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Posttranscriptional Regulation of Intestinal Epithelial Integrity by Noncoding RNAs

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Abstract

Maintenance of the gut epithelial integrity under stressful environments requires epithelial cells to rapidly elicit changes in gene expression patterns to regulate their survival, adapt to stress and keep epithelial homeostasis. Disruption of the intestinal epithelial integrity occurs commonly in patients with various critical illnesses, leading to the translocation of luminal toxic substances and bacteria to the blood stream. Recently, noncoding RNAs (ncRNAs) have emerged as a novel class of master regulators of gene expression and are fundamentally involved in many aspects of gut mucosal regeneration, protection, and epithelial barrier function. Here we highlight the roles of several intestinal epithelial tissue-specific microRNAs, including miR-222, miR-29b, miR-503, and miR-195, and long ncRNAs such as *H19* and *SPRY4-IT1* in the regulation of cell proliferation, apoptosis, migration, and cell-to-cell interactions and also further analyze the mechanisms through which ncRNAs and their interactions with RNA-binding proteins modulate the stability and translation of target mRNAs.

Keywords

Intestinal epithelial homeostasis; gut epithelial barrier; microRNAs; long noncoding RNAs; RNA-binding proteins; polyamines

Introduction

The mammalian gut epithelium is a rapidly self-renewing tissue in the body, which is a complex process including active proliferation of intestinal stem cells near the base of the crypts, migration of these newly divided cells up along the crypt-villus axis with differentiation, and apoptosis.^{1,2} Under physiological conditions, integrity of the intestinal epithelium fully depends on a dynamic balance among intestinal epithelial cell (IEC) proliferation, migration, differentiation, and apoptosis.^{3,4} The human epithelium of the small intestine undergoes $\sim 10^{11}$ mitoses per day and this rapid rate of cell proliferation is

counterbalanced by apoptosis, which is tightly regulated at multiple levels through many extracellular and intracellular factors. Apoptosis occurs at the crypt area to maintain the balance in cell number between newly divided and surviving cells, and it also occurs on the luminal surface of the intestine, where differentiated cells are lost.⁵ After acute injury, the intestinal mucosa exhibits a spectrum of responses and repairs itself rapidly to restore the epithelial integrity.⁶ Disruption of the gut epithelial integrity occurs commonly in patients with critical diseases such as trauma, burns, hemorrhage, and sepsis or after massive surgical operation, leading to the translocation of luminal toxic substances and bacteria to the blood stream.⁷

Maintenance of the intestinal epithelial integrity under stressful environments requires epithelial cells to rapidly elicit changes in the patterns of gene expression to modulate their survival, adapt to stress and keep epithelial homeostasis.^{8,9} Although the gene expression programs that control the gut epithelial integrity are strongly regulated at the transcription level, the essential contribution of posttranscriptional events is also increasingly recognized.^{10–12} In particular, changes in mRNA stability and translation play an important role in regulating the intestinal mucosal homeostasis.^{13,14} Control of mRNA stability and translation requires specific mRNA sequences (*cis*-element) that directly associate with *trans*-acting factors including RNA-binding proteins (RBPs)^{15,16} and noncoding RNAs (ncRNAs) such as microRNA (miRNAs).^{17,18} Several RBPs, including human antigen R (HuR, encoded by *Elavl1*), CUG-binding protein 1 (CUGBP1, also named as CELF1, CUGBP and embryonic lethal abnormal vision-like factor 1), AU-binding factor 1 (AUF1), and TIA-1 related protein (TIAR), are highly expressed in IECs and shown to regulate the turnover and translation of mRNAs encoding proteins regulating IEC proliferation and apoptosis such as p53, XIAP, MEK-1, ATF-2, cyclin-dependent kinase 4 (Cdk4), Myc, and JunD, thus modulating the intestinal epithelial integrity as summarized and reviewed recently.^{19,20}

The mammalian genome is majorly transcribed to a large transcriptome of ncRNAs, whereas protein-coding sequences only account for a minority of the cellular transcriptional output.^{21,22} The functional relevance of ncRNAs in the regulation of intestinal epithelial functions and homeostasis is particularly evident for miRNAs.^{23,24} However, other ncRNAs, such as small nucleolar RNAs, PIWI-interacting RNAs, transcribed ultraconserved regions, and large intergenic ncRNAs and overall, the heterogeneous groups of long ncRNAs (lncRNAs), are also involved in many aspects of cellular processes, physiology, and human diseases.²⁵ Here we briefly highlight the importance of several intestinal epithelial tissue-specific ncRNA including miRNAs and lncRNAs in IEC proliferation, apoptosis, migration, and cell-to-cell interaction, and further analyze in some detail the mechanisms through which ncRNAs regulate the stability and translation of their target mRNAs. We also summarize the biological functions of interactions between the RBPs and ncRNAs in posttranscriptional gene regulation and intestinal epithelial integrity under various pathophysiological conditions.

miRNAs in Intestinal Epithelial Homeostasis and Diseases

miRNAs are small ncRNAs of ~22 nucleotides and posttranscriptionally repress the expression of various genes by directly interacting with the 3'-untranslated region (UTR) of target mRNA.^{17,26} Several high-throughput and functional studies show that miRNAs are crucial for distinct cellular functions and play an important role in the pathogenesis of various human diseases such as cancers, heart failure, tissue injury/repair, and inflammation. For example, disrupted expression patterns of miRNA-21, miRNA-26, miRNA-31, miRNA-141, miRNA-145, miRNA-196, and miRNA-200 occur commonly in colon cancers, and they modulate cell motility and cancer invasiveness.²⁷ The settlement of colon cancer cells in the liver parenchyma is also suppressed by miRNA-493.²⁸ In addition, the expression levels of Dicer1, a key factor required for the process of miRNA biogenesis, decrease during the progression in gastric cancer.²⁹ Recently, miRNAs have also emerged as master biological regulators of the gut epithelial homeostasis and regeneration.^{24,30} We have profiled global miRNA expression in cultured IECs and the gut mucosa in mice and found that several miRNAs, including miRNA-222, miRNA-29b, miRNA-322, miR-503, and miR-195, are highly expressed in the intestinal epithelium *in vitro* as well as *in vivo* and that food starvation or polyamine depletion dramatically alters expression levels of these gut epithelial tissue-specific miRNAs. Our further studies demonstrate that control of cellular levels of these miRNAs and their affinities for target mRNAs plays a critical role in the maintenance of normal intestinal epithelial homeostasis and integrity.^{31,32}

