UNIVERSITY OF CALIFORNIA, SAN DIEGO

Intestinal epithelial cell derived microRNAs regulate type I interferon following infection with RNA virus

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in

Biology

by

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LIST OF ABBREVIATIONS

dsRNA, double stranded ribonucleic acid

miRNA, microRNA

RV, rotavirus;

HCV, hepatitis C virus

RIG-I, retinoic acid-inducible gene I

MDA5, melanoma differentiation-associated protein 5

IRF, interferon regulatory transcription factor

TNF, tumor necrosis factor

IFN, interferon

ISG, IFN-stimulated gene

SeV, sendai virus

poly(I:C), polyinosinic:polycytidylic acid

Ago2, protein argonaute-2

UTR, untranslated region

MOI, multiplicity of infection

qPCR, quantitative polymerase chain reaction

HAU, haemagglutinating units

siRNA, small interfering RNA

CDS, coding sequence

pfu, plaque-forming units

IECs, intestinal epithelial cells

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ABSTRACT OF THE THESIS

Role of epithelial microRNAs following infection with RNA virus in intestinal epithelial cells

by

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MicroRNAs are small (~22 nt) noncoding RNAs which function is to regulate mRNA levels in multicellular organisms. Several host-derived miRNAs have been shown to play an important role during viral infections by regulating the expression of genes encoding key immune mediators. We sought to investigate the ability of miRNA to regulate the intestinal epithelial cell (IEC) innate immune response. Using siRNA we downregulated the expression of the 2 key microRNA processing enzymes Drosha and Dicer-1 in HCT116 and HT29 and then infected with Sendai virus, Rotavirus, or stimulated the cells with the synthetic dsRNA mimic polyinosinic-polycytidylic acid (poly(I:C)). We observed that Drosha-silenced, but not Dicer-silenced cells, induced a significant increase in IFN-β expression and secretion, and also up-regulated the expression of ISG-15, which caused reduced viral load in infected cells. The cells showed also an increased expression of inflammatory cytokines TNF-α and IL-6. These responses were not due to changes in levels of phosphorylated IRF3 or phosphorylated IκBα. Top candidate miRNAs endogenous to IECs and predicted to target IFN-β, TNF-α, and IL-6 were found.

Introduction

Public health importance of intestinal RNA viruses

Intestinal RNA viruses such as the Rotavirus and Norovirus can infect epithelial cells that line the surface of the small intestine, causing gastroenteritis. Enteric viruses are a major medical problem, hospitalizing millions a year worldwide and causing morbidity, death, and economic burden.

Rotavirus, a dsRNA virus, remains the leading cause of acute diarrhea in children worldwide and is responsible for up to half of hospital admissions for diarrhea around the world. By age 5, nearly every child will have an episode of Rotavirus gastroenteritis regardless of whether they live in a high or low resource setting, indicating that usual hygienic measures do not stop the spread of the virus.[1] The elderly and immunocompromised are also more susceptible to infection. Despite the introduction of an anti-rotavirus vaccine in 2006, approximately half a million deaths still occur worldwide each year, mostly in developing countries.[2] Furthermore, prior infection with Rotavirus is thought to partially contribute to the pathogenesis of Celiac disease, an autoimmune disorder of the small intestine, by shifting the individual towards Th1-mediated immunity leading to loss of tolerance for gluten.[3]

Norovirus, a ssRNA virus, is the most common cause of acute and epidemic gastroenteritis. Because of its low infectious dose, Norovirus contamination is the leading cause of food and waterborne illness in the US, with 23 million cases reported a year. Outbreaks are common in cruise ships, restaurants, nursing homes, and schools

because of the virus's stability in the environment.[4-5] Individuals who are infected maintain immunity for 4-6 months, but do not develop long term immunity.[6] The lack of long term immunity and the inability to culture human Norovirus make vaccine development problematic at this time.

Hepatitis C Virus is a ssRNA virus classified by the WHO (World Health Organization) as one of the six oncogenic viruses.[7] It is one of the most significant chronic infections worldwide, affecting about 3% of the world's population, and leads to cirrhosis and liver failure.[8] Although HCV primarily infects liver cells, recent studies have indicated that HCV can also infect small intestine epithelial cells, which identifies the intestine as a potential reservoir.[9-10] Current standard treatment for HCV includes interferon.[11]

Human host recognition of dsRNA, innate immune response, and the role of IFN-β

After cell entry, RNA viruses generate dsRNA. Viral nucleic acid can be sensed by several host pattern recognition receptors (PRRs), including Toll-like receptors (TLRs) and the cytoplasmic RIG-I-like receptors. The RIG-I-like receptor family is comprised of RIG-I and MDA-5. They are RNA helicases that unwind dsRNA by hydrolyzing ATP. They contain a DExD/H sequence for RNA recognition and a caspase activation and recruiting domain (CARD) to relay the signal.[12-13] RIG-I has been shown to be critical for mounting a host immune response to Sendai virus and influenza by recognizing shorter, viral replicating dsRNA, while MDA-5 recognizes longer segments of dsRNA and artificial nucleic acids such as polyinosine-polycytidylic acid (poly I:C).[14]

Recognition by either RIG-I or MDA-5 results in a downstream signaling cascade

through MAVS (also known as IPS-1, VISA, or CARDIF), while TLRs that sense viral nucleic acid relay the signal to MyD88 or TRIF.[15-17] Both sensor pathways lead to activation of interferon regulatory factor (IRF) 3 and 7 as well as nuclear factor-kb(NF-kB), resulting in the induction of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and type I interferons (IFNs). Type I IFNs are a family of genes that exhibit potent antiviral activities, and include IFN-α and IFN-β.[18] IFN signaling acts in an autocrine as well as a paracrine manner to warn neighboring cells to activate antiviral mechanisms.[20] Secreted IFN-β binds to IFN receptors, which are ubiquitously expressed on most mammalian cells, and initiates the JAK/STAT signaling pathway, leading to expression of hundreds of interferon stimulated gene (ISG) products, including both protein-coding and non-coding miRNA genes.[19] ISG products work synergistically to create an antiviral state and promote apoptosis of infected cells. The IFN system is a potent defense mechanism and requires tight regulation in order to stimulate cells only when under viral attack. An overactive interferon signature is associated with systemic lupus erythematosus (SLE), Sjögren's syndrome, and other autoimmune diseases. IFN regulation is critical and there are several IFN regulatory mechanisms, one of which is miRNA, which can regulate post-transcriptionally. IFN-regulated microRNAs and microRNAs that regulate IFN may play a key role in this antiviral innate immunity.

