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Role of Translation Initiation in Regulation of Epithelial Junctions and Cell Motility

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science
at Virginia Commonwealth University

By

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LIST OF ABBREVIATIONS AND SYMBOLS

α : Alpha

β : Beta

γ : Gamma

AJ: Adherens Junction

TJ: Tight Junctions

AJC: Apical Junction Complex

ZO: Zonula occluden family

JAM-A: Junctional Adhesion Molecule-A

SM-22: Smooth muscle marker 22

α -SMA: Alpha smooth muscle actin

EMT: Epithelial Mesenchymal Transition

TGF- β : Transforming growth factor β

IFN γ : Interferon Gamma

TNF α : Tumor Necrosis Factor Alpha

HIEC: Human small intestinal epithelial cell

ROS: Reactive Oxygen Species

IL: Interleukin

MAGUK: Membrane-Associated Guanylate Kinase

ECM: Extra Cellular Matrix

MAPK: Mitogen-Activated Protein Kinase

mTOR: Mechanistic Target of Rapamycin

Met-tRNA^{iMet}: methionyl-initiator tRNA

eIF: Eukaryotic translation Initiation Factors

eIF4F: Eukaryotic Translation Initiation Factor 4 F

eIF4G: Eukaryotic Translation Initiation Factor 4 G

eIF4E: Eukaryotic Translation Initiation Factor 4 E

4EGI-1: eIF4E/eIF4G interaction inhibitor 1

PIC: 43S preinitiation complex

PABP: Poly (A) Binding Protein

tRNA: Transfer RNA

RNAi: RNA Interference

MNKs: MAPK-interacting Kinase

IBD: inflammatory bowel disease

CD: Crohn's diseases

TEER: Transepithelial Electrical Resistance

CAR: coxsackievirus and adenovirus receptor

TAMP: tight junction-associated MARVEL proteins

IBC: Inflammatory Breast Cancer

NPC: Nasopharyngeal Carcinoma

IRES: Internal Ribosomal Entry Site

CRISPR/CAS9: Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR associated.

FAK: Focal Adhesion Kinase

NSCLC: Non-Small Cell Lung Cancer

EMT: Epithelial–Mesenchymal Transition

TGFB: Transforming Growth Factor Beta

4EGI-1: eIF4E/eIF4G interaction inhibitor 1

CSCs: Cancer Stem Cells

RNAi : RNA interference

qRT-PCR: quantitative Real Time-PCR

PVDF: Polyvinylidene Difluoride

SDS-PAGE: Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis

TBS-T: Tris-Buffered Saline-Tween-20

DTT: Dithiothreitol

FA: Focal Adhesions

Abstract

Role of Translation Initiation in Regulation of Epithelial Junctions and Cell Motility

By Fahda Fawaz Alsharief, Master of Science

This thesis is submitted in partial fulfillment of the requirements for the Master of Science degree at Virginia Commonwealth University.

Virginia Commonwealth University, 2017

Thesis Director: Dr. Andrei Ivanov, Professor, Department of Human and Molecular Genetics

The integrity and barrier properties of intestinal epithelium are determined by specialized adhesive structures known as intercellular junctions; composed of adherens junctions (AJs), tight junctions (TJs) and focal adhesions that mediate cell-cell and cell matrix interactions, respectively. These two types of epithelial cell adhesions regulate each other during disruption and restitution of the epithelial barrier. Inflammatory cytokines such as interferon gamma ($IFN\gamma$) and tumor necrosis factor alpha ($TNF\alpha$) are elevated during intestinal inflammation. The most notable effects of $IFN\gamma$ and $TNF\alpha$ on intestinal epithelial homeostasis involve disruption of apical junctions and attenuation of cell migration. Although molecular mechanisms underlying these effects remain poorly understood, expressional downregulation of different adhesion proteins may play a major role in the cytokine-dependent disruption of the intestinal epithelial barriers. This thesis is based on the hypothesis that inhibition of the protein translation initiation machinery promotes the disruption of the intestinal epithelial barrier and attenuates epithelial restitution during mucosal inflammation. This study was focused on two eukaryotic translation initiation factors, eIF4G1 and eIF4G2, which play essential roles in the regulation of cap-dependent protein translation. Expression of both translation initiation factors was dramatically downregulated in model intestinal epithelial cell monolayers treated with $IFN\gamma$ and $TNF\alpha$ in parallel to cytokine-induced disruption of the epithelial barrier. siRNA or shRNA-mediated downregulation of either eIF4G1, or eIF4G2 increased permeability of well-differentiated SK-CO15 intestinal epithelial cell monolayers and decreased expression of different adherens junction and tight junction proteins. Furthermore depletion of these translation initiating factors inhibits different modes of migration (wound healing and transfilter migration) of stem-cell like

and well-differentiated intestinal epithelial cells. These findings suggest that eukaryotic translation initiation factors of the eIF4G family play unique roles in regulating integrity and restitution of the intestinal epithelial barrier. Downregulation of these translation initiating factors may mediate disruption of the intestinal epithelial barriers during mucosal inflammation.

1. INTRODUCTION

1.1 Role of Intracellular Junctions

Intercellular junction plays a crucial role by forming the barriers and keeping the tissue integrity in a multicellular organism. Epithelial barriers protect the body interior of mammalian species from external environments, and working as defending line between invading pathogens and the immune system of the host. This is true in the gastrointestinal tract, especially in the intestines, where the epithelial barrier plays a vital role in transporting nutrients and water, and preventing contamination which results from microbial intestinal tissues [5, 6]. As mentioned before the epithelial barrier is formed by intercellular junctions which are created by plasma membrane. Intercellular junctions are composed of scaffolding and adhesive proteins that are attached to different types of cytoskeletal structures such as microtubules, actin filaments, and intermediate filaments. These intercellular adhesive structures include various types of junctions: Tight Junctions (TJ), Adherens Junctions (AJ), Gap Junctions (GJ), and Desmosomes [7]. AJs, TJs, and desmosome are the more abundant junctions present in the apical side of the epithelia; these together form what is called apical junction complex (AJC) [7, 8]. The AJC maintains the characteristic of the epithelia by controlling permeability, polarity, adhesion and cell growth [9]. Both TJ and AJ are composed of transmembrane proteins and peripheral membrane proteins. Transmembrane proteins involve claudins, occludin, JAM-A, cadherin, and nectin, which are involved in homotypical trans-interaction. Whereas, peripheral membrane proteins ‘cytosolic plaque’ consists of the catenin, afadin and “zonula occludens” (ZO) family of proteins which stabilize the adhesive component of the AJC [7, 10]. Epithelial barrier is disrupted during the acute inflammatory phase thus amplifying inflammation, while the barrier is restored during the remission step, which helps to reduce inflammatory events [7]. The invading pathogenic microorganisms release a variety of agents which disrupt the epithelial barriers on the apical side of epithelial layers, such as, bacterial lipopolysaccharide, poreforming toxins, and cytoskeleton modifying proteins. However, on basal side, the barrier disruption will be increased by moving

activated immune cells to sites of pathogen invasion. The epithelial permeability will be increased by releasing reactive oxygen species (ROS) and proteases, or by secreting proinflammatory cytokines—such as, tumor necrosis factor (TNF), interleukin (IL), and interferon (IFN)—through mucosal immune cells. Increasing the permeability of the epithelial barrier is well-documented in different immune diseases and gastrointestinal diseases, such as inflammatory bowel disease (IBD), irritable bowel syndrome, gastric ulcer, celiac disease, food allergies, asthma, sepsis, cancer (esophagus, and colorectal) and type 1 diabetes [11, 12]. Accordingly, understanding the mechanisms that control the disruption of epithelial barrier is the main key to investigate molecular targets of mucosal inflammation.

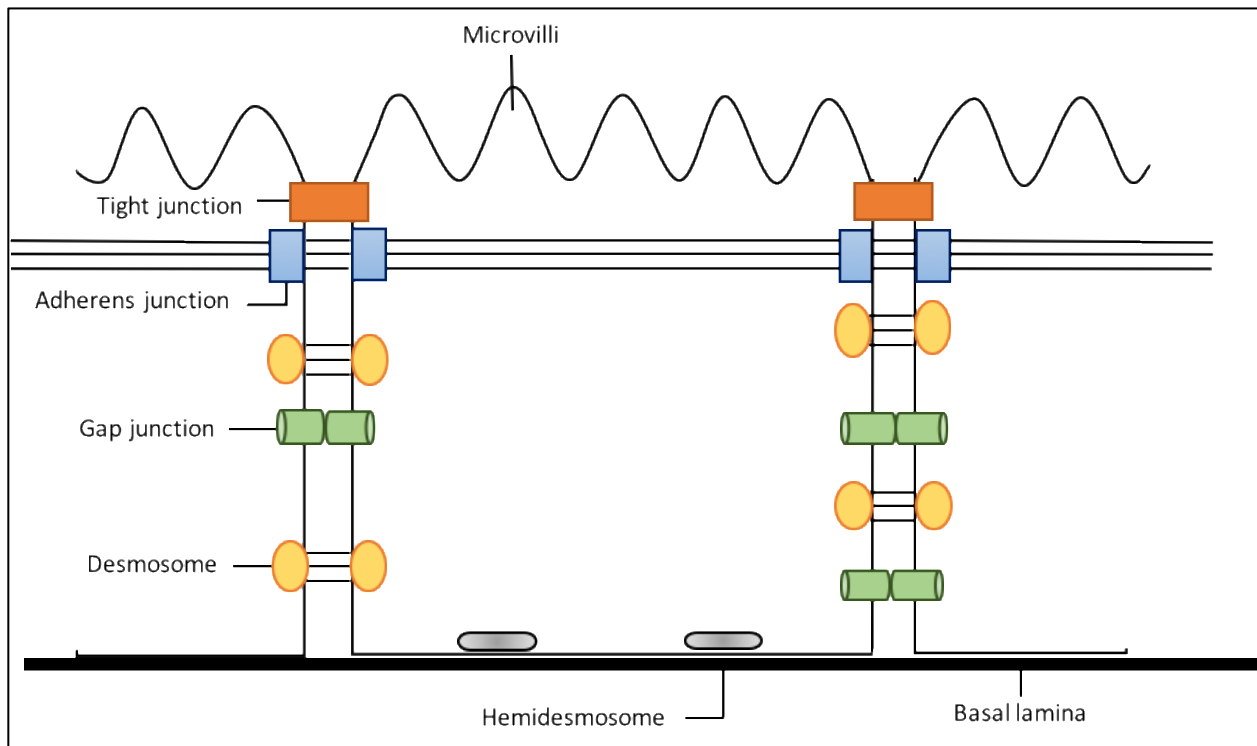


Fig 1.1: - Important Components of Intercellular Junctions in Mammalian Cells. Intercellular adhesions are mediated by specialized plasma membrane structures termed junctions; TJ, AJ, GJ and, Desmosomes. Adapted from Tsukita S. Nat Rev Mol Cell Biol, 2001

1.2 Molecular Compositions of Epithelial Junctions

1.2.1 Tight Junction

TJs are present in all types of epithelial cells, involving epithelial and endothelial cells. They are placed next to AJs in the most apical part of junctional complexes of epithelial cells [13]. Under the electron microscopy, TJs appear as zones of adjacent intercellular contacts sealing the paracellular space [11]. The TJs play the key role in regulating the movement of ions and solutes intercellularly and intracellularly [10]. They also establish the apico-basal cell polarity, and create the paracellular barrier [11]. Permeability and permselectivity are the main functions of TJs which can be determined in the lab by specific experiments. Permeability is a quantitative characteristic of TJs which can be measured by transepithelial electrical resistance (TEER), while the permselectivity is a qualitative feature of TJs which determines the transports of either cations or anions. For example, gastrointestinal epithelia classified depending on their permeability on three main categories: leaky, moderately, and tight. Leaky epithelium, which presents in the small intestine, has TEER under 200 W cm². Moderately leaky epithelium, which found in colon, has TEER between 300-1000 W cm². Whereas, tight epithelium barrier has TEER above 1000 W cm and presents esophagus. To study the regulation of TJs in vitro, human colonic carcinoma derived epithelial cell lines can be used. For instance, Caco 2 cells can be used to study the moderately leaky, while T84 and SK CO15 have been used to study tight barriers [11].

As many intercellular junctions, the molecular composition of TJs have been identified as transmembrane proteins (integral membrane proteins), and peripheral membrane proteins (cytoplasmic scaffolding plaque). The transmembrane proteins, which mediate cell-cell adhesions and create the paracellular barrier, consist of major types of integral proteins: claudin family; tight junction-associated MARVEL proteins (TAMP) family which includes occludin, tricellulin, and marvelD3; and immunoglobulin-like proteins such as junctional adhesion molecule (JAM)-A and coxsackievirus and adenovirus receptor (CAR)[11].

Occludin and claudin are considered as a core of TJ and regulate the permeability and selectivity of paracellular pathway of ions between adhering cells [10, 11, 13]. The peripheral membrane proteins, which form cytosolic scaffolding plaque and regulate TJ stability and remodeling, have five molecular component proteins: Zonula occluden family (ZO-1, ZO-2, ZO-3), cingulin, and afadin. Zonula occluden family is considered as prototypical components of the cytosolic plaque of TJ and has membrane-associated guanylate kinase (MAGUK) which binds to C-terminal of JAM-A, claudin, coxsackievirus and adenovirus receptor (CAR), and occludin by PDZ and SH3 domains [10, 11, 13]. Zonula occluden family and claudin are essential components to form TJ strands in epithelial cells, while other transmembrane proteins such as occludin help to regulate TJ strand formation with claudins [11, 13].

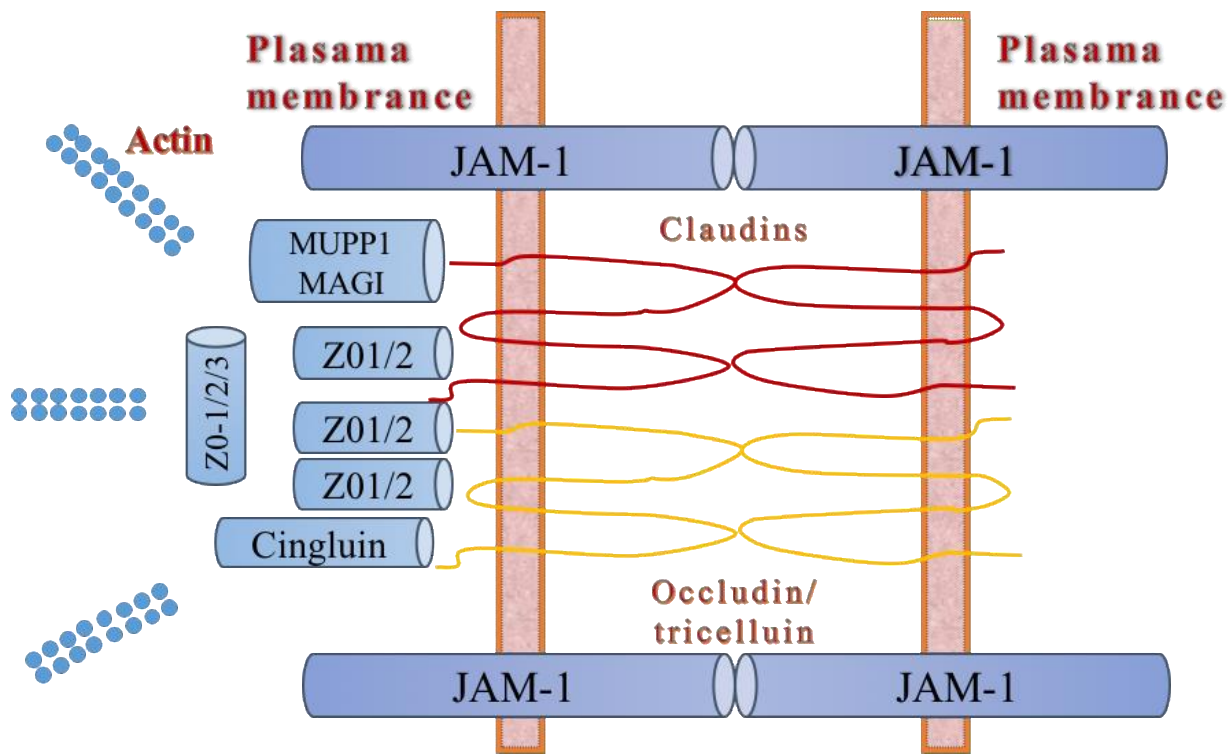


Fig 1.2: - Molecular Composition of Tight Junctions. TJ transmembrane proteins (claudins, occludin, JAM-A), cytosolic plaque proteins (ZO proteins, cingulin, MAGI), they associate with underlying actin filaments. Adapted from **Journal of Investigative Dermatology** Volume 127, Issue 11, [2]

1.2.2 Adherens Junction

Adherens junctions establish cell-cell contacts, regulate the maturation, and maintain the connecting network[10]. Their proteins perform several functions: they control the formation and assembly of tight junctions, which seal the paracellular space [14]; they regulate the initiation of intracellular signaling, membrane receptor, transcription, oncoproteins, and the actin cytoskeleton; and they stabilize the cell-cell adhesion [10, 15]. The complex of adherens junctions are composed of the transmembrane proteins cadherin and nectin, which interact with adjacent cells and with intracellular cytosolic plaque, such as catenin and afadin. Cadherin and nectin are the core of transmembrane protein of epithelial adherens junction [7, 10, 16]. Together, these proteins regulate the function of adherens junctions [10, 17]. Cadherin is considered as the large superfamily of calcium-dependent adhesion cell molecules, which contains multiple copies of extracellular cadherin domain (EC) in the extracellular region. This EC initiates trans-cadherin interactions between adjacent cells and helps to form the adherens junctions [10]. Cadherins regulate the cell-cell interaction and cell movement and maintain the tissue morphogenesis to keep the cell architecture in the adult. Also, they play a significant role in the development by controlling the cell segregation and formation of the interfaces in distinct tissue [16, 18, 19]. Increasing invasive phenotype of tumor cells is often correlated to loss or downregulation of cadherins [18]. E-cadherin is the main transmembrane glycoproteins and one of Ca^{2+} dependent adhesion proteins [7, 10]. It is necessary for localization and binding to catenins which is the part of cytoplasmic proteins that connect the cadherin proteins to actin cytoskeletons[10]. The cytosolic plaque of the AJ is composed of two armadillo family proteins: catenin and afadin [7]. The Catenin family involves alpha-catenin, beta-catenin, and p120-catenin. These cytosolic plaque proteins regulate local actin assembly by interacting with intercellular domains of E-cadherin through beta-catenin, which binds directly to alpha catenin and p120 catenin [14]. The decreasing in the expression of E-cadherin is responsible for incorrect localization of catenins and other TJ proteins [7, 10].

