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Cryptosporidium parvum* induces SIRT1 expression in host epithelial cells through downregulating *let-7i

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Abstract

Epithelial cells along human gastrointestinal mucosal surface express pathogen-recognizing receptors and actively participate in the regulation of inflammatory reactions in response to microbial infection. The NAD-dependent deacetylase sirtuin-1 (SIRT1), one member of the sirtuin family of proteins and an NAD-dependent deacetylase has been implicated in the regulation of multiple cellular processes, including inflammation, longevity, and metabolism. In this study, we demonstrated that infection of cultured human biliary epithelial cells (H69 cholangiocytes) with a parasitic protozoan, *Cryptosporidium parvum*, induced SIRT1 expression at the protein level without a change in SIRT1 mRNA content. Using real-time PCR and Northern blot analyses, we found that *C. parvum* infection decreased the expression of *let-7i* in infected H69 cells. Down-regulation of *let-7i* caused relief of miRNA-mediated translational suppression of SIRT1 and consequently, resulted in an increased SIRT1 protein level in infected H69 cell cultures. Moreover, gain- and loss-of-function studies revealed that *let-7i* could modulate NF-κB activation through modification of SIRT1 protein expression. Thus, our data suggest that *let-7i* regulates SIRT1 expression in human biliary epithelial cells in response to microbial challenge, suggesting a new role of *let-7i* in the regulation of NF-κB-mediated epithelial innate immune response.

Keywords

let-7i; SIRT1; *C. parvum*; Epithelium; NF-κB

1. Introduction

Epithelial cells provide the first line of defense against microbial infection at the human mucosal surface [1]. Epithelial cells along the gastrointestinal tract express several types of Toll-like receptors (TLRs), which are critical to the host's defense against microbial infection. Upon ligation, TLRs can activate downstream signal pathways, such as the NF-κB signaling pathway, and initiates a series of host cell defense reactions against pathogens,

including parasites [2]. In most cells, NF- κ B exists in a latent state in the cytoplasm bound to inhibitory proteins (collectively called I κ Bs) that mask its nuclear localization signal [3]. The latent form can be activated by inducing agents that result in phosphorylation and subsequent degradation of I κ Bs. NF- κ B is then free to translocate to the nucleus, bind to specific DNA elements, and regulate gene transcription. The products of which activate mucosal inflammatory and immune responses and alter epithelial functions [4].

Cryptosporidium parvum is a coccidian parasite of the phylum *Apicomplexa* [5]. This parasite infects the gastrointestinal, biliary, and, occasionally, respiratory epithelium of humans and animals [5]. Infection causes an acute, self-limited diarrheal disease in immunocompetent individuals but potentially life-threatening syndromes in immunocompromised patients worldwide [5,6]. Clinically, biliary infection by *C. parvum* has been implicated in the development of secondary sclerosing cholangitis, which eventually leads to liver transplantation [7,8]. *C. parvum* is also a biological threat to water supplies because of its resistance to chlorine and its presence in domestic mammals which serve as reservoirs [5,9]. However, host epithelial immune responses against *C. parvum* are still poorly understood and there is currently no fully effective medical therapy. Therefore, a better understanding of the molecular mechanisms of immune responses in epithelial cells, including cholangiocytes (epithelial cells lining the biliary tract), is critical to the development of new therapeutic strategies for *C. parvum* infection.

MicroRNAs (miRNAs) regulate gene expression at post-transcriptional level by binding to the 3' untranslated regions (UTRs) and/or the coding regions of their target mRNAs [10–12]. With respect to the immune system, miRNAs may be involved in all facets of immune system development by fine-tuning the cellular responses to the environment and may function as key regulators of host antimicrobial immune response [13]. Indeed, miRNAs have been implicated in the regulation of TLR/NF- κ B signaling pathway via translational suppression of their targeted mRNAs [2,14]. SIRT1 (also known as Sirtuin 1 or NAD-dependent deacetylase sirtuin-1) is a member of the sirtuin protein family [15]. SIRT1 can inhibit NF- κ B activity through promotion of p65 deacetylation [16]. Furthermore, SIRT1 has been identified as a target for more than 16 miRNAs in both malignant and non-malignant human cell types [17]. In epithelial cells, miR-200a suppresses SIRT1 expression and subsequently, regulates epithelial to mesenchymal transition-like transformation [18].