MicroRNA biogenesis

miRNAs are an endogenous, abundant class of small (19-25 nucleotides long), non-coding RNAs that play crucial regulatory roles in innate immunity as well as other biological processes such as organ development and oncogenesis.[21] To date, hundreds of

miRNAs have been identified in plants, animals, and viruses, and they are highly conserved among eukaryotes. They regulate gene expression post-transcriptionally generally by binding to the 3'UTR of complementary mRNAs , causing mRNA destabilization, degradation or translation inhibition.[22-24] In mammals, it's estimated that most (≥84%) of reduced protein output caused by miRNAs is accounted for by lowered mRNA levels (i.e. through degradation).[25] In certain instances, a miRNA may target the amino acid coding sequence (CDS) or the 5'UTR of its target mRNA.[26] A single miRNA may have hundreds of target genes, and miRNAs collectively have been suggested to influence the expression of 30% of human genes.[27] miRNAs are essential for fine-tuning gene expression in many important cellular events, as loss of Dicer1, a key protein for miRNA biogenesis, leads to embrionic lethality in mice.[28]

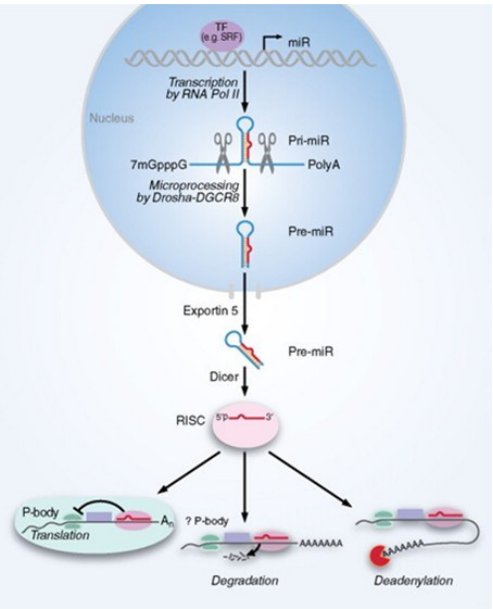


Figure 1. Schematic representation of miRNA biogenesis and function. (Kordes et al, Circulation Research 2009)

Mammalian miRNAs are often encoded in intergenic regions but also exist in exonic and intronic regions of genes.[29] In mammalian cells, miRNA biogenesis (see Fig. 1) begins with transcription by RNA polymerase to form a pri-miRNA and consequent processing by the RNAse III enzyme Drosha (complexed with the dsRNA binding protein DGCR8) in the nucleus, yielding a pre-miRNA hairpin.[30] The pre-miRNA is recognized by Exportin-5, which transports it into the cytoplasm via a Ran-GTP dependent mechanism.[31] Next, the cytosolic RNase III enzyme Dicer cleaves the pre-miRNA into the mature ~22nt sequence. One strand becomes incorporated into the RNA-induced silencing complex (RISC) as the guide strand, while the other miRNA strand (miRNA*) is degraded.[32] Thus, Dicer and Drosha are key enzymes in the generation of mature miRNAs.

MicroRNAs' role in innate immunity

miRNAs and their role in the immune system have been studied extensively in the last decade since their discovery in mammalian cells. Many endogenous miRNAs have been shown to play a beneficial role to the host during infection by enhancing the host innate immune response or by directly targeting the virus. For example, Dicer knockout mice were found to be more susceptible to VSV infection due to the loss of miR-24 and miR-93, which suppress VSV replication.[33] Additionally, upon vesicular stomatitis virus (VSV) infection of mouse macrophages, mir-155 is induced and targets a negative regulator of IFN, SOCS1, therefore increasing type I IFN production.[34] In humans, miR-32 can target proteins of the Retrovirus primate foamy virus type 1 (PFV-1), inhibiting its replication.[35] Likewise, miR-28, mir-29a, miR-125b, miR-150, miR-233, and miR-382

are all capable of targeting HIV mRNA.[36] Notably, mir-29a is induced by type I interferon.[37] Many other miRNAs, such as miR-21 in mice, control the innate immune response by dampening the inflammatory response and suppressing NF-κB signaling.[38]

Invading viruses can manipulate endogenous miRNA levels to enhance their own survival. One such case is VSV infection of mouse macrophages, which upregulates miR-146a expression, and results in an inhibition of RIG-I dependent type I IFN production by targeting of TRAF6, IRAK1, and IRAK2.[39] In an exceptional case, miR-122 in human liver was found to facilitate replication of HCV by binding to a noncoding region of the viral genome and physically protecting it from the host defense mechanisms of 5' exonucleases.[40-42] Yet, production of IFN-β in the liver by the host downregulates mir-122 levels.[43]

Moreover, some viruses encode their own miRNAs that can target host cell factors involved in viral defense. miRNAs deriving from DNA viruses have been found in adenovirus, retrovirus, and across the herpes family, including HSV, HCMV, EBV, and KSHV.[44] The viral miRNA encoded by HSV targets TGF-β1 and SMAD3, downregulating the TGF-β pathway and producing an anti-apoptotic effect.[45] To date, no viral miRNAs have been found to be encoded by RNA viruses, and no viral miRNAs have been found to target the IFN system.[46-47]

Results

Sendai virus upregulates type I IFN mRNA levels in IECs

I examined the immune response of the HCT 116 cell line to SeV to determine its fit as a model for viral infection in the gut. I focused on the expression of IFN-β because Type I IFN is a hallmark of host cellular response to viral infection. To optimize infection, I tested different infection length times and varying haemagglutinating units (HAU). HCT 116 contained the most IFN-β mRNA between 8-10 hours post- infection and after an infection at 200 HAU (Fig. 2A).

