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Research paper

Nuclear delivery of parasite Cdg2_FLc_0220 RNA transcript to epithelial cells during *Cryptosporidium parvum* infection modulates host gene transcription

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ABSTRACT

Intestinal infection by the zoonotic protozoan, *Cryptosporidium parvum,* causes significant alterations in the gene expression profile in host epithelial cells. The molecular mechanisms of how *C. parvum* may modulate host cell gene transcription and the pathological significance of such alterations are largely unclear. Previous studies demonstrate that a panel of parasite RNA transcripts are delivered into infected host cells and may modulate host gene transcription. Using *in vitro* models of intestinal *cryptosporidiosis*, in this study, we analyzed the impact of host delivery of *C. parvum* Cdg2_FLc_0220 RNA transcript on host gene expression profile. We found that alterations in host gene expression profile following *C. parvum* infection were partially associated with the nuclear delivery of Cdg2_FLc_0220. Specifically, we identified a total of 46 overlapping upregulated genes and 8 overlapping downregulated genes in infected cells and cells transfected with Full-Cdg2_FLc_0220. Trans-suppression of the DAZ interacting zinc finger protein 1 like (*DZIP1L*) gene, the top overlapping downregulated gene in host cells following *C. parvum* infection and cells transfected with Full-Cdg2_FLc_0220, was mediated by G9a, independent of PRDM1. Cdg2_FLc_0220-mediated trans-suppression of the *DZIP1L* gene was independent of H3K9 and H3K27 methylation. Data from this study provide additional evidence that delivery of *C. parvum* Cdg2_FLc_0220 RNA transcript in infected epithelial cells modulates the transcription of host genes, contributing to the alterations in the gene expression profile in host epithelial cells during *C. parvum* infection.

1. Introduction

Cryptosporidium is an important protozoan diarrheal pathogen in animals and humans (Checkley et al., 2015, Certad et al., 2017). Persistent watery diarrhea caused by *Cryptosporidium* infection has been reported as potentially fetal in youth, the immunocompromised (e.g., HIV/AIDS), and transplant recipients (Borad and Ward, 2010; Fishman, 2011; Acikgoz et al., 2012). Investigations on diarrheal etiologies in children also showed that *Cryptosporidium* is responsible for 15–25% of diarrheal cases (Chen et al., 2002; Checkley et al., 2015). Although asymptomatic infection of *Cryptosporidium* was observed in the majority of cases in humans and animals, colonization of this parasite can damage the intestinal barriers, affecting nutrition absorption, possibly causing persistent retardation of growth and impairing the immune response of host (Guerrant et al., 1999; Mondal et al., 2009; Squire and Ryan, 2017). More importantly, uneliminated oocysts excreted from these ignored hosts into environment could spread the infection to other hosts and would be an important source of waterborne outbreak of *cryptosporidiosis* (Checkley et al., 2015). At least 163 outbreaks of waterborne diseases were caused by *Cryptosporidium* infection (Karanis et al., 2007), and this protist has been listed as an indicator for water quality in the USA, UK, Australia and China. However, only one drug, nitazoxanide, has been approved by the American Food and Drug Administration (FDA) to treat *cryptosporidiosis*, and is efficacious in only 56–96% of immunocompetent hosts, but lacks efficacy in *cryptosporidiosis* patients with advanced AIDS (Rossignol et al., 2001; Amadi et al., 2002, 2009; Checkley et al., 2015).

Considering the close relationship between the severity of *cryptosporidiosis* and host status (e.g., immunity, nutrition, and age), exploring the mechanism of interaction between *Cryptosporidium* and host is key to developing resolution strategies for controlling *Cryptosporidium*. After internalization and residence in mature parasitophorous vacuoles at the apex of the host cells (Marcial and Madara, 1986), a direct connection (the feeder organelle) is formed between *Cryptosporidium*

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and host cell cytoplasm at the host cell-parasite interfaces, and is an important structure for regulating transportation of nutrition, molecular effectors and drugs (Marcial and Madara, 1986; Tzipori and Griffiths, 1998; Perkins et al., 1999; Huang et al., 2004; O'Hara and Chen, 2011; Wang et al., 2017a,b). Genomic and transcriptomic analyses showed that various protein-coding genes encoded in the *Cryptosporidium parvum* genome were released and involved in host-parasite interaction, and parasite intracellular development (Abrahamsen et al., 2004; Huang et al., 2004; Puiu et al., 2004; Wastling et al., 2009; Certad et al., 2017). A comprehensive transcriptomic analysis of the intracellular stages of *C. parvum* revealed a cascade of gene expression consistent with unique biologies for each developmental stage following parasitization of intestinal epithelial cells; many of these putative developmental stage-specific genes are of unknown function (Mauzy et al., 2012). In 2011, one hundred eighteen "orphan" RNA transcripts were identified in the sporozoites of *C. parvum* (Yamagishi et al., 2011). Our previous study indicated that several of them could be selectively delivered into the nuclei of infected host epithelial cells (Wang et al., 2017a). Further study revealed that nuclear delivery of parasite Cdg7_FLc_0990 RNA (GeneBank ID: FX115678.1) (Yamagishi et al., 2011) into infected intestinal epithelial cells suppresses transcription of the *LRP5*, *SLC7A8*, and *IL33* genes through histone modification-mediated epigenetic mechanisms (Wang et al., 2017b). The parasite Cdg2_FLc_0220 RNA, a transcript from a hypothetical protein gene (GeneBank ID: FX115592.1) located at the Chromosome 2 (Yamagishi et al., 2011), is delivered into the nuclei of infected host epithelial cells (Wang et al., 2017a). In the present study, the distinct role of nuclear delivery of Cdg2_FLc_0220 RNA in modulating transcription of host genes, such as the DAZ interacting zinc finger protein 1-like (*DZIP1L*), in intestinal epithelial cells infected with *C. parvum* was addressed.

