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Role of Extracellular Signal-Regulated Kinase (ERK) in Regulation of Intestinal Epithelial Tight Junctions

Abstract

Evidence indicates that MAP kinase (ERK1/2) is involved in regulation of epithelial tight junctions. There are different opinions expressed by investigators as to whether ERK disrupts the junctions or protects them. ERK has also been demonstrated to mediate the EGF-caused protection of the intestinal epithelial tight junctions (TJ) from hydrogen peroxide. Studies using pharmacological inhibitors have shown that EGF increases Thr-phosphorylation of occludin by a MAP kinase-dependent mechanism. This study aimed at looking at the role of ERK in regulation of tight junctions using pharmacological and molecular techniques.

Hypothesis: ERK protects tight junctions in differentiated Caco-2 cells, while it is disruptive to tight junctions in under-differentiated cells, and that in differentiated cells, ERK regulates the activity of PKC ζ to enhance Thr-phosphorylation of occludin.

Specific Aims: 1. To determine if MAP kinase activity induces contrasting effects in under-differentiated and differentiated intestinal epithelium and 2. To determine if ERK enhances Thr-phosphorylation of occludin by modulating the activation of PKC ζ .

Technical Approach: 1. The role of ERK in EGF-mediated Thr-phosphorylation of occludin was determined by evaluating the effect of MEK inhibitor (U0126) on: a) the phosphorylation and translocation of PKC ζ and PP2A, b) the association of PKC ζ and PP2A in occludin immunocomplex in under-differentiated and differentiated Caco-2 cell monolayers, and c) by *in vitro* incubation of recombinant PKC ζ and PP2A with activated ERK. Additionally, the effect of RNA interference was evaluated to determine the influence of ERK on tight junction assembly and maintenance of tight junction integrity. 2. To determine that ERK activity produces contrasting effects in under-differentiated and differentiated cells, Caco-2 cells were transfected with wild type (WT), dominant negative (DN) and constitutively active (CA) forms of MEK in an inducible vector (GFP-tagged pTRE2hyg). The expression of MEK and thus ERK was regulated in the Caco-2 cells on various stages of cellular differentiation, and its effect on tight junction integrity evaluated by measuring TER, inulin flux, immunofluorescence localization of TJ-proteins. The role of ERK in the regulation of tight junctions was also studied in mouse ileal epithelium by localization of occludin in detergent-soluble and insoluble fractions, and by immunofluorescence for tight junction proteins in the intestinal epithelial cells.

Results: Reduced expression of ERK by RNA interference enhanced the assembly and integrity of tight junctions. ERK does not directly phosphorylate occludin, but it enhances Thr-phosphorylation of PKC ζ in differentiated Caco-2 cells as well as *in vitro* assays. PKC ζ in turn, phosphorylates occludin on threonine residues. Thus ERK indirectly regulates Thr-phosphorylation of occludin, and preserves the integrity of tight junctions. ERK prevents hydrogen peroxide-induced translocation of PP2A to the TJs and thus preventing dephosphorylation of occludin by PP2A. Expression of CA-MEK in under-differentiated cells leads to disruption of tight junctions, while it enhances the tight junction integrity in differentiated cells. The opposite effects were observed with the expression of DN-MEK. In mouse ileum, ERK protected the tight junctions against oxidative stress produced by hydrogen peroxide.

Conclusions: ERK has a contrasting effect on tight junctions in differentiated and under-differentiated Caco-2 cells. This effect is produced by modulating the activation of PKC ζ and translocation of both PKC ζ and PP2A.

Document Type

Dissertation

Degree Name

Doctor of Philosophy (PhD)

Program

Biomedical Sciences

Research Advisor

Radhakrishna Rao, Ph.D.

Keywords

ERK, Tight Junction, MAPK

Subject Categories

Medical Biochemistry | Medical Cell Biology | Medical Sciences | Medicine and Health Sciences

**ROLE OF EXTRACELLULAR SIGNAL-REGULATED KINASE (ERK) IN
REGULATION OF INTESTINAL EPITHELIAL TIGHT JUNCTIONS**

A Dissertation
Presented for
The Graduate Studies Council
The University of Tennessee
Health Science Center

In Partial Fulfillment
Of the Requirements for the Degree
Doctor of Philosophy
From The University of Tennessee

By
Sudhir Aggarwal
December 2010

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DEDICATION

I dedicate this dissertation to the everlasting memory of my late grandfather
Lala Niranjan Lal Mahajan
to whose great vision and wisdom I attribute my quest for knowledge and excellence.

ACKNOWLEDGEMENTS

I would like to take this opportunity to express my gratitude for my mentor Dr. R.K. Rao, and my graduate committee members Dr. Polly A. Hofmann, Dr. Anjapravanda P. Naren, Dr. Trevor W. Sweatman and Dr. Christopher M. Waters who have guided me throughout my project for this dissertation. Their guidance, meaningful suggestions and constructive criticism has very certainly helped to make this work much more appealing. I am also grateful to my present and previous colleagues in Dr. Rao's lab- Dr. Geetha Samak, Dr. Takuya Suzuki, Dr. Parimal Sheth, Dr. Ankur Seth, Dr. Bertha Elias, Dr. Suneet Jain and Ms. Mitzi Dunagan for providing a healthy and cordial environment in the lab, and thus facilitating this work.

I also thank Dr. Gabor J. Tigyi Chair of the Physiology Department and personnel in his lab for helping in this project in several ways. I am also grateful to Dr. Willam Taylor and his staff at the Molecular Resources Center for providing logistical and intellectual support for some of the studies. Special thanks are due to Dr. Ramesh Ray who has been a guiding spirit all along my journey towards the completion of this work. I am also grateful to Dr. Richard Peppler, Dr. Cheryl Scheid, Dr. Pat Ryan and Dr. Don Thomason, who in their administrative capacities at the College of Graduate Health Sciences have been very generous with their encouragement and support for me whenever required. I would also like to thank Mr. Larry Tague for his continued help and guidance. My sincere thanks to Ms. Shirley Hancock and her group for helping in format review of this dissertation. I am also grateful to the administrative and secretarial staff of the Physiology department and the graduate school for all their help and cooperation.

I would also like to thank my father Mr. D.D. Aggarwal, my late mother Mrs. Raksha Aggarwal and other family members back home in India, who have been a continuous source of inspiration and motivation to me in this arduous journey. I am also thankful to my wife Ritu and loving sons Adamyia and Arsh whose unflinching love and support has been like a beacon light and a reason for me to keep going.

ABSTRACT

Evidence indicates that MAP kinase (ERK1/2) is involved in regulation of epithelial tight junctions. There are different opinions expressed by investigators as to whether ERK disrupts the junctions or protects them. ERK has also been demonstrated to mediate the EGF-caused protection of the intestinal epithelial tight junctions (TJ) from hydrogen peroxide. Studies using pharmacological inhibitors have shown that EGF increases Thr-phosphorylation of occludin by a MAP kinase-dependent mechanism. This study aimed at looking at the role of ERK in regulation of tight junctions using pharmacological and molecular techniques.

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Conclusions: ERK has a contrasting effect on tight junctions in differentiated and under-differentiated Caco-2 cells. This effect is produced by modulating the activation of PKC ζ and translocation of both PKC ζ and PP2A.

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LIST OF ABBREVIATIONS

ALP	Alkaline phosphatase
AS-ERK	Asntisense specific for ERK
BSA	Bovine serum albumin
CA-MEK	Constitutively active MAPK-ERK kinase
D	Differentiated
DMEM	Dulbecco's modified eagle medium
DN-MEK	Dominant negative MAPK-ERK kinase
dsRNA	Double stranded RNA
EDTA	Ethylenediamine tetraacetic acid
EGF	Epidermal growth factor
EGTA	Ethylene glycol tetraacetic acid
ERK	Extracellular signal-regulated kinase
FITC-inulin	Fluorescein isothiocyanate inulin
GFP	Green fluorescent protein
GSK	Glycogen synthase kinase
GST	Glutathione S-transferase
HPLC	High performance liquid chromatography
JAM	Junctional adhesion molecule
MAPK	Mitogen activated protein kinase
MEK	MAPK-ERK kinase
MS oligo	Missense oligo
NS-RNA	Non-specific RNA
OCT compound	Optimal cutting temperature compound
PBS	Phosphate buffered saline
p-ERK	Phospho-ERK
PKC ζ	Protein kinase C zeta
PLC	Phospholipase C
PMSF	Phenylmethylsulfonyl fluoride
PP1	Protein phosphatase 1
PP2A	Protein phosphatase 2A
si-ERK	siRNA specific for ERK
TBST	Tris buffer solution with tween
TER	Transepithelial electrical resistance
Thr	Threonine
TJ	Tight junctions
Tyr	Tyrosine
UD	Under-differentiated
WT-MEK	Wild type MAPK-ERK kinase
ZO	Zona occludens

CHAPTER 1: TIGHT JUNCTIONS: AN INTRODUCTION

1.1 Intercellular Junctional Complex

The epithelial cells perform various specialized functions in multicellular eukaryotic organisms. An important function among those is the barrier function. To act as effective barriers, the epithelial cells form adhesions with each other, thus forming a junctional complex.¹ This intercellular junctional complex helps the cells maintain homeostasis between the extra cellular and intracellular environments as also between various compartments or cavities of the body.^{2,3} This function can be attributed to the capability of the tight junctions to prevent movement of substances across the paracellular route. Thus the movement of all the substances that have to be transported across the epithelial layer has to occur by diffusion or active transport across the plasma membrane (trans-cellular route). This helps the cells to select and regulate the passage of various substances that can cross the epithelial layer.⁴

The junctional complex, in the vertebrates, consists of tight junctions, adherent junctions, desmosomes and gap junctions. A diagrammatic representation of the epithelial junctional complex is shown in **Figure 1.1**. Tight junctions are present at the interface of the apical and basolateral surface of the epithelial cells. They form a selective barrier against solutes and thus help maintain the polarity of the epithelial cells. Adherens junctions are present at the adjoining surfaces of the cells, basal to the tight junctions. They are present either as Zonula adherens encircling the cells or as adhesion plaques, as points of attachment to the extra cellular matrix. They provide a strong mechanical attachment between the adjacent epithelial cells. Thus they hold the adjacent cells together, and are also considered responsible for contact inhibition.⁵⁻⁷ Desmosomes are spot like adhesions present at the junctional surfaces of the adjacent cells. They are randomly distributed along the surface and protect the cells from shearing forces. They also help in holding the adjacent cells together.⁸ Gap junctions are also present between the adjacent epithelial cells. They connect the cytoplasm of the adjoining cells and facilitate movement of small intracellular signaling molecules from one cell to another.⁹⁻¹¹ Our interest, from among all the components of the epithelial junctional complex, is in the tight junctions, and these will be reviewed in greater detail in the following sections.

1.2 Morphology of Tight Junctions

As stated earlier, tight junctions in the vertebrate epithelial cells perform an important function of providing a barrier to passage of substances across the paracellular spaces in the epithelial layer. The requirement and significance of this barrier function determines the organs or types of cells where the tight junctions are located in the body. In other words, tight junctions are present in those organs or cell types in which it is critical to maintain a separation between the environments on the apical and basolateral the cell surfaces. Few examples of such organs are the gastrointestinal tract, kidneys etc.

Figure 1.1: Epithelial tight junction complex. Source: Denker, B.M. & Nigam, S.K. Molecular structure and assembly of tight junction. *Am. J. Physiol. Renal Physiol.* **274**, F1-F9 (1998).¹⁸⁸ (Reprinted with permission.)

Besides being present between the polarized epithelial and endothelial cells, the tight junctions also help in the formation of blood brain barrier in the oligodendrocytes and choroids plexus in the nervous system, blood testis barrier in the sertoli cells, in the organ of corti and collecting tubules of the kidney. Among all the sites where tight junctions are present, our focus is on the intestinal epithelium.

The mucosal layer of intestinal epithelium performs a critical function of selectively allowing the nutrients to pass through from the luminal side of the gastrointestinal tract, while restricting the passage of pathogens, toxins and allergens through the same route.^{12,13} In addition to performing this all-important barrier function, tight junctions also help the cells maintain polarity between their apical and basolateral sides. This characteristic is called the ‘fence function’ of tight junctions.^{2,14,15} The considerable amount of work done on tight junctions during the last several years has transformed the way the tight junctions are looked at now. Besides the ‘lipid model’ and ‘protein model’ of tight junction structure proposed earlier,¹⁶ now tight junctions are known to be associated with at least 50 different proteins that include both structural and signaling proteins. Thus tight junctions, instead of being just the barriers to paracellular permeability, have become an arena for the study of the interesting interplay between numerous signaling molecules and cascades.

Tight junctions cannot be seen well with a light microscope. So transmission and freeze fracture electron microscopy have been used to study the structure of tight junctions. Transmission electron microscopy has revealed that tight junctions are a series of contact points present on the apical side of adjacent epithelial cells (**Figure 1.2**). Under freeze fracture electron microscope, they appear as an anastomosis of fibrillar strands which seal the intercellular space¹⁷ (**Figure 1.3**).

Each strand of this network is composed of several transmembrane proteins embedded in the plasma membrane of the adjacent cells. This structural depiction of tight junctions is significantly different from the earlier descriptions when tight junctions were believed to be rigid structures made up of some molecules connecting the neighboring cells by calcium links. With the advent of newer imaging techniques, this opinion has changed and tight junctions are now considered to be dynamic structures. This opinion has stemmed from the observations that the degree of tightness of the tight junctions varies with the location of the tight junctions and the physiological function of the organ they are present in. For example, the junctions are tighter in distal tubule of the nephrons than in the proximal tubules,¹⁸ and the vascular endothelial barrier present in brain (Blood Brain Barrier) is certainly tighter than the similar barriers present in the capillaries elsewhere in the body.¹⁹ This variation in the tightness of the barrier made by the tight junction determines the selectivity of that particular barrier to the substances that can move across the epithelial layer. Structurally, this variation is a function of the number of sealing strands present across the plasma membranes of the two adjacent cells. Therefore, the tightness and thus the selectivity of the barrier is directly proportional to the number of such strands.^{18,19} Another factor that has led the researchers to believe in the dynamic nature of tight junctions is the association of numerous signaling molecules with various tight junction proteins.²⁰⁻³³

Figure 1.2: A transmission electron microscopic image of the epithelial tight junction complex. (TJ, Tight junction.) Source: Schneeberger E.E. & Lynch R.D. Structure, function and regulation of cellular tight junctions. *Am. J. Physiol.* **262**, L647-L661 (1992).² (Reprinted with permission.)

Figure 1.3: A freeze fracture electron microscopic image of the epithelial tight junction complex. (TJ, Tight junction.). Source: Schneeberger E.E. & Lynch R.D. The tight junction: a multifunctional complex. *Am. J. Physiol.* **286**, 1213-1228 (2004).³ (Reprinted with permission.)

1.3 Function of Tight Junctions

Tight junctions perform three important functions, namely cell-cell adhesions,³⁴ maintaining the distinct polarity between the apical and basolateral surfaces of the cell membrane,¹⁴ thus maintaining the surface specific functions of the cells, and regulation of transport of fluids and solutes across the epithelium.³⁵ The movement of substances across the epithelium occurs through the two pathways- transcellular and paracellular. Transcellular transport involves movement of water and solutes across the plasma membrane. This is an active process and is mediated by various specific ion channels and transporters present on the plasma membrane. On the contrary, paracellular transport is movement of solutes and water across the intercellular space. This is a passive process which is governed by the osmotic gradient produced by the transcellular transport. Paracellular transport is characterized by the quality of selective permeability, *i.e.* the permeability of the substances that moves across this route is determined by their molecular size or the ionic charge or both. Tight junctions regulate this paracellular permeability, and thus form an important barrier against entry of toxins, allergens, pathogens and even malignant cells across the epithelial layer in the gastrointestinal tract.^{12,36} Thus they protect the gastrointestinal tract against several diseases, edema, diarrhea, jaundice and metastasis by hematological route.³⁷ Obviously, this barrier function of the tight junctions is compromised in various gastrointestinal diseases such as inflammatory bowel disease (IBD), Crohn's disease, infectious enterocolitis and colon cancer.^{38,39} In addition, the tight junctions also help to define the polarity of epithelial cells by distinguishing the apical side of the cells from the basolateral side, thus working as a fence.^{2,14}

1.4 Role of Tight Junctions in Disease States

Disruption in the structure and function of tight junctions can be attributed to a host of diseases in human beings. Primary biliary cirrhosis, primary sclerosing cholangitis, malabsorption, celiac disease, multiple sclerosis, endotoxemia and some types of diarrhea are among the diseases which owe their etiology to the disruption in the barrier function of the tight junctions.³⁷ This compromised barrier function is also suspected to have an association with the causation of the inflammatory bowel disease like ulcerative colitis and Crohn's disease.^{4,37} On the other hand, loss of fence function of the tight junctions has been implicated in tumorigenesis and metastasis of various cancers in the humans.³⁷ Tight junctions are also understood to play a role in cell growth, proliferation and differentiation. Interaction between neighboring cells regulate epithelial cell proliferation and migration.²⁰ Tight junctions suppress proliferation and stimulate differentiation. Also the over-expression or down-regulation of certain tight junction components is known to play a role in epithelial-mesenchymal transformation^{40,41} and development of cancer.⁴²⁻⁴⁴ Some tight junction proteins are present in the nuclei of the dividing cells and thus regulate transcription, RNA processing and cell proliferation.^{41,45}

1.5 Molecular Structure of Tight Junctions

Tight junctions are composed of several proteins, which can be broadly classified into the following types: 1. Structural transmembrane proteins like Occludin,⁴⁶ Claudins,^{47,48} Junctional Adherens Molecules (JAM)^{3,49} and tricellulin.⁵⁰ 2. Intracellular Plaque proteins like Zona occludens ZO-1, ZO-2, ZO-3,^{51,52} Cingulin,^{53,54} Symplekin⁵⁵ etc. and 3. Signaling molecules / proteins like various kinases^{21,24,25,27,29,31,56,57} and phosphatases⁵⁷⁻⁶⁰ etc.

1.5.1 Transmembrane Proteins

1.5.1.1 Occludin

Occludin is a 63 kDa protein as seen on SDS-Page gel, and is one of the major transmembrane proteins of the tight junction complex.⁴⁶ Chemically, it is a phosphoprotein, and structurally is composed of four transmembrane and two extra cellular loops (**Figure 1.4**). Both the C-terminus and the N-terminus are located in the cytoplasm. The 150 amino acid long C-terminus of occludin as well as its first extra cellular loop are significantly conserved. The first extra-cellular loop of occludin is important in cell to cell binding, and it is rich in tyrosine and glycine residues. The second extra cellular loop on the other hand is rich in tyrosine residues, and plays a role in maintaining the paracellular transport pathway. The C-terminal region of occludin has an abundance of serine, threonine and tyrosine residues, which are substrates for numerous kinases and phosphatases acting together to regulate the integrity of tight junctions. Occludin is located mainly at the epithelial and endothelial cell junctions. Tight junction integrity is known to be directly proportional to its expression on these sites. The C-terminal region and the second extra cellular loop in the structure of occludin hold a place of importance in the regulation of tight junction integrity by occludin.^{46,61,62} It has been shown that the presence of occludin without its C-terminus leads to permeable junctions in the cells.⁶³ A similar effect is produced by treatment of cells with a synthetic peptide corresponding to the second extra cellular loop of occludin.⁶⁴ Even though occludin is considered to be a major structural protein in the tight junctions, it is not completely indispensable for the tight junctions. Some cells which do not express occludin at all, like the embryonic stem cells are known to form tight junctions too.^{2,3,62,65} Saitou *et al* have been able to generate occludin knockout mice and have reported that even though such mice had histological abnormalities in several tissues like hyperplasia of intestinal epithelium, calcifications in brain, testicular atrophy, thinning of compact bone etc., the tight junctions are normal morphologically. The electrophysiologically assessed function of the intestinal epithelium too was found to be normal.⁶⁶ This evidence directs us towards some other transmembrane proteins which may be structurally different, but can perform the same function as occludin.

Figure 1.4: The proteins forming the tight junction complex. (JAM, Junctional Adhesion Molecule). Source: Schneeberger E.E. & Lynch R.D. The tight junction: a multifunctional complex. *Am. J. Physiol.* **286**, 1213-1228 (2004).³ (Reprinted with permission.)