Drosha regulates IFN-β, TNF-α, and IL-6 mRNA levels and IFN-β secretion in IECs

To investigate the potential involvement of miRNAs during gut infections, I knocked down the dicer and drosha miRNA processing enzymes using small interfering RNA (siRNA). Following infection by SeV, HCT 116 cells that received drosha siRNA had increased levels of IFN-β mRNA compared to cells that received non-targeting or no siRNA (Fig. 3A). Cells that had received dicer siRNA had similar IFN-β mRNA levels to those receiving none. Secreted amounts of IFN-β from the supernatants of each treatment were consistent with their respective mRNA levels (Fig. 3B). The efficacy of gene silencing by siRNA was confirmed with mRNA levels of both dicer and drosha at <20% than that of untreated cells (Fig. 3, C and D). Protein levels of dicer and drosha were likewise depleted following siRNA treatment (Fig. 3E). Drosha-silenced cells also had increased mRNA levels of the pro-inflammatory cytokines TNF-α, IL-6, and ISG-15 compared to untreated cells (Fig. 3, F-H). A similar phenotype of increased IFN-β

mRNA after drosha knockdown was observed when cells were stimulated synthetic dsRNA analog poly(I:C) (Fig. 4). To see if the reverse would apply under drosha overexpression, HCT 116 cells were transfected with a drosha expression vector. As expected, after infection with SeV, expression of IFN-β mRNA was lower than in mock-treated cells (Fig. 5).

Drosha silencing reduces viral load of IECs

I investigated whether the increased production of IFN-β by drosha-silenced cells gave them a physiological advantage during viral infection. Drosha-silenced HCT 116 cells exhibited decreased SeV replication, consistent with their higher IFN-β output (Fig. 6, A-F).

Drosha silencing does not affect IFN-β or TNF signaling

At the end of the signaling cascade initiated by the viral RNA sensors RIG-I and MDA-5, IRF3 is phosphorylated and forms heterodimers, translocating into the nucleus and binding to interferon stimulated response elements (ISREs) to induce type I IFN and ISGs.[48] Likewise, downstream of RIG-I/MDA-5, IκBα can be activated by phosphorylation. Once phosphorylated, it unbinds from NFκB, allowing NFκB to translocate into the nucleus to start transcription of an array of inflammatory cytokines such as TNF-α and IL-6. To determine how Drosha or products of Drosha may be targeting IFN-β and TNF-α production, I analyzed a few components of these signaling pathways before and after drosha silencing. After infection by SeV, HCT 116 cells that

were silenced for dicer and drosha did not show any differences from untreated cells in level of p-IRF3 or p-IκBa protein (Fig. 7).

Candidate colonic miRNAs

The prior results suggested that there may be an intestinal epithelial miRNA that targets IFN-β and TNF-α directly rather than components of their signaling pathways. I identified several miRNAs present in HCT 116 an HT-29 that have capabilities, according to their seed sequences, to target IFN-β, TNF-α, and IL-6 (Table 1).

Drosha silencing increases IFN-β and decreases viral load following infection by rotavirus in colonic cells

Preliminary data of rotavirus as a model reflects the same pattern that emerged during SeV infection. IFN-β mRNA was produced in a dose-dependent manner (Fig. 8A) and levels peaked around 12 hours (Fig. 8B). INF-β mRNA was upregulated in Drosha-silenced cells following infection with RV (Fig. 8C) and resulted in decreased viral load (Fig. 8D).

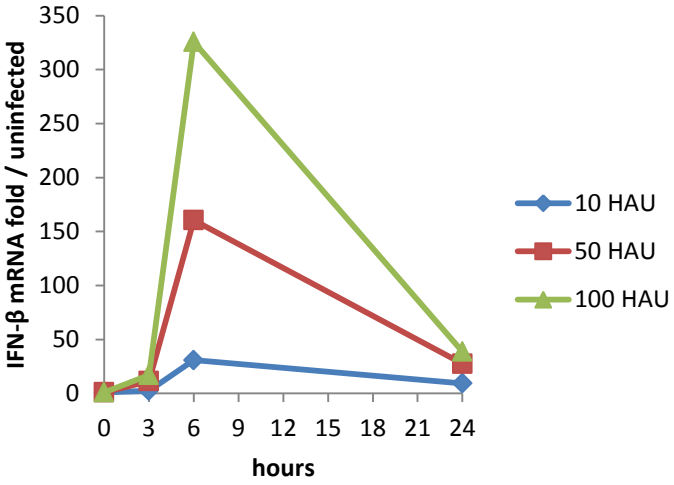


Figure 2. HCT 116 colonic cells produce IFN-β in response to SeV in a dose-dependent manner.

2 x 10^6 HCT 116 was infected with SeV at 10, 50, and 200 HAU. mRNA was collected after 0, 3, 6, and 24 hrs and measured for IFN-β.

