream via peripheral molecules, such as ZO proteins. Such inter-junction connection at the functional level is also found between TJs and other nearby junctions, therefore achieving a high level of junction networking and co-ordination. In addition to these physiological functions, TJs also involve in liver diseases, such as hepatitis infection and HCC development. Certain TJ molecules act as coreceptors for HCV and play a paramount role in hepatitis infection, in particular viral entry across the cell barrier. While most of the TJ molecules having reduced expression in HCC, certain TJ molecules are indeed involved in hepatocarcinogenesis. Limited studies have been performed to decipher the involvement of TJs in these liver diseases and many questions remain to be addressed. For instance, it is not known whether blocking hepatic TJs would have therapeutic effects in treating or preventing HCV infection in humans. In addition, whether other hepatitis viruses utilized such mechanisms for their entry into hepatocytes remains unknown. For HCC, it is not ascertained if TJ molecules can serve as biomarkers. Also, it remains to be investigated regarding the cellular mechanism involving TJ molecules that trigger HCC development? Therefore, further investigation should be performed regarding to TJs and liver diseases. Hopefully, the answers to some of these queries might form the foundation for future development of reliable biomarkers for disease indication and targeted molecules for disease treatment.

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CHAPTER 9

THE BLOOD-FOLLICLE BARRIER (BFB) IN DISEASE AND IN OVARIAN FUNCTION

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Abstract: The blood-follicle barrier (BFB) is one of the blood-tissue barriers in mammalian body found in developing follicles in the ovary. The BFB, besides the tight junction (TJ)-permeability barrier of the endothelial cells in the microvessels that surround the developing follicle, is constituted and contributed significantly by the basement membrane of the developing follicle which alters its composition rapidly during follicle development. While the concept of the BFB and its ultrastructure were described more than six decades ago, fewer than 20 reports are found in the literature that were dedicated to investigate the biology, regulation, and function of the BFB either in health or in disease. Furthermore, detailed analysis of the adhesion protein complexes and the regulation of the junction dynamics at the BFB are still missing in the literature. The goal of this short chapter is to provide an update on this important blood-tissue barrier, it is obvious that future investigation is much needed in the field to understand this ultrastructure better in order to treat and better ovarian disorders including ovarian cancer.

INTRODUCTION: THE CONCEPT OF THE BLOOD-FOLLICLE BARRIER (BFB)

Follicular fluid fills the follicular antrum and surrounds the ovum in an ovarian follicle, which in turn provides a unique microenvironment for follicular development and oocyte maturation.[1] Based on the morphological studies conducted in the 1950s and 1960s, it is known that ovarian follicles are surrounded by a network of capillaries in the theca interna, the follicular compartment per se is nonvascular, and each follicle is separated from the surrounding microvessels by a unique basement membrane,[2-4] illustrating the likely presence of a blood-tissue barrier at the site, which was designated

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the "blood-liquor barrier" in 1958.[5] However, the term BFB was first used in the literature in 1973 when the protein composition and concentration between the follicular fluid and blood serum or plasma were found to be different,[6] consistent with a subsequent report using follicular fluids and serum samples from women undergoing IVF.[7] This work was further expanded using a proteomic approach, and it was found that human follicular fluid contained several acute inflammatory phase proteins including transferrin, afamin, ceruloplasmin, hemopexin, haptoglobin, and plasma amyloid protein in levels different from the plasma, which also supported the notion that ovulation is similar to an inflammatory response.[8] The same study also found some antioxidant enzymes, including superoxide dismutase, glutathione transferase, catalase, paraoxonase, and heat shock protein 27 which can help to protect human follicles from toxic injury mediated by oxidative stress.[8] It is believed that proteins in the follicular fluid are similar to the blood plasma, providing important growth factors for follicular development and oocyte maturation.[8] In fact, with respect to low-molecular-weight proteins, the components of follicular fluid is similar to the blood plasma.[6,9] However, the protein content of human follicular fluid is different from that of the blood plasma due to the selective BFB which serves as a "molecular sieve".[6,10] For instance, in a study using ferritin (MW 500 kDa) and colloidal gold (MW 1000 kDa) as tracers to assess the BFB permeability, it was found that BFB served as a molecular sieve which was permeable to proteins < 500 kDa.[11] Subsequently, the BFB was found to be both charge- and size selective in mouse ovaries.[12]

COMPOSITION OF THE BFB

In order to transport from the theca into the follicular antrum, proteins/peptides, sugars, electrolytes, and ions in fluid must pass through the BFB which is composed of the vascular endothelium, sub-endothelial basement membrane, the thecal interstitium, the follicular basement membrane, and the membrana granulosa (see Fig. 1).[13]

The Permselectivity of the BFB

A recent study using the 'in vivo cryotechnique' (IVCT) to examine mouse ovaries morphologically and immunohistochemically,[13] it was shown that the immunostaining of albumin (~69 KDa, referred to as a low molecular size protein) was localized in the blood vessels, the interstitium, and developing follicles, suggesting that proteins with low molecular size can pass through the BFB towards developing follicles. While the immunostaining of mid-sized molecules, such as IgG1 (~150 kDa), IαI (inter-α-trypsin inhibitor, 220 kDa), and fibrinogen (340 KDa), were significantly reduced inside ovarian follicles, suggesting that although proteins with middle molecular size can pass through the blood vessels towards the interstitium, the follicular basement membranes inhibit the permselectivity of these molecules. For high molecular weight proteins, such as IgM (~900 kDa), its immunostaining was mostly restricted to the blood vessels, suggesting that proteins with high molecular size are blocked by the endothelial TJ-barrier of blood vessels. These findings suggest that the BFB is present from early stages of folliculogenesis and it has selective roles during follicular development until ovulation.[13]

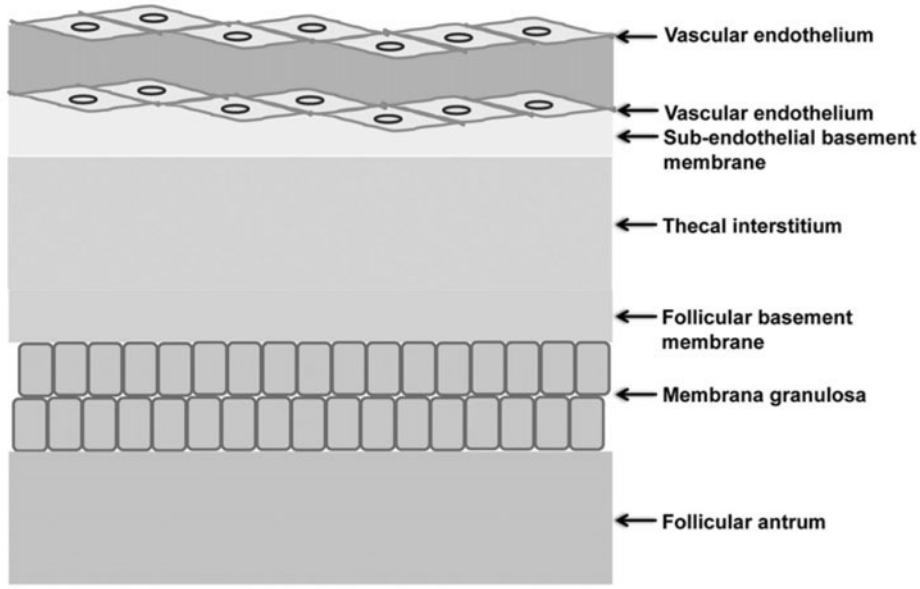

Figure 1. Schematic drawing illustrating the different components that constitute the blood-follicle barrier.

Endothelial Cells of Blood Vessels

The endothelial cells of blood vessels, mostly capillaries, contribute to the BFB which act as a permeability filter regulating the passage of serum proteins from blood vessels to the surrounding tissue, and such passage is tightly controlled by the dynamic nature of the junctional complexes, such as tight and adherens junctions and desmosomes, between endothelial cells.[14,15] In the ovary, such as mouse ovaries, the intercellular junctional complexes between endothelial cells are not well characterized, but the blockade of mid-sized proteins by the endothelial TJ-barrier has been shown to be regulated by nitric oxide.[16] It is also noteworthy that the BFB was found to be compromised as the result of a loss of ovarian superoxide dismutase activity in diabetic mice that led to ovulation defects, illustrating the critical role of reactive oxygen species to the BFB function.

Subendothelial Basement Membranes

Dependent on the pore size and charge selectivity of the components including Type IV collagen, laminin, and heparan sulfate proteoglycans, the basement membrane of blood vessels such as glomerular basement membrane can served as a molecular sieve.[17]

Follicular Basement Membranes

Since the permselectivity of the subendothelial basement membranes and the follicular basement membranes is different, it is suggested that this may be due to

the presence of unique extracellular matrix components. During folliculogenesis, the follicular basement membrane is a dynamic ultrastructure with its components rapidly change over time.[18] In primordial and pre-antral stages, collagen IV alpha 1-6 is present and later on in antral and attretic stages, but only alpha 1 and alpha 2 chains are present in the follicular basement membrane in cows[18] and mice.[19] On the contrary, perlecan, nidogen 1 and nidogen 2 are the predominant follicular basement membrane components in the pre-antral stage and thereafter in cows.[20,21] Laminin alpha 1, beta 2 and gamma 1 are also detected in the follicular basement membrane during follicle development in cows.[18] It is possible that such dynamic changes in the follicular basement membrane components contribute changes to the basement membrane permeability during folliculogenesis.

THE BFB IN POLYCYSTIC OVARY AND OVARIAN CANCER

Polycystic ovary syndrome (PCOS) is one of the most common female hormonal disorders characterized by anovulation, leading to irregular menstruation, infertility and polycystic ovaries.[22,23] In PCOS, follicle development is put on hold at an early follicular stages.[24] Since BFB regulates the composition of the follicular fluid by determining its component proteins, which in turn regulates follicle development, PCOS can be affected, at least in part, by the permselectivity of the BFB. Previous studies have shown that insulin-like growth factor binding proteins,[25] soluble Fas ligand[26] and inhibins A and B[27] are dysregulated in the follicular fluids in PCOS patients.

Using the IVCT in a mifepristone-induced PCO model, the morphology and the permselectivity of the BFB was examined in mouse ovaries,[28] in which the blood vessels were found to be enlarged along with an increase in blood flow, and follicular cysts were formed with thinner membrana granulosa in the blood vessels, the interstitium, and developing follicles.[28] The immunostaining of low and high molecular weight proteins, albumin and IgM, respectively, in the ovaries of PCO model mice were similar to that in normal mice. Albumin was detected in the blood vessels, the interstitium, and developing follicles and IgM was mostly retained inside the blood vessels.[28] On the contrary, the immunostaining of mid-sized molecules such as IgG, ITI, and fibrinogen had different pattern in the PCO model. The follicular basement membranes blocked the passage of both IgG and ITI from the interstitium to the follicles in PCO model. Moreover, fibrinogen was mostly restricted within the blood vessels and surrounded by the endothelial cells in the PCO mice.[28] These findings suggest that the permselectivity of BFB mediated by the endothelial cells of the microvessels and the follicular basement membrane may play important role in the pathogenesis of PCOS.[28]

Ovarian cancer is the most lethal of all gynecological malignancies.[29,30] Approximately 90% of the malignant ovarian tumors are originated from changes in the surface epithelium or surface epithelial inclusion cysts of the ovary.[31,32] It is possible that dysregulation of the BFB contributes to: (i) the abnormal changes of the surface epithelium, and/or (ii) the formation of surface epithelial inclusion cysts, of the ovary, which, in turn, leads to cancer progression. This possibility regarding the role of BFB in ovarian cancer must be carefully evaluated in future studies.

THE PERMEABILITY BARRIER FUNCTION OF THE BFB: SELECTIVE TRANSPORT PROCESSES VERSUS MERE FILTRATION

Similar to other blood-tissue barriers, such as the blood-brain barrier and the blood-testis barrier,[33-36] the BFB is now known to be more than a "molecular sieve" in the ovary by restricting the transcellular transport of solutes and macromolecules across the various membranes ultrastructures to reach the developing follicles entirely based on their molecular sizes. For instance, IαI (inter-α-trypsin inhibitor, 240 kDa) and pre-α-trypsin inhibitor (125 kDa) were found to be absent from the follicular fluid until an ovulatory stimulus (e.g., during LH surge) was given, and both of these protein were found in the antrum of mature follicles, usually within minutes, and integrally associated with the newly synthesized hyaluronic acid-rich cumulus extracellular matrix.[37,38] Subsequent studies have shown that these changes in permeability function at the BFB are also mediated by nitric oxide (NO),[16] consistent with earlier findings that NO regulates vascular function and permeability[39-41] as well as the Sertoli cell TJ-barrier permeability function.[42]

CONCLUSION AND FUTURE PERSPECTIVES

As briefly summarized above, the BFB is an important ultrastructure in the ovary by limiting the access of foreign compounds and/or harmful substances (e.g., toxicants, drugs) to the developing follicles. It is intimately related to follicle development and it also regulates the composition of the follicular fluid via subtle changes in its basement membrane composition during folliculogenesis, so that different components (e.g., proteins, peptides, electrolytes, ions, sugars, and others) can be precisely and rapidly recruited to the follicular fluid in response to the needs of developing follicles. A recent study has shown that some therapeutic drugs, such as doxorubicin, an anticancer drug, can permeate the BFB to induce ovarian failure by causing apoptosis of germinal vesicle occytes,[43] illustrating much work is needed to understand this blood-tissue barrier so that the reproductive health of women under chemotherapy can be maintained. While the concept of BFB was depicted more than six decades ago and the termed BFB was used almost 40 years ago, yet, less than 20 reports are found in the literature dedicated to study the biology and regulation of the BFB. It is likely that proteins found in the follicular fluid behind the BFB can be putative candidates for diagnostic markers for follicle and/or oocyte maturation and to assess oocyte quality. It is equally possible that follicular fluid proteins can sever as diagnostic, therapeutic and/or prognostic markers for various ovarian diseases including PCOS and ovarian cancers. There are pressing questions remain to be addressed. For instance, what initiates or regulates the formation of follicular fluid? Does this involve LH and/or FSH? What are the changes in cell-cell junctions that facilitate the formation of follicular fluid during folliculogenesis?

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CHAPTER 10

PHYSIOLOGY AND PATHOPHYSIOLOGY OF THE EPITHELIAL BARRIER OF THE FEMALE REPRODUCTIVE TRACT

Role of Ion Channels

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Abstract: The epithelium lining the female reproductive tract forms a selectively permeable barrier that is responsible for creating an optimal luminal fluid microenvironment essential to the success of various reproductive events. The selective permeability of the epithelial barrier to various ions is provided by the gating of epithelial ion channels, which work together with an array of other ion transporters to drive fluid movement across the epithelium. Thus, the luminal fluid is fine-tuned by the selective barrier with tight regulation of the epithelial ion channels. This chapter discusses the role of epithelial ion channels in regulating the epithelial barrier function and thus the fluid volume and ionic composition of the female reproductive tract; physiological factors regulating the ion channels and the importance of the regulation in various reproductive events such as sperm transport and capacitation, embryo development and implantation. Disturbance of the fluid microenvironment due to defects or abnormal regulation of these ion channels and dysregulated epithelial barrier function in a number of pathological conditions, such as ovarian hyperstimulation syndrome, hydrosalpinx and infertility, are also discussed.

INTRODUCTION

The epithelium lining the female reproductive tract, including the vagina, cervix, uterus and oviduct, forms a selectively permeable barrier that separates the luminal fluid environment from that of the blood and tissues. The epithelial layer, however, is not merely a

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physical barrier but actively involved in regulating the luminal microenvironment, on which various reproductive events, such as sperm transport, capacitation, fertilization, embryo development and implantation, critically depend. This luminal fluid microenvironment is determined by the coordinated or regulated transport of ions, amino acids, macromolecules as well as fluid, which is driven by osmotic pressure and ion gradients, across the epithelial layer through both the paracellular and transcellular pathways. While the paracellular pathway is mainly regulated by the dynamic changes of the epithelial tight junctions, the transcellular pathway is governed by the secretory and absorptive activities of the epithelium involving an array of different cellular mechanisms, such as ion channels and transporters.

The ionic composition of the luminal fluid of the female reproductive tract is a major determinant of the fluid volume, pH and osmolarity. The selective permeability of the epithelial barrier to various ions is regulated by the gating properties of the epithelial ion channels, which work in concert with other ion transporters, to enable directional transport (secretion or absorption) of ions across the epithelial layer. Importantly, the epithelial ion channels are regulated by a range of physiological factors, thereby providing a fine-tuned luminal fluid microenvironment optimal for various reproductive events. Defects in the expression or function of the ion channels may result in dysregulated epithelial barrier function and disturbance of the luminal fluid microenvironment, leading to disorders and infertility, as highlighted by cystic fibrosis (CF), hydrosalpinx and ovarian hyperstimulation syndrome (OHSS). While most of other chapters in this book focus on epithelial and/or endothelial junctions in different tissues, this chapter will focus on the selective barrier imposed by the epithelial ion channels of the female reproductive tract and discuss their physiological roles in regulating the luminal fluid microenvironment and various reproductive events. Possible involvement of defective expression/function of the ion channels and thus dysregulated epithelial barrier function in pathological conditions and infertility are also discussed.

THE LUMINAL FLUID MICROENVIRONMENT AND SELECTIVE EPITHELIAL BARRIER

Sperm enter into the female genital tract through the vagina, cervix and swim upward to the uterus and Fallopian tube (oviduct) where they meet and fertilize the egg. During their transit through the uterus and oviduct, sperm undergo a process termed capacitation, through which sperm acquire their fertilizing capacity. After fertilization, the zygote undergoes cell cleavage and early embryo development in the oviduct and is eventually transported to the uterus for implantation. A suitable but dynamically changing luminal fluid microenvironment is vital to the reproductive events taken place in different regions of the female genital tract.

Luminal Fluid Microenvironment in Different Segments of the Female Genital Tract

Vaginal fluid is acidic, with a pH ranging from 3~5 as documented by many reports.[1-5] This acidic pH is thought to contribute to the antimicrobial capacity of the vaginal fluid.[6] The ionic composition of human vaginal fluid is reported to be different from that of the

blood, low in Na^+ (75-82 mM), but high in K^+ (30 mM), with little fluctuation during the menstrual cycle.[7]

Human cervical fluid has a pH ranging from 5.4~8.[8,9] It has been reported that the pH of human cervical mucus varies during the menstrual cycle, with the highest values being found at the time of ovulation;[10-12] however, some others found no cyclic variation.[13] It has been suggested that this variation in pH may be brought about by the variation in the HCO_3^- content in the mucus. While the low endocervical pH is reported to impair the sperm-mucus interaction and lead to reduced fertility,[14-16] douching of vagina with sodium-bicarbonate improves the penetration of sperm into the cervical canal,[17,18] suggesting that HCO_3^- may reduce the viscosity of the cervical mucus.

In contrast to the acidic environment in the vagina, uterine and oviductal fluids are alkaline, with a pH as high as 7.6 to 8 depending on species.[19-26] It is believed that the HCO_3^- ion in the rabbit oviduct and uterus is two to four folds over that in the blood.[24,26] Mass et al reported that the oviductal fluid in rhesus monkey during follicular phase had an average pH of 7.2, corresponding to approximately 35 mM HCO_3^-, but became more alkaline during ovulation with an average pH of 7.6, corresponding to 90 mM of HCO_3^-[20].

Uterine fluid contains ions, macromolecules and organic solutes that are unique to the circulating blood system of the body. Similar to the vaginal fluid, the luminal K^+ concentrations (16-30 mM) of rat, pig and human uterine fluids were reported to exceed that in the plasma by 10-20 mM, while the level of Na^+ (115-120 mM) was 20-30 mM lower in the uterine fluid than that found in the plasma.[27-32] A lower Ca^{2+} ion and comparable Cl^- content compared to that in the plasma was also recorded in human uterine fluid.[27] The composition of oviductal fluid has been well documented in the human, rabbit and pig.[19,23,33-37] It has also been reported to be rich in K^+ in comparison to the plasma.[33,34,38]

Apart from the variations in the fluid composition and pH, the fluid volume of the female reproductive tract also undergoes dynamic and cyclic changes during the menstrual/estrous cycle. About 90 years ago, large fluctuations in uterine fluid volume were first observed during the estrous cycle of the rat, with a maximal fluid volume observed during proestrous and estrous and a minimum at diestrous.[39] Recently, we have also observed maximal uterine wet weight at estrous and minimal at diestrous in the mouse.[40] In humans, much greater uterine fluid volumes were observed at midcycle (83-180 μL) than that in the midluteal phase (5-35 μL).[41] Similar cyclic changes in fluid volume were also observed in the cervical mucus of humans.[42] It is believed that the maximal fluid volume in the midcycle facilitates sperm transport, while the minimal fluid volume in the luteal phase is important to the closure of the uterus and embryo implantation.

As the luminal fluids in different regions of the female reproductive tract exhibit an ionic composition significantly different from that of the plasma (Table 1), it strongly indicates that the epithelial layer not only acts as a barrier separating the luminal environment from the blood, but also actively transports ions and fluid across the selective epithelial barrier.

The Selective Barrier—Transport of Ions and Fluid

Although dynamic and cyclic changes in the fluid volume and composition along the female genital tract have been recorded for several decades, it is not until the recent 15 years that the origin and the underlying mechanism of the fluid formation are elucidated.

The dynamic changes in fluid volume and ionic composition indicate that there are active secretion and absorption of ions and fluid across the female reproductive tract, which depend on the epithelial cells lining the luminal surface. The tight junctions at

Table 1. Ion concentrations and pH in different regions of the female reproductive tract. (The data are from humans unless otherwise specified)

Region	Menstrual Phase	Na^+	K^+	Cl^-	HCO_3^-	pH	Reference
Vagina	Secretory	75 ± 5	30 ± 2	72 ± 7			Wagner 1980[7]
	Proliferative (at ovulation)	82 ± 4	30 ± 2	79 ± 4			
						4.0-4.2 (4.5-4.8)	Zuck 1939[5]
						3.5-4.2	Master 1959[2]
						3.9-5.8	Peeters 1972[4]
						4.4-4.8	Cailloutte 1997[1]
Cervix	-	100-175	3-21				Lebech 1970[157]
	-	72-195	15-48				Kopito 1973[42]
						5.4-8.0	Eggert-Krus 2000[8]
						6.4-8.0	WHO[9]
Uterus	Proliferative	128-158	6-9				Edwards 1968[158]
	Proliferative	124 ± 3	18 ± 3				Clemetson 1973[28]
	Secretory	108 ± 4	34 ± 3				
	Proliferative	106	4				Kar 1968[159]
	Secretory	130	3				
					44.8 ± 0.9 (rabbit)	7.7 ± 0.1	Murdoch 1968[24]
						6.6-7.6	Maas 1983[160]
Oviduct	Proliferative	140 ± 3	10 ± 2				Lippes 1972[161]
	Secretory	140 ± 2	8 ± 1				
		130	21.1	132			Borland 1977[162]
		145	6.7	119.5		7.7	David 1973[163]
						7.5-8.0	Leese 1988[19]
					20 ± 7.22	7.2-7.7 (hydrosalpinx)	Strandell 1998[164]
	Follicular				35	7.1-7.3	Maas 1977[20]
	Luteal (rhesus monkey)				90	7.5-7.8	
Plasma		133-150	3.5-5.5		25	7.2	Lippes 1972[161]

the luminal side form a tight seal at the cell junctions and separate the lumen (apical) from the blood side (basolateral). Although this barrier prevents free movement of molecules, it is selectively permeable to a variety of molecules including ions and water. Apparently, the fluid movement depends on the osmolarity difference established by the directional ion transport across the epithelium, which gives rise to the region-specific fluid microenvironment of the female reproductive tract.

In order to study the ion transport mechanism in the female reproductive tract, we established a primary culture of mouse endometrial epithelium, which could be assessed by the short-circuit current (Isc) measurement and a number of other techniques to determine the ion transport and related cellular mechanisms.[43] The reconstituted endometrial epithelium showed a transepithelial potential (PD) of 2.7 ± 0.2 mV, with the apical side negative with respect to the basolateral side. Under unstimulated condition, the basal Isc, which reflects the net active ion transport across the epithelium, could be reduced by 85% in Na^+-free solution and by 13% in Cl^--free solution, suggesting a predominant and continuous Na^+ absorption from the luminal side to the blood side.[43] Application of amiloride, an inhibitor of the epithelial sodium channel (ENaC), on the apical side substantially reduced the basal current by 57.7%, indicating the involvement of apical ENaC in the Na^+ absorption process.[43] Na^+ absorptive characteristic under unstimulated condition has also been observed in cultured human endometrial epithelium.[44] The basal Isc of the cultured mouse endometrial epithelium could also be reduced by $80.3 \pm 3.5\%$ by basoleteral addition of oubain, the Na^+-K^+-ATPase inhibitor, suggesting the involvement of Na^+-K^+-ATPase in Na^+ absorption by pumping out Na^+ from the basolateral membrane.[43] The involvement of Na^+-K^+-ATPase in Na^+ absorption is also supported by the observation that Na^+ absorption was enhanced by the insulin-induced increase in Na^+-K^+-ATPase activity in porcine endometrial epithelium.[45]

The cultured mouse endometrial epithelium also exhibits predominant anion (both Cl^- and HCO_3^-) secretory activities upon stimulation with various secretagogues.[43,46-50] Apical addition of ATP, or basolateral addition of bombesin, Arg^8 vasopressin or vasoactive intestinal peptide (VIP) led to transient increases of Isc, which could be inhibited by the nonspecific Cl^- channel blocker, Diphenylamine-2-carboxylic acid (DPC) or Ca^{2+}-activated chloride channel (CaCC) blocker, 4,4′-diisothiocyano-2,2′-stilbenedisulfonate (DIDS).[43,47] Basolateral addition of adrenaline, PGE_2, as well as forskolin induced sustained increase of Isc, which could be inhibited by apical addition of DPC, 5-Nitro-2-(3-phenylpropylamino) benzoic Acid (NPPB) and glybenclamide, which have been shown to have inhibitory effect on the cystic fibrosis transmembrane conductance regulator (CFTR),[51-53] but not the CaCC blocker, DIDS.[43,47,49,50,54] The adrenaline, PGE_2 and forskolin-induced anion secretion was further demonstrated to be mediated by the cAMP-dependent pathway, involving possible activation of CFTR.[49,50] Functional expression of CFTR in mouse endometrial epithelial cells was later confirmed using the patch-clamp technique demonstrating time and voltage-independent characteristic CFTR whole-cell currents with linear I-V relationship.[55] The sustained Cl^- secretion was also shown to depend on the basolateral Na^+-K^+-$2Cl^-$ cotransporter, which carries Cl^- into the cell, and basolateral K^+ channel, which provides the recycling pathway for K^+ to maintain the activity of Na^+-K^+-$2Cl^-$ cotransporter, as well as hyperpolarizes the cell to drive Cl^- secretion at the apical membrane.[50,54]

Apart from Cl^-, the cultured mouse endometrial epithelium also actively secretes HCO_3^-, which may be responsible for the high HCO_3^- content observed in the uterine fluid. A number of secretagogues also stimulate HCO_3^- secretion. When the cultured

epithelium was bathed in Cl^- free solution, under which condition HCO_3^- was the only permeable anion, forskolin or genestein could induce a sustained Isc increase, which could be substantially inhibited by DPC and glybenclamide on the apical side, but only slightly by DIDS, suggesting that the HCO_3^- secretion was mediated by CFTR in the apical membrane.[56] Interestingly, apical addition of PGE_2 was found to preferentially induce HCO_3^- secretion, rather than Cl^- secretion induced by basolateral PGE_2,[49] suggesting possible involvement of different receptor subtypes or transport mechanisms on both sides of the epithelial barrier. Since HCO_3^- is an alkaline ion, its transport can also be assessed by pH measurement. Wang et al established the fluorescent dye-based intercellular pH measurement on the confluent mouse endometrial epithelial cell culture in a two-sided perfusion system.[56] While the study showed that CFTR and a Cl^--HCO_3^- exchanger, most likely Slc26a6, are responsible for the HCO_3^- extrusion from the apical membrane, another study demonstrated that the basolateral Na^+- HCO_3^- cotransporter (NBC) and Na^+-H^+ exchanger (NHE) are responsible for the HCO_3^- entry and H^+ exit, respectively, from the basolateral membrane.[57,58] Taken together, we have proposed an ion transport model across the endometrial epithelial barrier based on the work on the mouse (Fig. 1). As shown earlier, the transport processes are subject to acute or tight regulation by neuronal and local factors.

Unlike the endometrium, which exhibits predominant absorptive activity under unstimulated condition, the oviductal epithelia exhibit predominant secretory activity under unstimulated condition. In a primary culture of human Fallopian epithelial cells, amiloride was found to have small effect on the PD and Isc, indicative of low absorptive activity.[37] On freshly isolated rabbit oviduct epithelium, Brunton and Brinster detected a PD with the apical side negative with respect to the basolateral side, and a basal Isc, which was suggested to be mediated by Cl^- flux from the basolateral to the apical side.[59] Active Cl^- secretion was confirmed by Gott et al in rabbit oviduct on a vascular perfusion setting,[60] as well as by Dickens[61] and Downing[37] on a confluent monolayer of the primary culture of rabbit and human oviductal epithelial cells. The basal PD, Isc, tranepithelial Cl^- flux or fluid secretion could be reduced by basolateral addition of Cl^- channel blocker SITS, Na^+-K^+-$2Cl^-$ cotransporter inhibitor bumetanide, or Na^+-K^+-ATPase inhibitor ouabain in rabbit oviduct epithelia.[59,60] Several secretagogues can increase the secretory activity in oviductal epithelia. Apical or basolateral application of ATP induced a transient increase in PD and Isc in human oviductal epithelial cell culture, which could be inhibited by Cl^- channel blockers.[37] Similar effect of ATP was also observed in bovine oviductal epithelium, which was demonstrated to be a Ca^{2+}-dependent Cl^- secretion.[62] Adrenergic agonists applied to the basolateral compartment, including adrenalin, noradrenalin, isoproterenol and phenylephrine, induced an increase in PD, Isc, Cl^- flux and also fluid secretion in rabbit oviductal epithelia.[59,61,63] However, it was unexpected that cAMP or cAMP agonist (cholera toxin, forskolin, etc.) elicited an inhibition instead of stimulation of Cl^- and fluid secretion in human or rabbit oviductal epithelial cells.[36,60,61,63] Leese et al suggested that the cAMP-activated Cl^- channel, CFTR, is substantially open at basal cAMP level, therefore, additional cAMP can not further increase Cl^- transport and that the effect of adrenergic agonist is more likely to be mediated by Ca^{2+}-dependent mechanism rather than by cAMP.[19] It may also be possible that apart from the activation of CFTR-mediated Cl^- secretion, cAMP also activates Cl^- absorption via other transporters, therefore masking the Cl^- secretion process. Supporting the notion that CFTR is open under basal condition, Leung et al observed a smaller PD and basal Isc in the reconstituted oviductal epithelia of

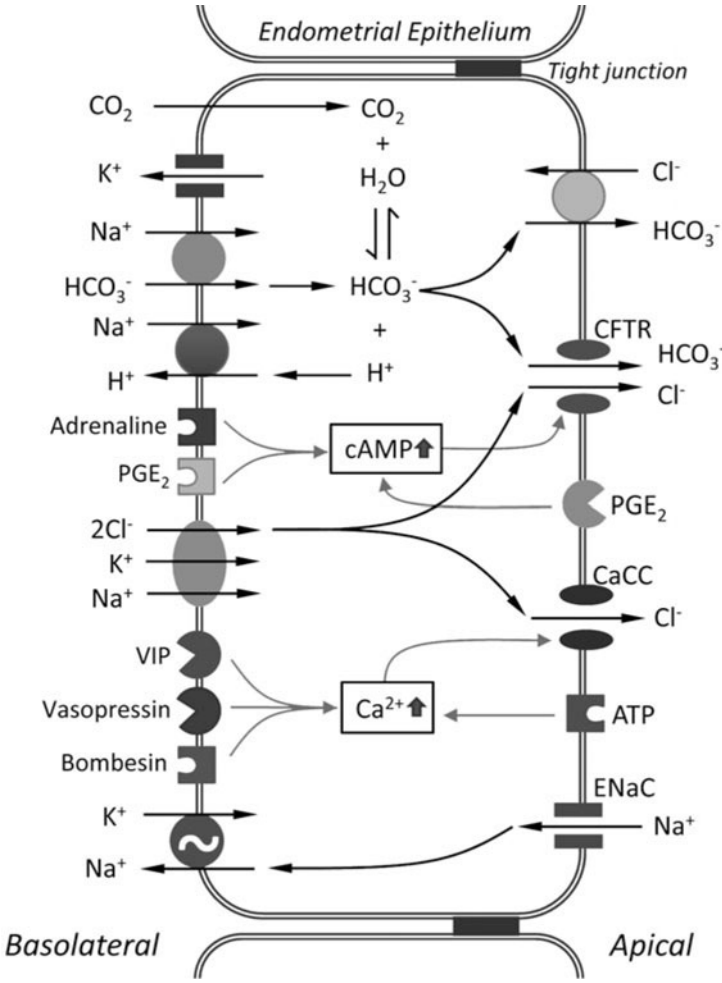

Figure 1. Proposed model for ion transport across endometrial epithelium. The model includes several transport processes: 1) Na^+ absorption, Na^+ enters via apical ENaC and is extruded by basolateral Na^+-K^+-ATPase; 2) Cl^- secretion, Cl^- enters via basolateral Na^+-K^+-$2Cl^-$ cotransporter with the Na^+ gradient provided by the Na^+- K^+-ATPase, and exits via apical CFTR; 3) HCO_3^- secretion, HCO_3^- either enters via basolateral Na^+- HCO_3^- cotransporter or is converted from CO_2 and H_2O by carbonic anhydrase, and exits via apical CFTR or an anion exchanger SLC26A6. Basolateral K^+ channel is required for providing a recycling pathway to maintain the activity of K^+-associated transporters. Basolateral Na^+-H^+ exchanger may be required for basolateral H^+ extrusion accompanying the apical extrusion of HCO_3^- to maintain intracellular pH. Possible influence of neuronal and hormonal factors on the transport processes and related signaling pathways are also illustrated.

CFTR knockout mice compared to that of wild-type control.[64] In the same study, however, forskolin could further stimulate a slight increase in Isc in wild-type mouse oviductal cell culture, but not in the CFTR knockout mice, indicating the activation of CFTR-mediated anion secretion upon cAMP stimulation.[64] Recently, we have observed a forskolin-induced increase in Isc in pig oviductal epithelia, which was demonstrated to be the CFTR-mediated HCO_3^- secretion rather than Cl^- secretion,[65] suggesting that

oviductal CFTR may not play a major role in Cl^- secretion, but is important to oviductal HCO_3^- secretion, which may be responsible for the high HCO_3^- concentration observed in oviductal fluids. The discrepancy observed in different species also suggests that the transport properties of the oviductal epithelial barrier may vary with species, especially where CFTR is concerned.

The directional ion transport, predominantly Cl^- and HCO_3^- secretion and Na^+ absorption, establishes the ion gradients across the epithelium, which drives water secretion or re-absorption in the female reproductive tract. Previously, it was believed that water is secreted or absorbed via the paracellular pathway. However, it was later demonstrated that water also moves via transcellular pathway through the water channels—aquaporins.[66] In the endometrial epithelium, aquaporin 2 and aquaporin 5 are found to be expressed in various species, with the highest expression at late luteal phase, corresponding to the time of embryo implantation, suggesting its role in fluid re-absorption during implantation.[67-70] In the oviduct, aquaporin 5 and 9 are expressed in pig oviductal epithelia,[71] and aquaporin 9 is detected in rat oviductal epithelia.[72] Aquaporin 9 is found to be localized in the apical membrane of pig and rat oviductal epithelia and increase in late follicular phase and early luteal phase in the pig or proestrous and estrous in the rat, suggesting that it may be responsible for the abundant oviductal fluid secretion important for sperm and early embryo transport along the oviduct.[72,73]

REGULATION OF ION CHANNELS AND SELECTIVE PERMEABILITY OF THE EPITHELIAL BARRIER IN REPRODUCTION

The ion selectivity and rate of transport across a wide range of epithelial barriers are known to depend on the rate-limiting apical ion channels, such as CFTR and ENaC, which determine the transepithelial secretory and absorptive activities, respectively.

CFTR belongs to the ATP-binding cassette (ABC) transporter family and functions as a cAMP/PKA-activated anion channel conducting both Cl^- and HCO_3^-. It is widely expressed in the apical or luminal surface of a variety of epithelia, including the airway, pancreas, gastrointestinal tract, sweat gland, as well as reproductive tract,[74] and non-epithelial cells including lymphocytes,[75] neurons[76] and endothelial cells.[77] The importance of CFTR in electrolyte and fluid secretion is highlighted by cystic fibrosis (CF), an autosomal recessive genetic disease caused by mutations of CFTR with hallmark defects in electrolyte and fluid transport in almost all exocrine glands/tissues.[78] CFTR can be activated by neuronal and hormonal factors that elicit intracellular increase in cAMP, such as β-adrenergic agonists and PGE_2 as mentioned earlier. We have recently discovered that CFTR has mechanosensitive gating properties promoting Cl^- secretion under mechanical stimulation.[79] CFTR has been reported to interact or regulate quite a number of proteins, including ion channels, such as ENaC,[80] and transportors, such as NHE[81] and NBC.[82] Most recently, CFTR has been shown to interact with proteins of apical compartments, including those in the tight junction complex.[83]

ENaC, also known as sodium channel non-neuronal 1 (SCNN1) or amiloride sensitive sodium channel (ASSC), consists of α, β and γ subunits (α, β and γ-ENaC), which are encoded by the SCNN1 genes within degenerin/ENaC (DEG/ENaC) superfamily.[84] Located in the apical membrane of a variety of polarized epithelia, ENaC has been well accepted to be the rate-limiting factor in transepithelial Na^+ absorption which occurs via the two-step mechanism, where Na^+ enters the epithelium via apical ENaC and is then

extruded by the basolateral Na^+/K^+-ATPase.[84] The electrogenic absorption of Na^+ creates an electrochemical driving force for K^+ secretion and builds up a transepithelial osmotic gradient, which drives water absorption across the epithelium. The essential role of ENaC in salt and water homeostasis has been well documented.[84-86] α-ENaC knock-out mice die soon after birth due to the failure in clearing the fetal liquid filling the lungs.[87] In the kidney, ENaC is expressed in distal nephron where the mineralocorticoid hormone aldosterone affects the expression of ENaCs and controls the sodium re-absorption.[88] Mutations in β and γ-ENaC causing hyper-active channels have been found in patients with Liddle's syndrome,[89] which is featured by an abnormally high Na^+ re-absorption in the distal nephron leading to expansion of extracellular fluid volume and arterial hypertension. Mutations causing a reduced channel activity of ENaC are also known to be associated with pseudohypoaldosteronism Type 1,[90] where symptoms of hypotension, hyponatremia and hyperkalemia are observed.

While the role and regulation of CFTR and ENaC in other epithelia have been the subjects of extensive studies, similar studies on the female genital tract are limited. Nevertheless, accumulating evidence suggests that these ion channels in the female reproductive tract are under tight regulation, which is essential to the success of a number of reproductive events.

Hormonal Regulation of Ion Channel Expression and Fluid Secretion/Absorption

It is well known that the female reproductive tract is under the influence of ovarian hormones, namely estrogen and progesterone, the blood levels of which vary during the ovarian cycle. Interestingly, ovarian hormones also appear to affect the selectivity of the female reproductive tract epithelial barrier by regulating CFTR and ENaC expression, thereby controlling fluid secretion and absorption of the epithelial barrier. Using in situ hybridization, we found that CFTR is expressed in the entire mouse female reproductive tract, including the vagina, cervix, uterus and oviduct, in an estrous cycle-dependent manner, with maximal expression at proestrous and estrous, but minimal expression at metestrous and diestrous.[48] Interestingly, the expression of ENaC also exhibits an estrous cycle-dependent pattern, but reciprocal to that of CFTR.[48] The cyclic expression patterns of these two ion channels appear to be due to their difference in response to ovarian hormones.[91-94] Estrogen is shown to stimulate CFTR expression but downregulate ENaC expression, as evidenced by the Isc measurements showing decreased Na^+ absorption and increased Cl^- secretion in estrogen-treated endometrial epithelia.[48] The role of estrogen in upregulating CFTR is further supported by the large forskolin-induced Isc observed from uterine epithelia freshly isolated from proestrous and estrous mice but not from the later stages of the cycle.[40] On the other hand, progesterone, which is secreted at late stage of the cycle, suppresses CFTR expression,[91] but may promote ENaC expression as demonstrated in the uterus,[94] as well as the lung and kidney.[95-97] The hormonal control of the ion-fluid transport is further demonstrated by Salleh et al using an in vivo uterine perfusion system by cannulating rat uterus. They showed that the basal fluid absorption, accompanied by Na^+ and Cl^- abosorption, in ovariectomied rats was inhibited after estrogen treatment, but greatly enhanced after progesterone treatment,[94] which is consistent with the previously described cyclic changes in uterine fluid volume.[39,41] The progesterone-enhanced absorption could be inhibited by amiloride, and progesterone treatment apparently increased the expression and

apical localization of the α subunit and γ subunit of ENaC, consistent with the role of ENaC in uterine fluid absorption during the progesterone-dominant luteal phase and implantation period.[94] Although Salleh et al only found weak expression of CFTR in the apical membrane by immunohistochemistry at estrous stage, raising the doubt on the importance of CFTR in fluid secretion, Ajonuma et al using an infection-induced hydrosalpinx model showed that the *C. trachomatis*-induced fluid accumulation in the uterus, as well as in the lung, observed in the wild-type mice was absent in the CFTR knockout mice, strongly indicating that CFTR is essential to the fluid secretion across epithelial barriers.[98]

The physiological consequence of the out-of-phase expression of CFTR and ENaC in the uterus is the changing permeability of the epithelial barrier to these ions and thus the fluid flow. As depicted in Figure 2, while maximal CFTR expression at the estrous (or midcycle) may enable a higher rate of uterine fluid production to facilitate sperm transport and sperm capacitation (see below), downregulation of CFTR and upregulation of ENaC at metestrous and diestrous (or late cycle) may promote fluid absorption thereby reducing the fluid volume in the lumen to enhance close contact between the endometrial surface and the embryo to facilitate implantation. In fact, on the day before implantation, CFTR is undetectable in the endometrial epithelia, with ENaC reaching a maximal level at this time.[99] The observed downregulation or absence of CFTR in uterine epithelium ensures that the uterine secretory activity is at a minimum during implantation. Furthermore, since CFTR has been shown to be a negative regulator of ENaC,[100] the absence of CFTR may remove its inhibition on ENaC and enable maximal ENaC activity and fluid absorption necessary for the success of embryo implantation (see below).

Oviductal fluid production is also estrous cycle-dependent and under the influence of steroid hormones. In rabbits, oviductal fluid production is maximal in estrous, while it declines during pseudopregnancy.[60,101,102] In monkeys, fluid secretion increases at ovulation.[103] Similar phenomena are also observed in cows.[104] Estrogen treatment increases fluid secretion in ewes and rabbits, whereas progesterone antagonizes the effect of estrogen.[105,106] Gott et al demonstrated, in a vascular perfusion preparation, that rabbit oviduct exhibited Cl^- secretion and oviductal fluid secretion, which could be blocked by Cl^- channel inhibitors, suggesting that the oviductal fluid secretion was driven by Cl^- secretion,[60] although the molecular identity of the Cl^- channel involved was not clear. We have recently demonstrated that CFTR expression is the highest at estrous in rat oviduct.[107] Moreover, Chen et al and Leung et al have also demonstrated that CFTR is actively involved in anion secretion in pig and mouse oviduct under stimulated conditions,[64,65] indicating that CFTR may be involved in driving the estrous cycle-dependent oviductal fluid production.

Apart from the uterus and oviduct, the expression of ENaC and CFTR in the cervix and vagina suggests that both ENaC and CFTR work closely together to maintain an optimal cervical and vaginal fluid microenvironment for sperm movement and optimal antimicrobial activity. Although high level of ENaC and low level of CFTR expression are detected in the vagina and cervix, suggesting a primarily absorptive role of this region, the higher level of expression of CFTR at proestrous may serve to lubricate the cervical and vaginal lumen and reduce the viscosity of the mucus for sperm movement towards the oviduct for successful fertilization. Taken together, the hormonal regulation of CFTR and ENaC expression in different regions of the genital tract and at different

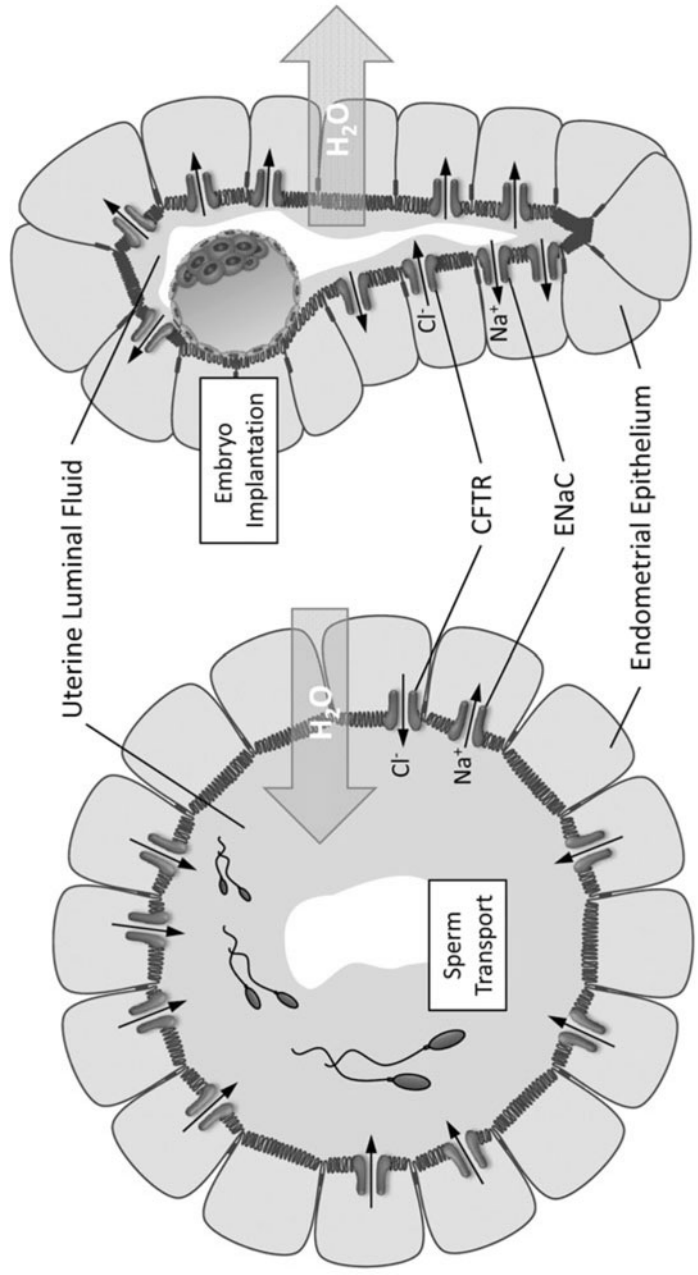

Figure 2. Schematic drawing showing dynamic change of uterine fluid volume as a result of differential expression of CFTR and ENaC at different stages of the cycle. High expression of CFTR and low ENaC expression at mid-cycle promotes active Cl^- and fluid secretion to facilitate sperm transport, while reciprocal expression at late stage of the cycle promotes Na^+ and fluid re-absorption leading to the "closure" of the uterine lumen to facilitate embryo implantation.

stages of the cycle provides a mechanism controlling the fluid volume and composition along the female reproductive tract, which is important for various reproductive events.

Interaction between ENaC and Cl^--Channels—A Switching Mechanism

The ion selectivity of the epithelial barrier is not only regulated by the ovarian hormone-induced differential expression of ENaC and CFTR during the estrous cycle, but also by their interaction at protein level, thereby providing a mechanism that enables the switching from basal Na^+ absorption to primary Cl^- secretion upon stimulation. While CFTR has been shown to inhibit ENaC activity in other tissues,[108,109] activation of endometrial Cl^- channels, including CFTR and CaCC, was observed to have inhibitory effect on the amilorid-sensitive Isc.[100,110] In the established mouse endometrial epithelial cultures, we found that extracellular ATP induced a reduction in basal Isc, which could be abolished by amiloride or apical removal of Na^+, suggesting that the ATP-induced downward Isc represents inhibition of Na^+ absorption. With the presence of amiloride to remove the contribution of Na^+ absorption to the Isc, ATP induced an upward, rather than downward, Isc, representing Cl^- secretion. The ATP-induced inhibition of Na^+ absorption was mimicked by UDP (a potent P2 purinergic receptor agonist) or ionomycin (Ca^{2+} ionophore) and blocked by treatment of the cells with reactive blue (P2 receptor atagonist), or EGTA (Ca^{2+} chelator). Pretreating the cells with DIDS, the blocker of CaCC, inhibited both the ATP-induced inhibition of Na^+ absorption and the Cl^- secection in the presence of amiloride. This suggests that activation of CaCC by ATP could inhibit ENaC in the mouse endometrial epithelia.[110] However, whether this is due to a direct interaction between CaCC and ENaC is still not known. In another study,[100] we found that after forskolin-induced Cl^--dependent Isc increase, the amiloride-sensitive Isc representing ENaC-mediated Na^+ absorption was reduced. Both the forskolin induced a Cl^- secretion and inhibition of ENaC activity were blocked by the Cl^- channel blocker DPC. Since the forskolin-induced DPC-sensitive Cl^- secretion had been demonstrated to be mediated by CFTR, an inhibition of ENaC activity by the activation of CFTR in the mouse endometrial epithelial cells was suggested.[100] On the other hand, enhancing ENaC expression and activity by growing the cells on matrigel was found to decrease CFTR[111] and CaCC[112] activity in the endometrial epithelial cells. Although the exact mechanism underlying the interaction between ENaC and CFTR in the endometrial epithelial cells is not clear, their interaction does provide a means to augment secretion while suppress absorption, or vice versa, across the epithelial barrier.