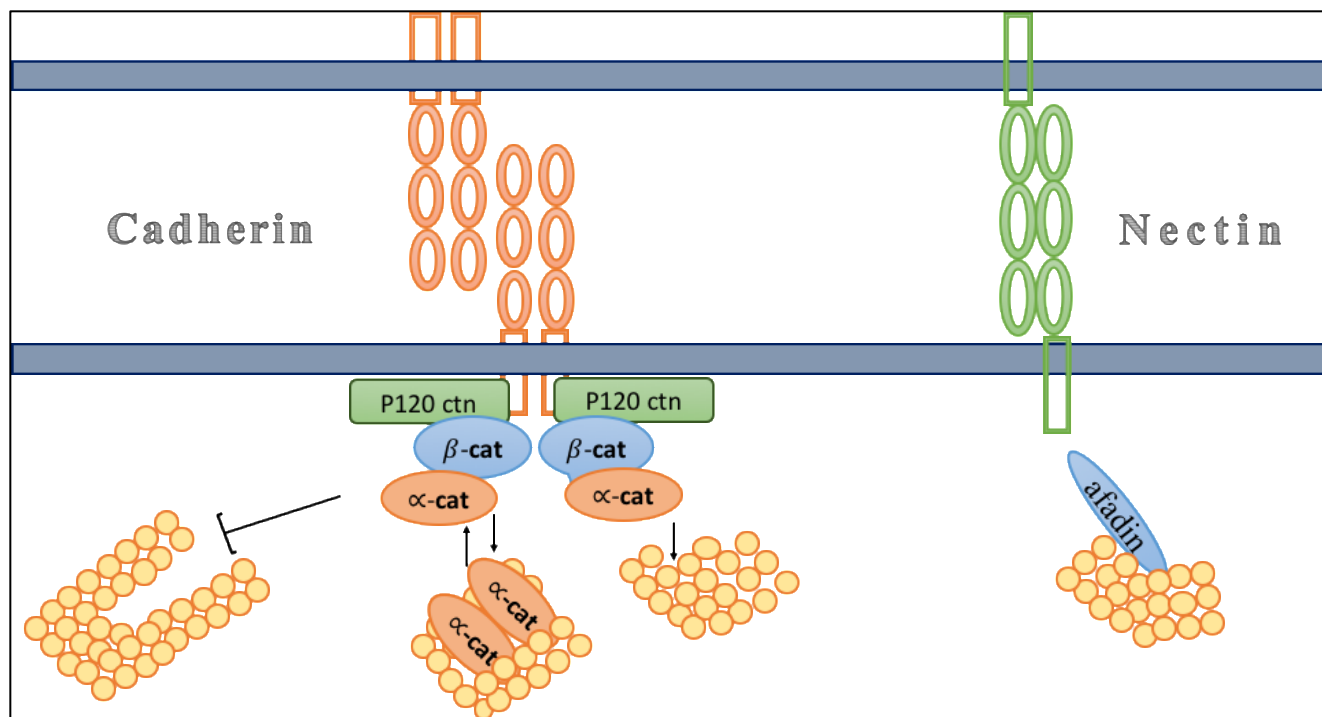


Fig 1-3: - Molecular Composition of Adherens Junctions. AJ formed by transmembrane proteins (cadherins, nectins), cytosolic plaque proteins (catenins, afadin), they associate with underlying actin filaments. Adapted from **Journal of Investigative Dermatology** Volume 127, Issue 11, [2]

1.3 Basic Mechanism of Cell Motility

Cell motility, a complex and highly integrated process, is essential for multiple biological processes, such as the development of organisms (morphogenesis), neurogenesis, wound healing, and immune response. Also, it drives the disease progression in cancer metastasis, mental retardation, arthritis, and atherosclerosis [20, 21]. The mode of the cell migration, which determines if the cell migrates individually (amoeboid or mesenchymal) or collectively (migration of cohesive multicellular units) depends on cytoskeleton organization, type of cell-matrix interaction and force generation, and modification of tissue structure imposed by migrating cells[22]. These two modes of cell motility are known as Collective Cell Migration and Individual cell migration respectively. The first mode of the cell migration is when the cells move collectively in a sheet like structure, in the presence of cell-cell junctions; this type of movement is observed during wound healing in epithelial cells. In contrast, another type of cell motility is Individual Migration, which happens by separation of cells from each other and move as a single cell by suppression of intercellular junctions and downregulation of cadherins. In the case of individual cell migration, there are two main types of the cell morphology, which use the different mechanism of migration. The first one is elongated, fibroblast-like cells, which undergo mesenchymal migration. The mesenchymal migration of the elongated morphology cells depends on integrin-mediated adhesion with substrate and degradation of extra cellular matrix (ECM) by the matrix-degrading protease which presents in the leading edge of migrating cell. The second type of the individual cell morphology is rounded cells which undergo amoeboid migration. Because the cell- substrate adhesion is weak, cells appear as a rounded morphology. Rounded cells move by amoeboid migration in an integrin-independent adhesion, and they do not require the ECM degradation. By changing the shape and squeezing themselves through the gap in ECM, the rounded cells can migrate through it. Thus, the transition from collective migration to individual migration is called the Epithelial-Mesenchymal transition. The basic cell motility depends on the reorganization of actin cytoskeleton and consists of four main processes: protrusion, adhesion, translocation, and retraction. First, cells motility is triggered by external or internal signaling which interacts with the extracellular receptors and activates the downstream signaling. The stimulated asymmetric cells and de novo actin filaments polarization occur at the leading edge. Then, the formation of membrane protrusion such as filopodia and lamellipodia

occurs by polarized actin filaments which push against the cell membrane. Secondly, this protruded membrane attaches to ECM and initiates the novel integrin dependent cell substrate adhesion formation at the leading edge. The adhesion complexes which recently formed are stabilized and mature into focal adhesion. Thirdly, translocation of the nucleus and cell body activated by acto-myosin contractile forces distort the cells into an elongated shape. Finally, disassembly of focal adhesion and release of cell-substrate rear adhesion allows the tail retraction [1, 23-27].

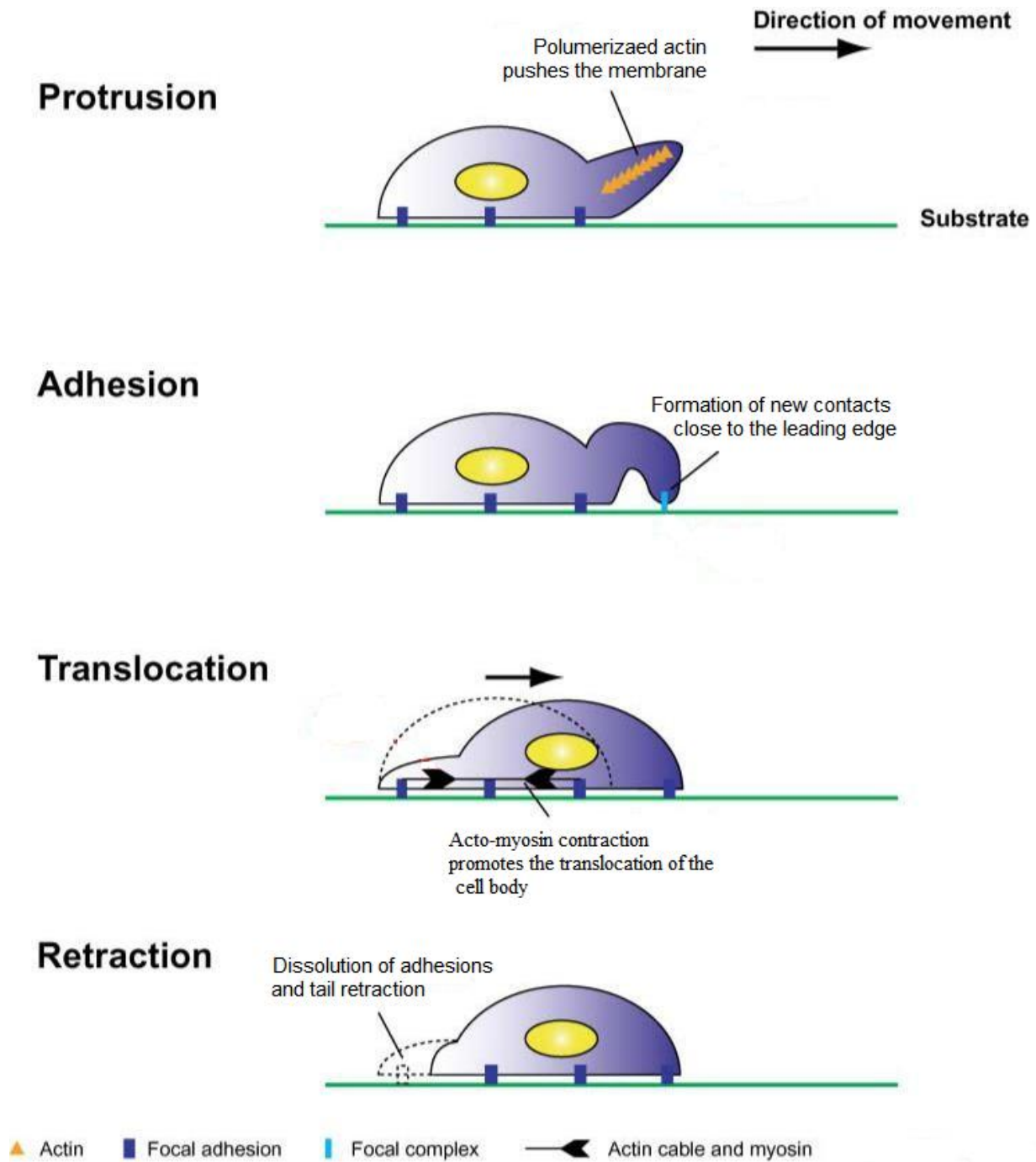


Fig 1-4: - Basic Cycle of Cell Motility. [1] *Cancer Science* Volume 96, Issue 7, pages 379-386, 29 JUL 2005 DOI:10.1111/j.1349-7006.2005.00062.x

1.4 Mechanism of Eukaryotic Translation

Translation of mRNA into protein is one of the multiple levels of regulated gene expression. By allowing more rapid changes in the cellular concentration of encoded protein, the translation process maintains the homeostasis in cell physiology and fate. The process of translation is composed of four main steps: initiation, elongation, termination, and ribosomal recycling. Multiple different protein factors involved in the translation mechanism interact with mRNA, transfer RNA (tRNA), and 40S ribosome, where the codon is located, and 60S ribosome. These interactions promote each stage of the translation process and ensure a mRNA is accurately translated into protein. Generally, during the initiation stage, the elongation-component 80S ribosome is formed by recruiting the mRNA to the 40S ribosome and the 60S ribosome. In the elongation step, the information in each nucleotide triplet (or codon) will be translated into amino acids by migrating 80S ribosome along the mRNA. These translated amino acids will be incorporated into a growing polypeptide chain. During the termination, the newly formed protein is released by recognizing the stop codon. Lastly, in ribosome recycling, the mRNA and 80S ribosome is released. The 80S ribosome will be separated into its original components: the 40S and 60S subunits, which can then begin the translation cycle again [28, 29].

1.4.1 Eukaryotic Translation Initiation Step

Translation initiation is considered as the rate-limiting step of protein synthesis, and as such, it is the most highly regulated phase of translation [28-30]. The variance of the initiation rates can be due to differences in regulatory features of mRNA, such as highly structured 5' un-translated region (UTR). Another variance could be the regulation of initiation factors by signaling cascades, such as mitogen-activated protein kinase (MAPK) pathway and mechanistic target of rapamycin (mTOR). Together, these variances have an effect on availability and activity of factors which alter the rate of translation [28]. Compared to prokaryotes, the initiation of protein synthesis in eukaryotes consists of the recruitment of a ribosome initiator tRNA complex to mRNA initiation codon. Whereas, in prokaryotes, this process occurs by direct interaction of ribosomal RNA with the mRNA. Eukaryotes have a highly regulated mechanism that relies on the interaction between the proteins and between protein and the mRNA.

As a result, the eukaryotic translation initiation has at least eleven different initiation factors which are required for translation initiation to occur accurately. Together these factors ensure that the methionyl-initiator tRNA (Met-tRNA^{iMet}), which is placed in the ribosomal P site, is base paired with the initiator AUG codon of an mRNA. Conceptually, the eukaryotic initiation process, which leads to formation of 80S ribosome, consists of multiple linked steps which are mediated by eukaryotic translation initiation factors (eIF). This process can be divided into five main stages: First, the eIF4F cap-binding complex binds to mRNA; second, a formation of the 43S preinitiation complex (PIC); third, recruitment of mRNA to the ribosome; fourth, localization of in initiation codon; finally, 80S ribosome will be formed by joining of a 60S ribosome and 40S ribosome. First, to recruit the target mRNA to the 40S subunit, the mRNA should be prepared by recognizing and binding to eIF4F complex and PABP. The eIF4F complex is composed of: three main subunits (eIF4E, eIF4A, eIF4G); and eIF4B as an auxiliary factor. Together these subunits promote unwinding of secondary structure in 5' UTR by eIF4A. The 40S subunit is prepared to recruit to mRNA by several eIF factors which bind individually, or as a multifactor complex. These binding factors include: eIF1, eIF1A, eIF2, eIF3, and eIF5. The eIF2 binds as a ternary complex which includes eIF2-TC with GTP and the initiator tRNA. Through direct interaction between eIF4G and eIF3, mRNA is directed to the ribosome. Following the step of recruitment, 40S subunit begins scanning for initiation codon by traveling along mRNA in 5' to 3' direction, and sampling codons on the mRNA with initiator tRNA anticodon. After recognition of initiation codon by base-pairing, GTP in eIF2-TC is hydrolyzed by eIF5. The eIF2-GDP complex and other initiation factors from 40S are released. By eIF2B, eIF2 recharges with GTP, and new initiator tRNA is recruited to reform the eIF2-TC. Now, the initiation factors are free to begin the new round of translation on different mRNA. Until eIF5B-GTP recruits and binds 60S subunits, eIF1A interacts with the 40S subunit. In the end, both of eIF1A and eIF5B-GTP are released following the GTP hydrolysis. Lastly, the newly 80S ribosome is formed and then enters the elongation cycle, which is followed by the termination and the recycling steps which results in the release of free 40S and 60S subunits. These released subunits are prepared for future rounds of translation [28, 29, 31, 32].

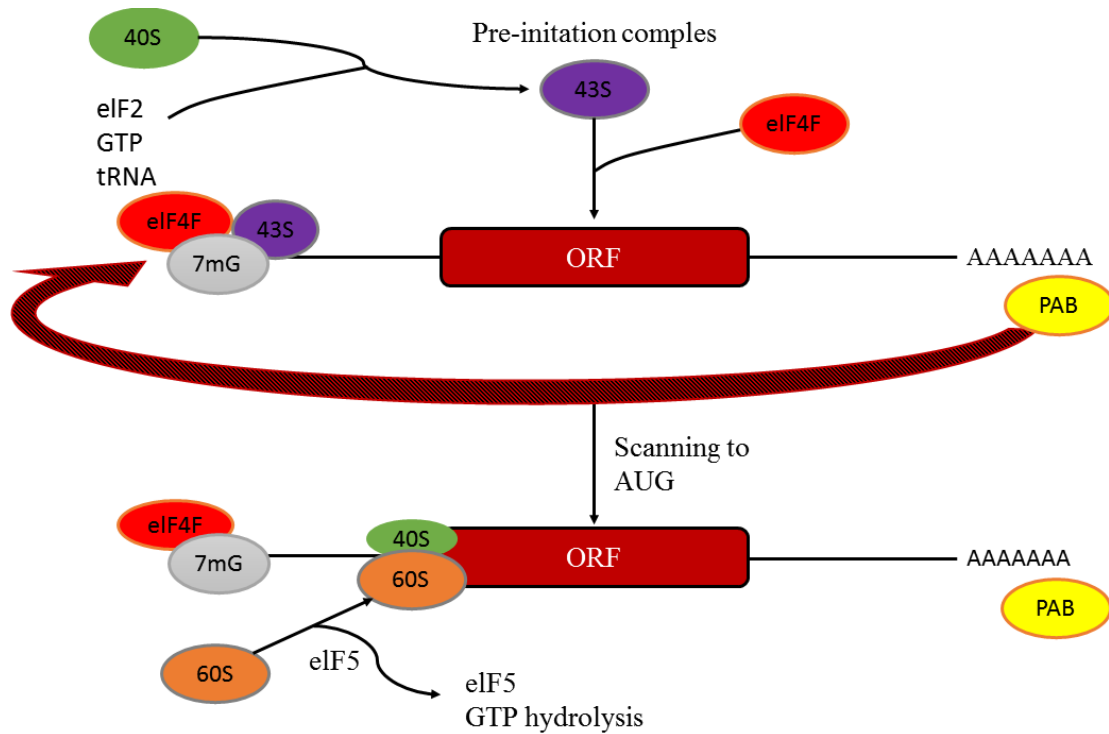


Fig 1.5: - Translation Initiation Mechanism. To form the 43S pre-initiation complex (purple), the 40S ribosomal subunit (green) interacts with eukaryotic initiation factor 2 (eIF2), GTP and initiator methionyl tRNA. The eIF4F complex (red), which composed of eIF4E, eIF4G, eIF4A, and eIF4B as an auxiliary factor. Together these factors bind with 43S, is then recruited to the 5' cap. Poly(A) binding protein (PAB; yellow) bound to 3' end of mRNA poly(A) tail in order to facilitate translation initiation by communicating with the 5' end of the mRNA through its interaction with eIF4G, as a result, the 5' and 3' ends of the mRNA are joined. Then, the 43S complex scans the mRNA until the AUG codon is recognized. This stimulates eIF5 to hydrolyze GTP, as a result of GTP hydrolysis, the eIFs dissociate and the 60S subunit joins. In the end, a fully functional ribosome is ready to begin peptide synthesis. Adapted from Nat Rev Genet. 2003 Aug;4(8):626-37.[4]

1.4.2 Importance of eIF4G Family in Translation Initiation Mechanism

There are two fundamental features in which the mRNA is selected for translation: 7'-methyl-guanosine cap binds to 5' end of mRNA; and poly(A) tail is added to 3' end of mRNA. These two characteristics are protecting mRNA from the degradation and are promoting the translation initiation. The eukaryotic translation initiation factor 4F (eIF4F) is a heterotrimeric factor, which is composed of three subunits eIF4E, eIF4G, and eIF4A. These subunits work as cap binding, molecular scaffold, and DEAD box helicase (ATP-dependent RNA helicase), respectively. Also, it interacts with 7' methyl-guanosine, and 3' poly (A) tail directly or indirectly. The fundamental role of eIF4F is to place in 5' end of mRNA through eIF4E (cap recognition) and to ensure the 5' end of mRNA will bind to the 40S ribosome. The eIF4G is considered as the largest and core component of eIF4F and is sufficient for efficient ribosomal attachment to capped mRNA. The eIF4G contains the binding domains for mRNA, eIF4A, eIF4E, eIF3, poly(A) binding protein (PABP), and MAPK-interacting kinase (MNKs). By these binding domains, eIF4G works as molecular scaffolding which regulates and recruits the activation of other eukaryotic translation initiation factors. It also increases the affinity of the interaction between 5' mRNA cap structure to eIF4E, and 3' mRNA poly(A) tail to PABP. The eIF4G promotes the ability of eIF4A to stimulate the translation by recruiting eIF4A and activating its ATP-dependent RNA helicase activity. Beside the stabilizing the affinity, eIF4G has two additional essential functions in mRNA recruitment to the ribosome. This recruitment occurs through interaction between eIF4G and eIF3. Additionally, eIF4G works as the bridge in 43S PIC and eIF4F-mRNA complex by interacting with eIF3. The eIF4G interacts with eIF4A (DEAD box helicase) and holds its active confirmation; this interaction is required to recruit this helicase to mRNA. DEAD box helicase facilitates the ribosome recruitment and scanning through unwind secondary structure of mRNA located in 5' UTR in an ATP-dependent manner. It is believed that the eIF4G directly recruits the 43S PIC through the physical interactions with eIF3 and eIF5 in the PIC [28, 32-36].