miR-222

miR-222 (also referred as miR-222-3p) is highly conserved in many species, which is clustered with miR-221 in tandem on the X chromosome.³³ Several studies have demonstrated that miR-222 modulates distinct cellular processes and functions in various cell types.^{34,35} During cancer development, miR-222 can act as either tumor-suppressive or oncogenic, depending on cellular content and tumor type. Although miR-222 plays a positive feedback loop to enhance expression of RelA and STAT3 in human colorectal cancer cells,³⁶ it represses the expression of estrogen receptor- α (ER- α) in 3T3-L1 adipocytes.³⁷ Our studies show that increased levels of miR-222 by fasting or polyamine depletion inhibit normal IEC proliferation at least partially by repressing Cdk4 expression.³¹ Cdk4 is necessary for the activation of cell progression through the cell cycle, whereas Cdk4 silencing inhibits the transition from G1 to S phase after mitogenic stimulation.³⁸ The coding region (CR) of the *Cdk4* mRNA contains two binding sites of miR-222 as confirmed by RNA pull-down assays using biotin-labeled miR-222.³¹ Ectopically expressed miR-222 inhibits Cdk4 expression, and this repression occurs at the translation level. Induced miR-222 decreases the abundance of *Cdk4* mRNA associated with actively translating fractions of polyribosome gradients and reduces the levels of newly synthesized Cdk4 protein, although it does not affect total *Cdk4* mRNA level. Studies using luciferase reporter constructs containing *Cdk4* CR and its 3'-UTR reveal that increased miR-222 inhibits the activity of Cdk4-CR luciferase reporter (containing miR-222 site) but fails to alter the activity of Cdk4-3'UTR reporter (without miR-222 site), indicating that miR-222 represses translation of the *Cdk4* mRNA through interaction with the Cdk4 CR, rather than its 3'-UTR. Interestingly, the miR-222 association with the *Cdk4* mRNA is inhibited by cellular

polyamines, and increasing the levels of polyamines by transfection with the *Odc* gene, encoding the key enzyme of polyamine biosynthesis,³⁹ inhibits the formation of miR-222/*Cdk4* mRNA complex and enhances Cdk4 translation, thus stimulating IEC proliferation. In contrast, polyamine depletion increases association of miR-222 with the *Cdk4* mRNA and inhibits Cdk4 expression at the translation level, leading to the inhibition of IEC division.

To determine the *in vivo* functions of miR-222 in the intestinal mucosa, a gain-of-function transgenic approach was used by generating intestinal epithelial tissue-specific miR-222 transgenic (miR222-Tg) mice. As shown,⁴⁰ miR222-Tg mice exhibit overexpression of miR222/GFP bicistronic RNA specifically in the mucosal tissues of the small intestine, colon, and caecum but it is undetected in stomach mucosa, liver, spleen, kidney, and thymus. There are no significant differences in body weight and gastrointestinal gross morphology in miR222-Tg mice compared with control littermates. The first phenotype observed in miR222-Tg mice is that the miR-222-elevated epithelium displays a significant mucosal atrophy in the small intestine, as evidenced by a decrease in the rate of DNA synthesis and the lengths of villi and crypts. Abnormal histological features of the small intestinal mucosa are also observed as indicated by disrupted crypt structure and increased proliferation of connective tissue in the crypt area in miR222-Tg mice. Consistent with observation *in vitro*,³¹ the levels of Cdk4 and other cell proliferation markers such as PCNA and Ki67 in the small intestinal mucosa also decrease in miR222-Tg mice. The second phenotype detected in miR222-Tg mice is that ectopically expressed miR-222 increases the susceptibility of intestinal epithelium to induced apoptosis after exposure to TNF- α , although local miR-222 overexpression does not directly induce cell death. Third, the miR-222-elevated intestinal epithelium is vulnerable to pathological stress, since increased levels of miR-222 not only delay mucosal repair after mesenteric ischemia/reperfusion (I/R)-induced injury but also exacerbate the induced gut permeability in mice exposed to cecal ligation and puncture (CLP). Increased miR-222 also inhibits expression of the Wnt receptor Frizzled-7 (FZD7) in the mucosal tissue. Studies further show that the *Fzd7* mRNA is a novel target of miR-222, and induced miR-222/*Fzd7* mRNA association represses FZD7 translation without effect on *Fzd7* mRNA stability. These results clearly show that miR-222 functions as an important negative regulator of normal intestinal epithelial regeneration and protection by down-regulating Wnt signaling pathway through targeting the *Fzd7*. These findings also suggest that increased miR-222 contributes to the pathogenesis of gut mucosal atrophy, delayed healing of damaged mucosa, and intestinal epithelial barrier dysfunction.

miR-29b

miR-29b, a member of the miR-29 family, targets several mRNAs and is involved in many aspects of cellular functions. miR-29b interacts with the *dnmt3a* and *dnmt3b* mRNAs and represses their expression posttranscriptionally, thus modulating DNA methylation-related reprogramming events.⁴¹ miR-29b also enhances fibrosis by regulating expression of collagen isoforms.⁴² It has been reported that cell proliferation and apoptosis is also regulated by miR-29b in different cell types, and miR-29b also plays a critical role in the development of abdominal aortic aneurysm in mouse.⁴³ The abnormal expression of miR-29b occurs commonly during tumorigenesis and cancer progression, and disrupted expression of miR-29b affects the tumor microenvironment to slow-down metastasis.⁴⁴ In

addition, patients with advanced liver cirrhosis exhibit a decreased level of miR-29a in the serum. Deregulation of miR-29 expression is also implicated in the pathogenesis of type-1 diabetes, and in patients with irritable bowel syndrome, miR-29b targets interferon- γ by interacting with its mRNA, thus altering innate and adaptive immunity and gut permeability.⁴⁵ Our recent study shows that fasting or polyamine depletion increases mucosal miR-29b levels in the small intestine, which associates with an inhibition of mucosal growth.³² In contrast, decreasing the levels of miR-29b by silencing its gene expression stimulates mucosal renewal in the small intestine. Our efforts to identify miR-29b target mRNAs implicated in these processes reveal that miR-29b interacts with the 3'-UTR of the mRNA encoding Cdk2, rather than CDK4, and represses *Cdk2* mRNA translation.³²