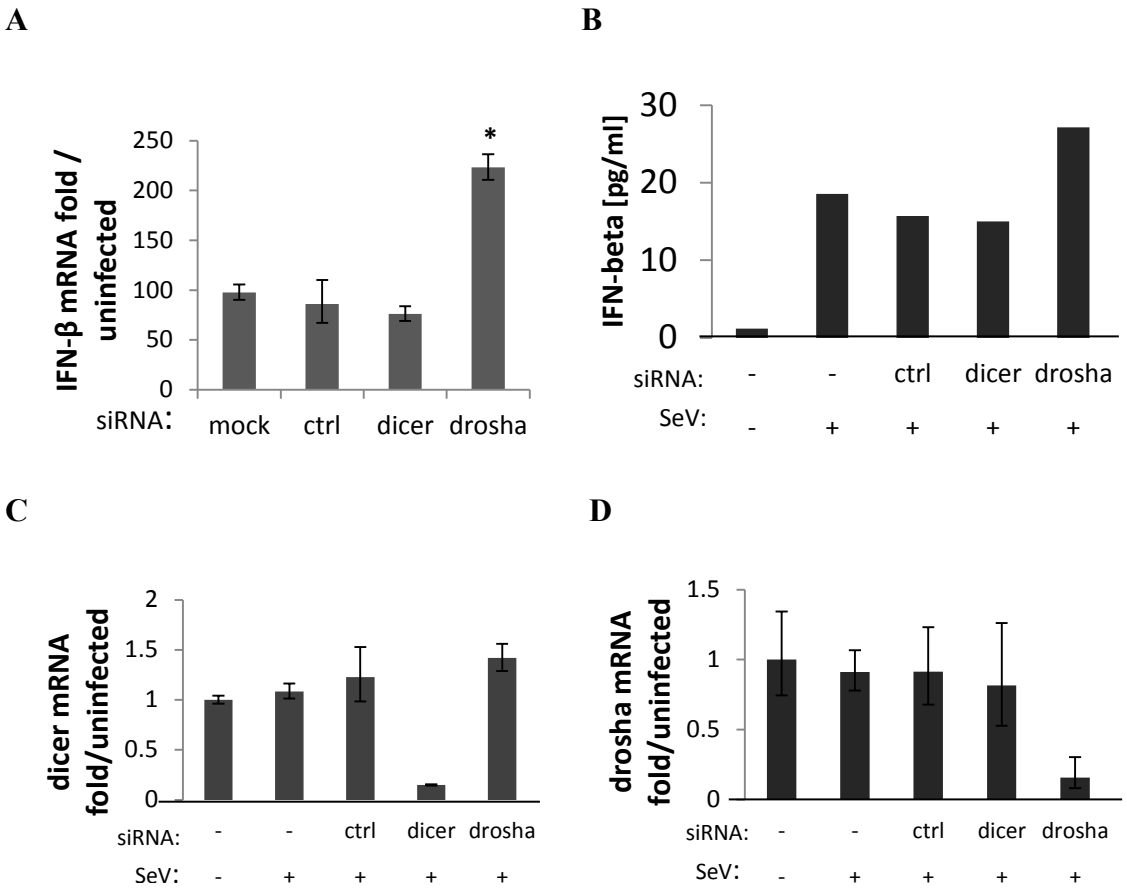
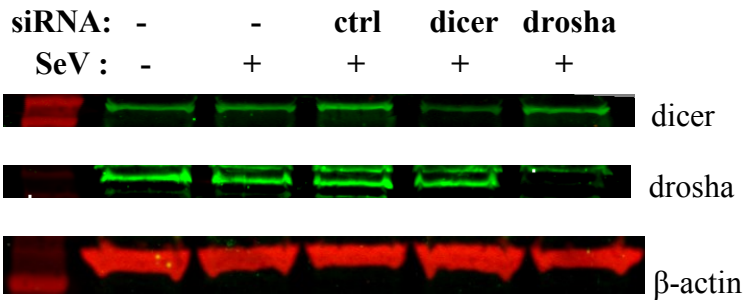


Figure 3. Silencing drosha, but not dicer, causes an increase in IFN-β production and subsequent induction of ISG-15, TNF, and IL-6 by colonic cells during SeV infection

5 x 10^5 HCT 116 cells were transfected with control, dicer, or drosha siRNA at a concentration of 100nm. After 24 h cells were infected by SeV at HAU 100. A, 6 h post-infection, mRNA was collected and IFN-β mRNA was measured as described in Figure 2A. Similar results were obtained in three independent experiments, *$p \leq 0.05$. B, At 24 h, IFN-β in supernatant was collected and measured by ELISA. C, At 6 h, Dicer and (D) drosha mRNA were measured. E, At 6 h, Dicer and Drosha were detected by immunoblot. F-H, At 6 h ISG-15, TNF, and IL-6 mRNA were measured.

E



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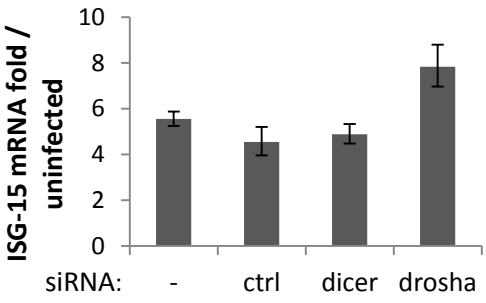

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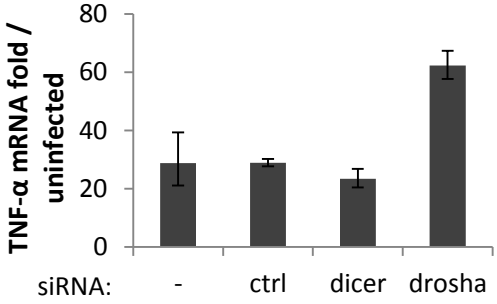

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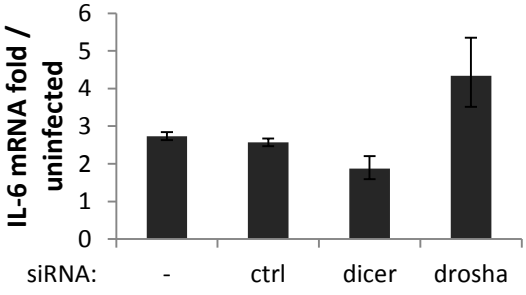

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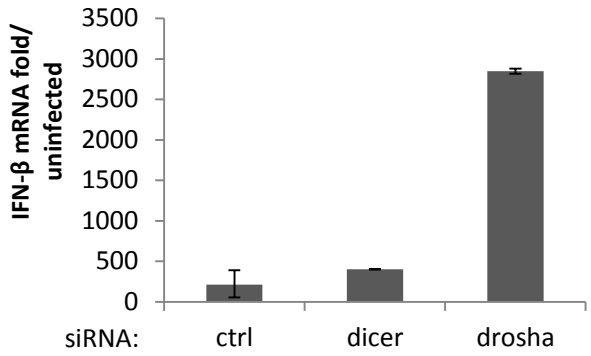


Figure 4. Silencing drosha, but not dicer, causes an increase in IFN-β production by HCT 116 during stimulation with poly(I:C).