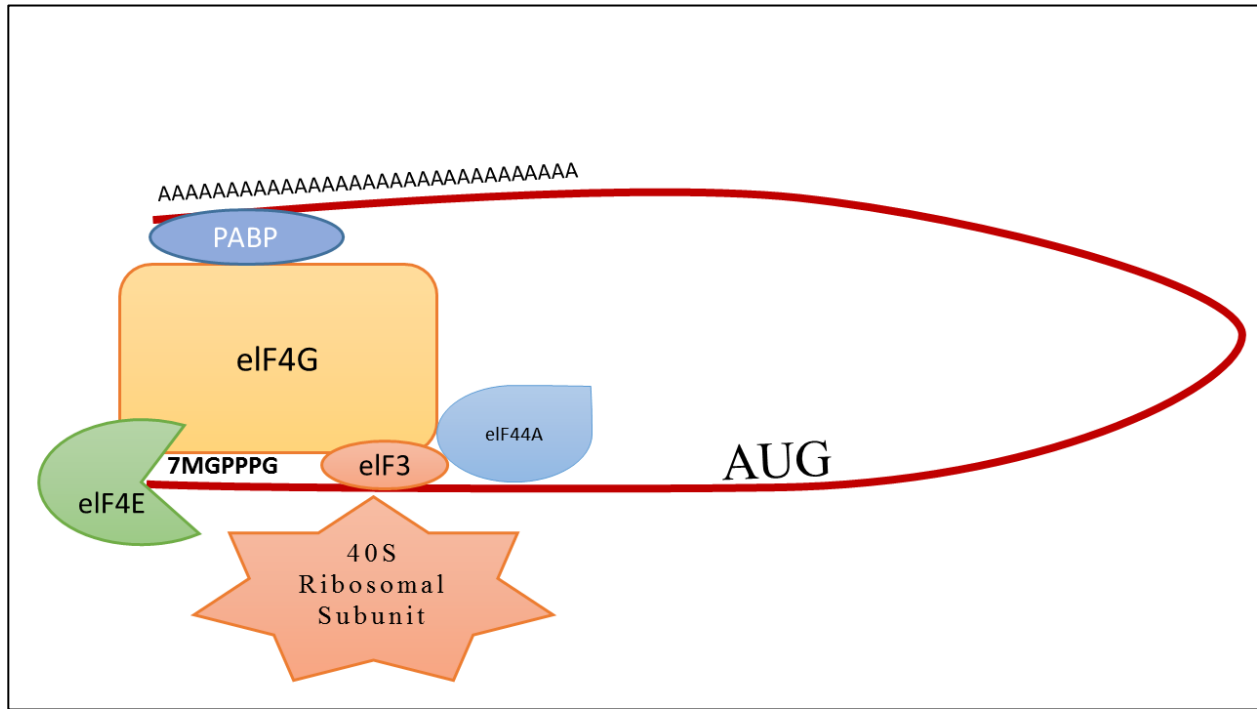


Fig 1.6: - Main Role of eIF4G family protein in Translation initiation Stage. eIF4G subunits are considered the largest component of the eIF4F family; these subunits contain the binding domain for mRNA, PABP, eIF4A, eIF4E, and eIF. They also stabilize the interaction of eIF4E with cap structure; and PABP with the poly A tail. Additionally, they directly interact with eIF3, component of 43S Ribosome, and work as bridge between eIF4F – mRNA complex, and 40S Ribosome. Adapted from ***Biol Res.* 2005;38(2-3):121-46.[3]**

1.5 Why we Study the Role of Translation Initiation in Regulation of the Epithelial Junctions and Cell Motility?

In this study, the overall goals were to investigate the role of eukaryotic translation initiation factor (eIF4G) in regulating structure and functions of apical junctions, and motility of intestinal epithelium and ECM adhesion; and to analyze the roles of eIF4G downregulation in cytokine induced barrier disruption and inhibited epithelial restitution. There are several reasons for focusing on translation factor eIF4G on regulating of epithelial junctions and cell motility. First, epithelial barrier is disrupted during the acute inflammatory phase thus exaggerating inflammation. Disruption of AJ, TJ, and FA plays a key role in the breakdown of the intestinal epithelial barrier during the mucosal inflammation [37-39]. For instance, proinflammatory cytokines relevant to IBD are known to trigger AJ and TJ disassembly and attenuate wound healing in model intestinal epithelial cell monolayer [40]. Decreased expression of adhesion proteins has been reported in IBD mucosa and cytokine-treated epithelial cells [41, 42], which plays a significant role in mediating breakdown of the intestinal epithelial barrier[43]. Second, mechanisms of cytokines-dependent downregulation of adhesion proteins remain poorly understood, although evidence suggests that they may involve posttranscriptional events. Protein translation is an attractive mechanism since it is known to be down-regulated by inflammatory stimuli in colonic and pancreatic epithelium[6, 44]. Third, little is known regarding the roles of eIF4G1 and eIF4G2 in regulating the expression of the epithelial adhesion proteins. The eIF4G1 was previously implicated in the control of p120 catenin expression in inflammatory breast cancer [45]. Furthermore, eIF4G1 was found to be enriched at the leading edge of migrating fibroblasts [46, 47] and to regulate tumor cell invasion[45, 48]. However, nothing is known about the roles of eIF4G translational initiator in regulating the integrity and restitution of the intestinal epithelial barrier under normal conditions or during mucosal inflammation. Based on this, we hypothesized that the process of translation initiation plays essential roles in regulation of the intestinal epithelial apical junctions and cell motility in intestinal models.

1.6 Specific Aims

Aim 1: To investigate the roles of eIF4G-dependent translation of AJ and TJ proteins in the maintenance and disruption of model intestinal epithelial barrier.

Aim2: To analyze the involvement of translation initiation factors in intestinal epithelial cell migration and ECM adhesion.

Hypothesis: The process of translation initiation plays essential roles in regulation of the intestinal epithelial apical junctions and cell motility in intestinal models.

2. MATERIALS AND METHODS

2.1 Cell Culture

Different intestinal epithelial cells that were used were maintained in standard T-flasks. HIEC, SC-CO15, T84, and HT29 were plated either onto 6 or 24 well plates depending on the experiment. Cells were allowed to grow in nutrient media until a confluency of 60-80%. The table provided describes the different components of a nutrient medium which were used for cell growth.

Cell Type	Basic Medium	Source	Serum Used	MEM-NEAA	HEPES	Pen-step	Cat #
SK-CO15	DMEM	ATCC	FBS 10%	5 ml	7.5 ml	5ml	30-2002
HT 29	DMEM	ATCC	FBS 10%	5 ml	7.5 ml	5ml	30-2002
T84	DMEM/F12	Corning	FBS 20%	----	----	5ml	10092CV
HIEC	OptiMEM 1	Invitrogen	FBS 5%	5ng/ml EGF	0.05 M	----	22600-134

2.2 Total Cell Lysates

In order to make lysate, cells were washed three times with HANK's+ on ice. After adding RIPA buffer, cells were scraped from 6-well dishes by using cell scraper. The RIPA buffer, which is formed by (20mM Tris, 1% Sodium Deoxycholate, 1% Triton, 50mM NaCl, 20mM EGTA, 2mM EDTA), should contain protease inhibitors (1:100) and phosphatase inhibitors cocktail (1:200)—both were obtained from Sigma-Aldrich to protect protein's integrity and protein phosphorylation. Cell were homogenized using Dounce Homogenizers. Then homogenized samples then were centrifuged for 20 mins at 14,000G, 4°C, and supernatant was diluted in the equal amount of 2x SDS sample buffer after adding 0.1M Dithiothreitol (DTT). Lastly, samples were boiled for 6 mins in 100°C, and then stored in -80°C refrigerator. By using BCA protein assay kit, (ThermoFisherScientific) the protein concentration of total lysate is determined.

2.3 Western Blotting/Immunoblotting

Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis (SDS-PAGE) was used to separate individual proteins. An equal amount of total protein was loaded into each lane (10-20ug). After the electrophoretic separation, proteins were transferred by electroblotting technique onto polyvinylidene difluoride (PVDF) or Nitrocellulose membranes according to the molecular weight of the proteins. The membrane was then blocked with 5% Non-fat dry milk, diluted into TBS-T (Tris-Buffered Saline-Tween-20) for an hour. After that, the primary monoclonal or polyclonal antibodies were added and incubated overnight in a cold room. On the second day, the membranes treated with primary antibodies were washed three times with TBS-T for 5 minutes each to remove all excess primary antibodies. Then HRP conjugated secondary antibodies were added to the membrane for an hour at 1:5000 concentration. Then, membranes were washed with TBS-T three times, followed by three more times washing with TBS for five minutes each. Finally, membranes were developed by using ECL reagent from GE-Healthcare. The images were taken on standard x-ray film by using SRX-101A image processor from Konica Minolta.

2.4 siRNA–Mediated Protein Knockdown

Cells cultured on 6-well plates were transfected with gene specific siRNA in order to obtain transient protein knockdown. Eif4G1 and Eif4G2 gene silencing was obtained using specific On-Target⁺ siRNA from GE-Lifesciences. The siRNA is specifically designed to reduce off target effects. As per the mechanism stated by the manufacturer, the sense strand is modified to prevent interaction with RISC-complex and thus favors anti-sense strand uptake while the anti-sense strand is modified to destabilize off-target effects and enhance target specificity (Manufacturer's manual, GE-Lifesciences). All knockdowns were performed as per manufacturers protocol. Control siRNA duplex#2 obtained from GE-Lifesciences was used as control in all experiments.

2.5 Generation of Stable Cell Lines with Small Harpin (sh)RNA-Mediated eIF4G1 and eIF4G2 Knockout

HIEC, and SK-CO15 cell lines with stable shRNA-mediated knockdown of either Eif4G1 or Eif4G2 were generated using the Sigma-Aldrich MISSION shRNA Lentiviral Transduction system according to the manufacturer's instructions (Sigma-Aldrich, Saint Louis, MO). All cell lines were transduced with either Eif4G1 or Eif4G2 shRNA-containing lentiviral particles, and stable cell lines were selected using puromycin (3 µg/mL for HIEC and 5 µg/mL for other three lines, respectively). A non-silencing shRNA lentiviral particle (RHS4346), lacking complementarity to any human gene, was used as a control.

2.6 Antibodies and other reagents

The following commercially-available primary polyclonal (pAb) and monoclonal antibodies (mAb), which have already been tested in our laboratory, will be used in the proposed study: anti eIF4G1, eIF4E (Cell Signaling Technology Danvers, MA), Eif4G2 (Bethyl Laboratories, Montgomery, Tx), anti-occludin, ZO-1, JAM-A, pAbs and anti-E- cadherin, occludin, ZO-1 mAbs (Life Technologies, San Francisco, CA); anti-β-catenin (BD Biosciences, San Diego CA.) Anti-puromycin mAb (Kerafast Boston, MA) Alexa-488-conjugated G-actin, rhodamine-phalloidin, as well as Alexa-488 or Alexa-568 dye-conjugated donkey anti-rabbit and goat anti-

mouse secondary antibodies will be obtained from Invitrogen (Eugene, OR); horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse secondary antibodies will be obtained from Jackson Immunoresearch Laboratories (West Grove, PA). All other reagents will be purchased from Sigma-Aldrich.

2.7 Immunofluorescence Labelling and Confocal Microscopy

Intestinal epithelial cells either transfected with siRNA against eIF4G1, and eIF4G2 or stable cell lines with shRNA mediated knockdown, were grown in a collagen-coated coverslip in 24 well dishes. Confluent cells on 4th-day post-transfection were fixed in 100% Methanol for 20 minutes at -20°C. After fixation, the cells were blocked by using Bovine Serum Albumin (BSA) in the dark for one hour. Combination primary antibodies, which were prepared by diluting them in BSA (1:300), were added to cells for an hour. Then, the cells were washed three times with BSA for five minutes each. Next appropriate secondary Antibodies diluted in BSA at 1:1000 were added. Next cells were washed three times with BSA for five minutes each. Lastly, coverslips were placed on the glass slide using Prolong Gold Antifade reagent (Applied Biosystems). Stained cells were examined by using a Zeiss LSM 700 Laser scanning microscope. The images were analyzed by using Zen Lite software (Carl Zeiss Microscopy).

2.8 Puromycin Incorporation Assay

Intestinal epithelial cells either transfected with siRNA against eIF4G1, and eIF4G2 or stable cell lines with shRNA mediated knockdown, were grown in a collagen-coated coverslip in 24 well plates. On the day of the experiment a using 200 ul pipette a scratch wound was created. Cell were allowed to move and 3h post-wounding samples were treated with DMEM containing 5 µM puromycin for 30 min prior to cell fixation. Cells were washed two times with HANK'S and proceeded to staining with anti-pyromycin antibody to detect the newly translated proteins.

2.9 Transepithelial Electrical Resistance (TEER)

Transepithelial electrical resistance (TEER) of cultured epithelial cell monolayers was measured using an EVOMX voltohmmeter (World Precision Instruments, Sarasota, FL). The resistance of cell-free collagen-coated filters was subtracted from each experimental point.

2.10 Wound Healing Assay/ Scratch Assay

Intestinal epithelial cells either transfected with siRNA against eIF4G1, and eIF4G2 or stable cell lines with shRNA mediated knockdown, were grown on 6-well plates. On the 4th day post-transfection, a horizontal thin scratch wound was performed in the confluent area of the cells monolayer by using a 200 μ l pipette tip. After making the scratch, the previous medium was removed and replaced by new one. Images of the scratch were capture in 0h, 24h, and 48h depending on the cell types. After that, the cell-free area was measured by using Image J software.

2.11 Spreading Assay

Intestinal epithelial cells either transfected with siRNA against eIF4G1, and eIF4G2 or stable cell lines with shRNA mediated knockdown, were grown on 6-well plates. On the 4th day post-transfection, cells were detached from plates by using TrypLE Express and counted with the hemocytometer after they were re-suspended in standard medium. First, 24 well plates were coated with 200 μ l of type-1 collagen and incubated overnight in order to dry. Then, 10^7 cells were seeded onto the plates. The cells were incubated for one hour to allow the cells to attach and spread over collagen type 1 at 37°C. Then, the cells were fixed with 70% ethanol for 10 minutes and stained by using DIFF stain kit. Lastly, the images of spread cells were taken by light microscope under 20x power and the area of spreading was measured by Image J software.

2.12 ECM Adhesion Assay

Intestinal epithelial cells either transfected with siRNA against eIF4G1, and eIF4G2 or stable cell lines with shRNA mediated knockdown, were grown on 6-well plates. On the 4th day post-transfection, cells were detached from plates by using TrypLE Express and counted with the hemocytometer after they were re-suspended in standard medium. Then, 10^7 cells were seeded onto 24 well plates which coated with type-1 collagen overnight. The cells then incubated for one hour at 37 °C to allow the attachment of the cells to ECM. After incubation, the Non-attached cells were removed by extensive washing up and down with 1ml of cell culture medium. Then the cells were fixed with -20°C methanol for 10 minutes.

Then the cells were stained by using a DIFF stain kit. The staining-adherent cells were examined by using light microscope under power 10x, and the number of adherent cells was manually counted.

2.13 Transfilter Migration Assay

In order to perform Transfilter migration assay 24 well plates each with Transwells® with 8.0µm pore size inserted in was used (Corning Incorporated, Costar®). The 50ul of the collagen type 1 was coated on the membranes and incubated overnight. Then, on the 4th day post-transfection, cells were detached from 6 well plates by using TrypLE Express and counted with the hemocytometer after they were re-suspended in 0.1 % FBS medium and added to the upper chamber of the . In contrast, the complete standard medium 10% FBS was added to the lower chamber of the Transwells which were previously prepared on the night before. The cells were incubated for 24h at 37°C to allow them to migrate overnight. After 24h, cells were fixed by using Methyl Alcohol from DIFF Stain Kit for 5 minutes. By using cotton swabs, the non-migrating cells were removed from the top of the filtered membrane. The cells in the bottom of the filtered membrane were stained with DAPI (nuclear stain), and the filters were mounted on microscope slides. By using the fluorescence microscope, the migrating cells were examined and counted manually.

