CHAPTER TWO

REVIE W OF LITERATURE
Epithelium is defined as the external layer of cells that covers all the free, open surfaces of the body including the skin and mucous membranes. On the other hand, the endothelium is the layer of cells lining the closed internal spaces of the body such as the blood vessels and lymphatic vessels. Epithelium is made up of epithelial cells and endothelium is made up of endothelial cells. Both these cell types have very common general structural features that include their assembly into interconnected sheets.

Epithelial cells are present in many organs as a specialized component. To fulfill their specific role of absorption or secretion, epithelial cells have specialized modifications on their surfaces. Due to their arrangement they create a physical barrier that prevents pathogens, toxins, and other unwanted materials from entering the body. Many of the physical properties of epithelial cells rely on their attachment to each other, which is facilitated by several types of cell junctions.

Epithelial and endothelial cell to cell interactions take place through complexes namely tight junctions (TJs), adherens junctions (AJs), desmosomes and gap junctions, which are specific in their morphology, composition and function [Figure: 2.1; Tokes et al, 2013]. Tight junctions are intercellular junctions adjoining the apical end of the lateral membrane surface. They have two functions, the barrier (or gate) function and the fence function [Sawada, 2013]. Tight Junctions are complexes made up of several proteins located at the apical ends of the lateral membranes of epithelial cells [Suzuki T, 2013] as well as endothelial cells [Mullin et al, 2005].
2.1: History of Tight Junction Proteins studies

Before the 20th century it was thought that intestinal epithelium formed a complete barrier which prevented transport between cells [Amasheh et al., 2002]. With the turn of the century, it was found that the paracellular spaces of epithelia allowed water and macrophages to cross. Later, it was found that variation in conductance between the apical and basal layers results in the transport of sodium ions which were thought to enter the cell across the apical membrane down its concentration gradient (later shown to be through Na\(^+\) channels) but transported in an energy-dependent manner out through the basal membrane. Later it was found that Cl\(^-\) ions follow Na\(^+\) ions in a passive way through the tight junction coupled to the electrical gradient generated by active Na\(^+\) transport [Ussing and Windhager, 1964].

Initially it was thought that TJs exist only in vertebrates, where they were first studied. But later on evidences suggested that they are also found in invertebrates and lower chordates. In lower chordates like tunicates, TJs have been seen in epidermis, pharyngeal and intestinal epithelia. Insects like cockroaches and arachnids like spiders and scorpions also display tight junctions [Cereijido and Anderson, 2001].

Different undamaged and normal epithelia exhibit a wide range of transepithelial electrical resistance (TEER). Epithelia can be classified as "leaky" or "tight" depending on the TEER value. Thus, the urinary bladder epithelium is around 2000 times tighter than that of jejunum [Crone and Christensen, 1981; Powell, 1981; Anderson and Van Itallie, 2009].

The TJs were first visualized ultrastructurally in 1963 by Farquhar and Palade [Farquhar and Palade, 1963]. They described the membrane junction complex to be resolved into three morphologically distinct links from the apical-most occludin or TJ (zonula occludens) followed by the cadherin-based adherens junction and the bottom-most desmosomes. The TJs are formed by a variable number of close cell–cell contacts or "kisses," which range from a single continuous contact (e.g., some endothelial cells) to a half dozen (jejunum) or dozens of contacts in the most extreme mammalian example (Sertoli cells).
The first protein of the tight junction complex to be identified was ZO-1 in 1986 [Stevenson et al, 1986] and was revealed to localize to the tight junction by immuno-electron microscopy (immuno EM). This was followed soon by the identification of ZO-2 [Jesaitis and Goodenough, 1994], ZO-3 [Haskins J et al, 1998] and cingulin [Citi et al, 1988]. Surprisingly, occludin was found to be unessential for barrier formation [Saitou et al 1998], which led to the subsequent discovery of many proteins of the claudin family [Furuse et al, 1998]. Claudins are the vital barrier forming proteins [Furuse, 2001].