5 x 10^5 HCT 116 Cells were transfected with 1 μg poly(I:C) 24 h after transfection with siRNA . IFN-β mRNA was measured at 6 h. Similar results were obtained in two independent experiments.

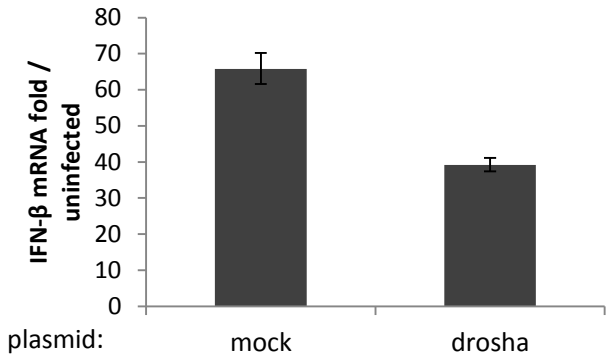


Figure 5. Drosha overexpression in HCT 116 cells reduces IFN-β expression during infection

5 x 10^6 HCT 116 cells were transformed with mock or drosha-expressing plasmid at a concentration of 1µg per well in a 24 well format. At 24 h, cells were infected with SeV at HAU 100. IFN-β mRNA was measured after 6 h as described in Figure 2A.

A-E

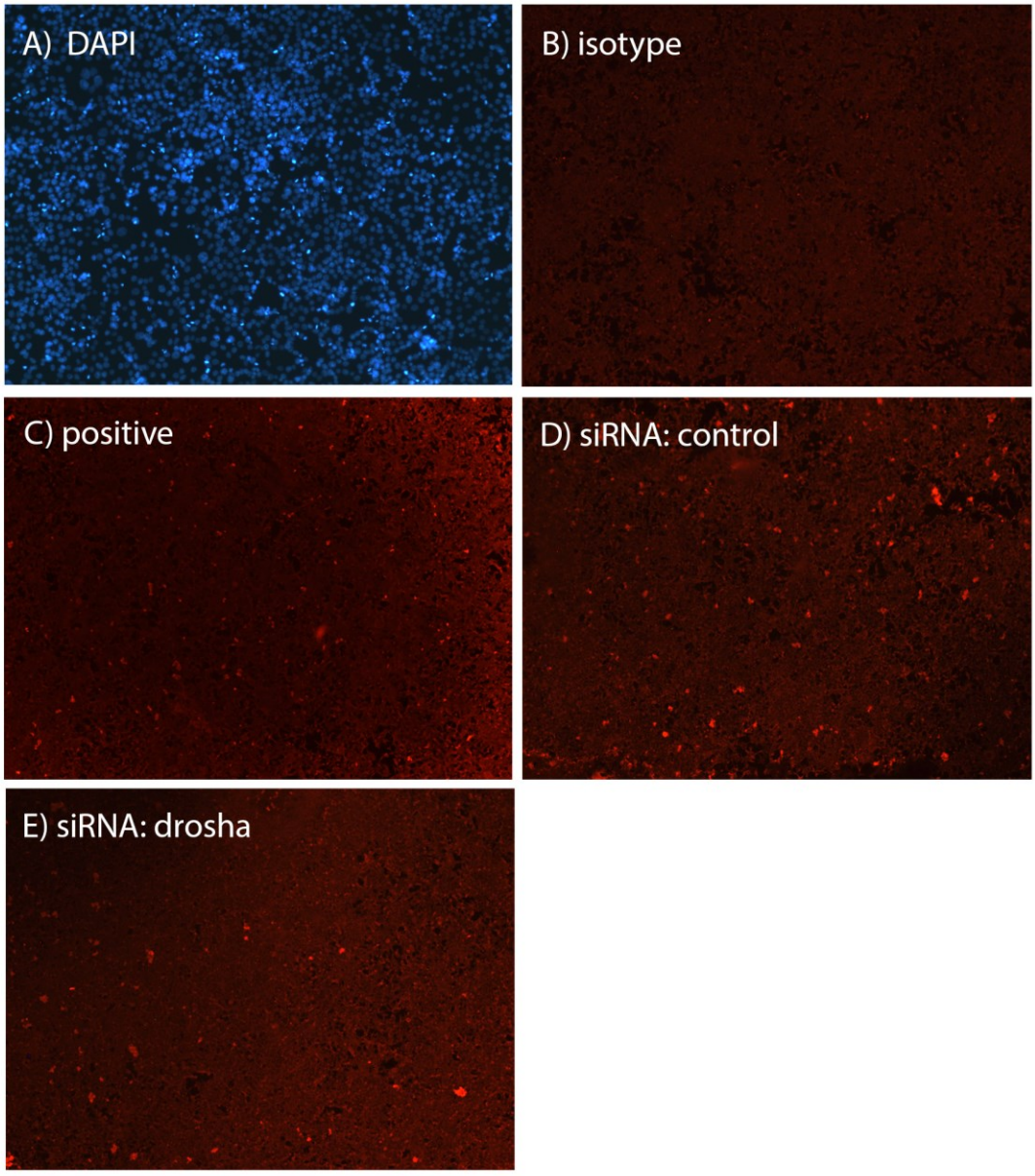
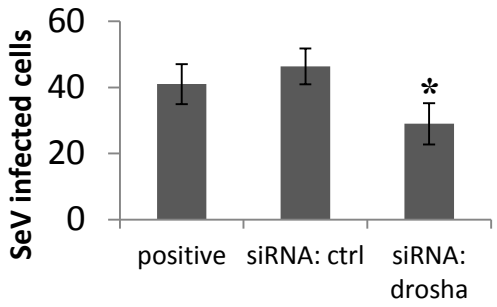


Figure 6. Drosha silenced colonic cells have decreased viral load

5 x 10^5 HCT 116 cells were silenced with (A-C) mock, D) control, or E) dicer siRNA and infected with SeV as described in Figure 2. HCT 116 cells were stained for goat IgG (B) and SeV (C-E), and infected cells were counted (F), $p<0.05$.