2.14 Cytokines and Pharmacological Inhibitors Treatment

For cytokine treatment, IFN γ (50 ng/ml) and TNF α (10 ng/ml) were added to both the cells in 6-well plates in order to harvest the cell lysate for immunoblotting, and in apical and the basolateral Transwell chambers of 24 well plates for 24h and 48 h to perform immunofluorescent staining. In the case of Pharmacological inhibitors treatment, which involves Ribavirin (20 µM) and 4EGI-1 (25µM), were added to 60-80% confluent cell in 6-well dishes and make wound after 2h post-treatment incubation. The images were obtained in 0h, 12h, and 24h post-wounded depending on the types of the cells. The appropriate dimethyl sulfoxide (DMSO) was added to control samples.

2.15 Quantitative Real-Time RT-PCR

Total RNA was isolated using an RNeasy mini kit (QIAGEN, Valencia CA), followed by DNase treatment to remove genomic DNA. Total RNA (1 µg) was reverse transcribed using an iScript cDNA synthesis kit (Bio-Rad Laboratories). Quantitative real-time RT-PCR was performed using iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories) and a 7900HT Fast Real-time PCR System (Applied Biosystems; Foster City, CA).

The following primers were used for Claudin 4: forward: 5'-CCCCAGTGACAAAAACCC-3'; and reverse: 5'-ACGGACTTAAACGTTTCGCA-3'.

β-Catenin: forward: 5'- ACAAACCTGTTTTGAAAATCCA-3'; and reverse: 5'-CGAGTCATTGCATACTGTCC-3'.

E-cadherin forward: 5'-TTACTGCCCCAGAGGAT-3'; and reverse: 5'-TGGAACGTCGTTACAGTG-3'.

Expression levels were normalized to the house-keeping gene GAPDH (forward: 5'-CATGTTTGTGATGGGTGTGAACCA-3'); reverse: (5'-AGTGATGGCATGGACTGTGGTCAT-3').

The threshold cycle number (Ct) for specific genes of interest and the housekeeping gene was determined based on the amplification curve representing a plot of the fluorescent signal intensity versus the cycle number. The relative expression of each gene was calculated with a comparative Ct method that is based on the inverse proportionality between Ct and the initial template concentration ($2^{-\Delta\Delta Ct}$), as previously described [49]. This method is based on the two-step calculation of $\Delta Ct = Ct \text{ target gene} - Ct \text{ GAPDH}$ and $\Delta\Delta Ct = \Delta Ct_e - \Delta Ct_c$ where index e refers to the sample from any control or eIF4G1 and eIF4G2 siRNA or corresponding miRNA treated cells, and index c refers to a sample from a control siRNA or control miRNA-treated cells assigned as an internal control.

3. RESULTS- PART I

3.1 Proinflammatory Cytokines Induce Disassembly of Intestinal Epithelial Tight and Adherens Junctions.

In order to investigate the effects of proinflammatory cytokines on the integrity of intestinal epithelial junctions, the polarized HT-29 cF8 (clone F8) cell monolayer was exposed for 48 h to either IFN γ alone (50 ng/ml) or in combination with TNF α (10 ng/ml). Integrity of AJ was evaluated by immunolabeling of HT-29 cells with β -catenin, whereas TJ integrity was examined by immunolabeling of ZO-1. Confocal microscopy shows that IFN γ induces focal disruption of characteristic AJs, and TJs labeling (arrowheads), indicating junctional disassembly. TNF α dramatically exaggerates this effect. Together, these observations indicate that proinflammatory cytokines triggers disassembly of AJ and TJ in intestinal epithelial cells monolayer (Fig. 3.1).

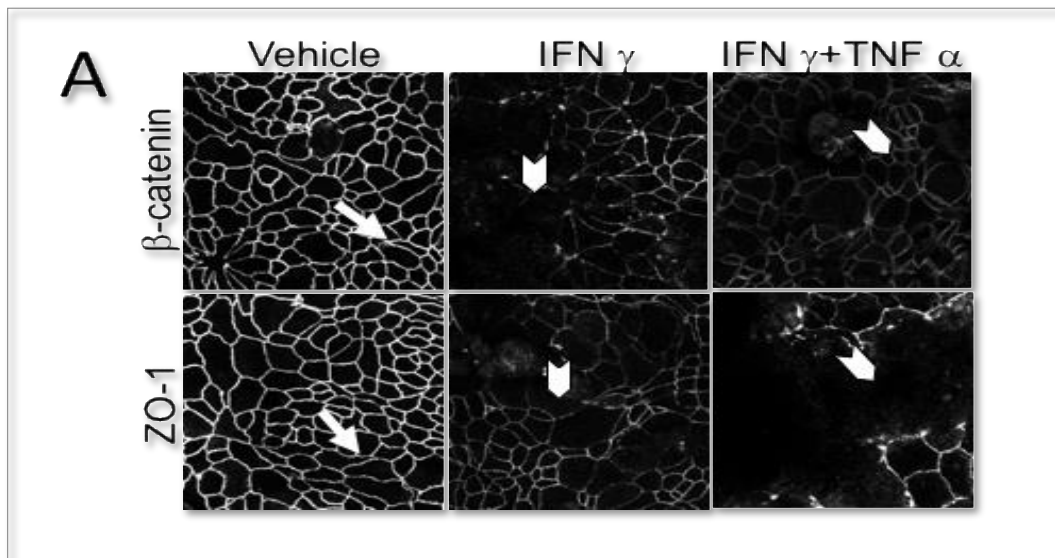


Figure 3.1. Proinflammatory cytokines induce disassembly of intestinal epithelial tight junctions and adherens junctions Confluent HT29 cell monolayers were treated for 48 h with either vehicle, IFN γ or a combination of IFN γ and TNF α . Localization of AJ and TJ proteins, β -cadherin and ZO-1, was determined by fluorescence labeling and confocal microscopy. IFN γ induces disappearance of AJ and TJ proteins from the areas of cell-cell contact (arrowheads), while TNF α potentiates IFN γ -driven junctional disassembly.

3.2 Proinflammatory Cytokines Decrease the Expression of TJs and AJs without Affecting their mRNA Levels.

Given microscopy data revealing disassembly of AJ and TJ treated with cytokines in Figure 3.1, the effect of proinflammatory cytokines on the expression of different AJ and TJ proteins was next investigated. Human HT29 and T84 colonic epithelial cells monolayer were treated with either vehicle (PBS) IFN γ alone (50 ng/ml), and in combination with TNF α (10 ng/ml). Total cell lysate were prepared at 24 h and 48 h of cytokine exposure. Immunoblotting revealed the significant downregulation of AJ proteins (E-cadherin and p120 catenin) and the TJ protein (occludin) in cytokine-treated cells (Fig. 3.2A). By contrast, quantitative RT-PCR analysis did not reveal changes in mRNA expression of selected junctional proteins in cytokine-treated epithelial cells (Fig. 3.2B). These results indicate that proinflammatory cytokines disrupt the integrity of the intestinal epithelial barrier via expressional down-regulation of different AJ and TJ proteins. Such downregulation can occur without inhibition of mRNA transcription.

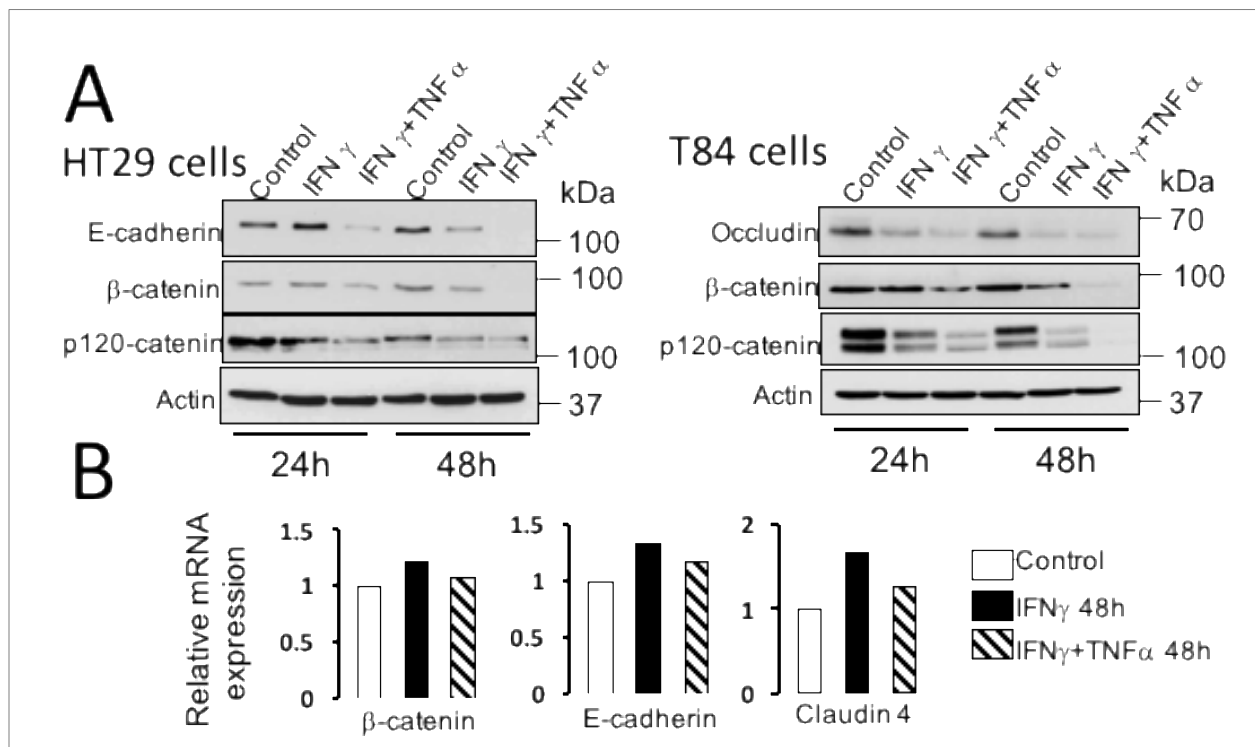


Figure 3.2. Proinflammatory cytokines decrease the expression of intestinal epithelial tight junctions and adherens junctions without affecting their mRNA levels

(A) Western blot analysis reveals that cytokines decrease expression of different AJ and TJ proteins in HT 29 and T84 human intestinal epithelial cell lines.

(B) Real time RT-PCR analysis of mRNA levels of selected junctional proteins in IFN γ and TNF α treated HT 29 cells.

3.3 Cytokines Decrease Expression of Different Translation Initiation Factors in Intestinal Epithelial Cell Lines

Our data suggest that proinflammatory cytokines disrupt the integrity of epithelial junctions by decreasing expression of different AJ and TJ proteins at post-transcriptional level. We rationalized that this mechanism may involve inhibition of protein translation and especially its rate-limiting step: translation initiation. Therefore, we next sought to investigate the effects of proinflammatory cytokines on expression of major translation initiation factors. To do that, again we compared expression of eIF4G1 and eIF4G2 isoforms in control and IFN γ /TNF α of treated both HT-29 and T84 cells, along with expression of other components of the eIF4F cap-dependent translation initiation complex (eIF4E). Cells were harvested for cell lysates on the 24h and 48h of the cytokine treatment. The immunoblotting analysis shows that exposure of T84 cell monolayers to either IFN γ or IFN γ plus TNF α resulted in dramatic downregulation of eIF4G1 and eIF4G2 protein expression (Fig. 3.3A). Likewise, this cytokine combination reduced the expression of both eIF4G isoforms in HT-29 cells (Fig. 3.3B). The same inhibitory effects were evident for eIF4E expression. This data suggests that proinflammatory cytokines deplete major components of protein translation initiation machinery in intestinal epithelial cells in vitro.

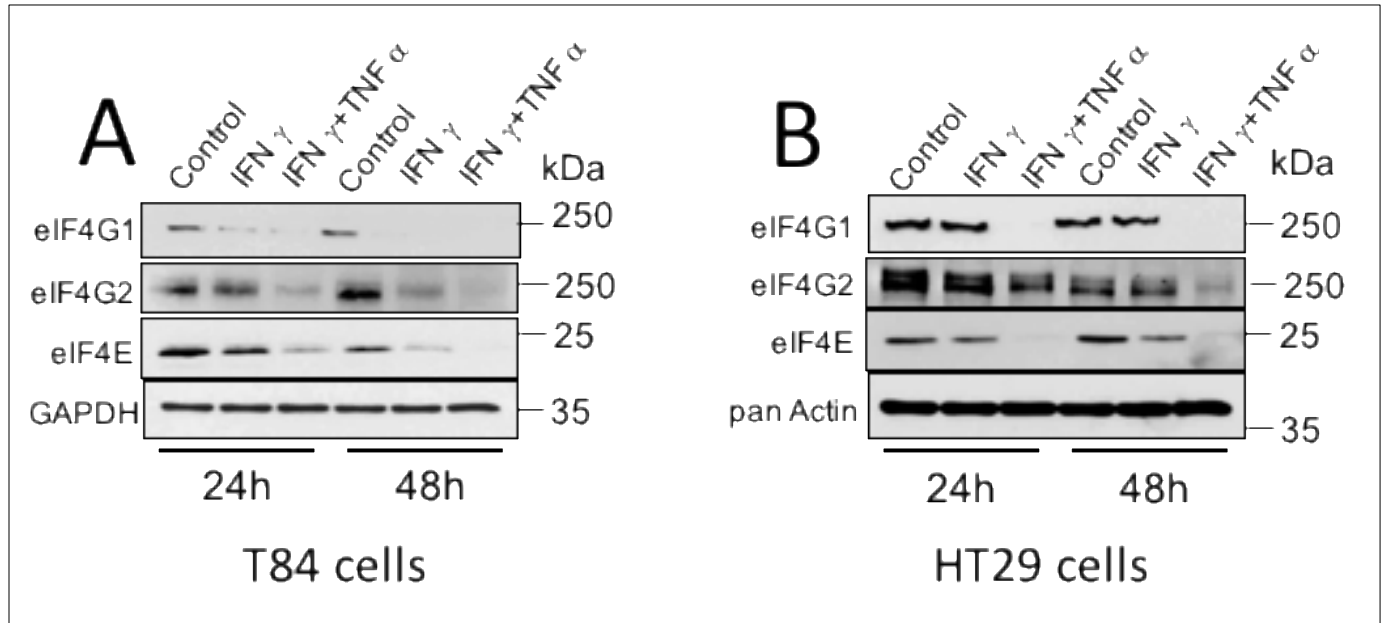


Figure 3.3. Cytokines decrease expression of different translation initiation factors in intestinal epithelial cell lines in vitro.

Representative immunoblots of HT 29 cells (A) and T84 (B) show that exposure of cells to either IFN γ or IFN γ plus TNF α markedly decreases levels of translation initiation factors eIF4G1, eIF4G2 and eIF4E.

3.4 Decreased Expression eIF4G2 in Intestinal Mucosa of IBD Patients

Given our results that proinflammatory cytokines decrease expression of different translation initiation factors in vitro, we sought to examine if similar events may also happened during intestinal inflammation in vivo. In order to do that we performed dual immunofluorescence labeling of eIF4G2 (green) and β -catenin (red) in affected colonic sections of patients with a B2 Montreal classification of Crohn's disease, as well as normal colonic mucosa of patients undergoing intestinal surgery. The banked tissue sections were provided by Dr. John Kuemmerle from the GI Division at VCU Medical Center. As shown in figure 3.4, in normal colonic mucosa, eIF4G2 was abundantly expressed in epithelial cells (Fig. 3.4, arrows). In contrast, labeling intensity of this translational initiator was decreased in IBD mucosa (Fig. 3.4 arrowheads). Overall these data are consistent with in vitro experiment result on figure 3.3, mucosal inflammation decreases levels of eIF4G1 and eIF4G2 in intestinal epithelium in vitro and in vivo (Fig 3.4).

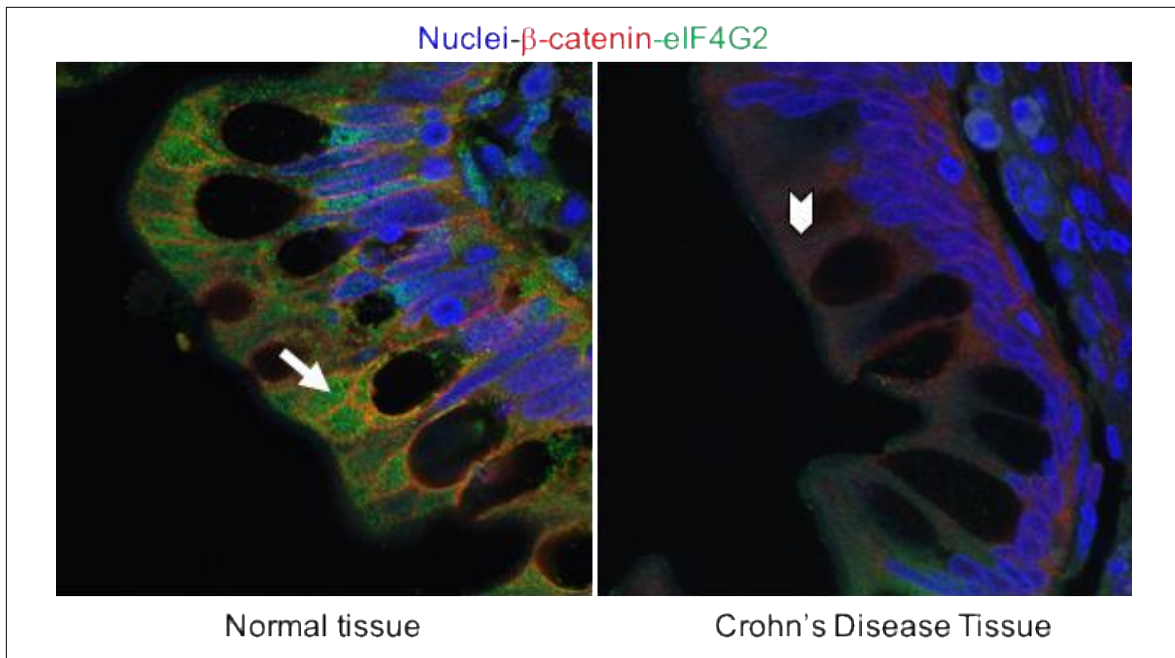


Figure 3.4. Decreased expression of a translation initiation factor in inflamed human intestinal mucosa in vivo.

Immunofluorescence images show diffuse eIF4G2 staining in normal colonic epithelia and marked reduction of eIF4G2 labelling in colonic mucosa of CD patient.