Regulation of ENaC and Physiological Implications

In addition to the observed regulation by ovarian hormones, ENaC expression or its channel activity is highly regulated by many other factors. In the kidney, for example, aldosterone, by activating nuclear mineralocorticoid receptors, triggers the transcription of many proteins related to Na^+ re-aborption, including ENaC.[113] In a study on mice, we demonstrated that low-Na^+ diet treatment caused significantly elevated mRNA levels of ENaC subunits in the uterus, which was likely to be due to elevated circulating aldosterone during the low Na^+ treatment.[114] Moreover, treating the primary culture of mouse endometrial

epithelial cells with aldosterone significantly increased the amiloride-sensitive Isc current representing ENaC activity.[114] The aldosterone-enhanced ENaC activity was found to be mainly due to the upregulation of γ-ENaC in the primary culture of mouse endometrial cells.[115] Therefore, it appears that similar to ENaC in the kidney, uterine ENaC is also sensitive to the levels of Na^+ intake and regulated by aldosterone, the key hormone controlling blood volume and pressure in the body through its action on promoting ENaC expression.[86,116] Interestingly, uterine ENaC activity is highly enhanced during implantation and pregnancy,[99] which may cause elevated Na^+ re-absorption across the uterine-blood barrier resulting in increases in blood volume/pressure during pregnancy. Therefore, the observed up-regulation of uterine ENaC during pregnancy may provide new insights into the cause of pre-eclampsia, a maternal disease featured by hypertension in pregnancy.

Interestingly, using the established mouse endometrial epithelial cell culture model, we found that both ENaC activity[117] and the expression of γ-ENaC[115] were enhanced by growing the cells on matrigel, which is believed to provide biologically active matrix materials resembling the basement membrane of many epithelia. Also, laminin, the major component of matirgel, was found to have similar effect on enhancing ENaC activity in the endometrial epithelial cells. Since matrigel consists of a mixture of proteins including a variety of growth factors, it may mimic the nutrient support from the uterine blood stream to the endometrium. Thus, endometrial ENaC may be regulated by nutrients from the blood stream under physiological conditions. Considering laminin is a fibrous protein present in the basal lamina of a variety of epithelia, the regulation of ENaC expression by laminin suggests that Na^+ and fluid absorption may depend on the polarity and integrity of the epithelial barrier. This is of physiological relevance since endometrial epithelium is deteriorated, transformed or rebuilt repeatedly with the menstrual cycles.

More recently, ENaC has been documented to be sensitive to mechanical force[118] and serine proteases,[119,120] which challenges the long-held belief that ENaC is a constitutively opened ion channel. In the female reproductive tract, especially in the uterus, this is of physiological importance since serine protease are abundantly found in embryo-uterus contact, released by the embryo and critically required for implantation. More importantly, ENaC is expressed in the apical membrane of the epithelium where lies the site for embryo adhesion, which may be activated by the proteases released by the implanting embryo. On the other hand, the implanting embryo may also produce mechanical disturbance that is known to activate ENaC. The activation of ENaC by both the mechanical stimulation and release of proteases could lead to enhanced Na^+ and fluid absorption thereby promoting luminal closure and embryo implantation.

Regulation of CFTR-Mediated Bicarbonate Secretion and Its Effect on Sperm Capacitation and Embryo Development

As mentioned earlier, a striking feature of the uterine and oviductal fluids is the presence of high HCO_3^- contents, which can be as high as 90 mM, two to four folds of that of the plasma.[20,24,26] However, the mechanisms regulating the formation of HCO_3^--rich uterine and oviductal fluids remain largely unknown. Recently, we have found that the maximal CFTR expression is concurrent with the highest HCO_3^- concentration or luminal pH in mouse uterus at estrous. We further demonstrated that several molecules involved in HCO_3^- production and HCO_3^- secretion, such as carbonic anhydrases (CA2 and CA12) that catalyze the conversion of CO_2 and H_2O into HCO_3^- and H^+, and the

Cl^-/HCO_3^- exchanger Slc26a6, another transporter responsible for HCO_3^- efflux from the apical membrane in addition to CFTR, exhibit maximal expression levels at estrous, indicating possible regulation of bicarbonate production/secretion by estrogen.[121] Indeed, exogenously administrated estrogen to ovariectomized female mice was found to enhance HCO_3^- dependent Isc measured across freshly isolated endometrial epithelia and treatment of the primary culture of mouse endometrial epithelial cells with estrogen also increased apical surface pH, consistent with a regulatory role of estrogen in uterine HCO_3^- secretion. Given that HCO_3^- is essential for sperm capacitation,[122,123] the upregulation of HCO_3^- secretion by estrogen at a time immediately prior to ovulation may enable sperm to acquire their fertilizing capacity in time to fertilize the egg (also see below).

It should be noted that under physiological condition, sperm capacitation mainly occurs in the oviduct, although some initial processes may take place in the uterus. The HCO_3^--secreting ability of the oviductal epithelial barrier has also been demonstrated by CFTR expression in the oviduct of rodents[48] and its involvement in oviductal HCO_3^- secretion in porcine oviductal epithelia.[65] Interestingly, early embryo development also takes place in the HCO_3^--rich oviductal fluid microenvironment. We demonstrated that mouse embryo development from 2-cell to morula or blastocyst stage was significantly inhibited in the absence of HCO_3^- or when cocultured with HCO_3^- secretion-deficient CFTR mutant cells as compared with the wild-type.[65] These results suggest that the selective permeability of the oviductal epithelial barrier to HCO_3^-, which is regulated by CFTR, is important for embryo development in addition to sperm capacitation.

DEFECTIVE ION CHANNEL EXPRESSION/FUNCTION AND DYSREGULATED BARRIER FUNCTION IN DISEASES

Since ion channels play a critical role in balancing the secretary and absorptive activities of the female reproductive tract epithelium, their dysfunction or abnormal expression may lead to dysregulated epithelial barrier function, resulting in diseases or disordered reproductive functions.

Defective Epithelial Bicarbonate Secretion and Infertility in CF

The importance of CFTR in female reproduction is highlighted by the reduced fertility in women with CF, a genetic disease caused by CFTR mutations with major defect in salt and water transport. However, the underlying cause remains obscure.

Abnormally thick cervical mucus, which forms a barrier for sperm penetration, has long been thought to be the primary cause for reduced fertility in CF women.[124] Comparing the physical properties and inorganic chemical composition of cervical mucus from women with CF to those of healthy controls, researchers found that the amount of water in the cervical mucus from CF women is 80% less than that found in normal subjects.[125] How does the defect in CFTR as a Cl^- channel lead to thick mucus? In fact, abnormally thick and viscous mucus is found in various organs of CF patients, such as the lung, intestine, pancreas, and uterine cervix.[126] It was thought that cervical mucus thinning and release during the female reproductive cycle relied mainly on fluid secretion and that the abnormal cervical mucus in CF women was attributed to the mucus dehydration resulting

from the basic defect in Cl^--dependent fluid transport. This notion led to the proposal that the dehydrated thick cervical mucus in CF women forms a barrier preventing sperm penetration, leading to infertility.[125]

It is not until recently that an alternative notion emerges to explain the formation of CF mucus. The novel notion focuses on another anion that CFTR transports, HCO_3^-, since abnormally reduced HCO_3^- secretion has been observed in various CF organs. Quinton et al recently demonstrated that mucus released from murine reproductive tract critically depends upon concurrent HCO_3^- secretion and functional CFTR.[127] In contrast, agonists-stimulated fluid secretion was not dependent on bicarbonate. Further more, stimulated mucus release was severely impaired in the cervix of CF deltaF508 mice. Thus, CFTR mutations and/or poor HCO_3^- secretion may account for the increased viscosity and lack of cyclic changes in cervical mucus long noted in women with CF. Similar findings were also obtained from the ileum and intestine.[128] A model is put forward to explain the effect of HCO_3^- in mucus discharge process. In normal mucin discharge, HCO_3^- competes with the fixed negative sites for Ca^{2+} and H^+ in mucus molecules, forcing the mucin to expand into a charged polymer that is easy to be removed from the lumen. In CF lumen, however, the lack of HCO_3^- keeps the fixed negative sites to be shielded by Ca^{2+} and H^+, resulting in mucin molecules adhering to each other and forming condensed and thick mucus in the lumen.[126] Although further investigations are required to validate this hypothesis, Quniton's findings have challenged the previous view and provided a new biochemistry basis for the role of CFTR and HCO_3^- in mucus formation.

Is the thick mucus caused by either defective fluid or HCO_3^- secretion the whole story to the reduced fertility in CF women? Clinical studies showed that pregnancy in some CF women undergoing assisted reproduction could not be achieved even after repeated sperm insemination, a procedure that would have overcome the thick cervical mucus long thought to the primary cause of infertility in CF women, but these patients did become pregnant after in vitro fertilization (IVF).[129] These clinical cases suggest other potential defect(s) beyond the cervix leading to infertility in CF women. It is interesting to note that sperm undergo capacitation process only in the female but not male genital tract and that a striking difference in the fluid microenvironment between the two tracts is the HCO_3^- contents, as low as 2-4 mmol/L in the epididymis,[130] where sperm are stored before ejaculation, while as high as 90 mmol/L in the female genital tract.[20,24,131] More importantly, HCO_3^- has been shown to be essential to sperm capaciation.[122,123] Obviously, a HCO_3^- permeability defect in the epithelial barrier of the female reproductive tract may impair the fertilizing capacity of sperm.

The demonstrated involvement of CFTR in mediating uterine HCO_3^- secretion (see above) and the critical role of HCO_3^- in spermatozoa function, especially sperm capacitation, have led us to the hypothesis that defective CFTR-mediated HCO_3^- secretion could lead to impaired sperm fertilizing capacity and thus reduced fertility seen in CF women. This hypothesis was tested in a mouse sperm-endometrial epithelial cell coculture system.[56] It was found that sperm motility was greatly enhanced when sperm was cocultured with endometrial epithelial cell, which had normal CFTR-mediated HCO_3^- secretion, as compared to that incubated with HCO_3^--free or endometrium cell-free medium.[56] In addition, the percentage of capacitated sperm was significantly attenuated when CFTR expression in the cocultured endometrial epithelial cells was suppressed with antisense against CFTR, as compared to the sense-treated controls. IVF assays on zona-intact mouse eggs further demonstrated that the number of 2-cell embryos obtained with sperm

capacitated in conditioned medium from CFTR antisense-treated endometrial cells was significantly reduced as compared to that obtained from the sense-treated controls. Taken together, these results suggest that CFTR is involved in endometrial HCO_3^- secretion and that impaired HCO_3^- secretion caused by defective CFTR results in reduced sperm fertilizing capacity. These results have provided a possible cause, other than the thick cervical mucus, for reduced fertility seen in CF women. In addition to the demonstrated defect in sperm capacitation, defect in CFTR-mediated oviductal HCO_3^- secretion and early embryo development (see above) may also contribute to the reduced fertility seen in women with CF. Taken together, these results suggest that the CFTR-medicated selective permeability to HCO_3^- of the female genital epithelial barrier is of critical importance in reproduction.

Excessive CFTR-Dependent Fluid Accumulation in OHSS

For about 15% of couples worldwide who are suffered from infertility, assisted reproduction technology (ART) is probably the most effective treatment to circumvent the problem.[132] The increasing demand and access to ART also lead to the rising incidences of ART complications. OHSS is one of the most life-threatening and potentially fatal complications of ART, arising from excessive stimulation of the ovaries by exogenous gonadotropins administrated during IVF procedures.[133,134] Severe OHSS is characterized by ovarian enlargement, pleural effusion, ascites, oliguria, hemoconcentration and thromboembolic phenomena.[135] Although massive fluid accumulation in various organs is considered as the principal feature of OHSS, the process of fluid formation in OHSS remains obscure, and no definitive treatments are currently available.

Increased vascular permeability mediated by vascular endothelial growth factor (VEGF) has been suggested to be responsible for the massive fluid shift from the intravascular compartment into the peritoneal cavity, leading to the formation of ascites.[136-139] Expression of VEGF and VEGF receptor are upregulated during the gonadotropin stimulation phase, preceding hCG injection, which has been confirmed in both the rat[140] and human.[141] After hCG stimulation, VEGF receptors were also found throughout the corpus luteum[141] with peak expression observed approximately 48 h after hCG injection.[140]

VEGF and its receptors increase the vascular permeability by regulating endothelial junction proteins, especially cadherin and claudin 5. Using an in vitro model of OHSS that based on the human umbilical veins endothelial cells, hCG and VEGF caused changes in the actin fibers, indicating a subsequent increase in capillary permeability. Cadherin concentration was elevated as well when hCG and VEGF were added, but not with the addition of estradiol.[142] An augmentation in VEGF concentrations and vascular permeability was also observed in another study with hCG addition to luteinized granulosa and human umbilical vein endothelial cells. In this in vitro OHSS model, claudin 5 expression was significantly reduced in endothelial cells.[143]

Apart from VEGF, other substances such as growth factors, angiotensin and cytokines, have also been suggested to play major roles in the elevated vascular permeability in OHSS. None of these substances, however, has been demonstrated to be directly involved in the pathogenesis of OHSS. Moreover, there have been reports showing no correlation between plasma VEGF concentrations and OHSS,[136-139] which suggests that some mechanisms other than the elevated vascular permeability may be responsible for the pathogenesis of OHSS.

As mentioned earlier, fluid movements across secretary epithelia are secondary to ion movements. The importance of ion movements across epithelia lies in their coupling with the movement of water, which is not actively transported, but moves in response to osmotic gradients, largely established by the transport of ions. Rapid passage of fluid into luminal spaces, as seen in OHSS, may be a consequence of abnormal ion transport across the epithelia. Several pathological conditions, such as cholera-induced diarrhea, in which there are massive fluid fluxes across epithelial barriers, are mediated by altered expression and function of transepithelial ion channels, particularly CFTR.[144] Moreover, as discussed earlier, CFTR expression is known to be regulated by ovarian hormones,[92,93] upregulated by estrogen, and downregulated by progesterone. It has also been well established that estrogen levels are highly elevated during ovarian hyperstimulation, with excessively high levels observed in OHSS.[145,146] We tested the hypothesis that abnormally upregulated CFTR expression and function by the excessively high levels of estrogen may be the cause of OHSS.[145] In an OHSS rat model, OHSS symptoms, including increased uterine wet weight, as well as upregulated CFTR expression and enhanced CFTR channel activity were observed, which could be mimicked by administration of estrogen, but not progesterone, in ovariectomized rats. Administration of progesterone that suppresses CFTR expression or antiserum against CFTR to OHSS animals resulted in alleviation of the symptoms.[145] Furthermore, ovarian hyperstimulation did not induce detectable OHSS symptoms in CFTR mutant mice, confirming the involvement of CFTR in the pathogenesis of OHSS. These results have demonstrated a pathological condition caused by abnormally upregulated CFTR with increased channel activity leading to excessive fluid shift and accumulation in the peritoneal cavity and in different organs, a condition that is in great contrast to CF, a disease with hallmark defect in electrolyte and fluid transport in most exocrine glands due to defective CFTR.[78] Taken together, these results further confirm a critical role of CFTR in regulating the permeability of epithelial barriers to anions and fluid.

Infection-Induced CFTR Upregulation in Hydrosalpinx and Female Infertility

Chlamydia trachomatis infection is the most common cause of pelvic inflammatory disease (PID) leading to severe tubal damage, hydrosalpinx (abnormal fluid accumulation in the Fallopian tubes), ectopic pregnancy and infertility.[147,148] Wolner-Hanssen et al recovered *C. trachomatis* from the uterus and Fallopian tubes of women with acute salpingitis.[149] Serological studies on women with salpingitis have demonstrated a strong association of tubal factor infertility with past *C. trachomatis* infection.[150] It should be noted that although hydrosalpinx accounts for about 30% of tubal factor infertility, the cause for the abnormal fluid accumulation in the tubes remains unknown. Witkin et al found increased levels of chlamydial heat shock proteins (HSPs) in women with previous chlamydial infections.[151] Antibodies to these HSPs were more prevalent in women with hydrosalpinx and tubal occlusion than in women with male factor infertility.[152] These proteins are also thought to be responsible for the induction of local immune responses that lead to an inflammatory reaction, impaired implantation and immune rejection after embryo transfer. The presence of cervical IgA antibody to chlamydia in hydrosalpinx has been strongly correlated with unsuccessful outcome after embryo transfer.[151]

Despite the long recognition of *C. trachomatis* as the most important cause of PID worldwide, the sequence of events linking chlamydia to hydrosalpinx formation and subsequent infertility or poor ART outcome has not been elucidated to a satisfactory extent. It should be noted that post-infectious hydrosalpinx pathology showed disarranged tubular epithelial barrier with atrophy of mucosal folds, marked exfoliation and loss of epithelial cells.[153] These disorders may affect the expression of epithelial membrane transporters and ion channels and subsequently ion and fluid transport, leading to the formation of hydrosalpinx fluid.

Interestingly, significant increase in CFTR expression has been observed in Fallopian tubes from hydrosalpinx patients compared with those of normal individuals. Thus, abnormally upregulated CFTR may be one of the underlying mechanisms for abnormal fluid secretion and accumulation in hydrosalpinx.[154] Moreover, our recent studies have shown that *C. trachomatis* inoculated into healthy Sprague-Dawley rat uteri induced uterine infection, massive uterine fluid accumulation (as seen in hydrosalpinx) and increased CFTR mRNA expression.[98] These data support the notion that infection may lead to abnormal upregulation of CFTR with concomitant changes in ion flux across the Fallopian tubal epithelium accompanied by increased fluid accumulation. The CFTR involvement in hydrosalpinx was further confirmed using CFTR mutant mice showing absence of fluid accumulation after *C. trachomatis* infection.[98,155] These results suggest that abnormally upregulated CFTR is responsible for the fluid formation in hydrosalpinx. Abnormal upregulation of CFTR with fluid accumulation upon bacterial infection, especially at the time of implantation when CFTR expression level and luminal fluid volume are normally at their lowest, may prevent 'closure' of the luminal surface and hence, embryo implantation failure. We have tested this hypothesis by administrating a large dose of estrogen to mice 24 h before implantation, which induced 100% implantation failure with obvious fluid accumulation in the uterus.[40] Therefore, accumulation of fluid in the Fallopian tubes and its regurgitation into the uterine cavity may be a contributing factor to the observed infertility induced by *C. trachomatis* infection, and the impaired implantation or endometrium receptivity of transferred embryos during IVF post-infection.[156]

Taken together, the evidence presented indicates that dysregulated epithelial barrier function due to defective or abnormal expression of epithelial ion channels in the female genital tract may have pathological consequences.

CONCLUSION

Accumulating evidence has indicated the important roles of ion channels, ENaC and CFTR in particular, in regulating the selective permeability of the epithelial barrier and the physiology of the female reproductive tract, as depicted in Figure 3. These ion channels, and thus the selective permeability of the epithelial barrier, are tightly regulated by the factors from both sides of the barrier, such as luminal factors (e.g., proteases and mechanical stimuli) or factors from the blood or basement matrix (e.g., ovarian hormones, mineralocorticoid and nutrition factors). Other autocrine/paracrine factors (e.g., noradrenaline or ATP released from nerve endings) may also regulate ion channel activity. Within the epithelium, interaction between ENaC and other Cl^- channels also provides a means to control directional movement of ions and fluid across the epithelial barrier. The temporal and spatial tight regulation of the ion channels governs the selective permeability of the epithelial barrier, thereby providing fine-tuning of the luminal fluid

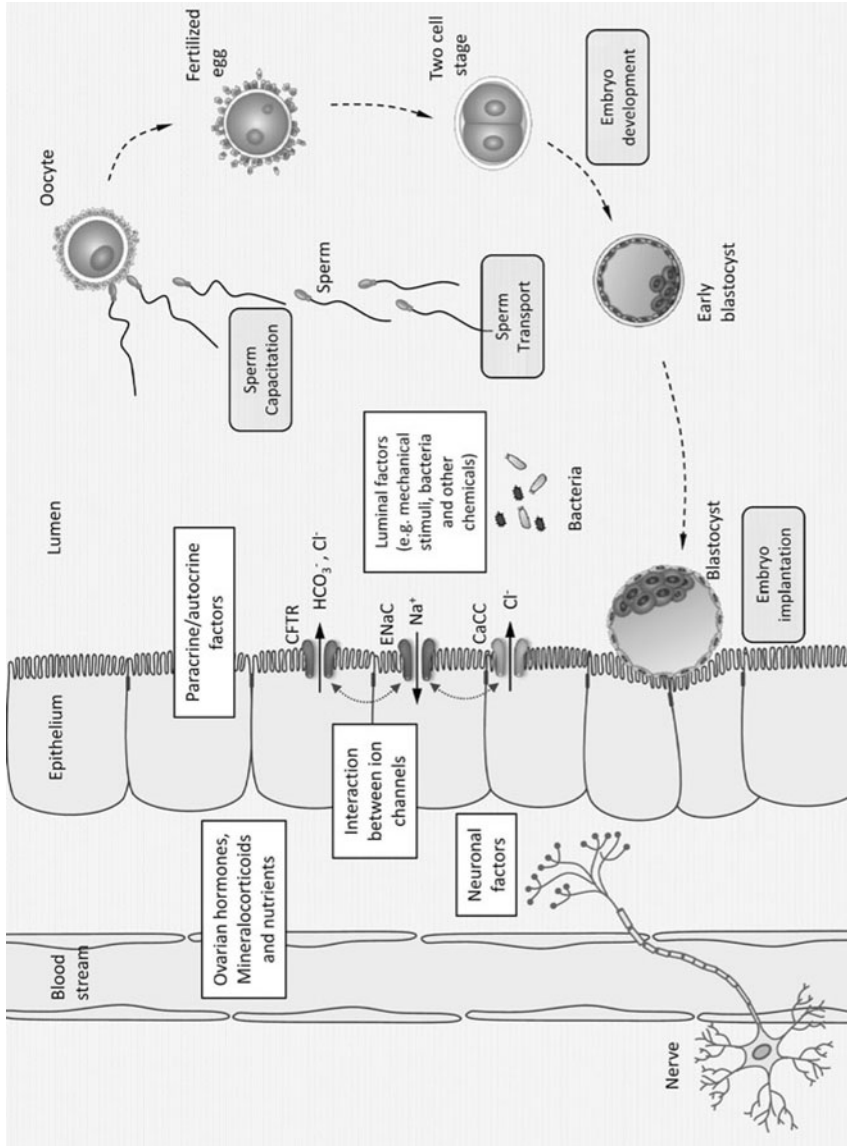

Figure 3. Schematic drawing showing multiple mechanisms for the regulation of ion channels and the selective permeability of the epithelial barrier of the female reproductive tract, as well as physiological implications in various reproductive events, sperm transport, capacitation, embryo development and implantation.

microenvironment crucial to various reproductive events taken place in the female reproductive tract, disruption of which may result in disorders and infertility. This raises the possibility that these ion channels may be the targets of new contraceptives, diagnosis and treatment of reproductive disorders and infertility.

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CHAPTER 11

THE BLOOD-EPIDIDYMIS BARRIER AND HUMAN MALE FERTILITY

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Abstract: Spermatozoa undergo a posttesticular maturation in the epididymis to acquire motility and the capacity to fertilize. Sperm maturation depends in part upon the creation of a specific microenvironment within the epididymal lumen. This environment is conditioned by proteins secreted by the epithelium and by exchange of molecules between the lumen and the blood circulation. These exchanges are selectively regulated by the blood-epididymis barrier. The blood-epididymis barrier is comprised of apical tight junctions between adjacent principal cells. Adherens junctions, which are necessary for cell adhesion, can also be found at the junctional complex present between adjacent principal cells. Progress has been made on the understanding of cellular interactions in the epididymis as well as the regulation of the luminal microenvironment and its importance for sperm maturation in rodents and humans. Clearly, changes in the function of cellular junctions in the human epididymis are associated with male infertility.

INTRODUCTION

Testicular spermatozoa are physiologically immature and must transit through the epididymis to become mature by acquiring motility and the ability to fertilize. The epididymis is the major component of the testicular excurrent duct system. Testicular input to the tissue is conveyed via the efferent ducts, which anastomose to form a single, highly convoluted epididymal duct. The studies of Orgebin-Crist[1] and Bedford[2] revealed that functional sperm maturation in the epididymis was the result of their exposure to the luminal environment. Thus, the ability of the epididymis to provide the appropriate milieu for sperm maturation is critical. This milieu is created by several processes, most

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notably the highly absorptive and secretory activities of the epithelial cells that line the duct and the presence of the blood-epididymis barrier which is formed by tight junctions between epithelial cells that line the lumen of the epididymis.

STRUCTURE OF THE HUMAN EPIDIDYMIS

The human epididymis is a highly convoluted duct which connects the efferent ducts to the vas deferens.[3] The epididymis is subdivided into segments according to functional and morphological differences, as well as the localization of regionally expressed genes. In the human, it is generally divided into three main regions: the caput, corpus and cauda epididymidis (Fig. 1). This is somewhat different from most animals which also have a distinct initial segment between the efferent ducts and the caput epididymidis. In the human, the caput epididymidis has an initial segment-like epithelium and is partially comprised of efferent ducts suggesting that it has many of the same functions as those found in other animals.[4] The basic structure of a tubule includes a well-defined lumen, stereocilia and a pseudostratified columnar epithelium containing mostly principal and basal cells. Principal cells are involved in secretion and absorption, while basal cells are thought to have a role in immunity or to regulate principal cell functions.[5-7] Previous reviews describe the different epididymal cell types in greater detail.[8,9]

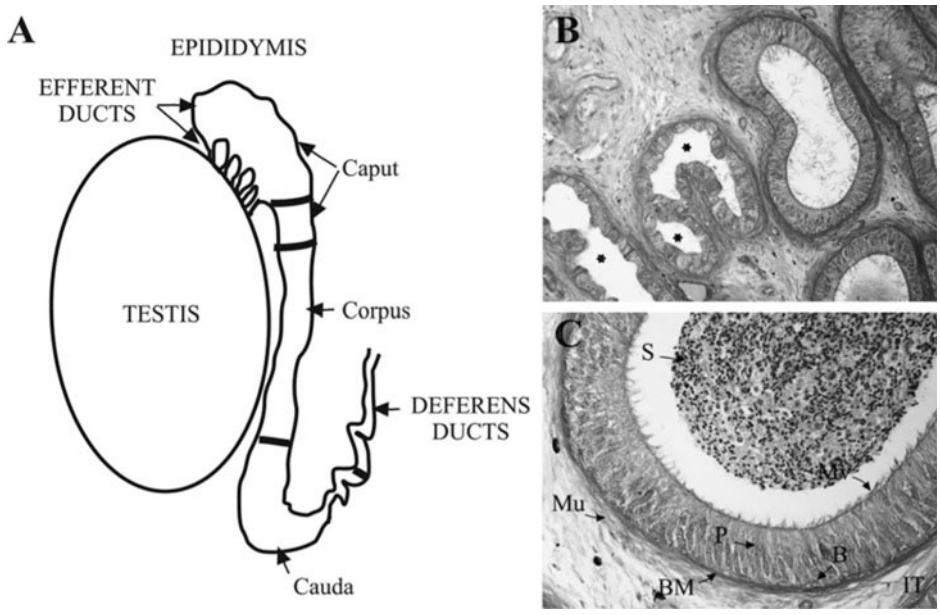

Figure 1. Structure of the human epididymis. A) The epididymis is generally divided into three regions: the caput, the corpus and the cauda. B) Cross-section of the human caput epididymidis shows that efferent ducts (*) can also be found in this region of the human epididymis. C) The pseudostratified epithelium lining the epididymal lumen is mainly composed of basal and principal cells. Principal cells are characterized by long microvilli. The tubules also have a thick muscular coat which plays an important role in sperm movement through the epididymis. S, spermatozoa; P, principal cells; B, basal cells; Mv, microvilli; Mu, muscular coat; BM, basement membrane; IT, interstitial space.

INVOLVEMENT OF THE HUMAN EPIDIDYMIS IN SPERM MATURATION

In the epididymis, spermatozoa undergo different processes including concentration, maturation, storage in a quiescent state and are protected from the immune system.[7-10] Transit time through the human epididymis varies between approximately 2 to 10 days.[7,11] Several studies have demonstrated that during epididymal transit, human spermatozoa acquire progressive motility, a higher ability to fertilize as well as an increasing ability to undergo the acrosome reaction and to bind the oocyte, revealing that human spermatozoa undergo a functional maturation in the epididymis.[12-18] A key element of sperm maturation, protection and storage is the presence of a highly specialized luminal microenvironment, which changes along the epididymal duct.[19] Recent data demonstrate the existence of regionalized gene expression in the human epididymis, reflecting the segment-specific secretion of proteins observed in the lumen.[20-24] Moreover, both the creation and maintenance of the luminal microenvironment are tightly regulated by the blood-epididymis barrier.

CHARACTERISTICS OF THE BLOOD-EPIDIDYMIS BARRIER

The ultrastructure of the blood-epididymis barrier was first described by Friend and Gilula[25] in the rat. This highly developed junctional complex is comprised of tight junctions between principal cells (Fig. 2). The physiological demonstration of a tissue barrier in the epididymis was reported by Howards et al[26] in the cauda and it was later confirmed in the entire epididymis by Turner et al.[27] This barrier has been described in the mouse,[28] monkey,[29] mink,[30] stallion[31] and dog.[32] Furthermore, the junctional complex varies in number and in geometrical organization along the rat epididymal duct with a larger number of tight junctional strands in the caput than in the cauda.[33] Cyr et al[34] also demonstrated that the length of the junctional complex is reduced along the lateral plasma membrane of principal cells in the corpus and cauda, as compared with the caput epididymidis, whereas the number of desmosomes increases along the epididymal duct. In this study, it was also revealed that junctions are dynamic structures with renewal of junctional proteins by turnover of plasma membranes.

While tight junctions between epithelial cells have a barrier and a fence function, these junctions also control the movement of water, ions and proteins between the blood circulation and, in this case, the epididymal lumen to create specific microenvironments.[35] Hoffer and Hinton[36] studied the integrity of the blood-epididymis barrier and found that lanthanum and inulin could not penetrate the rat blood-epididymis barrier. The permeability characteristics of this barrier in the rat caput epididymidis were later studied by Hinton and Howards[37] using micropuncture techniques. They showed that small molecular weight compounds, can cross the blood-epididymis barrier more quickly than larger molecular weight compounds which usually reach less than 7% of the blood plasma concentrations. Studies have shown differences in concentrations of certain components not only between the blood circulation and the epididymal lumen, but also within the epididymal lumen between the different regions, suggesting that the characteristics of the barrier could also be changing along the duct.[38] Moreover Chan et al[39] demonstrated, using transepithelial resistance measurements, that the blood-epididymis barrier was tighter in the cauda than in the proximal epididymis, which may be important for the storage of spermatozoa in a quiescent state. There have been few studies on the human blood-epididymis barrier, despite the fact that several studies have confirmed the existence of epididymal tight

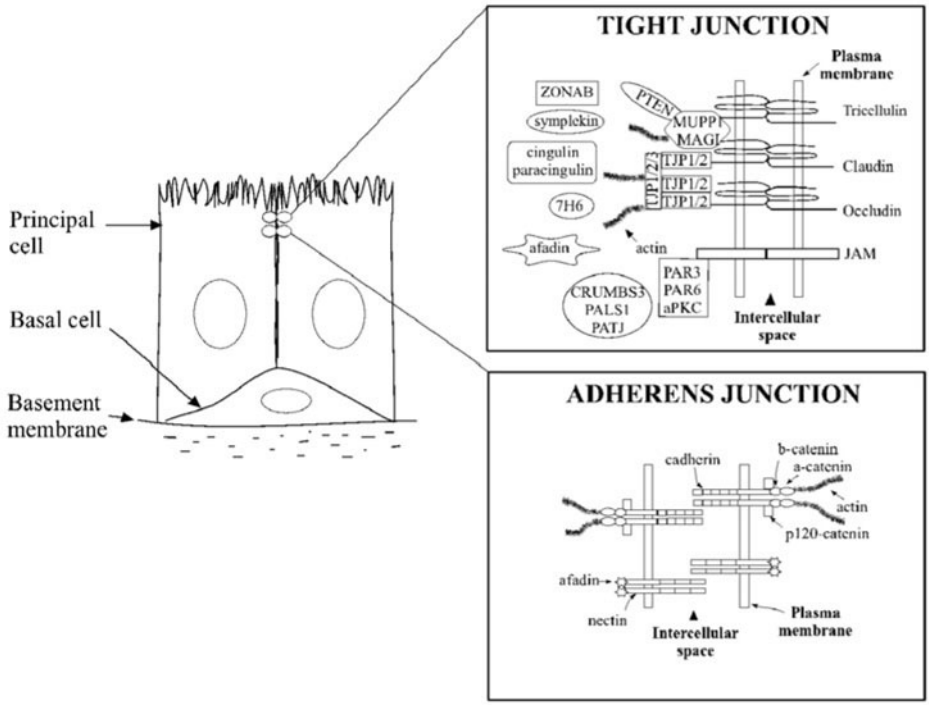

Figure 2. Organization of tight and adherens junctions. Tight junctional complexes are found between adjacent principal cells and are comprised of several transmembrane and peripheral proteins while adherens junctions are comprised of two multiprotein complexes, the nectin-afadin complex and the cadherin-catenin complex.

junctions in vivo and in vitro.[22,40-42] One of the features of the human epididymal junctional complex is that, unlike in rodents, the extent, or length, of the complex does not vary between epididymal regions.[22]

COMPOSITION OF EPIDIDYMAL TIGHT JUNCTIONS

Tight junctions are comprised of transmembrane proteins that interact with peripheral proteins which have specific roles in the assembly, maintenance and/or regulation of the tight junctional complex (Fig. 2).

Integral Proteins of Tight Junctions

Different classes of integral proteins are localized at tight junctions. The first class of proteins includes occludin, tricellulin and claudins (CLDN), all of which have a similar structure of four transmembrane domains with two extracellular loops with both N- and C-terminals localized in the cytoplasm. The second class of integral proteins includes the junctional adhesion molecules (JAM), which are single-pass proteins belonging to the immunoglobulin superfamily.

Occludin and Tricellulin

Occludin was the first integral protein identified as part of the tight junctional complex in a number of endothelia and epithelia.[43] This protein is present along the mouse, rat, rabbit, dog and human epididymis. In the adult epididymis, occludin is exclusively localized to apical tight junctions between principal cells, except in the proximal initial segment of the mouse, where occludin was found only in association with narrow cells.[22,44-47] In the developing mouse epididymis, although occludin is expressed by embryonic day 13.5, only by embryonic day 18.5 is it targeted to the apical region between principal cells.[44] Even if occludin binds to several peripheral proteins such as TJP1, 2 and 3,[48-50] its role is still unclear as tight junctions in many cell types may be formed in the absence of occluding.[51] Recent data suggest that another tight junctional protein, tricellulin, may compensate for occludin barrier and fence functions. Tricellulin mRNA is expressed in the human and mouse epididymis (Table 1). Tricellulin is localized at tricellular junctions.[52] Tricellulin can form heteromeric complexes with occludin.[53] In vitro knockdown of occludin results in the mis-localization of tricellulin to bicellular tight junctions.[54] A new tight junction associated protein of the occludin family called MARVELD3 has recently been discovered[55,56] but its expression in the epididymis is unknown.

Claudins

The presence of tight junctions in the absence of occludin led Tsukita et al to discover a new family of proteins of 24 members they named CLDNs.[57] CLDNs are the molecular basis of tight junctions. Indeed, exogenous expression of CLDNs in fibroblasts can induce the formation of tight junctional strands,[58] as well as create charge-selective paracellular pores that enable the passive diffusion of ions between cells.[59,60] CLDNs are found in all epithelia but their tissue distribution is specific and varies between species. In addition, owing to their ability to undergo heterophilic interactions and their ion selectivity, the CLDN composition of tight junctions determines the specific permeability properties of epithelia.[61] In the rat, mouse and human epididymis, *CLDNs 1* to *9* are expressed, suggesting a degree of conservation in the composition of tight junctions and similar roles for these CLDNs within the junctional complex.[22,62-65] In addition, *Cldn10* and *11* are expressed in the rat epididymis[66] and *CLDNs 10 to 12*, *14* to *19* and *23* in the human epididymis.[22] Several CLDNs are differentially expressed along the rat (*Cldn10* and *16*), mouse (*Cldn2, 3, 8* and *23*) and human epididymis (*CLDN2, 5, 8, 10, 16* and *23*) suggesting different roles for these *CLDN* genes along the duct.[22,63,64,67] Immunofluorescence studies in the rat epididymis revealed that Cldn1, –2, –3, –4, –6, –7, –8 and –10 proteins were localized to epididymal tight junctions, whereas Cldn5 was expressed in endothelial cells.[62-65] In the human epididymis, CLDN1, 3, 4, 8 and 10 are localized in the apical tight junctional complex.[22] However, CLDN1, 3 and 4 are also present along the lateral plasma membranes between neighboring principal cells and between principal and basal cells throughout the human epididymal duct.[22] This was also observed for Cldn1, 7 and 10 in the rat epididymis.[64,65] CLDNs present on lateral membranes may either form a pool of proteins for apical tight junctions or serve to reinforce cell adhesion. In addition, CLDN8 exhibits segment-specific localization in the human epididymis.[22] The fact that the CLDN composition of tight junctions and thus the permeability characteristics of the blood-epididymis barrier, varies along the epididymal duct could be a key element of the changing environment along the duct.

Table 1. Relative expression of genes encoding tight junctional proteins and nectins in the epididymis

	Relative Expression		
Gene Name	Caput	Corpus	Cauda
Tricellulin (MARVELD2)	++ (h) + (m)	++ (h) + (m)	++ (h) + (m)
JAM-2 (JAM-B)	++ (h) + (m)	+ (h, m)	+ (h, m)
JAM-3 (JAM-C)	+ (h, m)	– (h) + (m)	+ (h, m)
ESAM	+ (h, m, r)	+ (h, m, r)	+ (h, m, r)
PAR-3 (PARD3)	+++ (h) + (r)	– (h) + (r)	+++(h) + (r)
PAR-6 (PARD6A)	+++ (h) + (r, m)	+++ (h) + (r, m)	+++ (h) + (r, m)
MAGI-1	++ (h) + (m)	++ (h) + (m)	++ (h) (m)
MAGI-2	+ (m, r)	+ (m, r)	+ (m, r)
MAGI-3	+ (r)	+ (r)	+ (r)
MUPP1 (MPDZ)	+ (h, m)	– (h) + (m)	+ (h) + (m)
CINGULIN (CGN)	+ (h, m)	– (h) + (m)	+ (h, m)
JACOP/Paracingulin (CGNL1)	++ (h) – (m)	++ (h) – (m)	++ (h) – (m)
Symplekin (SYMPK)	+++ (h) ++ (m) + (r)	+++ (h) ++ (m) + (r)	+++(h) ++ (m) + (r)
Nectin-1/PVRL1	++ (m) + (r)	++ (m) + (r)	++ (m) + (r)
Nectin-2/PVRL2	+ (h, m, r)	+ (h, m) ++ (r)	+ (h, m) ++ (r)
Nectin-3/PVRL3	+ (h)	+ (h)	+ (h)
Nectin-4/PVRL4	++ (h) + (m)	++ (h) + (m)	++ (h) + (m)
Afadin	++ (r, m)	++ (r, m)	++ (r, m)

Expression levels were extracted from gene expression databanks (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc = GSE23812 and http://mrg.genetics.washington.edu). +, weakly expressed (0-100); ++, moderately expressed (100-250); +++, highly expressed (250 and more). h, human; m, mouse; r, rat.

Junctional Adhesion Molecules

To date, six JAMs have been identified: JAM-1 (JAM-A), JAM-2 (JAM-B), JAM-3 (JAM-C), CAR (Coxsackie adenovirus receptor), ESAM (endothelial cell-selective adhesion molecule) and JAML (also known as JAM-4 in the mouse). *JAM-2, -3* and *ESAM* are expressed in the human epididymis (Table 1). JAMs are known to interact with many

tight junction associated proteins.[68] JAMs may therefore indirectly regulate tight junction formation by targeting other proteins to the tight junctional complex. Indeed, expression of JAM-1 has been shown to promote localization of TJP1 and occludin at points of cell contact.[69,70] In addition, JAMs binds to the cell polarity complex, consisting of PAR-3, -6 and aPKC, which is involved in tight junction formation.[71,72] *PAR-3* and *PAR-6* are differentially expressed along the human epididymis (Table 1). Several studies suggest that JAM proteins could be implicated in epithelial barrier function since inhibition of JAM-1 affects tight junction assembly and transepithelial resistance.[73,74]

Peripheral Proteins of Tight Junctions

Tight junctions contain numerous cytoplasmic proteins that either serve as adaptors linking the transmembrane proteins to the actin cytoskeleton, thus stabilizing the tight junction structure, or that play roles in transcription, cell polarity, or other signaling functions. These proteins include the zonula occludens proteins (TJP1, TJP2 and TJP3), the membrane-associated guanylyl kinase inverted (MAGUK) proteins (MAGI-1, MAGI-2 and MAGI-3), the multi-PDZ domain protein 1 (MUPP1), the partitioning defective proteins (PAR-3 and PAR-6), the protein associated with Lin-7 (PALS1) and the PALS1-associated tight junction (PATJ), cingulin, symplekin and 7H6 antigen.[35,75]

Zona Occludens Proteins

TJP1 (also known as ZO-1) was the first protein identified as a tight junction constituent. In the human and rat adult epididymis, TJP1 is localized exclusively to tight junctions.[22,45] The genes encoding TJP2 (ZO-2) and TJP3 (ZO-3), which were discovered as TJP1-binding proteins,[49,76] are similarly expressed along the human epididymis.[22] Moreover it has been shown that TJP1 can interact with JAMs[75] and that TJP1 and TJP2 are important for the targeting of CLDNs to tight junctions, the formation of strands and the barrier function.[77,78] TJP1 also serves as a link between adherens and tight junctions. While it is currently unknown whether or not the TJPs play a role in the targeting of tight junctional proteins in the human epididymis, DeBellefeuille et al[79] reported co-immunoprecipitation of TJP1 and β-catenin, an adherens junction protein, especially in young animals during the formation of epididymal tight junctions, suggesting that TJP1 may play a role in the apical positioning of tight junctions.

Other Peripheral Proteins

Other peripheral proteins are expressed in the human epididymis including MAGI proteins (MAGI-1, -2, -3), MUPP1, cingulin, paracingulin and symplekin. However these proteins have only been detected at the mRNA level (Table 1). MAGI proteins can interact with transmembrane proteins of tight junctions such as JAM-4.[75,80] MAGI-1 also binds to β-catenin, suggesting that it is involved in the formation of both adherens and tight junctions.[81] MUPP1 is concentrated at tight junctions and interacts with several transmembrane proteins including CLDN1, CLDN8 and JAM-1.[82,83] A recent study revealed that the loss of MUPP1 did not affect the formation and integrity of tight and adherens junctions in EpH4 cells, while Lanaspa et al[84] reported that a decrease in transepithelial resistance was observed following the knockdown of MUPP1 in renal IMCD3 cells.[85] Cingulin interacts with TJP proteins,

JAMs, actin and myosin. In fact, cingulin is recruited to junctions via its interactions with TJP1.[70,86,87] In addition, cingulin knockout mice showed alterations in the mRNA expression levels of several tight junction proteins, including CLDNs and occluding.[88] However, experiments with cingulin knockout and knockdown epithelial cells suggest that cingulin does not play a direct role in the structure and functions of tight junctions, since these cells had normal tight junctions.[88,89] Paracingulin, identified as cingulin-like1/CGNL1, has been localized, unlike cingulin, at both adherens and tight junctions.[90]

REGULATION OF EPIDIDYMAL TIGHT JUNCTIONS

The cytoplasmic plaque of tight junctions is also comprised of regulatory and signalling components such as tyrosine and protein kinases, small GTPases, phosphatases and transcription factors, involved in tight junction regulation.[91,92] A number of these genes are expressed in the human epididymis (Table 2) and may be involved in epididymal tight junction regulation. However most of the existing information on the regulation of epididymal tight junctions is based on animal studies.

Table 2. Relative expression of genes encoding proteins that could be involved in the regulation of epididymal tight junctions

	Relative Expression		
Gene Name	Caput	Corpus	Cauda
CDC42	+++ (h, m, r)	+++ (h, m, r)	+++ (h, m, r)
RAB13	+++ (h)	+++ (h)	+++ (h)
	++ (r)	+ (r)	+ (r)
	+ (m)	++ (m)	++ (m)
PTEN	++ (h)	++ (h)	++ (h, m)
	+++ (m)	+++ (m)	
PRKACA/PKA	+ + (h)	+ + (h)	+ + (h)
	+++ (m)	+++ (m)	+++ (m)
PRKCA	+ (r)	+ (r)	++ (r)
PRKCD	++ (h)	++ (h)	++ (h)
	+++ (r, m)	+++ (r, m)	+++ (r, m)
PRKCE	+ (h, m)	+ (r, m)	+ (h, r, m)
	++ (r)		
PRKCZ	+++ (h)	+++ (h)	+++ (h)
	+ (r, m)	+ (r, m)	+ (r, m)
PRKCI	+ (h)	++ (r, m)	+ (h)
	++ (r, m)		++ (r, m)
SNAI1/SNAIL	+ (h, r, m)	nd (h)	+ (h, r, m)
		+ (r, m)	
SNAI2/SLUG	++ (h)	++ (h)	++ (h)
	+ (m)	+ (m)	+ (m)

Expression levels were extracted from microarrays databanks (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc = GSE23812 and http://mrg.genetics.washington.edu). +, weakly expressed (0-100); ++, moderately expressed (100-250); +++, highly expressed (250 and more). h, human; m, mouse; r, rat.

Suzuki and Nagano[28] suggested that the formation and maintenance of tight junctions relies on testicular factors, since the orchidectomy of adult mice results in a reduced number of tight junctional strands in the caput epididymidis. In contrast, Turner et al[27] reported that, in the rat cauda epididymidis, the blood-epididymis barrier was not androgen-dependent. It was also demonstrated that Cldn1 was regulated by androgens and thyroid hormones only in the initial segment of the rat epididymis.[62,93] In addition, several other *Cldn* genes (*Cldn5*, *Cldn8*, *Cldn11*, *Cldn14*) were down-regulated in the proximal rat epididymis following efferent duct ligation, suggesting that these genes are regulated by testicular factors.[94]

Dufresne and Cyr,[95] using a cell line derived from the rat caput epididymidis, demonstrated that two transcription factors, SP1 and SP3, are implicated in the regulation of Cldn1. SP1 and SP3 have been shown to regulate CLDN3 and CLDN4 in ovarian cancer cells,[96,97] CLDN19 in kidney cell lines[98] and occludin in human brain endothelium.[99] A third tumor suppressor that may be involved in the regulation of epididymal tight junctions is p63, which is expressed by basal cells of the rat epididymis and which has been shown to regulate Cldn1 expression in primary mouse keratinocytes by interacting with the SP binding domain of the Cldn1 promoter.[100,101] Furthermore the transcription factor Snail, which is expressed in the human epididymis (Table 2), is known to down-regulate the expression of junctional proteins (E-cadherin, CLDNs and occludin), resulting in an increase in tight junction paracellular permeability and disruption of the barrier function.[102-104]

These studies demonstrate that the regulation of the blood-epididymis barrier is complex, multifactorial, segment- and age-specific.

INTERACTIONS OF TIGHT JUNCTIONS WITH ADHERENS JUNCTIONS

Formation of adherens junctions facilitates the assembly of tight junctions in several tissues, as reflected by multiple interactions between proteins of adherens and tight junctions. Adherens junctions consist of two adhesive multiprotein complexes: the nectin-afadin complex and the cadherin-catenin complex (Fig. 2). Cell-specific expression of cadherins and nectins determines the adhesive characteristics of adherens junctions.[105]

The Nectin-Afadin Complex

Nectins are a familly of IgG- like adhesion receptors consisting of four members, nectin-1 to -4, with multiple splice variants. Nectins bind to afadin, an actin-binding protein also known as AF-6, providing a direct link to the cytoskeleton.[106] Afadin is thought to be involved in signalling pathways.[107] In epithelial cells, nectins and afadin are localized to adherens junctions.[108] Several in vitro studies demonstrated that nectins are implicated in cadherin-dependent cellular adhesion and in the assembly of tight junctions by recruiting cadherins to the nectin-based cell–cell adhesion sites through afadin and catenins[109] and then JAMs, CLDNs and occludin to adherens junctions to form tight junctions.[110,111] Indeed, inhibition of nectins and afadin interferes with the formation of adherens and tight junctions.[112-114] Although nectins and afadin have been poorly studied in the epididymis, gene expression studies have revealed that several nectins and afadin are expressed in the rat, mouse and human epididymis (Table 1).

