REVIEW ARTICLE

Non-coding RNAs in epithelial immunity to *Cryptosporidium* infection

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SUMMARY

Cryptosporidium spp. is a protozoan parasite that infects the gastrointestinal epithelium and causes diarrhoeal disease worldwide. It is one of the most common pathogens responsible for moderate to severe diarrhoea in children younger than 2 years. Because of the 'minimally invasive' nature of *Cryptosporidium* infection, mucosal epithelial cells are critical to the host's anti-*Cryptosporidium* immunity. Gastrointestinal epithelial cells not only provide the first and most rapid defence against *Cryptosporidium* infection, they also mobilize immune effector cells to the infection site to activate adaptive immunity. Recent advances in genomic research have revealed the existence of a large number of non-protein-coding RNA transcripts, so called non-coding RNAs (ncRNAs), in mammalian cells. Some ncRNAs may be key regulators for diverse biological functions, including innate immune responses. Specifically, ncRNAs may modulate epithelial immune responses at every step of the innate immune network following *Cryptosporidium* infection, including production of antimicrobial molecules, expression of cytokines/chemokines, release of epithelial cell-derived exosomes, and feedback regulation of immune homoeostasis. This review briefly summarizes the current science on ncRNA regulation of innate immunity to *Cryptosporidium*, with a focus on microRNA-associated epithelial immune responses.

Key words: *Cryptosporidium*, ncRNAs, miRNAs, lincRNAs, epithelial cells, immunity.

INTRODUCTION

Cryptosporidium spp. infects the gastrointestinal epithelium of vertebrate hosts and is an important protozoan parasite for humans; in particular, in HIV/AIDS patients and young children (Striepen, 2013). Infection in humans is characterized by self-limited diarrhoea and abdominal pain that usually last several days, but that can be chronic and life-threatening in immunocompromised hosts. The majority of human cryptosporidial infections are caused by *Cryptosporidium parvum* and *Cryptosporidium hominis*. In a recent massive clinical and epidemiological undertaking, the Global Enterics Multicenter Study (GEMS) demonstrated that four pathogens account for the bulk of diarrhoea-associated mortality in small children. After rotavirus, *Cryptosporidium* is the most common pathogen responsible for moderate-to-severe diarrhoea in children younger than 2 years, and particularly in infants (Kotloff *et al*. 2013). Infection with *Cryptosporidium* shows significant association with mortality in this age group and appears to predispose children to lasting deficits in age-appropriate body growth and cognitive development (Goodgame, 1996; Pierce and Kirkpatrick, 2009; Putignani and Menichella, 2010). The options for treatment are severely limited; only a single drug with limited efficacy is currently approved by the FDA (nitazoxanide, marketed under the name Alinia). There currently is no vaccine to prevent cryptosporidiosis.

The self-limiting nature of *Cryptosporidium* infection in immunocompetent subjects suggests that the host activates an efficient immune response to eliminate the infection. Evidence from *in vitro* and *in vivo* studies indicates that both innate and adaptive immunity are involved in the resolution of cryptosporidiosis and resistance to infection (Pantenburg *et al*. 2008; Petry *et al*. 2010; McDonald *et al*. 2013). After entry into host epithelial cells, the parasite resides within a unique intracellular but extracytoplasmic niche, separating the parasite from direct interaction with other cell types (Tzipori and Griffiths, 1998). Therefore, innate immune responses

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by epithelial cells are critical to the host's defence against infection.

Recent advances in genomic research have revealed the existence of a large number of non-coding RNAs (ncRNAs) in mammalian cells (Guttman *et al*. 2009). Two classes of ncRNAs, microRNAs (miRNAs) and the long intergenic ncRNAs (lincRNAs), have been shown to play key regulatory roles in diverse biological functions (Mercer *et al*. 2009). miRNAs are small regulatory RNAs that mediate either mRNA cleavage or translational suppression (Bartel, 2004). LincRNAs are long non-coding transcripts (> 200 nt) from the intergenic regions of annotated protein-coding genes (Ulitsky and Bartel, 2013). Emerging evidence supports a key regulatory role for lincRNAs across diverse biological functions, including gene transcription (Mercer *et al*. 2009; Lee, 2012). Both miRNAs and lincRNAs have been demonstrated to be regulators in host antimicrobial immune responses (Zhou *et al*. 2011; Carpenter *et al*. 2013). A better understanding of the role of ncRNAs in epithelial immunity to *Cryptosporidium* will provide new insights for the potential development of novel therapeutic strategies. Here, we briefly summarize the current understanding of ncRNA regulation of innate immunity to *C. parvum*, with a focus on miRNA-associated epithelial immune responses. For recent advances in general features of innate and adaptive immunity to *C. parvum*, readers are referred to more comprehensive reviews on the topic (Borad and Ward, 2010; Petry *et al*. 2010; McDonald *et al*. 2013).