Using an *in vitro* model of human biliary cryptosporidiosis, in this study, we provide data demonstrating that miRNA *let-7i* is decreased in cholangiocytes upon *C. parvum* infection. The decreased expression of *let-7i* contributes to *C. parvum*-induced upregulation of SIRT1 expression in infected cells. Furthermore, suppression of *let-7i* increases SIRT1 expression, which correlates with a reduction of NF- κ B activity. Our results suggest a new role of *let-7i* in the regulation of NF- κ B-mediated epithelial anti-*C. parvum* response.

2. Materials and methods

2.1. *C. parvum* and infection model

C. parvum oocysts of the Iowa strain were purchased from a commercial source (Bunch Grass Farm). H69 cells (a gift of Dr. D. Jefferson, Tufts University) are SV40-transformed

human biliary epithelial cells originally derived from normal liver harvested for transplant [14,19]. Before infecting cells, oocysts were treated with 1% sodium hypochlorite on ice for 20 min, followed by extensive washing with Dulbecco's modified Eagle medium (DMEM)-F12. Infection was done in a culture medium (DMEM-F12) containing viable *C. parvum* oocysts (oocysts with host cells at a 5:1 to 10:1 ratio), as described elsewhere [14,19].

2.2. Western blot

Whole cell lysates were obtained using the M-PER Mammalian Protein Extraction Reagent (Pierce) plus several protease inhibitors (1 mM PMSF; 10 µg/ml leupeptin, 2 µg/ml pepstatin). Cell lysates were then loaded (40 µg/lane) in a 4–12% SDS–PAGE gel to separate proteins and transferred to nitrocellulose membrane. Antibodies to SIRT1 (H-300, 1:500) and β-actin (Sigma–Aldrich, 1:1000) were used for detection. The optical density of the SIRT1 and β-actin bands was quantified using the ImageJ software. The values are presented as the ratio of SIRT1 and Actin optical density. Data are representative of three independent experiments.

2.3. Real-time PCR

Total RNAs were extracted using Trizol reagent (Ambion) and PCR reactions were carried out in triplicate using the SYBR Green PCR master mix (Applied Biosystems) [20]. The primers were: 5'-TTTATGCTCGCCTTGCTGTAG-3' (forward) and 5'-GAGAGATGGCTGGAATTGTCC-3' (reverse) for human SIRT1; 5'-TGCAGCTCTGTGTGAAGGTG-3' (forward) and 5'-ACAACCCTCTGCACCCAGTT-3' (reverse) for human IL-8; and 5'-TGCACCACCAACTGCTTAGC-3' (forward) and 5'-GGCATGGACTGTGGTCATGAG-3' (reverse) for human GAPDH. The *Ct* values were analyzed using the comparative *Ct* (–*Ct*) method. The amount of target was obtained by normalizing to the endogenous reference (GAPDH) and relative to control (untreated cell) [21].

For analysis of *let-7i*, total RNA was isolated from cells with the mirVana™ miRNA Isolation kit (Ambion). Comparative real-time PCR was performed by using the Taqman Universal PCR Master Mix (Applied Biosystems). Specific primers and probes for mature *let-7i* and snRNA RNU6B were obtained from Applied Biosystems. All reactions were run in triplicate. The amount of *let-7i* was obtained by normalizing to snRNA RNU6B and relative to control (untreated cell) as previously reported [14,20].