Figure: 2.1 Molecular distribution of TJP (Adapted from Suzuki, 2013)

2.2: Molecular structure of TJs
TJs which are located at the apical ends of the lateral membranes of epithelial and endothelial cells are complexes made up of several proteins. Occludin [Furuse et al, 1993], claudins [Furuse et al, 1998], junctional adhesion molecule (JAM) [Martin-Padura et al, 1998], and tricellulin [Ikenouchi et al, 2005], are the main transmembrane proteins, which interact with cytosolic scaffold proteins, like zonula occludens (ZO) proteins, that attach them to the actomyosin ring [Suzuki, 2013].
ZO proteins were the first tight-junction proteins described [Lee, 2015]. ZO-1, 2 and 3 [Gumbiner et al, 1991; Haskins et al, 1998; Willot et al, 1993] have been identified till date. They are members of the MAGUK (membrane associated guanylate kinase homologs) family of proteins. These are multi-domain proteins carrying three post-synaptic density 95/Drosophila disc large/zona-occludens (PDZ) domains, a Src homology-3 (Sh3) domain and a region of homology to guanylate kinase (GuK) homolog from the side of the N-terminus. The proline rich C-terminal region of ZO-1 and ZO-3 bind F-actin, whereas ZO-2 binds the actin-associated protein 4.1R. Many TJ proteins bind to the N-terminal half of ZO proteins.

Claudins bind to the PDZ1 domain, JAM-A binds to the PDZ3 region, whereas occludin binds to either GuK region or whole N-terminal region of ZO depending on the homologue [Figure: 2.2; Hartsock and Nelson, 2008; Lee, 2015]. Also, crystal structures and NMR data have exposed an important role for the dependence of JAM-A binding to PDZ3 domain of ZO-1 [Nomme et al, 2011].
Occludin, a 65 kDa molecule is a transmembrane 4 superfamily protein made up of four transmembrane domains, two extracellular loops, and one intracellular loop: a short N-terminal and a long C-terminal domain extended into the cytoplasm. The extracellular loops allow small ions to pass, but create a barrier against macromolecules [Furuse et al, 1994; Suzuki, 2013]. The C-terminal domain is required for interaction of occludin with several intracellular TJ proteins, like ZO proteins, which link it to actin [Lee, 2015]. During epithelial cell migration, occludin has been reported to localize at the leading edge of migrating cells and controls the direction of the migrating cell [Du et al, 2010].

Claudins form a charge and size-selective barrier as a result of the adhesive contacts between cells [Colegio et al, 2002; Furuse et al, 1998; Morita et al, 1999; Van Itallie et al, 2008]. Claudins were first revealed by Furuse and Tsukita in 1998 [Furuse et al, 1998; Anderson and Van Itallie, 2009]. They are a large group of proteins of 20–27 kDa size. They are also tetraspanin membrane proteins with one intracellular and two extracellular loops, and C-terminal and N-terminal cytoplasmic domains [Hartsock and Nelson, 2008; Anderson and Van Itallie, 2009; Lee, 2015; Suzuki, 2013]. Claudins are essential constituents and form the backbone of TJs even when they were expressed in fibroblast cells [Furuse et al, 1998; Lee, 2015]. They are a multigene family having around 24 isoforms. In spite of being structurally similar, different claudins perform different functions. Being crucial components of TJ strands, claudins are essential for electrical resistance, permeability, including size, electrical resistance, and ionic charge preference. They can be divided into two types: those involved in barrier formation (decreasing permeability) namely claudin-1, -3, -4, -5, -8, -9, -11, and -14 and those involved in channel pores (increasing permeability) i.e. claudin -2, -7, -12, and -15 [Alexandre et al, 2007; Amasheh et al, 2002; Amasheh et al, 2005; Basuroy et al, 2006; Colegio et al, 2002; Hou et al, 2006; Inai et al, 1999; Tsukita and Fu, 2000; Van Itallie et al, 2001; Van Itallie and Anderson, 2006; Anderson and Van Itallie, 2009; Yu et al, 2003].