Men1 mRNA, encoding menin protein, is also a target of miR-29b, and miR-29b/*Men1* mRNA association down-regulates menin expression posttranscriptionally in IECs. Overexpression of miR-29b lowers modestly the levels of *Men1* mRNA but reduces robustly the *de novo* synthesis of menin protein, whereas antagonization of miR-29b enhances menin protein synthesis and induces its steady-state levels.⁴⁶ This inhibition of miR-29b is mediated through a single binding site in the CR of *Men1* mRNA, since point mutation of the site abolishes miR-29b-induced repression of menin translation. Moreover, induced levels of cellular menin by miR-29b silencing increase the sensitivity of IECs to apoptosis induced by TNF- α plus cycloheximide (CHX), which is prevented by decreasing menin abundance. These observations suggest that miR-29b regulates IEC apoptosis by repressing translation of *Men1* mRNA, thus affecting the intestinal epithelial homeostasis.

We have recently reported that mRNAs, encoding Wnt co-receptor LRP6 and RBP HuR, also associate with miR-29b and their expression levels are tightly regulated by miR-29b at the posttranscriptional level.⁴⁷ miR-29b directly interacts with both *Lrp6* and *HuR* mRNAs via their 3'-UTRs, and ectopically expressed miR-29b destabilizes *Lrp6* and *HuR* mRNAs and represses their translation. This greater repressive effect of miR-29b on LRP6 and HuR expression is mediated through a single binding site within the *Lrp6* or *HuR* 3'-UTR, and deletion mutation of this site prevents miR-29b-induced repression of LRP6 and HuR expression. Repression of HuR by miR-29b, in turn, also contributes to miR-29b-induced LRP6 inhibition, since *Lrp6* mRNA is a target of HuR and association of HuR with the *Lrp6* mRNA enhances LRP6 expression.⁴⁸ In support of the notion, ectopic overexpression of HuR restores LRP6 expression to near normal level in cells overexpressing miR-29b.

To define the exact mechanism underlying the control of cellular miR-29b abundance, JunD was recently found to enhance miR-29b transcriptionally and post-transcriptionally in IECs.⁴⁹ Through its function as an activating protein-1 (AP-1) transcription factor, JunD is implicated in many aspects of cellular processes and inhibits IEC proliferation.⁵⁰ Ectopically expressed JunD specifically increases the levels of primary and mature forms of miR-29b, whereas JunD silencing inhibits miR-29b expression. JunD physically interacts with the miR-29b1 promoter via specific AP-1-binding sites, while mutation of these AP-1 sites from the miR-29b1 promoter blocks JunD-induced transcriptional activation of the *miR-29b1* gene. JunD also enhances formation of the Drosha microprocessor complex that is necessary for the cleavage of primary miRNAs into precursor miRNAs,⁵¹ thus also increasing miR-29b biogenesis. In polyamine-deficient cells, JunD silencing prevents the increased

levels of miR-29b, suggesting that cellular polyamines modulate miR-29b expression at least partially by altering JunD levels. Interestingly, JunD-induced repression of IEC proliferation is blocked by miR-29b silencing. These findings indicate that JunD-activated miR-29b expression contributes to the inhibitory effect of JunD on IEC proliferation and plays a role in the gut epithelium homeostasis. On the other hand, miR-29b transcription is also modulated by other transcription factors such as Myc, hedgehog, and NF- κ B via different binding sites within the miR-29b promoter.⁵²

miR-503

miR-503 is clustered with miR-322 on chromosome X and is highly conserved among many species. miR-503/322 had been well studied in myogenesis previously, and their levels increase during muscle differentiation, which associates with a decrease in the level of Cdc25A.⁵³ Deregulation of miR-503 expression also contributes to the pathogenesis of impairment of endothelial function under diabetic condition and reparative angiogenesis after limb ischemia.⁵⁴ It has been reported that the vascular smooth muscle cell phenotype and neointimal formation are tightly regulated by miR-503/322 and that miR-322 also alters osteogenesis by targeting Tob2 and *Osx*.⁵⁵ Our miRNA expression profiling studies reveal that small intestinal mucosal atrophy induced by polyamine depletion or food starvation is associated with a significant decrease in the levels of miR-503,³¹ suggesting its pro-proliferative role in the regulation of gut mucosal growth.

Further studies demonstrate that miR-503 enhances IEC proliferation and subsequent gut mucosal growth by increasing Myc through down-regulation of CUGBP1 expression.^{56,59} In addition to its well-defined role as a regulator of splicing, CUGBP1 destabilizes and represses translation of target transcripts by interacting with GU-rich element.⁵⁸ CUGBP1 is involved in many aspects of embryo-development and cellular processes such as responses to stress, proliferation, and apoptosis.⁵⁷ Target deletion of CUGBP1 is generally embryonic lethal, but the few CUGBP1-deficient mice that are born exhibit decreased cell viability, growth retardation, and spermatogenesis defects. CUGBP1 is also implicated in several other human diseases including cancer, fragile-X tremor/ataxia syndrome, and liver dysfunction. In mice fasted for 48 h, CUGBP1 levels in the small intestinal mucosa increases, which associates with an induction in CUGBP1/*Myc* mRNA complex and Myc repression.⁵⁹ In cultured IECs, CUGBP1 was found to bind the *Myc* 3'-UTR, and elevating the level of CUGBP1 leads to repression of Myc translation but has no effect on total *Myc* mRNA levels. Moreover, CUGBP1 represses Myc translation primarily by decreasing *Myc* mRNA association with HuR. Increased CUGBP1 results in G1-phase growth arrest of IECs, whereas CUGBP1 silencing enhances IEC proliferation. Ectopically expressed miR-503 reduces the levels of newly synthesized CUGBP1 protein in IECs, while miR-503 silencing enhances CUGBP1 biosynthesis and induces its abundance.⁵⁶ Overexpression of miR-503 increases *CUGBP1* mRNA levels in P-bodies, while silencing P-body resident proteins such as Ago2 or RCK reduces miR-503-mediated repression of CUGBP1 expression. This effect is also tightly regulated by cellular polyamines, since polyamine depletion decreases the levels of endogenous miR-503 and enhances CUGBP1 expression. Furthermore, miR-503-mediated CUGBP1 repression in turn alters the expression levels of