2.4. let-7i Precursor and antisense oligonucleotide

To manipulate cellular function of *let-7i*, we utilized an anti-sense approach to inhibit *let-7i* function and a precursor transfection approach to increase *let-7i* expression in H69 cells. Cells were grown up to 80% confluence and treated with *let-7i* antisense 2-methoxy oligonucleotide (Ambion) or the *let-7i* precursor (Ambion) using the lipofectamine™ 2000 reagent (Invitrogen). Experiments were typically performed 48 h after transfection.

2.5. Northern blot

Total RNAs harvested as above were run on a 15% Tris/Borate/EDTA (90 mM Tris/64.6 mM boric acid/2.5 mM EDTA (pH 8.3)) urea gel (Invitrogen) and transferred to a Nytran

nylon transfer membrane (Ambion). A LNA DIG-probe of *let-7i* (Exiqon) was hybridized using UltraHyb reagents (Ambion) according to the manufacturer's instructions with blotted snRNA RNU6B as a control.

2.6. Luciferase reporter constructs and luciferase assay

Transfection and luciferase assay were performed as described previously [14,22]. Briefly, cells were transiently co-transfected with the pGL3-BasicIL-8 promoter reporter construct (a gift of Dr. Nicholas F. LaRusso, Mayo Clinic at Rochester) and the anti-control or anti-*let-7i* for 24 h. Transfected cells were then treated with the SIRT1 inhibitor (5 mM Nicotinamide, Sigma–Aldrich), followed by assessment of luciferase activity. Luciferase activities were measured and normalized to the control β -gal level. The luciferase activity of each construct was compared with that of the promoterless pGL3 basic vector. All transfections were performed in triplicate and repeated at least twice.

3. Results

3.1. *C. parvum* infection induces expression of SIRT1 protein without a change in SIRT1 mRNA levels in H69 cells

We first assessed SIRT1 expression in H69 cells in response to *C. parvum* infection. When H69 cells were exposed to *C. parvum* for up to 24 h, a significant increase of SIRT1 protein content was detectable in H69 cells following *C. parvum* infection (Fig. 1A). Interestingly, no significant change of SIRT1 mRNA levels was detected by real-time PCR analysis (Fig. 1B). In contrast, as a positive control, a significant increase of IL-8 mRNA was found in cells following exposure to *C. parvum* (Fig. 1C), which is consistent with results from previous studies [23].

3.2. *C. parvum* infection decreases *let-7i* expression in H69 cells

The inconsistency of SIRT1 expression between the message level and its protein level in H69 cells following *C. parvum* infection suggests the potential of posttranscriptional mechanisms. miRNA-mediated posttranscriptional gene regulation may be involved in this process. Of those miRNAs that have been identified in H69 cells [21], we identified a potential targeting site within the coding region of SIRT1 mRNA by *let-7i* (Fig. 2A). To test whether *let-7i* may be involved in *C. parvum*-induced SIRT1 expression, we first detected miRNA *let-7i* expression in H69 cells following *C. parvum* infection. Using a probe detecting *let-7i* by Northern blot (Fig. 2B), we detected a significant decrease in *let-7i* levels in H69 cells following exposed to *C. parvum* for 12 h (Fig. 2B). Furthermore, a significant decrease in *let-7i* was confirmed in H69 cells following *C. parvum* infection by real-time PCR analysis specific for *let-7i* (Fig. 2C).

3.3. Manipulation of *let-7i* function results in reciprocal alterations in SIRT1 protein levels in H69 cells

We then treated H69 cells with anti-*let-7i* or *let-7i* precursor for 72 h and measured SIRT1 protein expression by Western blot. Transfection of H69 cells with the *let-7i* precursor caused a dose-dependent decrease in SIRT1 protein content (Fig. 3A). No significant change in SIRT1 mRNA levels was found between the control cells and cells treated with *let-7i*

precursor (Fig. 3B), suggesting no effect on cellular SIRT1 mRNA levels. Conversely, an increase in SIRT1 protein content was identified in H69 cells treated with anti-*let-7i* (Fig. 3C). However, no significant change in SIRT1 mRNA levels was found between the control cells and cells treated with anti-*let-7i* (Fig. 3D). These data suggest that SIRT1 is a target for *let-7i* in human cholangiocytes.