The Cadherin-Catenin Complex

Cadherins are part of a large protein family comprised of classical cadherins (Type I), protocadherins, desmosomal cadherins, atypical cadherins (Type II) and cadherin-related proteins. The expression of cadherins is ubiquitous and these proteins are involved in a variety of functions, including initiation and stabilization of cell–cell adhesion, regulation of the actin cytoskeleton, intracellular signaling and transcriptional regulation.[115]

In the human, rat and mouse epididymis, several classical and protocadherins are expressed.[66,67,93] In contrast to classical cadherins, protocadherins have weak adhesive properties, but they may regulate the adhesion properties of classical cadherins.[116] Studies on the epididymis have focused on the classical cadherin CDH1 (E-cadherin). In the rat and human epididymis, CDH1 is expressed by principal cells.[117,118] CDH3 (P-cadherin) is also expressed in the rat epididymis.[119] In the human epididymis, other cadherins that are expressed include *CDH3, CDH16, CDH22* and *CDH24*.[22] Moreover a study on the effect of efferent duct ligation on gene expression has revealed that several genes encoding cadherins (*CDH1, CDH15*) and protocadherins (*PCDHGA1, PCDHGA2, PCDHGA10, PCDHGA12, PCDHGB5, PCDHGB7, PCDHGA8, PCDHGA7, PCDHGA11, PCDHGA9, PCDHGC3*) are regulated by testicular factors in the rat proximal epididymis.[94] CDH1 was also shown to be regulated during rat epididymal development at both the mRNA and the protein levels.[117,119]

The cytoplasmic domain of classical cadherins is highly conserved and binds to the peripheral proteins β-catenin and p120catenin that provide a link to the cytoskeleton. Indeed β-catenin interacts with α-catenin, resulting in the association of CDH1 with the actin cytoskeleton.[120] It has become clear that β-catenin not only associates with the cadherin tail, but also translocates to the nucleus and participates in the Wnt signaling pathway.[121] Moreover, knockdown of CDH1 causes a delay in the correct localization of α-, β-catenin and TJP1.[122] However, p120-catenin is stable in the cytosol when unbound by the cadherin tail. In fact, association of p120-catenin with CDH1 has been proposed to stabilize CDH1 at the plasma membrane during the formation of cell–cell contacts and to prevent its degradation.[123] In addition, TJP1 can bind α-catenin and often colocalize with CDH1 in early cell-cell contacts and then shift apically as tight junctions form.[124] The TJP1-binding region of α-catenin appears to be important for strong cadherin adhesion. DeBellefeuille et al[79] demonstrated that several catenins were expressed in the rat epididymis. β-, α- and p120-catenin were found along the lateral plasma membranes between adjacent epithelial cells, with highest expression in the corpus and cauda epididymidis for α-catenin and β-catenin. An immunoprecipitation study confirmed that CDH1, p120- and α-catenin were associated with β-catenin in the adult rat epididymis. In addition, the authors demonstrated that α- and β-catenins and not p120-catenin, were regulated by androgens in the adult rat epididymis as well as during postnatal development. In the human epididymis, several catenins are expressed, including members of the α-catenin family (*CTNNA1, CTNNA2, CTNNA3*), the β-catenin family (*CTNNB1*) and the p120catenin family (*CTNND1, CTNND2*), as well as *CTNNAL1,* also known as alpha-catulin.[22,93] CTNNAL1 has been shown to be involved in Rho and NF-kappaB signalling pathways, suggesting a role for these signaling events in the regulation of human epididymal junctions.[125,126] In the rat epididymis, *Ctnnal1* is regulated by testicular factors in the proximal epididymis.[94]

Numerous studies have demonstrated that cadherins can induce signal transduction in the cytoplasm and nucleus through interactions with a variety of intracellular binding partners.[127] Not only do adherens junctions contribute to the assembly of other specialized cell junctions, but increasing evidence suggests that tight junction components influence cadherin-based adherens junctions. Knockdown of TJP2 results in delayed formation of adherens and tight junctions, which would suggest a role for TJP2 in the dynamic process of adherens junction maturation.[128] Exogenous expression of the N-terminal of TJP3 can also delay the localization of CDH1, β-catenin and TJP1.[129] Lioni et al.[130] also demonstrated a role for CLDN7 in the regulation of CDH1.

Further studies are needed to understand the specific role of each junctional protein on segment-specific human epididymal function.

THE BLOOD-EPIDIDYMIS AND HUMAN PATHOLOGIES

Human Male Infertility

Human male infertility is a major health problem affecting 25% of infertile couples.[131] Male infertility can result from multiple causes such as endocrinological or genetic disorders, testicular dysfunction, infections and immunological problems. However, a number of infertile men (18%) are diagnosed with idiopathic infertility, in which no clear cause can be found. Several lines of evidence, such as the presence of sperm-reactive antibodies in infertile men, suggest a direct role of epididymal dysfunction in male infertility. It is well-established that spermatozoa are antigenic and anti-sperm antibodies are detected in at least 9% of infertile couples.[132] In addition, some of the antibodies are directed against proteins secreted by the epididymal epithelium.[133] Furthermore, infiltration of immune cells in the epididymal epithelium and higher levels of anti-sperm antibodies can also be detected following vasectomy.[134] It has recently been shown that the expression of several genes encoding tight junctional proteins, such as CLDN8 and CLDN10, are altered in the epididymis of vasectomized patients.[135] A disruption of tight junctions could result in the exposure of sperm antigens to the immune system and in the production of anti-sperm antibodies. In ageing rats, in which a higher number of halo cells has been observed, the integrity of the blood-epididymis barrier is compromised and segment-specific changes in the localization and expression of occludin, Tjp1 and Cdh1 have been reported.[45] In addition, Hermo et al[136] showed, in cathepsin-A deficient mice, that reduced expression and mis-localization of Cldn1 and Cldn3 were associated with altered sperm motility. In the human epididymis of infertile patients suffering from non-obstructive azoospermia, the mis-localization of CLDN1, CLDN10 and TJP1 suggest that the blood-epididymis barrier is compromised.[137] In these patients, several membrane channels and transporters, which have been suggested as potential regulators of tight junctions,[138] were also down-regulated.[137] It suggests the possibility that the luminal environment may be altered in azoospermic patients. Alterations to the blood-epididymis barrier have also recently been reported in obstructive azoospermic patients but seem to be dependent on the localization of the obstruction in the epididymis.[139] While the condition may be rectified by epididymostomy in which the obstruction is removed surgically and that epididymal tubule reattached, some patients have still low levels of fertility.[140] Alterations include the altered expression of *CLDN1, 4* and *10*. Furthermore, some of the epididymal principal cells begin to express vimentin

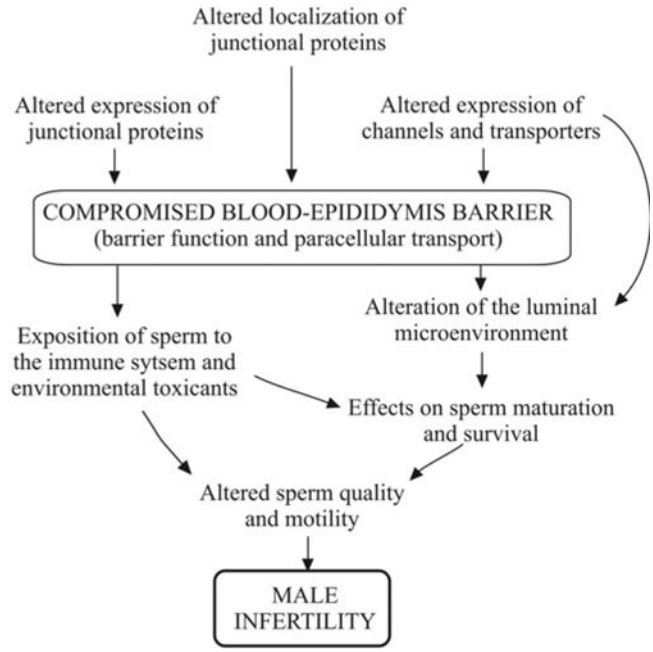

Figure 3. Implication of the blood-epididymis barrier in male infertility.

suggesting that they may undergo epithelial-mesenchymal transformation.[139] Male infertility due to epididymal dysfunction could thus result from multiple causes: the disruption of the blood-epididymis barrier, of the paracellular pathway and of the transcellular pathway (Fig. 3).

Other Pathologies

While there is increased information on the role of the epididymis in human fertility, there is limited information on other human pathologies in relation to the blood-epididymis barrier.

Von Hippel Lindau (VHL) disease, due to a mutation in the VHL gene, has been associated with the development of renal tumors as well as cystadenomas in the proximal region of the epididymis.[141,142] Interestingly, in the kidney, the loss of function of VHL gene results in the disassembly of intercellular junctions and an epithelial-mesenchymal transformation following the activation of the hypoxia-inducible factor (HIF). Indeed, HIF downregulates several junctional components including occludin and CLDN1.[143] Whether or not this is the case in the epididymis remains to be shown but, given the importance of CLDN1 in maintaining epididymal tight junctions,[144] it is likely that alterations in the expression of CLDN1 is to have an effect on barrier function. Interestingly, HIF1 subunits are expressed by principal cells in the adult rat epididymis[145] and in human VHL associated epididymal tumours.[146]

Infections of the male reproductive tract account for approximately 15% of male infertility cases. Spermatozoa can be affected by such infections during epididymal

transit.[147] Epididymitis is an inflammation of the epididymis that mostly result from a bacterial infection.[148] Several studies have shown, notably in the testis, that the production of cytokines can result in altered expression of tight junctional components.[149,150] Even if it is currently unknown, it is fairly likely that the blood-epididymis barrier is altered during epididymitis especially since pathogens, such as viruses and bacteria, can target and alter cellular junctions.[151,152]

A better understanding of the role of each CLDN in the blood-epididymis barrier and their implication in human male fertility could be important in the development of novel therapeutic strategies for infertility.

BIOLOGICAL TOOLS TO STUDY THE BLOOD-EPIDIDYMIS BARRIER

In the past years, several epididymal cell lines have been developed in rodents.[153-157] However while there are many similarities between the epididymis of laboratory animal models and that of the human, there are also many differences. The difficulty in obtaining human epididymal tissue has created a need to develop new tools to circumvent this problem and better understand the regulation of the blood-epididymis barrier. Recently human epididymal cell lines were developed from patients with proven fertility.[144] The cells retained many epididymal characteristics functions including the expression of multiple CLDNs, CDHs and TJPs proteins. The knockdown of CLDN1, 3, 4 and 7 in one of the cell lines resulted in the inability of the cells to form tight junctions.[144] These observations indicate that while epididymal tight junction are made up of a large number of CLDNs, the loss of a single CLDN may be sufficient to compromise the barrier function of epididymal tight junctions. The development of cell lines from the caput epididymidis of an obstructive azoospermic patient confirmed that the blood-epididymis barrier was altered in this type of infertility.[139] These cells express both cytokeratin and vimentin, but few markers of epididymal function and few cell adhesion or tight junction proteins. In fact, the cells are incapable of forming functional tight junctions.

CONCLUSION

The blood-epididymis barrier is critical for maintaining spermatozoa in a specific luminal environment essential for proper maturation, protection and survival. It is clear that in both non-obstructive and obstructive azoospermia, the proteins that are responsible for the maintenance of this barrier are altered. One of the unique features of epididymal tight junctions is that it is composed of a very large number of CLDNS. Yet data using human epididymal cell lines indicate that the loss of a single CLDN may be sufficient to compromise the barrier function of these junctions. Therefore, the mistargeting of certain CLDNs observed in the epididymis of azoospermic patients is likely to result in loss of barrier function. This not only implicates the epididymis as a component contributing to human infertility but also reinforces the notion that the function of the epididymis needs to be taken into consideration in the surgical treatment of azoospermia. Likewise, obstructive azoospermia appears to involve the epithelial-mesenchymal transformation of epididymal epithelial cells. Whether or not this is a reversible effect is currently unknown but these changes have been shown to be associated with loss of cellular junctions, thus indicating

that the function of the blood-epididymis barrier is compromised in these patients. While studies to date have focused almost exclusively on the barrier function of epididymal tight junctions, we know almost nothing of the paracellular transport across epididymal junctions and the role of CLDNs in this process. Certainly the regional expression of CLDNs along the human epididymis is suggestive of specific regionalized ion transport. The fact that the regional localization of certain CLDNs is maintained in rats and humans suggest a very specific and, possible an evolutionary maintained function. The recent development of human cell lines and increased availability of transgenic mice lacking specific CLDNs should allow us to address these issues in the future. Finally, while there now exist mounting evidence of a de-regulation of epididymal Cldns in human infertility, we need a much better understanding of the regulation of these proteins as well as other junctional proteins. The interaction between cellular junctions and the regulation of the proteins implicated in the formation of these junctions needs to be addressed in order to develop strategies to reinitiate epididymal junction formation in infertile men undergoing epididymostomy for azoospermia.

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CHAPTER 12

BLOOD-TISSUE BARRIERS

Morphofunctional and Immunological Aspects of the Blood-Testis and Blood-Epididymal Barriers

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Abstract: The blood-testis barrier (BTB) is known for its ability to create an immune privilege site in the seminiferous epithelium, but less is known of the blood-epididymal barrier (BEB). It is already established that the fully functional BTB and BEB are much more complex and consist of anatomical/physical (tight junctions, basolateral and apical membranes), physiological and immunological components, which are all necessary to make a functioning barrier in the testis and epididymis. However, comparative data for metazoans suggest that an effective Sertoli cell barrier is not entirely necessary for the development of germ cells during spermatogenesis or that our knowledge about the barrier structure/function in metazoans is still immature. This chapter compares the unique barrier formed by the Sertoli cells of the testis to that formed by the apical junctional complexes of the epididymal epithelium.

INTRODUCTION

Immune privileged sites, typically associated with blood-tissue barriers, are places in the body where auto-immunogenic or foreign antigens are tolerated without inducing detrimental immune responses. Examples of such barriers are found in the blood-brain barrier, blood-ocular barrier, gastrointestinal barrier, blood-testis and blood-epididymal barrier.[1,2] It is believed the testis is immune privileged in order to protect the developing

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germ cells since they possess a profile of novel surface antigens that if detected by the immune system would result in their destruction.[3,4] The blood-testis barrier (BTB) participates in creating an immune privilege environment in the seminiferous epithelium, but less is known of the blood-epididymal barrier (BEB). Originally, these barriers were only thought of in terms of the epithelial tight junctional complex. In nontesticular polarized epithelia, such as the epididymis, tight junctions are formed as circumferential bands of the plasmalemma near the apical surface (Fig. 1). However, in the seminiferous epithelium, tight junctions are formed between Sertoli cells as circumferential bands near the basement membrane (Fig. 1).[5,6] It was thought that the sole permeability properties of the BTB and BEB were due to the junctional complexes between Sertoli cells and between epididymal epithelial cells. In part, this is true because they do contribute to permeability properties of the testicular and epididymal epithelia, but these complexes are only a fraction of the permeability properties barrier of these two tissues.

Perhaps the earliest mention of a physiological barrier in the male reproductive tract was the study by Setchell et al (1969),[7] although it had been known before this study that blood-borne molecules were not able to penetrate the seminiferous epithelium and enter the lumen.[8] A year later, the paper by Dym and Fawcett (1970)[9] provided clear evidence for the lack of permeability of the junctional complexes between Sertoli cells, although the tight junctional complexes had been observed before this study.[10] Therefore, the

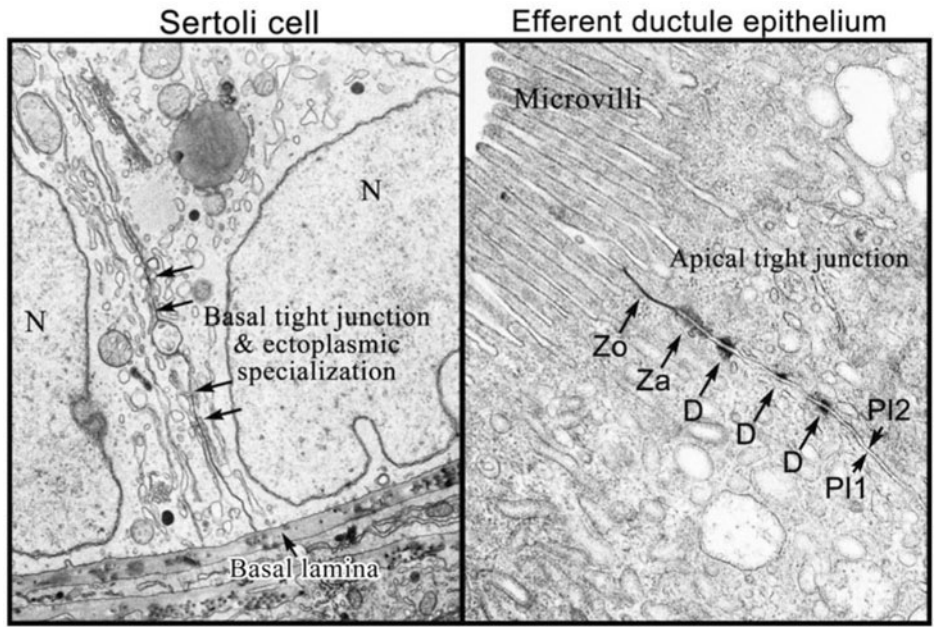

Figure 1. Differences in the spatial arrangement of the tight junction in the seminiferous epithelium and the efferent ductule epithelium. The blood-testis barrier is formed between two Sertoli cells (arrows), in association with the ectoplasmic specialization located near the basal lamina. N, Sertoli cell nucleus. In the efferent ductule epithelium (as well as in the other segments of the excurrent duct) tight junctions are restricted to the apical regions of adjacent epithelial plasmalemmas (Pl1 and Pl2). This apical tight junctional complex consists of zonula occludens, Zo; zonula adherens, Za; and desmosomes, D. Microvilli are seen protruding into the lumen.

link between the anatomical barrier (tight junctions) and the permeability/physiological barrier in the testis had been established. This link continues with the BEB,[11] that the anatomical and the physiological barriers have been considered to be synonymous with the tight junctional complexes. The junctional complexes do indeed restrict the movement of molecules from the blood/interstitium to the lumen of the seminiferous tubule and epididymal duct. However, this is not the only direction in which the movement of molecules is restricted. Junctional complexes also restrict movement from the lumen to the interstitium/blood, and presumably this allows for the maintenance of a specialized luminal fluid milieu that is hypothesized to play an important role in spermatogenesis in the testis and sperm maturation in the epididymis.

The immunological component of the BTB participates in the overall immune privilege in the testis as it forms a barrier that limits access of the immune system to the adluminal compartment of the seminiferous epithelium and sequesters the majority of the auto-antigenic germ cells. However, even though the terms BTB and immune privilege are often used synonomously, immune privilege in the testis is more complex and involves both the BTB and local production of immunomodulatory factors that combined create an environment throughout the whole testis that suppresses harmful immune responses to the auto-antigenic germ cells.

The fully functional BTB and BEB consist of anatomical/physical (tight junctions, basolateral and apical membranes), physiological and immunological components which are all necessary to make up functioning barrier in the testis and epididymis. The key aspect is how these three barriers interact.[12] In this context, in the present chapter we aimed to provide comprehensive and updated information related to the morphological, physiological and immunological aspects involved in the blood-testis and blood-epidydimal barriers.

THE MORPHOLOGICAL ASPECTS OF THE BLOOD-TESTIS BARRIER

History of the Sertoli Cell Barrier Discovery

The first descriptions of a BTB are dated from the beginning of the last century in pioneer experiments showing that injected dyes were not observed inside the seminiferous tubules.[13,14] However, only several decades later this barrier was for the first time called the 'blood-testis barrier', which involved the permeability barrier formed by Sertoli cells within the seminiferous epithelium, isolating the majority of germinal elements from many constituents of the circulatory and lymphatic systems.[15] Using electron microscopy, the BTB was confirmed by several investigators showing that its morphological basis was an extensive tight junctional network (zonula occludens) between adjacent Sertoli cells.[7,9,16-18] Additionally, injecting various electron-opaque particulates in the guinea pig and chinchilla testis interstitium, Fawcett and colleagues (1970)[19] observed that larger particles were excluded from the seminiferous tubules apparently by the surrounding layer of contractile cells (peritubular myoid cells), that were considered the primary barrier to penetration of the seminiferous tubules. When it breached, specialized Sertoli-to-Sertoli junctions within the epithelium constituted a secondary barrier to passage of materials into the testicular fluid. Therefore, there is no barrier between the blood and the seminiferous tubule. However, unfortunately the term 'blood-testis' barrier is a catchy one and has been used frequently over a long period. In fact, this term often leads to confusion since inappropriate parallels are frequently made between it and the blood-brain barrier.

In this context, the term 'male germ cell barrier' or 'male germ cell protective barrier' was proposed by Abraham (1991).[20]

The Sertoli cell is solely responsible for the formation of the BTB, therefore, the phrase 'Sertoli cell barrier' was properly coined by Russell and Peterson (1985).[21] In fact, Sertoli cells create two permanent (basal and adluminal) and one transient (intermediate) compartment within the seminiferous epithelium. Basal compartment cells include spermatogonia and young spermatocytes up to early leptotene phase of meiosis, and only these cells have direct access to the blood-borne substances.[9,10,22] Numerous tight or occluding junctions between Sertoli cells at their baso-lateral surfaces demarcate the basal from the adluminal compartment and these Sertoli cells provide a means to permit the movement of germ cells (leptotene) from the basal to the adluminal compartment (through the intermediate compartment) without destroying the barrier.[23] Subsequently, the BTB barrier has come to include not only the tight junction, but also the basal ectoplasmic specialization, which is an atypical adherens junction in the testis.[5]

Certain susbstances, including toxins and toxicants, must either breach the barrier or pass through the Sertoli cell to affect adluminal germ cells (more advanced spermatocytes and spermatids) directly. Nevertheless, as will be discussed in another section of this chapter, beyond compartmentalizing the seminiferous epithelium, the Sertoli cell barrier participates in forming a functional immunologic barrier. In a broader context, it could be considered that the BTB has three functional components:[24] (i) a physicochemical barrier consisting of continuous capillaries, Sertoli cells in the seminiferous epithelium connected together with narrow tight junctions and a layer of peritubular myoid cells around the seminiferous tubule; (ii) an efflux-pump barrier that contains P-glycoprotein in the luminal capillary endothelium and on the peritubular myoid cells, and multidrug-resistance associated protein 1 located basolaterally on Sertoli cells; and (iii) an immunological barrier consisting of the Sertoli cell barrier and immunomodulatory factors.

Development and Establishment of the Sertoli Cell Barrier

Sertoli cells play a key role in testis differentiation during fetal life[25] and their proliferation period in the mammal ends just before puberty, being regulated mainly by FSH (proliferation) and thyroid hormones (differentiation).[26,27] Although chronologically variable in mammals (e.g., 2-3 weeks in mice and rats to 11-14 years in humans), the period of Sertoli cell maturation/differentiation is coincident with the appearance and extensive proliferation of primary spermatocytes, tubular fluid secretion and flow, and lumen formation (Figs. 2-4).[28-30] In mammals, all these important functional events also coincide with the formation and development of the Sertoli cell barrier.[28-30] Thus, there is a clear correlation between Sertoli cell maturation/differentiation and the barrier establishment (Figs. 2-4). In fact, the physiological barrier is not intact in the *W/Wv* testis, showing a lack of Claudin 5 in areas devoid of germ cells, but the barrier is established and Claudin 5 is expressed following germ cell transplant.[31,32] Therefore, the germ cell component seems to be important for the Sertoli cell barrier effectiveness and, at least in mammals, the meiotic phase of spermatogenesis depends upon the establishment of a competent BTB.[7,9,33]

Differentiated Sertoli cells are protected by the anti-apoptotic protein *Bclw* and apoptosis is rarely observed in Sertoli cells of sexually-mature males.[34,35] Although it is considered that mammalian Sertoli cells are terminally differentiated and nondividing postpuberty,[36] this dogma is being challenged for several species, including rats, mice, hamsters, horses and humans, investigated under experimental and physiological (seasonal)

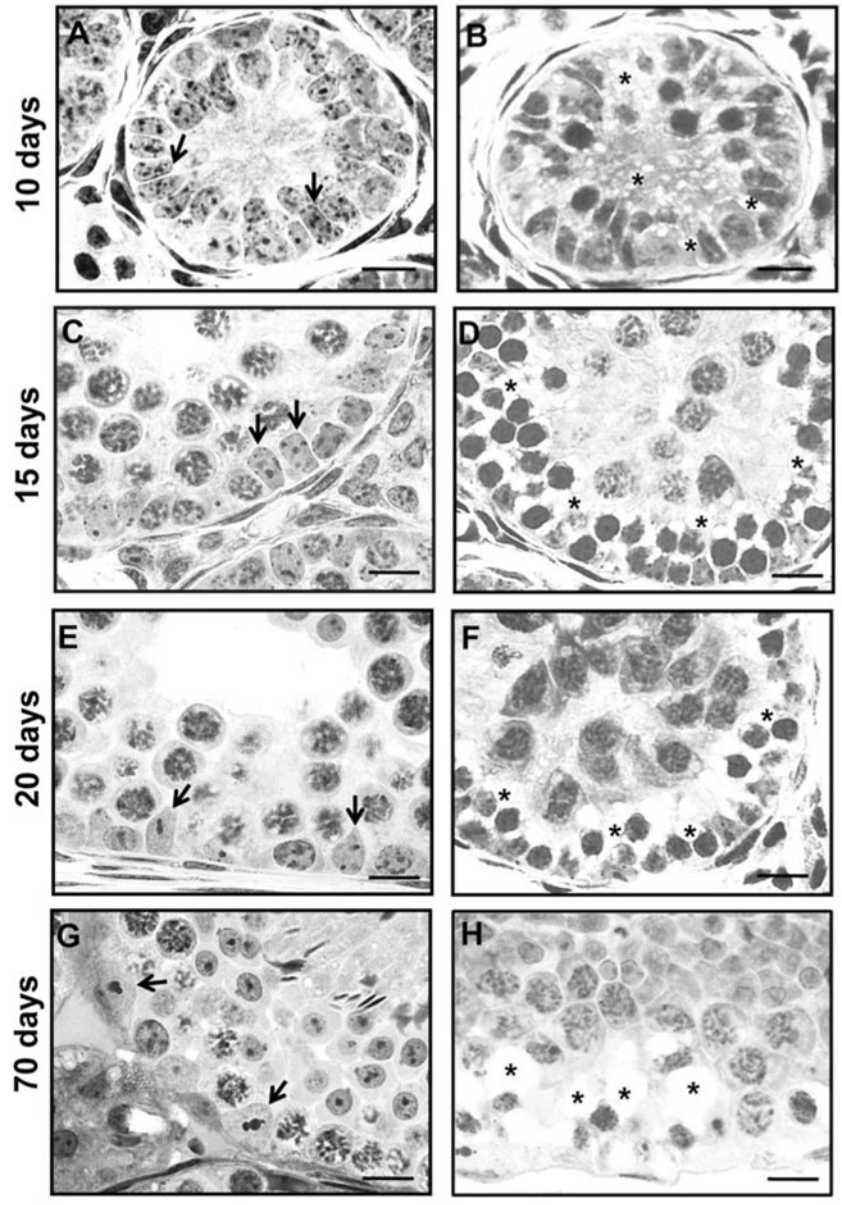

Figure 2. Development of the blood testis barrier (Sertoli cell barrier) in seminiferous cord/tubules cross-sections in control mice (A,C,E,G) and mice perfused at different ages with hypertonic fixative (B,D,F,H). As it can be observed, whereas there is no restriction to the entry of the hypertonic fixative into the center of the seminiferous cords until 10 days of age (asterisks in B), from day 15 to 70 days of age the damage (asterisks) caused by the fixative is restricted to the basal compartment due to the formation of the Sertoli cell barrier (D and F) that is fully functional in adult mice (H). Therefore, at these ages, the adluminal compartment is protected by this barrier. In another aspect, the arrows in the panels at the left side for the control mice indicate the status of Sertoli cells nuclei maturation, where typically immature (A), differentiating (C,E), and fully mature (G) Sertoli cells nuclei are shown. Scale bars: 15 μm (A,B) and 10 μm (C-H).

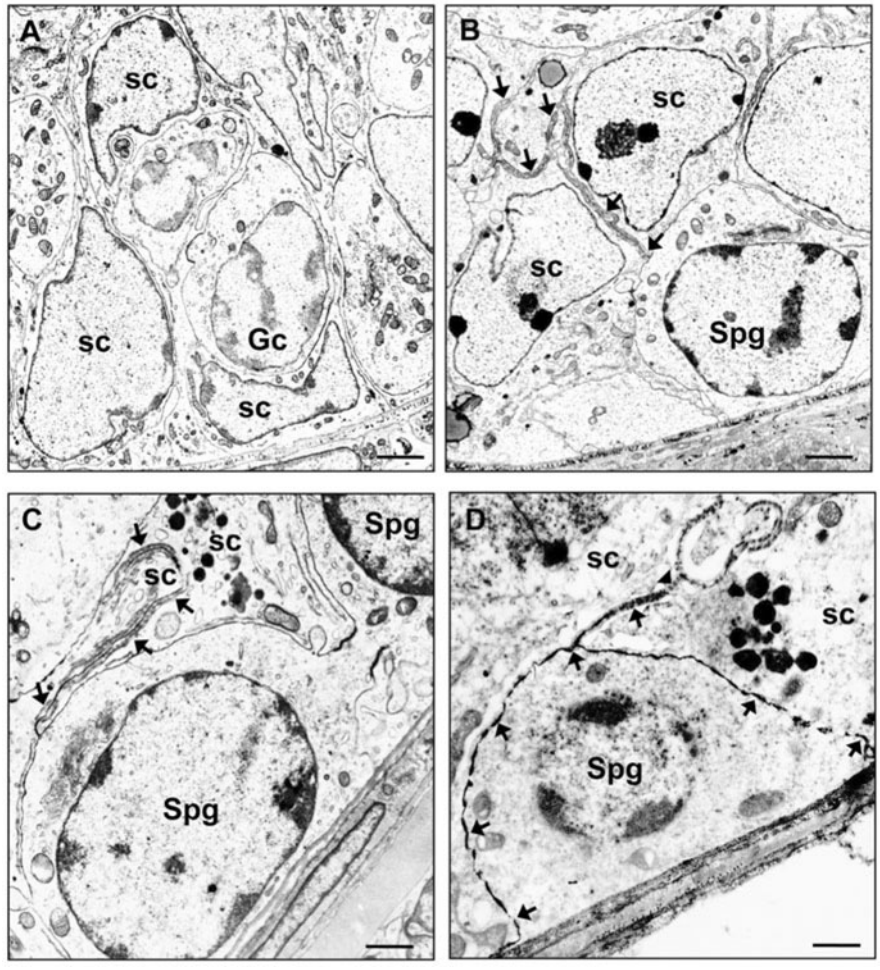

Figure 3. Establishment of the blood testis barrier (Sertoli cell barrier) in rats shown by transmission electron microscopy. Note that, whereas no tight junctions are observed between adjacent immature Sertoli cells (A), this junction (arrows) is being formed between differentiating Sertoli cells (B) or it is fully functional in adult animals (C,D). Therefore, as illustrated in Figure 2D, the lanthanum tracer (arrows) is present only between the plasma membranes of a contiguous Sertoli cell and a spermatogonia located in the basal compartment, and is prevented from reaching the adluminal compartment due to the tight junction (arrowhead) formed between adjacent Sertoli cells. SC = Sertoli cell; Gc = germ cell; Spg = spermatogonia. Scale bars: 2.6 μm (A,B); 1.7 μm (C,D).

conditions.[37-41] This important functional aspect related to Sertoli cells proliferative activity in adult animals could reflect on the Sertoli cell barrier dynamics and may explain the results showing that during the nonbreeding period the testis barrier is not effective[42] in mammals. Alteration in the barrier integrity could also be observed in sexually mature animals, in which Sertoli cells are not fully mature/differentiated due to pathological conditions such as for instance testicular dysgenesis.[43,44] Also in this context, Sertoli cell maturation and barrier formation are disrupted in the SCARKO mice, in which there has

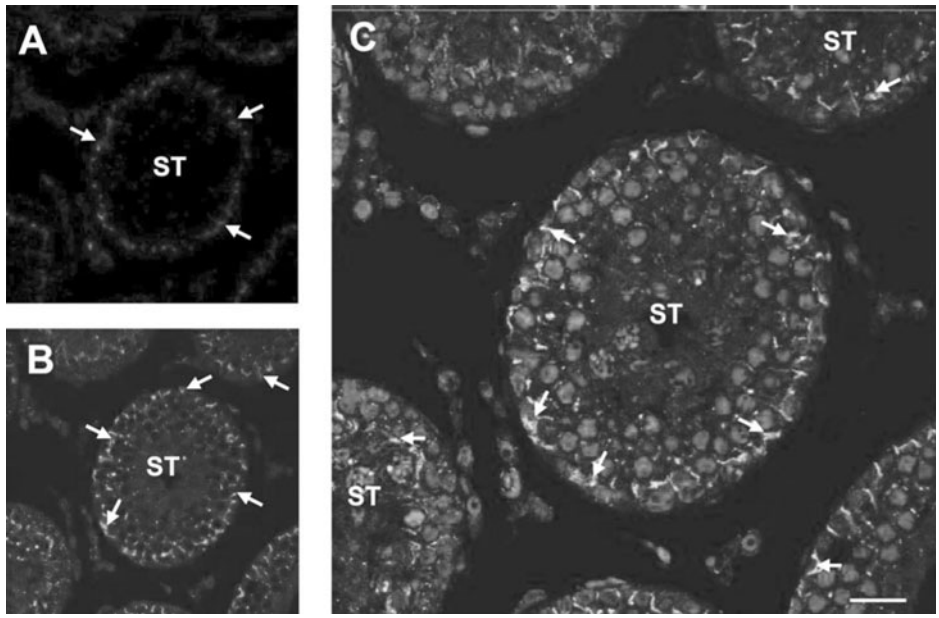

Figure 4. Confocal images from a 25 days old rat showing that all differentiating Sertoli cells were immunopositive (arrows) for occludin (red in A) and for ZO-1 (green in B), resulting in a yellow color when merged (C). Therefore, as shown in the previous figure the blood testis barrier (Sertoli cell-barrier) is already formed at this phase of development. Scale bar: 20 μm. A color version of this figure is available at www.landesbioscience.com/curie.

been a selective ablation of the androgen receptor in Sertoli cells.[45] These results strongly suggest that functional androgen receptors are important in Sertoli cells for the creation of the nurturing environment for germ cell development. Perhaps not by coincidence, androgen levels are very low during the nonbreeding period in seasonal species in which it is claimed that the Sertoli cell barrier is not effective.[46]

Recent data suggest that the cyclic Sertoli cell barrier dynamics, particularly the translocation of leptotene from the basal to the adluminal compartment (through the intermediate compartment), is disrupted in ether-lipid-deficient mice.[47] In some seasonal teleost fish species, the seminiferous epithelium shifts from a Sertoli cell monolayer containing some spermatogonia (nonreproductive season), to an active spermatogenic arrangement composed of spermatocysts and spermatozoa that fill the tubular lumen (reproductive season).[48-50] These structural changes have direct effects on the types and distribution of cell junctions on the seminiferous epithelium such as adhesive junctions and gap junctions,[51] and connexins,[52] and probably would affect the Sertoli cell barrier effectiveness.

Comparative Aspects of BTB in Invertebrates and Vertebrates

There are few studies related to the BTB along phylogenesis. The Table 1 summarizes, using morphological evidences, that the nature/combination of the constituents of the junction complexes differs according to the species and the stage of development at which the developing germ cells reach the "tight compartment".

Table 1. General characteristics of the Sertoli cell barrier (Blood-testis barrier) along phylogenesis in different metazoans

Metazoans	Characteristics	Reference
Phylum Porifera	The male germ cell makes contact with a choanocyte (carrier cell) which engulfs it and holds it within a membrane-bound cavity—the spermiocyst. The cell layer develops later, during maturation divisions.	20
Phylum Cnidaria	Presence of septate junction that is a long and tortuous pathway of intercellular space between the epidermal cells in the testis region.	91
Phylum Platyhelminthes	Parietal cells act as a barrier especially by the amount of septa and the possible cation-binding properties of the interseptal material.	92
Phylum Nematoda	The "tight compartment" is much reduced, and the open one is very long and this feature might be related to the long-lasting diploid growth phase.	93
Phylum Annelida	The walls of the testis are formed by a layer of parietal cells and a thick basement membrane. No specialized intercellular occluding junctions are found between adjacent parietal cells.	94
Phylum Crustacea	The presence of thick basal lamina and branching Sertoli-like cells, which may hinder free diffusion of molecules from the hemolymph to the germ cells, provide some form of barrier.	95
Class Insecta	The barrier is formed by the inter Sertoli septate junctions but small tight junctions are observed and they might be the real Sertoli cell (blood-testis barrier) barrier.	53,94,96
Phylum Echinodermata	No permeability barrier is observed between the spermatogenic cells and the hemal system or the somatic cells in general.	20
Phylum Chordata	In general, the morphological basis of Sertoli cell (blood-testis barrier) barrier in vertebrates consists of junctional complexes between the Sertoli cells, which constitute the wall of the testicular tubules, and between the peritubular myoid cells that surround them.	18,97
Class Pisces	Presence of tight junctions and desmosomes. Phase of spermatogenesis where the barrier is present: spermiogenesis (early spermatids).	20,57
Class Amphibia	Presence of tight junctions and desmosomes. Phase of spermatogenesis where the barrier is present: spermiogenesis (early spermatids).	98,99,100
Class Reptilia	Presence of tight junctions, subsurface cisternae, gap junctions, desmosome-like junction, septate-like junctions. Phase of spermatogenesis where the barrier is present: spermatocytary (zygotene).	53,101
Class Aves	Presence of tight junctions, septate-like junctions, subsurface cisternae, desmosomes. Phase of spermatogenesis where the barrier is present: spermatocytary (zygotene).	53,102,103
Class Mammalia	Presence of tight junctions and gap junctions. Phase of spermatogenesis where the barrier is present: spermatocytary (zygotene).	18,104,105

As it is shown in this table, more complex or elaborated structures are observed only in vertebrates, where inter-Sertoli tight junctions establish an effective barrier that appear either when meiosis is complete (teleosts and amphibians—both with cystic spermatogenesis) or immediately after the onset of meiosis (reptiles and bird—both having testes consisting of seminiferous tubules).[20,53] In the cystic arrangement of spermatogenesis, where Sertoli cells envelope a clone of germ cells that develop synchronously from initial spermatogonia to sperm,[54,55] tight junctions are regularly associated with desmosomes (Fig. 5). More specifically in fish, both cartilaginous and teleosts, tight junctions between Sertoli cells apparently form a functional barrier only when haploid germ cells are present (Fig. 5).[56,57] Therefore, different from mammals, fish meiotic germ cells are not shielded from the vascular system.[58] Interestingly, as

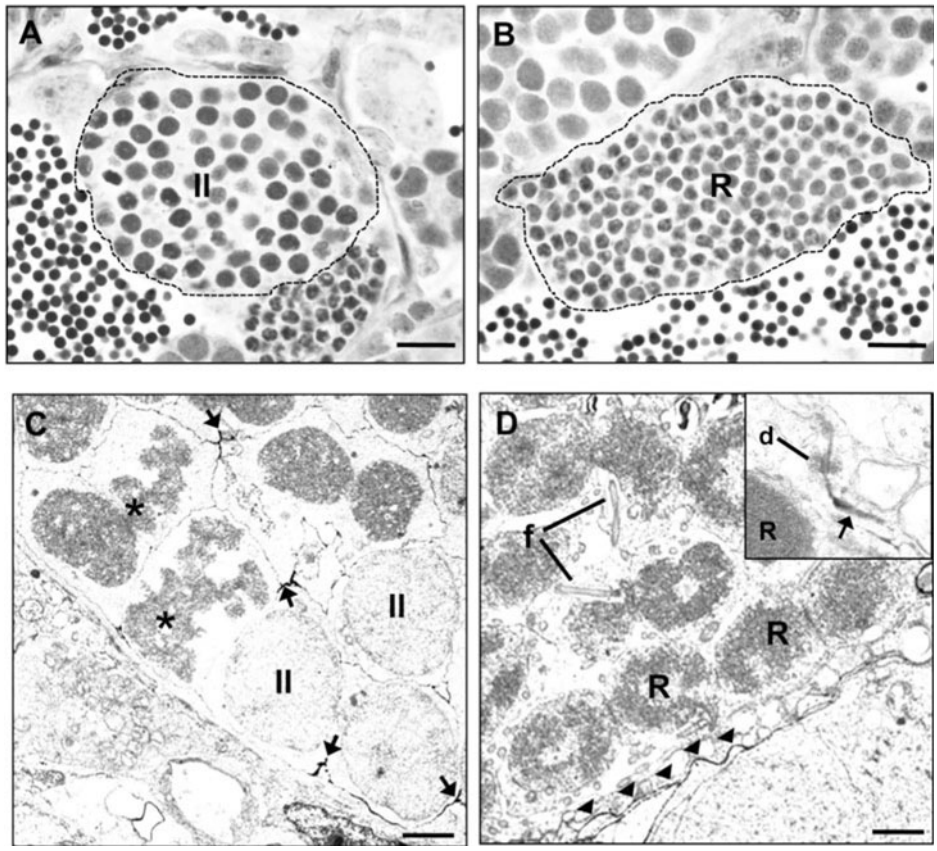

Figure 5. Typical germ cell cyst of secondary spermatocyte (II in A and C) and early spermatids (R in B and D) in zebrafish, investigated using light (A,B) and electron (C,D) microscopies. Observe in Figure 4C that the lanthanum tracer (arrows) is present inside the secondary spermatocyte cyst which also depicts meiotic divisions (asterisk). Therefore, the blood testis barrier (Sertoli cell barrier) in zebrafish is still not formed at the end of meiosis. However, no tracer is observed between early spermatids (R in D) and it is present (arrowheads) only in this germ cell cyst wall. Also depicted in Figure 4D are the spermatids flagellum (f). The insert in Figure 4D shows a junctional complex in fish where an occlusive/tight junction (arrow) and a desmosome are illustrated. Scale bars: 10 μm (A and B); 1.6 μm (C) and 1.3 μm (D). Parts of this figure were originally published in Leal et al., Biol Reprod 2009.[57]

Sertoli cells in fish seems to be not terminally differentiated they show mitotic activity even when they are associated with spermatids. However, they proliferate more in spermatogonial cysts, tending to stabilize their number per cyst during meiosis, just before the barrier is formed.[57,59] Based on the results summed up in Table 1, we could speculate that an effective Sertoli cell barrier is not entirely necessary for the development of germ cells during spermatogenesis or, most probably, that our knowledge about the barrier structure/function is still rather incipient. A possible evidence for this reasoning is the fact that even without a functional barrier, in the zebrafish a tracer (lanthanum) used to investigate the barrier effectiveness was never found in the seminiferous tubule lumen.[57]

THE PHYSIOLOGICAL ASPECTS OF THE BLOOD-TESTIS AND BLOOD-EPIDIDYMAL BARRIERS

The junctional complexes restrict the movement of molecules from the blood/interstitium to the lumen of the seminiferous tubule and epididymal duct. However, this is not the only direction in which the movement of molecules is restricted. Junctional complexes also restrict movement from the lumen to the interstitium/blood, and presumably this allows for the maintenance of a specialized luminal fluid milieu that is hypothesized to play an important role in spermatogenesis in the testis and sperm maturation in the epididymis. This bidirectional regulation is a key point that will be discussed in more detail below, and it is a major function of the physiological barrier for both the testis and epididymis.

In addition to the tight junctional complexes mentioned above, the other two places that physically impeded molecules from either entering or exiting the lumen and cells are the basolateral membrane and the apical membrane. Hence, from an anatomical point of view, the tight junctions, the basolateral and apical membranes would equal the anatomical BTB and BEB. However, the tight junctions in the testis would be more correctly termed as the Sertoli cell barrier as defined by Russell and Peterson (1985).[21] With each structure being highly restrictive in nature to blood-borne molecules, the testis and epididymis, like many other tissues must express specific transporting proteins on their basolateral and apical membranes that allow for the entry or exit of specific molecules. It is these transporters or carriers that define the remaining part of the physiological barrier. In addition, not only do the anatomical and physiological components of the barriers provide a specialized fluid milieu, but they also aid in the protection of the developing and maturing germ cells. Transporters can be used to prevent the entry of xenobiotics and harmful agents but they can also be used to eliminate and move such harmful agents out of the testis and epididymis. Interestingly, such transporters could be harnessed to move specific contraceptive agents into the testis and epididymis.

The experiments described by Setchell et al (1969)[7] demonstrated very clearly that the epithelium of the rete testis restricted the movement of higher molecular weight molecules such as inulin and albumin, whereas urea, water and bicarbonate entered readily. Slower rates of uptake were observed with ions, creatine and galactose. Much of this was later confirmed for the seminiferous tubule and epididymal duct.[60-64] Thus confirming the whole idea of a permeability barrier in the testis and epididymis. The transporters located on the basolateral membrane were found to be quite specific for

their substrates, for example, D-glucose, as measured by the uptake of radiolabeled 3-O-methyl-D-glucose was readily transported into the seminiferous tubule and epididymal duct[61,65] whereas L-glucose was restricted similar to that of inulin. Amino acids are also readily transported across the basolateral membrane.[66] Another example is the transport of L-carnitine across the basolateral membrane of very specific regions of the epididymis[67,68] and studies have revealed this transporter to be OCTN2 or Slc22a5.[69] Interestingly, there is also specific transport of L-carnitine out of the epididymal duct, across the apical membrane.[70] Another example of a transporter that has been identified on the apical membrane of the epididymis is CE11 that encodes a putative 12-transmembrane domain cotransporter that is homologous to a thymic stromal cotransporter.[71] Aquaporins have recently received considerable interest in their expression along the epididymal duct[72-75] but again, more detailed physiological studies are needed to define their precise transporting role in the epididymis. Many transporters have been identified in the epididymis using microarray analyses and the challenge now is to return to basic physiological studies to determine whether these are actively participating in the transport of substrates.

A key function of the physiological barrier is to maintain a specialized luminal fluid environment that is hypothesized to play an important role during spermatogenesis in testis and sperm maturation in the epididymis. The composition of testicular and epididymal luminal fluid has long been recognized to be distinctly different to that of blood plasma.[6,76-78] However, experiments designed to test the hypothesis that the luminal fluid composition plays an important role towards sperm maturation has been difficult to prove until recently. Some elegant studies by Breton and colleagues have shown the importance of transporters such as the Na/H exchanger, NHE3, the Na-HCO_3 cotransporter NBC, carbonic anhydrase II and the vacuolar H^+ATPase[79-81] that regulate the acidification of epididymal luminal fluid. Further studies have shown the importance of clear and narrow cells in this process as well as the novel finding that basal cells send projections to the lumen where they can act as sensors.[82] Ion transport into the epididymal lumen is important for male fertility as shown in mice that were null for the forkhead transcription factor Foxi1.[83] This factor plays an important role in the expression of vacuolar H^+ ATPase, carbonic anhydrase II and pendrin, a Cl^-/HCO_3 transporter.

Another example showing the importance of the epididymis providing an appropriate luminal fluid milieu for normal fertility is in the c-Ros (ROS1) null mouse.[84] In these animals the initial segment fails to develop resulting in the inability of spermatozoa to volume regulate, which in turn leads to deformed (kinked) spermatozoa when exposed to hypotonic environment in the female tract. The deformity is sufficient to cause failure of the spermatozoa to reach the egg resulting in infertility.[85,86] Studies have shown differences in the luminal fluid concentration of glutamate, inorganic phosphate and pH between c-Ros null and wildtype epididymides that maybe responsible for the observed phenotype.[87-89]

Another key difference between the anatomical and the physiological/permeability is that the latter is highly dynamic although it is well known that the tight junctions between Sertoli cells are undergoing remodeling[23,30] as spermatogenesis proceeds. In the testis the Sertoli cells are highly three-dimensional structures[36,90] that constantly change their characteristics during spermatogenesis and spermiogenesis. This would mean that during each phase of spermatogenesis, the needs, for example nutrient, of the Sertoli cell and the germ cells would change from one stage to the next. Therefore,

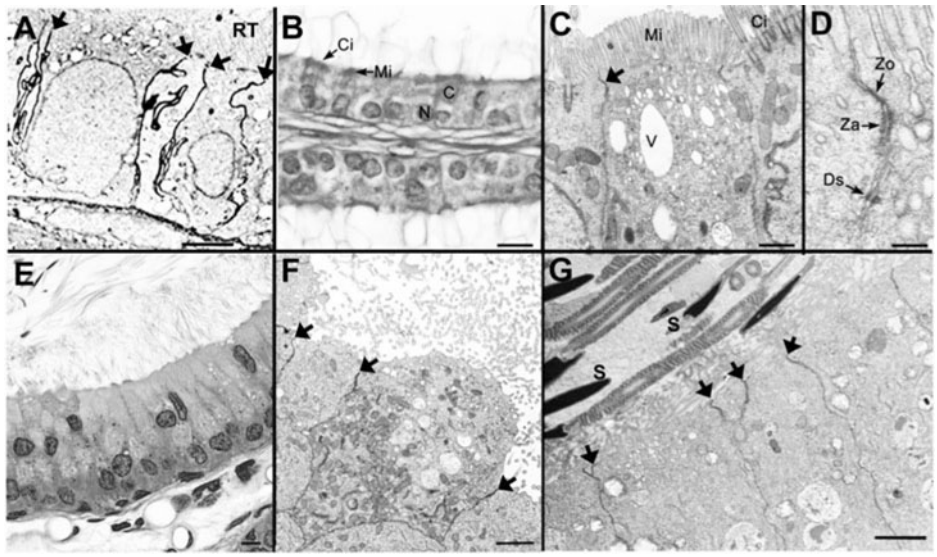

Figure 6. Excurrent duct in laboratory rodents where the rete testis (A), efferent ductules (B-D) and the epididymis (E-G) are illustrated, using electron (A,C,D,F,G) and light (B,E) microscopies. Note that, contrary to the seminiferous tubules, in all of these segments the junctional complex is located in the apical region. Therefore, as shown in Figure 5A the lanthanum tracer (arrows) is barred from entering into rete testis (RT) lumen at the apical side. The junctional complex (arrows) is also illustrated by transmission electron microscopy in the apical region of the efferent ductule (C) and the epididymis initial segment (F) and cauda (G). Figure 5D illustrates in more detail the junctional complex with the zonula occludens (Zo) and adherens (Za), and the desmosome (Ds). Ci = cilium; Mi = microvilli; N = nucleus; C = ciliated cell; V = vacuole; S = sperm cell. Scale bar: 3 μm (A); 10 μm (B); 1 μm (C); 0.25 μm (D); 15 μm (E); 2 μm (F); 1 μm (G). A color version of this figure is available at www.landesbioscience.com/curie.

it would be predicted that during each stage of spermatogenesis, similar and different molecules would be transported into and out of the lumen. This is quite a remarkable feat to regulate and co-ordinate the movement of many molecules across the epithelium so that the luminal fluid environment is kept optimal for each spermatogenic stage. Likewise in the epididymis; the feat here is that as spermatozoa progress along the epididymal duct they are exposed to a constantly changing luminal fluid milieu. Hence, the tight junctional complexes remain but that the permeability properties of the basolateral and apical membranes must change from one epididymal region to next (Fig. 6). In fact, it would change from one cell to the next. This would allow precise control of the luminal fluid composition along the duct. How this is co-ordinated is not known.

In summary, the physiological barrier in the testis and epididymis reflects the combined permeability properties of the tight junctional complexes, the basolateral membrane and the apical membrane. The permeability properties reflect the activity of transporters located on the basolateral and apical membranes. In both the testis and epididymis, the physiological barrier is highly dynamic, always changing to meet the needs of the developing and maturing spermatozoa as well as the Sertoli cells in the testis and the different epithelial cell types in the epididymis.