The JAM family belongs to the immunoglobulin (Ig) superfamily and is described as having two extracellular Ig domains, one transmembrane
domain, and one intracellular C-terminal domain. JAM-A, which is 43 kDa in size, contributes in the control and maintenance of the TJ barrier [Martin-Padura et al, 1998; Suzuki et al, 2011] through homophillic interactions. JAM-A [Martin-Padura et al, 1998], -B [Cunningham et al, 2000] and -C [Arrate et al, 2001] (or JAM-1, -2, and -3) have class II PDZ-binding motifs in the intracellular C-terminal domain, which interact with ZO-1 and Par-3, which is a polarity related protein. JAM-4 [Hirabayashi et al, 2003], coxsackievirus and adenovirus receptor (CAR) [Bruewer et al, 2005], endothelial selective adhesion molecule (ESAM) [Hirata et al, 2001], and the brain- and testis specific immunoglobulin superfamily (BT-IgSF) [Suzu et al, 2002] have class I PDZ-binding motifs. JAMs are capable of regulating cell proliferation, migration and invasion [Bazzoni et al, 2005; Mandell and Parkos 2005; Mandell et al. 2005]. JAM-A is essential for the internalization of integrins, a pre-requisite for cell movement [Cera et al, 2009]. CAR is a 46 kDa protein that concentrates at cell-cell contacts and co-localizes with ZO-1 in intestinal T84 cells [Cohen et al, 2001].

Tricellulin is a 64 kDa protein, specially localized at junctions of three cells, though it has also been found at bicellular connections. It is a tetraspan membrane protein with one intracellular and two extracellular loops [Ikenouchi et al, 2005; Krug et al, 2009]. When tricellulin is exogenously expressed at high levels, it localizes at all TJs (bicellular and tricellular), and decreases their permeability to both macromolecules and small ions.

2.3: Tight Junctions and cytoskeleton

Actomyosin filaments and microtubules are crucial in tight junction composition and functioning. Attachment of TJP to the perijunctional cytoskeleton is required for constitution of the junction and for maintenance of the barrier. In polarized epithelial cells, the actomyosin cytoskeleton exhibits marked three-dimensional organization in the apico-junctional complex. F-actin that forms a limiting belt plays an important role in controlling paracellular permeability and cell-cell adhesion [Gorfinkiel and Blanchard, 2011]. Physiological and pathological modification of the barrier is associated
with alteration of the junction-associated actin cytoskeleton. Several actin polymerizing proteins like Arp2/3, N-WASP, cortactin and WASP as well as actin regulating proteins such as the Rho GTPase family have been shown to be associated with tight junctions [Van Itallie and Anderson 2014].

**Figure: 2.3 Molecular interactions leading to perijunctional actomyosin ring**
(Adapted from Gorfrinkiel and Blanchard, 2011)

Activation and regulation of different cellular properties conferred by AJC proteins are controlled by their interaction with small Rho GTPases or with GEFs and GAPs. RhoA signaling is inhibited when ZONAB, Cingulin and Paracingulin sequestrate GEFH1 at the TJ. p114RhoGEF upon binding to cingulin, activates RhoA which in turn controls junction formation and epithelial morphogenesis. Rac1 activation during junction formation is through the association of Paracingulin with Tiam. PDZGEF1 and ZO-2 interact directly but indirectly with JAM-A which leads to activation of Rap2c which in turn regulates epithelial permeability by inhibiting Rho signaling. Rho, Rac1 and Cdc42 are inhibited when Bves and ZO-1 attach to GEFT and RhoGEF11. Association of ZO-1 with Tuba, leads to activation of Cdc42 shaping the cell junctions. SH3BP1 and MgcRacGAP are the two TJ GAPs that associate with paracingulin. The latter also binds to cingulin to either inhibit Rac signaling or regulate Cdc42 to control junctional assembly and epithelial morphogenesis [Figure 2.3; Gorfrinkiel and Blanchard, 2011]. Rho, Rac and Cdc42 signaling promotes AJC and perijunctional actomyosin cytoskeletal assembly. Cingulin and ZO-1 recruit p114RhoGEF and
RhoGEF11 at the TJ respectively. p114 RhoGEF binds to NM II and ROCK, creating a RhoA activation complex. These signaling events facilitate assembly of contractile actomyosin filaments that lead to the establishment of the perijunctional actomyosin ring and AJC maturation.

Thus, not only is there a mutual dependence of Rho GTPase(s) control of AJC assembly, the AJC also influences recruitment and activation of these GTPases.