3.4. Transfection of *let-7i* precursor abolishes *C. parvum*-induced SIRT1 protein expression

To confirm that relief of miRNA-mediated SIRT1 translational repression is required for *C. parvum*-induced SIRT1 protein expression, we transfected H69 cells with various doses of *let-7i* precursors for 48 h and then exposed them to *C. parvum* for 24 h followed by Western blot for SIRT1 protein. The *let-7i* precursor significantly inhibited up-regulation of SIRT1 protein in H69 cells induced by *C. parvum* infection in a dose-dependent manner (Fig. 4). Thus, *let-7i* precursor can abolish the up-regulation of SIRT1 protein in cholangiocytes in response to *C. parvum* infection. Coupled with the down-regulation of *let-7* in cells following *C. parvum* infection, the above data suggest that the relief of *let-7i*-mediated translational repression is required for *C. parvum*-induced SIRT1 protein expression.

3.5. Inhibition of SIRT1 restores anti-*let-7i*-reduced NF- κ B activation

The SIRT1 protein has emerged as a key physiological negative regulator of NF- κ B activity [24]. To test whether downregulation of *let-7i* can influence NF- κ B activity through induction of SIRT1, we treated H69 cells with a NF- κ B-driven IL-8 luciferase reporter construct [21] and measured the luciferase activity in the presence of anti-*let-7i* or a SIRT1 inhibitor. Intriguingly, we found that suppression of *let-7i* through transfection of cells with the anti-*let-7i* significantly inhibited the NF- κ B-driven IL-8 luciferase reporter activity (Fig. 5A). Inhibition of SIRT1 increased anti-*let-7i*-reduced luciferase activity (Fig. 5A). To confirm that anti-*let-7i* inhibits NF- κ B-driven IL-8 luciferase reporter activity through induction of SIRT1, we co-transfected cells with the NF- κ B reporter construct and anti-Ctrl or anti-*let-7i* in the absence or presence of the SIRT1 inhibitor. Transfected cells were then exposed to LPS to induce NF- κ B activity, followed by luciferase activity assay. Interestingly, we detected that knockdown of *let-7i* through transfection of cells with the anti-*let-7i* significantly inhibited LPS-induced IL-8 reporter activity (Fig. 5B). SIRT1 inhibitor could partially restore IL-8 reporter activity suppressed by anti-*let-7i*. Thus, downregulation of *let-7i* may inhibit NF- κ B activity through relief of miRNA-mediated translational repression of SIRT1.

4. Discussion

In this study, we demonstrated that (1) posttranscriptional repression of SIRT1 exists in human cholangiocytes; (2) *C. parvum* infection downregulates the expression of *let-7i*; (3) targeting of SIRT1 by *let-7i* results in posttranscriptional repression of SIRT1; (4) relief of *let-7i*-mediated translational repression may be involved in *C. parvum*-induced expression of SIRT1 expression in cholangiocytes; and (5) *let-7i* can influence NF- κ B activity through regulation of SIRT1 expression. These data suggest that miRNA regulates SIRT1 expression

in cholangiocytes, a process that may be associated with the regulation of inflammatory responses in epithelial cells during *C. parvum* infection.

SIRT1 is a multiple functional protein which participates in the development of cancer, metabolic disorders, and aging. Due to its functional importance, cellular expression level of SIRT1 is tightly controlled through both transcriptional and post-transcriptional mechanisms. Currently, there are more than 16 miRNAs that have been identified to regulate SIRT1 translation in various cell types for different biological processes. Of these miRNAs, miR-34a is the most well studied which has emerged as a tumor suppressor miRNA in neuroblastoma [25]. In addition, targeting of SIRT1 by miR-34a has been reported in colorectal cancer, prostate cancer, hepatocarcinoma, glioma, and pancreatic cancer [26–31]. Here, we reported that SIRT1 is a target of *let-7i* in human cholangiocytes. Notably, the potential targeting site of SIRT1 mRNA by *let-7i* is at its coding region. Our data show that *C. parvum* infection increased SIRT1 expression at protein level but not mRNA level, indicating post-transcriptional mechanisms. Overexpression of *let-7i* caused a decreased expression of SIRT1 at the protein level. Conversely, an increased expression of SIRT1 protein was identified in cholangiocytes treated with anti-*let-7i*.