F



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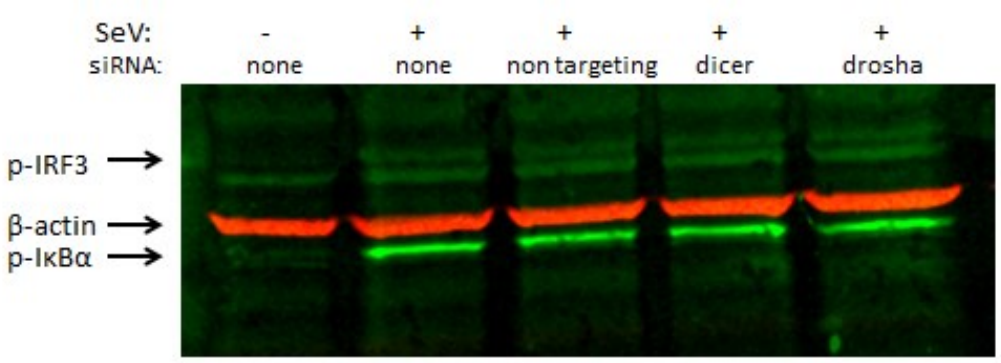

Figure 7. Drosha silencing does not affect phosphorylation of IRF3 or IκBα

5 x 10^5 HCT 116 cells were silenced with control, dicer, or drosha targeting siRNA, and infected with SeV as described in figure 2A. At 6 h, protein was collected and phospho-IRF3 and phospho-IκBα were detected by immunoblot.

Table 1. Candidate human miRNA regulators of IFN-β, TNF, and IL-6 found in HCT 116 and HT-29.

Human miRNAs predicted to target IFNB, TNF, and IL-6 were found using four different miRNA prediction sites on miRWalk, and those endogenous to both HCT 116 and HT 29 were selected as candidate miRNAs.

Target gene	Predicted miRNA	# detections out of 4
IFNB1	hsa-miR-548c-3p	3
	hsa-miR-557	3
	hsa-miR-563	2
	hsa-miR-607	2
	hsa-miR-145†	2
TNF	hsa-miR-181c	4
	hsa-miR-549	4
	hsa-miR-577	4
IL-6	hsa-miR-548d-3p	3
	hsa-miR-655	3
	hsa-miR-568	3
	hsa-miR-587	2
	hsa-miR-607	2
	hsa-let-7a‡	2
	† indicates found only in HT 29	
	‡ indicates found only in HCT 116	