3.5 Knockdown of eIF4G1 and eIF4G2 Decreases Expression of Different AJ and TJ Proteins in Human Colonic Epithelial Cell Monolayers

In order to investigate the role of eIF4G subunits on the integrity of epithelial junctions, the expression of two major eIF4G isoforms in epithelial cells was downregulated by using RNA interference (RNAi). To do that, eIF4G1 and eIF4G2 specific siRNA individual duplexes (D1-D4) were used. SK-CO15 human colonic epithelial cells were transfected with the control, eIF4G1, and eIF4G2 siRNA. The Expression of different AJ and TJ proteins was examined on day 4 post-transfection. Figure 3.3 shows that loss of these translation initiation factors decreases the expression of several junctional proteins. Interestingly, eIF4G1 appears to regulate the expression of both AJ and TJ proteins (Fig. 3.5A), whereas eIF4G2 was found to be a more selective regulator of TJ constituents (Fig. 3.5B).

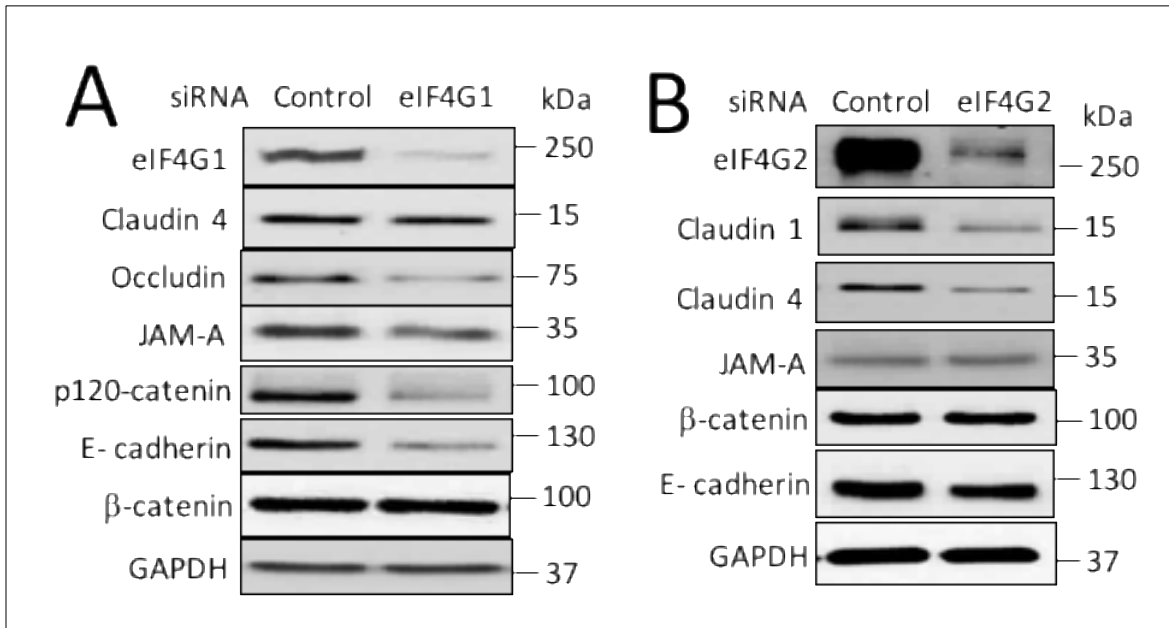


Figure 3.5. Knockdown of eIF4G1 and eIF4G2 decreases expression of different AJ and TJ proteins. SK-CO15 cells were transfected with either control, eIF4G1 or eIF4G2 siRNAs. Four days after the knockdown effects of eIF4G isoform knockdown on expression of different AJ and TJ proteins was determined by immunoblotting analysis.

3.6 siRNA-Mediated Depletion of eIF4G1 Prevents Formation of the Paracellular Barrier

Since previous experiments indicate that, depletion of eIF4G1 and eIF4G2 downregulates the expression of different AJ and TJ proteins, we next sought to investigate the roles of these translation initiation factors on paracellular barrier formation and assembly of epithelial junctions. The initial series of experiments involved transient siRNA-mediated knockdown of eIF4G1. Paracellular permeability was examined by measuring Transepithelial Electrical Resistance (TEER) from 2nd day through 4th day post transfection. We observed that loss of eIF4G1 in SK-CO15 cells significantly attenuate development of decreased TEER as compared to control siRNA-transfected cells (Fig. 3.6A). After measuring TEER, the cell monolayers were fixed and immunolabelled for β -catenin, occludin, claudin 4 (red) to visualize both AJ and TJ, and eIF4G1(Green) to visualize areas of its efficient knockdown. As a result, Figure 3.6B shows that depletion of eIF4G1 disrupted β -catenin and claudin-4 localization at apical junctions without having effect on occludin localization. Overall, these results suggest that eIF4G translation initiation factors play an essential role in the assembly of apical junctions and regulation of the intestinal epithelial barrier.

Subsequent experiments were aimed at establishing SK-CO15 cell lines with stable shRNA-mediated knockdown of either eIF4G1 or eIF4G2. These cell lines were created using protein-specific shRNA plasmids cloned into a lentiviral vector. Ready to use lentiviral particles were obtained from Sigma, SK-CO15 cells were infected with these lentiviral particles and stable cell lines were generated after puromycin selection. Control, eIF4G1 or eIF4G2 depleted SK-CO15 cells were plated on Transwell membrane filters, and TEER was measured on days 1-3 after the cells reached confluency. The results of this TEER measurement indicate that loss of either Eif4G1 or Eif4G2 impaired the development of the paracellular barrier (fig. 3.7).

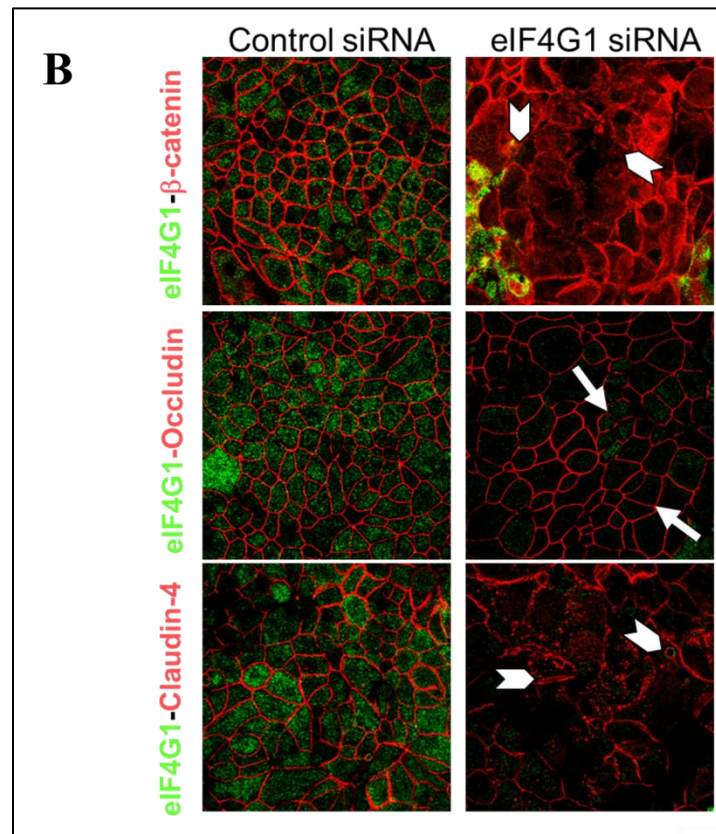
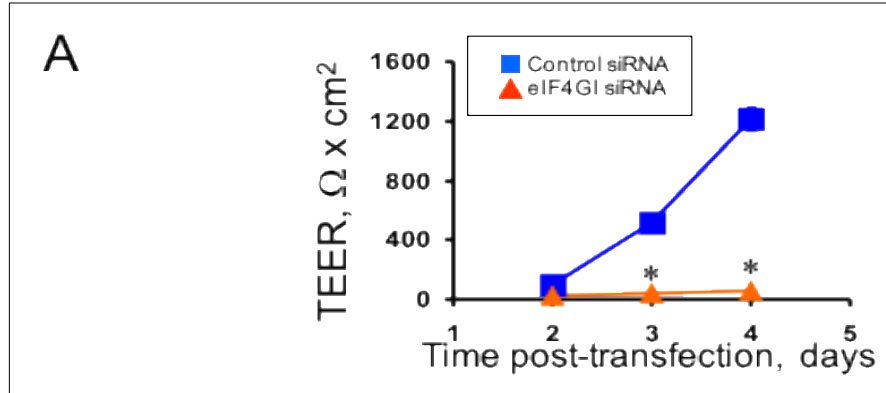


Figure 3.6. siRNA-mediated depletion of eIF4G1 prevents formation of the paracellular barrier and attenuates AJ and TJ assembly.

(A) TEER measurements demonstrate that eIF4G1-depleted SK-CO15 cell monolayers fail to develop the paracellular barrier. Data are presented as mean \pm SE, $n=3$ * $p<0.01$.

(B) Immunofluorescence labeling shows formation of normal β -catenin-based AJs and occludin and claudin 4-based TJs in control SK-CO15 cell monolayers on day 4 post-transfection. By contrast, eIF4G1-depleted cells have significant intracellular accumulation of β -catenin (arrowheads) and fragmented claudin4-based TJ strands (arrowheads).

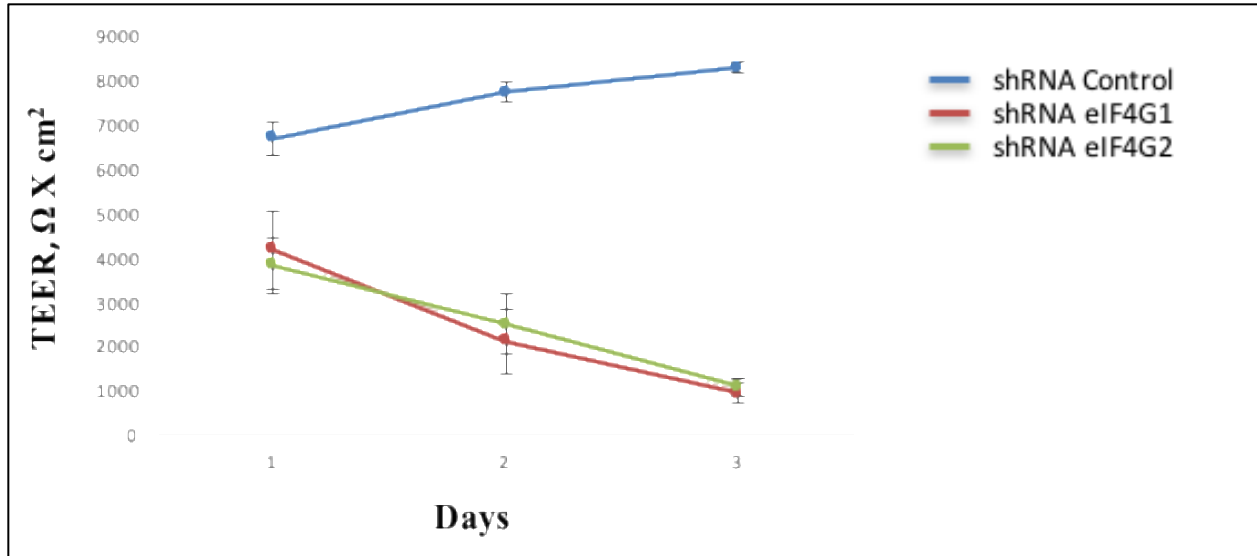


Figure 3.7. shRNA-mediated depletion of eIF4G1 and eIF4G2 prevents the formation of the paracellular barrier in SK-CO15 cell lines. Control, eIF4G1 or eIF4G2-depleted SK-CO15 cells were plated on membrane filters and TEER was measured at indicated time points after cell reached confluency. Data are presented as mean \pm SE, n=3 *p<0.01.

4. RESULTS- PART II

4.1 Newly Synthesized Puromycin-Positive Nascent Polypeptide Chains are Present at the Leading Edge of Migrating Cells.

Results of prior studies suggest that protein translation machinery could be localized at the leading edge of migrating individual cells such as fibroblasts [47, 50]. Based on these observations we started our study of the roles translation initiation in migration of intestinal epithelial cells by visualizing de novo protein translation in migrating cell monolayers. In order to do that, we used Puromycin Incorporation Assay. This assay is based on structural similarity between puromycin amino acyl-transfer RNA (aminoacyl-tRNA), which allows for puromycin incorporation into a newly synthesized elongated polypeptide chain by forming a peptide bond[51]. Confluent HT-29 cell monolayers were wounded and allowed to migrate into the wound for 3 h. Then, 1µg/ml puromycin was added for additional 5 min. Thereafter, cells were fixed and stained with anti-puromycin mAb to assess localization of de novo protein translation in migrating cell monolayer [46]. Such pulse labeling with puromycin of the active sites of protein synthesis revealed that translation occurs exclusively at the leading edge of migrating epithelial sheet (Fig. 4.1).

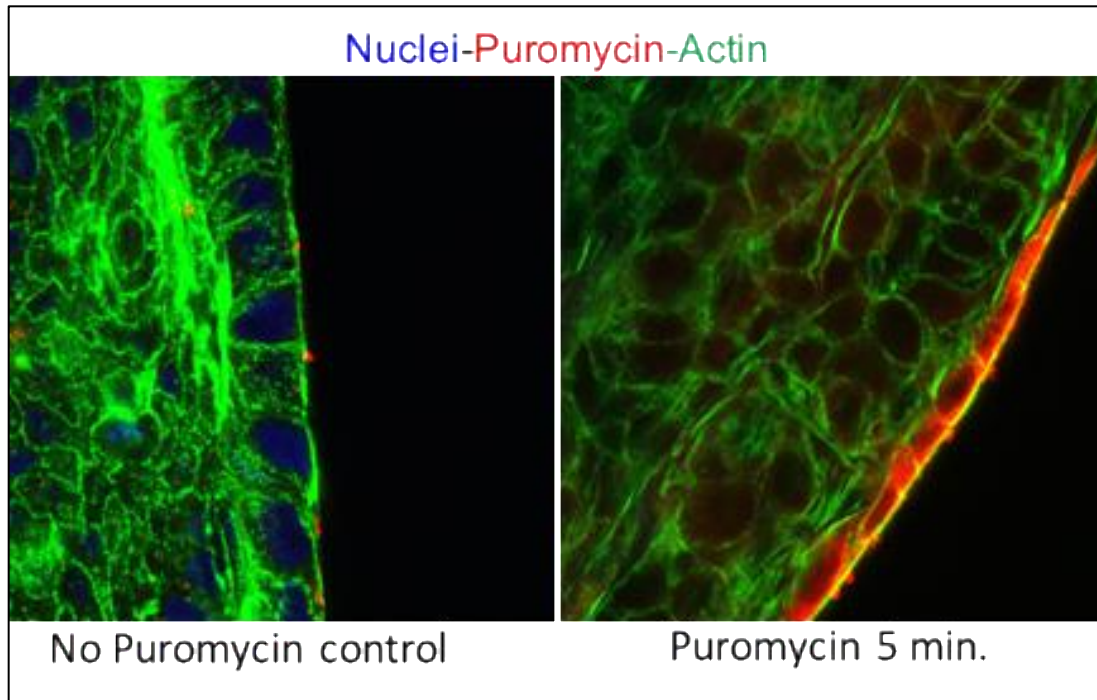


Figure 4.1. Newly synthesized puromycin-positive nascent polypeptide chains are present at the leading edge of migrating epithelial cells. Wounded HT 29 cell monolayers were, incubated for 5 minutes with 1 μ g/ml puromycin, with subsequent fixation with anti-puromycin mAb (red). The cells were counterstained for filamentous actin (green).

4.2 Cell Migration Alters Intracellular Localization of eIF4G2 in Intestinal Epithelial Cells

Because the results of previous experiments presented in figure 4.1 indicate that the nascent polypeptide chains are present at the leading edge of migrating cells, we next examined localization of eIF4G2 in stationary and migrating human intestinal epithelial cells. In this experiment, we used immortalized non-transformed HIEC cells, which are poorly differentiated stem cell like intestinal epithelial cells with high expression of eIF4G2. Immunoblotting analysis of eIF4G2 HIEC cells with a rabbit polyclonal antibody reveal a single band of correct size, which disappeared after eIF4G2 knockdown, which indicates specificity of this antibody (data not shown). Surprisingly, we found that eIF4G2 was localized at nucleus in stationary cells intestinal epithelial cells (figure 4.2A). However, wound healing resulted to dramatic relocalization of this translation initiation factor that become abundant in the cytosol and leading edge of the migrating cells (Figure 4.2B). Together our immunolabeling experiments suggest that motility of intestinal epithelial cells could be driven by local translation of essential regulators of cell motility at the leading edge of migrating cells.