THE IMMUNOLOGICAL ASPECTS OF THE BLOOD-TESTIS AND BLOOD-EPIDIDYMAL BARRIERS

Immune Privilege in the Testis

The immunological component of the BTB acts as a physical barrier that prevents leukocytes and immunoglobulins from entering the seminiferous tubules of the testis.[106-110] Under normal conditions, leukocytes including macrophages, T cells, and dendritic cells are present in the interstitial space. However, they are excluded from the basal and adluminal compartments of the seminiferous tubules.[108-110] Likewise, antibodies are inhibited from crossing the BTB. The immunoglobulin concentration in fluid collected from rete testis tubules was approximately 0.2% the concentration in serum.[106] The amount of immunoglobulin in seminiferous tubule fluid was much lower, as it was not detectable in an assay that compared the levels of immunoglobulin in seminiferous tubule fluid with those in rete testis fluid.[111] Consistently, anti-sperm antibodies injected into the testis were detected bound to sperm in the rete testis but not in the seminiferous tubules.[112]

The BTB is also thought to sequester the auto-antigenic germ cells from the immune system. Advanced male germ cells (pachytene spermatocytes, spermatids and spermatozoa) located within the adluminal compartment of the seminiferous epithelium, behind the BTB (Sertoli cell barrier), express germ cell specific antigens.[3,4] These antigens if detected by the immune system can elicit an immune response, supporting the role of the BTB in immunologically sequestering the germ cells.[3,4] However, 7 to 10 days after immunization of mice with syngeneic testis homogenates, immunoglobulin was deposited on preleptotene spermatocytes within the basal compartment of the seminiferous epithelium, outside of the BTB and by 10 to 15 days after immunization lesions similar to experimental autoimmune orchitis developed in these mice.[113] Additionally, antigens expressed only by germ cells located in the adluminal compartment, were detected in immune deposits outside of the BTB in the interstitial compartment.[114] For example, LDHc4 (testis specific isoform of Lactate Dehydrogenase), which is expressed only by haploid germ cells in the testis[115] was detected in Sertoli cells and immune complexes within the interstitium.[114] It was proposed that this could allow for the induction of immunologic tolerance to these germ cell auto-antigens.[114] Thus, as stated by Tung "The idea of complete sequestration of testis autoantigens as the pre-eminent basis of immunologic unresponsiveness no longer holds".[116] Instead testicular immune privilege must be more complex.

Further evidence that there is more to testicular immune privilege than just the BTB comes from transplantation studies. Allogeneic or xenogeneic tissues, such as fragments of skin, parathyroid or pancreas, transplanted into the interstitium of the testis survived for a prolonged period of time when compared to tissues transplanted in non-immune privileged sites.[117-122] For example, allogeneic parathyroid grafts transplanted into the interstitial space of MHC incompatible rats survived and remained functional (able to normalize serum calcium levels) for an extended period of time (mean survival time = 41 days) with 1/3 of grafts surviving over 100 days.[120] Similar tissue transplanted to the kidney, a non-immune privileged site, was rejected in an average of 12.8 days. Since the transplanted tissues were located in the interstitium and not inside of the seminiferous tubules, this indicates that the whole testis, not just the adluminal compartment, is immune-privileged.

More recently transplantation of germ cells and fragments of testes have been performed between genetically different individuals.[123,124] Even though the majority of the germ cell transplantation studies and all of the testis tissue fragment transplants used immune deficient rodents as recipients, and therefore ignored the issue of immune compatibility, a few of the germ cell transplants were performed into immune competent recipients. Transplantation between syngeneic donors and recipients, which are genetically identical, indicates that the transplanted auto-immunogenic germ cells can migrate into the basal compartment of the seminiferous epithelium without evoking an autoimmune reaction.[125,126] In rodents, transplantation of germ cells as allografts (mouse to mouse or rat to rat) or xenografts (mouse to rat) into immune-competent hosts was not as successful as transplants in immune-deficient hosts. There was limited colonization without immune suppression and in the allogeneic model immune suppression was required to generate offspring.[127-129] Thus, it was surprising that germ cell transplantation has been successful without the use of immune suppression between genetically different individuals in several immune competent large animals including pigs, goats, cattle, dogs and sheep.[130-137] Several of these experiments even resulted in live offspring.[133,137]

Overall, the results of germ cell transplantation are intriguing with studies performed in higher animals providing further evidence for testis immune privilege. However, it is difficult to make strong conclusions since in some cases limited animals were used and even when genetically compatible or immune compromised recipients were used success rates were variable, probably due to the complexity of creating the optimal germ stem cell niche, which is critical.[124,127] Clearly more studies designed to specifically examine the immune aspects of germ cell transplantation in various models are needed to clarify these differences.

Immune privilege in the testis appears to be an active process where locally produced immunomodulatory factors control the overall immune response. Consistently, fluid collected from the interstitium and seminiferous tubules (maximal at Stages II-VIII) is immunosuppressive and inhibits lymphocyte proliferation[138,139] and culture media collected from cells isolated from the testis, Sertoli cells,[140-142] Leydig cells,[143] germ cells[144] and testicular macrophages[145] inhibits lymphocyte proliferation in vitro. Additionally, most of the cells located within the testis produce immunoregulatory molecules that can inhibit immune responses and induce tolerance. For example, testicular cells have been shown to express anti-inflammatory cytokines, complement inhibitors, apoptosis inhibitors and other immunoregulatory molecules.[146-149] For more information on these factors and their role in testicular immune privilege refer to these recent reviews.[146-149]

In general the immunoregulatory molecules expressed by testicular cells cause a deviation from the typical proinflammatory and destructive immune response to one that is immunosuppressive and tolerogenic. For instance, after transplantation of allogeneic islets into mouse testes, memory T cells within the testis were killed by apoptosis and at the same time the number of $CD4^+/CD25^+$ regulatory T cells in the testis and lymphoid organs increased.[150,151] Consistently, testicular macrophages have immunosuppressive activity[152] and dendritic cells isolated from normal rat testes were immature and tolerogenic.[153] However, this does not mean immune cells within the testis are unable to elicit an immune response. The testis can become inflamed and chronic inflammation results in maturation of dendritic cells that can activate T cells.[153] Moreover, autoimmune reactions against the germ cells can develop and result in

autoimmune orchitis. This can occur even when the BTB remains intact (possibly due initially to immune cell recognition of germ cell antigens located outside of the BTB). Furthermore, survival of allogeneic and xenogeneic tissue transplanted into the interstitium is highly variable and the transplanted cells can be rejected.[117-119] Thus, there is a delicate balance that must be maintained.

What cells are responsible for creating this immune privileged environment? When Leydig cells, germ cells or complete spermatogenesis were depleted, islet and parathyroid grafts remained viable.[154-157] This indicates that while Leydig cells, germ cells and complete spermatogenesis may be involved, they do not appear to be required for immunoprotection in the testis in vivo. Moreover, these and other studies demonstrate that steroidogenesis is not required.[155,156,158] Although Head et al found that treatment with estrogen to decrease testosterone production resulted in graft rejection.[159]

The importance of Sertoli cells in immune protection was demonstrated by transplantation experiments where isolated Sertoli cells prolonged the survival of co-grafted allogeneic and xenogeneic cells.[146] When allogeneic Sertoli cells were co-transplanted with islets under the kidney capsule of diabetic rats, over 75% of the rats remained normoglycemic for over 100 days.[160] However, in this study, a three-day course of immune suppression with cyclosporine was required. Modification of the Sertoli cell isolation and culture procedure by Korbutt et al, improved graft protection such that 100% of the allogeneic islet grafts survived and normalized blood glucose levels over 100 days without immunosuppressive treatment.[161] The protection provided by the Sertoli cells was likely due to the secretion of immunoregulatory factors and not the physical Sertoli cell barrier as most of the co-transplanted islets were located near, but not surrounded by, the Sertoli cells at the graft site (Fig. 7A-D).[146,162,163]

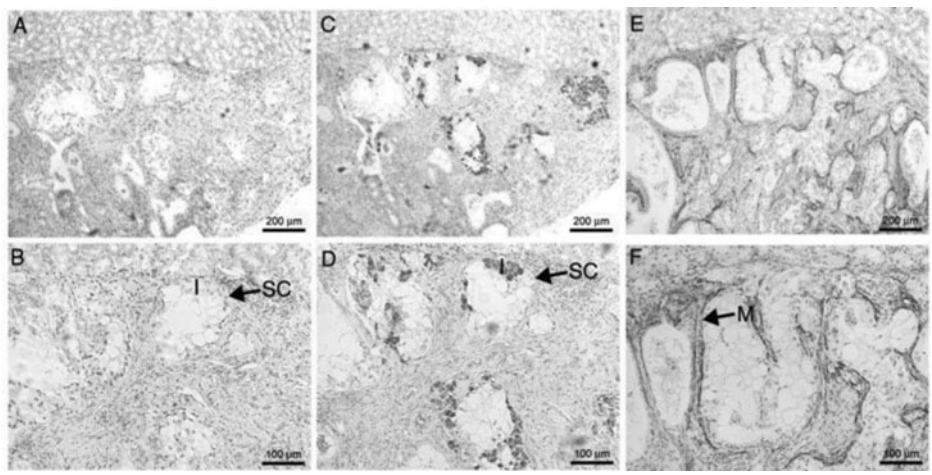

Figure 7. Three million BALB/c Sertoli cells and 500 BALB/c pancreatic islets were mixed together and transplanted underneath the kidney capsule of diabetic C3H mice. After 100 days the grafts were collected from normoglycemic mice and tissue sections were immunostained for GATA-4 (A and B), insulin (C and D) or smooth muscle alpha actin (E and F) to detect Sertoli cells, islets and peritubular myoid cells, respectively. Sections were counterstained with hematoxylin. I, islet; SC, Sertoli cell; M, myoid cell. A color version of this figure is available at www.landesbioscience.com/curie.

The potential involvement of peritubular myoid cells has not been studied thoroughly and should be examined further. Peritubular myoid cells express several immunoregulatory factors (TGFβ, MCP-1, LIF)[164] and interactions between Sertoli cells and peritubular myoid cells can influence their functions.[165] Moreover, one of the improvements initiated by Korbutt et al, resulted in a decrease in the purity of the Sertoli cell preparation to contain approximately 75% Sertoli cells.[161] While they did not specifically look for peritubular myoid cells in their preparations, we have subsequently found that our successful preparations contain 2-8% peritubular myoid cells and we have identified these cells surrounding the Sertoli cells at the graft site 100 days posttransplantation (Fig. 7E,F).[163] Peritubular myoid cells may also inhibit lymphocytes as spleen cells isolated from mice that had been injected i.v. with peritubular myoid cells had a reduced response to allogeneic and xenogeneic cells compared to control mice that were not transplanted.[166]

Another cell with an important role in testis immune privilege is the testicular macrophage. There are several subsets of macrophages located in the interstitium of normal testes: $ED1^+$ cells presumed to be circulating inflammatory monocytes; $ED2^+$ cells presumed to be immunoregulatory resident macrophages; and a third subset that expresses TGFβ and is immunosuppressive.[145,152,164] Testicular macrophages maintain the functions of traditional macrophages but also have a Type 2 phenotype associated with decreased production of proinflammatory cytokines and increased immunoregulatory function. The activity of these macrophages can be regulated by factors expressed by other testicular cells, such as Leydig cells, Sertoli cells and pertitubular myoid cells.[152,164] In general the co-operative interaction between testicular cells, testicular macrophages and other immune cells results in establishment and maintenance of the immune privileged milieu.

Overall, immune privilege in the testis is complex. The BTB impedes antibodies and immune cells from entering the seminiferous tubules and also sequesters the majority of the germ cells within the tubules. This allows for controlled antigen exposure/presentation and decreases the immune response to the germ cells. In addition, there is co-operative production of anti-inflammatory molecules and immunosuppressive factors by cells within the testis. Under normal circumstances this results in immunological tolerance, which leads to creation of an effective immune-privileged environment throughout the testis that protects the auto-antigenic germ cells from immunological destruction.

Immunological Barrier in the Epididymis

In the epididymis, the BEB forms an immunological barrier that restricts contact between the immune system and the auto-antigenic sperm. While monocytes/macrophages, $CD4^+$ T cells, and $CD8^+$ T cells can be found throughout the edidydimal epithelium and interstitial space[167,168] the BEB acts as a physical barrier that blocks their entry into the epididymal lumen and so immune cells are normally not detected within the lumen.[167] Immunoglobulins are also prevented from crossing the BEB.[4,169,170] Under normal conditions less than 2% of serum IgG levels were detected within the lumen of the cauda epididymis.[171] This small amount of immunoglobulin can be accounted for by leakiness of the rete testis.[171] Besides preventing entry of immune components into the duct, the BEB also prevents sperm antigens and auto-antigenic sperm from escaping the duct. This limits the interaction between the sperm and immune cells and prevents induction of an autoimmune reaction.[172]

Very few studies have examined the overall immune-privilege of the epididymis, although these few studies suggest the interstitial space of the testis is more immunologically privileged than the interstitium of the epididymis. When spermatozoa or testicular germ cells were injected into the interstitial space of the epididymis spermatic granulomas were formed.[173] In contrast, when spermatozoa or testicular germ cells were injected into the interstitium of the testis, no infiltrate or granulomas were detected. Moreover, i.v. injection of *Bordetella pertussigens*, resulted in inflammation and leukocyte infiltration in the epididymis, while no infiltrate was detected in the testis.[174] Despite the inflammatory reaction within the epidiymal interstitium, the epididymal ducts were not damaged indicating the BEB provided a sufficient immunological barrier.[174]

Despite numerous studies on transplantation of foreign tissue grafts into the testis, only one published study describes the survival of cells transplanted into the interstitium of the epididymis.[175] When allogeneic parathyroid tissue was transplanted into the epididymis all grafts were rejected with mean graft survival of 7.57 ± 0.9 or 12 ± 2.68 days if the lymphatics drained directly to the regional lymph nodes or bypassed the regional lymph nodes, respectively. Only if both the spleen was removed prior to transplantation and the lymphatic drainage bypassed the regional lymph nodes was a considerable prolongation in graft survival (21 ± 6.22 days) observed. Even so, the survival of allogeneic parathyroid tissue transplanted into the epididymis was much shorter than the survival of similar tissue transplanted into the testis.[118,119,121,146,175]

On the other hand, several immunoregulatory factors are expressed by cells in the epididymis,[176] and recently, Da Silva et al, demonstrated the presence of dendritic cells in the epididymis.[177] These dendritic cells were located primarily at the base of the epididymal epithelium and in the interstitial segment and proximal regions, the cells contained numerous projections that extended towards the lumen. The authors speculated that these cells "could be involved in the establishment and maintenance of immune tolerance to maturing spermatozoa".[177] Moreover, there is now evidence that regulatory T cells are important for tolerance induction to sperm after sperm antigen exposure in the epididymis of vasectomized mice.[178] The current data suggest that the BEB forms an effective immunological barrier and while in the testis the immunological barrier includes the interstitial space, further study is needed to determine how effective immue privilege is in the interstitium of the epididymis.

CONCLUSION AND FUTURE PERSPECTIVES

Overall, the BTB and BEB are composed of three aspects: the morphological, physiological and immunological components. Together these three aspects interact to make the fully functional barrier, which is necessary for maximal function of the testis and epididymis. The morphological barrier together with the physiological barrier regulates the entry and exit of molecules. This allows for the creation of a controlled milieu within the tubules/ducts that aids in the development and maturation of the germ cells. On the other hand, the immunological barrier in combination with the overall immunoregulatory environment results in a complete immune-privileged site that protects the germ cells from immunological destruction. While much is known in mammals about the specific molecules/mechanisms involved, the interactions are complex and there is still much which requires further study.

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CHAPTER 13

GAP JUNCTIONS AND BLOOD-TISSUE BARRIERS

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Abstract: Gap junction is a cell-cell communication junction type found in virtually all mammalian epithelia and endothelia and provides the necessary "signals" to coordinate physiological events to maintain the homeostasis of an epithelium and/or endothelium under normal physiological condition and following changes in the cellular environment (e.g., stimuli from stress, growth, development, inflammation, infection). Recent studies have illustrated the significance of this junction type in the maintenance of different blood-tissue barriers, most notably the blood-brain barrier and blood-testis barrier, which are dynamic ultrastructures, undergoing restructuring in response to stimuli from the environment. In this chapter, we highlight and summarize the latest findings in the field regarding how changes at the gap junction, such as the result of a knock-out, knock-down, knock-in, or gap junction inhibition and/or its activation via the use of inhibitors and/or activators, would affect the integrity or permeability of the blood-tissue barriers. These findings illustrate that much research is needed to delineate the role of gap junction in the blood-tissue barriers, most notably its likely physiological role in mediating or regulating the transport of therapeutic drugs across the blood-tissue barriers.

INTRODUCTION

Intercellular communication is an important means to maintain tissue homeostasis in multi-cellular organisms. In animals, gap junction communication (GJIC) plays a crucial role to maintain the homeostasis of different types of epithelia as well as endothelia. Gap junctions (GJ) are sometimes compared to plasmodesmata in plants as they both allow direct transport of solutes across cells.[1] However, besides intercellular transport of

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solutes via gap junction channels, GJ can also mediate solute transport between cells and extracellular space through GJ hemichannels.[2-4] GJ proteins are the basic building blocks of GJ and include connexins in vertebrates, innexins in invertebrates and pannexins in both vertebrates and some invertebrates.[5-7]

In this chapter, we discuss the roles of gap junctions in regulating the junction dynamics in tissue barriers in mammals based on the latest findings in the field. It is noted that pannexins are more closely related to innexins than connexins[8,9] and only form hemichannels.[5,7] Pannexin-based hemichannels are found more recently in vertebrates and there are very few reports in the literature studying the relationship of pannexins and junction barriers. This chapter thus focuses on the functional relationships between connexins and blood-tissue barriers in mammals. Particular focus will be put on how GJ provides the crucial crosstalk between different junction types coexisting at the blood-testis barrier so that the immunological barrier can be maintained during blood-testis barrier restructuring at the time of preleptotene spermatocytes, many of which are connected by intercellular bridges in clones,[10,11] in transit at the site.

CONNEXINS—THE BASICS: STRUCTURES, FUNCTIONS AND REGULATION

Connexin Family

Connexon is a functional unit of gap junctions and made up of a hexamer of gap junction proteins either of the same (monomeric) or different connexins (heteromeric). A connexon on the cell surface by itself is called a hemichannel while gap junction channel refers to connexons coupled between apposing cells[2,3] (Fig. 1).

There are at least 20 connexins identified in humans and rodents (Table 1). Each mammalian cell type only expresses certain members of the connexin family. These tetraspan proteins are highly conserved in their intracellular N-terminal tail, four transmembrane domains and two extracellular loops, which are for recognition and coupling.[2,3,12] The variability of connexins in terms of both length and sequence lies mostly in their intracellular loop and C-terminal tail. The C-terminal tail is the region where connexins interact with different modulators and interacting partners. Most phosphorylation sites of connexins (e.g., Cx43) are found on the C-terminal tail.[13]

Gap junction channel can be assembled between cells of the same cell type or of different cell types for heterocellular communication and the interaction of connexons can also be homotypic or heterotypic. Thus, a wide combination of gap junction channels can be formed between epithelial and endothelial cells.[2,3]

Despite the similarities between different connexin family members and expression of different connexins in the same cell type, different connexins seem to process unique functions as well as shared functions,[14,15] as illustrated in different knockout and/or knockin mouse models and genetic diseases in humans (Tables 1 and 2). For instance, while *Cx43*$^{-/-}$ mice die at birth, homozygous Cx43 knockin Cx26 mice are viable at birth. Even though the percentage of homozygous Cx43 knockin Cx26 mice born is less than the expected Mendelian ratio and has lower survival rate, it illustrates that Cx26 can at least partially compensate for the loss of Cx43. But some vital functions of Cx43 cannot be compensated by Cx26 as gametogenesis in both homozygous males and females becomes impaired, resulting in infertility.[15]

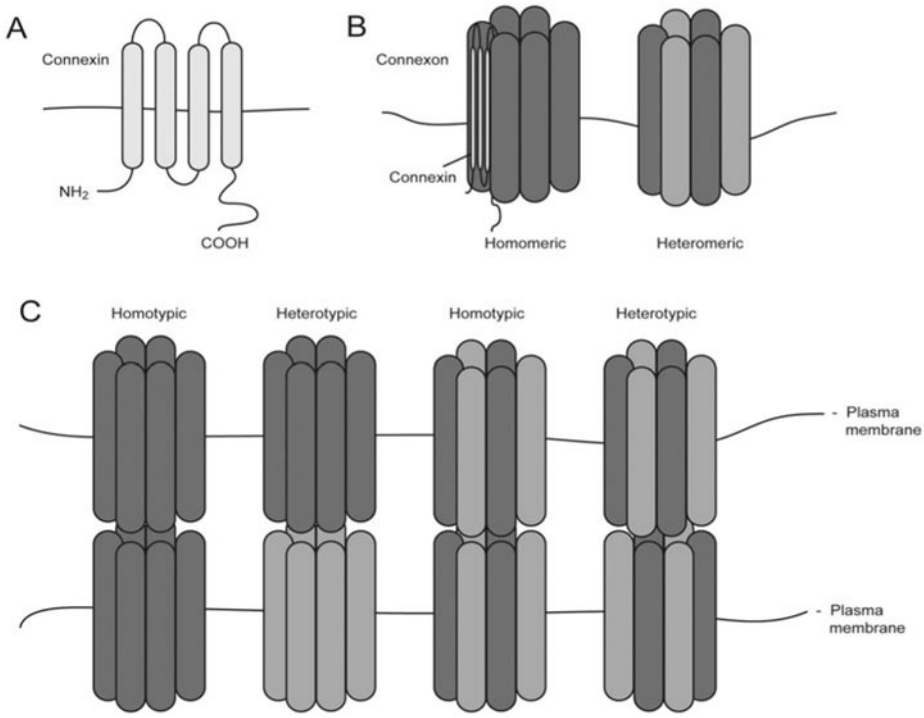

Figure 1. Schematic illustrations of the structure and organization of GJ. A) A connexin consists of four transmembrane domains, two extracellular loops and one intracellular loop. The variability of connexins lies mostly on the C-terminal tail that comes in different length and sequence and carries sites for phosphorylation and binding of interacting partners. B) Six connexins constitute a functional connexon. A connexon can be made up of the same type of connexins (homomeric) or of different types (heteromeric). An uncoupled connexon can also be called a hemichannel. C) GJ channel is formed between two compatible connexons on adjacent to that create a functional communication channel. The interaction of connexons could be homotypic or heterotypic, depending on the compatibility of individual connexins in a connexon.

Nomenclature of Connexins

Two systems of connexin nomenclature are currently used in parallel (Table 1). The conventional one names connexins according to their molecular sizes (in kDa).[16] For example, Cx43 means a connexin protein of 43 kDa. This is a commonly used system which is also used in this chapter. This system nonetheless has its drawbacks due to the differences in molecular sizes in connexin orthologs even between humans and mice. The second system involves grouping connexins according to their sequence similarities and length of their cytoplasmic tails.[16] Connexins are assigned into one of the several groups, namely α, β, γ, δ and ε,, and a number according to the order of discovery. Cx43 is named GJA1 in the second system, which marks it as the first member discovered in the alpha group of gap junction proteins.

Table 1. Connexin family members and associated defects due to mutations

	Human	Mouse	Human Hereditary Disease(s)	Phenotype(s) of Knockout Mice
GJA1	Cx43	Cx43	Oculodentodigital dysplasia,[113] cardiac defects,[114] hearing loss[115]	Neonatal lethality (lethal at birth) with abnormal cardiac development,[116,117] osteoblast dysfunction[118]
GJA3	Cx46	Cx46	Cataract[119]	Cataract[120]
GJA4	Cx37	Cx37	/	Female sterility[121]
GJA5	Cx40	Cx40	Cardiac defects[122]	Cardiac defects[123,124]
GJA6	/	Cx33	/	/
GJA8	Cx50	Cx50	Cataract[125]	Microphthalmia, cataract[126]
GJA9	Cx59	/	/	/
GJA10	Cx62	Cx57	/	Reduction in visual field in retina[127]
GJB1	Cx32	Cx32	Charcot-Marie-Tooth disease (CMTX)[128,129]	Decreased glycogen mobilization,[130] increased liver carcinogenesis,[131] mild myelination defects[132]
GJB2	Cx26	Cx26	Hearing loss,[133-135] epidermal disease[135,136]	Embryonic lethality[137]
GJB3	Cx31	Cx31	Non-syndromic hearing loss,[138,139] epidermal disease[83]	Placental dysfunction[140]
GJB4	Cx30.3	Cx30.3	Epidermal disease,[85] hearing loss[115]	/
GJB5	Cx31.1	Cx31.1	/	/
GJB6	Cx30	Cx30	Hearing loss,[141] ectodermal dysplasia[84]	Hearing loss,[142] behavioral changes to novel environment[143]
GJB7	Cx25	/	/	/
GJC1	Cx45	Cx45	/	Embryonic lethality due to vascular and cardiac defects[72,144]
GJC2	Cx46.6/ Cx47	Cx47	Pelizaeus-Merzbacher–like disease[145]	Mild myelination defects[146]
GJC3	Cx31.3/ Cx30.2	Cx29	Hearing loss[115,147]	/
GJD2	Cx36	Cx36	/	Visual transmission defects[148]
GJD3	Cx31.9	Cx30.2	/	Increase in cardiac impulse propagation[149]
GJD4	Cx40.1	Cx39	/	/
GJE1	Cx23	Cx23	/	/

Information about the recommended names of connexins and their corresponding molecular sizes in human and mouse is extracted from the UnitProtKB database (http://www.uniprot.org, accession date: 11 May 2010). Molecular sizes of connexins in mouse are the same as those in rats although some members are yet to be identified in rats. "/", not identified.

Table 2. Modulation of connexins and their effects on barrier integrity

	Modulation	Barrier Integrity	Other Observations	Tissue or Cell Line
Cx26	Overexpression	/	Prevent Na^+/K^+ ATPase inhibitor ouabain-induced TJ barrier disruption, even in the presence of gap junction blockers 18β-glycyrrhetinic acid or oleamide; Increase in Cldn14 expression	Human transformed bronchial epithelial cell line Calu-3[150]
	Overexpression	Increase	Increase in Cldn4 expression; the increase in barrier integrity can be disrupted by oleic acid, taurocholic acid and 18α-glycyrrhetinic acid	Human colonic cell line Caco-2[151]
	Ectopic expression with epidermis-specific promoter	Decrease	Disruption of epidermal barrier acquisition during development and recovery of epidermal barrier after wounding; increase in ATP release	Epidermis of genetically modified mice[88]
	Carriers of R134W *Cx26* allele (loss of function mutant)	/	Increase in epidermal thickness	Population study of human epidermis[87]
	Overexpression of R134W mutant (loss of function mutant)	/	Increase in epidermal thickness	Coculture of human keratinocyte cell line nTERT and human cervical cancer cell line HeLa[89]
	Overexpression	/	Increase in invasion of enteric pathogen *Shigella flexneri* bacteria	Coculture of human keratinocyte cell line nTERT and human cervical cancer cell line HeLa[89]
	Carriers of 35delG *Cx26* allele (loss of function mutant)	/	Increase in epidermal thickness	Population study of human epidermis[86]

continued on next page

Table 2. Continued

	Modulation	Barrier Integrity	Other Observations	Tissue or Cell Line
	Overexpression	/	Increase the dissemination of enteropathogenic bacteria *Shigella flexneri*; increase in ATP release	Human cervical cancer cell line HeLa[152]
Cx30	Knockout	Decrease	Independent of gap junction channel activity as GJ structures are lacking in normal mice	Instrastrial fluid–blood barrier in cochlear of *Cx30*$^{-/-}$ mice[153]
Cx32	Overexpression	No change	Induction of TJ strands and occludin level	Mouse hepatocyte cell line CHST8[154]
	Ectopic expression	Slight increase	Increase in levels and/or localization at cell borders of occludin, claudin-1, ZO-1 and ZO-1, which can be reversed by 18β-glycyrrhetinic acid; these observations are absent in Cx26 or Cx43 transfectants	Immortalized Cx32-deficient mouse hepatocytes[155]
Cx43	Conditional knockout	/	Acceleration of wound closure	Epidermis of *Cx43*$^{Cre\text{-}ER(T)/fl}$ mice[91]
	Knockdown with antisense oligo DNA	/	Acceleration of wound closure	Mice epidermis[92]
	Overexpression of Cx43K258Stop	Defective	Doubled half-life in Cx43K258Stop, which does not carry the C-terminal tail, as shown in HeLa cells	Epidermis of Cx43K258stop knockin mice[90]
	Knockdown with siRNA	No change	Disruption of barrier integrity only with a concurrent knockdown of Cx43 and desmosome protein plakophilin-2	Primary culture of Sertoli cells[60]

Life Cycle of Connexins

Connexins typically have short half-life of about 1.5-6 hours in mammalian cells.[17] Majority of connexins, with the exception of Cx26, are translated in the rough endoplasmic reticulum (ER)[18] and then oligomerize to form connexons in the

ER, ER–Golgi intermediate compartment or trans-Golgi network, depending on the individual connexins.[18,19] Cx26, however, could be synthesized outside of the Golgi-based secretory pathway and inserted directly to the plasma membrane via microtubules.[20] All connexins are capable of forming monomeric channels. When cells express more than one connexin at one time, heteromeric connexons may be formed but the compatibility of connexins to form heteromeric connexons depends on individual protein structure. Thus, it is not surprising to find heteromeric connexons consisting of connexins in the same subgroup. For instance, Cx43 and Cx46 in the α subgroup[21] and Cx26 and Cx30 in the β subgroup[22] have been demonstrated to form heteromeric channels.

After oligomerization, connexons are inserted into the plasma membrane.[18,23] Gap junction channels are assembled upon docking of connexons with compatible connexons on the apposing cell surface. Connexons until then remain "closed" to avoid unregulated and unwanted flow of materials from the intracellular compartment or between cells. The regulation of the opening of hemichannels and gap junction channels are to be discussed below. As mentioned above, connexon interactions can be homotypic or heterotypic. Connexons would sometimes aggregate to form gap junction plaques, which can contain up to thousands of connexons, between adjacent cells.[18,19] Connexons can also be targeted to specific domains on the membrane,[19,23] such as to cell adhesion site by microtubules.[24]

In gap junction plaques, gap junction channels that need to be metabolically degraded are internalized at the central region as double membrane vesicles into one of the apposing cells while new connexons are recruited to the periphery of the plaque. The internalized structure is called annular gap junction or connexosome and is targeted to lysosomes for degradation.[18]

Gating of Connexons

Connexons or pannexons alike have gating mechanisms to avoid unwanted flow of solutes.[7] Their openings are not "all or none" but are graded so that there are different levels of conductance, ranging between the fully "open" and "closed" state.[7,25] Different connexins display different regulatory mechanisms. The factors regulating the opening of connexins include intracellular and extracellular calcium concentration, voltage, mechanical stress, intracellular pH, redox potential and phosphorylation status of connexins.[7,12]

Phosphorylation of connexins serves as an important means for protein kinases in different signaling pathways to regulate the connexin gating.[13,26] Studies have shown different phosphorylation patterns of connexins under various physiological conditions.[27,28] For Cx43, many phosphorylation sites have been identified at its intracellular C-terminal tail.[13] Phosphorylation of Cx43 by kinases such as c-Src, MAPK, protein kinase C would result in a decline in GJIC whilst protein kinase A and casein kinase 1 can phosphorylate Cx43 to induce an increase in GJIC. A shift in the phosphorylation level of Cx43 at Ser-368, an inhibitory form, has been detected at different phases of cell cycle[28] and during development from embryonic stage to adulthood in mice.[27] Hence, changes in the phosphorylation status of connexins plays a role to induce changes in GJIC under different physiological conditions.

Selective Permeabilities of Connexins

Gap junction channels had been viewed as channels allowing passive nonspecific diffusion of solutes less than 1.0 to 1.5 kDa in molecular mass,[29,30] ranging from

inorganic ions, ATP, cyclic nucleotides, siRNA, glucose to polypeptides.[31] However, gap junction channels made of different connexins have been shown to process selective permeabilities (also called permselectivity) even towards similar solutes.[2,31-35] These selectivities include ionic charge and molecular size, while other factors are still being identified. Early studies by Goldberg et al.[33,36] provide a clear demonstration of this. Cx43 channels are more permeable to metabolites like ADP and ATP than Cx32 channels while Cx43 channels are less permeable to adenosine and calcein than Cx32 channels. Another example is the in vitro passage of siRNA through Cx43 channels, but not Cx26/Cx32 channels.[37] The rate of transfer is also inversely proportional to the length of siRNA.

For heteromeric channels, permeabilities are determined by their parental connexins. An early study has demonstrated the differences in permeabilities of homomeric Cx32 and heteromeric Cx26/Cx32 hemichannels.[38] While homomeric Cx32 hemichannels are similarly permeable to cAMP and cGMP, heteromeric Cx26/Cx32 hemichannels are more permeable to cGMP than cAMP. Heteromeric Cx43/Cx45 channels have also been shown to have unitary conductances that vary from their respective homomeric channels.[39] These functional diversities of connexins and connexons thus provide an explanation for the existence of a large number of connexins and human genetic diseases due to mutations in connexins (Table 1).

Modulators of Gap Junction Communication for Functional Studies

To perform functional studies of gap junction channels or hemichannels, modulation of their activities seems to be a necessity. Inhibition by RNAi or gene knockout model remains useful for the functional study of individual connexins but chemical modulators can also serve as convenient tools for functional studies (see Table 3). However, specificity of chemical modulators remains a concern.[7,40] Some widely used modulators, such as 18α-glycyrrhetinic acid and oleamide, have indirect actions on connexins and likely affect other signaling pathways besides GJIC.[40] In addition, these inhibitors inhibit connexin and pannexins channels at similar concentrations.[7] A comprehensive list of the effective concentration of these pharmacological inhibitors on connexin channels, hemichannels, pannexins hemichannels or other membrane channels, namely $P2X_7$ ATP channel and volume-regulated anion channel, has been provided by D'hondt et al.[7]

Additionally, mimetic peptides of connexins and pannexins were shown as specific inhibitors to study GJIC.[40] But they were later shown to exert steric inhibition rather than sequence-specific inhibition.[41] Cross-reactivity is another concern using mimetic peptides for functional studies since pannexins mimetic peptide were shown to inhibit Cx46 channels as well as pannexins hemichannels.[41] Therefore, much caution is needed to interpret results derived from studies using pharmacological inhibitors or mimetic peptides.

Assessment of Gap Junction Activity

GJIC or permeability of gap junctions is assessed by measuring the unitary conductance by patch-clamp technique or the flow of fluorescent or radioactive probes across gap junction channels or hemichannels.[25,31] While unitary conductance is measured with the patch-clamp technique,[42] the dye transfer assay has more varieties including the choice of dyes with different properties and different ways to introduce

Table 3. Gap junction blockers and their effects on barrier integrity

Chemical Modulator(s)	Concentration	Barrier Integrity	Other Observations	
18β-glycyrrhetinic acid	10 μM	/	No observable changes in distribution of occludin, ZO-1 and NCAM of TJ and N-cadherin and β-catenin of AJ	Primary culture of embryonic chicken lens epithelial cells[156]
Oleic acid and taurocholic acid	3 mM and 4.5 mM respectively	Decrease	/	Human colonic cell line Caco-2[151]
18β-glycyrrhetinic acid or oleamide	5-20 μM or 25-100 μM respectively	Decrease	No significant change (in terms of protein level or distribution) in Cx40, Cx43, occludin, claudin-5, JAM-A, JAM-B, JAM-C and ZO-1	Primary porcine brain microvascular endothelial cells[74]
18β-glycyrrhetinic acid	20 μM	Decrease	No significant change (in terms of protein level or distribution) of claudin-1 and ZO-1	Rat lung endothelial cell line RLE:rtTA:CL1[74]
Octanol or 18α-glycyrrhetinic acid	500 μM or 35 μM respectively	/	Reduction of monocyte/macrophage transmigration across a blood brain barrier model induced by TNFα and IFNγ	Cocultures of human fetal astrocytes and human umbilical vein endothelial cell HUVEC and freshly isolated human monocytes[157]
Oleic acid (oleamide) or 18α-glyceric acid	10 μM or 10 μM respectively	/	Decrease in enterocyte migration which is necessary for restitution of mucosal barrier	Primary culture of mouse intestinal epithelial cells[158]

the dye. Commonly used cell membrane impermeable dyes such as Lucifer yellow and neurobiotin, which are of different sizes and charges, can be introduced into selective cell or area of cells by microinjection, electroporation or scrape-loading.[32,35,39,41,43,44] A cell membrane permeable dye named calcein AM can also be used to label epithelial cells in vitro. This dye is converted in living cells into cell membrane impermeable calcein.[45] The transfer of dye between cells can be assessed by incubating labeled cells with unlabeled ones[45] or using the fluorescence recovery after photobleaching technique.[46] To investigate the selective permeability towards specific metabolites, metabolites labeled with radioactive probes are also used.[33,36]

CONNEXINS/GJ AND BARRIER FUNCTION

The physiological importance of connexins in various systems is demonstrated by the defects caused by mutations or ablation of connexins (Table 1). Our discussion in this section is limited to the roles of connexin-based gap junctions in maintaining blood-tissue barrier integrity. Readers are encouraged to consult other reviews for the roles of connexins in other systems, including the cardiac, neuronal and reproductive systems.[4,47-49]

Interaction of Connexins and Junction Associated Proteins

Multiple junction proteins, including integral membrane proteins, scaffolding proteins and cytoskeletal proteins have been shown to interact with connexins.[23,50] This information illustrates that connexins are part of the multiprotein junction complexes, suggesting thatgap junctions may modulate different junction types in different epithelia.[51]

Interaction of tight junction members, such as occludin, claudin-1, claudin-5, ZO-1 and ZO-2, with connexins were mostly demonstrated by colocalization and co-immunoprecipitation.[50] Direct association of Cx43 with ZO-1 and ZO-2 was also demonstrated.[52,53] The interaction of ZO-1 and Cx43 is important for the stabilization of Cx43 in the plasma membrane. The association of ZO-1 and F-actin binding protein drebrin with Cx43 was suggested for anchoring gap junction plaques to the actin cytoskeleton.[23] c-Src and ZO-1 also bind competitively to the C-terminal tail of Cx43.[54] The binding of c-Src to Cx43 and hence dissociation of ZO-1 from Cx43 was shown to drive the internalization of gap junction plaque from the cell membrane[55] and inhibit GJIC.[56,57]

For adherens junction, an early study showed that the assembly of adherens junction and gap junction are interdependent. Although adherens junction are assembled at the cell-cell interface prior to gap junction formation, addition of antibodies against either N-cadherin or Cx43 could abolish the assembly of both junction types.[58] Cx43 can be transported to N-cadherin at existing adhesion site[24] and it was shown to colocalize and co-immunoprecipitate with AJ proteins N-cadherin and β-catenin.[59,60] These reports thus supported a close physical and functional association between AJ and GJ.

GJ is also working closely with desmosomes in heart and reproductive organs. For instance, arrhythmogenic right ventricular cardiomyopathy is a hereditary disease of heart muscle caused by mutations in desmosomal proteins including plakoglobin and plakophilin-2.[61] Patients with this disease had a lower level of Cx43 at the cell surface.[62,63] The knockdown of plakophilin-2 by RNAi was shown to cause a reduction in Cx43 level and GJIC[63,64] while plakophilin-2 can physically associate with Cx43.[60,64] In the ovary and testis, junction complexes bearing ultrastructural properties of both desmosomes and GJ have been identified and are named desmosome-like or desmosome-gap junction.[65-68]

In short, connexins interact with various junction proteins and their associated scaffolding and signaling proteins in a tissue-dependent manner. The implications of these associations in regulating the homeostasis of barrier integrity are discussed below.

Endothelial Blood-Tissue Barrier

Various blood-tissue barriers are formed by TJ barrier between endothelial vascular cells and these include the blood-brain barrier, inner blood-retinal (also known as blood-ocular) barrier.[69,70] Additional reinforcement by epithelial cells, such as pericytes, also contribute to the blood-brain barrier.[70] Some barriers, such as blood-aqueous barrier

and blood-retinal barrier, consist of more than one layer of TJ barrier formed by both vascular and epithelial cells.[71]

As shown in knockout animals, some connexins are required for proper vascular development. For instance, Cx45 is present in the endothelial cells and smooth muscle cells of all blood vessels. Embryonic lethality in *Cx45*$^{-/-}$ mice was accompanied by defects in vascular development.[72] Perinatal death was noted in *Cx37*$^{-/-}$/*Cx40*$^{-/-}$ double knockout mice, but not in single *Cx37*$^{-/-}$ or *Cx40*$^{-/-}$ knockout mice. Vascular defects in *Cx37*$^{-/-}$/*Cx40*$^{-/-}$ double knockout mice were exhibited by hemorrhages in certain tissues, in particular testis and intestine.[73] These studies illustrate the importance of connexins in vascular development. In addition, studies have suggested the importance of gap junction activity in the maintenance of tight junction barrier integrity of vascular cells. In primary cultures of vascular cells from porcine brain, addition of GJ blocker, 18β-glycyrrhetinic acid (5-20 μM) or oleamide (25-100 μM), can significantly inhibit the barrier integrity.[74] In another study of rat blood-brain barrier, the reversible barrier disruption induced by ultrasound was accompanied by a redistribution of gap junction plaques. An increase in size of Cx43 and Cx36 based-GJ plaques was observed during blood-brain barrier disruption.[75] These reports illustrate the importance of connexins and GJ in vascular development and maintenance of endothelial vascular barrier.

Blood-Testis Barrier

Blood-testis barrier is notably different from the endothelial blood-tissue barrier mentioned above (see Fig. 2). This barrier is constituted by adjacent Sertoli cells residing in the seminiferous epithelium, near the basement membrane in adult mammalian testes, instead of endothelial vascular cells found in the interstitium. Secondly, it is formed at the basal, instead of the apical, side of Sertoli cells. Thirdly, its structural components include not only tight junctions, but also atypical adherens junctions (basal ectoplasmic specialization), desmosome-gap junctions and GJ.[66,76,77] Major functions of this barrier include providing immunological protection to developing germ cells and creating a microenvironment for the development of postmeiotic male germ cells, known as the apical compartment, during spermatogenesis[77,78] (Fig. 2).

Of the various connexins expressed by Sertoli cells,[48] Cx43 is a promising candidate that regulates the integrity of blood-testis barrier. While spermatogenesis defects were not reported in mice with Cx31, Cx32, Cx37, Cx40, or Cx46 knockout,[48] Sertoli cell specific Cx43 knockout causes impaired spermatogenesis, leading to infertility in homozygous male mice.[79] Further analysis reveals that these Sertoli cells without Cx43 stay in the proliferative phase without differentiation.[80] Since the establishment of functional blood-testis barrier has been associated with differentiation of Sertoli cells,[81] it is tempting to speculate that Cx43 may be a prerequisite for the establishment of blood-testis barrier.

A recent report from our research group indicated that Cx43 co-operates with desmosomal protein plakophilin-2 in the maintenance of the blood-testis barrier integrity. Simultaneous knockdown of both Cx43 and plakophilin-2, but not either one alone, would disrupt the barrier integrity in primary culture of Sertoli cells.[60] In addition, a dual-knockdown of desmoglein-2 and desmocollin-2, which are integral membrane proteins of desmosomes, would perturb the TJ-permeability integrity in primary Sertoli cell cultures, partly via enhancing the rate of endocytosis of TJ protein CAR.[82] We

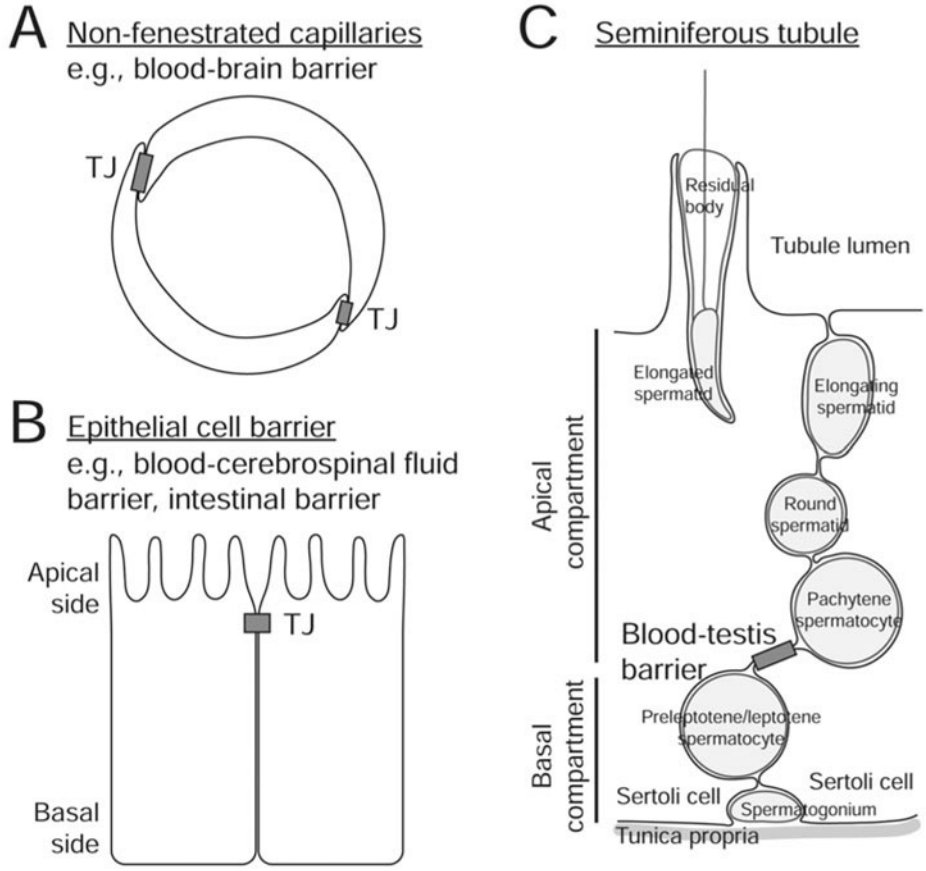

Figure 2. This figure illustrates the major morphological features of various types of blood-tissue barriers. A) Blood-tissue barriers, including blood-brain barrier, could be constituted by TJ formed between endothelial vascular cells in nonfenestrated capillaries. B) Blood-tissue barriers could also be formed by epithelial cells. At the blood-cerebrospinal fluid barrier, the blood vessel is fenestrated (without TJ) and TJs formed at the apical region of adjacent choroid plexus epithelial cells constitute the barrier. C) The blood-testis barrier is located in the seminiferous epithelium of the seminiferous tubule, which is formed near the basal region of adjacent Sertoli cells. Different junction complexes have been identified at this site, including TJ, basal ES (an atypical AJ), desmosome-like junction and GJ. The blood-testis barrier also segregates the seminiferous epithelium into the basal and apical (or adluminal) compartment, so that meiosis and the entire events of postmeiotic germ cell development (i.e., spermiogenesis) take place behind this immunological barrier in a specialized microenvironment.

postulate that when primary spermatocytes are in transit at the blood-testis barrier, such as at Stage VIII of the seminiferous epithelial cycle, the AJ, GJ and desmosome formed between Sertoli cells would be replaced by those between Sertoli cell and spermatocytes (Fig. 3). From these reports, a reduction of GJ and desmosome between adjacent Sertoli cells would induce blood-testis barrier disruption. This includes a decline in the steady-state levels of TJ and AJ proteins at the Sertoli cell surface, which is partly mediated by an increase in endocytosis of junction proteins. Thus, it resulted in an increase in the permeability at the apical region of the translocating spermatocytes to

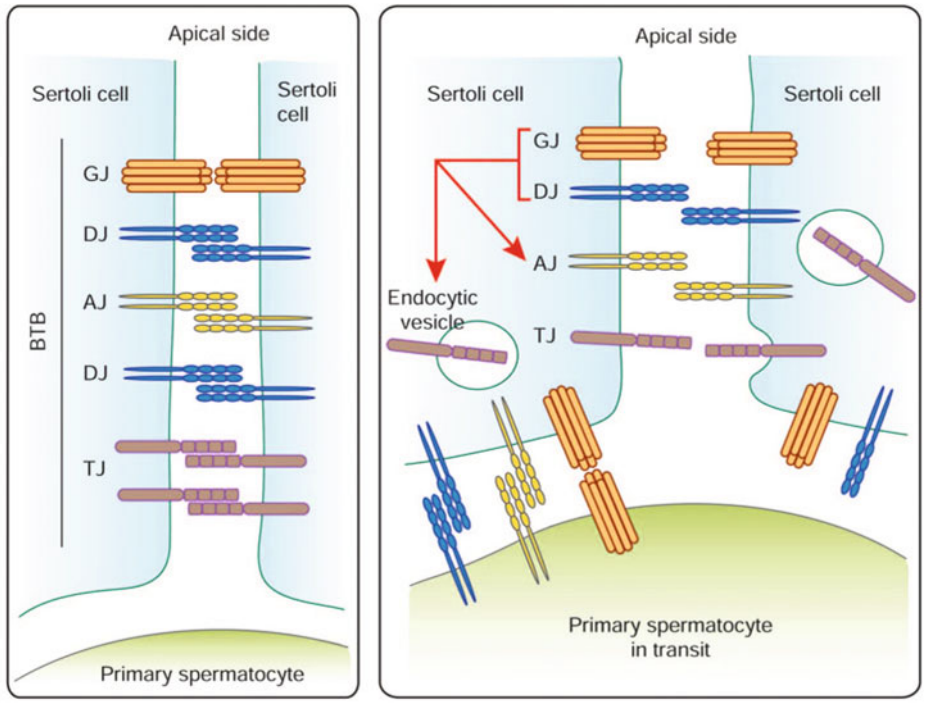

Figure 3. Schematic illustration of the roles of GJ and desmosome at the blood-testis barrier. The blood-testis barrier (BTB) remains intact at most stages of the seminiferous epithelial cycle and consists of coexisting TJ, AJ, GJ and desmosome-like junction (DJ) (left panel). Primary spermatocytes migrate across the BTB at Stage VIII of the seminiferous epithelial cycle in the rat testis (right panel). The AJ, DJ and GJ formed between Sertoli cells are likely replaced by those between Sertoli cell and spermatocyte since it is now known that many of AJ, DJ and GJ proteins are also found in germ cells, such as spermatocytes.[76-77] A reduction of GJ and DJ between adjacent Sertoli cells would destabilize the BTB, inducing its disruption. This involves a decline in the steady-state levels of TJ and AJ proteins at the Sertoli cell surface, which is partly mediated by an increase in endocytosis of junction proteins. The net result is an increase in the permeability at the apical region of the translocating spermatocytes to facilitate their transit at the BTB. However, "new" TJ, AJ, DJ and GJ are formed behind the spermatocytes in transit before the "old" junctions are being disrupted, so that the immunological barrier can be maintained.

facilitate its translocation. Cx43 hence likely serves as a regulator of the blood-testis barrier homeostasis by maintaining the crucial crosstalk among different coexisting junction types at the blood-testis barrier (Fig. 3).