CUGBP1 target mRNAs such as *Myc*, thus increasing IEC proliferation and gut mucosal growth.

miR-503/322 also modulates IECs apoptosis via TGF- β /Smad2 signaling pathway by targeting Smad ubiquitin regulatory factor 2 (Smurf2) which is an E3 ubiquitin ligase. Following ligand binding, the activation of TGF- β receptors phosphorylates Smad proteins and enhances their nuclear translocation, thereby altering transcription of target genes.⁶⁰ To exert the functions of different ligands in a timely manner, the TGF- β -activated Smads must be rapidly removed through a process of ubiquitin-dependent degradation. It has shown that Smurf2 directly associates with phosphorylated Smads and mediates their rapid ubiquitination and degradation. By modulating TGF- β /Smad2 signaling, Smurf2 is implicated in many aspects of cellular processes and pathologies. For example, Smurf2 enhances senescence in human fibroblasts, promotes p53 degradation by stabilizing the E3 ligase MDM2, and represses tumor growth by regulating the chromatin landscape and genome stability via RNF20.⁶¹ We have recently reported that miR-503/322 posttranscriptionally regulate Smurf2 expression in IECs.¹⁴ Both miR-503 and miR-322 bind to the *Smurf2* mRNA via through a single binding site within its 3'-UTR, and this interaction inhibits Smurf2 translation without effect on total *Smurf2* mRNA levels. Increased levels of endogenous Smurf2 by decreasing miR-503 and miR-322 abundances inhibit TGF- β -induced Smad2 activation by enhancing phosphorylated Smad2 degradation. Forced expression of Smurf2 in miR-503/322-deficient cells increases the resistance to TNF α /CHX induced apoptosis, which is abolished by decreasing Smurf2 levels. These results indicate that miR-503/322 modulate TGF- β /Smad2 signals in IECs through inhibition of Smurf2 translation, thus regulating intestinal epithelial homeostasis by altering IEC apoptosis.

miR-195

miR-195 is highly abundant in normal tissue, but its levels decrease dramatically in cancers.^{62,63} Several studies show that miR-195 is a biological repressor of mucosal growth through distinct mechanisms by targeting multiple genes. miR-195 inhibits cell proliferation by targeting Cdk4, cyclin D1, Cdk6, and WEE1^{62–64}, promotes apoptosis by down-regulating Sirt1⁶⁵, and affects cell motility and cancer invasion by modulating ActRIIA expression.⁶⁶ We have found that miR-195 regulates early rapid intestinal epithelial restitution after wounding by modulating expression of stromal interaction molecule 1 (Stim1) at the posttranscriptional level.⁶⁷ Stim1 acts as the Ca²⁺ sensor in the endoplasmic reticulum (ER) and activates TRPC1- and Orai1-mediated Ca²⁺ influx after store depletion.⁶⁸ Stim1 is primarily located in the ER in unstimulated cells, but it rapidly translocates to the plasma membrane after Ca²⁺ store depletion or exposure to stressful environments. In the plasma membrane, Stim1 associates with and activates TRPC1 and Orai1 channels, leading to an induction in Ca²⁺ influx through store-operated Ca²⁺ entry (SOCE). Inhibition of the Stim1 expression reduces SOCE activation and inhibits IEC migration after injury, whereas increasing the levels of Stim1 at the plasma membrane region by overexpressing a constitutively active EF-hand motif mutant Stim1 enhances SOCE and stimulates rapid intestinal epithelial restitution.⁶⁹ Our recent studies show that cellular Stim1 levels in IECs is tightly regulated posttranscriptionally via factors that

associate with 3'-UTR of *stim1* mRNA.⁶⁷ miR-195 directly interacts with the *stim1* 3'-UTR through a specific binding site, since this association is abolished by deletion- and point-mutation of the miR-195 binding site from *stim1* 3'-UTR. Increasing the levels of miR-195 decreases *stim1* mRNA as a result of increased degradation rate, whereas decreased levels of endogenous miR-195 enhance *Stim1* expression by increasing the stability of *stim1* mRNA. Interestingly, ectopic miR-195 overexpression induces the co-localization of tagged *stim1* RNA with P-bodies where these transcripts are believed to be sorted for degradation and/or translational repression. Moreover, decreased levels of *Stim1* by silencing its expression or miR-195 overexpression inhibit IEC migration over the denuded area after wounding.