MUCOSAL EPITHELIAL CELLS ARE CRITICAL PLAYERS IN THE HOST'S INNATE IMMUNITY TO *CRYPTOSPORIDIUM* INFECTION

In addition to providing a natural barrier that limits infection, the gastrointestinal epithelium also plays a critical role in the initial recognition of parasites and the triggering of adaptive immunity. Epithelial cells are equipped with several defence mechanisms to guard against infection by pathogens. Recent studies indicate that epithelial cells express a variety of pathogen pattern recognition receptors (PRRs), such as the Toll-like receptors (TLRs) and nucleotide binding and oligomerization domain-like receptors (NLRs), which recognize pathogens or pathogen-associated molecular patterns (PAMPs) (Kawai and Akira, 2010). TLRs recognize microbes on the cell surface and in endosomes, whereas NLRs sense microbial molecules in the cytosol. Upon specific pathogen recognition, these receptors recruit adaptor proteins and activate downstream signalling cascades that regulate the activity of nuclear factor kappa-B (NF-κB), mitogen-activated protein kinases (MAPK), or caspase-dependent signalling pathways (Kawai and Akira, 2010). This activation induces the expression of several adhesion molecules, inflammatory mediators (e.g. cytokines/chemokines), and antimicrobial peptides, initiating innate epithelial immune responses against pathogen infection (Fig. 1).

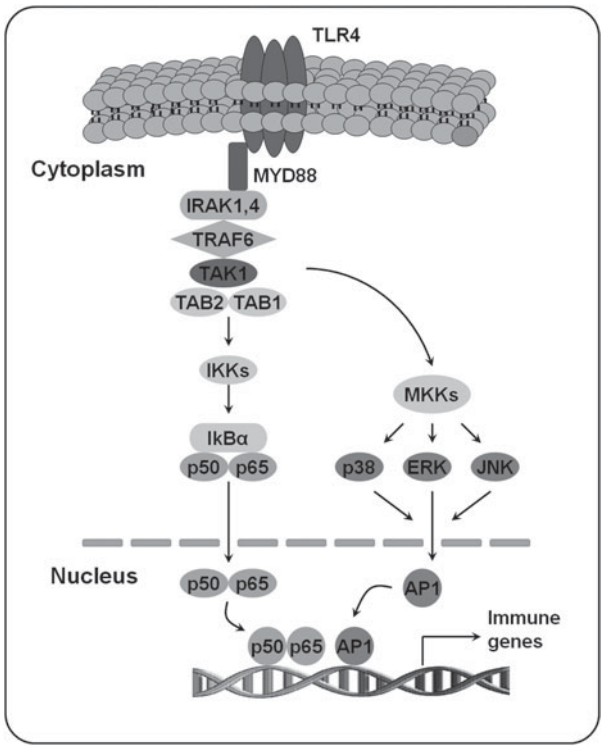

Fig. 1. TLR4/NF-κB signalling pathway and TLR4/MAPK signalling pathway in gastrointestinal epithelium. Epithelial cells express a variety of pathogen pattern recognition receptors (PRRs), such as TLRs, which recognize PAMPs. TLRs recognize microbes on the cell surface. Upon specific pathogen recognition, these receptors recruit adaptor proteins and activate downstream signalling cascades that regulate the activity of nuclear factor kappa-B (NF-κB), mitogen-activated protein kinases (MAPK), or caspase-dependent signalling pathways. This activation induces the expression of several adhesion molecules, inflammatory mediators (for example, cytokines/chemokines), and antimicrobial peptides, initiating innate epithelial immune responses against pathogen infection.