1.5.1.2 Claudins

Claudins are another group of transmembrane proteins, which contribute to the formation of tight junctions in epithelial cells. They are tetraspan proteins ranging from 20-25 kDa. This group of proteins is continuously expanding. At present, at least 24 of the claudins are known. Based on their sequence analysis, claudins have been classified into two groups- classic claudins (1-10, 14, 15, 17, and 19) and non-classic claudins (11-13, 16, 18, 20-24). Some claudins like Claudin-1 are ubiquitously expressed in the cells, whereas some others are more specific for certain cell types or period of development. Each claudin shows a unique crypt to villous expression pattern. They show diverse sequence variability. Like occludin, claudins too have a C-terminus and extra cellular loops. They all end in PDZ-binding motifs that interact with the PDZ domains in the scaffolding proteins (ZO-1) of the cell.⁶⁷⁻⁶⁹ Thus, claudins may have a role in maintenance of tight junction integrity in the absence of occludin. Claudin expression is very much tissue-type specific *e.g.* Claudins 1, 2, 3, 4, and 8 are found predominantly in kidney and liver tissue, claudin-5 in endothelial cells, claudin-11 in sertoli cells and brain myelin sheets, and claudin 1 and 2 in gastrointestinal tract.⁴⁷

1.5.1.3 Junctional Adhesion Molecules (JAM)

These proteins which belong to immunoglobulin superfamily have come to be known only recently (**Figure 1.4**). They do not have structural resemblance with the occludin or the claudins. It has been revealed by confocal and immunoelectron microscopy that they are localized in the apical region of the intercellular cleft. They have been demonstrated to co localize and interact with occludin, thus playing a role in the regulation of tight junction structure and function. JAM also has some role in transmigration of immune cells across the cell monolayer.^{49,69,70}

1.5.1.4 Tricellulin

It is a 70 kDa novel integral membrane protein containing four transmembrane domains (**Figure 1.5**). It is found in tricellular tight junctions where three cells join one another. It was identified as the first integral membrane protein which is localized at vertically oriented strands of tricellular tight junctions. Tricellulin has recently been reported to be present at bicellular tight junction too. Interestingly, whereas Tricellulin enhances junctional integrity in bicellular tight junctions (as demonstrated by increase in paracellular electrical resistance and permeability to ions and macromolecules) accompanied by enhanced ultrastructural integrity, in tricellular tight junctions, the ultrastructure was unaltered and permeability of macromolecules was decreased but that of ions was not affected.^{50,71}

Figure 1.5: Tricellulin. Source: Ikenouchi, J. *et al.* Tricellulin constitutes a novel barrier at tricellular contacts of tight junction strands. *Mol. Biol. Cell.* **171**, 939-945 (2005).⁵⁰ (Reprinted with permission.)

1.5.2 Plaque Proteins

1.5.2.1 Zona Occludens

These proteins, several of which are known to us, are present in the cytoplasm of the epithelial cells. They interact with the transmembrane proteins of the cell on one side and the actin cytoskeleton on the other. By virtue of their strategic location and interactions, these plaque proteins act as scaffolds between the transmembrane proteins, actin cytoskeleton and various signaling molecules regulating the tight junction dynamics in the cells. Some of the plaque proteins that have been identified are ZO-1, ZO-2, ZO-3, symplekin, cingulin, 7H6 and rab13.^{18,72,73} The Zona occludens (ZO) proteins are members of the MAGUK (Membrane Associated Guanylate Kinase) family, and are known to have three protein domains, at least one PDZ domain, an SH3 (Src Homology 3) domain and a GUK (Guanylate Kinase) homologous region, arranged in a sequential order.^{52,68,74} This structure of the ZO proteins is quite conserved. ZO-1, ZO-2 and ZO-3 interact with occludin, and ZO-1 is considered to bring occludin to the tight junctions.^{51,72} Even though presence of occludin is not absolutely essential for formation and maintenance of tight junctions, this interaction between occludin and ZO-1 may have some subtle and finer function in maintenance of the tight junctions. ZO-2 and ZO-3 do not have a substantial presence at the tight junctions even though they do interact with occludin.

1.5.2.2 Cingulin

Cingulin is a 140-160 kDa phosphoprotein that is localized on the cytoplasmic surface of the epithelial tight junctions. It forms coiled-coil parallel dimers, which further aggregate through intermolecular interactions, and interacts with both ZO-1 and ZO-2. Structurally, it consists of a head domain and a tail domain. Studies indicate that both head and the rod (tail) domain are required for the junctional localization of cingulin. Cingulin interlinks the submembrane plaque proteins of the tight junctions with the actomyosin cytoskeleton of the cells, and is considered to be a functionally important component of the tight junctions.⁷⁵

1.5.2.3 7H6 Protein

7H6 is a 155 kDa phosphoprotein found in the tight junctions of hepatocytes and epithelial cells. It is known to regulate the paracellular permeability in both epithelial and endothelial cells. It has been observed to be sensitive to the functional state of the cells. With the depletion of ATP in the cells, it dissociates from the junctional complex in a reversible manner. Expression of 7H6 progressively decreases as the cell state progresses from normal to dysplastic to carcinomatous.^{53,76}

1.5.2.4 Symplekin

It is a 126 kDa protein that has been localized in the junctional plaque. Symplekin is seen in the tight junctions of the sertoli cells but not in the endothelial cells. It is localized at the junctions as well as in the nucleus of the epithelial cells. As far as its

role is concerned, Symplekin is speculated to control nuclear events, like reporting the functional status of tight junction contacts.^{55,76}

1.5.3 Actin Cytoskeleton

Actin forms a major constituent of the cellular cytoskeleton. Even though actin is distributed all over in the cell, its interaction with the transmembrane proteins at the tight junction complex has a very significant role to play in tight junction integrity and stabilization and regulation. While an intact structural organization of actin filaments in the cell is responsible for maintenance of the tight junctions, a disruption of the same can lead to leaky junctions.^{20,61,72,77-85} Despite continued efforts by numerous investigators, our knowledge of the interactions between various junctional proteins and actin cytoskeleton is far from complete. Several modes have been proposed to describe the interactions between actin and occludin- the most common being the ZO-1 acting as a connector between the two. ZO-2 and ZO-3, besides occludin are also suggested to be interacting with F-actin, but this interaction is sideways rather than end to end.^{52,73}

1.6 Regulation of Tight Junctions by Signaling Cascades

Besides the junctional proteins, the plaque proteins and the actin cytoskeleton, there are several other proteins present in the vicinity of tight junction complex. Their role in the maintenance of tight junctions is more of a regulatory nature. These regulatory proteins/molecules are components of several different pathways, interplay of which is vital for maintenance and regulation of tight junction integrity. Some examples of such signaling molecules are MAP kinase, protein kinase C, protein phosphatase 2A, G-proteins, c-Src, phosphatidylinositol-3-kinase (PI3K) and phospholipase C γ . Modulation of the activity of these signaling molecules by pharmacological methods has demonstrated their role in regulation of TJ integrity in different epithelia.

1.6.1 Calcium and Cyclic-AMP

Myosin-generated tractions on the tight junction strands are considered to be the simplest model of regulation of tight junction permeability. It has been observed that myosin light chain kinase (MLCK) causes phosphorylation of the regulatory light chain of myosin and activates its contractions. This ATP-dependent contraction is regulated through a calcium-dependent mechanism.⁸⁶

c-AMP is also known to alter epithelial cell permeability by its interaction with the cytoskeletal system in gallbladder epithelium,⁸⁷ or proteasome-sensitive ubiquitination of occludin in inter-septal tight junctions.⁸⁸ c-AMP has also been demonstrated to influence microvascular permeability.⁸⁹

1.6.2 G-proteins

G-proteins have been demonstrated to be localized near the junctional complex, associated with at least one TJ protein, and to accelerate TJ biogenesis. Rho-subfamily of small GTP-binding proteins is known to coordinate functional state of actin. G-proteins, when activated, interact with their target proteins and regulate the dynamics of actin in the cells. They are also known to cause phosphorylation and inactivation of MLC phosphatase.^{22,23,32,33,76}

1.6.3 Protein Phosphatases

Protein phosphatases PP2A and PP1 directly interact with tight junctions. It is also known that enhanced PP2A activity induces dephosphorylation of the TJ proteins, ZO-1, occludin, and claudin-1, and is associated with increased paracellular permeability. Expression of PP2A catalytic subunit severely prevents TJ assembly. Conversely, inhibition of PP2A by okadaic acid promotes the phosphorylation and recruitment of ZO-1, occludin, and claudin-1 to the TJ during junctional biogenesis. Thus PP2A negatively regulates TJ assembly without appreciably affecting the organization of F-actin and E-cadherin.^{58-60,90}

1.6.4 Protein Kinases

Protein kinases play a very significant role in regulation of tight junction integrity. They are capable of phosphorylating specific (Serine, Threonine or Tyrosine) residues and bring about the changes in tight junction structure and function.

1.6.4.1 Tyrosine Kinases

Many Tyr-kinases like c-Src and c-Yes are involved in regulation of tight junction barrier function. The finding that oxidative stress-induced disruption of tight junction is mediated by the activation of c-Src, and that the dominant-negative expression of kinase-inactive c-Src delays the oxidative stress-induced disruption of tight junction and accelerates calcium-induced assembly of tight junction in Caco-2 cells demonstrated the role of c-Src in regulation of TJ.²⁵ There is also an evidence of Tyrosine kinase activity having a role in both assembly and disassembly of TJs. Occludin undergoes phosphorylation on Tyr residues during TJ disruption by oxidative stress. Studies have shown that tyrosine kinase inhibitors prevent oxidative stress induced disruption of TJ in Caco-2 cells. In these studies, phosphorylation of tyrosine residues on occludin, ZO-1, E-cadherin and β -catenin, and dissociation of these proteins from actin cytoskeleton lead to disruption of TJs. c-Src is also known to play a significant role in oxidative stress induced tyrosine phosphorylation of TJ proteins and decrease in TJ integrity. Tyr-phosphorylation of occludin also results in loss of its interaction with plaque proteins ZO-1 and ZO-3.^{27-29,31,91,92}

1.6.4.2 Serine/Threonine Kinases

Ser/Thr kinases such as ERK and PKC ζ , and Protein kinase C (PKC) are required for the proper assembly of tight junctions. Low concentrations of the specific inhibitor of PKC, calphostin C, markedly inhibit tight-junction biogenesis.²¹ Another study showed that oxidative stress increases the association of PI 3-kinase with occludin, and that PI 3-kinase activity is involved in oxidative stress-induced disruption of tight junction.²⁴ In another study, inhibition of PLC-gamma by PLC inhibitor, 3-nitrocoumarin was associated with an increase in TJ permeability.⁹³

1.6.5 Phosphorylation of Tight Junction Proteins

Kinases,^{21,56,94} along with protein phosphatases⁵⁸⁻⁶⁰ play a key role in maintaining the structure and function of tight junctions, by way of reversible phosphorylation of the tight junction proteins. Phosphorylation plays a very important role in regulation of various functions during a cell's life cycle, beginning from growth and differentiation to genetic regulation, signal transduction and the response to external stimuli. Protein kinases and phosphatases act in a complementary way to maintain various cellular proteins in a state of phosphorylation or dephosphorylation as the need may be in the cell. Any disruption of the delicate balance existing between these two sets of enzymes can be detrimental to the overall health of the cells and different biological processes going on therein. Activity of kinases and phosphatases, in turn, is influenced and regulated by several other regulatory molecules and proteins. Phosphorylation of intracellular and junctional proteins on serine, threonine and tyrosine residues has been demonstrated to be important in regulation of their functions viz growth, development, differentiation and migration. Any malfunction of these regulatory enzymes can lead to various diseases and abnormalities, like diabetes, hypertension, cardiac hypertrophy, genetic defects and cancer etc.^{95,96} Although the kinases and phosphatases involved in the regulation of Thr-phosphorylation of occludin are unknown, evidence suggests that PKC, MAPK, PP2A and PP1 are associated with TJ protein complex and regulate the integrity of TJs.^{58-60,97,98}

Occludin- one of the major tight junction proteins, is known to be present in the cells in both phosphorylated and non-phosphorylated forms. And it is also found in both the detergent-soluble and detergent-insoluble fractions of cellular proteins. The detergent (triton)-insolubility is an indication that occludin may not be present in isolation in the cells. It may rather be forming a complex with the actin cytoskeleton of the cell or other junctional proteins.⁹⁹

Another interesting fact is that on SDS-PAGE analysis, in addition to the bands corresponding to the regular molecular weight for occludin, some bands having higher molecular weight have also been noticed. The possibility that these higher molecular weight bands correspond to the phosphorylated form of occludin gets strengthened by the fact these bands disappear upon treatment of the samples by phosphatases.^{63,100,101} Further analysis has revealed that the phosphorylated form of occludin had undergone phosphorylation at serine and threonine residues.¹⁰²⁻¹⁰⁴

Studies have also found correlation between integrity of the tight junctions and the phosphorylation state of occludin. This comparison was made by incubating the cells in the presence of regular calcium containing or low calcium medium and then studying the bands of occludin in both cases. When the cells were subjected to low calcium medium leading to disassembly of tight junctions, occludin was seen in its unphosphorylated form and in the detergent soluble fraction. However, when the regular calcium containing medium was restored to the cells, leading to restoration of the tight junctions, the bands of phosphorylated occludin were seen in the detergent insoluble fraction. These observations have been corroborated by the immunofluorescence studies. Thus a logical conclusion that can be drawn out of this is that phosphorylation of occludin on serine and threonine residues is associated with intact tight junctions, whereas the unphosphorylated form of occludin that is seen in disrupted tight junctions is present in the form of a punctate appearance along the basolateral membrane of the cells.^{100,102,105}

Several theories have been postulated to explain the mechanism behind hyperphosphorylation of occludin and formation of the integrity of tight junctions. One school of thought is that hyperphosphorylated occludin is able to oligomerize through the extra cellular matrix, thus leading to stronger junctions between the cells.¹⁰⁰ Another opinion suggests that the hyperphosphorylation of occludin on serine and threonine residues helps it bind to the plaque proteins in the cell, thus bringing about the desired effect.^{51,106}

Phosphorylation of occludin has been observed over a wide spectrum of cell lines and species. But only a limited amount of information is available as to where specifically the occludin gets phosphorylated, which kinases are involved in the process and what molecules participate in regulation of this process. Some recent work done by various investigators points to the role of PKC and ERK in the phosphorylation of occludin on serine and threonine.⁹⁷ Studies have also shown that some of the phosphorylation sites in occludin are located in the ZO-1 binding domain of occludin while some others are present in domains responsible for targeting occludin to the tight junction complex.^{51,106} Since these studies suggest a crucial role played by Ser/Thr phosphorylation of occludin in TJ regulation, it is important to understand the role of this process during TJ assembly and disruption.

There are several serine threonine kinases present in the cell that are responsible for bringing about this dynamics to the tight junctions. Two examples are: MAP kinases, protein kinases. Serine-threonine kinases phosphorylate the –OH group of these amino acids which have similar side chains. The activity of these kinases is regulated by some events occurring during the life cycle of the cell, like DNA damage. Some chemical signals or signaling molecules like cAMP, cGMP, calcium, calmodulin and diacylglycerol etc. also have a role in spinning these kinases into action.^{107,108} Out of these, our interest is in looking at the role of MAP kinases in the regulation of tight junctions.

1.6.6 MAP Kinases and Their Role in Regulation of Tight Junctions

As stated above, they are a group of serine-threonine kinases, which are very important for the cell's normal functioning. They respond to extra cellular stimuli, and regulate various cellular activities, like gene expression, mitosis, differentiation, and cell survival/apoptosis. They can be further classified into 4 sub-groups:¹⁰⁹

1. **Extra cellular signal regulated kinases (ERK):** They are the classical MAPKs, and are activated by growth factors.
2. **c-Jun N-terminal kinases (JNK):** They phosphorylate c-Jun on Ser63 and Ser73, and respond to stress stimuli, such as cytokines, ultraviolet irradiation, heat shock, and osmotic shock.
3. **p38 isoforms:** This sub-group of MAPKs is responsive to stress stimuli.
4. **ERK5:** They are activated by growth factors and stress stimuli.

Among all the MAP kinases, the focus of this study is on ERK.

1.6.6.1 ERK

ERKs were initially discovered as kinases that were activated in response to Insulin and NGF.^{110,111} ERK1 and ERK2 are 44 kDa and 42 kDa molecules respectively, with 90% of sequence similarity in mammals. Both of them are expressed in most if not all mammalian cells. Studies have shown that in case of the experimental knock down of one, the other may step in to at least partially compensate for the loss.¹¹² Interestingly, to bring about the phosphorylation of their target substrate proteins on serine and threonine residues, these ERK proteins have to get activated themselves by a combined phosphorylation on threonine and tyrosine residues.¹¹³ Another group of kinases called MAPKK or MAP2K is responsible for bringing about this activation of MAPK.¹¹⁴ The activation /phosphorylation of ERK is kept in check by the protein phosphatases like PP2A and PP1, that remove the phosphates attached to the serine/threonine and tyrosine residues of ERK.¹¹⁵ The activity of ERK is influenced by two major factors:

1. Activation of transmembrane receptors: by various stimuli like ligands etc.¹¹⁶
2. Inactivation of phosphatases: as seen with oxidative stress.¹¹⁷

Recent studies have revealed that scaffolding may be a mechanism by which ERK cascade causes the signal transduction with a high efficiency and specificity.¹¹⁸ Downstream, ERK has several cytoplasmic and nuclear targets. In the cytosol, it activates the growth-factor responsive proteins.¹¹⁹ On the other hand, when it translocates to the nucleus, it regulates the gene expression and DNA replication by phosphorylating various transcription factors.¹²⁰

1.6.6.2 ERK in Regulation of Tight Junctions

Not much information is available on the exact role ERK plays in the regulation of tight junctions. The few studies that have tried to find out the role of ERK have only produced mixed results. In one study, ERK1/2 activation inhibited claudin-2 expression and transiently increased TJ integrity. This process was blocked by the ERK1/2 inhibitor U0126. This study provided evidence of control of TJ integrity by ERK1/2 signaling pathway by alterations in claudin composition within tight junction complexes.¹²¹ In another study, activation of PKC was shown to cause disruption of TJs through activation of MAP kinase.¹²² A recent study showed that bile in the intestinal lumen initiates ERK1/2-dependent signaling that is essential for normal expression of key TJ proteins and regulation of TJ integrity.¹²³ It was also demonstrated that bile acids influence the permeability of intestinal epithelium by autophosphorylating EGF receptors, dephosphorylating and rearranging occludin at the tight junctions.¹²⁴ Another study showed that a mutation in Ras (a signaling molecule upstream of ERK) is associated with increase in extra cellular signal-regulated kinase-2 phosphorylation and produces leakiness of tight junctions to certain types of solutes.¹²⁵ Similarly, Raf-1- another signaling molecule upstream of ERK has been implicated in causing disruption of epithelial TJs via downregulation of occludin.¹²⁶ Another study demonstrated that down-regulation of the MAPK signaling pathway causes the restoration of epithelial cell morphology and the assembly of tight junctions in Ras-transformed epithelial cells and that tyrosine phosphorylation of occludin and ZO-1 may play a role in some aspects of tight junction formation.¹²⁷ However, a recent study demonstrated that in differentiated Caco-2 cell monolayers, epidermal growth factor (EGF) protects the TJs against oxidative stress induced by H₂O₂. This protective effect of EGF was blocked by a MEK inhibitor, U0126, thus demonstrating that the action of EGF was mediated by ERK. It was further shown that activated ERK (phospho-ERK) was associated with TJ protein complex and was co-localized with occludin.⁹⁴ Thus these strikingly contrasting data poses a formidable challenge to define the precise role of ERK in regulation of tight junction integrity. This study is aimed at defining the role ERK plays in regulation of tight junctions and to find out the mechanism behind this contrasting influence.

1.7 Regulation of Tight Junctions by Inflammatory Mediators

1.7.1 Cytokines

Several inflammatory mediators like cytokines and TNF α , IFN γ and IL-6 bind to their receptors present on the basolateral surface of the intestinal epithelial cells. They activate the signal transduction pathway leading to increased expression as well as activation of myosin light chain kinase (MLCK) protein.¹²⁸ As explained above, activation of MLCK leads to contraction of actin cytoskeleton and tight junction strands, leading to permeable tight junctions.