C. parvum infection can activate the TLR4/NF- κ B signaling pathway and alter cholangiocyte miRNA expression profile in host epithelial cells. As one of the downregulated miRNAs in infected host cells, *let-7* appears to be a key regulator to control the activity of the TLR/NF- κ B signaling pathway in the host cells during *C. parvum* infection. Specifically, *let-7i* has been implicated in the negative regulation of TLR4 expression and contributes to cholangiocyte immune responses against *C. parvum* infection [2]. *let-7* has also been reported to downregulate the expression of cytokine-inducible Src homology 2-containing protein (CIS) in epithelial cells following LPS stimulation or *C. parvum* infection [14]. CIS enhances NF- κ B activation and binds to I κ B α . Data from the current study support a new mechanism by which *let-7i* may provide a feedback regulatory loop for NF- κ B signaling. SIRT1 can suppress NF- κ B activity by its ability to deacetylate p65 [16]. Thus, induction of SIRT1 through relief of *let-7i*-mediated posttranscriptional suppression in host epithelial cells during *C. parvum* infection may provide an inhibitory feedback to NF- κ B activity to avoid overreactions of inflammation in epithelial cells (Fig. 5C).

C. parvum usually only infects the epithelial cells along the gastrointestinal tract in immunocompetent individuals. In immunocompromised patients, infection can also result in extra-intestinal cryptosporidiosis, including biliary infection in AIDS patients and in liver transplant recipients under immunosuppressive drugs [32]. Biliary cryptosporidial infection has been implicated as a pathogenic factor for the development of secondary sclerosing cholangitis [7,8,33]. Comparing with intestinal epithelial cells, cholangiocytes express multiple TLRs and are more susceptible to TLR ligation, and thus, have been extensively used for the host-*C. parvum* interactions. Upon *C. parvum* infection, cultured human cholangiocytes displayed a significant alteration in miRNA expression profile, including upregulation and downregulation of certain miRNA genes through the activation of NF- κ B pathway [21]. Functional inhibition of a panel of upregulated miRNAs (e.g., miR-125b, miR-23b and miR-30b) in cholangiocytes increases *C. parvum* infection burden, indicating

that miRNAs are actively players in clearance of *C. parvum* infection [21]. In the current study, we demonstrated that *let-7i* plays a regulatory role for the NF- κ B pathway in cholangiocytes in response to *C. parvum* infection through regulating SIRT1 expression. Our findings provide new insights into the mechanisms of miRNA-mediated epithelial anti-*C. parvum* response. Given the importance of interferon- γ and human leukocyte antigens (HLAs) in anti-*C. parvum* immunity [34–36], it merits further investigation on the potential of interferon- γ and HLAs by miRNAs associated with *C. parvum* infection in epithelial cells.

In conclusion, our data support that *let-7* family miRNAs play a bidirectional effect in regulating NF- κ B activity. *let-7* family miRNAs may serve as a fulcrum in cholangiocytes linking the actions of *C. parvum* on TLR4 and NF- κ B signaling, simultaneously increasing TLR4 signaling while decreases NF- κ B activity, resulting in fine-tuning of inflammation response against *C. parvum* infection.

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Abbreviations

miRNA	microRNA
SIRT1	sirtuin 1, NAD-dependent deacetylase sirtuin-1
UTR	untranslated region
TLR	Toll-like receptor

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Fig. 1.