Activation of the NF-κB pathway through TLRs/NLRs is a common response in many epithelial cells in response to microbial infection. The NF-κB family of transcription factors consists of five members: p50, p52, p65 (RelA), c-Rel and RelB. In most cells, NF-κB exists in a latent state in the cytoplasm bound to inhibitory κBs that mask its nuclear localization signal (Hayden and Ghosh, 2008). Activation of NF-κB causes it to move into the nucleus and regulate the expression of a number of host genes, including miRNAs (Zhou *et al*. 2009, 2011). The transcription activation domain (TAD) necessary for the positive regulation of gene expression is present only in p65, c-Rel and RelB. Thus, promoter binding of p65, c-Rel and RelB usually is associated with gene transactivation. Due to their lack of TADs, p50 and p52 usually repress gene transcription, unless they are associated with a TAD-containing NF-κB family

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member or another protein capable of coactivator recruitment (Poppelmann *et al*. 2005).

After entry into host epithelial cells, *Cryptosporidium* spp. resides within a unique intracellular but extracytoplasmic niche, separating the parasite from direct interaction with other cell types. Therefore, *Cryptosporidium* spp. has been classified as a 'minimally invasive' mucosal pathogen (Chen *et al*. 2002). Because of the 'minimally invasive' nature of *Cryptosporidium* infection, mucosal epithelial cells are critical to the host's anti-*Cryptosporidium* immunity. Indeed, a recent study with newborn mice showed similar acute patterns of *C. parvum* infection in C57BL/6 wild-type and T and B cell-deficient $Rag2^{-/-}$ mice (Korbel *et al*. 2011). Upon *Cryptosporidium* infection, epithelial cells quickly initiate a series of innate immune reactions, including production of antimicrobial peptides (e.g. β-defensins and LL-37) and release of inflammatory cytokines/chemokines, such as interleukin-8 (IL-8) (Borad and Ward, 2010; Petry *et al*. 2010; McDonald *et al*. 2013). Production and secretion of antimicrobial peptides can kill *Cryptosporidium* or inhibit parasite growth. These cytokines/chemokines of epithelial cell origin can mobilize and activate immune effector cells (e.g. lymphocytes, macrophages and neutrophils) to the infection sites (Blikslager *et al*. 2007). Recent evidence implies that the release of epithelial exosomes may be an additional element of epithelial anti-*Cryptosporidium* defence (Hu *et al*. 2013).

NON-CODING RNAS ARE EMERGING REGULATORS IN HOST ANTIMICROBIAL IMMUNITY AND DEFENCE

It has become increasingly apparent that the non-protein-coding portion of the genome is of crucial functional importance for normal development and physiology, as well as for disease (Mercer *et al*. 2009). This non-protein-coding portion reflects these RNA transcripts that do not encode a protein product, so-called ncRNAs. ncRNA genes include highly abundant and functionally important RNAs, including transfer RNA (tRNA), ribosomal RNA (rRNA), snRNAs, miRNAs, siRNAs, piRNAs, and lincRNAs (Fig. 2). Among these ncRNAs, miRNAs and lincRNAs have received much attention as newly identified families of regulators in animal and plant cells. miRNAs comprise a large family of ~21-nucleotide-long RNAs that can bind to the 3′-untranslated region (3′UTR) of mRNAs based on perfect or nearly perfect complementarity. LincRNAs are thought to be longer than 200 nucleotides and have little or no protein-coding capacity. So far, a total of 1048 mature miRNAs and almost 3300 lincRNAs have been identified in humans (Kozomara and Griffiths-Jones, 2011; Schonrock *et al*. 2012). Of note, some viruses and parasite species also express miRNAs (Kincaid and Sullivan, 2012). However, it