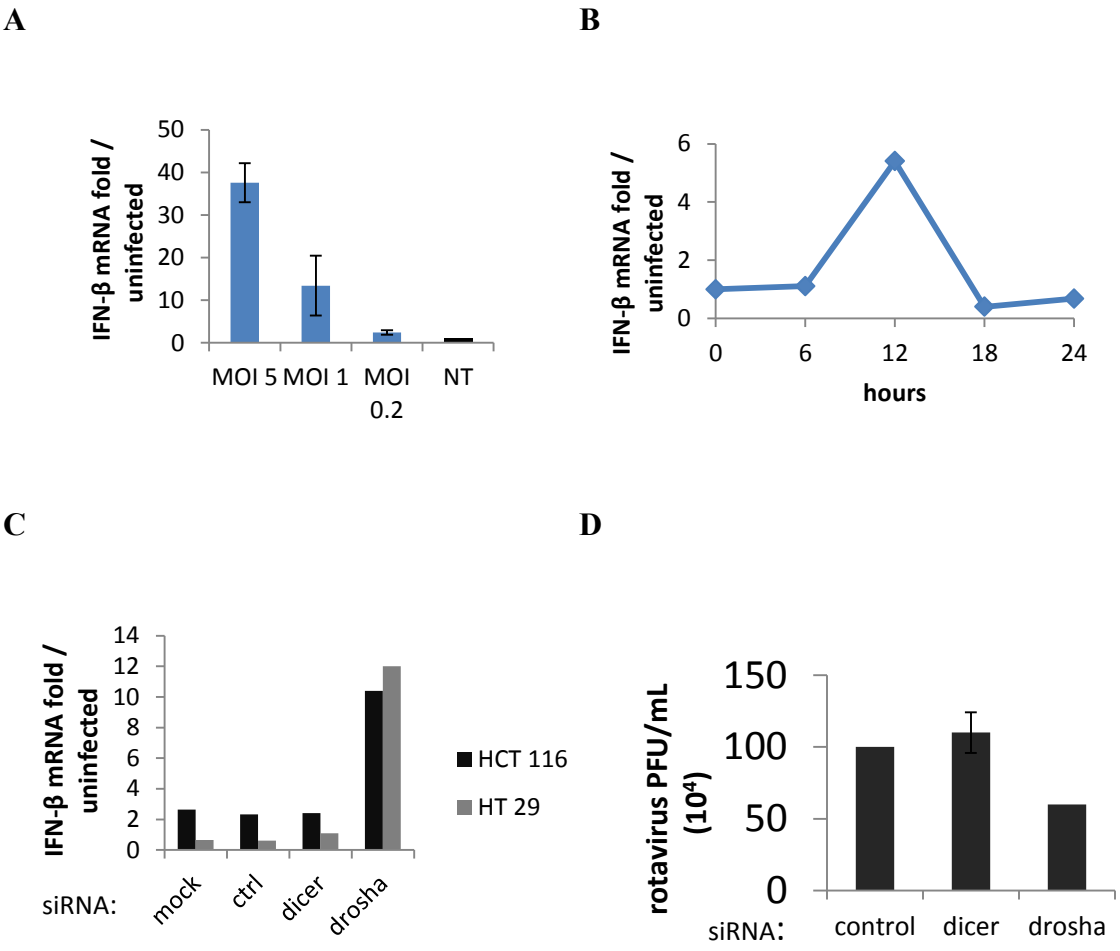

Figure 8. Preliminary findings with rotavirus are consistant with SeV results

A, 2 x 10^6 HCT 116 was infected with RV at MOI 5, 1, and 0.2, and mRNA was collected after 8 h and measured for IFN-β. B, 2 x 10^6 HCT 116 was infected with RV at MOI 2. After 6, 8, 10, and 24 hr, mRNA was collected and IFN-β mRNA was measured by q-PCR and normalized to the expression of GAPDH in each sample. C, 5 x 10^5 of HCT 116 and HT 29 Cells and were transfected with control, dicer, or drosha siRNA at a concentration of 50nm. At 48 h, cells were infected by SA11-4F RV at MOI 2. IFN-β mRNA was measured after 8 h as described in Figure 2A. D, 5 x 10^5 HT 29 cells were transfected with control, dicer, or drosha siRNA at a concentration of 50nm. At 48 h, cells were infected by SA11-4F RV at MOI 2. At 6 h post-infection, supernatant was collected and virus yield was measured by plaque assay. Results were consistent across two individual experiments.

Discussion

Host defense against viral invasion requires induction of appropriate innate immune responses, but needs to be tightly controlled because excessive induction can lead to hyperinduction of cytokines, tissue damage, and unreasonably strong inflammation leading to autoimmune and inflammatory disorders.[49] To do so, multiple negative regulators are expressed by immune and inflammatory cells following infection to control the amplitude of the response and turn down signaling. The abilities of certain cellular miRNAs to affect innate immune responses have been demonstrated in recent studies, but their roles have not previously been studied in IECs during infection by RNA virus. Given the importance of the gut as a mucosal surface continually exposed to enteric pathogens, a colonic microRNA that regulates IFN-β would be a valuable regulatory mechanism. This study observes the roles of miRNAs in IEC innate immune response to RNA virus.

I showed that human intestinal epithelial cell (IEC) lines HCT 116 mounts a significant, dose dependent innate immune response against virus by producing substantial levels of IFN-β following infection by RV and SeV. Broquet et. al (2011) also showed robust IFN-β induction following viral infection in the colonic cell line HT-29.[50] This renders those cell lines relevant as *in vitro* models of RNA infection of the intestinal epithelium. The level of IFN-β mRNA and protein in response to infection or poly(I:C) stimulation was increased upon Drosha depletion, but not Dicer depletion, suggesting the involvement of one or several miRNA produced by a Drosha-Dependent, Dicer-independent pathway.

While the classical pathway of miRNA biogenesis involves Dicer-1, numerous studies have shown miRNA biogenesis occurring in non-canonical fashions, which allow for production of functional miRNAs without processing by Dicer.[51] One such case is miR-451, completely conserved throughout vertebrates, which is processed by Drosha and then loaded into Ago2, sliced and trimmed, yielding a mature miRNA without the requirement of Dicer.[52-53] Deficiency in Drosha and Dicer do not always generate identical phenotypes, as shown in thymocytes after targeted gene deletion.[54] Drosha by itself was also found to recognize and cleave mRNA containing secondary stem loops, suggesting activity in an independent miRNA biogenesis pathway. Additionally, when Cummins and others (2005) compared expression of known miRNAs in wildtype HCT 116 with a HCT116 having a disrupted *Dicer* gene (HCT-Dicer^{ex5}) only 55 of 97 had differential expression.[55] Thus, the other 42 of the 97 miRNAs were able to mature in the absence of Dicer.

The findings suggest that silencing Drosha weakens the regulation on IFN-β production, allowing more IFN-β to be produced upon infection and resulting in lower viral replication. As expected, an increase of IFN-β production coincided with an increase of ISG-15 mRNA, demonstrating the influence of the IFN system on the anti-viral response. Surprisingly, TNF-α and IL-6, two inflammatory cytokines produced through the NFκB pathway, were also upregulated in Drosha-silenced cells . Silencing Drosha affects miRNA biogenesis, and miRNA targeting other genes, including TNF and IL-6 genes may have been affected as well.

It is important to note that Drosha-silenced cells did not affect the phosphorylation level of IRF3 or IκBα protein compared to control cells following infection. Since the IFN-β and the NFκB pathway appear to be unaffected by silencing, it is possible that the phenotype may be caused by direct regulation of IFN-β, TNF, and IL-6 mRNA. Previous studies have shown that human miR-26a, miR-34a, miR-145, and let-7b directly regulate IFN-β expression while themselves being IFNβ-inducible, contributing to a negative feedback loop.[56]

I identified several candidate miRNAs endogenous to IECs that have sequence similarity to IFN-β, TNF-α, and IL-6. However, prediction tools are not determinant and need experimental validation. A follow up experiment can be done *in vitro* by combining mimics of the miRNAs with their targets and observing potential knockdown.

In conclusion, my study has provided evidence of a drosha-dependent regulatory mechanism of IFN-β in IECs. It is possible that the observed immune response is a cumulative effect of many miRNAs working synergistically or as a system of counterbalances. In the future, it will be important to narrow down the list of candidate miRNA by selecting those that are downregulated in drosha-silenced cells, and to investigate whether their levels change after viral infection. Additionally, the ability of the miRNAs to downregulate their targets should be experimentally confirmed. Once the miRNA regulators of IFN-β have been confirmed, they may be potential therapeutic targets in viral infections. Designed locked nucleic acid (LNA)-modified oligonucleotides were shown to be able to therapeutically silence miR-122 in primates with HCV, leading to long-lasting suppression of the virus.[57] Intestinal diseases that

benefit from type I IFN therapy can also benefit from other novel miRNA-related treatment modalities that may arise, especially an efficient miRNA delivery system.

Materials and Methods

Cell culture

Human colonic cell lines HCT 116 and HT-29 were cultured in RPMI 1640 (Gibco); Embryonic African green monkey kidney cells MA-104 were cultured in Medium 199 (Gibco). All medium were supplemented with 10% FBS and 1% Penicillin/Streptomycin and cultured at 37^0 C and 95% air/5% CO2. Trypsin-EDTA 0.05%, from porcine pancreas, was used to detach cells and was from Sigma-Aldrich (Milwaukee, WI).