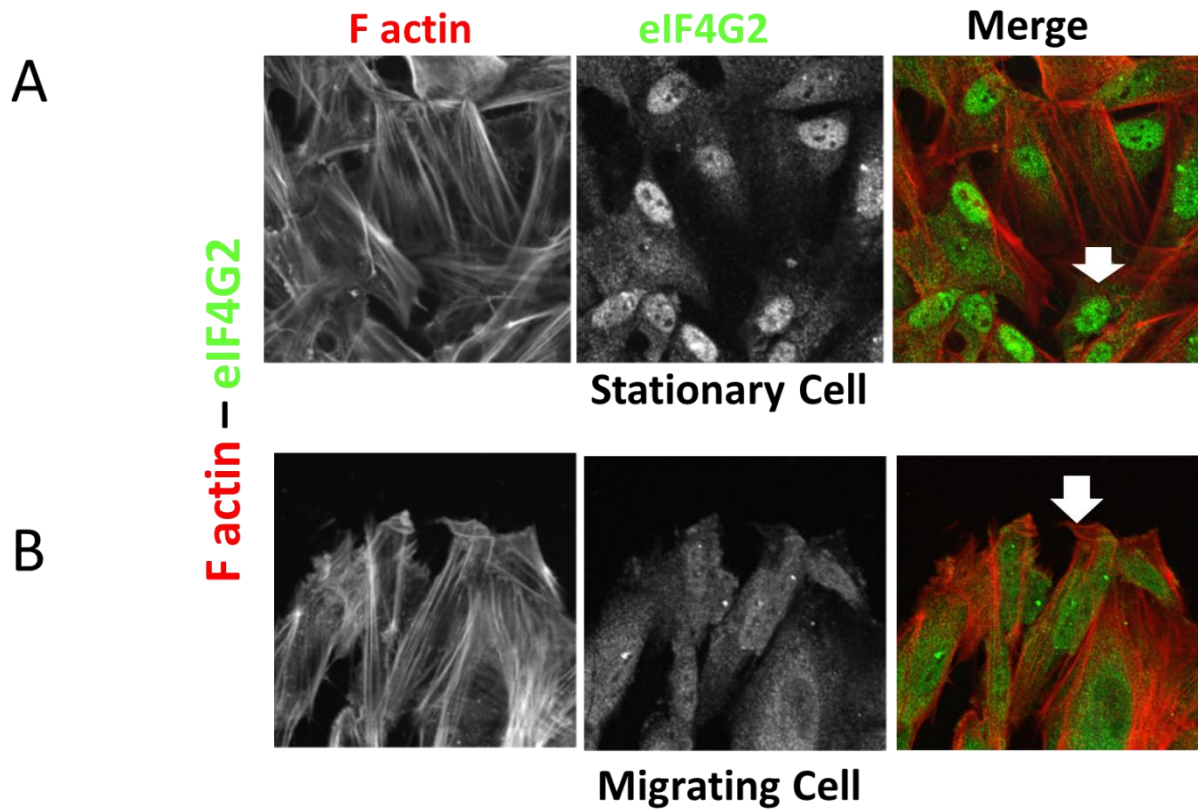
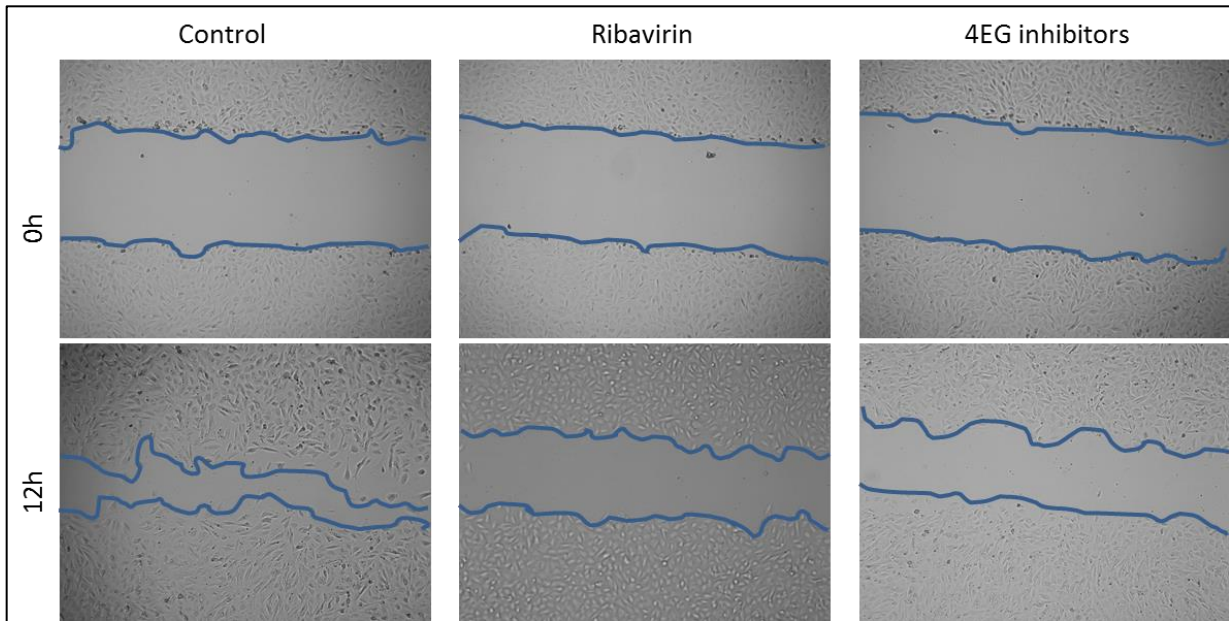


Figure 4.2. Confocal Images Showing Localization of eIF4G2 in Stationary (A) and Moving Cells (B). The confluent non-transformed HIEC cells were wounded by $10\mu\text{M}$ pipette. Then cells were fixed and dual labelled for eIF4G2 (green) and F-actin (red).

4.3 Pharmacological Inhibition of the eIF4E-Dependent Protein Translation Attenuated Motility of Intestinal Epithelial Cell Monolayers

The next series of experiments were designed to demonstrate causal roles of eIF4G-dependent translation initiation in the regulation of intestinal epithelial cell motility. Since eIF4G proteins regulate translation by interacting with eIF4E cap-dependent translation initiation factor, we used two pharmacological inhibitors that target different aspects eIF4E-dependent translation. One inhibitor is Ribavirin, an anti-viral drug, which is known to decrease the affinity of eIF4E to m⁷G cap of mRNA[52]. For the other inhibitor is 4EGI-1, a small molecule which inhibits the binding between eIF4G proteins and eIF4E resulting in inhibiting the cap-dependent translation initiation [53]. The experiments with pharmacological inhibition of protein translation initiation were conducted in both poorly differentiated HIEC and well-differentiated T84 intestinal epithelial cells. Confluent HIEC and T84 cell monolayers were wounded and then allowed to migrate to close the wound in the presence of either vehicle, Ribavirin, or 4EGI-1. Images were obtained for HIEC cells at 0 h when the wound initiated and 12 h of wound closure. For T84 cells, the images were taken at 0 h when the wound initiated and 24 h post wounding. As shown in Figures 4.3 and 4.4, a significant attenuation of wound closure by two inhibitors of translation initiation was observed in both cell lines. These results suggest that translation initiation is essential for collective migration of either stem cell-like or well-differentiated intestinal epithelial cells.

A



B

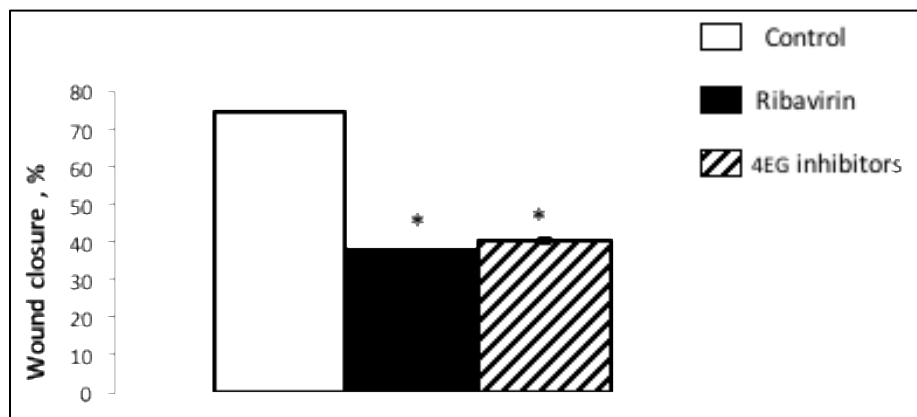


Figure 4.3. Ribavirin and 4EGI-1 attenuate the collective cell migration of HIEC cells. Effects of Ribavirin and 4EGI-1 inhibitors on the collective cell migration was determined by wound healing assay. HIEC cells were wounded and allowed to migrate in the presence of either vehicle, Ribavirin (20 μ M), or 4EGI-1 (25 μ M). The images were obtained in 0h and 12h post-wounding. Representative images of wounded monolayers (A) and quantification analysis of wound closure (B) are shown. Data are presented as mean \pm SE, n=3 *p < 0.01.

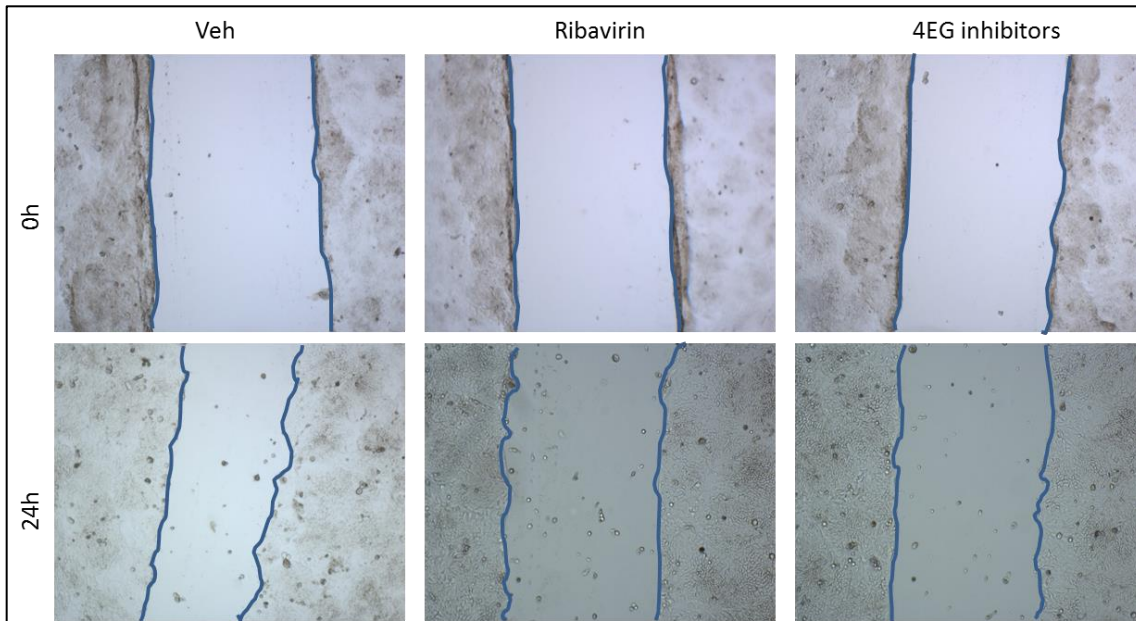
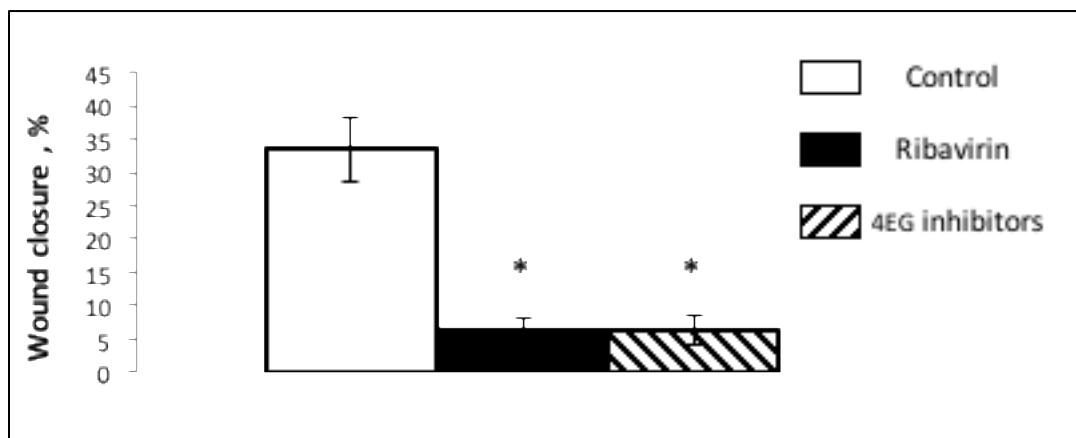
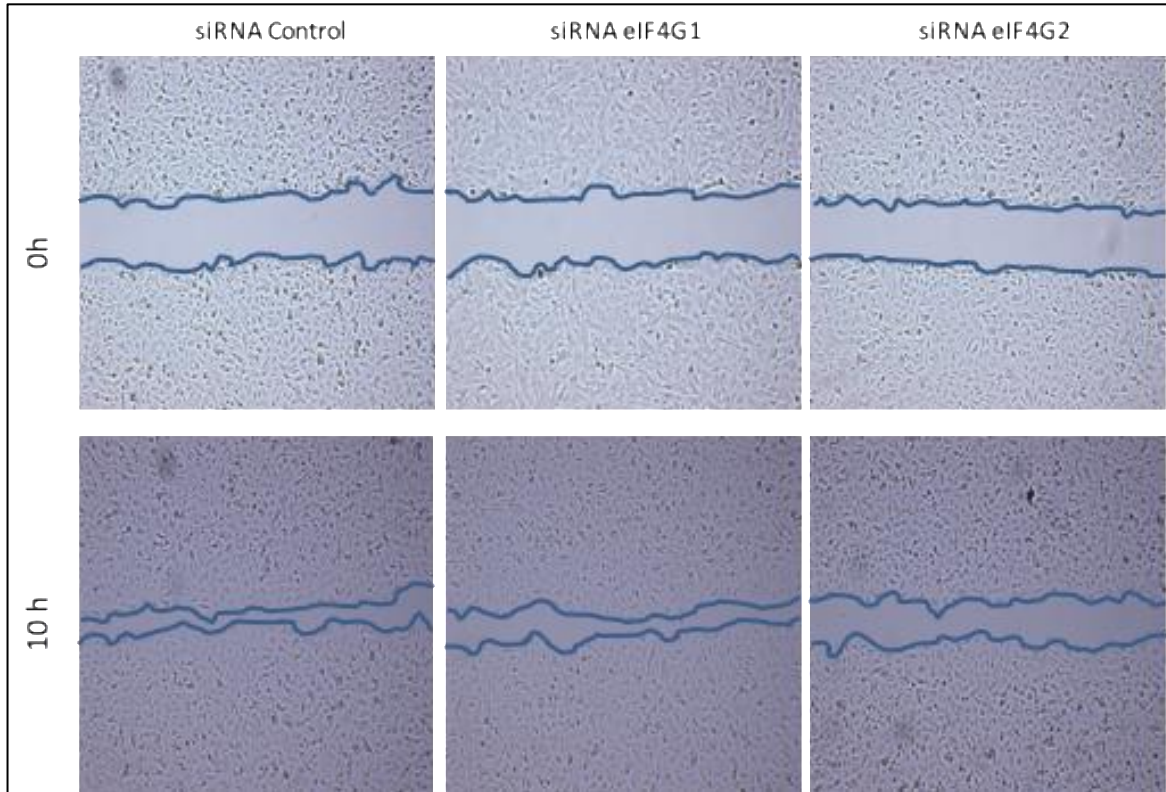
A**B**

Figure 4.4. Ribavirin and 4EGI-1 attenuate the collective cell migration in T84 cells. Effects of Ribavirin and 4EGI-1 inhibitors on the collective cell migration was determined by wound healing assay. Confluent T84 cells were wounded and allowed to migrate in the presence of either vehicle, Ribavirin (20 μ M), or 4EGI-1 (25 μ M). The images were obtained in 0 h and 24 h post-wounding. The images were obtained in 0h and 12h post-wounded. Representative images of wounded monolayers (A) and quantification analysis of wound closure (B) are shown. Data are presented as mean \pm SE, n=3 *p < 0.01.

4.4 Both eIF4G1 and eIF4G2 are Essential Regulators of Collective Migration of Intestinal Epithelial Cells

Since our experiments with pharmacological inhibition of protein translation initiation demonstrated essential role of this process in the regulation of intestinal epithelial cell migration, we next sought to investigate if eIF4G1 and eIF4G2 play unique or redundant roles in cell motility. To address this question, we transiently and specifically down-regulated expression of each translation initiation protein in either HIEC or T84 intestinal epithelial cells and determined the effects of such knockdown on epithelial wound healing. Interestingly, loss of eIF4G2 expression significantly attenuated collective migration of HIEC cells, whereas eIF4G1 knockdown was ineffective (figure 4.5). By contrast depletion of either eIF4G1 or eIF4G2 attenuated wound healing in T84 cell monolayers with similar efficiency (figure 4.6). These results highlight eIF4G2 as a universal regulator of intestinal epithelial cell motility independently of cell differentiation state. On the other hand, eIF4G1 appears to be dispensable for collective migration of stem like cells, but was required for migration of well-differentiated epithelial cells.

A



B

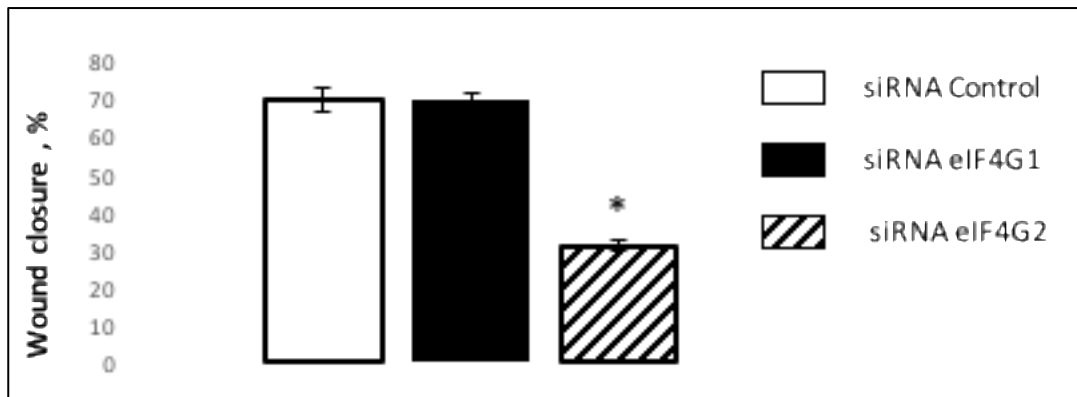


Figure 4.5. Depletion of eIF4G2 inhibits epithelial cell migration. Control, eIF4G1 and eIF4G2-depleted HIEC cell monolayers were mechanically wounded on day 4 post-transfection. To examine the rate of cell motility, the cell-free area was measured at 0 and 10 h after wounding. Representative images of wounded monolayers (A) and quantification analysis of wound closure (B) are shown. Data are presented as mean \pm SE, $n=3$ * $p < 0.01$.