Epidermal Barrier

Epidermal barrier of mammalian skin serves as the first line of defense against pathogens and other harmful substances.[1] At least nine connexins are expressed at different layers of epidermis except the uppermost layer called stratum corneum.[2] Influences of connexins on epidermal barrier integrity are exemplified by their effects on epidermal thickness and wound repair process. Multiple human hereditary diseases in skin with mutations in Cx26, Cx30, Cx30.3 and Cx31, have been discovered.[83-85] This illustrates

the necessity of connexins in maintaining the epidermis homeostasis and epidermal barrier. For instance, Cx30 mutants could result in hidrotic ectodermal dysplasia,[84] with symptoms including eczematous dermatitis.

Cx26 in particular has been the focus of much research since the loss of function mutants of Cx26 that lead to nonsyndromic hearing loss would give heterozygous individuals an advantageous edge of an increase in epidermal thickness.[86,87] An ectopic Cx26 overexpression in mice epidermis would however disrupt the epidermal barrier development and wound healing process.[88] Another study using cocultures of keratinocytes and HeLa cells demonstrated that the invasion of enteric pathogen *S. flexneri* could be enhanced by overexpression of Cx26, but not its loss of function mutant.[89] These reports collectively illustrate the inhibitory effect of Cx26 on the establishment, recovery and hence integrity of epidermal barrier.

Cx43, which displays a broad expression profile in epidermis, has been shown to regulate the epidermal barrier in animal studies even though Cx43 mutants are yet to be associated with skin abnormalities in humans. Knockin mice with Cx43 carrying no C-terminal tail (Cx43K258Stop) have perinatal death due to epidermal barrier defects. The truncated Cx43 mutant without C-terminal tail also form GJ channels and has a doubled half-life than Cx43.[90] Mice having an epidermis-specific Cx43 knockout or knockdown display an acceleration of wound closure.[91,92] These illustrate that a decline in Cx43 level is probably required for epidermal barrier establishment and wound repair.

CONNEXIN-MEDIATED BYSTANDER EFFECTS

Connexins Mediating Harmful Signals

The above discussion illustrates the regulatory roles of connexins on the homeostasis of different blood-tissue barriers. Most barriers serve primarily as selective permeablility barrier to isolate and protect cells behind the barriers from harmful substances such as pathogens.[1] While connexins could regulate barrier integrity, they could also be responsible for mediating harmful signals under pathological conditions. For instance, in intestinal barrier, Cx43 hemichannel was recently shown to mediate infection of enteric pathogen *Citrobacter rodentium*.[93] Water loss following *C. rodentium* incubation, as assessed by the water content in distal colon, was significantly reduced in heterozygous *Cx43*$^{+/-}$ mice. In addition, connexins have been implicated in tumorigenesis.[94] Tumor cell migration and attachment during metastasis was shown to be induced by Cx43.[95] This is probably due to the close structural association of connexins and adhesion molecules as discussed above so that an alteration of Cx43 would lead to changes in cell adhesion and cell migration.

Bystander Killing by Connexins

Due to their versatility, gap junction channels or hemichannels are capable of transferring harmful signals between neighboring cells. Bystander effect is a term used to describe the spread and amplification of harmful signals from cells directly exposed to insults to neighboring cells. These insults include radiation, inflammation and viral transfection.[96-99] Exposure to very low influences of α-particles could induce DNA damage in non-irradiated cells in skin and lung fibroblasts cultures.[99] After spinal cord

injury, Cx43 was upregulated. Rats with a knockdown of Cx43 by Cx43 antisense oligodeoxynucleotides showed reduced inflammation and a faster functional recovery.[98] GJIC was also shown to be responsible for mediating the transfer of apoptotic signals from HIV-infected astrocytes to non-infected ones.[97]

Bystander effect can be observed even across an intact barrier. A recent study has shown the damage caused by indirect exposure to cobalt-chromium nanoparticles in human fibroblast cells across an intact layer of BeWo cells.[100] The DNA damage resulted in fibroblast cells was reduced by gap junction mimetic peptide GAP26 while it was potentiated by antiarrhythmic peptide AAP10,[100] an upregulator of GJIC.[101] Furthermore, regional X-ray irradiation of the lower body part of mice induced DNA damage and apoptosis in mouse cerebella, which are behind the blood-brain barrier. The use of GJIC inhibitor 12-*O*-tetradecanoylphorbol-13-acetate could reduce these bystander effects.[102]

Potential Uses of Connexin-Bystander Effect

Apart from the bystander deaths mediated by gap junctions, bystander effect is beneficial under certain circumstances. Preconditioning in heart and brain involves exposing bystander cells to stress but not yet damaging stimuli, which results in better resistance towards higher and damaging levels of stimuli during subsequent exposures.[103,104] Studies utilizing Cx43-deficient mice reported the absence of preconditioning in heart and brain of Cx43-deficient mice, illustrating Cx43 as a prerequisite for preconditioning.[105,106] The role of connexins in preconditioning in heart has been recently reviewed.[107] In addition, the possibility of utilizing the bystander effect in cancer therapy has been explored. A recent review discussed the possibility of taking advantage of the bystander effect in radiation-related cancer therapy to amplify the harmful effects of radioactive isotopes or external radiation to tumor cells.[108] A potential gene therapy for cancer treatment involves the targeted introduction of thymidine kinase gene by virus into tumor cells, which is necessary for the processing of an antiviral drug named ganciclovir into its toxic phosphorylated form.[94,109] It has been shown that GJ would again increase the range of the toxicity due to its bystander effect.[96,110] These studies draw attentions not only to the safety of medical use of nanoparticles and radiation, but also to the potential uses of gap junctions to amplify signals, such as during cancer therapy.

CONCLUSION AND FUTURE PERSPECTIVES

Herein we summarize some of the latest findings in the field regarding the role of GJ and GJIC in the normal functioning of blood-tissue barriers. Earlier morphological studies have shown that in most blood-tissue barriers with the exception of the blood-testis barrier, GJs are present in discrete cellular localization at the paracellular site, being segregated from the tight and anchoring junctions.[1] Recent studies have shown that some GJ are present in the junctional complexes besides the GJ plaques to provide the necessary communications between cells to maintain the homeostasis of an epithelium including the TJ barrier function.[1] We also provide an updated molecular model regarding the crucial role of GJ in the blood-testis barrier dynamics by coordinating different *coexisting* junction types at the blood-testis barrier to facilitate the transit of primary spermatocytes, namely preleptotene spermatocytes, while maintaining the immunological barrier integrity (see Fig. 3).

Based on the latest findings that support this model, it is very likely that GJ is working beyond its "traditional" role of serving as a channel for the transport of chemical signals between cells. Perhaps other important biomolecules, such as electrolytes, ions, small molecular drugs, and paracrine factors are being actively transported across adjacent cells in a cell epithelium to synchronize cellular events. As a result, an entire epithelium can respond to the challenge of an external cue and/or stimulus during a complex molecular event, such as growth, differentiation, development and spermatogenesis.

In light of the recent advances in the role of GJ in blood-tissue barriers, such as the blood-testis barrier, which determines and/or dictates which drug(s) and how much of a drug can traverse the barrier to enter the apical compartment, it is also possible that GJ is working in concert with drug transporters, such as influx pumps (e.g., p-glycoprotein) or efflux pumps (e.g., Oatp3), at the blood-testis barrier. This possibility is important and it should be carefully evaluated in future studies to better understand the role of GJ in drug transport at the blood-testis barrier since such studies would have significant impacts to therapeutically manage illnesses. For instance, anti-viral drugs that effectively to reduce the AIDS/HIV-1 viral loads in the blood of AIDS patients fail to reduce the viral content in the semen,[111,112] making the male reproductive tract a safe haven for HIV-1 mutation. If these drugs could traverse the blood-testis barrier as effectively as in other organs, this would minimize the transmission of AIDS from infected individuals to their partners.

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CHAPTER 14

TRANSCRIPTIONAL REGULATION OF CELL ADHESION AT THE BLOOD-TESTIS BARRIER AND SPERMATOGENESIS IN THE TESTIS

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Abstract: Spermatogenesis involves precise co-ordination of multiple cellular events that take place in the seminiferous epithelium composed of Sertoli cells and developing germ cells during the seminiferous epithelial cycle. Given the cyclic and co-ordinated nature of spermatogenesis, temporal and spatial expression of certain genes pertinent to a specific cellular event are essential. As such, transcriptional regulation is one of the major regulatory machineries in controlling the cell type- and stage-specific gene expression, some of which are under the influence of gonadotropins (e.g., FSH and LH) and sex steroids (e.g., testosterone and estradiol-17β). Recent findings regarding transcriptional control of spermatogenesis, most notably target genes at the Sertoli-Sertoli and Sertoli-spermatid interface at the site of the blood-testis barrier (BTB) and apical ectoplasmic specialization (apical ES), respectively, involving in cell adhesion are reviewed and discussed herein. This is a much neglected area of research and a concerted effort by investigators is needed to understand transcriptional regulation of cell adhesion function in the testis particularly at the BTB during spermatogenesis.

INTRODUCTION

In mammalian testes, such as in rats, spermatogenesis is a highly co-ordinated event in which spermatogonia (2n) undergo a series of mitotic divisions and Type A spermatogonia differentiate into primary preleptotene spermatocytes. The primary preleptotene spermatocytes (2n) traverse the blood-testis barrier (BTB) which is created by adjacent Sertoli cells near the basement membrane and anatomically divides the

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seminiferous epithelium into the basal and apical compartment (Fig. 1), differentiating into leptotene and zygote spermatocytes, so that diplotene spermatocytes undergo two meiotic divisions and form haploid spermatids (1n) in the apical (adluminal) compartment of the seminiferous epithelium behind the BTB. Thereafter, spermatids undergo a series of extensive morphological changes known as spermiogenesis (steps 1 to 19 in the rat testis) and spermatozoa are released from the seminiferous epithelium at spermiation. The synchronous nature of spermatogenesis that involves mitosis, BTB restructuring, cell cycle progression, meiosis, spermiogenesis and spermiation, resulting in a specific pattern of cellular association at a given segment of the seminiferous tubule. Based on the unique cellular association pattern, the seminiferous epithelium can be classified into 12 stages and 14 stages in mouse and rat, respectively.[1,2] Throughout these stages, developing germ cells remain attached to the Sertoli cells via specialized cell junctions for structural and nourishment support, many of these junctions are uniquely found in the testis, such as ectoplasmic specialization (ES) and desmosome-like junction.[3] For instance, a specialized anchoring junction known as apical ES is restricted to the interface between Sertoli cells and spermatids (steps 8-19). Once apical ES appears at the Sertoli cell-step 8 spermatid interface, this is the *only* anchoring device to anchor developing spermatids until spermiation when apical ES begins to be engulfed by the Sertoli cell, analogous to "giant" endocytic vesicles undergoing internalization or endocytosis, forming an ultrastructure known as the tubulobulbar complex.[3] As noted above, the BTB also undergoes extensive restructuring at the Sertoli-Sertoli cell interface at Stage VIII of the epithelial cycle in the rat testis to accommodate the transit of preleptotene spermatocytes at the site. Thus, it is conceivable that different cell-cell interacting events occur at the Sertoli-Sertoli (i.e., BTB) and the Sertoli-germ (i.e., apical ES, desmosome-like junction and gap junction) cell interface at respective stages of the epithelial cycle. As such, precise temporal and spatial regulation of gene expression in Sertoli and germ cells occur stage-specifically in the seminiferous epithelium. In fact, recent studies using microarray analysis have revealed at least 80 stage-regulated gene probe sets whose expression is ≥3-fold higher in mature Sertoli cells than germ cells and certain stage-regulated pathways in Sertoli cells pertinent to cell migration during the seminiferous epithelial cycle have been also identified.[4] Therefore, transcriptional regulation of the cell-specific and stage-specific genes is essential to maintain the timely expression of specific genes during spermatogenesis.

TRANSCRIPTION FACTORS IN SERTOLI AND GERM CELLS CRUCIAL TO SPERMATOGENESIS

Some groups of transcription factors are ubiquitously expressed in Sertoli and germ cells throughout all stages of spermatogenic cycle that affect a relatively board spectrum of genes important for germ cell development. However, there are also differential expressions of selected transcription factors in Sertoli and germ cells crucial to exert stage-specific and cell type-specific gene regulation during spermatogenesis. Representative transcription factor families are summarized (Fig. 1) and briefly discussed in this chapter to illustrate how gene expression is co-ordinated in stage-specific and cell-specific manners in the seminiferous epithelium. However, emphasis is placed on transcriptional regulation of genes pertinent to maintain cell adhesion function at the BTB and the apical ES during spermatogenesis. However, it is noted that there

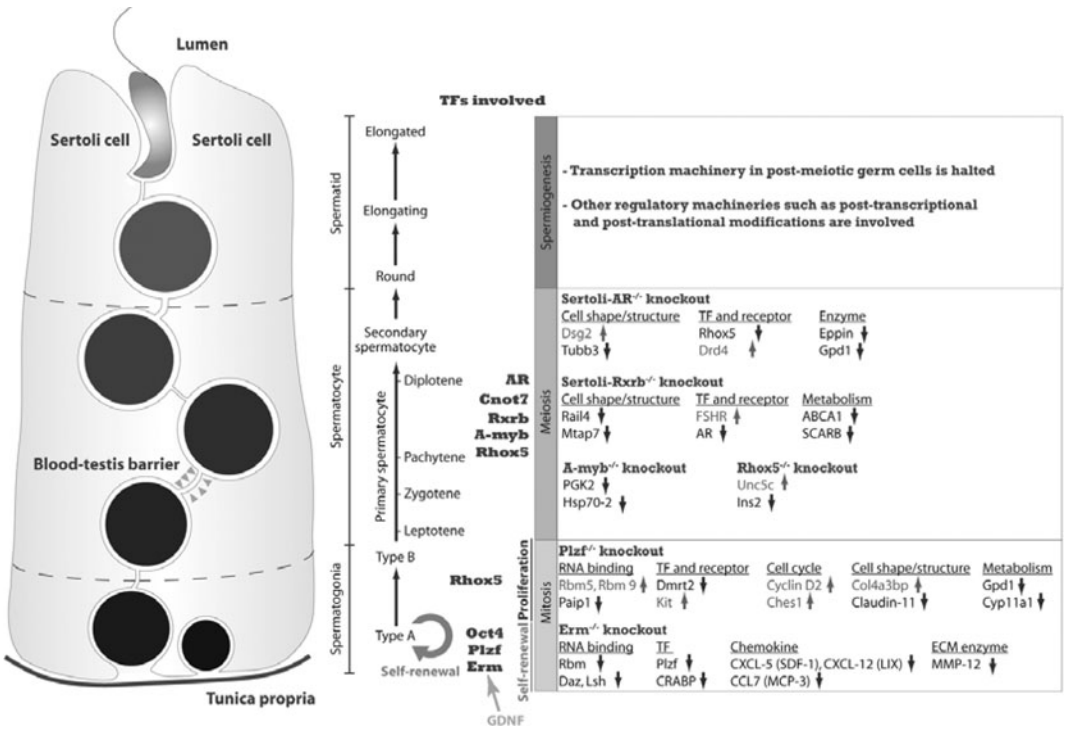

Figure 1. A schematic representation of developing germ cells engulfed by two adjacent Sertoli cells in the seminiferous tubule. Spermatogonia undergo mitotic divisions for either self-renewal (for the maintenance of the stem cell pool at the niche*) or proliferation that gives rise to primary spermatocyte. Primary spermatocytes then undergo meiotic divisions behind the BTB, leading to the production of haploid round spermatids. These cells undergo the process of spermiogenesis to become elongated spermatids and are released to the seminiferous tubule lumen at spermiation. During spermatogenesis, numerous transcription factors expressed in Sertoli and/or germ cells, which are crucial for the development of mature spermatids. Tissue- or cell type-specific knockouts of these transcription factors in mice have shown that their loss would lead to male infertility. Alteration of gene profiles were observed in the knockout models and some representatives from a plethora of genes in the profiling studies are selected and illustrated herein.[5,7,10,31,34,42,46,49,50,54,55,57,58] Upward arrow means an increase in its expression level; downward arrow means a decrease in its expression level; TF, transcription factor.

are very few reports in the literature that examine the transcriptional regulation of adhesion protein complexes at the BTB and the apical ES. The goal of this chapter is to highlight and discuss some of these findings. But more importantly, this chapter attempts to provide some helpful guides in this much neglected area of research that deserves attention in future studies.

Reproductive Homeobox X-Linked (Rhox) Homeodomain Proteins

Reproductive homeobox X-linked (Rhox) genes clustered on the mouse X chromosome encode transcription factors that are selectively expressed in reproductive tissues.[5] The twelve related homeobox genes (Rhox1-12) are selectively expressed in male reproductive tissue.[5] However, most X-linked genes are inactivated in germ cells during spermatogenesis,[6] but all Rhox genes are expressed in somatic cells in the testis.[5] In particular, the expressions of Rhox1, 4 and 11 are high in Leydig cells; whereas all Rhox1-12 are expressed in Sertoli cells. The temporal and spatial expressions of different Rhox genes in the testis suggest that some Rhox genes might perform distinct cell type-specific functions during specific phases of spermatogenesis.[5,7]

Rhox5 is the best studied Rhox gene. Rhox5$^{-/-}$ males were sub-fertile with reduced number of mature spermatids in the seminiferous epithelium. This decline in mature spermatid number was due to an increase in apoptosis among spermatogonia and spermatocytes.[5] Knockout of Rhox5 altered the expression of other genes in the testis as well,[5,8] such as an increase in Unc5c expression. Unc5c is known to promote germ cell apoptosis, since Unc5c$^{-/-}$ mice are having significantly fewer seminiferous tubules with apoptotic germ cells.[9,10] These results thus suggest that Rhox5 negatively regulates Unc5c expression in Sertoli cells. These findings also implicate the likely transcriptional involvement of Rhox5 and Unc5c in germ cell apoptosis, possibly at the specialized junctions at the Sertoli-germ cell interface, such as desmosome-like junctions, gap junctions and apical ES since gap junctions may provide the necessary signaling information between Sertoli and germ cells to mediate the events of cell apoptosis, which should be explored in future studies.

Similar to Rhox2, 3, 10 and 11, Rhox5 is responsive to testosterone, thus it is a candidate to mediate testosterone-dependent events of spermatogenesis.[5,7,11] Other studies have shown that testosterone is crucial to maintain the integrity of junctional complexes in the testis,[12] in particular BTB integrity,[13] such as the re-assembly of occludin-based "new" TJ-fibrils behind preleptotene spermatocytes in transit at the BTB at Stage VIII of the seminiferous epithelial cycle via recycling transcytosis[14,15] and/or de novo synthesis.[16,17] It is important to determine if Rhox5 is involved in the transcriptional regulation of integral membrane proteins at the BTB, such as occludin, claudins, JAMs, N-cadherin, nectins, in future studies. On the other hand, Rhox1 is dominantly expressed in Sertoli cells in neonatal rats when they are still actively dividing, but its expression diminishes considerably when Sertoli cells enter terminal differentiation phase at day 10-15 postpartum in mice[5] when BTB is established by day ~15. Such expression pattern suggests that Rhox1 might promote Sertoli cell proliferation and its reduced expression might be critical for the assembly of the BTB.

Androgen Receptor and Cell Adhesion Regulation at the Blood-Testis Barrier and Apical ES

Androgens are crucial in the maintenance of spermatogenesis.[18] Testosterone and its metabolite, 5α-dihydrotestosterone (DHT) mediate their effects via binding to intracellular androgen receptor (AR).[19] AR belongs to nuclear receptor superfamily which is found in Sertoli, germ, Leydig and peritubular myoid cells in the testis, and it acts as a ligand-dependent transcription factor that mediates androgen-dependent gene regulation through binding to the androgen response element (ARE) of the promoter region.[20] Apart from the classical testosterone-intracellular AR signaling pathway, recent studies have shown that testosterone also mediates its effects via nonclassical signaling cascades such as c-Src and MAPK in different cell types including testicular cells.[21,22] Instead of binding to intracellular AR, testosterone has been shown to bind to membrane-associated AR, triggering a cascade of signaling events, resulting in the stimulation of calcium influx and the activation of MAP kinase pathways.[23-25] Nonetheless, the activation of nonclassical testosterone action still requires nuclear AR.

The role of AR in the testis has been evaluated using various male total and conditional AR knockout mice models. The male total AR knockout mice (T-AR$^{-/y}$) displayed female phenotype and had undescended testes with severe interruption in germ cell development.[26,27] While AR specific knockout in germ cells (G-AR$^{-/y}$) illustrated no apparent influence in fertility with normal spermatogenesis in the testis; however, AR knockout in peritubular cells (PM-AR$^{-/y}$) led to reduced sperm count and drastic reduction in testis weight and these mice were infertile.[28,29] Additionally, Sertoli cell function was impaired in PM-AR$^{-/y}$ mice with reduced seminiferous tubule fluid production and a decline in androgen-dependent gene expression.[29] Thus, AR apparently is not the crucial transcription factor in germ cells to maintain male fertility but to fine-tune spermatogenesis, yet it is critically important in peritubular myoid cell function and its loss would impede spermatogenesis. Two lines of cell type-specific AR knockout mice (AR$^{-/y}$), namely Sertoli (S-AR$^{-/y}$) and Leydig cell (L-AR$^{-/y}$) specific knockouts, displayed testicular dysfunction with meiosis arrest, leading to infertility.[30-33] For instance, diplotene spermatocytes fail to develop in S-AR$^{-/y}$ mice, while round spermatids fail to enter spermiogenesis in L-AR$^{-/y}$ mice.[30-33] These results suggest that AR in Sertoli cells play a crucial role in the event of meiosis I and AR in Leydig cells are important to maintain spermiogenesis possibly through its effects on steroidogenic function.

Using microarray gene profiling, an array of genes was detected to be up- or down-regulated in prepubertal S-AR$^{-/y}$ mice compared to the wild-type.[34] For instance, serine protease inhibitor (Eppin), dopamine receptor 4 (Drd4) and glycerol-3-phosphate dehydrogenase 1 (Gpd1) are down-regulated in prepubertal S-AR$^{-/y}$ mice (postnatal day 10) *versus* normal mice.[34] Genes encoding two cell junction proteins including claudin-11 and laminin α5 are also down-regulated in S-AR$^{-/y}$ mice at postnatal day 10.5.[35] However, it remains to be determined whether AR exerts a direct effect on the gene transcription of these two junction protein genes.

TRANSCRIPTION FACTORS INVOLVED IN THE MAINTENANCE OF SPERMATOGONIAL STEM CELLS

A_{single} spermatogonia were originally conceived to be the possible spermatogonial stem cells (SSC) in the testis.[36] However, two recent studies have unequivocally demonstrated that not all the spermatogonia A_{single} are 'true' SSC in rodent testes and it was estimated that there are only about 2,000 to 3,000 SSC among the 35,000 A_{single} spermatogonia per testis.[37,38] Thus, it is apparent that only ~10% of the the spermatogonia are 'true" SSC in the testis. Nonetheless, understanding the mechanism regarding the self-renewal process of spermatogonia (and/or SSC) during spermatogenesis is crucial and might provide new insights for treatment of male infertility. It is also important to determine if 'true' SSC express unique sets of genes (i.e., specific SSC markers) *versus* other spermatogonia including A_{single} spermatogonia.

Promyelocytic leukemia zinc-finger (Plzf) and B-cell lymphoma 6 member B (Bcl6b) are members of the bric-à-bractramtrack broad complex/pox viruses and zinc fingers (BTB/POZ) domain transcriptional repressors that are expressed in spermatogonia.[39,40] Plzf and bcl6b regulate spermatogonial stem cell renewal.[41,42] Transplantation studies have shown that $Plzf^{-/-}$ spermatogonia are unable to repopulate the testis via mitosis in germ cell-depleted recipient testis,[42] suggesting that Plzf is crucial to maintain spermatogonial stem cells. An array of genes involved in cell metabolism, cell cycle and cell differentiation was found to be significantly reduced in purified $Plzf^{-/-}$ spermatogonia. Microarray studies have shown that genes such as testis specific X-linked Knockout of plzf showed a more pronounced effect on spermatogenesis since bcl6b null mice were shown to have offsprings of smaller litter size.[40] Male mice lacking Plzf showed a progressive loss of spermatogonia upon aging, leading to infertility eventually.[42] Genes such as Tsx and cyp11a1 and transcription factor including doublesex and mab-3 related transcription factor 2 (Dmrt2) are also down-regulated significantly *versus* normal mice.[42] Some proteins, such as cyclinD2 and Ches1, however, are upregulated in $Plzf^{-/-}$ mice. CyclinD2 is normally expressed in differentiated spermatogonia, spermatocytes and spermatids, but not in spermatogonial stem cells and it plays a role in spermatogonial differentiation.[43,44] However, questions such as whether Plzf acts directly to alter the transcription machineries on these genes remain enigmatic. Except one, kit gene encoding the transmembrane receptor of stem cell factor, is the direct Plzf target gene identified so far.[45] Kit is the hallmark of differentiating spermatogonia, Plzf-mediated kit repression is believed to be crucial to maintain the population of spermatogonial stem cells. In fact, Plzf represses kit gene transcription via the binding to the Plzf binding site located upstream of the exon 1 of the kit promoter in spermatogonia.[45]

It is no doubt that transcription factors expressed by germ cells are crucial for SSC renewal, recent studies have also showed that transcription factors expressed exclusively by Sertoli cells are also important regulators for this process.[46] Male mice with targeted disruption of Ets related molecule (ERM) displayed testicular atrophy with tubules devoid of germ cells, but having morphologically normal Sertoli cells at the age of 10 weeks postpartum.[46] Surprisingly, the lack of germ cells in the $ERM^{-/-}$ mice was not due to the interruption of spermatogenic differentiation process since the first wave of spermatogenesis progresses normally and spermatogonia finally give rise to spermatids. In fact, the exhaustion of SSC after the completion of the first wave of spermatogenesis was the cause of germ cell depletion in the Erm knockout, illustrating Erm is crucial for SSC renewal.[46]

Although Erm is a transcription factor exclusively expressed in Sertoli cells, a loss of Erm would impede changes in the expression of multiple genes, some of which are not *restricted* to Sertoli cells. In fact, a plethora of genes expressed in spermatogonial germ cells was altered in Erm knockout mice. A list of genes in Sertoli cells was found to be down-regulated (9- to 25-fold reduction) in $Erm^{-/-}$ mice , illustrating a disruption of Erm in Sertoli cells would impede gene expression in germ cells by microarray analyses including stromal cell-derived factor (SDF-1), chemokine ligand 5 (CXCL5), chemokine ligand 7 (CCL7) and matrix metalloproteinase 12 (MMP-12).[46] A recent study has confirmed that Erm directly binds to Ets binding site (EBS) of SDF-1 promoter region and is responsible for fibroblast growth factor 2-mediated SDF-1 gene activation in Sertoli cells and TM4 cells.[47] Based on studies in other systems, it is known that chemokines and MMP are crucial signaling molecules to maintain the stem cell niche. For instance, they play an important role in the recruitment of hematopoietic stem cells to the bone marrow.[48] Sertoli cells, the somatic supporting cells within seminiferous epithelium, might utilize these chemokines as niche signaling molecules to support spermatogonial stem cell renewal. In fact, alteration of Erm expression in Sertoli cells was shown to affect the expression of spermatogonia-specific genes such as Plzf (promyelocytic leukemia zinc finger) and Stra8 (stimulated by retinoic acid gene 8).[49] Apparently, Sertoli cell-specific transcription factors are as important as spermatogonia-specific counterparts in the maintenance of spermatogonial stem cell niche. Further investigations are warranted to elucidate how Erm mediates transcription of other genes and how spermatogonial stem cells and Sertoli cells cross-talk with each other via the actions of Erm-regulated genes. Current studies have shown that some of the above-mentioned transcription factors such as Erm are under the control of glial cell line-derived neurotrophic factor (GDNF).[50]

While SSC is restricted to the SSC niche in the testis, which is adjacent to the Sertoli cells, basement membrane and the interstitium, there is virtually no report in the literature that examines the cell-cell or cell-matrix junction which is essential to maintain the homeostasis of the SSC niche. A recent report using microarray pathway analyses have identified the most affected pathways during SSC differentiation are those involved in adherens junction, gap junction and actin cytoskeleton, illustrating there are functional cell junctions at the SSC niche, possibly at the SSC-SSC and the Sertoli cell-SSC interface.[51] Junction proteins at these sites, analogous to BTB and apical ES, must also be transcriptionally regulated, which should be carefully examined in future studies.

TRANSCRIPTION FACTORS INVOLVED IN GERM CELL DIFFERENTIATION AND SPERMIOGENESIS

DNA replication takes place in preleptotene spermatocyte and condensation of chromosomes commences in leptotene spermatocyte. Pairing of homologous chromosomes occurs in pachytene spermatocyte which allows the exchange of genetic material between homologous chromosomes. Pachytene spermatocyte further differentiates into diplotene spermatocytes (tetraploid, 4n) and each of which gives rise to two diploid secondary spermatocytes. Each secondary spermatocyte then enters the second meiotic division immediately and produces two haploid round spermatids (1n). Round spermatids undergo spermiogenesis with extensive morphological changes such as acrosome formation and tail elongation. Those events are tightly regulated by the expression of unique regulatory proteins that are expressed temporally and spatially in Sertoli and germ cells.

Some transcription factors are of particular importance in germ cell differentiation and spermiogenesis. CREM, a-myb, Cnot7 and Rxrb are representative examples. There are recent reviews[52,53] on the role of CREM in spermatogenesis, we therefore encourage readers to read those reviews for a more comprehensive view of the topic.

A-myb that expresses predominantly in male germ cells regulates the expression of genes involved in meiotic phase of spermatogenesis. Male germ cells that entered meiotic prophase were found to be arrested at pachytene stage when A-myb gene was disrupted in mice,[54] indicating A-myb is crucial to meiosis I. An array of genes expressed in primary spermatocytes such as phosphoglycerate kinase 2 (PGK2) and heat shock protein 70-2 (Hsp70-2) was also down-regulated significantly.[54] Based on the sequence analyses, it is known that myb-binding sites are present in the promoters of PGK2 and Hsp70-2 genes.

Knockout of Cnot7/CAF1, a CCR4-associated transcription cofactor, caused infertility in male mice.[55,56] Unsynchronized development of germ cells was observed in the null mice. Although Cnot7 is expressed in both Sertoli and germ cells, spermatogenesis could be restored in *Kit* mutant mice transplanted with spermatogonial stem cells from Cnot7$^{-/-}$ mice. These results indicate that Cnot7 in Sertoli cells, but not in germ cells, is responsible for germ cell development. Studies have also shown that Cnot7 binds the AF-1 domain of retinoid X receptor beta (Rxrb, a nuclear receptor) and their association is important in Rxrb-mediated gene transcription in spermatogenesis. Mice with target inactivation of Rxrb in whole organism (Rxrb$^{-/-}$) or in Sertoli cells (Rxrb$^{Ser-/-}$) were sterile, indicating that Rxrb is crucial to maintain the functions of Sertoli cells essential for germ cell development.[57,58] In fact, an array of genes involved in cholesterol metabolism (e.g., ABCA1and SCARB1), cytoskeleton organization (e.g., Rai14 and Mtap7) and sex hormone signaling (e.g., FSHR and AR) have been found to be altered in their expression levels in the Rxrb$^{Ser-/-}$ mice.[58] Apparently, Cnot7 and Rxrb are two transcription factors that are inter-dependent and indispensable for spermatogenesis. Obviously, much research is needed to define the transcriptional and post-transcriptional regulation of genes in meiosis, such as the temporal and spatial expression of genes pertinent to meiotic regulation.

TRANSCRIPTION FACTORS INVOLVED IN ANCHORING JUNCTION DYNAMICS IN THE SEMINIFEROUS EPITHELIUM

During spermatogenesis, extensive cell junction restructuring take place between adjacent Sertoli cells at the BTB as well as between Sertoli cells and developing spermatids during spermiogenesis. For instance, the timely restructuring of BTB that occur at Stage VIII of the epithelial cycle is needed to allow the transit of preleptotene spermatocytes so that post-meiotic germ cell development can take place in a specialized microenvironment known as the apical compartment behind the immunological barrier conferred by the BTB.[3] On the other hand, spermiogenesis is accompanied by progressive transit of developing spermatids across the epithelium, so that fully developed spermatids that are found at the adluminal edge can be released and enter the tubule lumen at spermiation.[3] It is well-established that several biomolecules in the seminiferous epithelium including cytokines (e.g., TGF-β3, TNFα, IL-1α) and hormones (e.g., testosterone) are crucial regulators of junction dynamics and their concerted efforts maintain the BTB integrity

while facilitating the transit of preleptotene spermatocytes at the BTB [for reviews, see refs. 59,60]. More recent studies have shown that the differential expression and bioavailability of junction proteins at the cell-cell interface via different regulatory mechanisms, such as transcriptional, post-transcriptional and post-translational regulation, are important to regulate junction restructuring events at the BTB and the apical ES.[59,61-63] Thus, the required junction proteins at the BTB and/or Sertoli-germ cell interface can be temporally and spatially expressed in such a way that the restructuring events of cell junctions in the seminiferous epithelium could be highly co-ordinated to facilitate the timely movement of developing germ cells.

It is well-documented in various epithelial cells that the expressions of junction proteins along the epithelium are tightly regulated by transcriptional modification of junction protein genes. A spectrum of transcription factors identified to regulate the expression of junction proteins in other epithelial cells are listed in Table 1. In the testis, some transcription factors are recently shown to play important roles in regulating the expression of junction proteins such as claudin-11 and junctional adhesion molecules (Fig. 2). For instance, WT1 is a zinc-finger transcription factor that regulates the apical ectoplasmic specialization (apical ES, a specialized adherens junction formed between Sertoli cells and elongating/elongated spermatids). Using microRNA targeting WT1 in Sertoli cells, disruption of apical ES was reported, indicating that WT1 might be involved in modulating the expression of the junction proteins that constitute the apical ES.[64] Although the detailed mechanism(s) by which WT1 regulates apical ES protein expression is unknown, it is clear that apical ES dynamics during spermiogenesis could be contributed by altering gene expression at this stage.

Apart from WT1, Sp/KLF transcription factor family has shown to regulate the expression of several junction molecules in Sertoli cells. For instance, we have shown that Sp1 and Sp3 upregulate the transcription of nectin-2 and junctional adhesion

Table 1. Transcription factors control the transcription of genes encoding cell junction proteins in other epithelial cells*

Transcription Factors	Junction Proteins	References
Slug	Occludin, claudin-1, E-cadherin, integrins (e.g., α3, β1)	72-75
Snail	Occludin, claudins (e.g., claudin-1, -3, -4, -7), E-cadherin	72-74
Sp/KLF family	Claudins (e.g., claudin-1, -4), P-, E-cadherin, integrins (e.g., α2b, α3, α5), connexins (Cx40, Cx43)	74,76-81
Smad family	Claudin-1, E-cadherin	82,83
Nkx2.5	Connexins (Cx40, Cx43)	81,84
HNF	E-cadherin, connexin32	85,86
SIP1	Claudin-4, P-, E-cadherins, connexins (Cx26, Cx31)	74,87
GATA	N-cadherin, connexin40	84,88

*This table is prepared based on earlier reports and reviews, illustrating the transcriptional control of junction proteins in other epithelia. It is not intended to be exhaustive due to page limits, however, it highlights the development of this rapidly evolving field of research.

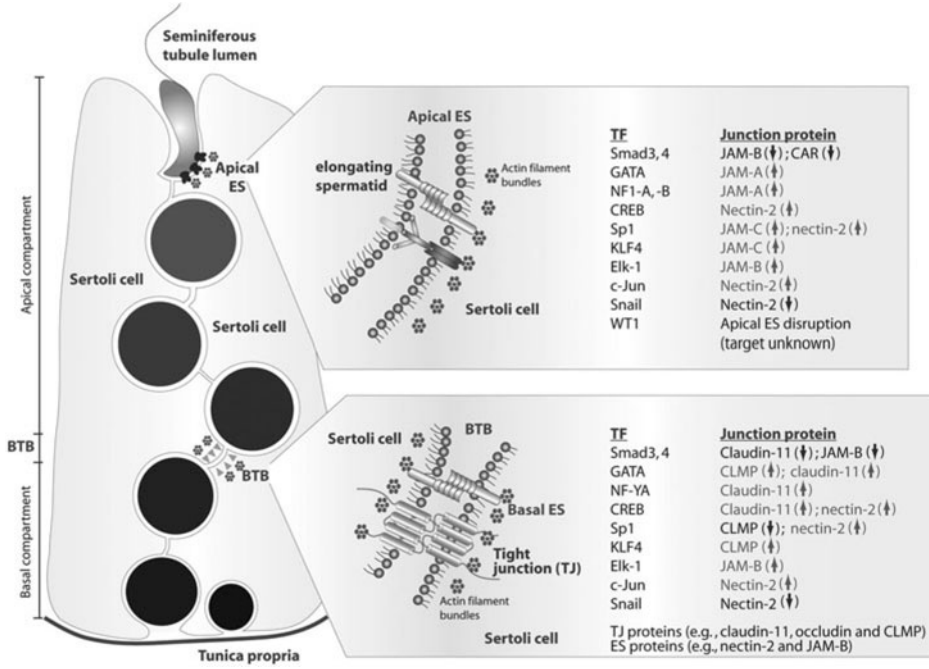

Figure 2. A schematic representation of different cell junction-associated proteins and their transcriptional regulation in the seminiferous epithelium in rodent testes. Different types of cell junctions can be found between testicular cells at the Sertoli-Sertoli and Sertoli-germ cell interface. The blood-testis barrier (BTB) formed at the basal compartment is constituted by tight junction and the basal ectoplasmic specialization (basal ES, a testis-specific atypical anchoring junction-type). Apical ES is restricted to the interface at Sertoli cells and elongating spermatids. Once apical ES appears in step 8 spermatids, it is the *only* anchoring device at the Sertoli-spermatid interface which persists until step 19 spermatids in rats and step 16 spermatids in mice, when it is undergoing endocytosis to be recycled for the formation of new apical ES and to prepare the elongated spermatids for spermiation and the 'degenerating' apical ES was called apical tubulobulbar complex (apical TBC) (for a review, see ref. 3). ES (both apical and basal) is typified by the presence of actin filament bundles found near the plasma membrane of the Sertoli cell. Gap junction and anchoring junction can also be found elsewhere between Sertoli and germ cells. Different types of junctions is made up of different junction proteins and transcription factors listed in the figure are involved in regulating particular junction proteins in the testis [64, 65, 66, 68, and Lui et al (unpublished data)]. Upward arrow, transcription activation; downward arrow, transcription inhibition. TF, transcription factor.

molecule-B (JAM-B) in Sertoli cells; whereas KLF4 is involved in regulating coxsackie and adenovirus receptor-like protein (CLMP).[65-67] CREB and GATA proteins have also been demonstrated to be the transcription factors that up-regulate the basal transcription of claudin-11 and nectin-2 genes in Sertoli cells, both of which are critical integral membrane components of the BTB.[65,68] Apart from controlling the basal expression of junction proteins in the seminiferous epithelium, several transcription factors were shown to interact with the promoters of junction protein genes upon cytokine stimulation so as to exert possible stage-specific regulation. For instance, TGF-β2 is known to activate Smad3 and Smad4 proteins and promotes the binding of Smad proteins onto the TGIF motif of the JAM-B promoter, resulting in JAM-B gene repression.[67] Since JAM-B is one of the integral membrane proteins at the BTB to confer the barrier function,

this TGF-β2-induced JAM-B repression thus leads to a loss of TJ-associated integral membrane proteins (including JAM-B and perhaps other proteins such as ZO-1, an adaptor protein of JAM-B) at the Sertoli cell BTB, leading to its transient disruption of the Sertoli cell TJ-permeability barrier as earlier reported when the Sertoli cell epithelium was exposed to TGF-β3.[69,70] The findings regarding the role of Smad proteins on JAM-B repression are highly significant since previous immunostaining analyses have shown that there is a shift in the localization of Smad proteins from the cytosol to the nucleus in the Sertoli cell epithelium during specific stages of the seminiferous epithelial cycle.[71] In short, by interacting stage-specific transcription factors, such as Smad3, with a respective promoter, stage-specific expression or repression of junction proteins during the seminiferous epithelial cycle can be achieved. Such cyclic shuffling of specific transcription factors from the cytosol to the nucleus provides a precise but efficient mechanism to control stage-specific expression or repression of junction proteins in the seminiferous epithelium at the Sertoli-Sertoli and Sertoli-germ cell interface. The identification of testis- and stage-specific transcription factors might open a new window for the design of novel contraceptives that target specific germ cell differentiation process. For instance, contraceptives that can interfere with the cyclic shuffling of a particular stage-specific transcription factor to the nuclei to exert its function in regulating gene expression pertinent to a particular event of spermatogenesis would become novel compounds to disrupt spermatogenesis without perturbing the hypothalamic-pituitary-testicular axis.

CONCLUSION

Transcription factors reviewed and discussed herein play indispensable roles in spermatogenesis as their functions have been identified by both in vitro RNA knockdown and promoter studies and knockout animal studies. With recent advances in genomic and proteomic research, an array of genes and proteins, such as transcription factors, has been identified to be altered in knockout animals with the phenotypes displaying disrupted spermatogenesis and/or infertility. However, in many cases, it is not known whether those altered genes are the direct targets of the respective transcription factor(s). Besides, numerous scattered reports indicate that there are several transcription factors that can exert their effects in mediating spermatogenesis, in particular their role in regulating cell adhesion function at the BTB. However, the detailed mechanisms by which these transcription factors regulate spermatogenesis have not been studied. Perhaps a systematic approach is needed to investigate the role of different transcription factors in regulating blood-testis barrier dynamics and spermatogenesis, and how transcription factors regulate different sets of genes in an orderly manner in the seminiferous epithelium, coinciding with the stages of the seminiferous epithelial cycle of spermatogenesis.

ACKNOWLEDGMENTS

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CHAPTER 15

c-Src AND c-Yes ARE TWO UNLIKELY PARTNERS OF SPERMATOGENESIS AND THEIR ROLES IN BLOOD-TESTIS BARRIER DYNAMICS

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Abstract: Src family kinases (SFKs), in particular c-Src and c-Yes, are nonreceptor protein tyrosine kinases that mediate integrin signaling at focal adhesion complex at the cell-extracellular matrix interface to regulate cell adhesion, cell cycle progression, cell survival, proliferation and differentiation, most notably in cancer cells during tumorigenesis and metastasis. Interestingly, recent studies have shown that these two proto-oncogenes are integrated components of the stem cell niche and the cell-cell actin-based anchoring junction known as ectoplasmic specialization (ES) at the: (1) Sertoli cell-spermatid interface known as apical ES and (2) Sertoli-Sertoli cell interface known as basal ES which together with tight junctions (TJ), gap junctions and desmosomes constitute the blood-testis barrier (BTB). At the stem cell niche, these SFKs regulate spermatogonial stem cell (SSC) renewal to maintain the proper population of SSC/spermatogonia for spermatogenesis. At the apical ES and the BTB, c-Src and c-Yes confer cell adhesion either by maintaining the proper phosphorylation status of integral membrane proteins at the site which in turn regulates protein-protein interactions between integral membrane proteins and their adaptors, or by facilitating androgen action on spermatogenesis via a nongenomic pathway which also modulates cell adhesion in the seminiferous epithelium. Herein, we critically evaluate recent findings in the field regarding the roles of these two unlikely partners of spermatogenesis. We also propose a hypothetical model on the mechanistic functions of c-Src and c-Yes in spermatogenesis so that functional experiments can be designed in future studies.

Biology and Regulation of Blood-Tissue Barriers, edited by C. Yan Cheng.

INTRODUCTION

In the mammalian testis such as in rodents, spermatogenesis takes place in the seminiferous epithelium of the seminiferous tubule via an intricate process in which a diploid spermatogonium is theoretically capable of producing 4096 haploid spermatids via cycles of mitosis and meiosis[1-4] even though ~75% of germ cells undergo apoptosis to avoid overwhelming the fixed number of Sertoli cells per testis.[5] Spermatogenesis refers to the development of spermatozoa from spermatogonial stem cells (SSC) and spermatogonia, which can be divided into several discrete cellular events, which include: (1) SSC/spermatogonial self-renewal via mitosis, (2) mitotic proliferation and differentiation of spermatogonia, and differentiation of Type B spermatogonia into preleptotene spermatocytes, (3) cell cycle progression in spermatocytes, (4) meiosis including meiosis I and II that form secondary spermatocytes and spermatids, respectively, (5) spermiogenesis and (6) spermiation. These events are supported exclusively by the Sertoli cell since Sertoli and germ cells are the *only* cellular components that constitute the seminiferous epithelium in the mammalian testis[6-9] (Fig. 1). The seminiferous epithelium, however, is anatomically segregated into the basal and the adluminal (apical) compartments by specialized junctions between adjacent Sertoli cells near the basement membrane that create the blood-testis barrier (BTB), so that meiosis I and II and postmeiotic spermatid development can take place in a specialized microenvironment (i.e., the adluminal compartment), segregated from the host's systemic circulation (Fig. 1). Moreover, preleptotene spermatocytes transformed from Type B spermatogonia must traverse the BTB while differentiating into leptotene spermatocytes at Stage VIII of the epithelial cycle to enter the apical compartment to prepare for meiosis I and II which occur at Stage XIV of the cycle in the rat testis. Thereafter, round spermatids (step 1 spermatids) are transformed into elongated spermatids (step 19) via spermiogenesis so that spermatozoa can be released into the tubule lumen at spermiation,[10] and all of these cellular events also involve the movement of developing germ cells across the seminiferous epithelium[8,11] (Fig. 1). It is therefore conceivable that tremendous restructuring events are taking place in the seminiferous epithelium throughout spermatogenesis, especially at the BTB during the transit of preleptotene spermatocytes into the adluminal compartment, the movement of spermatids across the epithelium and at the luminal edge when spermatozoa are released from the Sertoli cell epithelium at spermiation.[10-13] Interestingly, the events of spermiation and BTB restructuring that take place simultaneously at Stage VIII of the cycle but at opposite ends of the seminiferous epithelium (Fig. 1), were recently shown to be tightly regulated during spermatogenesis.[11,14]

The BTB is one of the tightest blood-tissue barriers found in the mammalian body to protect developing spermatocytes to undergo meiosis I and II, as well as spermatid development during spermiogenesis from deleterious immune factors, toxicants and/or unwanted substances/hormones.[11,13,15,16] Unlike other blood-tissue barriers which are created by endothelial tight junctions (TJs) of the capillaries (e.g., the blood-brain barrier, the blood-ocular/retina barrier), the BTB is constituted by multiple coexisting junction types, that is, TJ, basal ES [basal ectoplasmic specialization, a testis-specific atypical adherens junction (AJ) type], gap junction (GJ) and desmosome and is in proximity of the basement membrane (a modified form of extracellular matrix in the testis).[11,17,18] Also, unlike other blood-tissue barriers (e.g., the blood-brain barrier) which create a tightly "sealed" ultrastructure, the BTB undergoes extensive restructuring at Stage VIII of the seminiferous epithelial cycle to allow the transit of preleptotene spermatocytes to enter

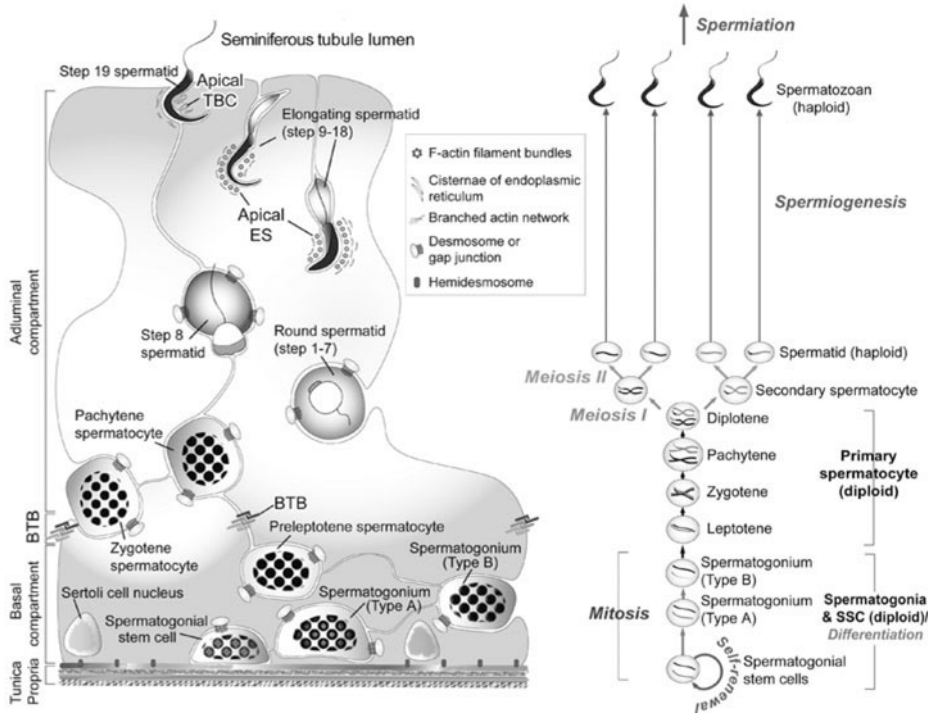

Figure 1. A graphic representation of the cellular events that take place in the seminiferous epithelium during spermatogenesis. Shown on left panel are relative locations of different germ cell types and their intimate association with the two adjacent Sertoli cells that constitute the seminiferous epithelium, overlying the tunica propria of the seminiferous tubule in the rat testis. The blood-testis barrier (BTB) formed by *co-existing* tight junction (TJ), basal ectoplasmic specialization (basal ES), gap junction and desmosome between adjacent Sertoli cells divides the seminiferous epithelium into two compartments: the basal and the adluminal (apical) compartments.[11,14,15] At the basal ES, spermatogonial stem cell (SSC) and early undifferentiated Type A spermatogonia undergo self-renewal via mitosis, to be followed by their differentiation into differentiated Type A and then Type B spermatogonia, which are the germ cells to be transformed to preleptotene spermatocytes. Preleptotene spermatocytes are the only germ cells that are in transit at the BTB to enter the adluminal compartment while differentiating into leptotene spermatocytes at Stage VIII of the epithelial cycle, to be followed by zygotene and pachytene spermatocytes to prepare for meiosis I and II (see right panel) which takes place at Stage XIV of the epithelial cycle. Apical ES is the *only* anchoring device found at the Sertoli cell-elongating spermatid interface (step 8-19 spermatids) to maintain spermatid adhesion, cell polarity (orientation) *and* cell-cell communication. Apical ES is typified by the presence of actin filament bundles sandwiched in between cisternae of endoplasmic reticulum and the apposing plasma members of the Sertoli cell and the spermatid, whereas desmosome and gap junction provide spermatogonia/spermatocytes/step 1-7 spermatids mechanical adhesion onto Sertoli cells and cell-cell communication, respectively. Immediately prior to spermiation, apical ES undergoes extensive endocytic vesicle-mediated protein trafficking (e.g., endocytosis, transcytosis, recycling and endosome-/ubiquitin-mediated protein degradation) and transforms to an ultrastructure visible under electron microscopy and fluorescent microscopy at high resolution as the result of invagination of plasma membrane formerly designated apical tubulobulbar complexes (apical TBC), preparing for the "degeneration" of the apical ES to facilitate the release of sperms at spermiation.[10,11,25,26] Underneath the seminiferous epithelium is the tunica propria, which is composed of four layers, beginning with the basement membrane, Type I collagen layer, the peritubular myoid cell layer and the lymphatic endothelium, sequentially.[21,142] Between Sertoli cells and the basement membrane is an intermediate filament-based cell-matrix anchoring junction type called hemidesmosome. Recent studies as described in *text* and summarized herein have shown that c-Src and c-Yes are involved in multiple cellular events pertinent to spermatogenesis, such as SSC/spermatogonia self-renewal, BTB restructuring to accommodate the transit of preleptotene spermatocytes, apical ES dynamics to facilitate spermiogenesis and spermiation.

the adluminal compartment for further development. Yet, the BTB integrity cannot be compromised, even transiently, during the passage of preleptotene spermatocytes to avoid the host's immune system from mounting an immune response to specific antigens that arise transiently during meiosis I and II and spermiogenesis. Recent studies have shown that this is possibly mediated via the intricate but coordinated actions of cytokines and testosterone, in which cytokines disrupt the "old" BTB above preleptotene spermatocytes in transit while androgens promote the assembly of "new" TJ-fibrils below migrating spermatocytes, likely by regulating the kinetics of endocytosis of integral membrane proteins (e.g., occludin and N-cadherin).[11,14]

Similar events come to pass at the apical ES, an *atypical* AJ type similar to the basal ES at the BTB but restricted to the interface of Sertoli cells and developing spermatids (step 8-19 in the rat testis) at spermiation—involving the breakdown of apical ES and the release of mature spermatozoa from Sertoli cell cytoplasmic crypts to the tubule lumen, which occurs synchronously with the translocation of preleptotene spermatocytes across the BTB.[10,11,14,19] Apical ES is the only anchoring device once it appears between Sertoli cells and step 8 spermatids which persists through step 19 and 16 in rats and mice, respectively[7,19] to maintain cell adhesion and spermatid polarity (orientation)[20] and structurally it is a hybrid AJ type, possessing the properties, as well as the constituent proteins of AJ, focal contact, TJ and GJ found in other epithelia.[21-23] Although adhesion protein complexes such as the N-cadherin/catenin complex can be found at both the apical and basal ES, other protein complexes, such as $\alpha 6\beta 1$-integrin/laminin333 and JAM-C/ZO-1, are restricted to the apical ES[11,20] and biologically active laminin fragments generated at the apical ES were shown to regulate the BTB restructuring at Stage VIII of the epithelial cycle,[24] thereby coordinating these two cellular events, namely spermiation and BTB restructuring, that take place at the opposite ends of the seminiferous epithelium. Prior to spermiation, the apical ES undergoes extensive restructuring to prepare for its degeneration so that adhesion protein complexes can be recycled via endocytic vesicle-mediated protein trafficking involving endosomes[25-27] and it is metamorphosed into an endocytic device formerly designated as apical tubulobulbar complex (TBC), manifested by tubular invaginations in the Sertoli cell cytoplasm which provides a complementary cuff for the docking of spermatid head.[11,27-29] In short, the role of the apical TBC is to recycle the endocytosed junctional components, such as the $\alpha 6\beta 1$-integrin/laminin333 complex, and the JAM-C/ZO-1 complex, from the "old" apical ES to be reused for the "new" apical ES that arises during spermiogenesis.[11]

In this chapter, based on recent findings in the literature, we provide a critical evaluation on how two well studied members of SFKs (Src family kinases), namely c-Src and c-Yes, which are known to perform considerably overlapping but also discrete cellular functions in several epithelia and are mostly restricted to the cell-matrix interface at the focal contacts,[30,31] work together to regulate spermatogenesis via their effects on: (1) spermatogonial stem cell/spermatogonial self-renewal and (2) cell adhesion which is mediated via their actions on cell adhesion protein complexes at the apical ES and/or BTB, as well as nongenomic androgen action. Initially, we provide a brief comparison of c-Src and c-Yes structurally and functionally and their divergent roles on cell adhesion during carcinogenesis since this information will be helpful in designing functional experiments for reproductive biologists.