miR-122a and miR-146a

IECs line the intestinal mucosa and form an epithelial barrier against a wide array of noxious substances and bacteria present in the lumen. The epithelial barrier is a specialized structure that consists of different intercellular junctions including tight junctions (TJs) and adherens junctions (AJs).⁷⁰ The TJ is the apical-most element of the junctional complex and it seals IECs together in a way that prevents even small molecules from leaking between cells. The TJ continuously undergoes remodeling and its constituent proteins are high dynamic and tightly controlled at multiple levels by numerous factors. The maintenance of normal levels of TJ proteins is important for the epithelial TJ barrier function, whereas decreased levels of TJ proteins increase gut TJ permeability. Several miRNAs are shown to modulate the stability and translation of mRNAs encoding TJ proteins and play a critical role in the regulation of the epithelial barrier function. Treatment with TNF- α increases expression of miR-122a in enterocytes, cultured IECs, and intestinal mucosal tissue, and induced miR-122a decreases the levels of mRNA and protein of TJ occludin, thus increasing intestinal TJ permeability.²⁴ On the other hand, miR-122a silencing by its specific antisense oligoribonucleotide prevents repression of occludin expression and rescues the gut barrier function in TNF- α -treated mice. In cultured IECs, miR-122a was found to directly bind to the occludin 3'-UTR and be sufficient to induce degradation of occludin mRNA. miR-146a is also highly expressed in a variety of gut tissues and negatively regulates gut barrier function and mucosal immunity.⁷¹ Target deletion of miR-146a enhances intestinal barrier function and protects mice against DSS-induced colitis. Consistently, mucosal tissues from patients with ulcerative colitis also exhibit elevated colonic miR-146a.

miR-429 and others

miR-429 is recently shown to impair intestinal barrier function in diabetic mice.⁷² The levels of mucosal miR-429 in mice with type 1 diabetes increase, which associates with a decrease in the levels of TJ occludin and zonula occludens-1 (ZO-1) in the intestinal mucosa. In studies *in vitro*, ectopically expressed miR-429 represses occludin expression posttranscriptionally via direct interaction with its 3'-UTR, although it does not alter ZO-1 level. Treatment with exogenous agomiR-429 in normal mice down-regulates occludin and induces barrier dysfunction, while exogenous antagomiR-429 up-regulates the TJ expression and improves the barrier function in diabetic mice. In addition, let-7, miR-9, miR-21, miR-145, miR-155, miR-192, and miR-374 are also implicated in the regulation of TJ expression, gut permeability, mucosal immunity, and inflammation as summarized and discussed in our recent publications.¹⁹

LncRNAs in Gut Barrier Function and Epithelial Integrity

LncRNAs are defined as transcribed RNAs spanning >200 nucleotides in length and lack protein-coding capacity.⁷³ LncRNAs are different from well-characterized structural RNAs, such as rRNAs, tRNAs, snRNAs, and snoRNAs or small regulatory RNAs. LncRNAs share many features with mRNAs, and both classes of RNA possess 5'-methyl-guanosine cap and 3'-poly(A) tail, and they can be transcribed from multiexonic genes. Most lncRNAs are dynamically expressed in tissue- and cell type-specific patterns, although some lncRNAs are ubiquitous.⁷⁴ An increasing body of evidence shows that lncRNAs are involved in distinct cellular processes, physiologies, and pathologies by modulating gene expression at different levels. Several lncRNAs are shown to regulate chromatin remodeling, transcriptional and posttranscriptional control, and protein metabolism. LncRNAs modulate numbers of gene transcription processes, as indicators of transcription factor activity, functional guides for ribonucleoprotein (RNP) complexes, decoys that titrate away RBPs, and scaffolds for the assembly of functionally related proteins. LncRNAs also regulate a limited number of posttranscriptional processes including mRNA stability and translation, working jointly with miRNAs and RBPs.^{75,76} Although only a small number of tissue-specific and functional lncRNAs are identified and well characterized in the intestinal epithelium, some lncRNAs are shown to regulate intestinal epithelial barrier function, thus contributing to maintenance of the intestinal epithelial integrity.

LncRNA *H19*

LncRNA *H19* is a 2.3-kb capped, spliced, and polyadenylated ncRNA that is transcribed from the conserved imprinted *H19/igf2* gene cluster located on human chromosome 11p5.5. *H19* expression increases remarkably during embryogenesis but its levels are down-regulated after birth. Emerging evidence also indicates that increased levels of *H19* are commonly observed in a broad spectrum of pathological conditions such as malignancies⁷⁷ and after estrogen treatment⁷⁸ or exposure to hypoxia.⁷⁹ *H19* inhibits embryonic placental growth and *trans*-modulates a network of imprinted genes during fetal development, but the role of *H19* in cancer development depends on its cellular content and tumor types. Targeted *H19* deletion in mice stimulates growth as indicated by an increase in body weight, which is prevented by transgenic re-expression of the *H19* gene.⁸⁰ Several studies reveal that *H19* acts as a primary miRNA template for miR-675⁸¹ and it also functions as a molecular sponge for miRNA let-7.⁸²

We have recently reported that increasing the levels of *H19* specifically inhibits expression of the TJ ZO-1 and the AJ E-cadherin posttranscriptionally, but it fails to alter the expression levels of TJs ZO-2, claudin-1, claudin-3, and JAM-1 and the AJ β -catenin.⁸³ Elevation of cellular *H19* abundance disrupts the epithelial barrier function as evidenced by a decrease in transepithelial electrical resistance (TEER) values and an increase in the levels of paracellular flux of fluorescein isothiocyanate (FITC)-dextran. *H19* overexpression does not affect cell viability, and it only slightly inhibits IEC proliferation *in vitro*. To examine the mechanism underlying this process, *H19* overexpression was found to not only enhance the degradation *ZO-1* and *E-cad* mRNAs but also repress their translation. Since *H19* exon-1 encodes miR-675-5p and miR-675-3p and it can act as the primary miRNA transcript for

miR-675,⁸¹ it is likely that induced *H19* disrupts the epithelial barrier function through the release of miR-675. To test the possibility, our studies further show that ectopically expressed *H19* specifically induces the levels of miR-675-5p and miR-675-3p in IECs. Like *H19*, increasing the levels of either miR-675-3p or miR-675-5p also represses expression of ZO-1 and E-cadherin and leads to the epithelial barrier dysfunction. Although *H19* also acts as a molecular sponge to reduce the bioavailability of let-7,⁸² inhibition of let-7 by its specific antagomirs fails to reduce cellular abundances of ZO-1 and E-cadherin, indicating that the impact of *H19* on let-7 plays a limited role in the *H19*-induced repression of ZO-1 and E-cadherin expression. These findings support a novel idea that *H19* disrupts the gut barrier function by decreasing ZO-1 and E-cadherin as a result of specific induction in miR-675 processing from *H19*.