2. Materials and methods

2.1. Parasites and in vitro infection model

C. parvum oocysts used in this study were the Iowa isolate purchased from Bunch Grass Farm. Oocysts were firstly treated with 20% sodium hypochlorite at 4 °C for 20 min, and then washed twice with Phosphate Buffered Saline (PBS) and RPMI-1640. Viable oocysts were resuspended in RPMI-1640, and respectively used to infect human carcinoma intestinal epithelial HCT-8 cells (ATCC) and non-carcinoma small intestinal epithelial FHs 74 Int cells (INT) (ATCC) with a ratio of oocysts to host cells at 5:1 to 10:1 in the serum free mediums to establish *in vitro* infection models. Stable HCT-8-G9a$^{-/-}$ cells were generated through transfection of cells with the G9a-CRISPR/Cas9 KO$^{(h)}$ and G9a-HDR plasmids (Santa Cruz), as previously reported (Ming et al., 2017). Cells were washed with PBS to remove oocyst walls, oocysts and free sporozoites, and changed with complete culture medium at 4 h post infection.

2.2. Overexpression of C. parvum Cdg2_FLc_0220

Total RNA was isolated from *C. parvum* oocyst using Tri-reagent solution (invitrogen) and chloroform/isopropanol. The genomic DNA was removed from total RNA samples by DNase Treatment & Removal (ambion), and treated RNA was reverse transcribed into cDNA with M-MLV (invitrogen). *C. parvum* Cdg2_FLc_0220 was amplified using primers listed in Table S1, sub-cloned into the pcDNA3.1 vector (invitrogen) and transformed into *Escherichia coli* DH5α to construct the overexpression plasmid of Cdg2_FLc_0220 (named as Full-Cdg2_FLc_0220). INT (1×10^5) and HCT-8 (2×10^6) cells were respectively seeded into 24-well plates. After cultured for 24 h at 37 °C and 5% CO_2, each well with cells was transfected with 1 μg Full-Cdg2_FLc_0220 or empty vector pcDNA3.1 using the Lipofectamine 2000 Reagent (Invitrogen) and Opti-MEM (Gibco). Cells were changed with complete mediums and collected at 24 h and 48 h after transfection for further study.

2.3. Microarray analysis

The Agilent SurePrint G3 Human Gene Expression Microarray and service to process the samples were applied to genome-wide analysis. Briefly, INT cells were grown to 80% confluence and exposed to *C. parvum* infection or Full-Cdg2_FLc_0220 transfection, respectively. Total RNA of harvested cells was isolated with the RNeasy Mini kit (Qiagen). A mixture of equal amounts of total RNAs from each group was used as the reference pool. A total of 2 μg RNA from each sample was then labeled with the Agilent Gene Expression Hybridization Kit (Agilent). After hybridization, the slides were scanned with the Agilent Microarray Scanner (Agilent). The Feature Extraction software (version10.7.1.1, Agilent Technologies) was used to analyze array images to get raw data and Genesrping software was employed to finish the basic analysis with the raw data. Quantified positive signals were then extracted and analyzed by the LC Sciences, in accordance with MIAME guidelines. Protein coding genes differentially expressed after *C. parvum* infection and overexpression of Cdg2_FLc_0220 were screened by fold change > 1.3 and $P < .05$. The heatmaps for genes differentially expressed in both arrays after infection and overexpression were depicted by using MeV 4.9.0.

2.4. Total RNA extraction from whole cells and nuclear extracts and cDNA synthesis

Cells were directly cleaved by using Tri-reagent solution (invitrogen) to extract total RNA from whole cells, and cell pellets were collected by using trypsin-EDTA (Gemini Bio Products) and washed with PBS for isolation nuclear extracts. Cell pellets were then treated by 500 μl nuclear buffer A (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM MgCl2, 0.5% NP-40) for 10 min at 4 °C, centrifuged at 1000 rpm for 2 min at 4 °C, and resuspended in 500 μl nuclear buffer B (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM MgCl2). Nuclear extracts were collected by centrifugation at 2300 rpm for 2 min at 4 °C, and treated by Tri-reagent solution (invitrogen). Both total RNA samples of whole cells and nuclear extracts were extracted and reversely transcribed into cDNA as 2.2.

2.5. Real-time PCR

Realtime PCR was performed using the SYBR Green polymerase chain reaction master mix (Applied Biosystems) in ABI-Prism 7900HT (Applied Biosystems, Foster City, CA, USA) with primers listed in Table S1. Experiments were performed in triplicate and the values were normalized to GAPDH (for whole cell analysis) or U2 (for nuclear delivery analysis) and expressed as $2^{-\Delta\Delta Ct}$.

2.6. Western blot

Whole cell protein lysates were prepared using M-PER® Mammalian Protein Extraction Reagent (Thermo scientific) in the presence of protease inhibitors and measured using Bio-Rad *DC* Protein Assay Reagent (Bio-Rad). Proteins (20 μg) were loaded into 10% SDS–polyacrylamide electrophoresis gels and transferred to nitrocellulose membrane. The following antibodies were used for blotting: anti-DZIP1L (Santa Cruz Biotechnology), anti-H3 (Cell Signaling), anti-Cyclophilin A (Cell Signaling), and anti-GAPDH (Santa Cruz Biotechnology).

2.7. Formaldehyde cross-linking RNA immunoprecipitation (RIP)

Cell pellets were collected for HCT-8 cells infected with *C. parvum* oocysts at 24 h post infection (pi) and washed with PBS. The cross-linking reaction was performed with 0.3% of formaldehyde at 37 °C for