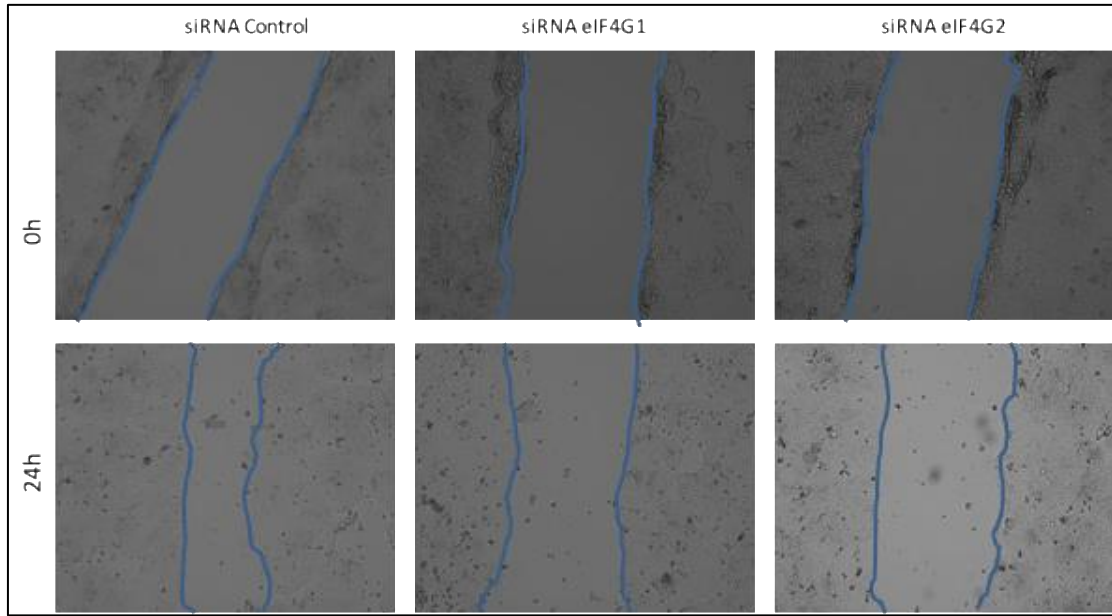
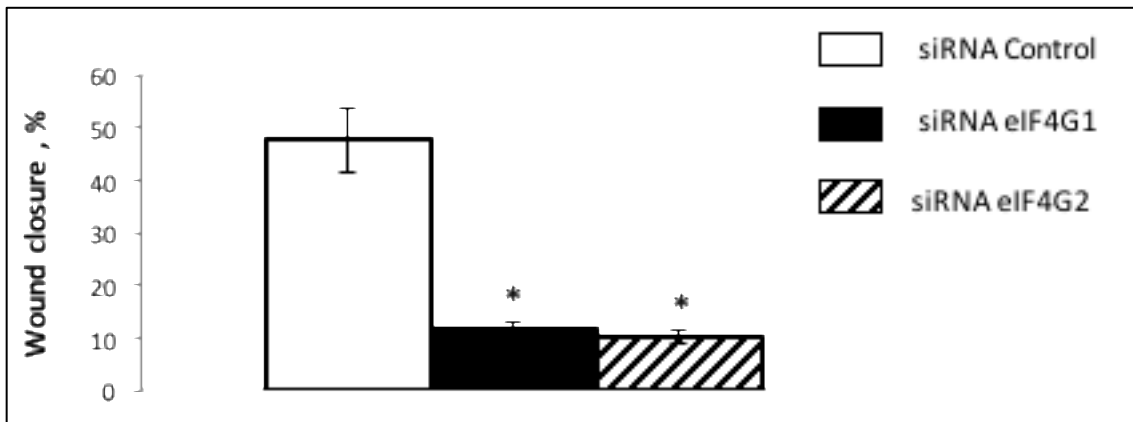
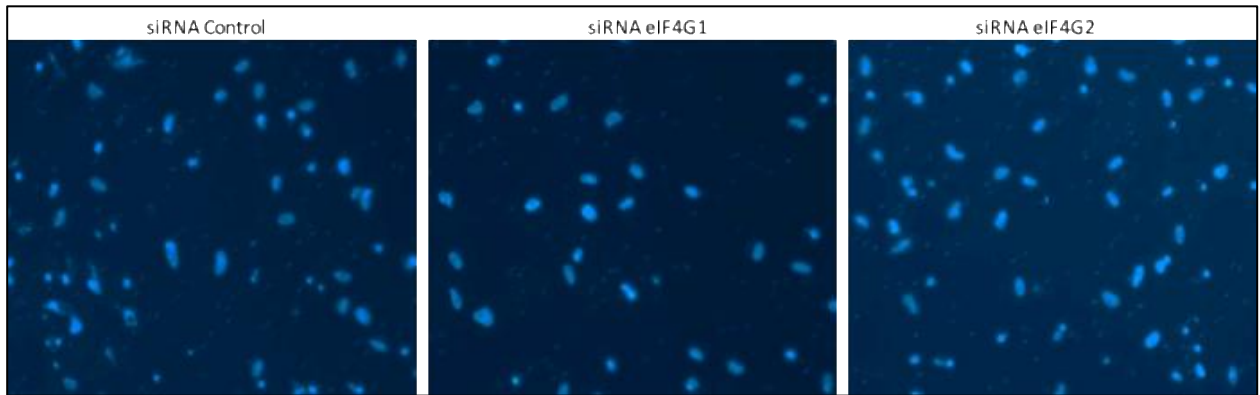
A**B**

Figure 4.6. Depletion of eIF4G1 and eIF4G2 inhibits epithelial cell migration. Control, eIF4G1 and eIF4G2–depleted T84 cell monolayers were mechanically wounded on day 4 posttransfection. To examine the rate of cell motility, the cell-free area was measured at 0 and 24h after wounding. Representative images of wounded monolayers (A) and quantification analysis of wound closure (B) are shown. Data are presented as mean \pm SE, n=3 *p < 0.01.

4.5 Depletion of eIF4G1 and eIF4G2 Decreases Individual Cell Migration of HIEC Cells.

Wound healing assays captures just a single mode of collective cell migration. Therefore, we sought to expand our study to examine the roles of eIF4G1 and eIF4G2 in regulation of individual cell motility that could be essential for tumor cell dissemination. Individual motility of control, eIF4G1, or eIF4G2-depleted HIEC cells was examined by monitoring transfilter cell migration in the Boyden chamber. We found that depletion of either eIF4G1, or eIF4G2 significantly decreased individual migration of HIEC cells with eIF4G1 depletion being more potent inhibitor of cell motility. Overall, these experiments suggest that eIF4G1 and eIF4G2 play non-redundant roles in regulating collective and individual migration of intestinal epithelial cells in vitro. eIF4G2 appears to be universal regulator of cell motility, whereas the involvement of eIF4G1 is more limited and depends on the experimental conditions(Fig.4.7).

A



B

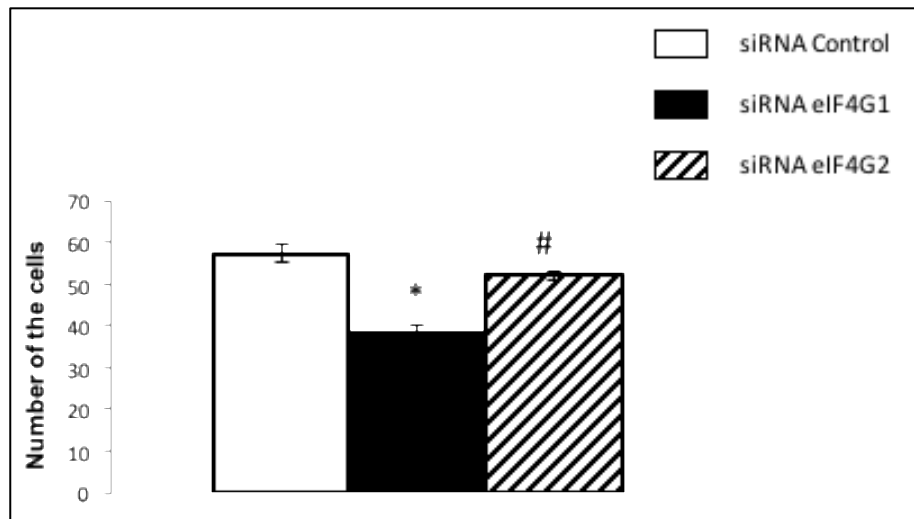
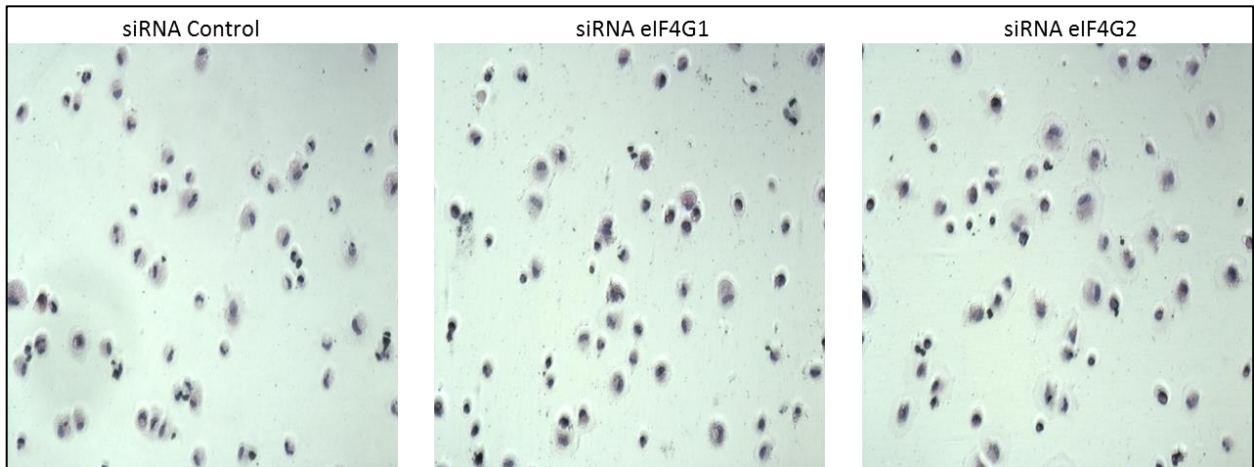


Figure 4.7. Knockdown of eIF4G1 and eIF4G2 decrease transfilter migration of HIEC cells. HIEC Cells were transiently transfected with Control, eIF4G1, eIF4G2 siRNA. As described in materials and methods, Control, eIF4G1, and eIF4G2 depleted HIEC cells were subjected to Transfilter migration assay. The bar graph represents the number of cells migrated between control, eIF4G1, and eIF4G2 depleted HIEC cells. Data presented as mean \pm SE, $n=3$ * $p < 0.01$, # $p < 0.05$.

4.6 eIF4G1 and eIF4G2 do not Regulate Cell- ECM Adhesion, while eIF4G2 is Essential Regulator of Cell Spreading

Since cell migration is a multistep process we next sought to investigate which steps could be regulated by eIF4G1 and eIF4G2 in motile intestinal epithelial cells. Cell adhesion to the ECM and cell spreading over ECM are initial steps of cell migration and are considered as the rate limiting steps, we investigated how these events could be affected by eIF4G1 and eIF4G2 knockdown in HIEC cells. Figure 4.8 shows that transient depletion of eIF4G1 and eIF4G2 had no effect on the adhesion of HIEC cells to collagen I matrix. Furthermore, loss of eIF4G1 did not affect cell spreading on collagen I (Fig. 4.9). By contrast depletion of eIF4G2 resulted in a dramatic increase in the surface area of attached HIEC cells, thereby indicating increased cell spreading (figure 4.9).

A



B

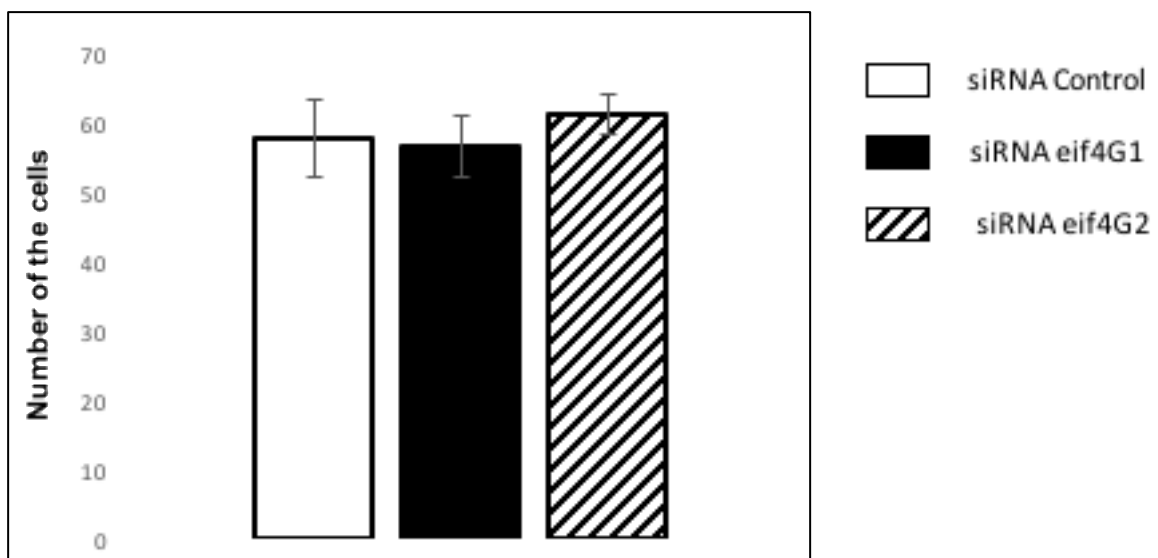
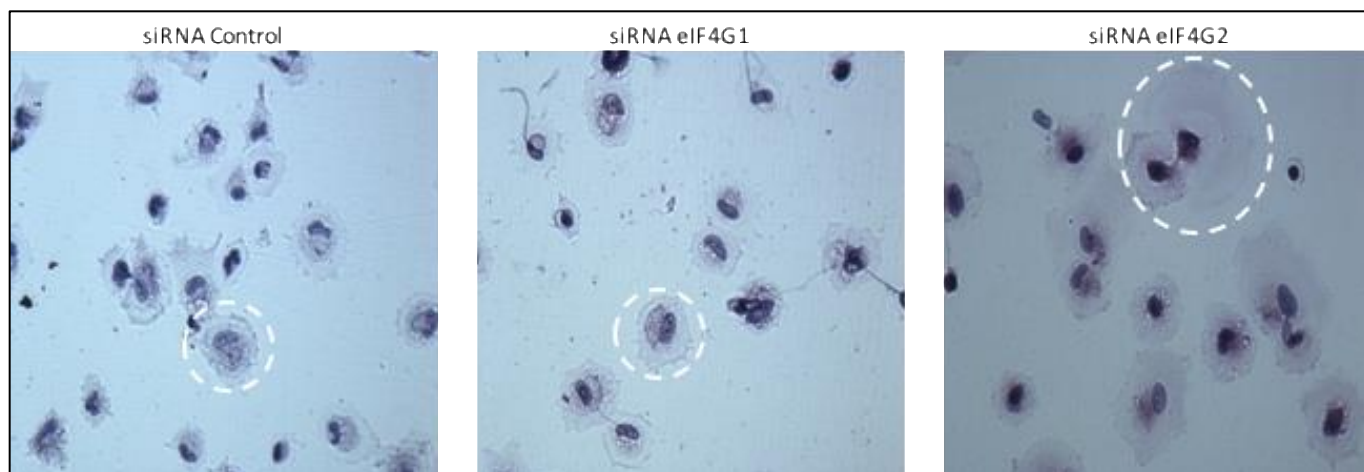


Figure 4.8. Knockdown of eIF4G1/eIF4G2 does not effect on epithelial cell-matrix adhesion. HIEC Cells were transiently transfected with Control, eIF4G1, eIF4G2 siRNA. The adhesion assay was performed using Control, eIF4G1, eIF4G2 knockdown cells on the 4th day post transfection. Note that there is no difference in the number of the cells adhered to the matrix between eIF4G1 and eIF4G2 cells as compared to control cells. Fig.14A shows the images for stained Control, eIF4G1, eIF4G2 knockdown cells. Fig.14B shows the quantification of the adhesion assay.

A



B

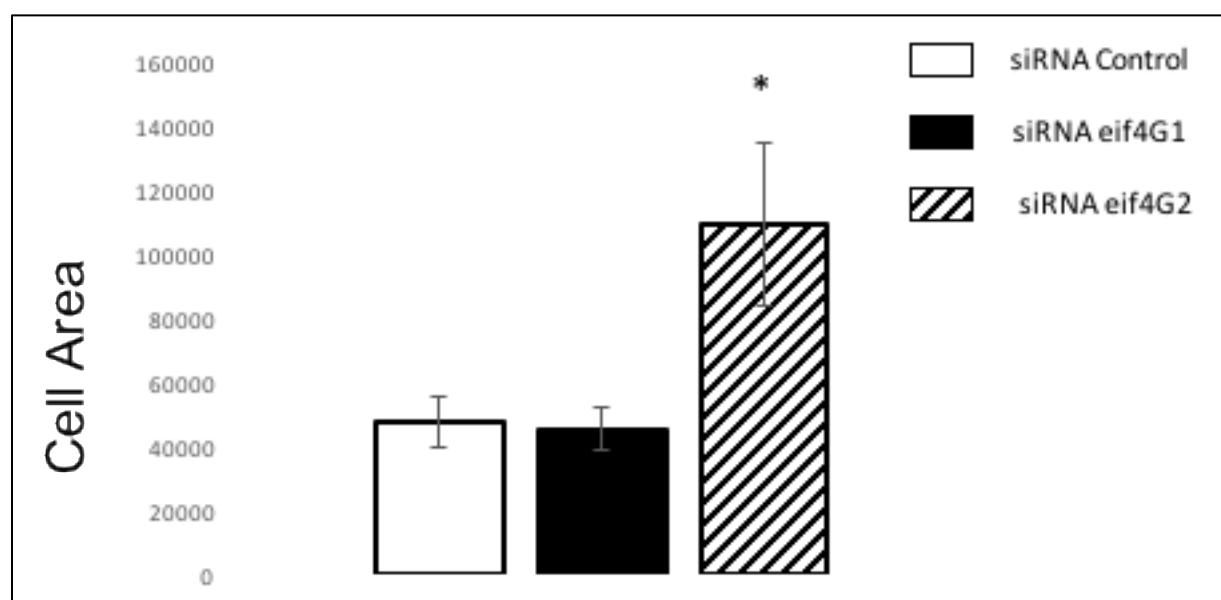
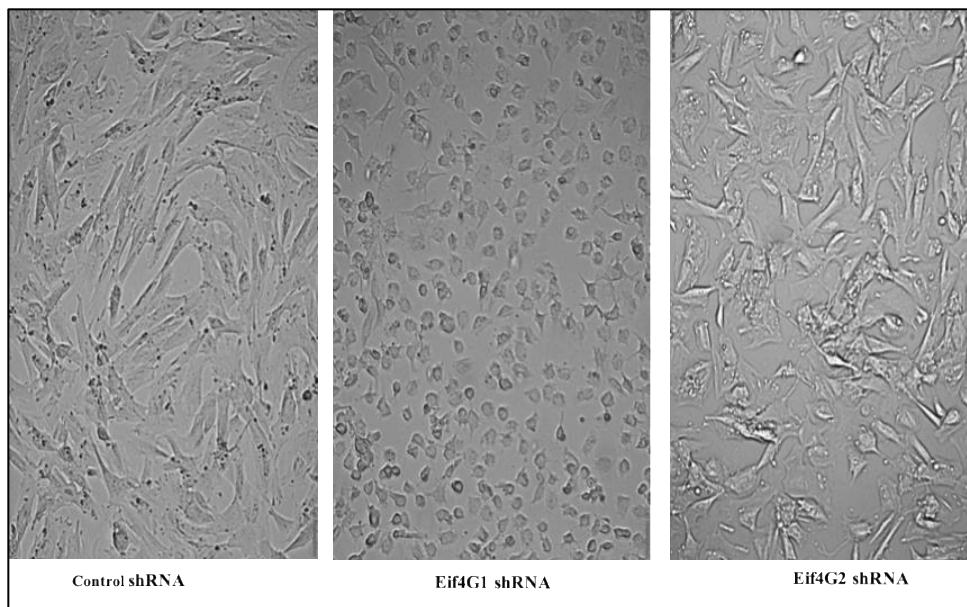


Figure 4.9. eIF4G1, eIF4G2 knockdown accelerate cell spreading in HIEC cells. HIEC cells were transiently transfected with Control, eIF4G1, and eIF4G2 siRNA. The cell spreading assay was performed on the 4th day post transfection. 10^7 cells were loaded onto 24-well plates which were coated with Collagen type-1 overnight and were allowed to settle for one hour. Images were obtained by using high power microscope. Note that the eIF4G2 knockdown cells spread faster as compared to the control cells. Data presented as mean \pm SE, $n=3$ * $p < 0.01$.