1.7.2 Oxidative Stress

Oxidative stress in the form as is caused by reactive oxygen species (ROS) like superoxide, hydrogen peroxide or acetaldehyde, leads to disruption of tight junctions, and increased paracellular permeability. The disruptive effect of superoxide or hydrogen peroxide on tight junctions is believed to be caused by their conversion to hydroxyl radical. The latter is even more reactive than superoxide and hydrogen peroxide, and is capable of causing injury to virtually every tissue of the body. That is why the reactive oxygen species have been implicated in causing several diseases like inflammatory bowel disease (IBD) and necrotizing enterocolitis.¹²⁹

Hydrogen peroxide causes disruption of epithelial tight junctions in the colonic, tracheo-bronchial, alveolar, retinal pigment epithelial and renal tubular epithelial cells. This process is mediated by a tyrosine kinase dependent mechanism. The intracellular signaling molecules like c-Src and PI3K are involved in this signal transduction pathway.²⁵ As stated above, oxidative stress leads to tyrosine phosphorylation of tight junction proteins, leading to disruption of the barrier function.

Hydrogen peroxide exhibits a biphasic dose response curve in its disruptive effect on tight junctions. At low concentrations varying from 5 to 50 μM , there has been observed a progressive increase in paracellular permeability. This effect diminishes at concentrations between 50 and 500 μM , only to come back at still higher concentrations which are lethal to cells.

1.8 Tight Junction Regulation by Mucosal Protective Factors

1.8.1 Epidermal Growth Factor (EGF)

EGF is a potent mitogen in several cell types both *in vivo* and *in vitro*. Structurally, it is a 53 amino-acid long single chain polypeptide. EGF is secreted in gastric, duodenal and small intestine secretions as well as in bile. In humans, the main source of EGF is the parotid gland. The biological activity of EGF present in the pancreatic juice and urine has been found to be very low.¹³⁰

EGF binds to its receptor (EGFR) present on the cell surface and increases the intrinsic tyrosine kinase activity of the receptor. This increased tyrosine kinase activity results in activation of a signal transduction cascade in the cells, leading to increased intracellular calcium, glycolysis, protein synthesis and gene expression. EGF has been demonstrated to protect the tight junctions against oxidative stress caused by hydrogen peroxide. This effect of EGF is brought about by activation of Ras-Raf-MEK-ERK pathway. Activation of MEK and thus ERK by EGF leads to phosphorylation of occludin on serine and threonine residues, thus enhancing tight junction integrity. The use of MEK inhibitors U0126 and PD98059 reverses this effect. EGF also reverses the

translocation of intracellular translocation of occludin brought about by hydrogen peroxide. This effect too was attenuated by MEK inhibitors.⁹⁴

1.8.2 Other Factors

Other factors like Transforming growth factor alpha are also known to have protective effect on the gastrointestinal epithelium. But their role in regulation of the tight junction regulation is still not clear.¹³¹

CHAPTER 2: HYPOTHESIS

Mitogen/Microtubule Activated Protein Kinases (MAPK) or ERK are a group of serine-threonine kinases, which regulate gene expression, mitosis, differentiation, cell survival and apoptosis.¹³² However, little is known about the exact role of ERK in the regulation of epithelial tight junctions. The few studies on this subject have only produced mixed results. While some of them held ERK responsible for disruption of tight junctions, others have tried to establish the protective role of ERK in maintaining the integrity of tight junctions. Our analysis of the literature has revealed that various studies demonstrating this contrasting effect of ERK on tight junctions were done using different cell lines. Caco-2 cells are known to differentiate, whereas several other cell lines used in the published studies of ERK's role in tight junctions do not differentiate at all. So we hypothesized that

1. MAPK/ERK protects the tight junctions in differentiated cells, whereas it is disruptive to tight junctions in under-differentiated Caco-2 cells.
2. This contrasting effect of ERK on tight junction integrity is produced by variable levels of active ERK present in under-differentiated and differentiated cells, and its effect on translocation of PKC ζ and PP2A to the junctional region and their binding with occludin. Owing to its role in cell growth, differentiation and maturation, active-ERK is present in higher quantities in under-differentiated cells, and so as to allow cells to proliferate, inhibits the formation of tight junctions. On the other hand, in differentiated cells the levels of active-ERK are lower. Another possible factor behind this contrasting effect may be the differences in downstream signaling of ERK producing stronger junctional localization of PKC ζ and occludin-phosphorylation to protect tight junctions in differentiated cells, and moving away from junctions in under-differentiated cells leading to disruption of tight junctions. ERK may also be producing the same effect on tight junction integrity by regulating localization of PP2A to tight junctions in a manner opposite to that of PKC ζ .

CHAPTER 3: MATERIALS AND METHODS*

3.1 Cell Culture

Caco-2 cells- the human intestinal epithelial cell line derived from a human colorectal carcinoma were used in these studies. They are very commonly used *in vitro* model for studying the tight junctions, and are considered to be the gold standard for these studies. The reason is that these cells are polarized, form tight junctions and have a viable brush border epithelium. Caco-2 cells possess a unique capability of differentiating spontaneously under standard cell culture conditions. They exhibit the qualities of the absorptive intestinal epithelium making them an excellent model to study intestinal epithelial structure and function.¹³³⁻¹³⁵

The cells were grown in DMEM containing 10% (v/v) fetal bovine serum (FBS) or newborn calf serum (NBCS), high glucose, L-glutamine, pyruvate, and fortified with penicillin, streptomycin and gentamicin. Sterility was maintained by filtration through 0.22 μ m filters. The medium was stored at 4°C, and prewarmed to 37°C before cell culture. The cells were grown as monolayers in 100mm petri dishes or 75cm² flasks. Experiments were conducted on cells grown in polycarbonate membrane transwell inserts (Costar, MA) of varying diameters *i.e.* 6.5 mm, 12 mm and 24 mm. Studies were conducted on days 3 or 4 (under-differentiated cells) and days 7-14 (differentiated cells).

3.2 Transwell Inserts

These are permeable membrane supports used to grow cells (**Figure 3.1**). They consist of an inner cylindrical structure that has a polycarbonate membrane base. The polycarbonate membrane has microscopic pores in it that are small enough not to let the medium seep through them. The cells are grown on the membrane in the insert, which is then placed into a multi-well plate that also contains the medium. Thus transwells serve as an excellent device to study both the surfaces of a cell monolayer, and also to study the potential difference, the transepithelial electrical resistance and paracellular flux across the two surfaces. The transwell inserts are commercially available in different sizes.

3.3 Measurement of Transepithelial Electrical Resistance (TER)

The TER was measured according to the method described by Hidalgo *et al*,¹³⁵ using the Millicell-ERS Electrical Resistance System (Millipore, Bedford, MA). The

* Source: Aggarwal,S., Suzuki,T., Taylor,W.L., Bhargava,A., Rao,R. Contrasting effects of ERK on tight junction integrity in differentiated and under-differentiated Caco-2 cell monolayers. *Biochem J.* [Epub ahead of print] (2010).¹⁹² (Adapted with permission.)

Figure 3.1: A diagrammatic representation of transwell insert.

resistance was calculated as ohms/cm^2 , by dividing the observed value by the surface area of the membrane (0.33 cm^2 , 1.13 cm^2 and 4.52 cm^2 for 6.5 mm, 12 mm and 24 mm size transwells, respectively). To ensure that the resistance observed was independent of the area or size of the membrane in the transwells, the resistance of the membrane ($\sim 30 \text{ ohms}/\text{cm}^2$) was subtracted from the observed resistance values.

The Millicell-ERS Electrical Resistance System (**Figure 3.2**) is a portable apparatus which is used to measure the TER. It consists of a main unit or meter and a set of electrodes which are connected to the main unit by a set of wires. The display screen on the main unit/console of the meter displays the numerical value of the TER in ohms. Each prong of the bi-pronged electrode goes into the medium on either side of the membrane supporting the cell monolayer in the transwell insert (**Figure 3.3**).

Before measuring the resistance, the electrode is sterilized by dipping it in 70% ethanol for 10 minutes, and after that it is allowed to equilibrate and normalize with the cell medium by dipping the electrode in the medium for 30 minutes at room temperature. The values of TER obtained using the Millicell-ERS Electrical Resistance System are very reliable.

Millicell-ERS uses alternating current (AC) to measure membrane resistance. AC source is preferred to the traditional direct current (DC) because with the former, there is minimal or no effect of the membrane voltage or voltage electrode offset. In addition, there is no effect of the electrochemical deposition of metals on the electrodes and a zero net charge of the cells, which as a whole prevents the detrimental effect of DC on the cell membranes.

In addition to the TER, this instrument is also capable of measuring the potential difference across the membranes. A silver/silver chloride (Ag/AgCl) pellet on each

Figure 3.2: Millicell-ERS electrical resistance system. Source: *Millicell[®]-ERS User Guide P17304*, 2. Rev. C, (2007).¹⁸⁹ Millicell is a trademark of Millipore Corporation Billerica, MA. The illustration is courtesy of Millipore Corporation. (Reprinted with permission.)

Figure 3.3: Measurement of transepithelial electrical resistance. Source: *Millicell[®]—ERS User Guide P17304*, 7. Rev. C, (2007).¹⁹⁰ Millicell is a trademark of Millipore Corporation Billerica, MA. The illustration is courtesy of Millipore Corporation. (Reprinted with permission.)

electrode is useful for measuring the voltage. Thus the resistance (R) can be directly measured, or it can be deduced from the values of potential difference (V) observed across the electrodes and the current (I) flowing through the circuit, using the Ohm's law:

$$V = IR \quad \text{and} \\ R = V/I$$

3.4 Cell Treatments

For studying the effect of various inhibitors, EGF and hydrogen peroxide, the cells were grown in transwell inserts. The medium was changed every alternate day. Experiments were performed on days 3 or 4 (under-differentiated cells) and days 12-17 (differentiated cells). On the day of the experiment, the cells were washed with the experiment medium (PBS-BSA-Glucose or plain DMEM without serum, glutamine or pyruvate). The cells were preincubated for 50 minutes in the presence or absence of specific inhibitors to be tested in the experiment medium. Wherever required, epidermal growth factor (EGF), to get a final concentration of 15 nM, was injected into both the apical and basal compartments of the transwells, and incubated for 10 minutes. Then the hydrogen peroxide, to get a final concentration of 10 μ M, was injected again into both the apical and basal compartments of the transwells.

3.5 Paracellular Inulin Flux Measurement in Cell Monolayers

The permeability of the cell monolayer was studied by measuring the paracellular permeability of fluorescein isothiocyanate-inulin (FITC-Inulin) across the membranes in the transwell inserts. Van Italie and Anderson have recently suggested that the tight junctions have pores that regulate passage of solutes across the epithelium without necessarily affecting the TER.¹³⁶ The size of these pores varies from 3.5-4 $\text{\AA}$. So, to effectively study the integrity of tight junction, we needed a substance which had a molecular size of more than what could pass through those tight junction pores. So we chose FITC-Inulin. Inulin is a cylindrical molecule having a semilength of 25 $\text{\AA}$ and radius of 10 $\text{\AA}$.¹³⁷ Thus, this molecule is incapable of passing through the pores in tight junctions, and its permeability across the epithelial layer is highly indicative of disruption of tight junctions.

A solution of FITC-inulin was added to the basal compartment of the transwell inserts in a final concentration of 50 μ g/mL. Cells were incubated at 37°C for the desired duration of the experiment. At timed intervals during the study, 100 μ L aliquots were taken of the medium from the apical as well as the basal compartments of the transwell inserts. Equal volume of the medium or the buffer bathing the cells constituted the blank controls. All the aliquots were transferred to a 96-well fluorescence reader assay plate, and the fluorescence was read with the help of an FLx800 Microplate Fluorescence Reader (BioTEK Instruments, Winooski, VT) using the KC junior software. The filter pair used in this case was an excitation filter at 485 nm and an emission filter at 538 nm.

The unidirectional flux of inulin into the apical compartment was calculated in terms of the percentage of inulin administered into the basal compartment per cm² surface area of the membrane, using the following equation:

$$\frac{\text{Apical reading} \times V1}{\text{Basal reading} \times V2} \times \frac{100}{t} \times \frac{1}{A} = \% \text{ flux / hr / cm}^2$$

Where

V1 = Volume of medium in the apical compartment of the transwell

V2 = Volume of medium in the basal compartment of the transwell

t = Duration of treatment of cells, before flux is read

A = Area of the monolayer / membrane in cm²

3.6 Calcium Switch Experiment

It is now known that when growing in the absence of calcium in the medium, the cells do not polarize or form tight junctions. On the contrary, when the calcium is restored in the medium, the cells not only polarize and form tight junctions; they also develop attachment sites and get oriented with their apices towards the surface.¹³⁸ Since the formation of tight junctions involves changes in the cellular architecture and a reorganization of cytoskeletal elements; the changes which are highly influenced by calcium, the calcium switch has been a long established and practiced method to study tight junction assembly and integrity.¹³⁹ Addition of calcium in the medium stimulates the complex signaling pathway involving kinases and phosphatases that leads to formation of tight junctions. In the process, first the adherens junctions are formed, and then the tight junctions. Two methods can be used to deprive the cells of calcium- either growing them in a low calcium medium while culturing them in the transwell inserts, and then inducing the tight junction formation by restoring the regular high calcium medium; or by removing the calcium out of the cells transiently by using the chelators like EGTA leading to disruption of tight junctions, and then facilitating their recovery by restoring the calcium containing medium. In our studies, we used EGTA chelation.

Caco-2 cells were grown to confluence in the transwell inserts in regular medium. Calcium was removed using 4 mM EGTA into both apical and basal compartments for about 10 minutes, while continuously measuring TER, and monitoring cell morphology under the microscope. Then the cells were washed three times with the regular medium to remove any traces of EGTA, and cells were incubated in regular medium for different periods of time. The tight junction recovery was monitored by measuring the TER and unidirectional flux of inulin, as described before. A normal assembly of tight junctions is indicated by an increase in the TER and a reduction in inulin flux across the cell monolayer.

3.7 Oligonucleotides for ERK

Knocking down of a particular gene or protein of interest, and thus reducing its expression has, over the time, become a widely used and accepted molecular technique to study the role of various proteins in the cells. Oligonucleotides are single stranded oligomers of nucleotide units, which are bound together by phosphodiester bonds between the 3'-hydroxyl group of one monomer and the 5'-hydroxyl group of the other monomer. In other words, they are short fragments of RNA or single stranded DNA. The antisense oligonucleotide act at the level of mRNA during protein synthesis. Being the complimentary to the coding sequence of mRNA, they bind to it and thus inhibit the translation of the protein coded by that mRNA sequence. This mechanism of their action accords a unique and high specificity to their use.¹⁴⁰

We designed the oligonucleotides against ERK1 and ERK2. To do this, first, the sequence of these genes was obtained from the NCBI human database in the PubMed archives by doing a blast search. To double check the uniqueness of the sequence thus obtained, it was subjected to a second blast search to make sure that it did not resemble any other known human gene in the database. Thereafter, the two sequences were aligned using the ClustalW program (European Bioinformatics Institute, Cambridge, UK). This enabled us to find the sequences common to both the genes, as well as a unique sequence for ERK1. To make sure again that the sequence areas chosen were unique to these genes, a thorough BLAST search of the known human genome was again performed. Since we did not find any other sequence matching the ones we had selected, that confirmed that the sequence we had chosen are unique for the genes concerned. Reverse compliments of the short sequences thus isolated were designed, and the following nucleotide sequences were used to reduce the expression of respective genes.

ERK1	GCCGCCGCCGCCGCAT
ERK1 and 2	AGACCATGATCACACAGGGT

A missense oligo with the following sequence was designed to serve as an appropriate control in the experiments.

Missense	TATACGATGGTACAGTGGAGT
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These oligonucleotides were custom synthesized from Sigma Genosys (St. Louis, MO) and Integrated DNA Technologies (Coralville, IA) in 0.2 μ mol scale, and were purified using HPLC technique. Since instability is a common occurrence with oligonucleotides, in order to provide them with more stability, they were custom synthesized in phosphorothioated form.

3.8 siRNA Specific for ERK

siRNA is a short (20-25 nucleotide long) sequence of double stranded RNA or DNA sequence, with 2-3 nucleotide overhangs on either side. Each strand has phosphate

on 5' end and a –OH on the 3' end. This structure is obtained by treating the double strand DNA with a dicer enzyme. siRNA causes a sequence specific degradation of its homologous single stranded RNA molecule. They are used in the RNA interference (RNAi) pathway. As is evident from their name, siRNAs interfere in the expression of genes they are specific for. This process is also known as post-transcriptional gene silencing.¹⁴¹ Even though their function is somewhat similar to that of oligonucleotides, the siRNA has some distinct advantages over oligonucleotides. One, siRNA is more stable, and second, it can be used in much lower concentrations as compared to the antisense oligos. Moreover, siRNA causes a highly selective inhibition of the synthesis of protein of interest. This makes siRNAs a significant tool for studying the function of various genes. However, siRNA is not without its own disadvantages, namely mounting of immune response by the cells, unintended off-targeting *i.e.* downregulation of genes with incomplete complementarity.

siRNA specific for ERK was very kindly provided by Dr. Aditi Bhargava (University of California, San Francisco), and was synthesized as per the following protocol:

ERK1 cDNA (from nucleotide 002745; accession number: NM_002745) was amplified by RT-PCR and cloned in TopoII vector (Invitrogen). The identity of the cloned product was verified by sequencing. The template was then digested with SpeI or XbaI to linearize it, and sense and anti-sense strands were obtained using T7 or SP6 polymerases respectively. β -globin cDNA was used to make control dsRNA. The sense and antisense RNA were synthesized from cDNA inserts cloned in plasmid vectors using MEGAscript RNA kit (Ambion). Equimolar concentrations of sense and antisense RNA were mixed, extracted with phenol: chloroform (1:1), precipitated and resuspended in 10mM Tris-Cl (pH 7.5) and 1mM EDTA (annealing buffer). RNA was boiled for a minute in a boiling water bath and the temperature of the bath was allowed to cool to room temperature over 12-16 h resulting in the formation of long dsRNA.¹⁴² Integrity of the long dsRNA was checked by gel electrophoresis. This long dsRNA was subsequently digested with RNase III to produce enzymatically cleaved pool of siRNA.

3.9 Transfection of Antisense Oligonucleotides or siRNA into Caco-2 Cells

Caco-2 cells cultured in 6-well cluster plates, when 30-50% confluent were used for transfection. Before transfection, cells were washed twice with Hank's Balanced Salt Solution (HBSS) containing Calcium, to remove all traces of antibiotics from the cells. Then, transfection with oligonucleotide was done using 0.9 mL of antibiotic free medium, 500 nM oligonucleotide, 3.5 μ L of Oligofectamine, and 1 μ L Plus reagent per μ L of the antisense oligonucleotide in each well of the 6-well cluster plate. Controls used were cells transfected with missense oligonucleotide sequence. Similarly, the transfection with siRNA involved 0.9 mL of the antibiotic free medium, 15 μ g of the siRNA per ml of medium (13.5 μ g per well), 3.5 μ L of Oligofectamine, and 1 μ L Plus reagent per μ g of the siRNA used in each well of the 6-well cluster plate. Controls in this case were cells transfected with non-specific siRNA.

Post-transfection, the cells were incubated at 37°C. After 6 hours of transfection, the transfection cocktail was removed from the cells; they were washed, and regular medium was introduced into the cell cultures. Cells were incubated overnight at 37°C. Next morning, the cells were trypsinized and seeded on to transwell inserts for conducting various studies. The TER and cell morphology under microscope were observed on a daily basis to monitor the health of the cells.

3.10 Preparation of Cell Lysates

After completion of experiments, the cell monolayers were washed twice with ice-cold PBS, and then lysed with heated Lysis Buffer D (SDS 0.3% w/v, Tris 10 mM, pH 7.4, containing Sodium vanadate 10 μ M, Sodium fluoride 100 μ M and Protease inhibitor cocktail 10 μ L/mL). The lysate thus made was sonicated and heated at 100°C for 10 minutes. An aliquot was taken for protein estimation, and then the lysate was mixed with equal volume of 2x Laemmli's sample buffer, heated at 100°C for 10 minutes, and stored at -20°C for immunoblot analysis.