LncRNA *SPRY4-IT1*

The lncRNA *SPRY4-IT1* is a 706-bp transcript that was originally identified in adipose tissue. Further study shows that *SPRY4-IT1* is broadly expressed in different tissues including the intestinal mucosa in human.⁸⁴ *SPRY4-IT1* is transcribed from the intronic region of the *SPRY4* gene, but *SPRY4-IT1* and *SPRY4* mRNA are two totally independent transcripts. *SPRY4-IT1* is highly expressed in human melanoma cells, and its subcellular levels are higher in cytoplasmic polysomes or ribosomal clusters than other regions. *SPRY4-IT1* silencing in melanoma cells inhibits proliferation, causes defect in differentiation, and induces apoptosis, indicating the importance of *SPRY4-IT1* in melanocytic transformation.⁸⁵

Xiao *et al* recently unveiled that *SPRY4-IT1* enhances the gut epithelial barrier function by increasing the TJ expression at the posttranscription level.⁸⁶ *SPRY4-IT1* is highly expressed in IECs and it is almost equally distributed in the cytoplasm and nucleus. *SPRY4-IT1* silencing inhibits expression of several TJ proteins including claudin-1, claudin-3, JAM-1 and occludin, but fails to alter the cellular levels of ZO-1, and E-cadherin, α -catenin or β -catenin. Consistently, *SPRY4-IT1* silencing also disrupts the epithelial barrier function in an *in vitro* model, which is rescued by overexpression of TJ proteins such as claudin-1 or occludin. To examine the *in vivo* function of *SPRY4-IT1* in the gut barrier function, levels of intestinal mucosal *SPRY4-IT1* are increased by infecting mice with the recombined *SPRY4-IT1* lentiviral expression vector (lenti-*SPRY4-IT1*).⁸⁷ Although elevation of *SPRY4-IT1* levels in the mucosa by lenti-*SPRY4-IT1* does not affect basal TJ levels, it protects the gut barrier function in mice exposed to septic stress induced by CLP. In mice infected with lenti-*SPRY4-IT1*, the degree of CLP-induced gut permeability is lower than that observed in mice infected with control lentivirus. Exposure of CLP also decreases the mucosal levels of claudin-1, claudin-3, occludin, and JAM-1 proteins in the small intestine, but this reduction is either completely prevented or significantly decreased by increasing *SPRY4-IT1* levels via treatment with lenti-*SPRY4-IT1*. Regarding the mechanism by which *SPRY4-IT1* regulates TJ expression, increased *SPRY4-IT1* stabilizes TJ mRNAs and enhances their translation.

To study the impact of *SPRY4-IT1*-regulated TJ expression on clinic, we examined changes in the levels of *SPRY4-IT1* in the colonic mucosa collected from patients with diagnosed with increased gut permeability (IGP). The colonic mucosal samples obtained from patients without significant changes in the gut permeability served as controls. Interestingly, mucosal

SPRY4-IT1 levels in patients with IGP decrease dramatically compared with those observed in control patients. Moreover, decreased levels of mucosal *SPRY4-IT1* in patients with IGP are associated with a significant decrease in the levels of claudin-1, claudin-3, occludin, and JAM-1 proteins as measured by immunostaining assays. These results strongly suggest that decreased levels of *SPRY4-IT1* and subsequent repression of TJ expression play a critical role in gut barrier dysfunction in human.

In addition, there are numerous studies showing the importance of several other lncRNAs in the regulation of embryonic development,⁸⁸ cell differentiation⁸⁹ and apoptosis⁹⁰ in various cell types and organs, but their expression and biological functions in the maintenance of gut epithelial integrity remain unknown.

Interaction between RBPs and NcRNAs in Intestinal Epithelial Integrity

Although many RBPs function as housekeeping factors, several RBPs, including HuR, CUGBP1, AUF1, TTP, TIA1, and TIAR, interact with specific subsets of mRNAs and control gene expression patterns after exposure to stress.^{91,92} HuR is the best studied RBP and it usually increases the stability and translation of target mRNAs by directly associating with AU-rich elements (AREs) located in the 3'-UTRs of target mRNAs.^{93–95} HuR contains two N-terminal RNA-recognition motifs through which it binds to mRNAs with high affinity and specificity. Several recent studies reveal that RBPs can function together with ncRNAs and jointly regulate expression of shared target mRNAs under biological and pathological conditions.⁹⁶ It has been reported that HuR recruits let-7/RISC to suppress the translation of *Myc* mRNA,⁹⁷ whereas the RBP Dnd-1 prevents miRNA access to target mRNA.⁹⁸ Our recent studies show that HuR and CUGBP1 interact functionally with ncRNAs to jointly regulate the intestinal epithelial homeostasis and integrity.

RBP/miRNA Interactions

Unlike miR-195, HuR interacts with and stabilizes *stim1* mRNA, thereby enhancing Stim1 expression in IECs.⁶⁷ Since both miR-195 and HuR interact with the *stim1* 3'-UTR, it is possible that miR-195 and HuR compete for association with *stim1* mRNA and thus regulate the stability of *stim1* mRNA antagonistically. To support the possibility, ectopic HuR overexpression prevents the miR-195-mediated repression of Stim1 expression and this effect results from an increased stability of the *stim1* mRNA. In cells overexpressing miR-195, the destabilization of the *stim1* mRNA is completely prevented by increasing HuR abundance. HuR stabilizes the *stim1* mRNA by interacting with its 3'-UTR as confirmed by results from biotin pull-down and luciferase reporter gene assays. Increased HuR also decreases the levels of miR-195/*stim1* mRNA complex, while HuR silencing increases the *stim1* mRNA association with miR-195. Furthermore, HuR silencing and miR-195 overexpression synergistically reduce the levels of cellular Stim1 by increasing *stim1* mRNA degradation.