C. parvum infection induces expression of SIRT1 protein without a change in SIRT1 mRNA levels in cholangiocytes. (A) Up-regulation of SIRT1 protein in human cholangiocytes following *C. parvum* infection. A representative Western blot from three independent experiments is shown in A. Actin was blotted as a loading control. Densitometric levels of SIRT1 signals were quantified and expressed as the ratio to actin. (B) No significant change in SIRT1 mRNA expression in human cholangiocytes in response to *C. parvum* infection. H69 cells were exposed to *C. parvum* oocysts followed by real-time PCR analysis for SIRT1 mRNA. (C) Expression of IL-8 mRNA in H69 cells in response to *C. parvum* infection. * $p < 0.05$ ANOVA vs non-infected controls.

Fig. 2.

C. parvum infection decreases expression of *let-7i* in cholangiocytes. (A) The schematic of SIRT1 mRNA shows a potential binding site in the coding region for miRNA *let-7i*. (B) *C. parvum* infection decreases *let-7i* expression in H69 cells as assessed by real-time PCR. * $p < 0.05$ ANOVA vs the controls. (C) *C. parvum* infection decreases *let-7i* expression in H69 cells by Northern blot analysis. RNU6B (U6) was used as a loading control.

Fig. 3.

Manipulation of *let-7i* function results in reciprocal alterations in SIRT1 protein expression in H69 cells. (A and C) Transfection of *let-7i* precursor or anti-*let-7i* induces a dose-dependent decrease or increase, respectively, in SIRT1 protein expression in H69 cells. H69 cells were treated with various dose of *let-7i* precursor (A) or anti-*let-7i* (C) followed by Western blot for SIRT1. (B and D) *let-7i* Precursor or anti-*let-7i* transfection does not affect SIRT1 mRNA levels. H69 cells were exposed to *let-7i* precursor or anti-*let-7i* followed by real-time PCR analysis for SIRT1 mRNA. Pre =precursor. * $p < 0.05$ ANOVA vs the nontreated control cells.

Fig. 4.

Transfection of *let-7i* precursor abolishes *C. parvum*-stimulated SIRT1 protein expression. Transfection of *let-7i* precursor inhibits up-regulation of SIRT1 proteins in H69 cells following *C. parvum* infection. Cells were transfected with the *let-7i* precursor and then exposed to *C. parvum* followed by Western blotting for SIRT1. Data are representative of three independent experiments. * $p < 0.05$, vs the nonstimulated control; # $p < 0.05$, vs the *C. parvum* infection group.

Fig. 5. miRNA *let-7i* reduces NF-κB activation via regulation of SIRT1 expression. (A) Suppression of *let-7i* inhibits NF-κB activation. Cells were transfected with pGL3-BasicIL-8 promoter and the Anti-*let-7i*. Luciferase activity, reflecting NF-κB activation, was then monitored. * $p < 0.05$ ANOVA vs the nontreated cells; # $p < 0.05$ ANOVA vs treated control cells. (B) Suppression of *let-7i* inhibits LPS-induced NF-κB activation. Cells were transfected with pGL3-BasicIL-8 promoter and the Anti-Ctrl or Anti-*let-7i* for 24 h and then exposed to LPS for 4 h. Luciferase activity, reflecting NF-κB activation, was then monitored. * $p < 0.05$ ANOVA vs the nontreated cells; # $p < 0.05$ ANOVA vs Anti-*let-7i* infected cells; † $p < 0.05$ ANOVA vs Anti-Ctrl + LPS treated cells; ‡ $p < 0.05$ ANOVA vs Anti-*let-7i* + LPS treated cells. (C) Model of *let-7i* regulation of NF-κB activity. miRNA *let-7i* is negatively regulated by TLR4/NF-κB signaling pathway and functions to repress multiple factors, including TLR4, CIS and SIRT1. CIS can enhance NF-κB activation by increasing degradation of IκBα, while SIRT1 is a NF-κB suppressor by its ability to deacetylate p65. (–) Represents negative regulation; (+) represents positive regulation.