3.11 Preparation of Detergent-soluble and Detergent-insoluble Fractions from Cellular Proteins

When participating in the formation of tight junctions, Occludin and other tight junctional proteins closely interact with the actin cytoskeleton. To study the specific role of these proteins at tight junctions, it is imperative to avoid interference from other intracellular proteins. The best way to do so is to separate out the actin cytoskeleton fraction of the cellular proteins to which our junctional proteins of interest are bound. The actin cytoskeletal fraction of cell proteins is detergent-insoluble. Thus, to separate this fraction out, a detergent based buffer was used as explained below. Even though this is an excellent technique to study the tight junctional proteins, it also has its associated downside. Triton-insoluble fraction may contain some lipid rafts bound to the actin cytoskeleton, thus necessitating a cautious interpretation of their results. However, this can be overcome by density-gradient centrifugation to separate the lipid rafts.

The cell monolayers, after completion of the experiment, were washed with ice-cold PBS. Cells were then lysed in Lysis buffer-CS (20mM Tris buffer containing 1% Triton X-100, 2 μ g/mL leupeptin, 10 μ g/mL aprotinin, 10 μ g/mL bestatin, 10 μ g/mL pepstatin-A, 2 mM vanadate, and 1 mM PMSF). The lysate was ultracentrifuged at 14,600 g for 4 minutes at 4°C, to sediment highly dense actin cytoskeleton. The supernatant was transferred to a new tube with the help of a transfer pipette, and this was labeled as Detergent-soluble fraction. The sedimented actin cytoskeleton pellet was suspended in Lysis buffer CS, and the sediment was homogenized by sonication. This was labeled as the detergent-insoluble fraction. Aliquots were taken for protein estimation and another set of aliquots was mixed with equal volume of 2x Laemmli's sample buffer, heated at 100°C for 10 minutes, and stored at -20°C for immunoblot analysis.

3.12 Protein Estimation

Various protein samples- total cell lysates or the detergent-insoluble fractions prepared from experimental studies were taken in 10 μ L aliquots in a clear 96 well assay plate. Varying concentrations (100-1000 μ g/ μ L) of protein BSA solution were used as standards to compare the protein values of the experimental samples. Protein estimation was done using 200 μ L of BCA Lowry's reagent (Reagent A and B mixed in the ratio of 40:1) for each sample. The samples were then incubated at 37°C for 10 minutes, and then read at 562 nm in a Spectramax 190 plate reader (Molecular Devices, CA) using Soft Max Pro software (Molecular Devices, CA). Amount of protein present in various samples was then calculated.

3.13 Immunoprecipitation of Cellular Proteins

Immunoprecipitations were done from the whole cell lysate or the detergent-insoluble fraction of the cellular proteins, which were obtained as mentioned above. We used the whole cell lysate that contained denatured proteins to study phosphorylation of a particular residue (*e.g.* Threonine, serine or tyrosine). Whereas, in some studies, where protein-protein interactions were required to be studied, we used the native (non-denatured) form of protein, extracted in lysis buffer CS, to immunoprecipitate one protein and then immunoblot it for the other.

The volume of protein solution/suspension containing 400 μ g of protein was mixed with equal volume of 2x Immunoprecipitation buffer, and incubated with 2 μ g of Rabbit polyclonal anti-phosphothreonine or Rabbit polyclonal anti-occludin antibodies as required. The tubes containing the cell extracts and the antibody were incubated at 4°C on the rocker overnight. The next day, the antibody bound protein was conjugated with Protein-A sepharose beads by incubating at the rocker at 4°C for 1 hour. The beads were then washed with 1x immunoprecipitation buffer, by centrifugation, three times at 4°C. The beads were then suspended in 20 μ L of 1 $\times$ concentrated Laemmli's sample buffer and heated at 100°C for 10 min. The tubes were spun down at high speed for 10 minutes, and the supernatant transferred to freshly labeled tubes for immunoblot analysis.

3.14 Western Blotting

The denatured protein extracts prepared from the experiments were mixed with equal volume of 2x Laemmli's sample buffer and heated at 100°C for 10 minutes. 20 μ g from each of the protein samples was then loaded on to 7% SDS PAGE pre-cast gels (Invitrogen), and the gel allowed to run in MOPS-SDS Running Buffer (containing SDS and Tris-Glycine) at 120 V for about 75-80 minutes until the loading dye reached the other end of the gel. In the meantime, a PVDF membrane was pre-charged with methanol for 45 seconds, and kept moist in the transfer buffer (Tris base, Glycine and Methanol).

Upon completion of the electrophoresis, the gel was washed, and transferred overnight to the PVDF membrane at 55 V in 4°C. The membrane was then blocked in blocking buffer (5% Milk for non-phospho proteins or 5% BSA for phospho- proteins) made in Tris Buffer solution with Tween or TBST (Tris base, Sodium chloride and Tween-20 dissolved in distilled water, and pH adjusted to 8.0). The blocking was done either for 3 hours at room temperature or overnight at 4°C. The blot was then probed for the proteins of interest using various primary antibodies like Rabbit anti-pERK (1:2000), Mouse ERK1/2 (1:500), Rabbit anti-PKC ζ (1:2000), Mouse anti-PP2A (1:2000), Rabbit anti-ZO-1 (1:2000), Mouse anti-occludin (1:3000), Rabbit anti-phosphothreonine (1:2000), mouse anti-actin (1:5000) etc. by incubation the membrane in the respective antibody solutions on rocker at 4°C overnight.

The membrane was then washed with TBST five times for 5 minutes each, and then incubated with Horse Radish Peroxidase (HRP) conjugated secondary antibody solution *i.e.* Anti-rabbit HRP or anti-mouse HRP in 1:10,000 dilution made in 4% milk or BSA solution for 1 hour on rocker at room temperature. The membrane was again washed with TBST 5 times for 5 minutes each. The blot was developed using the electrochemiluminescence (ECL) substrate for the HRP enzyme conjugated in the secondary antibody. ECL from Amersham/GE Healthcare was used in our experiments.

The mechanism behind this detection is that the secondary antibody conjugated with HRP binds to the primary antibody specific for the protein of interest. Upon addition of the substrate (ECL), HRP catalyzes the oxidation of luminol present in the substrate. Luminol thus gets transformed into its excited state and emits light signal, which can be detected on a sensitive x-ray film (**Figure 3.4**). The emission of light decays with the inactivation of the enzyme HRP with time. In case of very weak signals sometimes, the Visualizer or Hyper ECL (Upstate/Millipore) was used to enhance the emission of light. Enhancer increases the intensity of the chemiluminescence signal of the protein-antibody complex, and makes it easy to be detected on the x-ray film.

3.15 Stripping and Reblotting

The PVDF membranes can be reblotted for other proteins after blotting them for one. For this purpose, the primary and the secondary antibodies bound to the protein present on the membrane have to be removed and the surface of the protein exposed to allow the new primary antibody to bind. This can be achieved by stripping the membrane with a low pH buffer containing a detergent. So, the membranes were stripped with 1x Stripping Buffer (Glycine, SDS, Tween-20 mixed in distilled water, pH 2.5) for 30 minutes at room temperature, washed with TBST, reblocked and reprobed as explained above.

Figure 3.4: The principle of chemiluminescence for immunoblot analysis.

3.16 Immunocytochemistry/Confocal Microscopic Imaging

3.16.1 Fixing the Cells

The cells grown on transwell inserts were treated with various reagents to study their effect on the cells. Upon completion of the experiment, the cells were washed twice with ice-cold Phosphate buffered saline solution (PBS), and then fixed either in 3% paraformaldehyde solution or 1:1 acetone:methanol mixture pre-made and stored at 70°C. To fix with paraformaldehyde, cells were treated with paraformaldehyde for 10 minutes at room temperature, and then washed with PBS three times for 10 minutes each. The cells were then stored at 4°C in the PBS and sodium azide solution. To fix with acetone:methanol, cells were treated with cold acetone:methanol solution for 5 minutes, and then air dried for 5 minutes. The membranes thus fixed were stored at -70°C for immunostaining.

3.16.2 Permeabilization

The process of permeabilization uses a mild detergent solution to cause some pores in the cell membranes to facilitate the passage of the primary antibodies into the cells to bind the protein(s) of interest. To permeabilize the cells fixed with paraformaldehyde, the PBS-azide buffer was removed from the cells, and the cells washed with PBS at room temperature three times for 10 minutes each. The cells fixed in acetone:methanol were allowed to come to room temperature, and were rehydrated by adding PBS to the transwells at room temperature. The permeabilization was done by treating the fixed cell monolayers to 0.2% TritonX100 solution made in PBS for 5 minutes at room temperature. The monolayers were then washed thrice with PBS for 10 minutes each to remove all traces of TritonX100.

3.16.3 Blocking

The blocking was done using blocking buffer which was constituted of 4% milk solution made in TBST, for 30 minutes at room temperature.

3.16.4 Immunostaining

The blocked cell monolayers were then double labeled by incubating them with primary antibodies like mouse monoclonal anti occludin and rabbit polyclonal anti-ZO-1, mouse monoclonal anti-ERK1/2, rabbit polyclonal anti-pERK, rabbit polyclonal anti-PKC ζ or mouse monoclonal anti-PP2A. All these antibodies were used in a concentration of 2.5 $\mu\text{g/ml}$ (1:100 dilution) in the blocking buffer and their different combinations were used for double labeling. The monolayers were incubated in primary antibody solution for 1 hour in a humidifying chamber in the dark. Then the cell

monolayers were washed with a diluted blocking buffer (1% milk in TBST) three times for 10 minutes each. The monolayers were then incubated with the secondary antibody solution made in blocking buffer (1:100). Various secondary antibodies used were anti-rabbit IgG and anti-mouse IgG conjugated with either Alexafluor-488 or Cy-3. This incubation was also done for 1 hour in the humidifying chamber in the dark. Thereafter, the cells were washed thrice with PBS for 10 minutes each. The monolayers were mounted on glass slides, using mounting fluid (DABCO and glycerol). A cover slip was placed on the membrane containing a tiny droplet of the mounting fluid, and the cover slip was sealed by applying nail enamel on its edges. The labeled slides were stored in the dark at 4°C.

3.17 Microscopy/Imaging

The slides were viewed under a confocal laser-scanning microscope (Zeiss LSM510 PASCAL). Serial XY section each 1 µm thick were taken. The aperture, gain and laser intensity were modulated based on the intensity of fluorescence observed in control slides. The same set of settings was used to take images from all the slides for an experiment. Following sets of settings were generally used while imaging the slides:
Green Fluorescence: Alexa 488, Gain 400-450, Aperture 76, Laser Intensity 10%
Red Fluorescence: Cy3, Gain 350-400, Aperture 76, Laser Intensity 80%.

The images were stacked using the 'Image J' software (National Institutes of Health, Bethesda, MD) and the images were processed in the Adobe Phostoshop software (Adobe Systems, San Jose, CA). The vertical images were obtained by analyzing the Z-sections.

3.18 Densitometric Analysis of Images

All the images were analyzed by densitometry using the 'Image J' software. The images taken in the .jpg format were opened in 'Image J' software.

3.18.1 Analysis of Western Blots

For analysis of Western blots, a random square was plotted on the picture, and density of the background was analyzed by placing the square on an area outside of the bands. Then the same box/square was placed over each band in the blot, and the density analyzed. At least three blots for each experiment were thus analyzed. The average values for each protein band were calculated, and the net density of the protein band was calculated by subtracting the background value. This net density was then plotted in the form of a bar chart.

3.18.2 Analysis of Confocal Images

For analyzing the confocal images, a similar square was first placed on an area depicting no or little fluorescence to find the background value. Then the box was moved to the areas showing red or green fluorescence, and the fluorescence density was analyzed. At least four such readings were taken from each frame, and at least three fields or images were analyzed. Background value was subtracted, and the net fluorescence value was calculated and plotted. Even though the densitometry data obtained by using this method appeared to correctly represent the localization of proteins under study, the method had a drawback of the observer's bias. The areas to be analyzed either for the junctional localization of a specific protein or the subtraction of background were chosen by us while doing the analysis. Even though this selection was purely random, still it may not be termed as a completely objective densitometric analysis.

3.19 Making Tet-on and Tet-off Cell Lines

Caco-2 cells grown in 6-well cluster plates were transfected with commercially available pTet-on or pTet-off vectors (Clontech) (**Figure 3.5**), using lipofectamine LTX and plus reagent as explained above. The cells were then selected with G0418 sulfate (700 µg/ml). After 14 days, the colonies of selected cells were picked up and cloned using cloning cylinders, cultured and maintained in a lower concentration (350 µg/ml) of G0418 sulfate. These cell clones were then characterized by immunoblotting the whole cell lysate for Tet-R protein, and by luciferase assay.

Figure 3.5: pTet-on/pTet-off vector. Source: *pTet-On Advanced Vector Information* PR742221, 11, 1 (2007).¹⁹¹ Courtesy of Clontech Laboratories, Inc., a Takara Bio Co., Mountain View, CA © 2007 Clontech Laboratories, Inc. (Reprinted with permission.)

3.20 Preparation of MEK Constructs

GFP sequence having cohesive *Sall* and *EcoRV* sites was amplified by PCR, using the pAcGFP1-N1 plasmid (Clontech, Mountain View, CA) as template and the following primers (custom synthesized by Integrated DNA Technologies Inc, Coralville, IA):

5'-GACGCGTCGACATGGTGAGCAAGGGCG -3' (forward)
5'-CCGATATCTCACTTGTACAGCTCATC -3' (reverse)

The amplified PCR product for *GFP* was digested with *Sall* and *EcoRV*, and ligated into pTRE2hyg vector (digested with the same restriction endonucleases), to obtain pTRE2hyg-pAcGFP1-N1 vector (**Figure 3.6**). *MEK1* cDNA sequences for wild type MEK (WT-MEK), dominant negative *MEK1*_{K97M} (DN-MEK) and constitutively active *MEK1*_{S218E,S220D} (CA-MEK) in vector were obtained from Dr. Natalie Ahn University of Colorado, Boulder CO. *MEK* sequences having cohesive *Bam*HI and *Mlu*I sites was amplified by PCR, using the above cDNAs as templates and the following primers (custom synthesized by Integrated DNA Technologies Inc. Coralville, IA):

5'-GCGGATCCACCACCATGCCCAAGAAGAAG -3' (forward)
5'-GCACGCGTCGACGCCAGCAGCATG -3' (reverse)

The amplified PCR products for *WT-MEK*, *DN-MEK* and *CA-MEK* were digested with *Bam*HI and *Mlu*I, and ligated into pTRE2hyg-pAcGFP1-N1 vector (digested with the same restriction endonucleases) to obtain pTRE2hyg-pAcGFP1-N1-MEK constructs. The resulting DNA plasmids were then transformed by using DH5 α FIQ competent cells, and purified by using Qiagen maxi- or mini-prep kits. The plasmids were sequenced at

Figure 3.6: pTRE2hyg-AcGFPN-MEK1 vector.

the Molecular Resources Center facility of the university. The expression of various mutants of MEK upon their transfection into the cells was also analyzed using the RT-PCR.

3.21 Alkaline Phosphatase (ALP) Assay

To check whether the cells are differentiated or not, alkaline phosphatase assay is an important tool. This assay works on the principle that alkaline phosphatase converts p-nitrophenol phosphate to nitrophenol in an alkaline environment.¹⁴³

For this assay, on days 3 and 12 the cells were washed with 1x PBS twice. The cells were then lysed using 100 μ L of 1x cell lysis solution (part of the kit). The cell lysates were then transferred into microtubes. 10 μ L of each cell lysate was transferred into a clear 96-well assay plate in duplicates. 100 μ L of the ALP assay solution was added to each well mixed by gentle rocking. The plate was covered and incubated at 37°C to let the yellow color develop. Then, to each well 20 μ L of 1N NaOH was added to stop the reaction. Absorbance was read at 410 nm using Spectramax spectrophotometer, and plotted on a graph. The absorbance is directly proportional to the ALP activity present in the cell lysates.

3.22 *In vitro* Studies

3.22.1 Synthesis of C-terminal Occludin

The pGEX2T vector containing the insert for the 150 amino acid c-terminal occludin was transformed in the DH5 α FIQ strain of *E. coli*. The transformed cells were then cultured in the Lurea Broth medium and the protein was prepared. It was then purified using the dialysis bag.⁹²

3.22.2 Activation of ERK

The commercially available recombinant ERK was incubated at 37°C, with or without MEK and ATP (500 μ M) while shaking at 800 rpm.

3.22.3 Phosphorylation of Occludin

To check for its phosphorylation, the GST-tagged c-terminal occludin prepared as above was incubated with active or inactive ERK or PKC ζ in the presence or absence of ATP. The resulting proteins after the completion of incubation were subjected to immunoblot analysis as described above.

3.23 Mouse Tissue Experiments (*Ex vivo* Studies)

To study the effect of ERK *ex vivo*, on mouse intestinal epithelium, some mouse studies were conducted using a protocol approved by the Institutional Animal Care and Use Committee (IACUC). 10-12 weeks old young, healthy BL57 mice were used in the experiments. These mice were purchased from Jackson Laboratory, Bar Harbor, Maine. The mice were weighed and anesthetized with isoflurane. The mice were then decapitated and dissected to take the ileum out. Ileum was flushed with saline to clear it of all the food and fecal matter. Small (about 1 cm long) pieces of ileum were then incubated in a six-well cluster plate in DMEM, and subjected to oxidative stress with hydrogen peroxide in the presence or absence of EGF for varying lengths of time. Upon completion of the treatment, the tissue was washed with normal saline, and mucosa was scraped. Triton soluble and triton insoluble fractions were made from the protein so obtained, and immunoblotting was done according to the method described above. Another set of tissue pieces was frozen in OCT in liquid nitrogen. 1µm thick cryosections, on glass slides were made from these tissue pieces, which were later immunostained and imaged under confocal microscope, for various tight junction proteins as explained above.

3.24 Reagents and Chemicals

Cell culture medium (DMEM), fetal bovine serum (FBS) and antibiotics were procured from GIBCO-BRL (Grand Island, NY). Newborn calf serum was purchased from Invitrogen. Pure recombinant enzymes ERK1, ERK2, PKC ζ and PP2A were purchased from Upstate/Millipore. Rabbit anti-pERK was purchased from Cell Signaling. Mouse ERK1/2, rabbit or mouse anti-ZO-1, rabbit or mouse anti-occludin, rabbit anti-phosphothreonine antibodies were purchased from Zymed Laboratories/Invitrogen. Mouse anti-actin antibody, HRP conjugated anti-rabbit and anti-mouse secondary antibodies used in immunoblotting and Cy3 conjugated anti-rabbit and anti-mouse secondary antibodies used in immunostaining were purchased from Sigma. Rabbit anti-PKC ζ antibody was purchased from Upstate/Millipore. Mouse anti-PP2A antibody was purchased from BD Biosciences. Alexafluor-488 conjugated anti-mouse and anti-rabbit antibodies were got from Invitrogen. Anti-GFP antibody was obtained from Living Colors (Invitrogen). Alkaline phosphatase assay kit was purchased from the Biomedical Research Service Center at University of Buffalo, Buffalo NY. Most of the other reagents and chemicals were purchased either from Sigma Chemical Company or Fisher Scientific.

3.25 Statistical Analysis

The observed data in the two different groups was compared using Student's *t*-tests for grouped data. Significance in all tests was set at 95% or greater confidence level.

CHAPTER 4: RESULTS*

4.1 Effect of Reduced Expression of ERK on Integrity and Assembly of Tight Junctions in Caco-2 Cells

4.1.1 Rationale

A previous study has demonstrated that EGF protects the tight junctions against oxidative stress induced by hydrogen peroxide.⁹⁴ U0126, a specific inhibitor of MEK was shown to inhibit this protective effect, thus indicating that ERK was involved in the process.¹⁴² But this study was based on use of pharmacological inhibitors. But use of pharmacological inhibitors has a disadvantage that the effects of inhibitors depend on the permeability of cells to the inhibitors. Pharmacological agents may also lack the selectivity in inhibiting the target enzymes. It may not be able to inhibit the enzyme being freshly synthesized in the cells. Thus, in order to further confirm the findings, and to do so more reliably, we employed the molecular technique of reducing the expression of ERK by using oligonucleotides and siRNA.