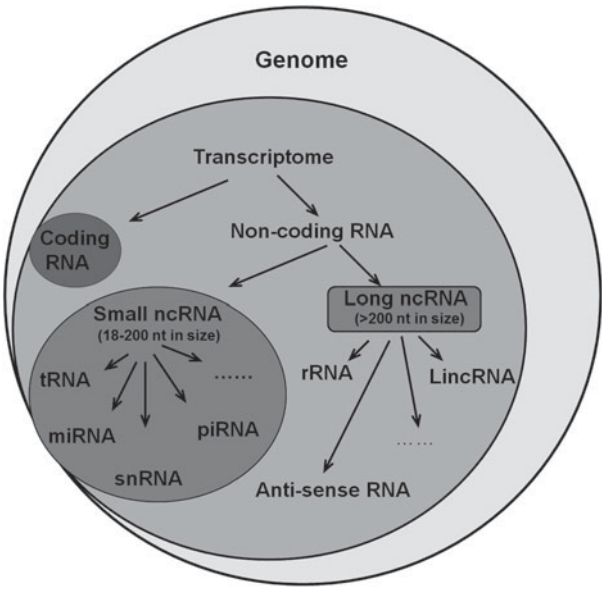

Fig. 2. Type of human non-coding RNAs. Almost 70% of the human genome is transcribed, and only 2% encode for proteins. ncRNAs are loosely grouped into two major classes based on transcript size: small ncRNAs and lincRNAs.

appears that *Cryptosporidium* spp. does not have the siRNA machinery and, thus, might not express miRNA molecules (Abrahamsen *et al*. 2004). Whether *Cryptosporidium* spp. expresses lincRNAs has not been investigated.

Accumulating data indicate that miRNAs and lincRNAs are an essential part of the complex regulatory networks that control various cellular processes, including differentiation and fate of cells, as well as immune responses in epithelial and immune cells (Zhou *et al*. 2011; Carpenter *et al*. 2013). miRNAs are predicted to promote fine-tuning of post-transcriptional regulation to >60% of mammalian protein-coding mRNAs (Liu and Olson, 2010), and have emerged as key post-transcriptional regulators of gene expression. LincRNAs can function *in cis*, recruiting protein complexes to their site of transcription, thus creating a locus-specific address (Chaumeil *et al*. 2006), and also *in trans* to regulate distantly located genes (Huarte *et al*. 2010). The recent discovery of lincRNAs in association with specific chromatin modification complexes, such as Polycomb Repressive Complex 2 (PRC2), which mediates histone H3 lysine 27 trimethylation (H3K27me3), suggests a role for lincRNAs in managing chromatin states in a gene-specific fashion (Rinn *et al*. 2007). LincRNAs can be induced in innate immune cells and may act as key regulators of the inflammatory response (Guttman *et al*. 2009; Carpenter *et al*. 2013). Pathologically, lincRNAs have been associated with human inflammatory diseases and malignant and neurological disorders (Huarte *et al*. 2010; Carpenter *et al*. 2013).

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ALTERATIONS IN NCRNA GENE EXPRESSION IN EPITHELIAL CELLS FOLLOWING *C. PARVUM* INFECTION

miRNAs are initially transcribed as primary transcripts known as pri-miRNAs by RNA polymerase II (RNA pol II) and cropped into ~70- to 100-nucleotide-long hairpin precursors (termed pre-miRNAs) in the nucleus by the RNAse III Drosha (Lee *et al*. 2004). Pre-miRNAs are actively transported by exportin-5 to the cytoplasm, where they are cleaved by the enzyme Dicer to form mature miRNAs. This cleavage event gives rise to a double-stranded ~22 nt product composed of the mature miRNA guide strand and the miRNA* passenger strand. The mature miRNA is then loaded into the RNA-induced silencing complex (RISC), while the passenger strand is degraded. The RISC identifies target mRNAs by base-pair complementarity, resulting in mRNA cleavage and/or translational suppression (Winter *et al*. 2009). The majority of miRNA genes are located in intergenic regions or in antisense orientation to annotated genes, indicating that they form independent transcription units. Approximately 50% of human miRNA genes are expressed from non-protein-coding transcripts. Other miRNAs are located within introns of annotated genes, which may be transcribed as part of their 'host genes' (Saini *et al*. 2007).