GENERAL STRUCTURE OF SFKs AND A COMPARISON BETWEEN c-Src AND c-Yes

Currently, SFKs, such as Src, Yes, Fyn, Lck, Hck, Fgr, Lyn, Blk and Yrk, constitute a well-defined subfamily of cytoplasmic nonreceptor protein tyrosine kinases known to regulate various cellular events, such as cell differentiation, proliferation, migration and adhesion.[32-34] Genetic models of SFK-knockout mice were shown to display impaired immune function, developmental defects and deficiency in multiple epithelia (Table 1). Each member of the SFK family shares similar physicochemical characteristics, that is, they have an apparent molecular weight of 53-62 kDa and a backbone structure composed of four src-homology (SH) domains for protein-protein interactions (Fig. 2A): specifically, an N-terminal SH4 domain, followed by SH3 and SH2 domains and finally a kinase (SH1) domain near the C-terminus.[32,34,35] The myristoylation and palmitoylation sites near the N-terminus of SH4 domain in all SFKs are needed for membrane localization,[36,37] so that SFK kinases can be recruited to specific cellular domains/sites in response to changes in the environment, pathogenesis of a disease (e.g., tumorigenesis) and/or during development. Interestingly, c-Src and Blk are nonpalmitoylated since there is a cluster of basic amino acid residues in c-Src/Blk which is able to interact with phospholipids for membrane anchoring. Also, reversible palmitoylation has been shown to affect the distinctive localization, trafficking and activation of SFKs. For example, mono-palmitoylated c-Yes was found to accumulate at the Golgi pool of caveolin which was then transported to the plasma membrane through the exocytic pathway, whereas c-Src rapidly cycled between the plasma membrane and late endosomes/lysosomes.[38,39] In addition, palmitoylation probably increases the affinity of SFKs (e.g., c-Yes but not c-Src) for the lipid-enriched membrane microdomain at cell junctions, which plays a role in down-regulation of the SFK transforming activities and thus the functional differences between c-Src and c-Yes.[40-42] Summy et al, suggested that the unique subcellular localization of c-Yes modulated its effects on actin remodeling in Src-deficient fibroblasts.[43] It is noted that, although myristoylation exerts a positive effect on kinase activity, the resulting membrane association may negatively regulate intracellular stability of c-Src (and also Blk) because they may be more susceptible to ubiquitin-mediated degradation.[44,45] This seemingly contributes to the signaling specificity between c-Src and c-Yes, which will be further discussed below.

SH3 and SH2 domains are located behind the SH4 domain (Fig. 2A), which bind to proline-rich motifs and phosphotyrosine residues found in other proteins, respectively. Thus, these two domains are critical for mediating inter- and intramolecular protein-protein interactions. The SH1 domain at the C-terminus is the kinase/catalytic domain, harboring the autophosphorylation site, i.e., Tyr419 in c-Src[46] and Tyr424 in c-Yes[47] (Fig. 2A). Tyr530 in c-Src and Tyr535 in c-Yes at the C-terminal region, however, are inhibitory in nature since their phosphorylation lead to a blockade of the corresponding SFK activity. Endogenous negative regulators of SFKs, namely C-terminal Src kinase (Csk) and Csk-homologous kinase (Chk), keep SFKs in an "off" state in the absence of stimulation[48] and a conformational change as shown in Figure 2B would allow phosphorylation of the stimulatory tyrosine to activate SFKs.[33] It has been reported that the association of c-Src with the plasma membrane is relatively dynamic and is regulated by its intrinsic kinase activity and its SH2 domain.[49] These observations thus indicate that SFKs are capable of interacting with a large number of partner proteins and substrates via their inherent SH domains, which pave the way for their ability to integrate and transmit diverse cellular signals.

Table 1. Phenotypes in SFK-knockout mice*

Knockout(s)	Viability	Fertility	Phenotype(s)	Reference(s)
Triple knockouts				
src$^{-/-}$ *yes*$^{-/-}$ *fyn*$^{-/-}$	Embryonic lethality (E9.5 day)		Defects in cell-ECM signaling	143
hck$^{-/-}$ *fgr*$^{-/-}$ *lyn*$^{-/-}$	Increase in mortality vs. wild type		1. Hampered leukocyte recruitment 2. Impaired neutrophil phagocytosis 3. Altered macrophage activation	144,145
Double knockouts				
src$^{-/-}$ *yes*$^{-/-}$	Perinatal lethality		Reduced in body weight	146
src$^{-/-}$ *fyn*$^{-/-}$	Perinatal lethality		1. Reduced in body weight 2. Impaired keratinocyte cell adhesion	146,147
src$^{-/-}$ *hck*$^{-/-}$	Perinatal lethality		Osteopetrosis	148
yes$^{-/-}$ *fyn*$^{-/-}$	Viable		Renal disease	146
fyn$^{-/-}$ *lyn*$^{-/-}$	Increase in mortality vs. wild type		Severe lupus-like kidney disease	149
hck$^{-/-}$ *fgr*$^{-/-}$	Viable	Fertile	1. Defects in adhesion-dependent neutrophil functions 2. Impaired integrin signaling, altered cytoskeletal structure and reduced motility in macrophages	150-152
Single knockout				
src$^{-/-}$	Postnatal lethality	Infertile	1. Osteopetrosis 2. Defects in tyrosine phosphorylation of cytoskeletal proteins in osteoclasts 3. An increase in Hck levels in osteoclasts 4. VEGF fails to compromise the BBB 5. Suppression of VEGF-induced FAK phosphorylation and formation of a FAK/αvβ5complex 6. Reduction in tumor-induced VP and in spontaneous tumor metastasis 7. Resistance to tumor cell extravasation during metastasis 8. Defects in development of mammary gland, uterine and ovarian	60,67,148, 153-159

continued on next page

Table 1. Continued

Knockout(s)	Viability	Fertility	Phenotype(s)	Reference(s)
yes$^{-/-}$	Viable	Fertile	1. Defects in pIgA-pIgR transcytosis 2. VE-cadherin–β-catenin complex that maintained endothelial barrier function in lung did not dissociate after VEGF treatment 3. Resistance to tumor cell extravasation during metastasis	60,120,146
fyn$^{-/-}$	Viable	Fertile	1. Reduction in testis weight and presence of degenerated germ cells (3-4 weeks old) 2. Impaired TCR signaling 3. Neural dysfunctions 4. Defects in actin cytoskeleton 5. Increases c-Yes in oocytes	160-164
lck$^{-/-}$	Viable	Fertile	1. Defects in T-cell development and TCR signaling 2. Impaired B-cell function and BCR signaling 3. Retinal dysplasia	165-167
hck$^{-/-}$	Viable	Fertile	Impaired phagocytosis in macrophages	151
fgr$^{-/-}$	Viable	Fertile	Defects in eosinophil recruitment to the lung during allergic airway inflammation	151,168
lyn$^{-/-}$	Viable	Fertile	1. Autoimmune disease and myeloproliferation 2. Abnormal prostate gland morphogenesis	169-171
blk$^{-/-}$	Viable		Defects in T-cell development	172

*This table is not intended to be exhaustive. Instead, but it illustrates the importance of SFKs in immune function and development, especially in cell migration and cell adhesion. ECM: extracellular matrix; VEGF: vascular endothelial growth factor; BBB: blood-brain barrier; VP: vascular permeability; TCR: T-cell receptor; BCR: B-cell antigen receptor.

DIVERGENT ROLES OF c-Src AND c-Yes DURING CARCINOGENESIS

As a proto-oncogene, aberrant and elevated c-Src activity and its protein levels are often associated with the development and/or progression of miscellaneous human cancers as recently reviewed.[33,50,51] For instance, the activation of c-Src has been detected in more than 70% of human colorectal cancer patients but only ~50% for c-Yes.[52,53] During cancer cell invasion and metastasis, c-Src was found to mediate AJ disruption and cytoskeletal turnover by targeting cell adhesion-related molecules such as integral membrane proteins (e.g., E-cadherin), actin-binding proteins (e.g., villin), as well as

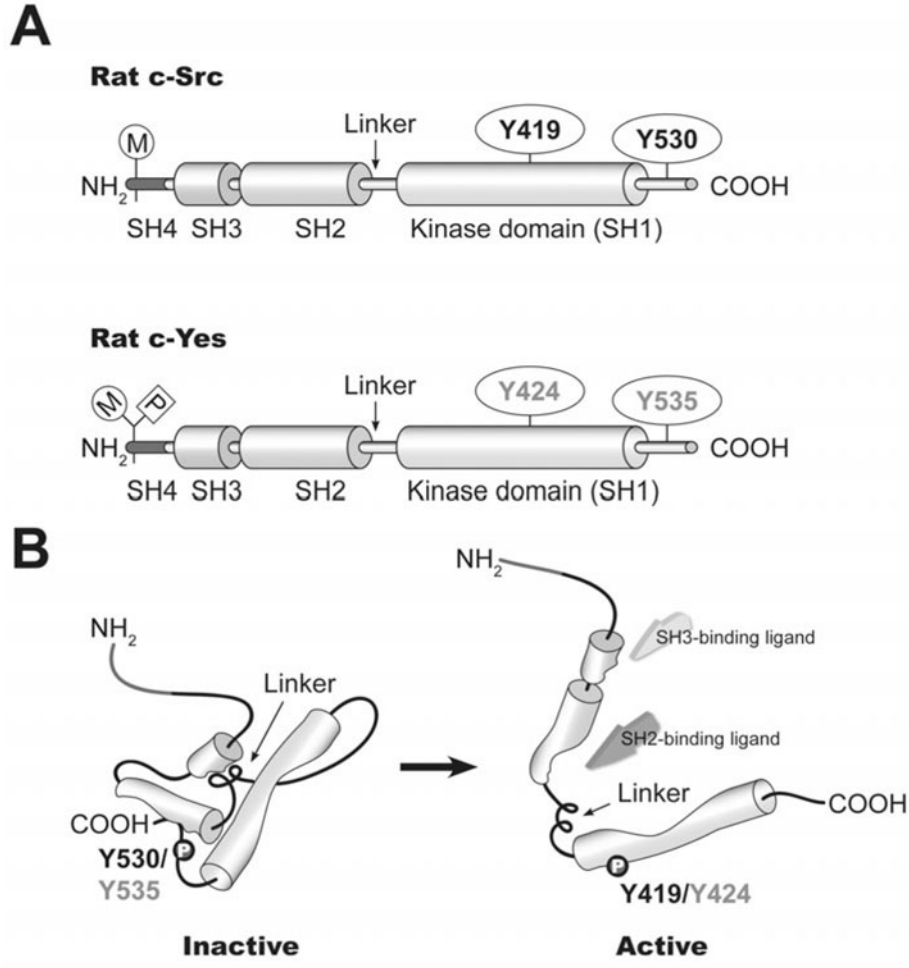

Figure 2. A schematic drawing illustrating structural and functional domains of rat c-Src and c-Yes and their activation in epithelial cells including the seminiferous epithelium. A) Members of Src family kinases (SFKs), such as c-Src and c-Yes, are composed of four characteristic src-homology (SH) domains: SH4 domain at the N-terminus, followed by SH3 and SH2 domains and finally a kinase/SH1 domain which is joined to the SH2 domain by a short polyproline Type II helix called SH2-SH1 linker. Within the SH4 domain of c-Src/c-Yes there is a penultimate glycine (Gly2) residue cotranslationally myristoylated (M) to be used for membrane targeting and signal transduction. In addition to the myristoylation, an cysteine residue (Cys3) near the N-terminus of c-Yes is posttranslationally palmitoylated (P) to be used for its subcellular trafficking, such as membrane association due to an increase in its hydrophobicity following palmitoylation. Subsequent to the myristoylation/palmitoylation motif, there is a unique domain (50-70 residues in length, shown in red at the N-terminus) that confer structural uniqueness to each member of SFKs because of its sequence divergence across the family. But the function of this region is not well defined. B) Under normal physiological conditions, SFK is auto-inhibited through intramolecular interactions in which the SH2 domain binds to the inhibitory phosphotyrosine at the C-terminal tail, while the SH3 domain interacts with the SH2-SH1 polyproline linker so that these two protein interacting motifs block the kinase domain and stabilize SFK in an inactive conformation (left). SFKs can be activated by binding of a ligand to the SH2/SH3 domain, or with dephosphorylation of the inhibitory phosphotyrosine by protein tyrosine phosphatases, thus leading to a conformational change which would allow the phosphorylation of the stimulatory tyrosine in the activation loop and to confer intrinsic kinase activity in kinase domain (SH1) (right).

activators of Rho GTPases (e.g., Tiam1), to promote cell migration.[54-56] Moreover, a c-Src/β-actin complex in integrin-mediated cell adhesion at focal contact was found to modulate peripheral actin dynamics in colon cancer cells.[57] Equally important, a blockade of c-Src activity was shown to maintain AJ/TJ barrier function and to suppress tumor dissemination.[58-60]

Despite the fact that c-Src is expressed predominantly in tumors and its overexpression and/or hyperactivation is most frequently observed in human epithelial and non-epithelial cancers,[61,62] distinctive profiles of c-Yes at the membrane lipid microdomains are found during carcinogenesis which cannot be substituted by c-Src. For instance, in human colorectal cancer HT29 cell line, c-Yes knockdown, but not c-Src, was found to induce cell apoptosis, and morphological changes, which also accompanied by E-cadherin/β-catenin accumulation at the AJ and, as such, an adaptor function unique to c-Yes other than its intrinsic kinase activity in cell migration was proposed.[63] However, the role of c-Yes in germ cell apoptosis remains to be investigated. In human melanoma cells, c-Yes, but not c-Src, became constitutively active after being treated with GD3 ganglioside in lipid rafts, thereby triggering the p130Cas and paxillin signaling pathway to facilitate cell growth and invasion.[64] These results are in agreement with those discussed earlier that the interaction of c-Src with membrane-associated targets are dynamic events and that activation of c-Src can cause it to associate with the plasma membrane while at the same time making it less stable.[39,44,49] Yet, the preferential localization of c-Yes at the membrane microdomains allows this SFK to function in specific cellular domains in response to changes in the environment during growth or pathogenesis. Additionally, the lysosomal localization of c-Src as earlier reported[38,65] probably distinguishes it from c-Yes even more. Abnormal aggregation of lysosomes at the perinuclear region which occurs during tumor invasion and metastasis can be induced by c-Src through its SH2 domain via a kinase activity-independent manner.[66] All of these findings clearly demonstrate that the distinct cellular localizations of c-Src and c-Yes likely confer their unique characteristics as regulators of membrane trafficking and junction dynamics during carcinogenesis, some of which are also applicable to spermatogenesis.

SFKs IN THE TESTIS

Several members of the SFK family, such as Src, Yes, Fyn, Lck, Hck, Fgr, Lyn and Blk have been found in rodent testes, except Yrk which is absent in mammals.[67-69] Interestingly, a different isoform of c-Src exists in the testis versus the one found in the nervous system and, in addition to the full-length kinases, some members of the SFK family are found in the testis as truncated variants (e.g., Fyn, Hck).[46,70,71]

By using the technique immunohistochemistry and an antibody prepared against c-Src at its C-terminus which partially cross-reacted with c-Yes, Fyn and Fgr due to their sequence similarity, c-Src was shown to be highly expressed in Sertoli and germ cells[72] and it localized both to the BTB and the apical ES in the adult rat testis (Fig. 3), consistent with an earlier report.[73] The expression of c-Src in the testis was also shown to be stage-specific, with a moderate decrease at the BTB during Stages VII-VIII of the epithelial cycle, but an increase at the apical ES at spermiation, in particular at the site where spermiation just occurs, suggesting that these are the structures of the residual bodies undergoing phagocytosis by Sertoli cells (Fig. 3). Recent studies have also shown that c-Src structurally associates with p-FAK, β1-integrin, laminin α3-, β3- and γ3 at the

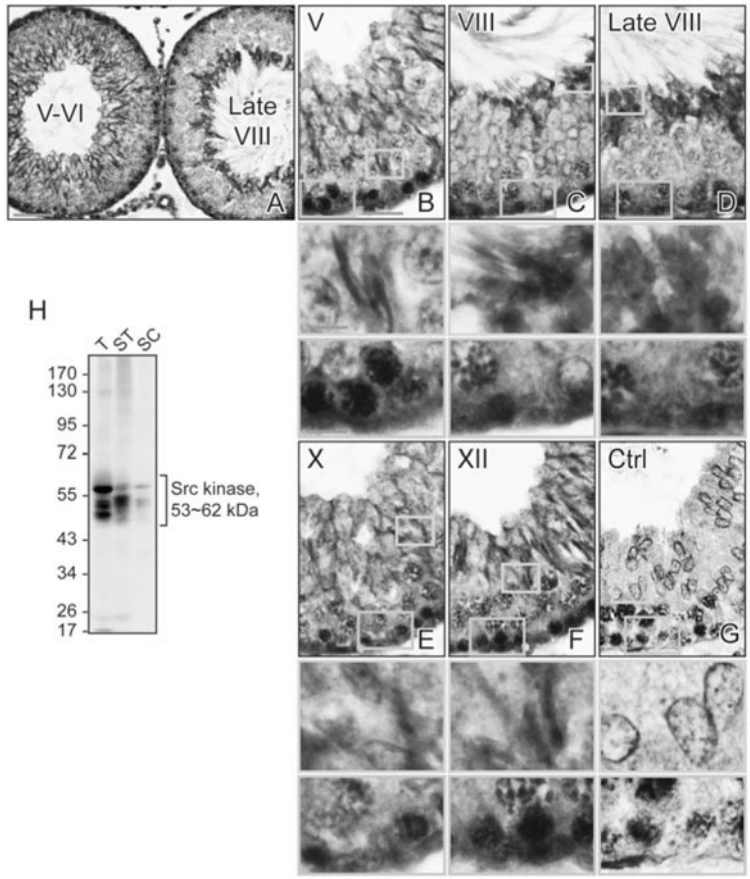

Figure 3. Distribution and cellular localization of c-Src in the seminiferous epithelium of adult rat testes. Paraffin sections of adult rat testes were used for immunohistochemistry using an antibody against c-Src (Santa Cruz, Cat. # sc-8056, Lot# K0507, 1:50 dilution) as shown in A-G, essentially as earlier described for c-Yes,[83] illustrating cellular localization and stage-specific expression of c-Src in the seminiferous epithelium. The specificity of this antibody was illustrated in an immunoblot shown in (H) using lysates of testes (T), seminiferous tubules (ST) from adult rats and Sertoli cells (SC, from 20-day-old rats following 4 days in cultures). As indicated by the manufacturer, this antibody specifically recognized c-Src, however, it also cross-reacted partially to c-Yes, Fyn and Fgr; however, based on the immunoblot shown in (H), this antibody cross-reacted strongly and mostly with c-Src. Immunoreactive c-Src was intense in the seminiferous epithelium throughout the epithelial cycle of spermatogenesis, localized in the basal compartment near the basement membrane consistent with its localization at the BTB *and* c-Src also associated with developing spermatids at the apical ES (A). At Stage VIII, the localization of c-Src at the BTB became considerably weakened, but it remained to be intensely localized near the luminal edge of the seminiferous tubule and c-Src was found to associate with the residual body derived from elongated spermatids/spermatozoa which have just departed the epithelium and the residual body appears being phagocytosed by Sertoli cells (D *vs.* B, C, E and F) [see also the magnified micrographs in the lower panels of B-G encircled with either a "yellow" or "blue" box derived from the encircled "yellow" and "blue" boxed areas of the corresponding micrograph, illustrating representative areas of the adluminal (apical) compartment (e.g., apical ES, residual bodies) and basal compartment (e.g., BTB)]. Representative tubules of Stages (V, VIII, late VIII, X and XII) correspond to B-F, respectively, *versus* control (Ctrl, E) which is a Stage X tubule and serves as a negative control in which mouse IgG was used in place of the anti-c-Src IgG to validate the specificity of the c-Src staining. Bar in A = 100μm; bar in B = 40 μm, which applies to C-G; bar in the micrograph behind b = 20μm which is an inset from B, this applies to all other insets. A color version of this figure is available online at www.landesbioscience.com/curie.

apical ES, as well as with CAR, desmoglein-2, connexin 43 and MTMR2 at the BTB.[74-82] Furthermore, c-Yes was found to structurally interact with occludin, FAK, N-cadherin, β-catenin, β1-integrin and actin, but not with CAR, JAM-A, paxillin and vimentin.[83] Although much work is needed to determine if other SFK members, besides c-Src and c-Yes, also interact with these proteins at or near the Sertoli-Sertoli and Sertoli-spermatid interface, the significance of SFKs, in particular c-Src and c-Yes, in cytoskeletal and cell junction dynamics is apparent.

SFK AND SPERMATOGONIAL STEM CELL PROLIFERATION

The initial step of spermatogenesis involves differentiation of spermatogonial stem cells (SSC) [which are a subset of A_{single} spermatogonia (A_s)[84,85]] to differentiated Type A spermatogonia (e.g., A_1, A_2, A_3, A_4), to be followed by Type B spermatogonia and then spermatocytes.[84,86] However, this also requires proliferation of SSC in the stem cell niche for self-renewal to maintain the proper population of SSC. The stem cell niche is located in the basal compartment of the epithelium, near the base of the Sertoli cell but above the basement membrane where several tubules meet and it borders the interstitial space.[87] Recent studies have shown that GDNF (glial cell line-derived neurotrophic factor)[88] released from the Sertoli cell into the stem cell niche binds onto its receptor GFRα-1, which, in turn, recruits Ret transmembrane receptor [note: GFRα-1 is expressed exclusively by A_s spermatogonia and possibly A_{paired} (A_{pr}) spermatogonia whereas Ret is expressed by all premeiotic germ cells[89-92]] to the site. Then, the activated GDNF/GFRα-1/Ret protein complex induces the c-Src/c-Yes complex at the stem cell niche, which in turn, activates PI3-K (phosphoinositide 3-kinase)/Akt (also known as PKB, protein kinase B, which is a nonreceptor Ser/Thr protein kinase) downstream to up-regulate *N-myc* (neuroblastoma derived myelocytomatosis viral related oncogene) expression (and other genes essential for self-renewal, such as *Bc16b, Erm, Lhx1*) to elicit SSC proliferation for self-renewal.[88,93] In short, GDNF-induced SSC self-renewal via mitotic division at the stem cell niche in rodent testes is mediated by c-Src/c-Yes, consistent with findings in the field regarding the role of SFK and stem cell proliferation.[94]

c-Src ON CELL ADHESION, BLOOD-TESTIS BARRIER AND SPERMATOGENESIS

c-Src was first found to be expressed by both Sertoli cells and germ cells at various stages of development and it was shown to be most abundantly associated with elongated spermatids in the adult rat testis.[95] c-Src expression,[96] in particular its activated form, p-c-Src-Tyr419,[74] was later found to peak at the luminal edge of the seminiferous epithelium at the site of the apical ES just prior to spermiation, suggesting a role of c-Src in the degeneration of the apical ES at Stage VIII of the epithelial cycle. This notion was subsequently assessed and elaborated in studies using the adjudin model, which is a potential male contraceptive that primarily targets the apical ES[97-101] to induce premature spermatid release from the epithelium in rat testes without compromising the BTB. c-Src was found to structurally interact with the N-cadherin/β-catenin complex and c-Src was found to be induced in both its protein level and intrinsic kinase activity during adjudin-induced AJ restructuring that led to germ cell loss.[72] It is likely that

c-Src alters the phosphorylation status of adhesion protein complexes at the apical ES, mediating a loss of protein-protein interactions at the apical ES as the result of an increase in protein endocytosis and possibly endosome-mediated phosphoprotein degradation. Indeed, c-Src is one of the SFK members that has been implicated to be involved in late endosomal-lysosomal intracellular trafficking[38] and there is evidence that loss of E-cadherin function in epithelial cells requires Src-dependent ubiquitination and lysosomal degradation, to cause a breakdown of cadherin-based cell adhesion.[102,103] Moreover, upon activation, c-Src is translocated to late endosomes[38] from the perinuclear region to the plasma membrane, where it becomes less stable and targeted for degradation.[44,45] This thus facilitates internalization of adhesion proteins. This concept on the involvement of c-Src in intracellular protein trafficking involving endocytic vesicles and phagosomes, and it is consistent with findings shown in Fig. 3 in which c-Src was intensely localized at the "degenerating" apical ES, associating with residual bodies shortly after spermiation has taken place in which c-Src was localized with phagosome-like structures (see Fig. 3C, D vs B, E and F).

Findings in other epithelia in which c-Src regulates cell adhesion via its effects on endocytic vesicle-mediated protein trafficking events are also consistent with recent studies that reported the involvement of c-Src in protein trafficking and junction restructuring events at the BTB. c-Src was found to form a functional complex with GJ protein connexin 43, desmosomal protein plakophilin-2 and basal ES proteins N-cadherin and β-catenin and was proposed to regulate the phosphorylation status and binding of occludin to ZO-1, to perturb the Sertoli cell TJ-barrier.[82] These findings are consistent with studies in other epithelia that activation of c-Src is believed to be involved in the dissociation of TJs. For example, two highly conserved tyrosine residues (Tyr398 and Tyr402 in humans) at the C-terminal region of occludin in epithelial cells could be phosphorylated exclusively by c-Src which would prevent the binding of ZO-1 and its homologs (e.g., ZO-2, ZO-3), but not F-actin, to occludin, thus negatively regulating TJ reassembly in Caco2 cells.[104] In keeping with this, it has been reported that decreased Ser/Thr phosphorylation and induced tyrosine phosphorylation of occludin occur at the same time during the disruption of epithelial TJ.[105] A study using the calcium switch model also revealed that during TJ biogenesis, upon Gα12-stimulated tyrosine phosphorylation of ZO-1/ZO-2 by c-Src, claudin-1 and occludin became disassociated from the ZO-1 complex, thereby destabilizing the TJ-barrier. These findings thus support the notion that c-Src activation can lead to a loss of TJ-barrier function.[106]

In addition, *c-src*$^{-/-}$ mice were found to be infertile (Table 1), however, it is not known at present if this is the result of a failure in SSC self-renewal since SFK signaling has been recognized as essential for self-renewal of mouse SSC[88,93,107,108] (see above). A study from our laboratory also showed that when a selective SFK inhibitor PP1 was administrated intraperitoneally to adult rats, there was a loss of spermatocytes and round spermatids.[72] Moreover, c-Src is intimately involved in nongenomic androgen action in the testis and androgen is known to be involved in spermatogenesis and to be essential for meiosis and germ cell development.[109,110] A more recent study has shown that the nongenomic action of androgen that regulates Sertoli-germ cell adhesion, mostly notably spermatid adhesion in the seminiferous epithelium using a novel cell adhesion assay involving seminiferous tubules, is mediated by c-Src, which can also modify androgen-regulated genes transcriptionally.[111]

c-Yes ON CELL ADHESION, BLOOD-TESTIS BARRIER AND SPERMATOGENESIS

Functional redundancy between c-Yes and c-Src in many epithelia examined to date, coupled with the lack of specific agonists and/or antagonists against individual kinases have made it difficult to discern the physiological function of c-Yes *versus* c-Src during spermatogenesis. A recent study has reported the cellular localization of c-Yes in the seminiferous epithelium of adult rat testes.[83] It has shown that c-Yes is present at the BTB in almost all stages of the epithelial cycle, with the highest expression occurring at Stages VIII-IX, coinciding with BTB restructuring to accommodate the transit of preleptotene spermatocytes at the site (Fig. 4). This pattern of stage-specific expression for c-Yes is somewhat different from c-Src in that the expression of c-Src at the BTB around stages VIII-IX was found to be considerably reduced. Thus, it is increasingly clear that c-Yes and c-Src, while both are members of the SFK family, are probably playing different roles in the seminiferous epithelium during the epithelial cycle of spermatogenesis. Notably, c-Yes was found to interact with occludin- and N-cadherin-based adhesion protein complexes at the BTB.[83] The involvement of c-Yes in TJ formation/maintenance has been reported in other epithelial cells. In T84 human intestinal cell line and MDCK II epithelial cells, c-Yes was shown to form a functional complex with occludin; and when the calcium depletion-repletion model was used to study the latter cell type, there was a temporary surge of tyrosine phosphorylation of occludin correlating with TJ reassembly.[112,113] Indeed, when BTB function was found to be enhanced during adjudin-mediated apical ES disruption at the Sertoli-spermatid interface in adult rat testis, the protein levels of several TJ and basal ES proteins, as well as c-Yes, were found to be induced at the BTB.[83,114]

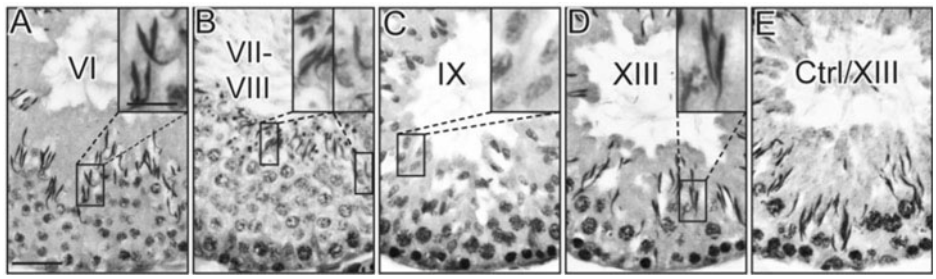

Figure 4. Distribution and cellular localization of c-Yes in the seminiferous epithelium of adult rat testes. c-Yes localization in the seminiferous epithelium of adult rat testes was examined by immunohistochemistry as described.[83] c-Yes was found to be expressed stage-specifically at the BTB and apical ES. c-Yes was localized at the BTB in almost all stages of the epithelial cycle, with the highest expression at Stages VIII-IX (B, C *vs*. A, D), which is the opposite to c-Src whose localization at the BTB was the lowest at Stages VIII-IX (see Fig. 3). c-Yes was found both at the convex and the concave sides of the apical ES (A, B and D), except that it rapidly diminished at the apical ES shortly before spermiation. In a Stage IX tubule (C), the level of c-Yes in the apical compartment was virtually nondetectable except that it peaked at the BTB in Stages VIII-IX at the time of BTB restructuring to facilitate the transit of preleptotene spermatocytes. (E) Cross-sections of testes stained with normal mouse IgG instead of the anti-c-Yes antibody, illustrating the staining shown in A-D was specific for c-Yes. Bar in A = 55μm, which applies to B-E, bar in inset in A = 25μm, which applies to all insets in B-E.

Other studies have shown that cytokines, such as TGF-β3 and TNFα, are crucial regulators of BTB dynamics.[115,116] During TGF-β3-induced loss of Sertoli cell-TJ barrier integrity, a significant decline in the c-Yes steady-state protein level was found to precede the reduction of other integral membrane proteins at the BTB, such as occludin and N-cadherin at the TJ and basal ES, respectively, which thus illustrates the importance of c-Yes in supporting TJ-barrier function.[83] More important, when c-Yes activity was blocked by a selective SFK inhibitor SU6656 preferentially to block c-Yes function, occludin and N-cadherin at the Sertoli-Sertoli cell interface were found to become mis-localized and to associate more with the endocytic vesicle protein clathrin,[83] further demonstrating the significance of c-Yes in conferring cell adhesion at the Sertoli cell BTB.

Besides at the BTB, c-Yes was also detected at the apical ES but subsided considerably to an almost nondetectable level by late Stage VIII just prior to spermiation, coinciding with the degeneration of the apical ES.[83] This pattern of expression of c-Yes is different from c-Src which, in contrast, most notably expressed at Stage VIII at both the apical ES and the BTB, but it remained intensely expressed at the "degenerating" apical ES site, associated mostly with ultrastructures of residual bodies following spermiation (see Fig. 3 versus Fig. 4). It is noteworthy that these differential expression patterns of c-Yes and c-Src around the time of spermiation presumably indicates their participation in the degeneration of apical ES, as well as the subsequent endocytic recycling events when the "old" apical ES under dissolution is being used to assemble the "new" one for newly differentiated step 8 spermatids during spermiogenesis (Fig. 5). As noted above, following biosynthesis, c-Yes is transported to the plasma membrane from the Golgi pool of caveolin in COS-1 cells[39] to be used for endocytic vesicle-mediated protein trafficking events, such as protein recycling since caveolin is a marker for protein transcytosis in cells including those in the testis.[117,118] In line with this possibility, c-Yes knockout mice revealed a deficiency in pIgA-pIgR transcytosis which would not be compensated by c-Src; and the formation of a pIgR-c-Yes-EGFR complex is necessary for the pIgA internalization and recruitment to the recycling endosome in polarized epithelial cells.[119,120] The possibility that c-Yes is involved in protein recycling events during spermatogenesis, such as for recycling of proteins from the "old" apical ES and the "old" BTB site to the "new" apical ES and "new" BTB under "construction" via its role in endocytic vesicle-mediated protein trafficking, such as endocytosis, recycling, transcytosis and endosome-/ubiquitin-mediated protein degradation (Fig. 5), must be carefully evaluated in future studies.

In this context, it is of interest to note that Par6, a component of the Par3/Par6 polarity complexes known to regulate the junction restructuring at the apical ES,[20] reaching its lowest expression level at the apical ES in misaligned and depleting spermatids following treatment of rats with adjudin, was found to have a tighter association with SFK member(s).[121] All of these results suggest that c-Yes or c-Src activity at the apical ES is being used to induce undesired phosphorylation of apical ES constituents such as that of Par6, to evoke protein endocytosis [note: Par6, Par5 (also known as 14-3-3) and Cdc42 (a component of the Par6-based protein complex[20,122]) were recently shown to be involved in regulating protein endocytosis at the Sertoli cell BTB,[123,124] thereby destabilizing the apical ES and leading to elongating/elongated spermatid loss from the epithelium.

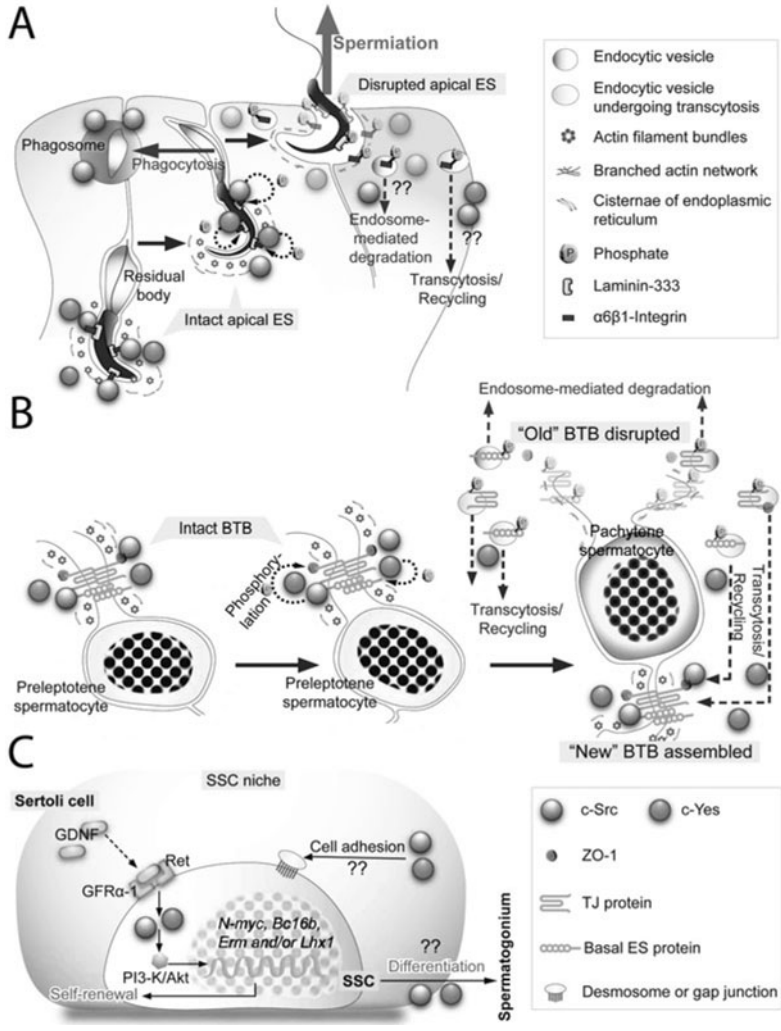

Figure 5. The involvement of c-Src and c-Yes in different cellular events during the seminiferous epithelial cycle of spermatogenesis. Based on recent findings in the field as summarized and discussed in the main text, c-Src and/or c-Yes are involved in at least three separate cellular events in the seminiferous epithelium during the seminiferous epithelial cycle of spermatogenesis as shown in (A), (B) and (C), namely (i) spermatid adhesion and the phagocytic events pertinent to the elimination of residual body at spermiation (and possibly the endocytic vesicle-mediated protein trafficking of the "old" apical ES at spermiation) (A), (ii) restructuring of the BTB to accommodate the transit of preleptotene spermatocytes across the BTB, with the "new" BTB being assembled before the "old" BTB is disrupted, so that pachytene spermatocytes can prepare for meiosis in the adluminal (apical) compartment (B) and (iii) self-renewal of spermatogonial stem cells (SSC) as well as the differentiation of SSC to spermatogonia (Type A) and the adhesion of SSC to the Sertoli cell at the stem cell niche via desmosomes and gap junctions (C). Since c-Yes was recently shown to be involved in the endocytic vesicle-mediated (e.g., clathrin) protein trafficking in the testis, in particular at the Sertoli cell BTB,[83] we postulate that c-Yes and c-Src may be involved in the endosome-mediated protein trafficking at the apical ES at spermiation (e.g., phagocytosis of residual body derived from step 19 spermatids while transforming to spermatozoa *and* degeneration of the apical ES to facilitate spermiation) and at the BTB during the transit of preleptotene spermatocytes, both events take place at Stage VIII of the epithelial cycle when c-Yes and c-Src was highest at the BTB and the apical ES, respectively (see also Figs. 3 and 4).

AN EMERGING MODEL BY WHICH SFKs REGULATE SPERMATOGENESIS IN PARTICULAR BLOOD-TESTIS BARRIER DYNAMICS IN THE SEMINIFEROUS EPITHELIUM

The model depicted in Figure 5 regarding the role of SFKs, most notably c-Src and c-Yes, in spermatogenesis, was prepared based on recently published studies in the rodent testis as described in the text above. First, c-Src and c-Yes play a critical signaling role to mediate GDNF-stimulated SSC proliferation for self-renewal of SSC via PI3K/PBK downstream (see also Fig. 1); without this, aspermatogenesis would result due to the lack of differentiated Type A and B spermatogonia. Second, c-Src and c-Yes are crucial for spermatid adhesion to the epithelium at the apical ES, possibly working in conjunction with FAK (focal adhesion kinase)[78] to maintain the proper phosphorylation status of proteins at the apical ES (e.g., integrins, laminins, nectins, JAM-C) via structural interactions between c-Src and/or c-Yes with these proteins[75,77,83,121] in response to testosterone[74,76,111,125] via the nongenomic pathway.[110,111] This action of SFKs, in concert with FAK,[126] maintain cell adhesion. This is also applicable to the Sertoli-Sertoli cell interface at the BTB via the structural interactions of c-Src with connexin 43[82] and desmoglein-2[79] and also c-Yes with occludin and N-cadherin,[83] so that c-Src and c-Yes can maintain the proper phosphorylation status of integral membrane proteins and their peripheral adaptors at the BTB. This, in turn, confers proper protein-protein interactions between integral membrane proteins and their adaptors at the BTB so that these proteins can be assembled to the TJ-fibrils at the BTB to maintain the immunological barrier integrity. For instance, it was shown that a loss of FAK, which is a component of the occludin-ZO-1 adhesion protein complex,[126] by RNAi using FAK-specific siRNA duplexes was found to induce dissociation between occludin and ZO-1, which was accompanied by unwanted phosphorylation of occludin.[127] Perhaps c-Src and c-Yes also regulate endocytic vesicle-mediated protein trafficking events (e.g., endocytosis, transcytosis, recycling) via the effects of c-Yes on actin filament dynamics.[83] It is obvious that the action of c-Src and c-Yes on different cellular events pertinent to spermatogenesis in particular their role in BTB dynamics as depicted in the model shown in Figure 5 will require additional investigation in the years to come, however, this model provides a framework for investigators in the field, including our laboratory, to design functional experiments. For instance, besides mediating the effects of GDNF to induce SSC proliferation, can c-Src and c-Yes confer adhesion of SSC to the Sertoli cell at the stem cell niche to maintain proper signaling between Sertoli cells and SSC to fine tune spermatogonial differentiation via gap junctions and/or desmosomes? If this is the case, what is the significance of cell adhesion conferred by SFK kinases to the maintenance of SSC population at the stem cell niche?

CONCLUSION AND FUTURE PERSPECTIVES

As briefly summarized above, it is increasingly clear that members of the SFK, most notably c-Src and c-Yes, are two unlikely partners that regulate spermatogenesis, most notably the BTB and the apical ES function. Both c-Src and c-Yes have been defined as crucial signaling molecules at the cell-extracellular matrix interface, similar to FAK, since they regulate focal adhesion (or focal contact) function, pertinent to cell migration during development (e.g., embryogenesis) and pathogenesis (e.g., tumorigenesis and metastasis), mediating integrin-based signaling functions;[128-130] thus, no studies were

found in the literature regarding the role of SFK members at the cell-cell interface in the seminiferous epithelium. In fact, their significance at the cell-cell AJ in the seminiferous epithelium was not known until the 2000s[131] when c-Src was first found to be a component of the apical ES at the Sertoli-spermatid interface,[72,96] most notably the integrin-based adhesion protein complex when examined by co-immunoprecipitation.[78] Since then, c-Src and its functional partner FAK, as well as c-Yes, were also found to be structurally associated with proteins at the BTB, such as occludin,[83] N-cadherin,[83] connexin 43,[82] CAR[80] and desmoglein-2.[79] Thus, these two SFKs have become two unlikely partners in regulating cell adhesion in the seminiferous epithelium. As discussed herein, members of SFK such as c-Src and c-Yes, are also involved in GDNF-mediated SSC proliferation for self-renewal to maintain the proper SSC/spermatogonia population at the stem cell niche. It remains to be investigated if c-Src and/or c-Yes are involved in other aspects of spermatogenesis, most notably, cell cycle progression, meiosis and germ cell apoptosis. Since, cancer cells are known to utilize c-Src signaling to regulate cell cycle progression during tumorigenesis[132,133] and other studies have shown that androgen-induced Src activation regulates cell cycle progression, apoptosis, migration and differentiation in cancer cells, such as in prostate and breast cancer;[134-136] thus, it is logical that future studies should include an investigation on the role of c-Src and c-Yes on cell cycle progression and meiosis in spermatocytes and cell apoptosis in spermatocytes and spermatids during spermatogenesis. Furthermore, much work is needed to delineate the role of c-Src and c-Yes in endocytic vesicle-mediated protein trafficking at the apical ES and the BTB, in particular how these nonreceptor protein tyrosine kinases work in concert with FAK to mediate protein endocytosis, recycling and transcytosis to maintain the homeostasis of apical ES and BTB function. For instance, endocytic vesicle-mediated protein trafficking events are known to involve SFK[137] and recent studies have demonstrated unequivocally the involvement of Src in protein endocytosis and/or recycling during carcinogenesis.[138-141] In short, we have provided a working hypothetical model to investigate the role of two unlikely partners, c-Src and c-Yes, on multiple cellular events pertinent to spermatogenesis, such as at the apical ES and the BTB.

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CHAPTER 16

ROLE OF P-GLYCOPROTEIN AT THE BLOOD-TESTIS BARRIER ON ADJUDIN DISTRIBUTION IN THE TESTIS

A Revisit of Recent Data

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Abstract: The blood-testis barrier (BTB) is one of the tightest blood-tissue barriers in mammals including rodents and humans. It is used to sequester meiosis I and II, postmeiotic spermatid development via spermiogenesis and the release of sperm at spermiation from the systemic circulation, such that these events take place in an immune-privileged site in the adluminal (apical) compartment behind the BTB, segregated from the host immune system. Additionally, drug transporters, namely efflux (e.g., P-glycoprotein) and influx (e.g., Oatp3) pumps, many of which are integral membrane proteins in Sertoli cells at the BTB also work cooperatively to restrict the entry of drugs, toxicants, chemicals, steroids and other xenobiotics into the adluminal compartment. As such, the BTB that serves as an important physiological and selective barrier to protect germ cell development also poses a "hurdle" in male contraceptive development. For instance, adjudin, 1-(2,4-dichlorobenzyl)-*1H*-indazole-3-carbohydrazide, a potential nonhormonal male contraceptive that exerts its effects on germ cell adhesion, most notably at the Sertoli cell-spermatid interface, to induce "premature" germ cell loss from the seminiferous epithelium mimicking spermiation, has a relatively poor bioavailability largely because of the BTB. Since male contraceptives (e.g., adjudin) will be used by healthy men for an extended period of his life span after puberty, a better understanding on the BTB is necessary in order to effectively deliver drugs across this blood-tissue barrier in particular if these compounds exert their effects on

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developing germ cells in the adluminal compartment. This can also reduce long-term toxicity and health risk if the effective dosing can be lowered in order to widen the margin between its safety and efficacy. Herein, we summarize latest findings in this area of research, we also provide a critical evaluation on research areas that deserve attention in future studies.