4.1.2 Transfection of Caco-2 Cells Using ERK-antisense Oligos

The antisense oligonucleotides against ERK, and siRNA specific for ERK were designed as described in Chapter 3 and custom synthesized in the phosphorothioate form for enhanced stability. These oligonucleotides and siRNA were transfected into Caco-2 cells using oligofectamine. Cell lysates were prepared from transfected cells on days 1, 3 and 7 after transfection. Western blotting of the cell lysates was done for ERK1/2, p38MAPK and β -actin to confirm the reduced expression of ERK in the cells post-transfection.

4.1.3 Reduced Expression of ERK by Antisense Oligos

On Western blotting, it was observed that on day 3 post-transfection, in the cells transfected with antisense oligos the expression of ERK was reduced to 55-75% as compared to cells transfected with missense oligos (**Figure 4.1A and C**). But on later days *i.e.* days 6 and 7, the levels of ERK were no different in cells transfected with antisense oligos as compared to cells transfected with missense oligos (**Figure 4.1B and D**), thus indicating the unstable nature of antisense oligonucleotides, and their short life-

* Source: Aggarwal, S., Suzuki, T., Taylor, W.L., Bhargava, A., Rao, R. Contrasting effects of ERK on tight junction integrity in differentiated and under-differentiated Caco-2 cell monolayers. *Biochem J.* [Epub ahead of print] (2010).¹⁹² (Adapted with permission.)

Figure 4.1: Knockdown of ERK using antisense oligos in Caco-2 cells. A & B: Caco-2 cells were transfected with antisense (AS) oligonucleotides specific for ERK1, ERK1/2 and missense (MS) oligonucleotides. Equal amounts (20 μ g) of protein from extracts prepared from transfected cells 3 days (A) and 7-days (B) post-transfection were immunoblotted for ERK1/2 and β -actin. **C & D:** Densitometric analysis of immunoblots shown in panels A and B. After comparing the band density for β -actin to be equal, band density of other samples is normalized with density of MS Oligo bands taken as 100. Values are mean $\pm$ sem (n = 3). Asterisk (*) indicates that values are significantly different from the values for missense (MS) oligos.

span in the cells. Because of this limitation of antisense oligonucleotides, we chose to use long double-stranded siRNA in our further studies because the long double-stranded siRNA has proven to be much more effective and longer-lasting as compared to small siRNAs.¹⁴²

4.1.4 Effect of Reduced Expression of ERK by Antisense Oligos on Tight Junction Integrity

The transfected cells were cultured on transwell inserts, beginning day 1 post-transfection. On day 3 or day 7 post-transfection, cells were washed twice with DMEM without serum, glutamine or antibiotics, and incubated for one hour in DMEM without serum, glutamine or antibiotics. FITC-inulin solution was added to the basal compartment of the transwells at this time. At the end of one hour incubation, TER and inulin flux were measured as described in Methods. Cells were washed with ice-cold 1x PBS twice, fixed in acetone: methanol, and immunostained for junctional proteins occludin and ZO-1.

It was observed that transfection with antisense oligos specific for ERK resulted in no significant increase in the basal TER in 3-day or 7-day cell monolayers (**Figure 4.2A**) compared to cells transfected with missense oligos. However, the transcellular flux of FITC-inulin, which is considered to be a superior indicator of tight junction integrity, from basal to the apical compartment of transwell inserts was significantly lower in cell monolayers with reduced ERK expression compared to control cell monolayers. The inulin flux in 7-day cell monolayers was, however, not significantly different between missense oligo-transfected and antisense oligo-transfected cell monolayers (**Figure 4.2B**). Immunostaining and confocal microscopy showed that transfection of cells with ERK antisense oligos resulted in enhanced junctional localization of occludin and ZO-1, compared to cells transfected with missense oligos (**Figure 4.2C and D**).

4.1.5 Effect of Reduced Expression of ERK by Antisense Oligos on Calcium-induced Assembly of Tight Junctions

To determine the role of ERK in the assembly of tight junctions, we evaluated the effect of reduced ERK expression by antisense oligos on calcium-induced assembly of tight junctions. Cells were preincubated in DMEM without serum, glutamine or antibiotics for one hour. Basal TER values were recorded. The cells were then treated with 4 mM EGTA for 10 minutes, while monitoring their TER. The TER was allowed to fall up to 25-30% of basal values. Cells were then washed with DMEM, and the regular calcium-containing medium was reintroduced into the transwells. TER and flux were monitored hourly to assess the reassembly of tight junctions, and restoration of barrier function.

Figure 4.2: Effect of knockdown of ERK by antisense oligos on epithelial barrier function in Caco-2 cell monolayers. **A & B:** Transepithelial electrical resistance (TER) (A) and inulin permeability (B) were measured in cell monolayers transfected with missense (MS)-Oligo or antisense (AS) oligo specific for ERK1 or ERK1/2 on days 3 and 7 after seeding on transwell inserts. Values are mean $\pm$ sem (n = 3). Asterisk indicates the values that are significantly different from the values for corresponding MS-oligo values. **C:** 3-day cell monolayers transfected with missense-oligos or anti-sense (AS) specific for ERK1 or ERK1/2 were fixed and stained for occludin and ZO-1 by immunofluorescence method. **D:** Fluorescence for intracellular protein was quantitated by densitometric analysis. Values are mean $\pm$ sem (n = 3). Asterisk (*) indicates the values that are significantly different from the values for corresponding MS-oligo values.

We observed that in 3-day under-differentiated cells transfected with antisense oligos specific for ERK, recovery of TER was significantly better at 150 minutes, as compared to missense controls (**Figure 4.3A**). The recovery of TER in non-transfected cells was similar to missense controls. The cells expressing reduced amounts of ERK also exhibited a significantly lower quantities of inulin-flux compared to missense controls (**Figure 4.3C**). No such effect of transfection with ERK-antisense oligos on tight junction assembly was seen in 7-day differentiated cells (**Figure 4.3B and D**).

4.1.6 Reduced Expression of ERK by RNA-interference

Since oligonucleotides get disintegrated in the cells, and have a short life-span, we used long double-stranded siRNA specific for ERK, which has the advantage of longer lasting stability in the cells, to reduce the expression of ERK. Immunoblot analysis revealed that transfection with ERK1/2 siRNA, but not control RNA, resulted in significant decrease in ERK levels in the cells on day 3 and day 7 (**Figure 4.4A and B**). The levels of p38MAPK, a closely related kinase remained unaffected between cells transfected with non-specific RNA or ERK specific siRNA thus confirming the specificity of ERK1/2 siRNA used. Although the 7-day old differentiated cells had a higher amount of p38MAPK compared to 3-day old under-differentiated cells.

4.1.7 Effect of Reduced Expression of ERK by RNA-interference on Tight Junction Integrity

We evaluated the effect of reduced ERK expression by RNA interference on tight junction integrity. It was observed that transfection with siRNA increased the basal TER in 3-day under-differentiated cell monolayers, whereas, it decreased the basal TER in 7-day differentiated cell monolayers (**Figure 4.5A**) compared to corresponding cells transfected with control RNA. In support of change in TER values, the basal inulin permeability in cell monolayers transfected with siRNA was lower in 3-day under-differentiated cell monolayers and higher in 7-day differentiated cell monolayers, as compared to inulin permeability in corresponding cells transfected with non-specific or control RNA (**Figure 4.5B**). Immunostaining and confocal microscopy showed that reduction of ERK expression by siRNA resulted in enhanced localization of occludin and ZO-1 at the intercellular junctions on day 3, (**Figure 4.5C**) whereas on day 7, it resulted in reduced localization of occludin and ZO-1 at the junctions (**Figure 4.5D**).

4.1.8 Effect of Reduced Expression of ERK by RNA-interference on Calcium-induced Assembly of Tight Junctions

We also evaluated the effect of ERK-siRNA on calcium-induced assembly of tight junctions. We observed that similar to antisense oligo effect, transfection of ERK-siRNA markedly accelerated the calcium-induced restoration of TER (**Figure 4.6A**) and development of barrier to inulin (**Figure 4.6B**) in 3-day under-differentiated cell

Figure 4.3: Effect of knockdown of ERK by antisense oligos on the assembly of tight junctions in under-differentiated and differentiated cell monolayers. **A & C:** Under-differentiated (UD) 3-day old or differentiated (D) 7-day old Caco-2 cells transfected with missense oligos or ERK-specific antisense (AS-ERK) oligos were subjected to calcium switch-mediated tight junction assembly. Transepithelial electrical resistance (TER) (A) was measured during EGTA (ethylene glycol tetraacetic acid) treatment and calcium replacement. Inulin permeability (C) was measured at 150 min after calcium replacement. **B & D:** Tight junction assembly was also studied in differentiated 7-day old missense-transfected or ERK-specific antisense (AS-ERK)-transfected cells. TER (B) and inulin permeability (D) were measured. Values are mean $\pm$ sem (n = 3). Asterisk indicates the values that are significantly different from the values for corresponding MS-oligo values.

Figure 4.4: Knockdown of ERK by RNA-interference in Caco-2 cell monolayers.

A: Equal amounts (20 μ g) of protein from extracts of 3-day under-differentiated (UD) and 7-day differentiated (D) Caco-2 cells transfected with control siRNA (NS-RNA) or ERK1/2-specific siRNA (si-ERK) were immunoblotted for ERK, p38MAPK and β -actin.

B: Densitometric analysis of immunoblots shown in panel A. After comparing the band density for β -actin to be equal, band density of other samples is normalized with density of NS RNA bands taken as 100. Values are mean $\pm$ sem (n = 3). Asterisk (*) indicates that values are significantly different from the values for NS-RNA.

Figure 4.5: Contrasting effects of knockdown of ERK by RNA-interference on epithelial barrier disruption in under-differentiated (UD) and differentiated (D) cell monolayers. **A & B:** Transepithelial electrical resistance (TER) (A) and inulin permeability (B) were measured in cell monolayers transfected with non-specific (NS)-RNA or ERK-specific siRNA on day 3 or 7 after seeding on transwell inserts. Values are mean $\pm$ sem (n = 6). Asterisk indicates the values that are significantly different from the values for corresponding NS-RNA values. **C & D:** 3 day (C) or 7 day (D) cell monolayers transfected with NS-RNA or ERK-specific siRNA were fixed and stained for occludin and ZO-1 by immunofluorescence method. (Data representative of three experiments.)

Figure 4.6: Contrasting effects of knockdown of ERK by RNA-interference on the assembly of tight junctions in under-differentiated (UD) and differentiated (D) cell monolayers. **A & B:** Under-differentiated 3-day old Caco-2 cells transfected with NS-RNA or ERK-specific siRNA (si-ERK) were subjected to calcium switch-mediated tight junction assembly. Transepithelial electrical resistance (TER) (A) was measured during EGTA (ethylene glycol tetraacetic acid) treatment and calcium replacement. Inulin permeability (B) was measured at 150 min after calcium replacement. **C & D:** Tight junction assembly was also studied in differentiated 7-day old NS-RNA or ERK-specific siRNA transfected cells. TER (C) and inulin permeability (D) were measured. Values are mean $\pm$ sem (n = 6). Asterisk indicates the values that are significantly different from the corresponding NS-RNA values.

monolayers, compared to those in control siRNA transfected cells. On the other hand, in 7-day differentiated cell monolayers siRNA transfection resulted in a significant delay in the recovery of TER (**Figure 4.6C**) and restoration of barrier function (**Figure 4.6D**) compared to those transfected with control RNA. Non-transfected cell monolayers demonstrated a similar pattern of recovery as NS-RNA transfected cells on both days 3 and 7.

4.1.9 Inference

These studies involving reduced ERK expression have demonstrated that ERK has a significant role to play in maintaining the integrity of tight junctions in Caco-2 cells. Not only that, ERK is also involved in the *de novo* assembly of tight junctions. Even though the studies involving antisense oligos did not provide us with an opportunity to compare these effects of ERK-knockdown between under-differentiated and differentiated cells, but with the help of more stable long double-stranded siRNA, we could establish the differential effect of ERK on tight junction integrity in under-differentiated and differentiated cells. While reduced expression of ERK enhanced junctional integrity and assembly in under-differentiated cells, ERK-knockdown resulted in disruption and delayed assembly of tight junctions in differentiated cells. The possible reason why we did not see the effect of oligonucleotides in differentiated cells is that oligonucleotides are less stable and are quickly degraded in the cells, thus producing a short-lasting effect. Similar recovery responses observed in non-transfected cells and missense oligo-transfected or NS-RNA transfected cells indicated that the process of transfection itself did not have any influence on the reassembly of tight junctions.

4.2 Role of ERK in the Effect of EGF on Hydrogen Peroxide-induced Tight Junction Disruption in Under-differentiated (3-4 Days Old) and Differentiated (12-14 Days Old) Caco-2 Cell Monolayers

4.2.1 Rationale

Extensive studies indicate that EGF protects the epithelial barrier function from a variety of insults including oxidative stress.^{28,94,144-157} However, EGFR activation can also disrupt cell-cell adhesion and promote cell migration.^{124,158,159} Therefore, it is crucial to define the conditions, which render differential EGF responses. Our hypothesis is that the response of EGF is mediated through the agency of ERK and is a function of differentiation status of the cells. Therefore, we evaluated the effect of EGF on hydrogen peroxide-induced barrier disruption in under-differentiated and differentiated cell monolayers. In order to confirm that the effect of EGF is mediated by ERK, we also studied the effect of inhibition of ERK activation by EGF, using a MEK-inhibitor, U0126.

4.2.2 Establishing the Difference in Differentiation Status between 3-4 Days and 7-14 Days Old Cell Monolayers

Identity of cell state (under-differentiated vs. differentiated) was confirmed by several methods, like measuring alkaline phosphatase activity, and villin expression, levels of phosphor-GSK-3 β and phospho-Akt and tight junction integrity as determined by TER and paracellular flux of FITC-inulin.

4.2.2.1 Alkaline Phosphatase (ALP) Assay

Expression of alkaline phosphatase, an enzyme present on the brush border of the intestinal epithelial cells increases as the cells mature and differentiate.¹⁶⁰⁻¹⁶² For the assay, 3 and 4 days old under-differentiated or 7 and 14 days old differentiated Caco-2 cells were washed with PBS and lysed in 1x cell lysis buffer. Varying amounts of lysates containing equivalent amounts (5, 10, 15 and 20 μ g) of protein were then mixed with 100 μ L of ALP assay solution in duplicates, and incubated at 37°C for 30 minutes. The reaction was stopped by adding 20 μ L of 1N NaOH to the wells. The absorbance, which is proportional to the ALP activity, was read at 410 nm. Our results show that alkaline phosphatase activity in 7 and 14 days old differentiated cells was 6-7 times higher than that in the 3 and 4 days old under-differentiated cells (**Figure 4.7A**).

4.2.2.2 Villin Expression

Villin is among several proteins in the cells whose levels are different in differentiated and under-differentiated cells. It is an actin-binding protein that is expressed mainly in the brush border of the epithelial cells in the vertebrates. Thus its levels are higher in differentiated cells than in under-differentiated ones.^{163,164} To study the levels of these two proteins and assess the differentiation status of Caco-2 cells, whole cell lysates from 3 and 4 days old (under-differentiated) or 7 and 14 days old differentiated Caco-2 cells were immunoblotted for villin. We observed that levels of villin were higher in 7 and 14 days old cells compared to the 3-4 days old cells (**Figure 4.7B**).

4.2.2.3 Expression of p-GSK-3 β

Glycogen synthase kinase 3 β is a serine threonine kinase. It is a component of Wnt signaling pathway and its phosphorylated form is known to play an important role in embryonic development, cell proliferation and differentiation.¹⁶⁵⁻¹⁶⁹ To study the levels of GSK-3 β , cell lysates of 3, 4, 7 and 14 days old Caco-2 cells were immunoblotted for p-GSK-3 β and total GSK-3 β . Our studies indicated that levels of p-GSK-3 β were higher in 7 and 14 days old differentiated cells compared to that in 3 and 4 days old under-differentiated cells. The levels of total GSK-3 β were, however, higher in under-differentiated cells as compared to differentiated cells (**Figure 4.7B**).

Figure 4.7: 7 or 14 days old cells are differentiated while 3-4 days old cells are under-differentiated. **A:** Alkaline phosphatase activity was measured in equal amounts of protein extracts (10 μg) from under-differentiated (UD) 3 or 4-day and differentiated (D) 7 or 14-day Caco-2 cell monolayers. Values are mean ± sem (n = 4). Asterisk (*) indicates that values are significantly different from the values for 3-day old cells. **B:** Extracts from Caco-2 cells on days 3, 4, 7 or 14 after seeding were immunoblotted for villin, p-GSK-3β, total GSK-3β, p-Akt, total Akt and E-cadherin. **C & D:** Differentiation status was also examined by measuring tight junction integrity. Transepithelial electrical resistance (TER) (C) and inulin permeability (D) were measured in 3, 4, 7 and 14 days old cell monolayers. Values are mean ± sem (n = 3). Asterisk indicates the values that are significantly different from the corresponding values in 3-day old cell monolayers.

4.2.2.4 Expression of p-Akt

Akt is another enzyme that is an important component of PI3K/Akt pathway. p-Akt is also considered to be a marker of differentiation and its expression has been shown to increase in differentiated Caco-2 cells.¹⁷⁰ Its inhibition is known to affect differentiation of olfactory bulb stem cells.¹⁷¹ Signaling by p-Akt is also known to prevent the cancer phenotype of some colorectal cancer cells.¹⁷² We also studied the levels of p-Akt and total Akt in 3, 4, 7 and 14 days old Caco-2 cells by immunoblot analysis. We observed that like GSK-3 β , levels of p-Akt too were higher in 7 and 14 days old differentiated cells compared to that in 3 and 4 days old under-differentiated cells. However, the levels of total Akt were unchanged (**Figure 4.7B**).

4.2.2.5 Expression of E-cadherin

E-cadherin is a component protein of adherens junctions, and its expression has been shown to be increased in differentiated Caco-2 cells.¹⁷⁰ We examined the levels of E-cadherin in cell lysates from 3, 4, 7 or 14 days old cells. We observed that the expression of E-cadherin was not significantly different on various days (**Figure 4.7B**). The probable reason for this deviation from other published data is that in those studies, the investigators have used sub-confluent cells, while in our studies we used fully confluent cell monolayers even on earlier days *i.e.* 3 or 4. Since these cells were forming junctions, they are differentiated to some extent but not completely, we have labeled them as under-differentiated.

4.2.2.6 Tight Junctional Integrity in Under-differentiated and Differentiated Cells

As the Caco-2 cells grow and differentiate, the tight junctions formed by them become stronger as measured by TER and paracellular permeability.¹³³⁻¹³⁵ We characterized the differentiation status of Caco-2 cells by doing serial examination of TER and paracellular flux of FITC-inulin in 3, 4, 7 and 14 days old confluent monolayers of Caco-2 cells. We observed that while the TER was lower (300-350 Ohms/cm²) in 3-4 days old under-differentiated monolayers, while it was higher (450-630 Ohms/cm²) in 7 or 14-days old differentiated cell monolayers (**Figure 4.7C**). We also observed that in coherence with the increase in the TER with increased differentiation of the cells, the paracellular flux of FITC-inulin followed a downward trend. The permeability of 3-4 days old under-differentiated monolayers was higher compared to 7 or 14 days old differentiated cell monolayers which had a significantly lower permeability (**Figure 4.7D**).

Thus, we established that 7-day or 14-day old Caco-2 cells are mature and differentiated, whereas 3-4 days old cells show full expression of some differentiation markers such as E-cadherin, but low expression of other markers like villin, p-GSK-3 β and p-Akt. Therefore, 3-4 days old cells can be defined as under-differentiated.

4.2.3 Effect of EGF and Hydrogen Peroxide on Tight Junction Integrity

We evaluated the effects of EGF on hydrogen peroxide-mediated disruption of barrier function and the role of ERK in EGF-mediated protection of tight junctions in 4-day under-differentiated and 14-day differentiated cell monolayers. Cells grown on transwell inserts were preincubated at 37°C in PBS-BSA-glucose with or without MEK inhibitor, U0126 for 50 minutes. EGF was added to the medium in both apical and basal compartments to achieve a final concentration of 30 nM. Oxidative stress was induced in the cells by administering 20 μ M hydrogen peroxide. Adequate controls were used where cells were not treated with EGF or hydrogen peroxide. Tight junction integrity was assessed by measuring TER and inulin flux at hourly intervals. After two hours, the cells were washed with cold 1x PBS and fixed in acetone: methanol for immunostaining.