To define the mechanism underlying miR-195 and HuR in the regulation of *stim1* mRNA stability in opposite directions, further studies show that miR-195 increases the levels of *stim1* mRNA associated with Ago2, suggesting an increase in the association of *stim1* mRNA with P-bodies.⁶⁷ Interestingly, the induced *stim1* mRNA association with Ago2 in

cells overexpressing miR-195 is completely prevented by increasing HuR levels. Induced HuR alone also decreases *stim1* mRNA presence in P-bodies, whereas HuR silencing increases the Ago2 association with the *stim1* mRNA. Studies using plasmids that expressed MS2-tagged *stim1* mRNA⁶⁷ demonstrate that increased miR-195 enhances the co-localization of *MS2-stim1* RNA and Ago2 and RCK signals and that miR-195-induced association of *MS2-stim1* RNA with P-bodies is lost in cells overexpressing HuR. Moreover, miR-195-induced repression of IEC migration over the denuded area after wounding is also abolished by increasing HuR levels. Together, these data provide new additional evidence that control of cellular Stim1 expression depends on the balance of the *stim1* mRNA associations with miR-195 and HuR, suggesting that interaction between HuR and miR-195 modulate the intestinal epithelial integrity via rapid restitution by jointly regulating *stim1* mRNA stability (Fig. 1).

In addition, both CUGBP1 and miR-222 mutually associate with *Cdk4* mRNA and down-regulate Cdk4 translation by recruiting the *Cdk4* mRNAs to P-bodies synergistically.³¹ This inhibitory effect is even more enhanced by depleting cellular polyamines, while silencing both CUGBP1 and miR-222 had a stronger effect on restoring Cdk4 expression in polyamine-deficient cells compared with silencing either miR-222 or CUGBP1 alone. Moreover, ectopically expressed CUGBP1 and miR-222 together have a synergistically inhibitory effect on IEC proliferation.

HuR/lncRNA *H19* Interactions

Recent studies reveal that HuR interacts with several lncRNAs to jointly regulate target transcripts antagonistically or synergistically.^{99,100} HuR binds and recruits *lncRNA-p21* to let-7/Ago, resulting in the destabilization of *lncRNA-p21*; HuR also competes with ncRNA *7SL* to modulate p53 translation. We have reported that HuR prevents *H19*-induced barrier dysfunction by blocking miR-675 processing from *H19*.⁸³ Similar to the findings observed in other cell types,¹⁰¹ HuR directly binds to the *H19* in IECs. Ectopically expressed HuR prevents an increase in the levels of both miR-675-5p and miR-675-3p in cells overexpressing *H19*, suggesting that HuR suppresses miR-675 processing from *H19*. Consistently, HuR overexpression also rescues expression of ZO-1 and E-cadherin and largely restores the epithelial barrier function in *H19*-transfected cells. On the other hand, cotransfection with the *H19* and HuR does not alter the expression levels of ZO-2, claudin-1, claudin-3, JAM-1, and β -catenin proteins.

In intestinal epithelial tissue-specific HuR knockout (IE-HuR^{-/-}) mice,⁸³ targeted deletion of HuR in IECs increases the basal levels of miR-675-5p and miR-675-3p in the small intestinal mucosa, although it only marginally reduces mucosal total *H19* level. Consistent with findings observed in cultured IECs, increased levels of miR-675 in the mucosa following HuR knockout are associated with a decrease in ZO-1 and E-cadherin expression. To examine if the miR-675-induced repression of ZO-1 and E-cadherin affected the gut barrier function *in vivo*, mice were exposed to stress induced by acute mesenteric I/R. As expected, mesenteric I/R induces a significant increases gut permeability to FITC-dextran, but the degree of I/R-induced gut barrier dysfunction is similar in IE-HuR^{-/-} and littermate mice. However, increased levels of miR-675 by HuR deletion delays the recovery of gut

barrier function after I/R-induced stress. Although the barrier function recovers quickly after I/R in littermates, this repair process is slow in the presence of increased miR-675 in IE-HuR^{-/-} mice. These results indicate that targeted deletion of HuR in IECs increases miR-675 processing from *H19*, thus repressing the recovery of gut barrier function after exposure to pathological stress.

HuR/*SPRY4-IT1* Interactions

HuR was reported to bind the 3'-UTRs of *claudin-1*, *claudin-3*, *occludin*, and *JAM-1* mRNAs and enhances their translation and stability.^{102,103} Since both HuR and *SPRY4-IT1* regulate TJ expression at the posttranscriptional level, we recently examined if *SPRY4-IT1* modulates the association of HuR with the TJ mRNAs. The direct interaction of *SPRY4-IT1* with HuR was measured in an *in vitro* RNA-RNA binding system using biotin-labeled *SPRY4-IT1*. As shown,⁸⁶ biotinylated *SPRY4-IT1* specifically binds to HuR but does not interact with other RBPs such as CUGBP1, TIAR, or AUF1. *SPRY4-IT1* also fails to directly associate with TJ proteins claudin-1, claudin-3, occludin, or JAM-1. *SPRY4-IT1* association with HuR is also further confirmed by ribonucleoprotein/immunoprecipitation assay using anti-HuR antibody. HuR/*SPRY4-IT1* association does not affect the stability of *SPRY4-IT1*. Interestingly, *SPRY4-IT1* silencing blocks HuR binding to *claudin-1*, *claudin-3*, *occludin*, and *JAM-1* mRNAs, although it does not alter total HuR levels or its subcellular distribution.

On the other hand, there are also multiple predicted *SPRY4-IT1* binding sites in the 3'-UTRs of mRNAs encoding claudin-1, claudin-3, occludin, and JAM-1. As measured by RNA-RNA pull down assays, the levels of these TJ mRNAs in biotinylated *SPRY4-IT1* pulldown materials are much higher than those observed in samples pulldown by biotin-labeled *Gapdh*.⁸⁶ HuR silencing only decreases, but not totally prevents, the binding of *SPRY4-IT1* to mRNAs encoding these TJs. These results indicate that *SPRY4-IT1* is also able to directly bind to the TJ mRNAs, which is independent from its association with HuR. Taken together, our findings suggest a novel model whereby *SPRY4-IT1* controls intestinal epithelial TJ permeability via interaction with HuR (Fig. 2). First, *SPRY4-IT1* interacts with HuR, and this association enhances the *SPRY4-IT1* binding to TJ mRNAs. Second, *SPRY4-IT1* also directly interacts with TJ mRNAs. Third, *SPRY4-IT1* and its interaction with HuR enhance the gut barrier function by increasing TJ expression posttranscriptionally.