INTRODUCTION

The blood-testis barrier (BTB) and the blood-brain barrier (BBB) are considered to be two of the tightest blood-tissue barriers in the mammalian body.[1-5] This consideration is based on studies performed more than a century ago when dyes were administered to laboratory animals, only the testis and the brain were found to remain unstained,[1] illustrating the presence of a barrier in these two organs. Structurally, however, the BTB and the BBB are quite different. For instance, the BTB is constituted by specialized junctions between adjacent Sertoli cells near the basement membrane of the seminiferous tubules in which microvessels located in the interstitium contribute relatively little permeability barrier function to the BTB. For the BBB, it is almost exclusively constituted by endothelial tight junction (TJ) in the capillaries near the base of the brain, with minor structural contributions by pericytes.[1,6] While TJ is also found at the Sertoli-Sertoli cell interface at the BTB, the typical TJ ultrastructure is being reinforced by co-existing basal ectoplasmic specialization [basal ES, a testis-specific adherens junction (AJ) type] limited only to the BTB in the mammalian testis, such as in rats.[1,7] At the BTB, tightly packed actin filament bundles that lie perpendicular to the Sertoli cell plasma membrane are sandwiched between cisternae of endoplasmic reticulum and the two apposing Sertoli cell plasma membranes.[1,7] Besides TJ, basal ES also coexists with gap junction. Collectively, these junctions coupled with desmosome constitute the BTB. While these unusual ultrastructural features serve well to protect the events of meiosis I and II, postmeiotic spermatid development and spermiation from drugs, chemicals and toxicants that may be found in the systemic circulation, they also pose a significant barrier to male contraceptives and/or therapeutic agents if these drugs exert their effects primarily behind the BTB.[1,8] Recent studies have shown that this is largely due to the presence of multiple drug transporters found in the testis, mostly restricted to the Sertoli cells that constitute the BTB, but also in different germ cell types ranging from spermatogonia, spermatocytes, spermatids and spermatozoa, to limit the entry and/or the distribution of drugs behind the BTB.[1,9] Herein, we summarize some recent findings in the field based on studies on adjudin, a potential male contraceptive, regarding its tissue distribution and bioavailability following its administration in rats. These findings are helpful in future studies to design better compounds and/or formulations to improve the bioavailability and distribution in the testis behind the BTB.

ADJUDIN AND ITS BIOAVAILABILITY IN THE TESTIS

When [^{3}H]-adjudin was administered to adult rats via gavage and the distribution of [^{3}H]-adjudin in different organs, including the testis, was monitored thereafter, it was found that few than 1% of administered adjudin was uptaken by the testis,[10] even though it was found to be a very potent drug to induce germ cell loss from the seminiferous epithelium in different treatment regimens performed over at least a 5-year period.[10,11]

Also, adjudin was found to be rapidly metabolized and cleared from the host body, such as in rats, usually within 20-24 hours either administered orally[10] or through i.v.[12] These findings thus illustrate that the narrow margin that was observed between its efficacy and safety in a subchronic toxicity study in rats could be "widened" if adjudin and other male contraceptives under development in the field, could be reformulated to improve their tissue distribution and thus bioavailability in the testis.[8]

DRUG TRANSPORTERS AND BLOOD-TESTIS BARRIER (BTB) DYNAMICS

For virtually any drugs, unless they belong to the classes of compounds that can bind to specific receptors, such as antagonists or agonists of cytokines (e.g., TGF-β and TNFα), hormones (e.g., FSH) and/or cytokines per se that can competitively bind onto the corresponding receptors of hormones (e.g., FSH receptors) or cytokines (e.g., TGF-β receptors, TNF receptors) in Sertoli cells, they are restricted from entering the adluminal compartment behind the BTB since one of the major functions of the BTB is to restrict paracellular flow of substances.[1-4,9] However, many of these compounds, including drugs, can penetrate the BTB to gain access to the adluminal compartment via drug transporters utilizing the transcellular route. However, premeiotic (e.g., spermatogonia, early spermatocytes) and postmeiotic (e.g., late primary and secondary spermatocytes, spermatids and sperm) germ cells are also equipped with efflux pump transporters to actively pump harmful, but also therapeutic, drugs and substances out of the adluminal compartment.[1,9,13] At present, ~800 drug transporters are known to date found in different epithelial/endothelial cells including cancer cells;[14-16] as well as Sertoli cells and almost all classes of germ cells in the testis.[1,9,17] Unfortunately, virtually all the reports found in the literature regarding the study of drug transporters in the testis were limited to cellular distribution/localization studies, investigating the cellular expression of different drug transporters in the testis without any pertinent functional studies in particular the transport of drugs across the BTB.[9,13,17]

In the testis, drug transporters can be broadly classified into two categories: namely the ATP-binding cassette (ABC) drug transporters and the SoLuteCarrier (SLC) drug transporters. ABC drug transporters in the testis are efflux pumps which either prevents drugs (e.g., adjudin) from entering the adluminal compartment or pumps drugs out of the adluminal compartment should they enter the immune-privileged site via influx pumps, and these drug transporters are ATP-dependent drug pumps. The best studied ABC transporters in the testis are: (1) P-glycoprotein (a multidrug resistance protein, also known as *Mdr*, or *Abcb1*), (2) multidrug resistance-related protein (*Mrp*) and (3) breast cancer resistance protein (*Bcrp*), all of which are found in Sertoli cells.[9,13] For SLC drug transporters in the testis, they are mostly influx pumps which pump drugs (e.g., adjudin) ATP-independently into the adluminal compartment behind the testis, and the energy required for transport is coming from gradient generated by a primary active transport system or drugs can pass through a SLC transporter through the built-in "pores".[1] The best studied SLC transporters in the testis are: (1) organic anion transporting polypeptide (OATP) transporters (e.g., Oatp3) and (2) organic anion transporter (OAT)/organic cation transporter (OCT)/organic cation/carnitine transporter (OCTN) (e.g., *Slc22a5* also known as OCTN2, a SLC organic cation transporter family member 5 and also known as Na+-dependent organic cation/carnitine transporter 2; *Slco6b1*, a SLC organic anion transporter family member 6b1 also known as a testis-specific transporter-1 (TST-1); *Slco6c1*, a SLC organic anion transporter

family member 6c1, also known as TST-2].[1] Study has shown that [^{3}H]-adjudin enters the adluminal compartment via these influx pumps as demonstrated in an in vitro system by culturing Sertoli cells on Matrigel-coated bicameral units with negligible germ cell contamination which formed a functional TJ-barrier that mimicked the Sertoli BTB in vivo.[13] For instance, a knockdown of either Oatp3 by RNAi or a knockdown of all four influx pumps: Oatp3, *Slc22a5*, *Slco6b1* and *Slco6c1* by RNAi using specific corresponding siRNA duplexes versus nontargeting control siRNA duplexes was found to significantly impede the flow of [^{3}H]-adjudin from the basal to the apical compartment.[13] In short, the distribution of [^{3}H]-adjudin is the net result of interactions of multiple drug transporters (perhaps in the 100s), both influx and efflux drug transporters, in which adjudin serves as a substrate to these drug pumps. These findings also illustrate the complexity of drug designs for male contraceptive development.

P-GLYCOPROTEIN REGULATES THE BIOAVAILABILITY OF ADJUDIN IN THE TESTIS

P-glycoprotein is one of the best studied efflux pumps in the testis.[9,13] A recent study has shown that P-glycoprotein is localized predominantly at the BTB in the seminiferous epithelium of adult rat testes in all stages of the seminiferous epithelium cycle.[18] Its expression at the BTB is noted to be the highest at Stages IV-VII of the seminiferous epithelial cycle, diminished somewhat at Stages VIII-IX[18] when the BTB undergoes restructuring to accommodate the transit of preleptotene spermatocytes at the site to enter the adluminal compartment for further development, suggesting P-glycoprotein may possibly play a role in regulating BTB dynamics. In studies based on the use of co-immunoprecipitation coupled with dual-labeled immunofluorescence analysis, P-glycoprotein was found to structurally interact with TJ-integral membrane proteins occludin, claudin-11 and JAM-A,[18] as well as FAK.[19] This latter observation is important since FAK is an important nonreceptor protein tyrosine kinase known to be involved in integrin-based signaling[20,21] and plays a crucial role in conferring junction permeability.[22,23] In fact, FAK was recently shown to be an integrated component of the occludin-ZO-1 protein complex at the BTB,[24] and it plays a critical role in regulating the Sertoli cell TJ-permeability barrier function.[25] Thus, the structural association of P-glycoprotein and FAK seemingly suggests that this efflux drug transporter may be functioning in some other ways via its interactions with the occludin-ZO-1-FAK protein complex besides being served as a drug transporter. Indeed, it was found that the silencing of P-glycoprotein in Sertoli cell epithelium in vitro, in which the Sertoli cell epithelium had a functional TJ-barrier with ultrastructures of TJ, basal ES, gap junction and desmosome,[26,27] led to a disruption of the TJ-barrier function,[19] that is, making the TJ-barrier "leaky" when P-glycoprotein was knockdown. Interestingly, pharmacokinetics analysis of the flux of [^{3}H]-adjudin across the Sertoli cell BTB has shown that the silencing of P-glycoprotein by RNAi did not render the Sertoli cell BTB to become completely "disrupted" and "freely" permeability to the transport of [^{3}H]-adjudin across the barrier, perhaps due to the presence of other efflux pumps at the site, such as *Mrp* and *Bcrp*, which are known to be present at the Sertoli cell BTB.[18,28,29] Interestingly, in a pharmacokinetics study to assess the flux of [^{3}H]-adjudin from apical to basal (A to B) or basal to apical (B to A) compartment using Sertoli cells cultured on bicameral units having the establishment of a functional TJ-barrier, it was found that while the silencing of P-glycoprotein by RNAi affected the flux of [^{3}H]-adjudin from B to A (i.e., making the BTB "leaky"), it failed to perturb the A

to B flux illustrating while P-glycoprotein guards the entry of [^{3}H]-adjudin to the adluminal compartment of the epithelium, it did not "eliminate" adjudin that was already "admitted" to the adluminal compartment via influx pump transporters.[19,30] These findings thus illustrate the intriguing role of P-glycoprotein in regulating the distribution adjudin in the testis. Of course, it remains to be determined if other efflux drug transporters, such as *Mrp* and *Bcrp*, are effective to pump adjudin that has reached the adluminal compartment out of the BTB, or how these three drug efflux pumps work cooperatively to regulate the distribution/bioavailability of adjudin in the adluminal compartment behind the BTB.

INTERACTIONS OF ADJUDIN WITH P-GLYCOPROTEIN IN RATS AND HUMANS: A MOLECULAR MODELING ANALYSIS

Introduction

In order to better understand the structural interactions between adjudin and P-glycoprotein so that second generation analogs of adjudin can be synthesized to improve their transport across the BTB to enter the adluminal compartment, so that they can induce "premature" germ cell depletion from the epithelium more effectively than adjudin, we sought to perform molecular modeling analysis to identify the putative interacting domain and/or amino acid residues between the two molecules.

Tertiary Structure Prediction for P-Glycoprotein of Rat and Human

The amino acid sequences of P-glycoprotein of *Rattus norvegicus* (UniProtKB ID: P43245) and *Homo sapiens* (UniProtKB ID: P08183) were retrieved from UniProt (http://www.uniprot.org). A BLASTp search[31] was carried out to find appropriate proteins with significant amino acid sequence and structural similarity to P-glycoprotein by searching the Protein Data Bank (PDB) database at http://www.rscb.org/pdb/.[32] The search was refined to find a suitable structural homolog for the modeling of P-glycoprotein in rat and human. The amino acid sequence of these two proteins and their template sequences were aligned using the Align Sequence to template, a sequence alignment tool in Accelrys Discovery Studio 3.1(DS3.1), (Accelrys Software, http://accelrys.com/). Based on the alignment generated, the tertiary structure of P-glycoprotein of rat and human were predicted using Build Homology Models tool from DS3.1. Discrete Optimized Protein Energy (DOPE) and Modeller Objective Function (MOF) scores of the resulting models were used to select the most reliable model. The predicted structures were energy minimized by Smart Minimizer algorithm in Discovery Studio 3.1. The minimization was carried out in 500 steps by applying CHARMm force field and then subjected to validation. Backbone conformation was evaluated by examining the Psi/Phi interactions in Ramachandran Plot, obtained from PROCHECK.[33] Based on the plot, residues in the disallowed regions were refined using Loop Refinement (MODELER) module, from DS3.1. The final refined model was tested for their stability and reliability using ERRAT.[34]

Molecular Docking of Rat and Human P-Glycoprotein with Adjudin

Active sites of proteins are often associated with structural pockets in the protein. The identification of such substrate binding sites in enzymes helps us to understand their

binding interactions with substrates and other small molecules. The drug binding site of P-glycoprotein was taken from the template structure that was used for homology modeling. The docking simulation tool, Glide (Schrödinger, Inc.) was used to perform docking with each of the two proteins and adjudin. The modeled proteins were prepared using the Protein Preparation Wizard, a workflow in the Schrödinger Suite of programs. Using this tool, all hydrogen atoms were added to the proteins, the protonation states for histidine residues were optimized and the entire protein was minimized using OPLS-2005 force field. The ligand, adjudin was prepared using Schrödinger Ligprep tool (version 2.3, Schrödinger, LLC, New York, NY, 2009). LigPrep was used to find stereoisomers and to perform energy minimization using OPLS_2005 force field. The binding site was defined in terms of two concentric cubes: the bounding box, which contains the center of any acceptable ligand pose and the enclosing box, which contains all ligand atoms of an acceptable pose. The prepared ligand was docked flexibly to the two P-glycoproteins using Simple Precision mode (SP) mode in Glide (Version 5.5, Schrödinger, LLC, New York, NY). To soften the potential for nonpolar parts of the ligand, the vdW (van der Waal's) radii of ligand atoms were scaled with partial atomic charge (absolute value) less than the specified cutoff. Default scaling factor was 0.8 and the partial cutoff value was 0.15. The Glide docking algorithm generated 5000 poses per ligand for the initial phase of docking and restricted to 400 poses for energy minimization. Upon completion of each docking calculation, the best docked structure was chosen using Glidescore (Gscore) function, a modified and extended version of the empirically based Chemscore function.

Homology Modeling

The amino acid sequence of P-glycoprotein of rat and human was used to find a suitable structural template using BLASTp against PDB database. The best homolog was selected based on similarity score and crystal structures with better resolution. The template crystal structure of multidrug resistance protein (PDB ID: 3G60), namely P-glycoprotein, from mouse[35] showed 82% and 87% identity with rat and human P-glycoprotein sequence, respectively. The structure of P-glycoprotein of rat and human was modeled and the best reliable models were subjected to loop refinement by DS3.1 and validation by ProCheck.

Validation for Modeled Structure of Rat and Human P-Glycoprotein

The empirical distribution of data points observed in rat (Fig. 1) and human (Fig. 2) P-glycoprotein were subjected to Ramachandran plot analysis (see Figs. 1 and 2) for structure validation, illustrating the theoretically "favored", "allowed" and "generously allowed" regions as defined by PROCHECK. The validation of the two modeled P-glycoprotein structures shows that the stereochemical geometry as well as the overall structural geometry of the models is good. For rat and human P-glycoprotein, the number of residues in most favored regions, additional allowed regions, generously allowed regions and disallowed regions are: 967 (86.6%), 114 (10.2%), 36 (3.2%) and 0 (0%); and 909 (87.7%), 107 (10.3%), 21 (2%) and 0 (0%), respectively. The secondary structure of the two modeled proteins was also generated by PDBsum (Fig. 3A,B),[36] in which the structure of P-glycoprotein of rat (Fig. 3A) and human (Fig. 3B) represents secondary structure elements (alpha-helices and beta-sheets) together

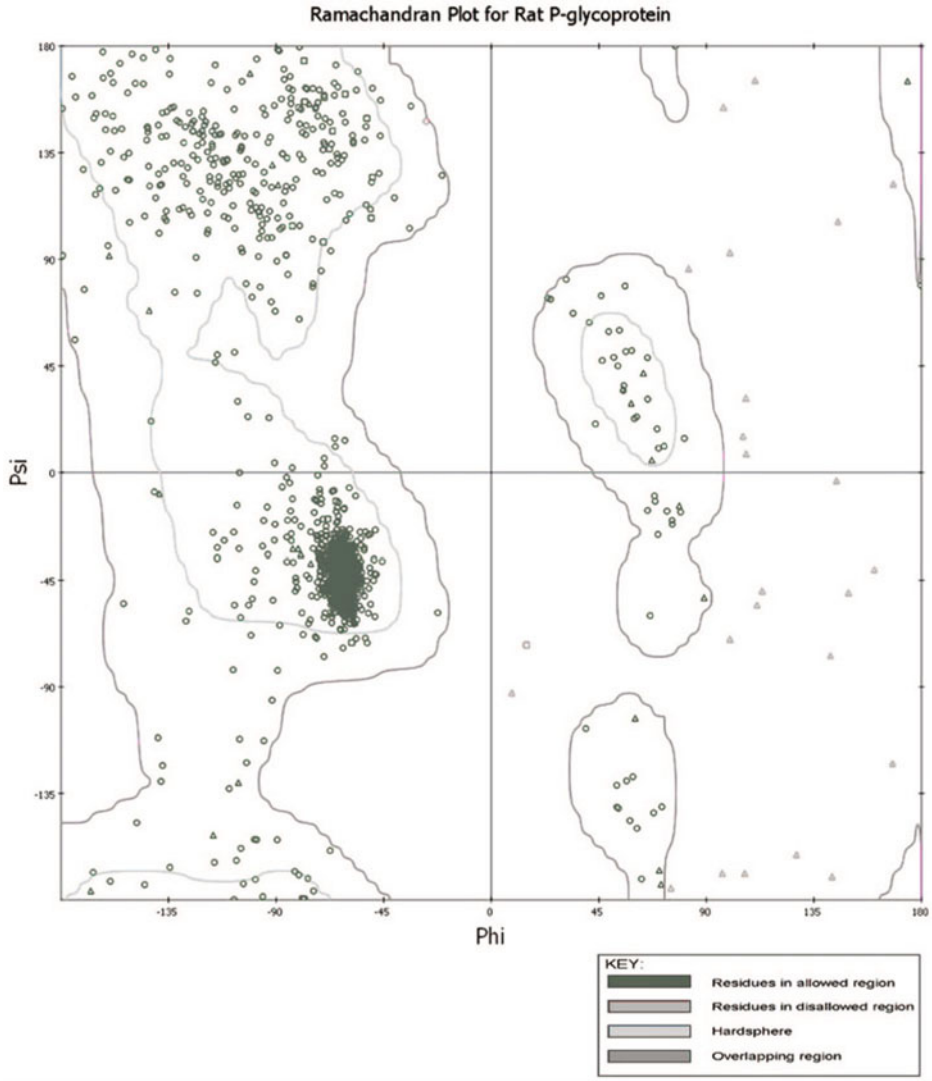

Figure 1. Ramachandran plot for the modeled rat P-glycoprotein.

with various structural motifs, such as beta- and gamma-turns, and beta-hairpins. The 3D model of P-glycoprotein of rat and human shows an inward-facing conformation closely representing a two-fold symmetry. The nucleotide binding domains (NBDs) are separated and the inward facing conformation is formed from two bundles of six helices. This results in a large internal cavity open to both the cytoplasm and the inner leaflet (see Figs. 4 and 5). The modeled structure for rat (Fig. 4) and human (Fig. 5) P-glycoprotein is consistent with the template crystal structure of mouse P-glycoprotein.[35] Thus, these modeled structures can be further used to study interactions with small molecules, such as adjudin.

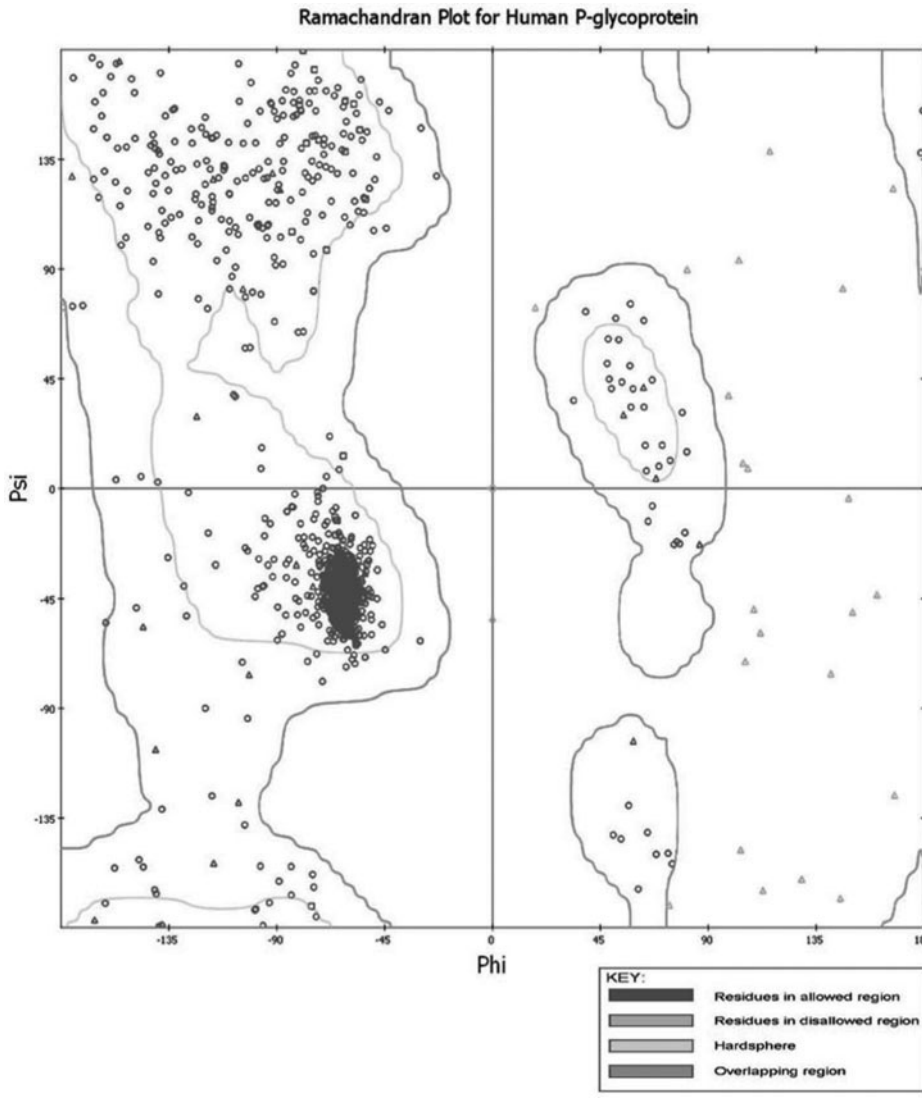

Figure 2. Ramachandran plot for the modeled human P-glycoprotein.

Molecular Docking Analysis

Molecular-docking was performed on the 3D model of P-glycoprotein, built by the homology modeling method (see above). The presumptive drug binding pocket of P-glycoprotein comprises mostly hydrophobic and aromatic residues.[35] The modeled structure reveals that the inward facing conformation is competent to bind drugs. The common residues involved in the drug binding cavity of inward facing crystal structure of mouse P-glycoprotein and the outward facing homology model from Sav1866 structure (note: Sav1866 is the first high-resolution structure of an ABC exporter of Sav1866 from

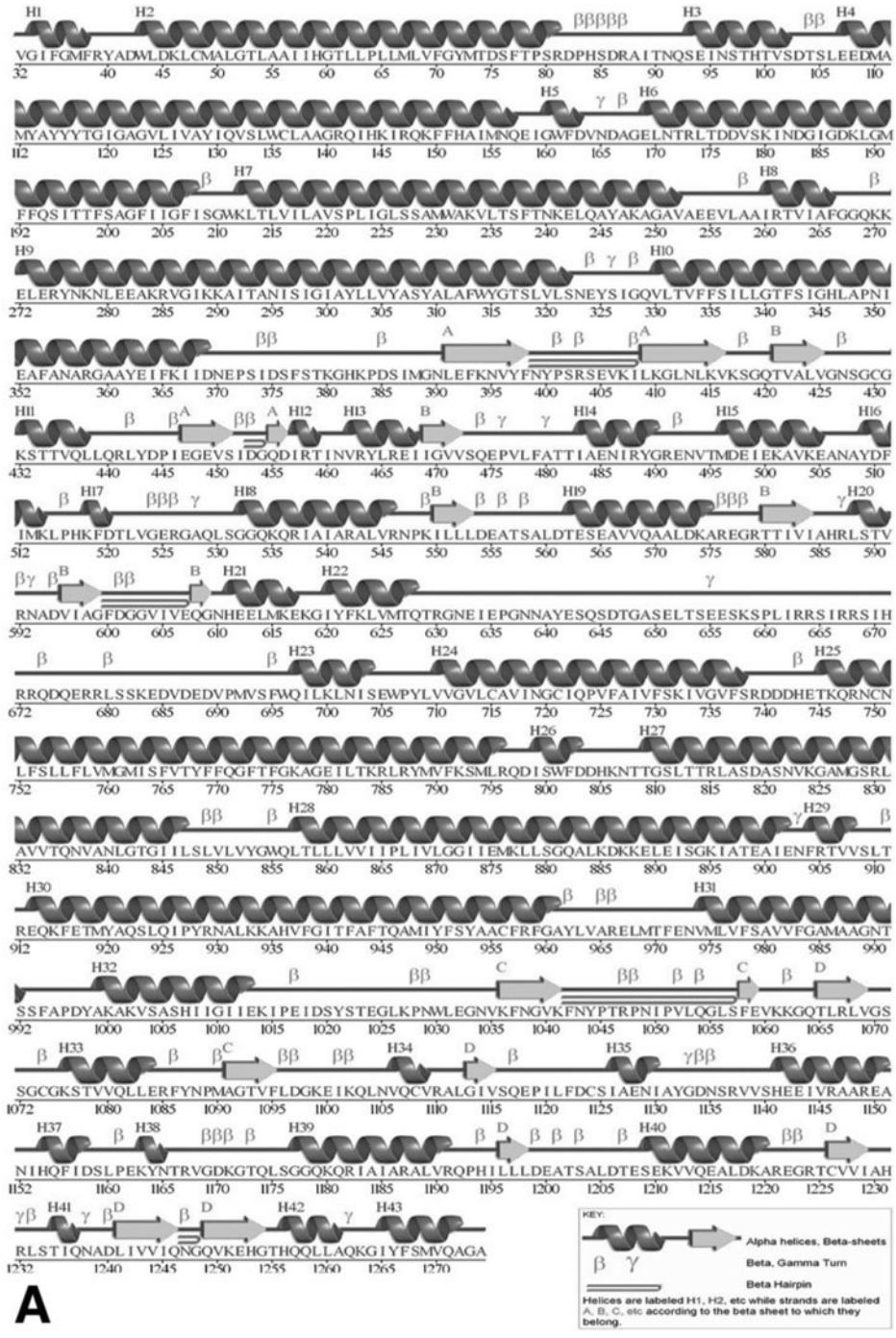

Figure 3. A) Secondary structure representation for modeled structures of rat P-glycoprotein. Figure continued on next page.

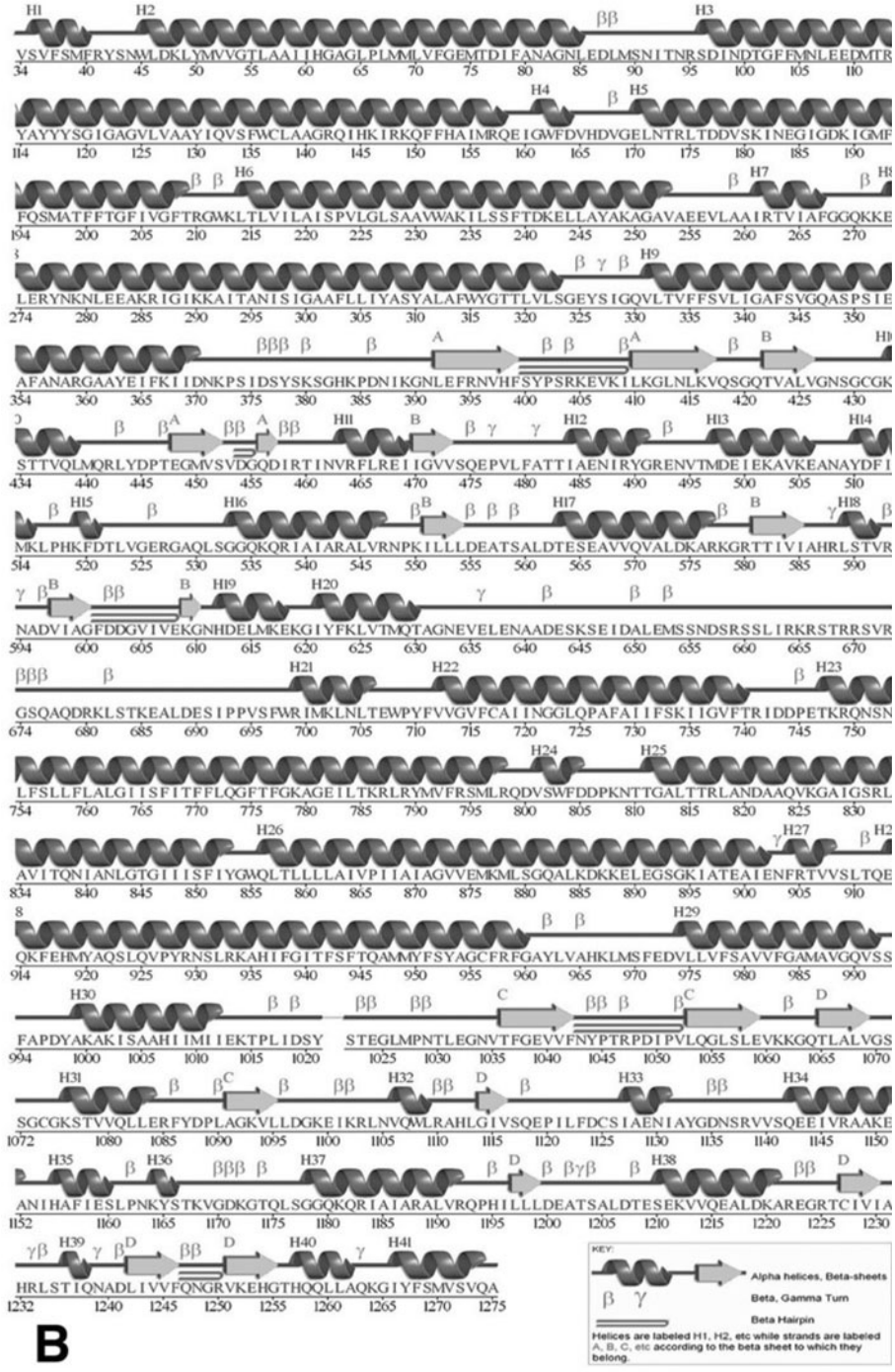

Figure 3, continued from previous page. B) Secondary structure representation for modeled structures of human at P-glycoprotein.

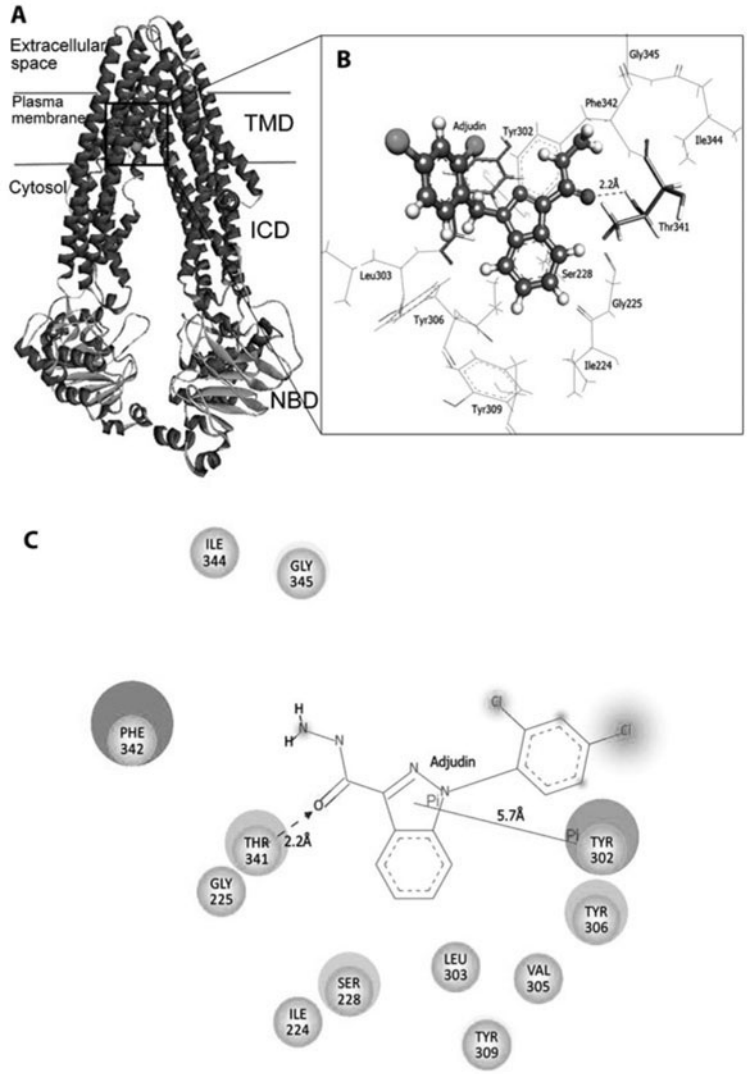

Figure 4. Docked complex of rat P-glycoprotein with adjudin. A) This depicts the 3-D modeled rat P-glycoprotein in ribbon format, docked with adjudin and its relative configuration across the Sertoli cell plasma membrane. TMD (transmembrane domain), ICD (intracellular domain) and NBD (nucleotide-binding domain where ATP binds). B) This represents an enlarged view of the interacting residues in CPK 3D conformation (CPK is a coloring scheme named after the CPK molecular models designed by Robert Corey and Linus Pauling and improved by Walter Koltun: "white" for hydrogen; "black" for carbon; "blue" for nitrogen; "red" for oxygen; "green" for chloride). C) Two-dimension representation of molecular interactions between adjudin and the rat P-glycoprotein. "Green" circles represent residues involved in van der Waals interactions; "Pink" circles represent residues involved in hydrogen bond, polar or charge interactions; "Blue" halo around residues represent solvent accessible surface of an interacting residue. "Orange" Line represents Pi-Pi interaction (Pi stacking interaction refers to noncovalent interactions between aromatic rings) between Tyr^{302} and adjudin and "blue" dotted line represents hydrogen bond formation between side chain of Thr^{341} and donor oxygen atom of adjudin. GLY (Gly, G), Glycine; ILE (Ile, I), Isoleucine; LEU (Leu, L), Leucine; PHE (Phe, F), Phenylalanine; SER (Ser, S), Serine; THR (Thr, T), Threonine; TYR (Tyr, Y), Tyrosine; VAL (Val, V), Valine. A color version of this figure is available online at www.landesbioscience.com/curie.

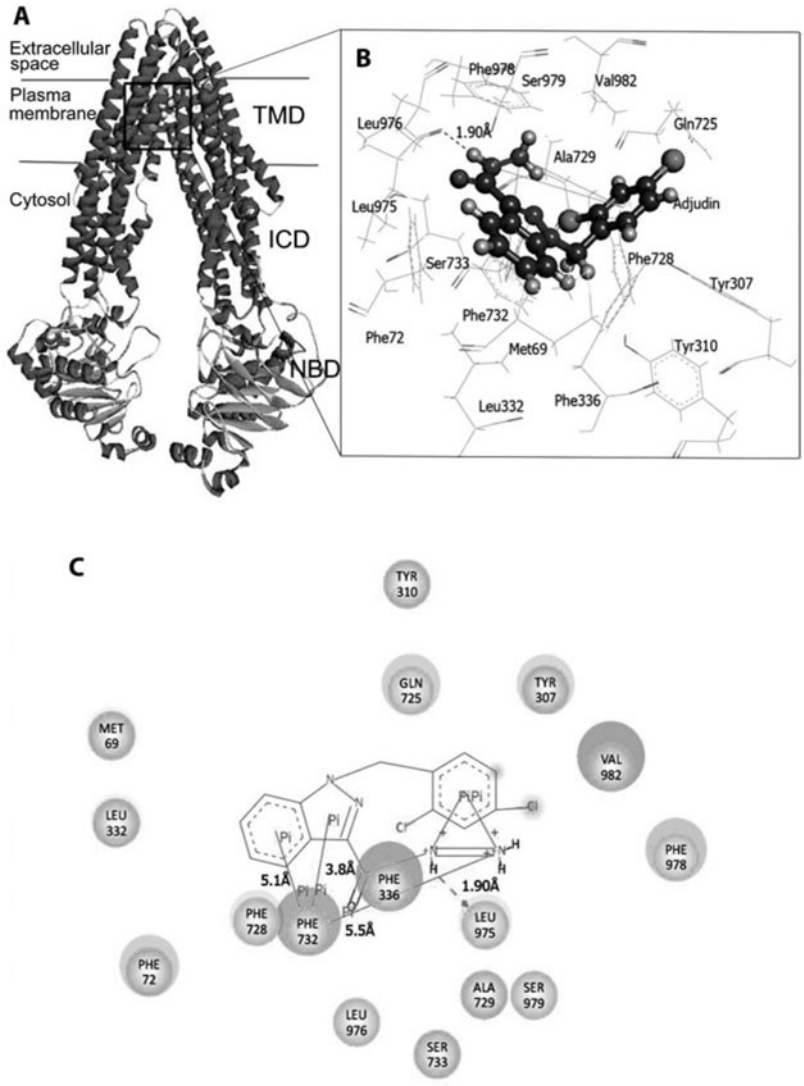

Figure 5. Docked complex of human P-glycoprotein with adjudin. A) The 3-D modeled human P-glycoprotein in ribbon format, docked with adjudin and its relative configuration across the Sertoli cell plasma membrane. TMD (transmembrane domain), ICD (intracellular domain) and NBD (nucleotide-binding domain where ATP binds). B) Enlarged CPK view of the docking site in (A) ("white" for hydrogen; "black" for carbon; "blue" for nitrogen; "red" for oxygen; "green" for chloride). Adjudin is depicted in scaled ball-and-stick model and interacting residues are in stick model and their interactions in its 3D conformation. C) Two-dimension representation of molecular interactions between adjudin and the human P-glycoprotein. "Green" circles represent residues involved in van der Waals interactions; "Pink" circles represent residues involved in hydrogen bond, polar or charge interactions; "Blue" halo around residues represent solvent accessible surface of an interacting residue. "Orange" lines represent Pi-Pi and Pi-cation interactions between Phe^{732} and adjudin. "Green" dotted line represents hydrogen bond formation with main chain of Leu^{975} with an estimated bond length of 1.90. ALA (Ala, A), Alanine; GLN (Gln, Q), Glutamine; LEU (Leu, L), Leucine; MET (Met, M), Methionine; PHE (Phe, F), Phenylalanine; SER (Ser, S), Serine; TYR (Tyr, Y), Tyrosine; VAL (Val, V), Valine. A color version of this figure is available online at www.landesbioscience.com/curie.

Staphylococcus aureus)[37-39] was used in defining the binding site for docking with adjudin. Since P-glycoproteins from rat and human share significant similarity in their sequence and structure, the same binding pocket residues with slight variation corresponding to their sequence position were specified for docking (Figs. 4 and 5). Many of these residues face the drug binding pocket and are highly conserved, suggesting a common mechanism of poly-specific drug recognition. The drug binding site of P-glycoprotein resides in the cell membrane and is formed by TM helices.[40]

Docking of P-Glycoprotein with Adjudin

The docking simulation of P-glycoprotein with adjudin shows that adjudin binds to rat P-glycoprotein with a docking score of -5.497kcal/mol and human P-glycoprotein with a docking score of -7.496 kcal/mol. The docking energy and van der Waal's energy involved in docking are tabulated in Table 1. The docked complex of rat P-glycoprotein illustrates that adjudin forms hydrogen bond with Thr^{341} and forms a Pi-Pi interaction with Tyr^{302} (Fig. 4A,B). The docked complex of human P-glycoprotein shows that Leu^{975} forms hydrogen bond with adjudin. Phe^{732} plays a major role in forming Pi interactions with two Pi-Pi interactions and a Pi-cation interaction with the ligand (Fig. 5A,B). Two dimension plot created using DS3.1 for both the docked complexes illustrates a detailed view of the types of molecular interactions between adjudin and P-glycoprotein of rat and human (Figs. 4C and 5C).

Table 1. Molecular interactions of P-glycoprotein with adjudin

Receptor	Docking Score (kcal/mol)	Docking Energy (kcal/mol)	Van der Waal's Energy (kcal/mol)	Hydrogen Bond Interacting Residues	Van der Waal's Interaction Residues	Pi Stacking Interaction Residues
Human P-glyco-protein	–7.496	-38.798	-38.170	Leu975 (1.90Å)	Met69, Phe72, Tyr307, Tyr310, Leu332, Phe336, Ala729, Phe732, Ser733, Val982	Phe732
Rat P-glyco-protein	-5.497	-31.679	-31.483	Thr341 (2.2Å)	Ile224, Gly225, Ser228, Tyr302, Leu303, Val305, Tyr306, Tyr309, Ile344	Tyr302

CONCLUSION

As reported in two recent studies illustrating that the entry of drugs (e.g., [^{3}H]-adjudin) to the adluminal compartment behind the BTB is mediated via the combined effects of influx (e.g., Oatp3, OCTN2, TST-1, TST-2)[30] and efflux (e.g., P-glycoprotein)[19] drug transporters since the silencing of these genes via the use of specific siRNA duplexes without any detectable off-target effects would impede the transport of [^{3}H]-adjudin across the Sertoli cell BTB. These findings also illustrate the significance of understanding the regulation of the BTB and how drug transporters regulate the barrier function via its interactions with FAK and some of the adhesion protein complexes, such as occludin-ZO-1 and JAM-A-ZO-1, at the BTB. More important, preliminary molecular modeling studies, such as those summarized above, have demonstrated the presence of putative interacting domains and the involving amino acid residues within the 3D structure of P-glycoprotein, both in rats and humans, that structurally engage adjudin via hydrogen bonds and van der Waal's force (see Figs. 4 and 5). This information should be helpful to design other analogs of adjudin that can have "lesser" interactions with efflux pumps but "better" interactions with influx pumps to optimize their transport across the BTB and their retainment in the adluminal compartment. In short, molecular modeling can be a powerful tool to design male contraceptive drugs based on some existing known molecular entitites, in particular those that have poor bioavailability and distribution due to their interactions with drug transporters at the BTB.

NOTE ADDED IN PROOF

An important recent paper from Reina Bendayan's Laboratory has demonstrated that ABC efflux drug transporters (e.g., P-glycoprotein) are also important to regulate the transport of antiretroviral drugs (e.g., atazanavir, mitoxantrone) across the BTB using TM4 cells (a Sertoli cell line)[41] as the study model, which is in agreement with our latest findings.[19]

ACKNOWLEDGMENTS

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CHAPTER 17

THE APICAL ECTOPLASMIC SPECIALIZATION-BLOOD-TESTIS BARRIER FUNCTIONAL AXIS IS A NOVEL TARGET FOR MALE CONTRACEPTION

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Abstract: The blood-testis barrier (BTB), similar to other blood-tissue barriers, such as the blood-brain barrier and the blood-retinal barrier, is used to protect the corresponding organ from harmful substances (e.g., xenobiotics) including drugs and foreign compounds. More importantly, the BTB allows postmeiotic spermatid development to take place in an immune privileged site at the adluminal (or apical) compartment to avoid the production of antibodies against spermatid-specific antigens, many of which express transiently during spermiogenesis and spermiation. The BTB, however, also poses an obstacle in developing nonhormonal-based male contraceptives by sequestering drugs (e.g., adjudin) that exert their effects on germ cells in the adluminal compartment. The effects of these drugs include disruption of germ cell cycle progression and development, apoptosis, cell adhesion, metabolism and others. Recent studies have demonstrated that there is a functional axis that operates locally in the seminiferous epithelium to co-ordinate different cellular events across the Sertoli cell epithelium, such as spermiation and BTB restructuring during the seminiferous epithelial cycle of spermatogenesis. Components of this functional axis, such as the apical ectoplasmic specialization (apical ES, a testis-specific atypical anchoring junction type) and the BTB, in particular their constituent protein complexes, such as α6ß1-integrin and occludin at the apical ES and the BTB, respectively, can be the target of male contraception. In this chapter, we

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highlight recent advances regarding the likely mechanism of action of adjudin in this functional axis with emphasis on the use of molecular modeling technique to facilitate the design of better compounds in male contraceptive development.

INTRODUCTION

Male contraception is an important alternative in family planning.[1-3] An ideal male contraceptive, such as a male pill, must be safe, effective, accessible, inexpensive, preferably long-acting yet reversible and culturally acceptable by couples in both developing and industrialized nations, similar to the "pills" for women. Unfortunately, this ideal has proven to be elusive after decades of research and efforts in the field. One of the major obstacles for the development of male contraceptives, unlike pills for women, is our incomplete understanding of spermatogenesis, namely the biology of sperm production in the testis. Even though the cellular events pertinent to spermatogenesis[4-8] and the hypothalamic-pituitary-testicular axis that regulates spermatogenesis[9-12] are well understood, the underlying mechanisms that regulate spermatogenesis at the molecular and biochemical levels remain largely unexplored.[8,13-17] Based on a recently completed Phase III multicenter trial in China,[18] it seems that the most promising lead of putting a male contraceptive on the market at present is via the use of monthly testosterone injections based on testosterone undecanoate[19,20] (a long-acting testosterone) that suppresses spermatogenesis. Since the level of intratesticular testosterone in men and rodents is about 100-fold higher than the peripheral concentration in the systemic circulation to maintain spermatogenesis,[21,22] administration of testosterone undecanoate to men via injections thus suppresses the release of GnRH from the hypothalamus, which in turn reduces pituitary LH production, disrupting Leydig cell steroidogenesis in the interstitium in the testis. The net result of these interactions lowers the proper intratesticular testosterone level to sustain spermatogenesis. However, in a recent Phase II clinical study based on the use of a combination of long-acting progestin (norethisterone enantate) and testosterone undecanoate conducted in the United States sponsored by World Health Organization (WHO) and CONRAD, the review panel in early 2011 has recommended discontinuation of this trial since risks of possible side effects outweigh the potential benefits to the male study participants (see www.conrad.org/news-pressreleases-63.html). This latest episode thus represents a major setback in the development of testosterone injection or hormonal-based "male pills" as male contraceptives since different ethnic groups, Asian *versus* Caucasian men, can have different response to exogenous testosterone to suppress spermatogenesis, in which Asian men were found to be more susceptible to testosterone-based contraception.[23,24] Yet the use of nonhormonal contraceptives also has its own obstacles.[25] First and foremost, the blood-testis barrier (BTB) in the testis poses a major obstacle to contraceptives[26,27] if these drugs exert their effects behind the BTB in the immune privilege site in the testis, namely the adluminal (apical) compartment (Fig. 1). The BTB also confers poor bioavailability to most nonhormonal contraceptives, such as adjudin.[28,29] Thus, even though adjudin is highly potent in the testis by exerting its effects behind the BTB to induce "premature" spermiation, this low bioavailability thus narrows the margin between efficacy and safety as illustrated in a subchronic toxicity study.[30] Second, the molecular and biochemical mechanisms that regulate the cellular events of spermatogenesis remain poorly understand. For instance, the regulation of spermatogonia/spermatogonial stem cell (SSC) renewal and their differentiation, germ cell cycle progression (such as via the actions of cyclins, vitamin A and its metabolites), meiosis, spermiogenesis and spermiation are not entirely

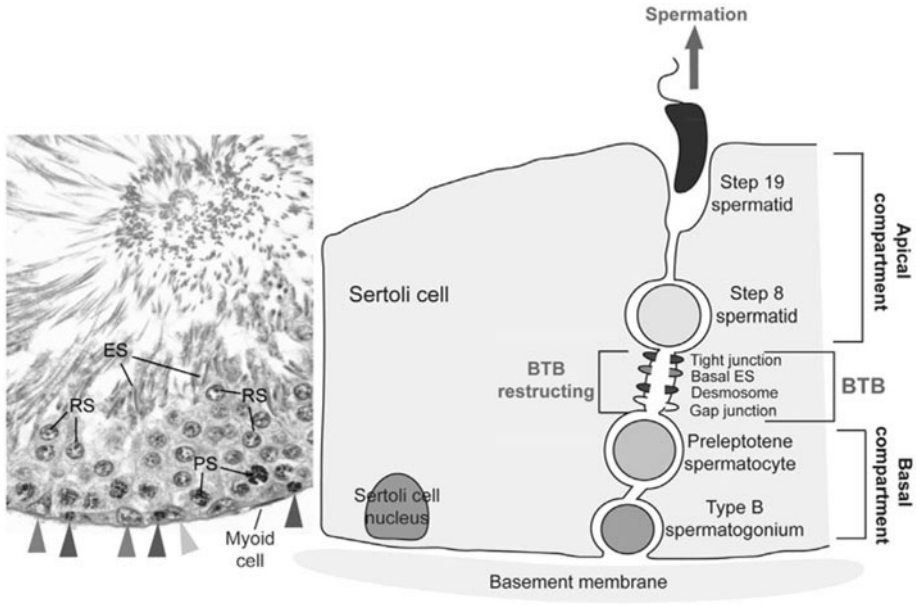

Figure 1. A schematic drawing illustrating the apical ES-BTB functional axis in the seminiferous epithelium of adult rat testis. The micrograph shown in the left panel is the cross-section of a Stage VIII seminiferous tubule from an adult rat testis illustrating the occurrence of spermiation at the luminal edge of the seminiferous epithelium. BTB also undergoes restructuring at this stage so that preleptotene spermatocytes (see "dark gray/green" arrowheads) that are differentiated from spermatogonia (see "light gray/orange" arrowhead) at this stage can be in transit at the BTB to enter the adluminal (apical) compartment to differentiate into pachytene spermatocytes (PS) to prepare for meiosis I and II which occur at Stage XIV of the epithelial cycle. Also shown are the Sertoli cell (see "medium gray/pink" arrowhead). RS, round spermatid; ES, elongated spermatid (step 19). The schematic drawing shown on the right panel illustrates that the BTB physically divides the seminiferous epithelium into the basal and apical compartment. However, as described in text, a functional axis exists between the apical ES and the BTB to co-ordinate the cellular events, namely spermiation and BTB restructuring, that occur at opposite ends of the epithelium at Stage VIII of the epithelial cycle. A color version of this figure is available online at www.landesbioscience.com

known[15,31-35] even though major advances have been made in the past decade. However, several new targets that could become the prime candidates for male contraception are forthcoming. One of these candidates is a newly identified local functional axis in the seminiferous epithelium known as the apical ES-BTB axis that co-ordinates the events of spermiation and BTB restructuring during spermatogenesis.[36,37] Given that adjudin, 1-(2,4-dichlorobenzyl)-*1H*-indazole-3-carbohydrazide, is a potential male contraceptive that exerts its effects by perturbing cell adhesion at the apical compartment behind the BTB in rats,[38] rabbits[39] and beagle dogs,[40] we review herein recent findings in the field regarding the possible use of adjudin to disrupt the apical ES-BTB axis and hence spermatogenesis. We also highlight the significance of putting more resources and efforts in research in order to understand the biology and regulation of the BTB, so that better approaches can be developed to deliver male contraceptives (and perhaps therapeutic drugs to treat germ cell tumors) behind this blood-tissue barrier in men. Obviously, these findings will also benefit the development of nonsurgical approach to control fertility in pets and even wildlife, thereby protecting our environment.