In both 4-day and 14-day old cells, hydrogen peroxide caused disruption of tight junctions as was evident from decreased TER (**Figure 4.8A,C**) and increased inulin flux (**Figure 4.8B,D**). Similar to a previous observation, on day 14, EGF significantly attenuated hydrogen peroxide-induced decrease in TER (**Figure 4.8A**) and increase in inulin flux (**Figure 4.8B**). Pretreatment of cells with U0126 significantly inhibited the effect of EGF on hydrogen peroxide-induced changes in TER (**Figure 4.8A**) and inulin permeability (**Figure 4.8B**). In contrast to 14-day cells, in 4-day cells, EGF exacerbated the effect of hydrogen peroxide on TER (**Figure 4.8C**) and inulin flux (**Figure 4.8D**). EGF-mediated potentiation of hydrogen peroxide effect on inulin permeability was prevented by U0126 (**Figure 4.8D**).

Immunostaining of fixed cell monolayers for tight junction proteins demonstrated that hydrogen peroxide treatment disrupts the junctional organization of occludin and ZO-1 in both 4-day under-differentiated and 14-day differentiated cell monolayers (**Figure 4.9**). In 14-day cells, EGF attenuated the hydrogen peroxide-induced redistribution of occludin and ZO-1 from the tight junctions, and U0126 treatment inhibited this protective effect of EGF (**Figure 4.9A**) indicating that this effect is mediated by ERK. In 4-day cell monolayers EGF enhanced the hydrogen peroxide-induced redistribution of occludin and ZO-1 (**Figure 4.9B**).

4.2.4 Inference

Alkaline phosphatase assay and expression of differentiation markers like Villin, p-GSK-3 β and p-Akt helped establish that 7 or 14-day cells are mature and differentiated, while the 3 or 4-day old cells are under-differentiated. Studies involving MEK inhibitor U0126 revealed that in differentiated cells, EGF protects the tight junctions from hydrogen peroxide-induced oxidative stress and this effect is mediated by ERK. On the other hand, in under-differentiated cells, EGF instead of protecting the junctions, potentiated the disruptive effect of hydrogen peroxide. Attenuation of the combined disruptive effect of EGF and hydrogen peroxide by pretreatment with U0126 demonstrated that the disruptive effect of EGF, like the protective effect, was mediated via ERK.

Figure 4.8: Contrasting effects of EGF on hydrogen peroxide-induced epithelial barrier disruption in under-differentiated and differentiated cell monolayers. Differentiated (14-day) (**A & B**) or under-differentiated (4-day) (**C & D**) old Caco-2 cell monolayers were pretreated with U0126 (10 μ M;) for 50 min prior to administration of EGF (30 nM). Hydrogen peroxide (20 μ M) was administered 10 min after EGF. Transepithelial electrical resistance (TER) (**A & C**) and inulin permeability (**B & D**) were measured at varying times. Values are mean $\pm$ sem (n = 6). Asterisk (*) indicates that values are significantly different from corresponding control values; Symbol @ indicates that values are significantly different from corresponding H₂O₂ values; Symbol # indicates that values are significantly different from corresponding EGF+H₂O₂ values.

Figure 4.9: Contrasting effects of EGF on hydrogen peroxide-induced disruption of tight junctions in under-differentiated and differentiated cell monolayers. A & B: Differentiated 14-day (A) or under-differentiated 4-day (B) old Caco-2 cell monolayers were pretreated with U0126 (10 μ M) for 50 min prior to administration of EGF (30 nM). Hydrogen peroxide (20 μ M) was administered 10 min after EGF. Cell monolayers were fixed and stained for occludin and ZO-1 by immunofluorescence method. Images were taken in X-Y plane, and processed as explained in Methods section. **C-F:** Intensity of intracellular fluorescence of Occludin (C & E) and that of ZO-1 (D & F) in 14-day or 4-day cell monolayers was quantified by densitometric analysis. Values are mean $\pm$ sem (n = 3). Asterisk (*) indicates that values are significantly different from corresponding control values; Symbol @ indicates that values are significantly different from corresponding H₂O₂ values; Symbol # indicates that values are significantly different from corresponding EGF+H₂O₂ values.

A Day 14 (Differentiated)

B Day 4 (Under-differentiated)

Figure 4.9 (continued).

4.3 Expression of Dominant Negative and Constitutively Active MEK-1 Exhibit Contrasting Effects on Tight Junction Integrity in Caco-2 Cells

4.3.1 Rationale

ERK is known to play an important role in cell growth, differentiation, maturation and apoptosis, in addition to regulation of tight junctions.^{112,173} For this very reason, reduced expression of ERK for a prolonged period of time has the ability to produce certain morphological changes in the cells, thus rendering them unsuitable for studying junctional barrier function. Even though we did not notice any such changes in our studies, illustrated above, using antisense oligos and siRNA for ERK, to verify our previous data, we studied the effect of regulated expression of MEK and thus activation of ERK in Caco-2 cells using an inducible vector. This technique has the advantage of providing the ability to induce the expression of our gene of interest on the desired day or stage of cell differentiation and for a brief period so as to avoid any undesirable effects of prolonged ERK expression or suppression.

4.3.2 Preparation of Tet-on and Tet-off Caco-2 Cell Lines

Caco-2 cells were transfected with commercially available pTet-on or pTet-off vectors as described earlier. The transfected cells were selected with G418 sulphate (700 µg/mL) and cloned using the cloning cylinders, and maintained in 300 µg/mL of G418 sulphate. Lysates from transfected and selected cells expressing the Tet-controlled transactivator (tTA-Advanced for Tet-off system or rtTA-Advanced for Tet-on system) were immunoblotted for the TetR protein, and the clones expressing highest amounts of TetR were chosen for further studies. Functioning of the Tet-on/off system in the cells was verified by Luciferase assay. Tet-on/off cells were transfected with Luciferase tagged pTRE2hyg reporter vector. The expression of the reporter vector was turned on by addition (Tet-on) or withdrawal (Tet-off) of doxycycline from the medium. Luciferase assay was done to check the expression of the reporter vector. The cell clones expressing high amounts of the reporter vector were chosen for further studies. These clones were the same as those expressing high amounts of TetR.

4.3.3 Design of pTRE2hyg-GFP Vector and Insertion of MEK Genes

GFP sequence was inserted between SalI and EcoRV unique restriction endonuclease sites in the commercially available pTRE2hyg vector. The GFP-tagged pTRE2hyg vector was digested using the unique restriction enzyme sites of BamHI and ClaI in the multiple cloning site. The wild type (WT), dominant negative (DN) and constitutively active (CA) isoforms of MAPK-ERK-Kinase (MEK) were isolated and ligated into the pTRE2hyg-pAcGFPN vector, in such a way that MEK gene was on N-terminus side of the GFP. The size of the resulting construct was verified by running the samples on a 1% agarose gel with ethidium bromide (EtBr). The resulting construct

expressing the GFP-MEK as a composite protein was sequenced. BLAST analysis of the observed sequence of the vector was done to compare it with its theoretical sequence. It was ascertained upon sequencing and BLAST analysis that the mutations for the DN (K97M) and CA (S218E and S220D) were in place in the vectors we made. The Tet-on or Tet-off Caco-2 cells generated as described earlier were transfected with the plasmids containing MEK (wild-type and mutants) using lipofectamine-LTX and plus reagent.

4.3.4 Expression of MEK Constructs

The expression of MEK (WT and mutants) was confirmed by expression of GFP in the cells at 6 hours after addition of doxycycline in 3-day (**Figure 4.10A**) as well as 7-day old cell monolayers (**Figure 4.10B**). Cells were fixed in acetone-methanol and immunostained for tight junction proteins occludin or ZO-1. Confocal microscopy revealed that on day 3, expression of WT-MEK and CA-MEK lead to decreased localization of occludin and ZO-1 at the intercellular junctions. The redistribution of junctional proteins was more pronounced with the expression of CA-MEK compared to WT-MEK (**Figure 4.10C**). In contrast, expression of DN-MEK produced increased junctional localization of occludin and ZO-1. On the other hand, on day 7, expression of WT-MEK and CA-MEK resulted in enhanced junctional localization of occludin and ZO-1, as compared to cells expressing vector alone, (**Figure 4.10D**) whereas the expression of DN-MEK produced a decreased junctional localization of occludin and ZO-1.

4.3.5 Inference

These studies using regulated expression of wild-type MEK and mutants further confirmed the findings of previously described studies using pharmacological inhibitor (U0126) and reduced ERK expression by antisense oligos and siRNA. While in undifferentiated 3-day old cells, expression of CA-MEK resulted in junctional disruption, expression of DN-MEK resulted in more robust junctional localization of occludin and ZO-1. This effect was in contrast to what was observed in differentiated 7-day cells. Expression of CA-MEK results in presence of active MEK in cells in greater quantities, thus producing more active ERK in the cells. Whereas, DN-MEK, when expressed in the cells competes with the innate MEK present in the cells, thus inhibiting activation of ERK. Thus, presence of higher amounts of active-ERK (activated by CA-MEK) resulted in stronger localization of junctional proteins in differentiated cells, while it lead to disruption of junctions in undifferentiated cells. The effects of expression of DN-MEK were in complete contrast to those observed with CA-MEK. These results corroborated the finding that ERK has contrasting effect on tight junctions in differentiated and undifferentiated cells. This study suffers from a limitation that we could not evaluate and compare the junctional integrity in terms of TER and inulin flux. That is because cells over-expressing MEK isoforms and thus influencing the active-ERK levels in the cells were very fragile. Even though we did not notice any morphological changes at any point of time, with any of the MEK isoforms, the cells tended to get off the transwell

Figure 4.10: Effects of expression of active and inactive MEK1 on tight junction integrity at different stages of differentiation. **A & B:** Caco-2 cells expressing Tet-on regulator were transfected with GFP or GFP-tagged MEK1, wild type (WT), dominant negative (DN) or constitutively active (CA) MEK in pTRE2hyg-AcGFP-N vector and the expression of GFP and MEK was induced by administering doxycycline on day 3 (A) or day 7 (B). Expression of GFP before and after doxycycline was visualized by immunofluorescence staining and confocal microscopy. **C & D:** Under-differentiated 3-day (C) and differentiated 7-day (D) old cell monolayers, in which MEK expression was induced by doxycycline were fixed and stained for occludin and ZO-1. **E-H:** Intensity of intracellular fluorescence of Occludin in 3-day cell monolayers (E) and 7-day cell monolayers (F), and that of ZO-1 in 3-day cell monolayers (G) and 7-day cell monolayers (H) was quantified by densitometric analysis. Values are mean $\pm$ sem (n = 3). Asterisk (*) indicates that values are significantly different from corresponding values in Vector transfected cells.

Figure 4.10 (continued).

membrane very easily during the procedures. Only a few colonies of cells were available in the end, which were used for immunostaining and confocal microscopy.

4.4 Role of ERK in Regulation of Thr-Phosphorylation of Occludin

4.4.1 Rationale

The studies mentioned above have demonstrated that ERK has contrasting effects on regulation of tight junctions in under-differentiated and differentiated cells. Evidence indicates that PP2A and PKC ζ are involved in the regulation of Thr-phosphorylation of occludin and that EGF prevents hydrogen peroxide-induced Thr-dephosphorylation of occludin in Caco-2 cell monolayers.^{56,59,60,94,97} Occludin is known to be highly phosphorylated on serine and threonine residues in intact junctions, whereas the same residues are dephosphorylated in disrupted junctions.^{97,100,102,105,106} ERK is a serine, threonine-kinase, but its role in phosphorylation of occludin is not known. It may possibly be exerting its protective effect on junctions by directly phosphorylating occludin on serine and threonine residues. Alternatively, it may be phosphorylating and thus influencing activation of another serine-threonine kinase like PKC ζ , which might in turn be phosphorylating occludin. ERK may also have some effect on protein phosphatases like PP2A and PP1 in the cells, resulting in dephosphorylation of occludin on serine and threonine residues, thus resulting in disrupted junctions. The following studies were aimed at determining the mechanism behind the contrasting influences of ERK in regulating tight junction integrity. We tried to determine whether differentiation dependent effect of ERK on tight junctions involved modulation of PKC ζ and/or PP2A.

4.4.2 Junctional and Signaling Proteins in 4-day and 14-day Cell Monolayers

As described in Chapter 1, the assembly of tight junctions involves numerous transmembrane, scaffold and plaque proteins. Additionally, numerous signaling molecules such as protein kinases and phosphatases are associated with tight junctions, and are known to regulate tight junction integrity. To find out if the differential effect of ERK on 4-day under-differentiated and 14-day differentiated cells is not caused by any differences in their expression of various junctional and signaling proteins, we assessed the expression and distribution of various junctional and signaling proteins in 4-day under-differentiated and 14-day differentiated Caco-2 cells.

4.4.2.1 Expression of Claudin-4, PP2A and PKC ζ in Under-differentiated and Differentiated Cells

Lysates from 4 and 14-day old Caco-2 cells were blotted for claudin-4, PKC ζ and PP2A. Our results show that levels of PP2A, PKC ζ and tight junction proteins such as claudin-4 are not different in 4-day under-differentiated and 14-day old differentiated cell monolayers (**Figure 4.11A and B**).

Figure 4.11: Expression of junctional and signaling proteins in differentiated and under-differentiated Caco-2 cells. **A & B:** Equal amounts (20 μ g) of protein from extracts of 4-day under-differentiated (UD) and 14-day differentiated (D) Caco-2 cells were immunoblotted for claudin-4, PKC ζ and PP2A-C α (A). After comparing the band density for β -actin to be equal, band density of other samples was normalized with the and density of the same protein on day 4 taken as 100. Values are mean $\pm$ sem (n = 3). Asterisk (*) indicates that values are significantly different from the values for day 4.

4.4.2.2 Activity and Distribution of p-ERK in Under-differentiated and Differentiated Cells

After establishing that the under-differentiated and differentiated cells were expressing similar levels of junctional and signaling proteins, we examined whether the level and distribution of ERK are different in under-differentiated and differentiated cells.

We analyzed the levels of active ERK (p-ERK) and total ERK in these cells by immunoblot and immunofluorescence methods. 4-day old under-differentiated and 14-day old differentiated Caco-2 cells were treated with or without 30 nM EGF for 2, 5, 10, 30 and 60 minutes. Cell lysates were immunoblotted for p-ERK and total ERK1/2. Cell monolayers were also fixed in acetone:methanol and immunostained for p-ERK.

The level of total ERK in 14-day differentiated cells was higher than that in 4-day under-differentiated cells (**Figure 4.12A and B**). In contrast, the level of p-ERK was greater in 4-day under-differentiated cells compared to that in 14-day old differentiated cells. EGF treatment rapidly and robustly increased p-ERK in 14-day old differentiated cells. EGF-mediated activation of ERK was much more pronounced in 14-day old differentiated cells compared to that in 4-day old under-differentiated cells (**Figure 4.13**). Immunofluorescence localization indicated that p-ERK is distributed predominantly in the intracellular compartment in 4-day old under-differentiated cell monolayers, while in EGF-treated 14-day old differentiated cell monolayers p-ERK was distributed more at the peri-junctional region (**Figure 4.14**).

4.4.2.3 ERK Regulates Tight Junctions Prior to Influencing Differentiation of Cells

It is widely known that in addition to its role in regulation of tight junction integrity, that is the subject of this study, ERK has a very significant role to play in cellular proliferation and differentiation.^{96,174,175} We determined if the reduced expression of ERK by RNA-interference or expression of CA-MEK effected any change in differentiation status of the cells. Towards this end, we analyzed the differentiation status of Caco-2 cells expressing reduced ERK or over-expressing CA-MEK on day 4 or day 8 post-transfection, by alkaline phosphatase assay and immunoblotting for villin expression. We observed that while on day 4 post-transfection, levels of alkaline phosphatase were significantly higher in cells with reduced ERK expression compared to control cells; the levels of alkaline phosphatase were not significantly different on day 8 (**Figure 4.15A**). Expression of differentiation marker protein villin too was higher in cells transfected with siRNA specific for ERK compared with cells transfected with non-specific RNA (**Figure 4.15B**). On the other hand, cells expressing CA-MEK did not exhibit any significant difference in alkaline phosphatase or villin levels before or after induction of expression on days 4 or 8 post-transfection (**4.15C-F**).

Figure 4.12: Differential distribution of ERK in Caco-2 cells. A & B: Equal amounts (20 μ g) of protein from extracts of under-differentiated (UD) 4-day and differentiated (D) 14-day Caco-2 cell monolayers were immunoblotted for total ERK and phospho-ERK (active), and the bands were quantitated by densitometric analysis. After comparing the band density for β -actin to be equal, the density of day 14 bands was normalized against the day 4 band density which was arbitrarily taken as 100. Values are mean $\pm$ sem (n = 6). Asterisk (*) indicates that values are significantly different from corresponding 4-day values.

Figure 4.13: Differential distribution of active ERK in EGF-treated cell monolayers. **A & B:** Under-differentiated (UD) 4-day and differentiated (D) 14-day Caco-2 cell monolayers were incubated with EGF for varying times and equal amounts (20 μ g) of protein from cell extracts were immunoblotted for phospho-ERK and total ERK1/2. **C & D:** After comparing the band density for β -actin to be equal, the density of '+EGF' bands was normalized against the '-EGF' band density which was arbitrarily taken as 100. Values are mean $\pm$ sem ($n = 3$). Asterisk (*) indicates that values are significantly different from corresponding '-EGF' values.

Figure 4.14: Differential localization of active ERK in EGF-treated cell monolayers. Under-differentiated 4-day and differentiated 14-day Caco-2 cell monolayers were incubated with EGF for varying times and the cell monolayers fixed and stained for phospho-ERK by immunofluorescence method. (Data representative of 3 experiments.)

Figure 4.15: ERK regulates tight junctions before influencing the differentiation status in under-differentiated Caco-2 cells. **A:** Alkaline phosphatase activity was measured in equal amounts (10 μg) of protein extracts from under-differentiated, day 3 post-transfection and differentiated day 7 post-transfection, Caco-2 cell monolayers transfected with NS-RNA or siRNA specific for ERK. Values are mean ± sem (n = 3). Asterisk (*) indicates that values are significantly different from the values for NS-RNA transfected cells. **B:** Equal amounts (20 μg) of protein extracts from Caco-2 cells transfected with NS-RNA or ERK siRNA on days 3 or 7 after seeding were immunoblotted for villin. **C & D:** Alkaline phosphatase activity was also measured in extracts from under-differentiated, day 4 post-transfection (C) and differentiated day 7 post-transfection (D) Caco-2 cell monolayers transfected with empty vector or CA-MEK. Values are mean ± sem (n = 3). **E & F:** Equal amounts (20 μg) of protein extracts from Caco-2 cells transfected with empty vector (V), WT-MEK (W), DN-MEK (D) or CA-MEK (C) on days 3 or 7 after seeding were immunoblotted for villin.

4.4.3 Role of ERK in Regulation of Occludin Phosphorylation in Caco-2 Cells

As mentioned earlier, PP2A and PKC ζ play a key role in regulating the phosphorylation state of occludin and thus influence the tight junction integrity. Previous studies demonstrated that PP2A directly interacts with occludin and regulates its phosphorylation on Thr residues.^{59,60} Therefore, we evaluated the effect of EGF and hydrogen peroxide on threonine-phosphorylation of occludin and co-immunoprecipitation of occludin with PP2A or PKC ζ in 4-day under-differentiated and 14-day differentiated cell monolayers.

4-day under-differentiated and 14-day differentiated Caco-2 cells grown on transwell inserts were pre-incubated with or without 10 μ M U0126, and then treated with or without 30 nM EGF for 10 minutes. Oxidative stress was induced by administering 20 μ M hydrogen peroxide for one hour. Cells were washed with cold PBS, and whole cell lysate was made in lysis buffer D. Phospho-threonine was immunoprecipitated from the cell lysates as explained in the Methods section, and was immunoblotted for occludin.