2.4: Other modes of Regulation of Tight Junctions

Several cytokines have been found to play important roles in regulation of epithelial tight junctions in pathophysiological conditions. A few growth factors have also been shown to play roles in protection and maintenance of TJ integrity. The redistribution and expression of TJ proteins and rearrangement of the actin cytoskeleton is brought about by increase in paracellular permeability in intestinal epithelial cells by interferon-γ [Bruewer et al, 2005]. Tumor necrosis factor-α, a proinflammatory cytokine, induces apoptosis and inflammatory response in intestinal epithelial cells [Schulze et al, 2006]. It leads to barrier breakdown by MLCK expression and MLC phosphorylation [Ma et al, 2005] and also through claudin-2 expression [Mankertz et al, 2009]. Recent studies show that Interleukin-1β causes increased intestinal TJ permeability by regulating expression of occludin and MLCK [Al-Sadi et al, 2009]. IL-6 increases paracellular permeability by increasing levels of the pore-forming claudin-2 in intestinal epithelial cells [Suzuki et al, 2011]. The protection of the intestinal barrier has also been attributed to IL-10 [Madsen et al, 1999; Sun et al, 2008].

The intestinal barrier is protected against noxious stimuli including oxidative stress, ethanol, and acetaldehyde by Epidermal Growth Factor [Basuroy et al, 2006]. Transforming Growth Factor-β also protects intestinal barrier function against deleterious stimuli like infectious agents Cryptosporidium parvum and enterohemorrhagic Escherichia coli [Howe et al, 2005].
Paracellular permeability of small, but not large ions is reduced by inhibition of MLCK and CK2. They also disturb the dynamic behavior of ZO-1, which is required to control epithelial barrier. Moreover, the actin-binding region of ZO-1 is essential for tight junction barrier regulation. Thus, the epithelial barrier is regulated when ZO-1 and its transmembrane binding partners, act as a mediators to receive signals from the actin cytoskeleton or other tight junction proteins [Shen, 2012].

2.5: Diseases associated with Tight Junctions

Several diseases have been described where TJs are disrupted. The fence function of TJs is perturbed in cancer, when oncogenes like Ras and Raf1 downregulate occludin and ZO-1 expression [Chen et al, 2000; Li and Mrsny, 2000]. Disturbances in TJs lead to Ca^{++} malabsorption [Selbach, et al, 2002] and also diabetic retinopathy [Igarashi et al, 2000; Sawada et al, 2003; Sawada, 2013]. Decrease in occludin expression had been reported in diabetes [Mullin et al 2005a]. Cytokines released during inflammation due to allergens also lead to TJ dysfunction. Functional changes and actin reorganization brought about by effectors of several pathogens leads to barrier breakdown.

2.6: Barrier breakdown due to infection

Many pathogens use and sometimes disturb the TJs in the apical barrier of the epithelium to infect a host and spread the infection [Bonazzi and Cossart, 2011]. Rotavirus disrupts the TJ using its toxins NSP4 and VP4 by depolarizing host cells [Nava et al, 2004]. This lends the virus access to integrins which are the viral receptors for attaching to host cells. The subsequent release of toxins results in delocalization of TJPs like ZO-1, occluding and Claudin-3 [Tafazoli et al., 2001; Beau et al., 2007]. The initial steps of internalization of Hepatitis C virus requires binding to occludin and CD81 [Ploss et al, 2009], whereas binding to claudin-1 is involved in the later steps of internalization. Invasion of host cells by Reovirus requires interaction
of its surface protein α-1 s with JAM-A [Barton et al, 2001; Guglielmi et al, 2007]. Adenoviruses use surface fiber proteins to bind to CAR proteins and disrupt CAR/CAR interactions at TJs [Kerr, 1999; Walters et al, 2002]. Similarly, coxsackievirus uses the CAR proteins as a co-receptor during infections [Bergelson et al, 1997].