4.7 Loss of eIF4G1 Triggered Loss of Myoepithelial Phenotype in Stem Cell-Like Intestinal Epithelial Cells

While working with HIEC cells depleted of either eIF4G1, or eIF4G2 we made a serendipitous observation that eIF4G1 deficiency caused dramatic alterations in cell shape. Indeed, control HIEC cells displayed an elongated spindle-like shape that is characteristic for either mesenchymal, or myoepithelial cells. By contrast, eIF4G1-depleted HIEC cells become orthogonal-shaped, which is more characteristic of epithelial cells (figure 4.10A). No significant alterations in cell shape was observed after eIF4G2 knockdown (figure 4.10A). In order to gain insights into molecular mechanisms that underline such dramatic cell shape changes, we performed immunoblotting analysis of expression of mesenchymal (vimentin) and myoepithelial (α -SMA, L-Caldesmon, and Sm 22) markers in these cells. Interestingly, eIF4G1 depletion did not change vimentin expression, but decreased the levels of all tested myoepithelial markers (figure 4.10B). Expectedly, no changes in expression of either mesenchymal or myoepithelial markers was observed in eIF4G2-depleted HIEC cells. Interestingly, loss of myoepithelial markers caused by eIF4G1 depletion was not accompanied by the induction of classical E-cadherin (data not shown), which suggests that we did not observe a myoepithelial-to-epithelial transformation. Instead, loss of eIF4G1 is likely to transform HIEC in some yet to be characterized poorly-differentiated state. Importantly, all tested myoepithelial markers are important organizers and regulators of the intracellular actin cytoskeleton. Loss of these proteins is likely to disrupt normal organization of actin filament thereby contributing to inhibited motility of eIF4G1-depleted HIEC cells.

A



B

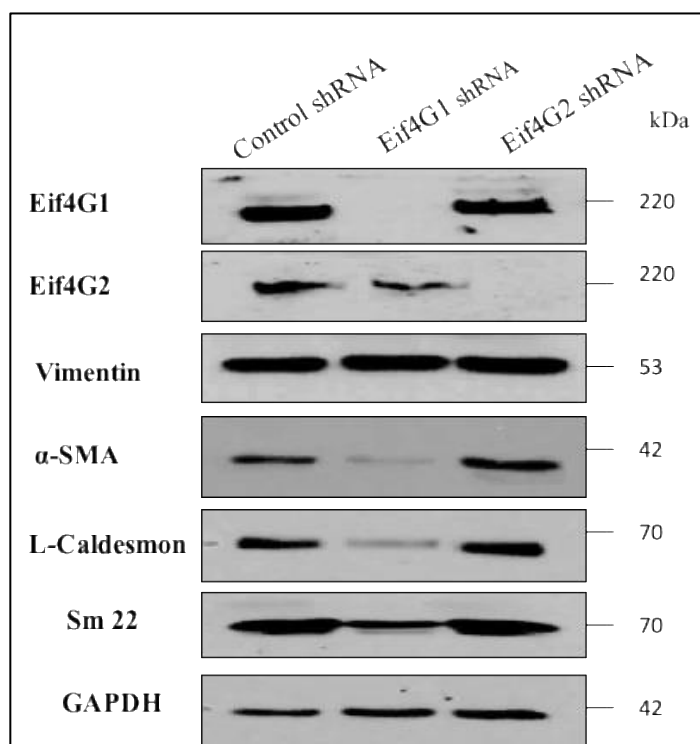


Figure 4.10. Altered cell shape and decreased expression of myoepithelial markers in eIF4G1-deficient HIEC cells. (A) The effect of eIF4G1, or eIF4G2 depletion on HIEC cell shape was examined by using bright-field microscopy. B) Immunoblotting analysis of expression of mesenchymal and myoepithelial markers in total lysates of control, eIF4G1, or eIF4G2-depleted HIEC cells.

5. DISCUSSIONS

5.1 Proinflammatory cytokines and their effect on translation of AJs and TJs proteins in intestinal epithelial cell monolayers; and on the protein translation initiation machinery.

A number of previous studies[41, 54], and our initial interesting finding results (Fig.3.1, 3.2A), strongly suggest that proinflammatory cytokines disrupt the integrity of the intestinal epithelial barrier via expressional down-regulation of different AJ and TJ proteins. Such downregulation can occur without inhibition of mRNA transcription or enhanced degradation of junctional proteins[55-57] and is shown in Figure 3.2B. These findings are consistent with conclusion of our previous study on pancreatic epithelial cells (HPAF-II) [6], which demonstrated that the treated HPAF-II cells with $IFN\gamma$ alone or the combination of $IFN\gamma$ plus $TNF\alpha$ weakens the pancreatic epithelial barrier by inducing the disassembly of pancreatic AJ and TJ proteins. Also, the expression of different AJs and TJs were downregulated by exposure to proinflammatory cytokines.

Then, we determined the effects of proinflammatory cytokines on the protein translation initiation machinery. We examined if cytokine-induced expressional downregulation of different AJs and TJs proteins is accompanied by inhibition of eIF4G-dependent translation initiation in the intestinal epithelium. Expression of eIF4G1 and eIF4G2 isoforms was compared in control and $IFN\gamma$ alone or combination with $TNF\alpha$, in treated HT-29 and T84 cells, along with expression of other components of the eIF4F cap-dependent translation initiation complex (eIF4E). We observed that expression of intestinal epithelial eIF4G1, eIF4G2 and eIF4E is downregulated by proinflammatory in vitro. Likewise, we demonstrated the relevance of these findings to human IBD, and we performed dual immunolabeling of eIF4G2 and β -catenin in affected colonic sections of patients with a B2 Montreal classification of Crohn's disease. We also used normal colonic section from patients undergoing intestinal surgery. We found that, in normal colonic mucosa, eIF4G2 was abundantly expressed in epithelial cells (Figure 3.4, arrows). In contrast, labeling intensity of this translational initiator was decreased in IBD mucosa (Figure 3.4 arrowheads). This finding is consistent with in vitro result in Figure 3.3, [58, 59] and

suggests that mucosal inflammation decreases levels of eIF4G1 and eIF4G2 in intestinal epithelium in vivo.

Our results (figures 3.3 and 3.4) are consistent with a previous study that demonstrated eIF4G1 control of p120 catenin expression in IBC (inflammatory breast cancer). Overexpression of eIF4G1 was observed in IBC, which is responsible for the pathological features of IBC such as, overexpression of E-cadherin, strong cancer cell interaction, tumor emboli formation, and IBC cell invasion. However, the downregulation of eIF4G1 will block formation of tumor cell emboli by impairing cap-dependent mRNA translation of p120 [45].

In addition, there are many evidences indicating that the overexpression of eIF4G1 has an oncogenic role and promotes the process of tumorigenesis. For example, overexpression of eIF4G1 has been seen in different types of cancers: lung cancer, hypopharyngeal cancer, nasopharyngeal carcinoma (NPC), and IBC [45, 48, 60, 61].

Since our finding shows inhibition of eIF4G1 and eIF4G2 protein expression in cytokine- treated epithelial cells, there are two possible mechanisms of inhibition which we will explore in the future. The first mechanism comprises transcriptional downregulation of eIF4G isoforms, whereas another mechanism involves proteolytic degradation of these translational initiators. These two possibilities are consistent with previous studies which demonstrated caspase and calpain-dependent cleavage of eIF4G1 and eIF4G2 under different experimental conditions [62-64].

5.2 eIF4G isoforms and their roles in regulating the structure and permeability of model intestinal epithelial barriers.

Given our previous published paper, [6] it has been shown that the proinflammatory cytokines induce permeability of human pancreatic epithelial monolayers, and that there is a decrease of the integrity of epithelial barrier which is revealed by decrease the TEER measurements. We want to investigate if the depletion of eIF4G isoforms, which correlates with downregulation in the expression of different AJs and TJs, has an effect on the integrity of intestinal epithelial barrier and permeability. The results of this finding strongly suggest that the eIF4G isoforms (eIF4G1 and eIF4G2) have the important role in: regulation of the integrity and assembly of

epithelial AJs and TJs; and permeability of model intestinal epithelial barriers. This result is supported by two lines of evidence. The first is a significant decrease the expression of AJ and TJ proteins in depletion of eIF4G isoforms in intestinal epithelial cell monolayers (Figures 3.5A and 3.5B). The second evidence is that the eIF4G isoforms depletion prevents the formation of the paracellular barrier and attenuates AJ and TJ assembly. Also, eIF4G isoforms are known to control the translation of not only AJ and TJ components, but other different proteins [29, 65, 66]. our experiments were designed to minimize the possible effects of altered cell motility and proliferation on junctional regulation. We have used two different approaches. One approach was involved in transient knockdown of eIF4G1 or eIF4G2 in SK-CO15 cells and HT 29 cells [67, 68]. The second approach has involved the creation of stable inducible knockdown of eIF4G isoforms in SK-CO15 cells using a lentiviral transduction system [69]. As described in our recent lab papers [6, 70], paracellular permeability was examined by measuring transepithelial electrical resistance (TEER) in siRNA transient knockdown for both eIF4G1 and eIF4G2, and shRNA-mediated knockdown for both eIF4G1 and eIF4G2. We found that TEER measurements demonstrate that the transient knockdown of eIF4G1 in SK-CO15 monolayers fails to develop the paracellular barrier and attenuates AJ and TJ assembly (figure 3.6). These findings agreed with the result of the shRNA-mediated depletion of eIF4G1 and eIF4G2 which shows that both depletion of eIF4G1 and eIF4G2 prevents the formation of the paracellular barrier in SK-CO15 cell lines (figure 3.7). Generally, these findings are supported previous studies which suggested that the IBD and different intestinal and colorectal cancers increase the intestinal permeability [12].

5.3 eIF4G isoforms localization.

The localization of translational initiation factors is considered as a significant mechanism by which proteins were derived to their functional sites within cells [47]. Given previous studies from various groups of researchers who found that the protein translation machinery localizes on the leading edge of migrating fibroblasts [47, 50, 71], we want to investigate if similar actions happen during migration of intestinal epithelial cell monolayers. The protein translation on the leading edge was assessed by pulse-labeling of the cells with puromycin and immunolabeling of newly-translated proteins with anti-puromycin mAb, [46] as shown in Figure 4.1. We confirmed this result by the subsequent experiment which used wounded HIEC cell monolayers and we

immunolabeled it for eIF4G2 using commercially available antibodies. We found that eIF4G2 is localized on the cytoplasm of the leading edge of the migrating cell (figure 4.2B) compared to the stationary cell which has eIF4G2 localized in the nucleus (figure 4.2A). This finding suggests that the translation initiation machinery is active on the leading edge of migrating cells.

5.4 Pharmacological inhibitors and their roles in cell migration and regulating the structure and permeability of model intestinal epithelial barriers.

There are several multiple inhibitors which either work by the interaction between eIF4G and eIF4E or between the 5' cap of mRNA and eIF4E [52, 72, 73]. Ribavirin is one of the pharmacological inhibitors, which is considered as antiviral drug and structurally mimics to 5' cap of mRNA thus competing with eIF4E binding. It has been established through various studies that Ribavirin attenuates the migration and invasion of lung and breast cancer cells in vitro by reducing the wound closure. Also, this drug reduces the metastasis of pulmonary cancer cells, and blocks the growth of tumor cells in vivo. Additionally, it suppresses the TGF β -induced EMT and changes the shape of cells to be more epithelial-like round morphology in both NSCLC and in breast cancer cell lines by downregulating the expression of mesenchymal markers [74-76]. Another pharmacological inhibitor is eIF4E/eIF4G interaction inhibitor 1 (4EGI-1), which is considered as an antitumor agent. It works allosterically by binding to eIF4E and prevents the interaction between eIF4E and eIF4G. Recent studies investigated the negative role of 4EGI-1 in breast CSCs (cancer stem cells) by inhibiting their proliferation and invasion. Also, 4EGI-1 inhibits breast CSC by inducing the apoptosis, and suppressing tumor angiogenesis and tumor growth in vivo [73, 77, 78]. In our study, we examined the effects of Ribavirin and 4EGI-1 on the collective cell migration of intestinal epithelial monolayers, which involves HIEC (poorly differentiated cells) and T84 (well differentiated cells). We found that both Ribavirin and 4EGI-1 decrease the collective cell migration in both cell lines compared to the control (figures 4.3 and 4.4). These findings agree with previous studies which indicate the negative role of Ribavirin on cell migration [74, 76].

5.5 eIF4G isoforms and their effect on motility, ECM adhesion, and morphology of intestinal epithelial cells.

Depletion of eIF4G isoforms proteins in intestinal epithelial cells was found to result in attenuated collective cell migration (fig. 4.5 and 4.6) and decreased transfilter migration of individual cells (fig. 4.7). In the case of collective cell migration, there are two different results depending on the type of intestinal epithelial cell lines. For the HIEC cells, which is stem-cell-like, the collective cell migration significantly decreased in knockdown of only eIF4G2. However, in T84 cell lines (well-differentiated cells), the wound closure was attenuated in depletion of both eIF4G1 and eIF4G2. These results agree with previously published study which reports that eIF4G1 significantly attenuates the wound closure of NSCLC cell [74]. Our findings regarding molecular mechanisms that underlie the inhibited migration of eIF4G isoforms knockdown in intestinal epithelial cells created unsolved questions. The first mechanism, loss of eIF4G1 or eIF4G2 can decrease cell motility via downregulation of different FA proteins. Another mechanism is perhaps by inhibiting of Rac1 GTPase and its down-stream kinase PAK1, which is known to phosphorylate paxillin along with Src and FAK [79]. Because the ECM adhesion and cell spreading are the rate limiting steps of cell migration, we have supported our finding results by examining the cell adhesion and cell spreading in HIEC cells. We found that there is no change in the ECM adhesion over collagen type 1 in depletion of eIF4G isoforms, whereas the cell spreading was significantly increased in eIF4G2 depletion (fig. 4.8 and 4.9). These findings maybe explain the decreased velocity of cell migration in HIEC cells. In order to investigate if knockdown of eIF4G isoforms has an effect on EMT, we examined the morphology of the confluent HIEC cells in shRNA-mediated knockdown of both eIF4G1 and eIF4G2. We found that in the case of eIF4G1, the cells become more epithelial-like rounded cells compared to control and eIF4G2 depletion, which appear as a more fibroblast-like spindle shape (fig. 4.10A). Accordingly, we examined the expression of different myo-epithelial markers by immunoblotting. We found that the decrease in the expression of myo-epithelial markers, which involve alpha-SMA, L-caldesmon, and sm22, in eIF4G1 depletion. However, there is no change in one of EMT marker vimentin (fig.4.10B). These findings are consistent with the previous study which suggest that knockdown of eIF4G1 in NSCLC cell lines is induced by changing the morphology of the cells to be more epithelial-like round morphology[74].

6. CONCLUSION

our study suggests that loss of intestinal epithelial eIF4G isoforms play essential roles in disruption of the mucosal barrier by decreasing the expression of different AJs and TJs proteins; and attenuating epithelial integrity and restitution by inducing disassembly of intestinal epithelial TJs and AJs. Furthermore, the loss of eIF4G isoforms markedly attenuates epithelial wound closure depending on the type of the cells. Also, the depletion of eIF4G isoforms decreases the transfilter migration of individual cells. Finally, our studies have shown that knockdown of eIF4G1 and eIF4G2 have no effect on ECM adhesion, whereas the depletion of eIF4G2 has a positive effect on cell spreading by increasing the cell surface area. However, further studies need to confirm this hypothesis.

7. Future Directions

In order to study the effect of eIF4G isoforms depletion with different intestinal epithelial cell lines, HIEC, HT-29, SK-CO15 and T84 with stable shRNA-mediated knockdown and lentiviral CRISP/CAS9 knockout system of either eIF4G1 or eIF4G2 will be used to perform all the mentioned assays in this thesis. Also, we will overexpress select AJ and TJ proteins (p120 catenin, E-cadherin, claudin-4, and some others) in eIF4G1 or eIF4G2-depleted epithelial cells using an internal ribosomal entry site (IRES) to determine if overexpression of junctional proteins can restore barrier function and junctional integrity in eIF4G- deficient cells. Additionally, we want to elucidate if overexpression of eIF4G isoforms ameliorates cytokine-induced defects of apical junctions and disruption of the epithelial barrier. These gain of function experiments have two major goals. One is to examine if the increased expression of eIF4G1 and eIF4G2 can enhance normal epithelial barrier. The other goal is to demonstrate a causal role of eIF4G isoform downregulation in the cytokine-induced breakdown of the model intestinal epithelial barrier. Also, we are interested in determining the effects of chronic mucosal inflammation on global protein translation and the translation initiation machinery, in vivo.

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