We observed that hydrogen peroxide treatment caused a significant decrease in threonine-phosphorylation of occludin in both 14-day differentiated and 4-day under-differentiated cell monolayers (**Figure 4.16A, B and C**). EGF effectively restored the occludin phosphorylation and this effect of EGF was attenuated by U0126 in 14-day old cell monolayers (**Figure 4.16A and B**). EGF or U0126 did not produce a significant influence on threonine-phosphorylation of occludin in 4-day old cell monolayers.

4.4.4 ERK Does Not Directly Phosphorylate Occludin

Having demonstrated that ERK is involved in the phosphorylation of occludin on threonine residues, we tried to determine if ERK, being a serine, threonine kinase, is directly responsible for phosphorylating occludin on threonine residues.

For this study, commercially available recombinant inactive ERK1 or 2 were activated using MEK and ATP as explained in the Methods section. The active-ERK was incubated with c-terminus 150 amino acid GST-occludin in the presence of ATP for one hour. The protein mix was thrombin cleaved to dissociate occludin and GST. Immunoblotting was done for phospho-threonine, ERK and PKC ζ .

We observed that active ERK did not phosphorylate c-terminus 150 amino acid GST-occludin on threonine (**Figure 4.17A**) or serine (**Figure 4.17B**) residues *in vitro*, although PKC ζ was seen to phosphorylate occludin on threonine (**Figure 4.17C**). This shows that while PKC ζ directly phosphorylates occludin on threonine residues, active-ERK does not do so. These results indicate that the role of ERK in threonine-phosphorylation of occludin is not direct, but is probably mediated through some other signaling molecules.

Figure 4.16: Effect of ERK on Threonine-phosphorylation of occludin in differentiated and under-differentiated Caco-2 cells. **A:** 14-day old differentiated (D) or 4-day old under-differentiated (UD) Caco-2 cell monolayers were pre-treated with U0126 (10 μ M) for 50 min prior to administration of EGF (30 nM). Hydrogen peroxide (20 μ M) was administered 10 min after EGF. phospho-Threonine was immunoprecipitated from cell lysates and immunoblotted for occludin. **B & C:** Densitometric analysis of occludin bands of 14-day old (B) and 4-day old (C) cell monolayers in experiment described in panel A. Band density is normalized with density of 'Control' band arbitrarily taken as 100. Values are mean $\pm$ sem (n = 3). Asterisk (*) indicates that values are significantly different from corresponding control values; Symbol # indicates that values are significantly different from the corresponding H₂O₂ values; Symbol @ indicates that values are significantly different from corresponding EGF+H₂O₂ values.

Figure 4.17: ERK does not directly phosphorylate occludin *in vitro*. **A & B:** Equal amounts (20 μ g) of recombinant inactive-ERK, active-ERK (activated using MEK and ATP) or PKC ζ was incubated with GST-tagged C-terminal 150 amino acid tail of occludin in the presence of ATP. Immunoblotting was done for phospho-Threonine (A) and phospho-Serine (B). (Data representative of 3 experiments.) **C:** GST-tagged C-terminal 150 amino acid tail of occludin was also incubated with or without PKC ζ in the presence of ATP, and immunoblotting was done for phospho-Threonine.

4.4.5 ERK Regulates PKC ζ and PP2A

It is evident from the previous study that ERK does not directly phosphorylate occludin on threonine residues. This led us to hypothesize that ERK interacts with other signaling molecules involved in the threonine-phosphorylation and dephosphorylation of occludin. PKC ζ and PP2A are two such molecules which have been shown to regulate phosphorylation of occludin on threonine residues.^{58,97,150} ERK could be involved in either phosphorylation, and thus activation of PKC ζ or phosphorylation and thus inactivation of PP2A. PP2A activation/inactivation is regulated by its phosphorylation on tyrosine residues. Since ERK is not a tyrosine-kinase, we tried to determine if ERK has a role in phosphorylation of PKC ζ .

4.4.5.1 ERK Phosphorylates PKC ζ *in vitro*

Commercially available recombinant PKC ζ was incubated with active or inactive ERK and ATP at 30°C for one hour and immunoblotted for phospho-threonine. We observed an increased threonine phosphorylation of PKC ζ in the presence of active-ERK compared to inactive ERK (**Figure 4.18**).

4.4.5.2 Regulation of PKC ζ and PP2A by ERK in Caco-2 Cells

Previous studies demonstrated that PP2A directly interacts with occludin and regulates its phosphorylation on Thr residues. Therefore, we evaluated the effect of EGF and hydrogen peroxide on co-immunoprecipitation of occludin with PP2A or PKC ζ in 4-day under-differentiated and 14-day old differentiated cell monolayers.

4-day old under-differentiated or 14-day old differentiated Caco-2 cells grown in transwell inserts were pre-incubated with or without 10 μ M U0126, then treated with or without 30 nM EGF for 10 minutes. Cells were subjected to oxidative stress by administering 20 μ M hydrogen peroxide for 30 minutes. Occludin was immunoprecipitated from detergent-insoluble fraction of cellular proteins, and immunoblotting was done for PKC ζ and PP2A.

We observed that hydrogen peroxide treatment caused a significant increase in co-immunoprecipitation of PP2A with occludin (**Figure 4.19A and B**) in 14-day differentiated cell monolayers, but not in 4-day under-differentiated cell monolayers. EGF effectively decreased PP2A association with occludin and this effect of EGF was attenuated by U0126 (**Figure 4.19A and B**). EGF or U0126 did not produce a significant influence on PP2A co-immunoprecipitation with occludin in 4-day old cell monolayers. In contrast to PP2A, PKC ζ co-immunoprecipitation with occludin was significantly reduced by hydrogen peroxide in 14-day old differentiated cell monolayers (**Figure 4.19A and C**). EGF pretreatment abrogated the hydrogen peroxide effect on co-immunoprecipitation of PKC ζ with occludin; and this effect of EGF was attenuated by U0126 (**Figure 4.19C**). In 4-day under-differentiated cell monolayers, however, hydrogen peroxide or EGF failed to influence PKC ζ association with occludin.

Figure 4.18: ERK phosphorylates PKC ζ *in vitro*. **A:** PKC ζ was incubated with active or inactive ERK in the presence of ATP, and immunoblotting was done for p-Threonine (p-Thr), PKC ζ and ERK1/2. **B:** p-Thr bands for PKC ζ incubated with inactive- or active-ERK were analyzed by densitometric analysis. Values are mean $\pm$ sem (n = 3). Asterisk (*) indicates that values are significantly different from corresponding values for inactive-ERK.

Figure 4.19: Differential effects of EGF on cellular distribution of PP2A and PKC ζ in under-differentiated and differentiated cell monolayers. **A:** 4-day under-differentiated or 14-day differentiated Caco-2 cell monolayers were pretreated with U0126 (10 μ M) for 50 min prior to administration of EGF (30 nM). Hydrogen peroxide (20 μ M) was administered 10 min after EGF. Occludin was immunoprecipitated from detergent-insoluble fraction of cellular proteins and immunoblotted for PKC ζ and PP2A-C α . **B & C:** Densitometric analysis of PP2A-C α (B) and PKC ζ (C) bands in experiments described in panel A. Band density is normalized with density of ‘Control’ band arbitrarily taken as 100. Values are mean $\pm$ sem (n = 3-5). Asterisk (*) indicates that values are significantly different from corresponding control values; Symbol # indicates that values are significantly different from the corresponding H₂O₂ values; Symbol @ indicates that values are significantly different from corresponding EGF+H₂O₂ values.

Immunostaining and confocal microscopy showed that hydrogen peroxide induced a redistribution of PP2A with predominant localization at the peri-junctional region in both 4-day and 14-day old cell monolayers. Pretreatment with EGF abrogated hydrogen peroxide-induced PP2A redistribution, and this effect of EGF was prevented by U0126. The responses to EGF and U0126 were similar in 4-day and 14-day old cells. Hydrogen peroxide did not affect the distribution of PKC ζ in both 4-day under-differentiated and 14-day differentiated cell monolayers, while EGF enhanced junctional distribution of PKC ζ in 14-day differentiated cell monolayers, but not in 4-day under-differentiated cell monolayers (**Figure 4.20**).

4.4.6 Inference

Since these studies have revealed that under-differentiated and differentiated Caco-2 cells do not differ in expression of important junctional and signaling proteins like claudin-4, PKC ζ and PP2A, the differential effect of EGF on tight junction integrity, mediated by ERK could be a result of differences in the levels of expression and intracellular distribution of phospho-ERK (active-ERK) and total ERK1/2 in the cells. EGF treatment produced much greater activation of ERK in differentiated cells as compared to the under-differentiated cells. Localization of ERK in cells in response to EGF stimulation was also observed to be different in differentiated and under-differentiated cells, pointing towards this being a possible reason for the contrasting effects of EGF on tight junction integrity in differentiated and under-differentiated cells. Further, a significant increase in alkaline phosphatase levels in Caco-2 cells with reduced expression of ERK, accompanied by a slight increase in villin expression on day 4 post-transfection indicated that knockdown of ERK may be causing the cells to differentiate. Since no such effect was observed on day 8 post-transfection, it may be inferred that since at this time cells are already differentiated, ERK knockdown does not influence their differentiation any further. No significant increases in alkaline phosphatase levels and villin expression upon induction of MEK expression in cells expressing WT-MEK and its mutants either on day 4 or 8 post-transfection indicated that the differentiation status of cells is not altered in these cells. The possible reason for these findings is that in under-differentiated cells reduced ERK expression enhances the integrity of tight junctions, and subsequently brings about the changes in their differentiation status. These findings point to the fact that ERK regulates tight junctions first and then influences the differentiation status of the cells.

Our studies to see the threonine-phosphorylation of occludin revealed that in differentiated cells EGF prevented the hydrogen peroxide induced dephosphorylation of occludin, and this effect was mediated by ERK. Interestingly, however, no influence of EGF or ERK was observed on threonine-phosphorylation of occludin in under-differentiated cells. The findings of *in vitro* studies involving pure recombinant ERK, revealed that ERK does not directly phosphorylate occludin on serine or threonine residues, whereas PKC ζ does so, when considered along with the finding that active-ERK phosphorylates PKC ζ point towards a complex interplay among these signaling molecules. The possible role of ERK could involve phosphorylation and activation of

Figure 4.20: Differential effects of EGF on co-localization of occludin with PP2A and PKCζ in under-differentiated and differentiated cell monolayers. Under-differentiated 3-day and differentiated 14-day old cell monolayers were pretreated with U0126 (10 μM) for 50 min prior to administration of EGF (30 nM). Hydrogen peroxide (20 μM) was administered 10 min after EGF. Cell monolayers were fixed and stained for PP2A or PKCζ by immunofluorescence method. (Data representative of 3 experiments.)

PKC ζ which would, in turn, phosphorylate occludin on threonine. But this theory only explains the protective role of ERK that was observed in differentiated cells. This theory does not explain the disruptive effect of ERK on tight junctions in under-differentiated cells.

This question led us to look for some other signaling molecule that could be interacting with these proteins and thus influencing the tight junction integrity. Our examination of the role of PP2A revealed that it indeed is a participant in this regulatory network of signaling molecules. In differentiated cells, PP2A was seen to disrupt the tight junctions by associating and localizing with occludin in response to oxidative stress, and reversal of this effect by MEK inhibitor indicated the role of ERK in preventing the disruption. Association of occludin and PKC ζ in response to oxidative stress and EGF treatment, on the other hand, demonstrated a pattern that was complete opposite of that seen with PP2A. Contrarily, inability to observe these effects of ERK in under-differentiated cells clearly points out that there are certainly some variations in how the signaling cascades and molecules are wired in under-differentiated and differentiated cells.

4.5 EGF Protects the Ileal Epithelial Tight Junctions in Mouse Intestine

4.5.1 Rationale

All the evidence gathered and presented so far to establish the role of ERK is based on studies conducted either *in vitro* or in cultured cells. Even though Caco-2 cells are considered to be the gold standard for studying epithelial tight junctions, cultured cells are known to sometimes exhibit responses or effects which may be different from those seen in physiological systems. To confirm that the role of ERK as seen in animal tissues was the same as seen *in vitro* or in cultured cells, we evaluated the effect of EGF and hydrogen peroxide in the following *ex vivo* studies using mouse ileum tissue.

Ileum was dissected out of the 10-14 weeks old BL-57 mice. Short segments, measuring ~1-2 cm, of ileum were preincubated in DMEM without serum, antibiotics or glutamine for 30 minutes. 30 nM EGF was administered to the medium for 10 minutes. The ileum segments were subjected to oxidative stress by administering different concentrations (100 μ M and 250 μ M) of hydrogen peroxide for one hour. Detergent-soluble and insoluble fractions were prepared from the mucosa of the segments, and immunoblotted for occludin. Another set of ileum segments was fixed in OCT to make cryosections and immunostaining.

Immunoblotting revealed that hydrogen peroxide treatment resulted in reduced occludin levels in the detergent-insoluble fraction (**Figure 4.21**). Pretreatment with EGF was seen to attenuate this effect of hydrogen peroxide and resulted in restoration of occludin to the detergent-insoluble fraction. Detergent-soluble fraction of the proteins

Figure 4.21: Role of EGF in protection of epithelial tight junctions in the mucosa of mouse ileum. Short segments of mouse ileum were pretreated with 30 nM EGF for 50 minutes before being subjected to oxidative stress using 100 μ M or 250 μ M hydrogen peroxide for one hour. **A:** Equal amounts (20 μ g) of protein from detergent-soluble and detergent-insoluble fractions of protein prepared from mucosal scrapings were immunoblotted for occludin. **B:** Densitometric analysis of occludin bands in experiment described in panel A was done with band values for detergent-soluble fraction normalized against those for detergent-insoluble fraction which was arbitrarily taken as 100. Values are mean $\pm$ sem (n = 3). Asterisk (*) indicates that values are significantly different from corresponding values for detergent-insoluble fraction.

exhibited a corresponding decrease in occludin levels with EGF pretreatment prior to oxidative stress.

Immunostaining and confocal microscopy of cryosections prepared from these ileal segments showed disruption of tight junctions and reduced junctional localization of ZO-1 by hydrogen peroxide in a dose dependent manner (**Figure 4.22**). Pretreatment with EGF was seen to restore the junctional localization of ZO-1 and thus protecting the tight junctions against disruptive effect of hydrogen peroxide.

4.5.2 Inference

This study was aimed at studying in a physiological system the effect of EGF as seen in cell cultures. As is evident, that in mouse ileal epithelium EGF protects the tight junctions against hydrogen peroxide induced oxidative stress. This response is similar to the one seen in differentiated Caco-2 cells, which is true because the epithelial cells in ileal mucosa are differentiated.

4.6 ERK Protects Tight Junctions against Osmotic Stress-induced Disruption

4.6.1 Rationale

Human intestine is constantly being exposed to osmotic stress. Some recent studies have shown the role of EGF in prevention of osmotic stress induced disruption of tight junctions.¹⁷⁶ Some other MAP kinases like JNK have been demonstrated to play a role in disruption of tight junctions produced by osmotic stress.¹⁷⁷ We tried to evaluate the role of ERK in the EGF-mediated protection of tight junctions against osmotic stress-induced disruption.

4.6.2 Expression of Constitutively Active MEK Attenuates the Osmotic Stress-induced Tight Junction Disruption in Differentiated Caco-2 Cell Monolayers

Differentiated Caco-2 cell monolayers expressing empty vector or various mutants of MEK *i.e.* WT-, DN or CA-MEK, cultured on transwell inserts, were subjected to osmotic stress by administering 0.35 M mannitol for 3 hours. The monolayers were fixed and immunostained for tight junctional protein occludin. We observed that compared to control cell monolayers, the cells expressing WT- or CA-MEK exhibited a better and stronger junctional localization of occludin even under osmotic stress. In contrast, the cells expressing DN-MEK demonstrated a redistribution of occludin from the junctions when subjected to osmotic stress (**Figure 4.23**).

Figure 4.22: EGF protects epithelial tight junctions in the mucosa of mouse ileum. Short segments of mouse ileum were pretreated with 30 nM EGF for 50 minutes before being subjected to oxidative stress using 100 μM or 250 μM hydrogen peroxide for one hour. Ileal segments were fixed in OCT and cryosections were labeled for ZO-1 by immunostaining. Fluorescence images were obtained by confocal microscopy. (Data representative of 3 experiments.)

Figure 4.23: Expression of constitutively active MEK attenuates the osmotic stress-induced tight junction disruption in differentiated Caco-2 cell monolayers. 7-day old differentiated Caco-2 cell monolayers expressing empty vector, DN-MEK, WT-MEK or CA-MEK were subjected to osmotic stress by administering 0.35 M mannitol for 3 hours. Cells were fixed and stained for occludin. (DN, Dominant negative; WT-Wild type; CA, Constitutively active; MEK, MAPK-ERK kinase.)

4.6.3 Expression of DN-MEK Prevents the EGF-mediated Protection of Tight Junctions from Osmotic Stress

A previous study had shown that EGF prevents the tight junction disruption induced by osmotic stress in Caco-2 cells. This effect of EGF was shown to be attenuated by MEK inhibitor, U0126 thus indicating a role of ERK in this protective effect.^{76,121,122,126} We examined the effect of EGF on osmotic stress-induced disruption of tight junctions in Caco-2 cell monolayers expressing DN-MEK.

With the help of immunofluorescence studies, we observed that while in cells expressing empty vector, EGF abrogated the osmotic stress induced redistribution of occludin from tight junctions, it failed in producing this protective response in cell monolayers expressing DN-MEK (**Figure 4.24**).

4.6.4 Inference

Inability of the cells transfected empty vector or DN-MEK to withstand osmotic stress, as also the ability of cells expressing WT-MEK or CA-MEK indicate that ERK is involved in protection of tight junctions from osmotic stress-induced disruption. Absence of the protective effect of EGF on tight junctions against osmotic stress in cells expressing DN-MEK indicates that this protective effect of EGF is mediated by ERK.

Figure 4.24: Expression of dominant negative (DN)-MEK prevents the EGF-mediated protection of tight junctions from osmotic stress. 7-day old differentiated Caco-2 cell monolayers expressing empty vector or DN-MEK were subjected to osmotic stress by administering 0.35 M mannitol for 3 hours, in the presence or absence of 30 nM EGF. Cells were fixed and stained for occludin.

CHAPTER 5: DISCUSSION*

ERK has a variable effect on the integrity of epithelial tight junctions; it can disrupt tight junction integrity in some cases^{121,122,125,126} whereas, it can also protect tight junction integrity in other cases.^{94,123} The mechanistic basis for this paradoxical ERK activity in tight junction regulation remains unclear. The literature on the subject provides contradictory information on the subject and thus, only adds to the confusion. The studies done so far to establish the role of ERK have been done in different cell types, and different sets of results have been published, and mechanisms put forward. Unfortunately, the subject has been suffering from inadequate attention of the investigators. No study has been done so far which could shed some light on the variable effects of ERK on regulation of tight junction integrity. Little attention seems to have been paid to the idea that stage of differentiation of cells may be a major factor in how ERK acts in regulation of tight junction integrity. In this study, we have made an attempt to address the controversial issue of differential role of ERK in regulation of tight junctions, and to find out the mechanism behind this enigmatic effect. We hypothesized that the state of cell differentiation is pivotal for ERK-induced differential regulation of tight junction integrity in intestinal epithelial cell monolayers. Towards this end, we show that ERK destabilizes tight junctions in under-differentiated epithelial cells, whereas, it protects tight junction integrity in differentiated epithelial cells. We conclude that differences in the spatial distribution of active-ERK and its influence on distribution of PP2A and PKC ζ contribute to the differential response to ERK in under-differentiated and differentiated Caco-2 cell monolayers.