THE APICAL ES-BTB FUNCTIONAL AXIS

Spermatogenesis takes place in the seminiferous epithelium of the seminiferous tubule, which is the functional unit that produces spermatozoa and estrogen, whereas Leydig cells in the interstitial space produce testosterone and estrogen. All these hormones together with FSH and LH released from the pituitary gland support and maintain spermatogenesis.[9-12,41,42] Interestingly, the seminiferous epithelium (see Fig. 1) is composed of only a monolayer of Sertoli cells, with each Sertoli cell supports up to ~30-50 germ cells at different stages of their development.[37] The seminiferous epithelium is anatomically segregated into the basal and the adluminal (apical) compartments by the BTB (Fig. 1) such that meiosis I and II, postmeiotic spermatid development, namely spermiogenesis, as well as spermiation all take place in the apical compartment behind the BTB. However, during the seminiferous epithelial cycle of spermatogenesis, distinctive cellular events take place across the epithelium at the opposite ends of the Sertoli cell. For instance, at Stage VIII of the epithelial cycle, spermiation that allows the release of fully developed spermatids (i.e., spermatozoa) takes place concurrently with the restructuring of the BTB that facilitates the transit of preleptotene spermatocytes at the site from the basal to the apical compartment, so that they can differentiate to zygotene, pachytene and then diplotene spermatocytes which enters meiosis I to be followed immediately by meiosis II at Stage XIV of the epithelial cycle.[7] Recent studies have shown that a functional axis operates locally in the seminiferous epithelium to co-ordinate cellular events such as spermiation and BTB restructuring.[36] Ectoplasmic specialization (ES) is a unique testis anchoring junction type[37,43] which is typified by the presence of actin filament bundles sandwiched in between cisternae of endoplasmic reticulum and the apposing plasma membranes of Sertoli cells. In the seminiferous epithelium, ES is found at the BTB and the apical compartment and is known as basal ES and apical ES, respectively. Basal ES is restricted to the Sertoli-Sertoli cell interface at the BTB[37,44] with the typical features of actin filament bundles and the cisternae of endoplasmic reticulum present on *both* sides of the two adjacent Sertoli cells. On the other hand, apical ES at the apical compartment is responsible for anchoring spermatids (step 8-19) at the Sertoli-spermatid interface.[37,44,45] Once it appears during spermiogenesis, apical ES is the only anchoring device that adheres spermatids (step 8-19) to the Sertoli cells in the epithelium and to confer spermatid polarity (orientation) until spermiation.[8,13,45,46] Unlike basal ES, apical ES has the typical actin filament bundles ultrastructure restricted to the Sertoli cell side and these ultrastructural features are not visible in the developing spermatids. These actin-based filament bundles are known to confer the unusual adhesive strength of cell adhesion at the apical ES.[17] $\alpha 6\beta 1$-Integrin which is restricted to the Sertoli cells and laminin-333 (laminin $\alpha 3$, $\beta 3$ and $\gamma 3$; note: a functional laminin ligand is a trimeric protein composed of an α, β and γ chain that can bind to an integrin receptor) which is limited to the spermatid constitute one of the best studied adhesion protein complexes at the apical ES.[43,47,48] This protein complex, together with N-cadherin-β-catenin and nectin-afadin complexes that use actin-based cytoskeleton are three of the known adhesion protein complexes that confer apical ES function to anchor developing spermatids to the Sertoli cell in the seminiferous epithelium.[8,14,16]

Matrix metalloproteinase-2 (MMP-2), a product of Sertoli cells, is highly expressed at Stage VIII of the epithelial cycle, and mostly restricted to the apical ES in the rat testis.[49] Laminin chains are known substrate of MMP-2, and fragments of laminin chains

following proteolytic cleavage by MMPs are also known to act as biologically active peptides to regulate various cellular functions in multiple epithelia.[50] Indeed, recombinant laminin fragments of laminin chains added to Sertoli cell epithelium with an intact tight junction (TJ)-permeability barrier that mimics the Sertoli cell BTB in vivo was found to perturb the TJ-barrier function dose-dependently. It was found that laminin fragments was able to reduce the steady-state level of occludin at the BTB, possibly mediated by an increase in occludin internalization via endocytic vesicle-mediated endocytosis.[36] Furthermore, overexpression of specific laminin fragments in Sertoli cell epithelium in vitro also displayed the same phenotype with a disrupted TJ-barrier function.[36] These findings thus illustrate the presence of a functional axis in the seminiferous epithelium between the apical ES and the BTB. In short, release of laminin chains mediated by the action of MMP-2 on the $\alpha 6\beta 1$-integrin-laminin-333 complex at the apical ES during spermiation can promote BTB restructuring, and thus co-ordinating the events of spermiation and BTB restructuring that occurs at the opposite ends of the seminiferous epithelium at Stage VIII of the epithelial cycle.

These findings, besides their physiological significance, are critical to male contraceptive development. If the biology of this apical ES-BTB axis is fully elucidated, components in this axis can become the prime targets for male contraceptive development. For instance, a blockade or an activation of MMP-2 action at the apical ES can "delay" or induce "unwanted/premature" spermiation, respectively. This can lead to malfunctioning sperm in the epididymis or spermatids being phagocytosed by Sertoli cells in the epithelium to disrupt spermatogenesis. Also, the underlying signaling mechanism(s) by which the biologically active laminin fragments exert their effects to perturb the BTB function, if known, can also be "blocked", which would disrupt BTB restructuring necessary for the timely transit of preleptotene spermatocytes to enter the apical compartment for further development. In both approaches, spermatogenesis will be disrupted, resulting in infertility. In the section below, we illustrate how adjudin is being developed as a candidate male contraceptive that exerts its effects on some of the components in this functional axis.

ADJUDIN

Adjudin, 1-(2,4-dichlorobenzyl)-*1H*-indazole-3-carbohydrazide, is an analog of lonidamine.[28] Lonidamine, however, is an anti-cancer drug that exerts its effects primarily by inhibiting hexokinase activity most potently in cancer cells following their exposure to radiation.[51] During the course of investigation to develop lonidamine as an anti-cancer drug in the 1970s,[51] it was found to possess potent anti-spermatogenic activity by depleting germ cells from the seminiferous epithelium in rodents.[52,53] Lonidamine was found to disrupt the actin filament bundles in Sertoli cells that are restricted almost exclusively to the ES in the seminiferous epithelium when examined by electron microscopy.[54] Thus, various analogs of lonidamine were synthesized and tested in our laboratory almost two decades ago to select the most potent analog with minimal toxicity, and having the activity to disrupt apical ES based on an assay that would induce testin expression at the site.[38,55] Subsequent studies have shown that adjudin disrupts actin filament bundles preferentially at the apical ES,[56,57] inducing premature spermiation, leading to defoliation of virtually all germ cells except spermatogonia/

spermatogonial stem cells.[58] Thus, the anti-fertility effect of adjudin is *reversible* since the remaining SSC can repopulate the epithelium similar to the initiation of spermatogenesis in immature rats.[28] In light of the potent effects of adjudin on cell adhesion in the testis, adjudin-treated rats have been used as an in vivo model to study anchoring junction regulation in the seminiferous epithelium by delineating the signaling pathways that regulate germ cell adhesion as well as the components of the adhesion protein complexes at the apical ES.[48,59-63] For instance, it is now known that adjudin initially affects the integrin-based adhesion protein complex at the apical ES, and it also has effects on other signaling and/or adaptor proteins at the site including FAK, p130Cas, DOCK180, RhoA, vinculin, LIMK, ROCK, Arp2/3 complex, Eps8, and PKB,[48,60,64-66] which in turn induces ERK1/2 downstream[48,60] that leads to the apical ES degeneration. This process likely mimics the events of spermiation that take place at Stage VIII of the epithelial cycle.[15,67-69]

DRUG TRANSPORT AT THE BTB

The BTB, similar to other blood-tissue barriers (e.g., blood-brain barrier, blood-retinal barrier) and the intestinal TJ-barrier, poses an important barrier to limit the entry of environmental toxicants (e.g., cadmium, mercury, bisphenol A, phthalates), drugs and other xenobiotics into the apical compartment behind the barrier (Fig. 1). This is mediated by the presence of efflux (ATP-dependent) and influx (ATP-independent) drug transporters at these barriers.[70-72] The BTB, constituted by *co-existing* TJ, basal ES, gap junction and desmosome between Sertoli cells near the basement membrane,[17,73,74] being one of the tightest blood-tissue barriers besides the BBB, limits the access of male contraceptives to the apical compartment. For instance, it was shown that >95% of adjudin administered to rats orally by gavage failed to reached the testis[28,30] even though adjudin was exceedingly potent to induce apical ES disruption in the seminiferous epithelium.[28,75,76] Recent studies have shown that the entry of adjudin was neither mediated by diffusion nor by paracellular transport, such as during the epithelial cycle at Stage VIII when the BTB is undergoing restructuring to facilitate the transit of preleptotene spermatocytes.[77,78] Instead, the entry of adjudin to the apical compartment is mediated by transcellular transport as a result of the combined efforts of multiple influx and efflux pump drug transporters.[77,78] For instance, even the knockdown of a major efflux drug transporter, such as P-glycoprotein, by RNAi failed to make the BTB freely permeable to [^{3}H]-adjudin,[77] because of the presence of the multiple efflux and influx drug transporters at the Sertoli cell BTB.[72,77,79] Interestingly, recent studies have shown that P-glycoprotein, besides serving as an efflux drug transporter, is an integrated component of occludin-based complex at the BTB.[79] Its expression is significantly induced following the exposure of testes and/or Sertoli cells to adjudin.[77,79] More importantly, P-glycoprotein was found to regulate occludin-ZO-1-based adhesion protein complex at the BTB via its effects on FAK, which altered phosphorylation status of occludin to confer changes in the kinetics of protein endocytosis, thereby destabilizing or stabilizing the BTB integrity.[77] Collectively, these findings demonstrate the complexity of drug transport at the BTB, illustrating much research is needed to understand the regulation of drug entry at the BTB for nonhormonal contraceptives in particular when these drugs exert their effects on germ cell adhesion, apoptosis, and/or development behind the BTB.

INTERACTION OF ADJUDIN WITH INTEGRIN AT THE APICAL ES AND OCCLUDIN AT THE BTB

Introduction

In earlier studies using adjudin-treated rats (e.g., adjudin at 50 mg/kg b.w., by gavage) or Sertoli cell epithelium, it was shown that adjudin perturbed apical ES[48,59,60] by compromising the adhesive function of the α6β1-integrin-laminin-333 complex initially, to be followed by desmosome.[75,80] This in turn led to an activation of PKB (protein kinase B) and ERK1/2 (extracellular regulated kinase 1/2) downstream,[60] causing "unwanted" premature release of spermatids from the seminiferous epithelium, mimicking spermiation.[47,81] Subsequent studies have shown that the α6β1-integrin-based adhesion protein complex is composed of p-FAK (phosphorylated focal adhesion kinase)-Tyr[397] and p-FAK-Tyr,[576] p-130Cas (p130 Crk-associated substrate), DOCK180 (dedicator of cytokinesis 180), RhoA (Ras homolog gene family, member A, a small GTPase), and vinculin (an adaptor at the apical ES). These proteins create an α6β1-integrin-pFAK-p-130Cas-DOCK180-RhoA-vinculin protein complex at the apical ES,[48] and it is one of the primary targets of adjudin. Collectively, these findings illustrate that α6β1-integrin may serve as a "receptor" protein complex for adjudin. To explore this possibility, molecular modeling was used to examine the presence of a docking pocket in α6β1-integrin for adjudin binding. Since adjudin is likely to enter the apical compartment through the BTB via drug transporters, and occludin is one of the major TJ-fibril building blocks in the testis, we also elected to perform this docking study between occludin and adjudin.

Molecular Modeling of α6β1-Integrin and Occludin with Adjudin

In rats, amino acid sequence of the two known α6 integrin variants, namely α6 integrin variant 1 [XP_001059353.2] and α6 integrin variant 2 [XP_215984.4], as well as β1 integrin [NP_058718.2] and occludin [NP_112619.2] of *Rattus norvegicus* were obtained from NCBI-Protein sequence database (http://www.ncbi.nlm.nih.gov/guide/proteins/) for molecular modeling. The amino acid sequence of β1 integrin was appended into α6 integrin variant 1 sequence for homology modeling of heterodimeric form of α6β1 integrin variant 1. Similarly, β1 integrin amino acid sequence was also appended into amino acid sequence of α6 integrin variant 2 for homology modeling of heterodimeric form of α6β1 integrin variant 2. The crystal structure of integrin αVβ3 [PDB ID: 3IJE] was found as a suitable template for the homology modeling of both α6β1 integrin variant 1 and variant 2 by NCBI-BLASTP search. We have used four different steps in our molecular modeling approach: (i) target-template sequence alignment, (ii) model building, (iii) loop refinement, and (iv) model validation. The programs of "Homology Modeling" modules such as Align123 and MODELER automodel of Discovery Studio 3.0 (Accelrys Software, http://accelrys.com/) were used for sequence alignment and model building, respectively. The alignment results show that αVβ3 integrin matches with α6β1 integrin variant 1 by 32.2% sequence identity and 52.5% sequence similarity. Similarly, αVβ3 integrin shows 32.5% sequence identity and 53.3% sequence similarity with α6β1 integrin variant 2 (Fig. 2). A total of five models were generated for each of the model building of integrin proteins. Among these, the model of lowest DOPE was selected for further process such

as loop refinement by MODELER loop-model and energy minimization by Smart Minimizer algorithm in Discovery Studio 3.0. The minimization was carried out in 500 steps by applying CHARMm force field. Occludin protein sequence was modeled based on multiple-threading alignments by I-TASSER server[82] since there is a lack of availability of suitable template structures for homology based modeling in PDB database. The confidence score (C-score) of the modeled occludin structure was –1.57. Finally, the predicted models were validated by analyzing stereo chemical properties by Ramachandran plot.[83] The plots (Fig. 3) illustrate that 99.6% amino acid residues of α6β1-integrin variant 1, 99.7% residues of α6β1-integrin variant 2 and 98.1% residues of occludin are distributed in the allowed region of the Ramachandran plot which are in the inside, hardsphere and overlap regions. This thus confirms that the modeled structures of α6β1-integrin for both variants and occludin are reliable.

The modeled structures of α6β1 integrin variant 1, α6β1 integrin variant 2 and occludin were used for the preparation of proteins by Maestro 9.0 protein preparation wizard. In which bond order assigned, and hydrogen added appropriately. These structures were optimized by exhaustive sampling and minimization methods by applying OPLS2005 force field. The structural co-ordinate of adjudin (CID: 9819086) was obtained from NCBI-PubChem database. LigPrep (version 2.3, Schrödinger, LLC, New York, NY, 2009) was used to generate 3D structure of adjudin. The active site residues of each of α6 integrin variant 1, α6 intergrin variant 2 and β1 integrin were identified by performing EMBOSS global pairwise sequence alignment (http://www.ebi.ac.uk/Tools/emboss/align/index.html) with αVβ3 integrin amino acid sequence. The alignment outputs show that ligand binding residues from Gly172 to Gly181 of αV integrin[84] matched with amino acid residues of both α6 integrin variant 1 and 2 from Gly183-Gly192 (Fig. 4A,B). Similarly, the ligand binding residues Asp119, Ser121, Ser123, Asp217 and Glu220 of β3 integrin[84] matched with Asp130, Ser132, Ser134, Asp226 and Glu229 of β1 integrin (Fig. 4C). Hence, the identified matching residues, namely from Gly183.A to Gly192.A, Asp130.B, Ser132.B, Ser134.B, Asp226.B and Glu229.B of both α6β1 integrin variant 1 and variant 2, were selected as centroid of the active residues for the generation of receptor grid. For occludin, the extracellular loop region from Ala88 to Ser108 was defined as active site residues. The Standard Precision (SP) mode of Glide was used for the flexible ligand docking. UCSF Chimera[85] was used for the visualization and analysis of the docking results.

The docking results of adjudin into α6β1 integrin variant 1, α6β1 integrin variant 2 and occludin are summarized in Table 1. The molecular docking of adjudin into α6β1 integrin variant 1 shows the formation of single hydrogen bond by Ser263.A in β-propeller domain of α6 integrin chain (i.e., in A-chain) and large number of hydrophobic contacts by Trp190.A, Tyr234.A, Leu235.A, Arg259.A, Ala260.A, His262.A, Ser292.A, Tyr175.B, Asp226.B, Pro228.B, His263.B, PHE264.B, Gly266.B, Asp267.B, Leu270.B residues in β-propeller domain of A-chain and βA domain of B-chain. It is interesting to note that adjudin molecule is interacting at the interface of both propeller domain and βA domain (Fig. 5). Generally, the biologically active integrin molecule is formed by the network of interactions of α and β integrin subunits. In this, N-termini of both α and β subunit (i.e., β-propeller domain and βA domain) form globular head of ligand binding domain for extracellular region.[84] Hence, the binding of adjudin in interface residues of α and β subunit would likely inhibit the signal transduction of this integrin protein across the cell membrane.

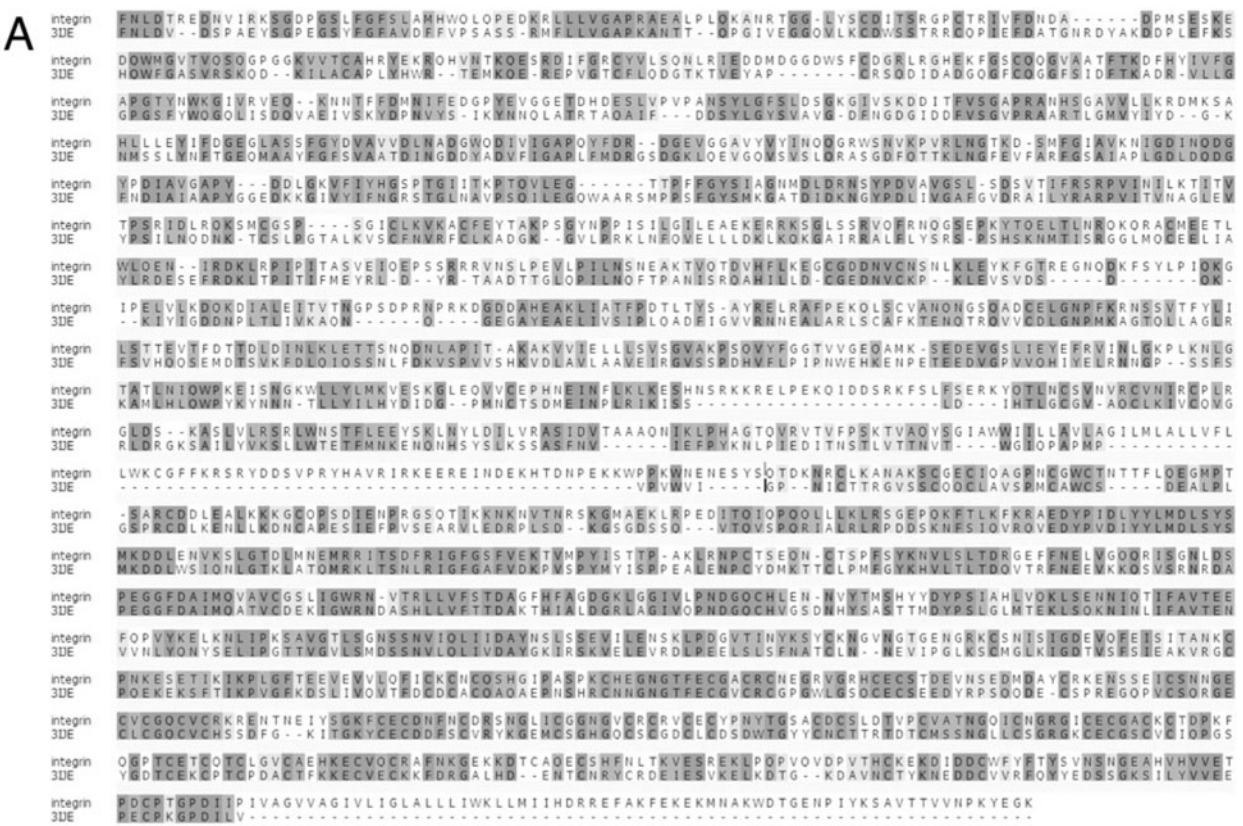

Figure 2. The sequence alignment results used for building 3-D models in heterodimeric form for the target sequences of (A) α6β1-integrin variant 1 and (B) α6β1-integrin variant 2 with template sequence of αVβ3-integrin [PDB ID: 3IJE]. In short, the α6-integrin sequence shown in (A) and (B) corresponds to variant 1 and variant 2 of α6-integrin of *Rattus norvegicus*, respectively; but the β1-integrin of *Rattus norvegicus* has no molecular variant. Continued on following page.

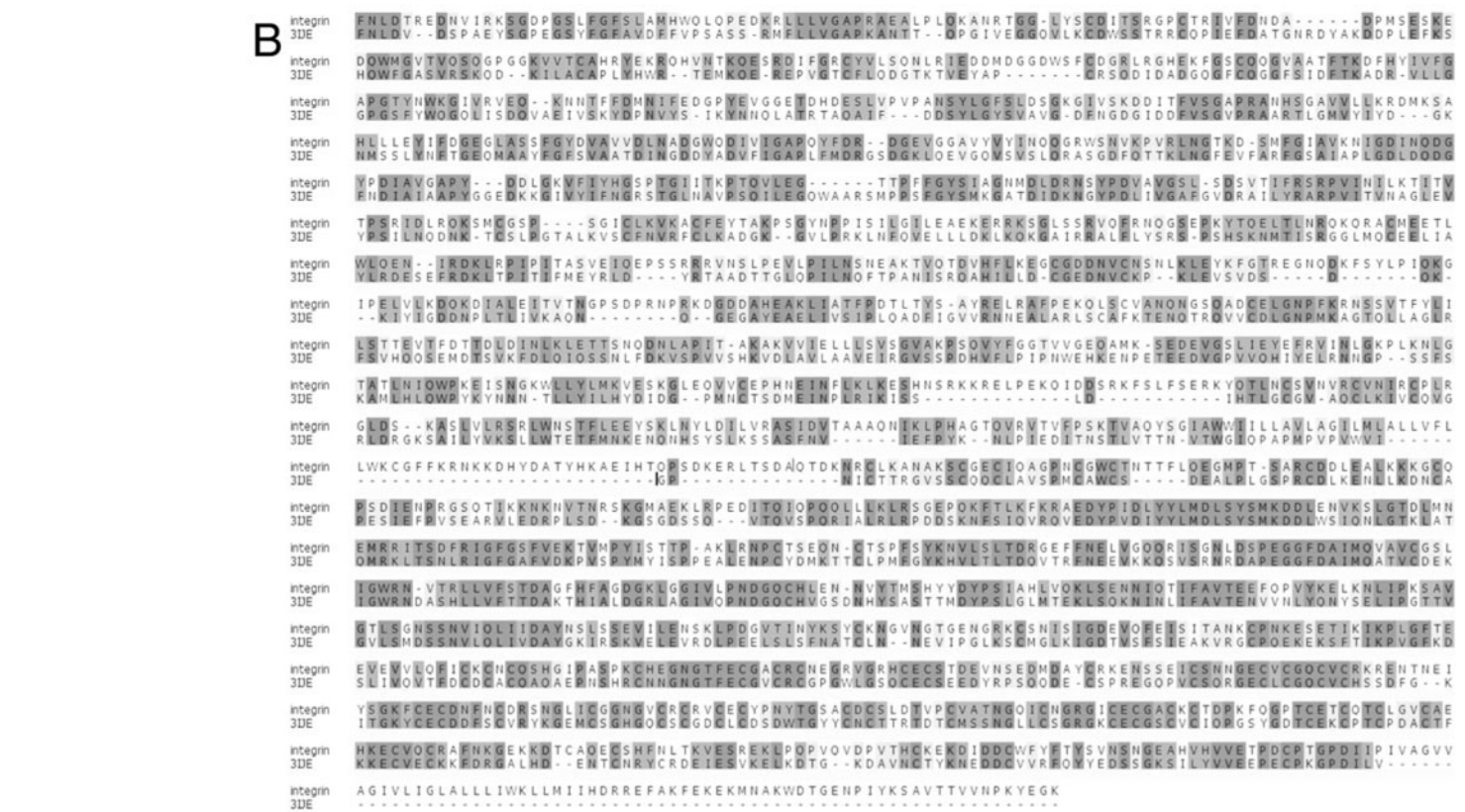

Figure 2, continued from previous page. The "gray/green" line in (A) or (B) denotes the N-terminal amino acid residue of β1-integrin of the α6β1-integrin receptor, where the "black/red" line in (A) or (B) denotes the N-terminal amino acid residue of β3-integrin of the functional αVβ3-integrin receptor. A color version of this figure is available online at www.landesbioscience.com

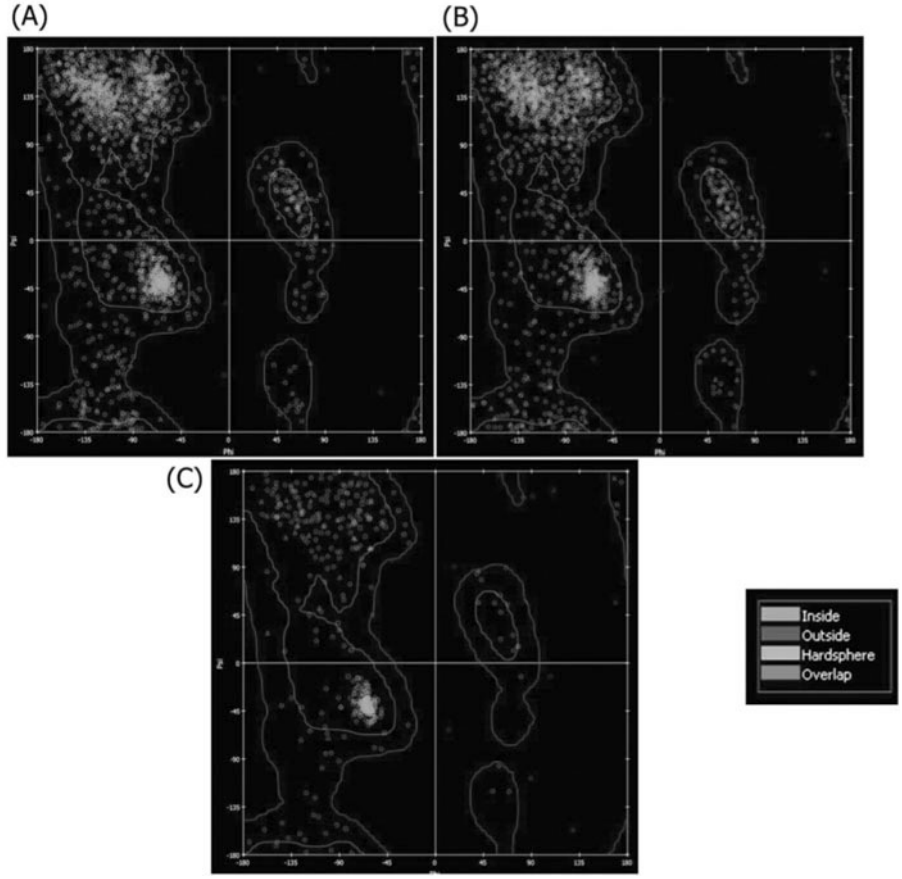

Figure 3. The Ramachandran plot for (A) α6β1-integrin variant 1, (B) α6β1-integrin variant 2 and (C) occludin. These Ramachandran diagrams are used to visualize backbone dihedral angles ψ against φ of amino acid residues in the corresponding protein structure of α6β1-integrin variant 1 and 2 and occludin.

Similarly, the molecular docking of adjudin into α6β1 integrin variant 2 resulted in the formation of two hydrogen bonds and large number of hydrophobic contacts (Table 1). The amino acid residues of β-propeller domain such as Leu226.A and Arg259.A are involved in hydrogen bond formation. In addition, Val227.A, Pro230.A, Ala231.A, Ser233.A, Ala260.A, Asn261.A, His262.A and Ser263.A of β-propeller domain and Ser227.B, Pro228.B, Gly261.B, Glu320.B, and Phe321.B of βA domain are also involved in hydrophobic contacts to make more stable interactions. Hence, it is observed that adjudin also makes strong interactions at the β-propeller-βA domain interface residues of α6β1 integrin variant 2 (Fig. 6). It is significant to note that the amino acid residues such as Arg259.A, Ala260.A, His262.A, Ser263.A and Pro228.B are the common residues involved in interactions with adjudin in both α6β1 integrin variant 1 and α6β1 integrin variant 2. In addition, α6 integrin is the only chain involved in hydrogen bond formation and makes more number of hydrophobic contacts in both α6β1 integrin variant 1 and α6β1 integrin variant 2. Hence, we conclude that adjudin

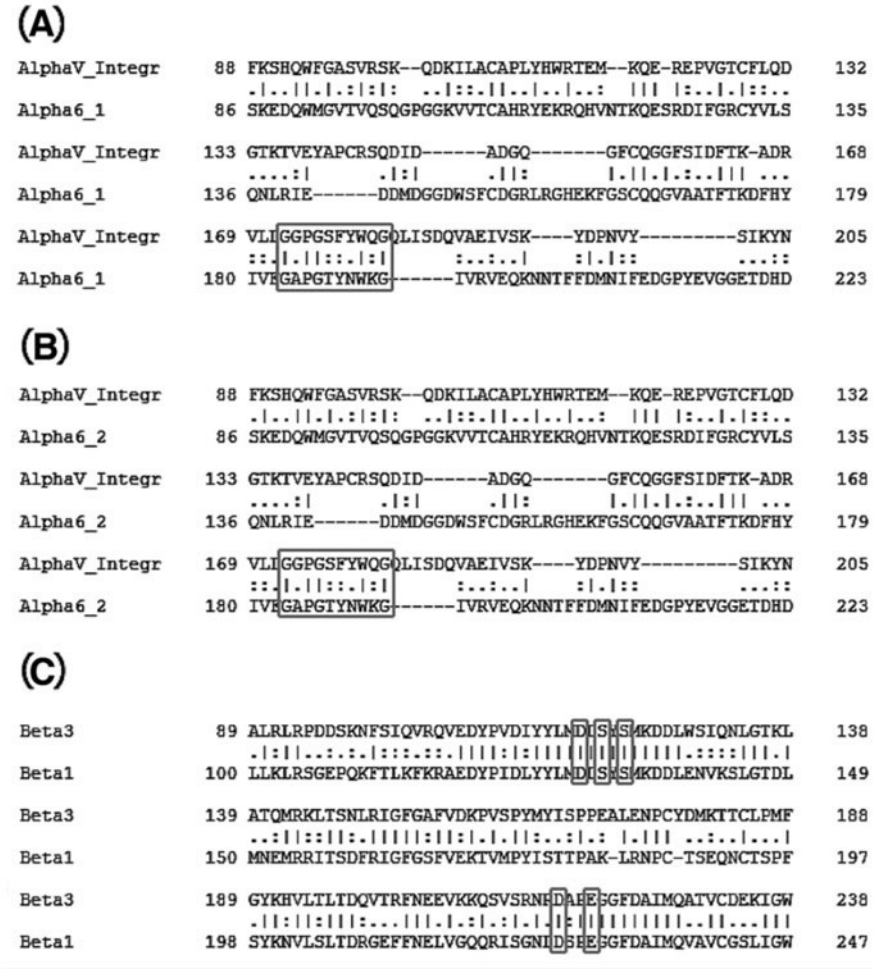

Figure 4. Pairwise sequence alignment results for predicting active site residues of (A) α6-integrin variant 1, (B) α6-integrin variant 2, and (C) β1-integrin. Active amino acid residues are tagged by red box. A color version of this figure is available online at www.landesbioscience.com

molecule is interacting at the main inter-subunit interface residues lying within the head between the β-propeller of α6 integrin and βA domain of β1 integrin.

The 'adjudin-occludin' docked complex shows three hydrogen bonds by Gly121, Gly123, and Lys380 residues, in addition to the active participation of Tyr93, Gly94, Gly96, Phe98, Phe117, Gly119, Tyr122, Gly125, Tyr126, Pro378, Thr384 and Glu400 residues for hydrophobic contact (Fig. 7). This docking result thus illustrates that adjudin molecule interacts with the extracellular residues such as Tyr93, Gly94, Gly96, Phe98, Pro378, Lys380, Thr384 and Glu400, in addition to the transmembrane helical residues such as Phe117, Gly119, Gly121, Tyr122, Gly123, Gly125 and Tyr126. Based on this molecular modeling study, adjudin molecule can alter the functional activity of occludin protein at the BTB.

Remarks and Summary

In short, the molecular modeling findings shown in Figures 5-7 clearly demonstrate that adjudin serves as a ligand that interacts with α6β1-integrin receptor at the apical ES and occludin at the BTB. These findings, in particular data shown in Figures 5 and 6, also yield some new insights on the mechanism by which adjudin induces signaling function involving ERK1/2 downstream of the α6β1-integrin-p-FAK-p130Cas-DOCK180-RhoA-vinculin complex. It is likely that the coupling of adjudin with the α6β1-integrin receptor (see Figs. 5 and 6) induces activation of the FAK-p130Cas-DOCK180-RhoA-vinculin complex, which relates the signaling function to ERK1/2, which in turn perturbs the integrin-based adhesive function at the apical ES.

Adjudin is also found to bind to occludin at the BTB (Fig. 7), and this coupling can likely lead to a disruption of the TJ-permeability barrier function at the BTB. However, the BTB is composed of *co-existing* basal ES, desmosome and gap junction, and recent studies have shown that the BTB has a unique mechanism in which a transient disruption of TJ does not lead to the widespread disruption of the other co-existing junctions (e.g., basal ES, desmosome) in order to maintain BTB integrity[86-88] during spermatogenesis unless it is exposed to an unusual acute concentration of a toxicant.[58] It was postulated that these co-existing junctions are usually "engaged" to maintain the BTB integrity, however, when the BTB is under assault, such as when exposed to toxicants, these co-existing junctions can be "disengaged" so that remaining junctions can maintain the immunological barrier transiently even though the TJ-barrier is temporarily disrupted.[86,89] These findings also illustrate the uniqueness of the BTB among other blood-tissue barriers in mammals.

THE APICAL ES-BTB AXIS IS A PRIME TARGET FOR MALE CONTRACEPTIVE DEVELOPMENT

The apical ES-BTB functional axis in the seminiferous epithelium as depicted in Figure 1, which coupled with the molecular modeling data shown in Figures 5-7 thus illustrate that many component proteins in this axis can be the target of male contraception. The modeling data shown in Figures 5-7 also illustrate that a second generation adjudin analogs can likely be synthesized to improve: (i) its potency at the apical ES, and perhaps (ii) its bioavailability in the apical compartment behind the BTB by disrupting the BTB integrity transiently for its "transit" at the BTB, mimicking the transit of preleptotene spermatocytes at the site at Stage VIII of the epithelial cycle. This possibility is supported by recent findings that when rats were treated with adjudin at an acute dose (i.e., 250 mg/kg b.w.), BTB was *irreversibly* disrupted.[58] On the other hand, the optimal dose (i.e., 50 mg/kg b.w.) that effectively induced male fertility *reversibly* in rats by depleting virtually all germ cells from the epithelium except spermatogonia/spermatogonial stem cells (SSC), BTB was found to remain intact when germ cells began to dislodge from the epithelium within 6-hr following its administration until all the tubules were devoid of germ cells except spermatogonia/SSC by ~2-wk.[58] Interestingly, the BTB was found to be transiently disrupted at this low but effective dose by ~6-wk and it was "re-sealed" by 20-wk.[58] Thus, adjudin can be chemically modified based on findings in Figure 7, such that the appropriately modified adjudin can be rapidly "docked" to occludin to induce BTB disruption within hours following treatment. This thus promotes its bioavailability in the apical compartment behind the BTB to exert its effects at the apical ES to dislodge spermatids from the epithelium.

Table 1. Molecular interactions of α6β1-integrin and occludin with adjudin

Docking Complex	H-bond (D—H…A)[a]	H-Bond Length (Å)	Hydrophobic Contact Residues	Docking Score	Glide Energy (kcal/mol)
α6β1-Integrin variant 1 and adjudin	OH (Ser263.A)…O	1.967	Trp190.A, Tyr234.A, Leu235.A, Arg259.A, Ala260.A, His262.A, Ser292.A, Tyr175.B, Asp226.B, Pro228.B, His263.B, PHE264.B, Gly266.B, Asp267.B, Leu270.B	–6.133	–43.739
α6β1-Integrin variant 2 and adjudin	NH…O (Leu226.A) NH…O (Arg259.A)	2.480 1.812	Val227.A, Pro230.A, Ala231.A, Ser233.A, Ala260.A, Asn261.A, His262.A, Ser263.A, Ser227.B, Pro228.B, Gly261.B, Glu320.B, Phe321.B	–5.459	–38.214
Occludin and adjudin	NH…O (Gly121) NH…O (Gly123) NH (Lys380)…N	2.181 2.283 2.334	Tyr93, Gly94, Gly96, Phe98, Phe117, Gly119, Tyr122, Gly125, Tyr126, Pro378, Thr384, Glu400	–3.987	–34.741

[a] D- Donor; H- Hydrogen; A- Acceptor.

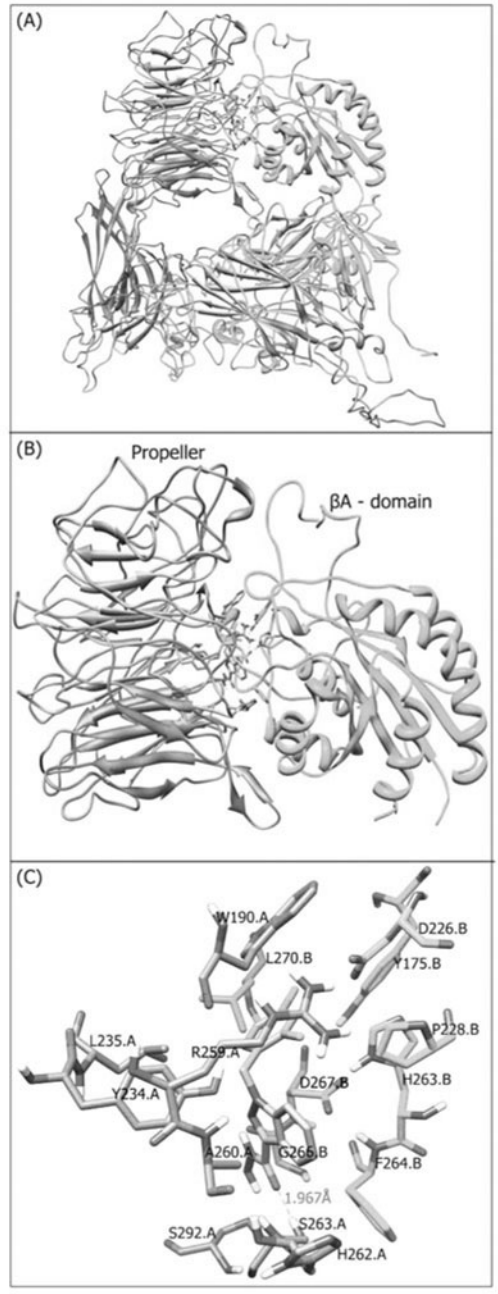

Figure 5. Illustration of docked complex for adjudin into α6β1-integrin variant 1. A) Ribbon drawing of full-length docked complex [shown in cyan (α6) and green (β1)], (B) ribbon drawing of α6β1-integrin interface domains that show the interacting adjudin molecule and (C) stick representation of interacting interface residues [shown in cyan (α6), green (β1) and orange (adjudin)]. Hydrogen bond are depicted by red dotted lines along with the bond length. This picture was generated by using the UCSF Chimera software package. A color version of this figure is available online at www.landesbioscience.com

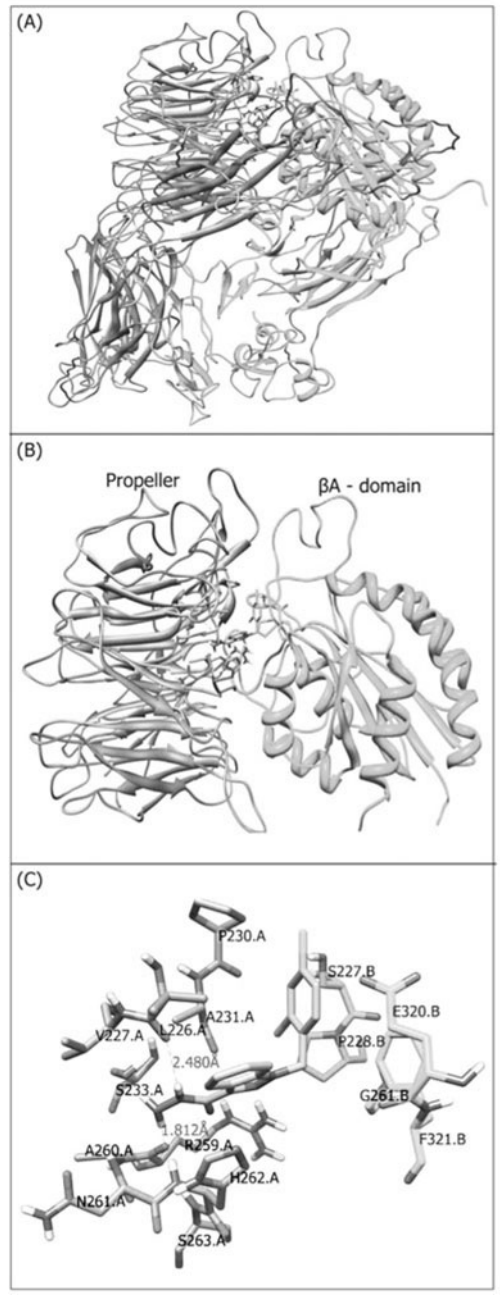

Figure 6. Illustration of docked complex for adjudin into α6β1-integrin variant 2. (A) Ribbon drawing of full-length docked complex [shown in cyan (α6) and green (β1)], (B) ribbon drawing of α6β1-integrin interface domains that show the interacting adjudin molecule and (C) stick representation of interacting interface residues [shown in cyan (α6), green (β1) and orange (adjudin)]. Hydrogen bonds are depicted by red dotted lines along with the with bond length. A color version of this figure is available online at www.landesbioscience.com

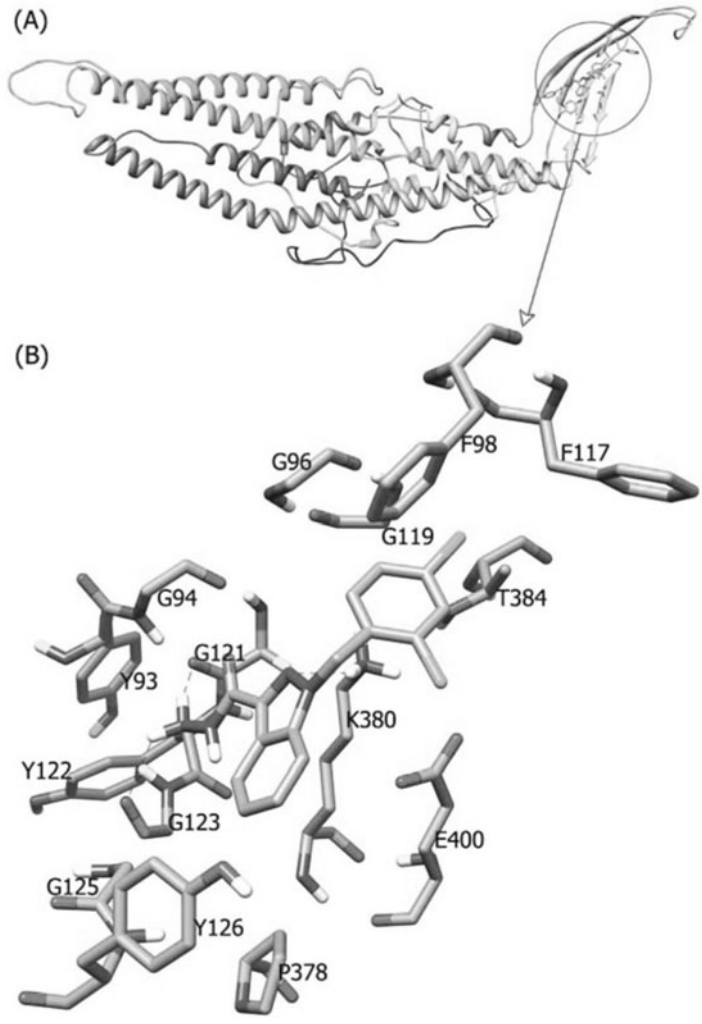

Figure 7. Illustration of docked complex of adjudin into occludin. (A) Ribbon drawing of full-length docked complex of adjudin into occludin. The interacting residues are tagged by a circle and (B) stick representation of interacting residues. Adjudin is shown in orange color. Hydrogen bonds are depicted by red dotted lines. A color version of this figure is available online at www.landesbioscience.com

THE ROLE OF BTB IN THE DIFFERENTIATION OF SPERMATOGONIA/ SPERMATOGONIAL STEM CELLS (SSC)

Treatment of adult rats (~270-300 gm b.w.) with adjudin at 50 mg/kg b.w. by gavage is known to induce *reversible* infertility since the remaining spermatogonia/SSC in the seminiferous tubule gradually repopulate the entire epithelium, re-initiating spermatogenesis.[28] A recent study, however, has shown that by treating adult rats (~270-300 gm b.w.) with an acute dose of adjudin at 250 mg/kg b.w. by gavage, the infertility induced by adjudin in these rats was *irreversible*, yet this was *not* the result of adjudin-induced spermatogonia/

SSC depletion from the seminiferous epithelium since the population of spermatogonia/SSC in the tubules of rats from this high-dose adjudin treated group remained similar to rats in the low-dose treated and normal control groups.[58] Detailed histological/morphological and functional analysis of the testes from these animals showed that the BTB in rats from the high-dose treated group was *irreversibly* disrupted whereas the BTB in low-dose treated group was only *transiently* disrupted by adjudin.[58] These findings thus demonstrate unequivocally that an intact BTB is critical for differentiation of spermatogonia/SSC to re-initiate spermatogenesis. This conclusion is also supported by genetics studies in which the knockout of connexin43 (Cx43) [note: Cx43-based GJ is an integrated component of the BTB[34,90] which co-exists with TJ, basal ES and desmosome to constitute the BTB[17]] led to infertility in mice.[91-94] Furthermore, Cx43 knockout also produced a number of phenotypes shared by rats from the adjudin-treated high dose group.[58] For instance, it was found that although adult $Cx43^{-/-}$ mice had similar population of spermatogonia/SSC in the tubules as compared to the wild type, the $Cx43^{-/-}$ mice displayed meiotic arrest in which spermatogonia/SSC failed to differentiate beyond Type A spermatogonia.[91] Also, Sertoli cells in these mice remained actively mitotic and proliferative such that clusters of Sertoli cells were shed from the epithelium and Sertoli cell clusters were found in the tubule lumen.[93,94] This thus contributes to the inability of the BTB to "seal" properly to maintain a functional BTB. Additionally, even though ultrastructures of TJ were visible at the BTB in these mice, the steady-state level of ZO-1 (a TJ-associated adaptor at the BTB) was significantly reduced and ZO-1 was also found to be mis-localized in the seminiferous epithelium[92] This in turn led to mis-localization of TJ- and basal ES-associated proteins at the BTB since many of these proteins (e.g., occludin, claudins, JAM-A, catenins, cadherins) interact directly or indirectly with ZO-1 at the BTB.[56,61,86] These findings thus suggest that the BTB in these $Cx43^{-/-}$ mice is not fully functional and intact. In short, an intact and fully functioning BTB is necessary for differentiation of spermatogonia/SSC to Type A and Type B spermatogonia, and spermatocytes, to initiate spermatogenesis.

CONCLUSION AND FUTURE PERSPECTIVES

Based on recently published findings in the field as summarized above, which coupled with the molecular modeling findings described here thus lead us to conclude that the $\alpha 6\beta 1$-integrin receptor at the apical ES is one of the primary targets of adjudin. Moreover, adjudin can also form a docking complex with occludin at the BTB. It is of interest to know note that the occludin-ZO-1-based protein complex that regulates the Sertoli cell TJ-permeability function can be modulated by FAK.[95,96] Additionally, this occludin-ZO-1-FAK complex was found to structurally interact with P-glycoprotein, an efflux drug transporter, at the Sertoli cell BTB.[78] Collectively, these findings thus illustrate that several component proteins along the apical ES-BTB functional axis[36] (e.g., $\alpha 6\beta 1$-integrin and occludin) can be the target of a male contraceptive, such as adjudin. Perhaps the most important of all, the molecular modeling findings as reported herein also illustrate that more potent adjudin analogs can be designed to enhance its "penetrability" at the BTB and its affinity to the $\alpha 6\beta 1$-integrin receptor, thereby activating its associated regulatory proteins (e.g., p-FAK, DOCK180, PKB) and the ERK1/2 signaling pathway downstream. This, in turn, perturbs germ cell adhesion more effectively in the seminiferous epithelium, leading to infertility. This also requires lesser amount of drug to be administered because of the better BTB penetrability, and since the hypothalamic-pituitary-testicular axis is not disrupted, side-effects should be minimal.

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