It has been shown that exposure to HIV-1 triggers decrease in transepithelial resistance across intestinal and genital epithelial monolayers in vitro. It also reduces TER with significant decrease in expression of tight junction protein and subsequent increased permeability. Treatment with HIV-1 envelope protein gp120 but not tat, a HIV-1 regulatory protein, also leads to barrier breakdown. Also, inflammatory cytokines including TNF-α, are produced by both intestinal and genital epithelial cells upon exposure of the epithelial monolayers to HIV-1. These cytokines are known to destabilize tight junctions. This was shown to cause a small but significant bacterial and viral translocation across epithelial monolayers. So, HIV-1 exposure at the mucosa directly leads to production of inflammatory cytokines by the mucosal epithelium and is independent of viral infection [Nazli et al, 2010; Schmitz 2002]. When observed microscopically, the colonic epithelial layer appears undamaged. However, intercellular tight junctions (TJ) have been found to be downregulated at the transcriptional and translational levels. Transcription of TJP genes decreases along the proximal-to-distal gut in HIV patients [Chung et al, 2014].

Enteropathogenic Escherichia coli (EPEC) and enterohemorrhagic E. coli (EHEC) effector Tir, is inserted into the host cell plasma membrane and acts as a receptor for the bacterial surface protein intimin [Figure: 2.4; Chen and Frankel, 2005; Guglielmi et al, 2007; Knodler et al, 2001; Hanajima-Ozawa et al, 2007] which upon a cascade of events leads to destabilization of TJs. The intracellular domain of Tir leads to the formation of actin based pedestals by activating the adaptor protein Nck, the actin regulators N-WASP and the Arp2/3 complex, and cytokeratin-18. Secretion of EspF, EspG and Map has an indirect effect on TJ integrity. These three effectors are able to activate the membrane-associated actin-binding protein ezrin, dephosphorylate occludin,
and activate myosin II light chain kinase (MLCK). Activated MLCK can in turn activate myosin II that might have a role in TJ destabilization by pulling on actin filaments.

**Figure: 2.4 Interactions of different bacteria in TJ breakdown** (Adapted from Bonazzi and Cossart, 2011)

*Helicobacter pylori* uses the type IV secretion system to inject its effector CagA into host cells and leads to breakdown of epithelial polarization and tight junctions. In the host cells, CagA, interacts with ZO-1 and JAM1, to form ectopic TJs at the bacteria–host interaction sites by sequestering these proteins away from cell–cell contacts [Amieva et al, 2003; Higashi et al, 2002; Selbach et al, 2002]. PAR1, a regulator of cell polarization, interacts with CagA to enable both SHP-2 interactions with CagA and the mislocalization of TJ proteins from cell–cell contacts.

*Vibrio cholerae* secretes a metalloprotease, called the hemagglutinin/protease (HA/P), which degrades the extracellular domain of occludin [Wu et al, 2000]. This leads to disruption of TJs and dissociation of ZO-1, brought about by conformational change of the cytoplasmic domain of occludin that remains associated with the plasma membrane.

*Clostridium perfringens* secretes the enterotoxin CPE that binds occludin [Singh et al, 2000] and claudin-3 and -4, inducing their degradation [Sonoda
et al, 1999] whereas *Cl. difficile* secretes toxin A and toxin B that monoglucosylate the small GTPases Rac, Cdc42, and Rho, thereby altering actin cytoskeleton [Nusrat et al, 2001]. These toxins also dissociate ZO-1, ZO-2, and occludin from TJs.

*Salmonella* and *Shigella* species invade non-phagocytic host cells by injecting T3SS effectors causing the host membrane to ruffle and resulting in engulfment of bacterial particles. *Salmonella* SPI1 effectors SopB, SopE, SopE2, and SipA alter TJs by decreasing ZO-1 expression levels and delocalization of occludin [Boyle et al, 2006]. The *Salmonella typhimurium* T3SS effector AvrA though, stabilizes TJs, by reducing expression of ZO-1, claudin-1, and occludins [Bonazzi and Cossart, 2011].

*Shigella* infections affect the expression levels of ZO-1, claudin-1, and the phosphorylation state of occludin, leading to a severe disruption of TJs. ZO-1 has also been found to be associated with the distal portion of actin filaments forming *Shigella* and *Listeria* comet tails as well as EPEC-induced actin pedestals [Hanajima-Ozawa et al, 2007; Sakaguchi et al, 2002].

Thus, pathogens like bacteria and viruses have exploited the primary defense machinery of hosts that is epithelial barriers to their advantage. They inhabit epithelia, invade host cells, or even disrupt host barriers by targeting host proteins associated with cell adhesion. Consequently, researchers have also used pathogens and their methods of subverting the host defense to study such vital properties.