5.1 Reduced Expression of ERK has Contrasting Effects on Tight Junction Integrity and Assembly in Caco-2 Cell

Our studies involving reduced expression of ERK by use of antisense oligos or long double-stranded siRNA revealed that while knockdown of ERK enhanced tight junction integrity in under-differentiated 3-day Caco-2 cell monolayers, it disrupted the junctions in differentiated 7-day cell monolayers. While the effect of antisense oligos in the cells was short lived (3-4 days) and did not provide an opportunity to compare the effect of ERK-knockdown in under-differentiated and differentiated cells, by using long double-stranded siRNA, we were able to show a significant reduction in ERK levels at both day 4 (under-differentiated) and day 8 (differentiated) post-transfection. Our study shows that knockdown of ERK increases TER and decreases inulin permeability in 3-day under-differentiated cell monolayers, indicating an enhancement of tight junction barrier function. In contrast, TER was reduced and inulin permeability was elevated in 7-day

* Source: Aggarwal,S., Suzuki,T., Taylor,W.L., Bhargava,A., Rao,R. Contrasting effects of ERK on tight junction integrity in differentiated and under-differentiated Caco-2 cell monolayers. *Biochem J.* [Epub ahead of print] (2010).¹⁹² (Adapted with permission.)

differentiated cell monolayers transfected with siRNA for ERK. Junctional distribution of occludin and ZO-1 was enhanced by ERK siRNA, but not control RNA or antisense oligos in 3-day old cell monolayers, whereas, it was reduced in 7-day old cell monolayers. These results demonstrate that ERK exerts disruptive influence on tight junctions in under-differentiated cells whereas, it enhances tight junction integrity in differentiated cell monolayers.

The influence of ERK on assembly of tight junctions in under-differentiated and differentiated cells, as studied by calcium switch method revealed that the calcium-mediated restoration of barrier function was significantly faster in ERK antisense oligo- or siRNA-transfected 3-day under-differentiated cell monolayers compared to that in missense or control siRNA transfected cells. In contrast, reassembly of tight junctions by calcium was delayed by knockdown of ERK in 7-day, differentiated cell monolayers. These observations suggest that ERK activity directly influences the mechanisms involved in tight junction assembly, and this influence is different in differentiated and under-differentiated cells.

These results indicate that the role of ERK in regulation of tight junction integrity and assembly varies with the differentiation status of the cells. Some of the previous studies that showed the disruptive effect of MAPK pathway on tight junctions used under-differentiated cells or the cell types that do not differentiate.^{121,122,125} On the other hand, highly differentiated cells were used when a protective response of MAPK pathway activation was observed on tight junction.⁹⁴ In the present study, we were able to demonstrate these opposing effects of ERK in the same cell type but at different stage of differentiation. Even though the signaling machinery in the cells is too complex to allow us to pinpoint a specific mechanism behind these opposing roles of ERK, it is possible that the target proteins or downstream signaling to ERK pathway may be different at different stages of differentiation.

The use of ERK knockdown as a tool to study its effect on tight junction integrity and assembly had a advantage of being a more specific and reliable method compared to the use of pharmacological inhibitors used in previous studies. Use of pharmacological inhibitors as a tool to study the function of any protein or enzyme has a drawback that the effect of inhibitors depends upon ability to permeate into the cells. Lack of selectivity is another issue with pharmacological inhibitors. Moreover, given the complex nature of signaling cascades in the cells, it is very much possible that a particular protein may be capable of getting activated by more than one pathway, and blocking its activation by inhibiting just one pathway (as is done by pharmacological inhibitors) may not necessarily be a very good strategy to study its role. This study is unique in the sense we have relied on the use of molecular techniques to study the role of ERK. By using antisense oligonucleotides and RNA-interference, we have tried to target the synthesis of ERK rather than its activation the cells. Even though we have used pharmacological inhibitors too, but their use in this study was very limited. This is not to say though that oligonucleotides or siRNA are perfect techniques. Use of oligonucleotides and RNA-interference too is not without its disadvantages like mounting of immune response in the cells, and unintended off-targeting caused by incomplete complementarity.

5.2 ERK Mediates the Contrasting Effects of EGF on Tight Junction Integrity in Differentiated and Under-differentiated Caco-2 Cells

In this study, we have also demonstrated the role of ERK in tight junction integrity in regular under-differentiated and differentiated cells (expressing normal levels of ERK). Our observation of a rapid decrease in TER and increase in inulin permeability by hydrogen peroxide indicated that hydrogen peroxide disrupts epithelial barrier function in both under-differentiated and differentiated Caco-2 cell monolayers. Additionally, hydrogen peroxide treatment resulted in redistribution of occludin and ZO-1 from the intercellular junctions in both under-differentiated and differentiated cell monolayers. Also, pretreatment with EGF attenuated the disruptive effect of hydrogen peroxide on tight junctions in differentiated cells, while EGF potentiated the hydrogen peroxide effect in under-differentiated cells. In both the differentiated and under-differentiated cells, MEK inhibitor U0126 neutralized the effect of EGF thus indicating that this effect is mediated by ERK.

Previous studies have shown that oxidative stress induced by hydrogen peroxide disrupts tight junctions in differentiated epithelial cells.^{56,59,60,91,94,178} Furthermore, EGF, functioning as a gastrointestinal mucosal protective factor attenuates hydrogen peroxide-induced disruption of tight junctions and barrier dysfunction.^{94,123} EGF is known to activate ERK1/2¹⁷⁹⁻¹⁸¹ via MAPK-ERK signaling pathway by activation of GRP, Ras and Raf and MEK.^{182,183} It has been shown to prevent hydrogen peroxide-induced barrier disruption by an ERK-dependent mechanism in Caco-2 cells.⁹⁴ But so far, we have not come across any evidence in the literature about ERK being disruptive to tight junctions in Caco-2 cells. Our findings that activation of MAPK signaling pathway by EGF produces the opposite effect on tight junction integrity clearly indicates that either the target proteins or downstream signaling in the pathway are different in under-differentiated and differentiated cells. These studies had an advantage that we were able to study and demonstrate the effects of oxidative stress and role of MAPK pathway in dealing with this stress in the same cell types but at different stages of differentiation. On the other hand, since we relied on the use of pharmacological inhibitor of MEK, U0126 in this study, it may have been affected by the same disadvantages of pharmacological inhibitors, namely their permeability in the cells and selectivity, as described before. But the fact that we could corroborate these findings in the other studies involving use of molecular techniques makes these results reliable.

5.3 Expression of Constitutively Active MEK and Dominant Negative MEK Produces Contrasting Effects on Tight Junctions in Differentiated and Under-differentiated Caco-2 Cells

Our studies involving use of Tet-regulated expression of MEK (wild type and mutants) in 3-day under-differentiated or 7-day differentiated cells revealed that expression of WT-MEK and CA-MEK on day 3 showed disrupted junctional organization of occludin and ZO-1 compared to that in vector transfected cells, while the expression of DN-MEK enhanced the organization of occludin and ZO-1 at the

intercellular junctions. In contrast, on day 7, expression of WT-MEK and CA-MEK enhanced junctional organization of occludin and ZO-1, and the expression of DN-MEK showed a small, but significant disruption of junctional occludin and ZO-1.

MEK being immediately upstream of ERK in the MAPK signaling pathway,¹⁸⁴ its activation is known to activate ERK. In this study we employed the technique of regulated expression of constitutively active (CA) and dominant negative (DN) mutants of MEK in the cells with the goal of regulating the activation of ERK in the cells. While CA-MEK directly brings about phosphorylation and thus activation of ERK, DN-MEK competes with the innate MEK synthesized in the cells and thus inhibits the activation of ERK. Since continuous expression of CA- or DN-MEK can cause a sustained activation or suppression of ERK in the cells, thus producing several undesirable changes in cell morphology and physiology, this technique of using a regulated expression system had the advantage of ability to turn on or turn off the expression of MEK in the cells, as and when required.

Some studies done previously have tried to establish the role of MAPK pathway in regulation of the expression of tight junction proteins. Studies have showed that Raf1, a signaling molecule upstream of ERK disrupts tight junctions by downregulation of occludin in rat salivary gland epithelial cells.^{126,185} In another study, down-regulation of MAPK pathway in Ras-transformed MDCK cells resulted in restoration of tight junction integrity and barrier function.¹²⁷ In all these studies, the cell lines used were either under-differentiated or the ones which do not differentiate at all. In our studies, we have used Caco-2 cell lines which are considered to be the gold standard for studying tight junctions.¹³⁵ Since these cells differentiate, we could evaluate the role of ERK in both under-differentiated and differentiated cells in the same cell type. Moreover, to our knowledge, this is the first study that has used the regulated expression of MEK and its mutants to study the role of ERK in regulation of tight junction integrity. MEK being immediately upstream of ERK in the signaling pathway gives an added advantage of ruling out the involvement of alternate pathways in activation of ERK, subsequent to mutations in Ras or Raf as was done by others.

Another important point was to see whether the knockdown of ERK or over-expression of MEK and its mutants alters the differentiation status of the cells and thus affects the tight junction integrity. Our ERK knockdown studies revealed that on day 4 post-transfection, the cells with reduced ERK expression demonstrated a significant increase in alkaline phosphatase levels and a slight increase in differentiation marker protein, villin. This indicated that the cells with reduced expression of ERK, in addition to exhibiting enhanced tight junction integrity, were getting better differentiated compared to cells with normal levels of ERK. On the other hand, the cells expressing DN-MEK only exhibited increased tight junction integrity and no effect on their differentiation status on day 4 post-transfection. This effect may be because of the longer duration of action of siRNA in the cells giving it enough time to bring about the changes in differentiation level of the cells after increasing the tight junction integrity. Since DN-MEK was expressed in cells over a shorter period of time (a few hours only), the only effect seen was on tight junction integrity and not on differentiation status of the cells.

These studies indicate that ERK regulates tight junctions first and then influences the differentiation. The effect of ERK knockdown or expression of DN-MEK was not seen on day 8 post-transfection since by this time cells are already differentiated.

While these results are exciting and have revealed a lot about differential role of ERK in regulation of tight junction integrity based on stage of differentiation of the cell, unfortunately, we still are in the dark about the precise mechanism behind it.

5.4 ERK Differently Modulates the Association of PP2A and PKC ζ with Occludin in Differentiated and Under-differentiated Caco-2 Cells

Hydrogen peroxide produced a significant decrease in threonine-phosphorylation of occludin in both 14-day differentiated and 4-day under-differentiated cell monolayers, which was effectively restored by EGF. Attenuation of this effect of EGF by U0126 in 14-day old cell monolayers indicates that ERK is involved in this process. EGF or U0126 did not produce a significant influence on threonine-phosphorylation of occludin in 4-day old cell monolayers, thus showing that under-differentiated and differentiated cells have different signaling mechanisms for ERK.

In differentiated cell monolayers, hydrogen peroxide enhanced co-immunoprecipitation of PP2A with occludin, whereas, it decreased the association of PKC ζ with occludin. This increased association of PP2A and reduced association of PKC ζ may explain the previous observation that hydrogen peroxide rapidly dephosphorylates occludin on Thr residues. In differentiated cells alone, EGF attenuated the hydrogen peroxide induced increase in PP2A association with occludin, and decreases PKC ζ association with occludin by an ERK-dependent mechanism. Such a regulation of PP2A and PKC ζ association with occludin by hydrogen peroxide and EGF was absent in under-differentiated cell monolayers. The effect of EGF on PP2A and PKC ζ redistribution was further confirmed by confocal immunofluorescence localization. Hydrogen peroxide increased the peri-junctional localization of PP2A in both under-differentiated and differentiated cell monolayers, and this effect was abrogated by EGF. In contrast, EGF induced an enhanced localization of PKC ζ at the intercellular junctions by an ERK-dependent mechanism in 14-day differentiated cell monolayers, whereas, such an effect was not seen in 4-day under-differentiated cell monolayers. In case of both PP2A and PKC ζ , the EGF effect was attenuated by MEK-inhibitor U0126.

The mechanism involved in differential response to ERK in under-differentiated and differentiated cell monolayers is unclear. It is likely that the downstream signaling events in response to ERK activation may be different in these cells. But these studies have not been able to conclusively determine what those downstream signaling molecules are. Our previous study showed that EGF prevented hydrogen peroxide-induced dephosphorylation of occludin by an ERK-dependent mechanism.⁹⁴ Occludin is known to be highly phosphorylated on Threonine residues in the epithelium with intact tight junctions,^{102,103,105} while it is dephosphorylated during the disruption of tight junctions by calcium depletion,²⁵ phorbol esters,¹⁸⁶ or acetaldehyde.^{27,156,157} Occludin

phosphorylation appears to be required for the assembly of tight junctions.^{102,103,105} Evidence indicates that PP2A^{59,60} and PKC ζ ^{97,150,153} play roles in the regulation of Thr-phosphorylation of occludin. A recent study demonstrated that hydrogen peroxide-induced occludin dephosphorylation is mediated by PP2A translocation to tight junctions.^{59,60} However, the effect of hydrogen peroxide on PKC ζ association with tight junctions is unknown. This study helped us understand the effect of hydrogen peroxide and EGF on threonine-phosphorylation of occludin and the association of PP2A and PKC ζ with occludin, and their distribution in 4-day under-differentiated and 14-day differentiated cell monolayers.

5.5 Differentiated and Under-differentiated Caco-2 Cells Exhibit Differential Distribution of Active-ERK

Analysis of ERK and p-ERK indicated that much higher level of active ERK is present in under-differentiated cells compared to that in differentiated cell monolayers, although the level of total ERK is greater in differentiated cells. EGF induced a rapid and robust increase in active ERK in differentiated cells. On the other hand, in under-differentiated cells, EGF induced only a slight increase in active ERK, which is already high without EGF stimulation. However, the levels of other signaling molecules like PP2A and PKC ζ or junctional proteins like Claudin-4 did not significantly vary between under-differentiated and differentiated cells. Immunofluorescence microscopy, revealed that active ERK in under-differentiated cells is distributed in the intracellular compartment while significant amount of active ERK is localized at the peri-junctional region in differentiated cell monolayers.

This differential distribution of active-ERK observed in under-differentiated and differentiated cells is a novel finding and has not been reported before. Therefore, the differences in sub-cellular distribution of activated ERK may contribute to different downstream signaling events in under-differentiated and differentiated cells. The differential distribution of p-ERK may be responsible for its distinct effects on PP2A and PKC ζ in differentiated and under-differentiated cells.

5.6 ERK Does Not Directly Phosphorylate Occludin, but Phosphorylates PKC ζ , Which in Turn Phosphorylates Occludin

Our *in vitro* studies using recombinant ERK and C-terminal 150 amino acid tail of occludin demonstrated that ERK does not directly phosphorylate occludin on serine or threonine residues. It was also seen that PKC ζ , another serine threonine kinase was directly phosphorylating occludin on threonine residues. ERK causes threonine phosphorylation of PKC ζ *in vitro*. However, ERK was not seen to phosphorylate PP2A *in vitro* since PP2A gets activated by phosphorylation on tyrosine residues. Rather, PP2A was seen to result in dephosphorylation of active or phospho-ERK, thus further confirming our previous observation of interplay among PP2A, PKC ζ and ERK in regulation of tight junctions.

As described before, phosphorylation of occludin and its dephosphorylation on serine and threonine residues is important for regulation of tight junction integrity. ERK has been shown to influence the threonine-phosphorylation of occludin.⁹⁴ PP2A is known to affect the tight junction integrity by causing dephosphorylation of occludin.^{59,60} But it is not known whether ERK directly brings about the phosphorylation of ERK or if it does so through another serine-threonine kinase. Even though PKC η ¹⁸⁷ has been reported to influence tight junction integrity by regulating occludin phosphorylation, not much known about the role of PKC ζ in occludin phosphorylation. In this study, we have established that PKC ζ phosphorylates occludin on threonine residues, at least *in vitro*, and ERK influences this process by regulating the phosphorylation of PKC ζ . The previous studies which looked at the role of PP2A in regulation of tight junction integrity only focused on its influence on occludin phosphorylation. Our study was a step further in determining the role of ERK in influencing association of PP2A with occludin and its localization at tight junctions. Once again, the unambiguous results about the association of PP2A with occludin and its junctional localization in response to oxidative stress and EGF treatment, in differentiated cells, while no significant differences seen in under-differentiated cells point to the differences in the target proteins and/or downstream signaling of ERK.

5.7 EGF, Similar to Its Effect on Tight Junctions in Differentiated Cells, Protects the Tight Junctions in Mouse Ileal Epithelium

All the studies we had conducted so far were either *in vitro* or were done on the cells. To substantiate our findings from studies in cultured cells or *in vitro* studies, we studied the effect of hydrogen peroxide and EGF *ex vivo* on mouse ileal epithelial tight junctions. Hydrogen peroxide was observed to be disruptive to junctions in a dose dependent manner, and EGF pretreatment was protective to junctions. This effect is similar to what we have demonstrated in differentiated Caco-2 cells. The possible reason for this finding is that the epithelial cells present at the villi of intestinal mucosa are differentiated and EGF protects the junctions there. Even though the cells in crypts are under-differentiated, we did not see, on immunostaining, the EGF effect similar to what was seen in under-differentiated Caco-2 cells. On immunoblotting of detergent-soluble and insoluble fractions made from mucosal scrapings, occludin was seen to be redistributed from detergent-insoluble fraction and EGF pretreatment effectively attenuated this redistribution. This effect was similar to what would be seen in differentiated cells. Since it was not possible to isolate just the crypts and study occludin localization there, we could not study the effect of hydrogen peroxide and EGF in crypt cells.

CHAPTER 6: SUMMARY AND CONCLUSION

These studies demonstrate that ERK has a disruptive influence on tight junctions in under-differentiated Caco-2 cells, while it has a protective role on tight junctions in differentiated cell monolayers. This difference in response may be caused by differences in ERK distribution and its downstream signaling leading to a difference in its influence on PP2A and PKC ζ association with tight junction proteins. While it is interesting to understand the role of ERK in regulation of tight junctions is dependent upon the stage of differentiation of the cells, it was much more perplexing as to what exactly makes ERK behave differently in under-differentiated and differentiated cells. Even though there is a complex interplay of various tight junction proteins like occludin, ZO-1, claudins etc., and signaling molecules that regulate the tight junction integrity in cells; the same molecules appear to be responsible for the regulation irrespective of the stage of differentiation of cells. This observation makes the whole story even more interesting and complicated. While occludin continues to be the major structural component of tight junctions, its phosphorylation or dephosphorylation at serine/threonine or tyrosine residues is an important factor in regulation of tight junction integrity. ERK is a serine-threonine kinase and our observations indicate it does not directly phosphorylate occludin on serine or threonine. That brings into the picture other signaling molecules, PKC ζ and PP2A. We have demonstrated that ERK phosphorylates PKC ζ , and enhances its localization at tight junctions in differentiated cells but not in under-differentiated cells. Similarly, we have shown that ERK is responsible for EGF mediated protection of tight junctions from hydrogen peroxide-induced oxidative stress. This effect is produced by reducing localization of PP2A and its association with occludin at tight junctions in differentiated cells. ERK, however, does not seem to influence this co-localization and association of occludin and PP2A in under-differentiated cells. While this study has been able to address the question of the differential role of ERK in regulation of tight junctions in differentiated and under-differentiated cells, it has left several questions unanswered. Some of those are:

1. What is the exact mechanism behind the differential influence of ERK on co-localization and association of PKC ζ and PP2A with occludin?
2. What are the other players involved in the process?
3. At what point of time during cell's maturation/differentiation, does the turnaround occur when ERK turns from disruptive to protective?
4. What is the signal or stimulus that brings about this change?
5. What is happening downstream of ERK in the signaling pathway and how is it different between differentiated and under-differentiated cells?

These questions pose a formidable challenge, but at the same time, can open up a whole new area of research that may attract the attention of future investigators. At this

time, we can only suggest that there may be differences in how various components of numerous signaling cascades are interlinked in differentiated and under-differentiated cells. Our surmise is that ERK is part of such a signaling cascade that changes its connections while cells are growing and differentiating. Thus, while being a part of one pathway in under-differentiated cells, it behaves in one manner, and when the cells are differentiated, some or all of its downstream signaling may change so as to produce the effect seen in differentiated cells. A considerable amount of work may be required before this mystery can be unraveled. The immediate next logical step would be to look for the signaling molecules downstream of ERK through which ERK produces its effects.

This study has a potential for future application in the field of cancer metastasis. It is known that cancer cells lose their ability to differentiate, and are highly under-differentiated. It is also known that tight junctions act as a significant barrier against metastasis of cancer, particularly those of gastrointestinal tract. These cancers spread after disrupting this barrier. Our studies demonstrate that when ERK expression is inhibited or reduced in under-differentiated cells, they form stronger tight junctions. Thus this study may hold a promise for halting the spread of numerous cancers if methods are devised to achieve a targeted inhibition or reduction of ERK expression in those cells. One possible immediate future study would be to determine whether the effect of ERK signaling on tight junctions is reversed by de-differentiation of cells during carcinogenesis.

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