Conclusions

ncRNAs and their target transcripts have an overwhelmingly broad spectrum of actions in gut development and mucosal physiology, and the limited portion of this huge network that has been discovered is still the tip of the iceberg. The results summered in Table 1 provide novel evidence showing the importance of several intestinal epithelial tissue-specific ncRNAs in the regulation of mucosal regeneration, protection, and repair after injury. Based on our studies and others, we propose a model delineating the posttranscriptional control of intestinal epithelial homeostasis and integrity through miRNAs, lncRNAs, and their interactions with RBPs (Fig. 3). In this model, IEC proliferation, apoptosis, migration, and cell-to-cell interactions are tightly controlled by two groups of ncRNAs: the negative

ncRNAs, including miR-222, miR-29b, miR-195, miR-122a, miR-675, miR-146a, miR-429, and lncRNA *H19*, and positive ncRNAs such as miR-503 and lncRNA *SPRY4-IT1*. The negative ncRNAs destabilize and/or inhibit translation of mRNAs encoding factors that up-regulate mucosal regeneration and protection, thus disrupting the intestinal epithelial integrity. In contrast, positive ncRNAs increase mucosal growth and protect the intestinal epithelial integrity. RBPs interact with both negative and positive ncRNAs and regulate their binding and biological functions either synergistically or antagonistically. Maintenance of the normal intestinal epithelial integrity under pathological conditions depends on a dynamic balance between positive and negative ncRNA.

Clearly, there are still many critical issues that remain to be investigated regarding this working model. First, it remains largely unknown how expression levels of these intestinal epithelial tissue-specific ncRNAs are regulated and controlled in response to stressful environments. Second, the molecular mechanisms by which altered ncRNAs and their interactions with RBPs modulate the stability and/or translation of target mRNAs remain to be fully investigated in the intestinal epithelium. Third, experiments examining the *in vivo* functions of ncRNAs by using intestinal epithelial tissue-specific ncRNA knockout or transgenic mouse models, particularly increasing or decreasing the levels of lncRNAs in the mucosal tissue, are badly needed and they will provide a crucial information about physiological functions of ncRNAs. Finally, translational studies in patients with disrupted mucosal growth/adaptation and gut barrier dysfunction are still limited and should be emphasized in the future study.

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Figure 1.

Schematic diagram depicting the regulation of *stim1* mRNA stability by miR-195 interaction with HuR. miRNA-195 and HuR compete for association with the *stim1* 3'-UTR and regulate *stim1* mRNA decay in opposite directions. ER, endoplasmic reticulum; PM, plasma membrane; TRPC1, canonical transient receptor potential-1.

Figure 2. Schematic diagram of proposed influence of *SPRY4-IT1* upon the epithelial TJ permeability. *SPRY4-IT1* interacts with HuR and forms *SPRY4IT1*/HuR complex that binds to the mRNAs encoding TJs. *SPRY4-IT1* also directly interacts with the TJ mRNAs. *SPRY4-IT1* and its interaction with HuR enhance the gut barrier function by increasing TJ expression.

Figure 3.

Model proposed to explain posttranscriptional regulation of intestinal epithelial integrity by ncRNAs. There are two groups of ncRNAs: negative ncRNAs that disrupt the intestinal epithelial integrity, and positive ncRNAs that increase mucosal growth and protect the barrier function. RBPs interact with both negative and positive ncRNA and regulate their biological functions synergistically or antagonistically. Normal intestinal epithelial integrity depends on the balance between positive and negative ncRNAs.

Table 1

Biological functions and targets of ncRNAs and RBPs in the intestinal epithelium

ncRNAs	Functions	Targets	Reference
miRNAs	miR-29b	↓ Proliferation and mucosal growth	Cdk2 mRNA-3'UTR 32
		↑ IEC apoptosis	Men1 mRNA-CR 46
			Lrp6 mRNA-3'UTR 47
			HuR mRNA-3'UTR 47
	miR-122a	↓ Gut epithelial barrier function	Occludin mRNA-3'UTR 24
	miR-146a	↓ Mucosal immunity and epithelial barrier function	mRNAs encoding anti- inflammation factors 71
	miR-195	↓ IEC migration and epithelial restitution	stim1 mRNA-3'UTR 67
	miR-222	↓ Proliferation and mucosal growth	Cdk4 mRNA-CR 31
		↓ Migration and mucosal repair	Fzd7 mRNA-CR 40
		↓ Tight junction barrier function	TJ mRNAs 40
LncRNAs	miR-429	↓ Epithelial barrier function	Occludin mRNA-3'UTR 72
	miR-503	↑ Apoptosis and proliferation	Cugbp1 mRNA-CR 56
	miR-322	↑ Sensitivity of IECs to apoptosis	smurf2 mRNA-3'UTR 14
	HI9	↑ Gut permeability	ZO-1 mRNA (via miR-675) 83
			E-cad mRNA (via miR-675) 83
	SPRY4-IT1	↑ Tight junctions and protects gut barrier function	Claudin-1 mRNA-3'UTR 86
			Claudin-3 mRNA-3'UTR 86
			Occludin mRNA-3'UTR 86
			Jam-1 mRNA-3'UTR 86
	CUGBP1	↓ Proliferation and mucosal growth	Cdk4 mRNA via interaction with miR-222 31
RBPs			Myc via interaction with HuR 59
		↓ Apoptosis	c-IAP1 and c-IAP2 56
	HuR	↑ Migration after injury	stim1 mRNA via interaction with miR-195 67
		↑ Mucosal growth	Lrp6 and Myc mRNAs 20,48
		↑ Gut barrier function	TJ and AJ mRNAs by interacting with SPRY4-IT1 and HI9 86,83