Gene-silencing with siRNA and transfection

Small interfering RNA (siRNA) for Dicer, Drosha, and non-targeting control pool (scrambled) was purchased from Dharmacon (Lafayette, CO). siRNA was transfected into HCT 116 cells at a concentration of 50 or 100nm using Dharmafect reagent (Thermo Scientific) according to manufacturer's protocol 24 h before infection with SeV or 48 h before infection with RV.

Plasmids

The pcDNA4/TO/cmycDrosha plasmid was purchased from Addgene (Cambridge, MA) (ID 10828). pcDNA3.1A was used as a control plasmid (Invitrogen). Plasmids were transformed into HCT 116 cells using TurboFect reagent (Thermo Scientific) according to the manufacturer's instructions.

Virus infection and cell stimulation with poly(I:C)

Rotavirus strain SA11-4F was provided by Dr. M. K. Estes (Baylor College of Medicine, Houston, TX). To infect with RV, cells were incubated in serum-free media while RV was activated by trypsin (10 μg/ml, 37^0C) 1 h before infection. RV was diluted with serum-free media to the desired MOI and 100 μL was placed drop-wise per well in a 24 well plate. Following 1 h of adsorption to the cell surface at 37^0C, virus inoculum was removed and cells were replenished with complete media.

RV was grown in MA-104 cells infected at 0.1 MOI, and incubated for 72 h in presence of trypsin (0.44 μg/ml), after which cells were lysed by freezing and thawing three times to achieve virus release. Extracted virus was titrated by plaque assay on MA-104 cells.

Sendai virus was diluted to desired HAU in TransfectaGro (CellGro) and 200 μL was placed drop-wise per well in a 24 well plate. Following 1 h of adsorption to the cell surface at 37^0C, virus inoculum was removed and cells were replenished with complete media.

Low molecular weight (LMW) Poly(I:C) was from InvivoGen (San Diego, CA) and diluted with PBS to 1 mg/mL before infection. pIC was used to infect cells cytoplasmically using Dharmafect (Thermo Scientific) according to manufacturer's directions. pIC was used to stimulate cells endosomally by placing 1 μg onto cells resting in complete media.

RNA extraction and quantitative RT-PCR

Total cellular RNA was extracted using RNeasy Mini Kits from Qiagen (Valencia, CA) followed by DNase I (Qiagen) treatment according to the manufacturer's instructions. 1 μg of total RNA was used for reverse transcription using iScript (Bio-Rad Laboratories, Hercules, CA), and the cDNA used as the template for qPCR with 2 x SYBR green Master mix (Applied Biosystems, Foster City, CA) with the following primers: IFN-β forward 5'-AAA CTC ATG AGC AGT CTG CA-3' and reverse 5'-AGG AGA TCT TCA GTT TCG GAG G-3'; GAPDH forward 5'-ATG GAA ATC CCA TCA CCA TCT T-3' and reverse 5'-CGC CCC ACT TGA TTT TGG-3'; IL-6 forward 5'-AGG GCT CTT CGG CAA ATG TA-3' and reverse 5'-AAG GAA TGC CCA TTA ACA ACA AC-3'; ISG-15 forward 5'-GAG AGG CAG CGA ACT CAT CT-3' and reverse 5'-CTT CAG CTC TGA CAC CGA CA-3'; TNF-α forward 5'-CCA TCA GAG GGC CTG TAC CT-3' and reverse 5'-GCA GCC TTG GCC CTT GA-3'; Dicer1 forward 5'-GGC CCC AAT CCT GGA CTT AT-3 and reverse 5'-AAG CCG CTC CAG GTT AAA TC-3'; Drosha forward 5'-TTC CCT CCC TTG GCC CAG CTT-3' and reverse 5'-CTA TAA AAG GCT CTC GGG CCG C-3';. All primers were purchased from Valuegene (San Diego). Denaturation was 5 min at 95^0C followed by 40 cycles of amplification at 95^0C for 30 s and 60^0C for 30 s using an ABI StepOnePlus (Applied Biosystems). Induction was calculated using the ΔΔCt method, and values were normalized to untreated cells.

Immunoblot analysis

0.2 x 10^6 IECs per well were seeded in 24-well plates and cultured over 2 nights. Following infection, the cells were lysed on ice with RIPA lysis buffer supplemented with PI, PMSF, and NaCl (Santa Cruz). Lysates were centrifuged at 15,000 x *g* for 5 min at 4^0C and equal amounts of protein were mixed with 6x SDS sample buffer, boiled and run on SDS-PAGE ready gels(Bio-Rad). Proteins were transferred onto nitrocellulose membranes and probed with primary Ab followed by secondary Ab. Antibodies were purchased from Cell Signaling.

Viral Plaque Assay

RV pfu was titered by plaque assay on MA-104 cells according to the protocol of Arnold and others (2009).[58]

ELISA

Human IFN-β of cell supernantants was assayed using the HuIFN-β ELISA kit (PBL Assay Science) according to manufacturer's directions.

Immunohistochemistry

For SeV staining, HCT 116 cells were seeded in a glass chamber. Cells were fixed in methanol for 5 min, washed, and permeabilized with PBS containing Triton (TPBS). Cells were washed, blocked with 10% goat serum, then incubated for 1 h in goat SeV Ab (abcam) or IgG isotype control Ab (Jackson). Cy3 donkey anti-goat (Jackson) was added for 1 h followed by counterstaining with Hoechst.

Microscopy analysis

SeV plaques were counted in 6 areas per slide and averaged. Samples were examined in our laboratory under an Olympus BX41 microscope, and digital images were captured using the PictureFrame Application 2.3 (Optronics).

miRNA target prediction

miRWalk database was used to predict human miRNAs with complementary seed regions to the IFNB1 mRNA 3' UTR, 5' UTR, and CDS. Predictions from miRanda, miRDB, RNA22, and Targetscan were also computed during miRWalk's analysis and candidate miRNAs with the top number of matches were selected. Selected candidates were then cross-checked with miRWalk's database of endogenous known or experimentally detected miRNAs in the HCT 116 cell line.

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