Recent studies indicate that transcription of miRNA genes in epithelial cells infected by *C. parvum* can be elaborately controlled through various regulatory mechanisms, including transactivation and transrepression by nuclear transcription factors associated with the downstream signalling pathways of TLR/NLRs; in particular, by the NF-κB pathway (Chen *et al*. 2005). Using an *in vitro* model of human biliary cryptosporidiosis, a significant alteration in miRNA expression profile has been identified in infected host epithelial cells following infection *in vitro* (Zhou *et al*. 2009). The up-regulated miRNAs include miR-23b, miR-30b, miR-30c, miR-125b, miR-15b, miR-16, miR-27b, miR-24 and miR-21; downregulated miRNAs include *let-7i*, miR-513, miR-98, and miR-214 (Fig. 3). Further exploration has identified that transcription of a subset of miRNA genes is regulated through NF-κB activation in human biliary epithelial cells in response to *C. parvum* infection (Table 1). Specifically, inhibition of NF-κB activation by SC-514, an IKK2 inhibitor, blocked *C. parvum*-induced up-regulation of a subset of pri-miRNAs, including pri-miR-125b-1, pri-miR-21, pri-miR-23b-27b-24-1 and pri-miR-30b. Moreover, direct binding of the NF-κB p65 subunit to the promoter elements of *mir-125b-1, mir-21, mir-23b-27b-24-1* and *mir-30b* genes was identified by chromatin immunoprecipitation analysis and confirmed by the luciferase reporter assay using the constructs covering the potential promoter elements of these miRNA genes. The data demonstrated that *mir-125b-1, mir-21, mir-30b* and *mir-23b-27b-24-1* cluster genes were transactivated through promoter binding of the NF-κB p65 subunit following *C. parvum* infection. In contrast, *C. parvum* transactivated *mir-30c* and *mir-16* genes in biliary epithelial cells in a p65-independent manner (Zhou *et al*. 2009).

In addition to the up-regulation of some miRNAs, NF-κB signalling may also be involved in the down-regulation of miRNA genes in response to *C. parvum* infection. Transcription of the *let-7i* gene in biliary epithelial cells in response to *C. parvum* infection has been reported to be suppressed through promoter binding by the NF-κB subunit p50 along with CCAAT/enhancer-binding protein beta (C/EBPβ) (O'Hara *et al*. 2010). Furthermore, a recent study showed that *C. parvum* infection suppressed transcription of the *mir-424-503* gene in an NF-κB-dependent manner. Increased promoter recruitment of NF-κB p50 associated with the *mir-424-503* gene was observed in infected epithelial cells (Zhou *et al*. 2013).

ANTIMICROBIAL MOLECULES FROM EPITHELIAL CELLS CONTROLLED BY MIRNAS FOLLOWING *C. PARVUM* INFECTION

Classic epithelial cell-derived antimicrobial peptides, such as β-defensin 2 and LL-37, have been shown to have anti-cryptosporidial activity *in vitro* (Chen *et al*. 2005; Dommett *et al*. 2005) and may play a role in mucosal anti-*C. parvum* defence *in vivo*. Nitric oxide (NO) has been shown to play an important role in epithelial defence against *C. parvum* in some models (Leitch and He, 1994; Gookin *et al*. 2006; Nordone and Gookin, 2010), while other models have failed to show similar effects (Hayward *et al*. 2000; Lean *et al*. 2003).

NO production in epithelial cells is mainly regulated by inducible nitric oxide synthase (iNOS) (Pautz *et al*. 2010). NO production in epithelial cells after *C. parvum* infection is increased through NF-κB-dependent mechanisms, with the involvement of miRNA-mediated stabilization of iNOS mRNA (Zhou *et al*. 2012). Specifically, the RNA stability of iNOS mRNA is enhanced in host epithelial cells following *C. parvum* infection. Increased stability of iNOS mRNA facilitates the protein synthesis of iNOS, promoting NO production in infected cells. Underlying mechanisms of iNOS mRNA stabilization are associated with the suppression of the KH-type splicing regulatory protein (KSRP), an RNA-binding protein that interacts with the mRNAs that have AU-rich elements (AREs) in their 3′UTR. Binding of these ARE-containing mRNAs by KSRP causes mRNA degradation; thus, KSRP is a key mediator of mRNA decay (Linker *et al*. 2005; Winzen *et al*. 2007). iNOS mRNA is one of the mRNA molecules regulated by KSRP; there are several

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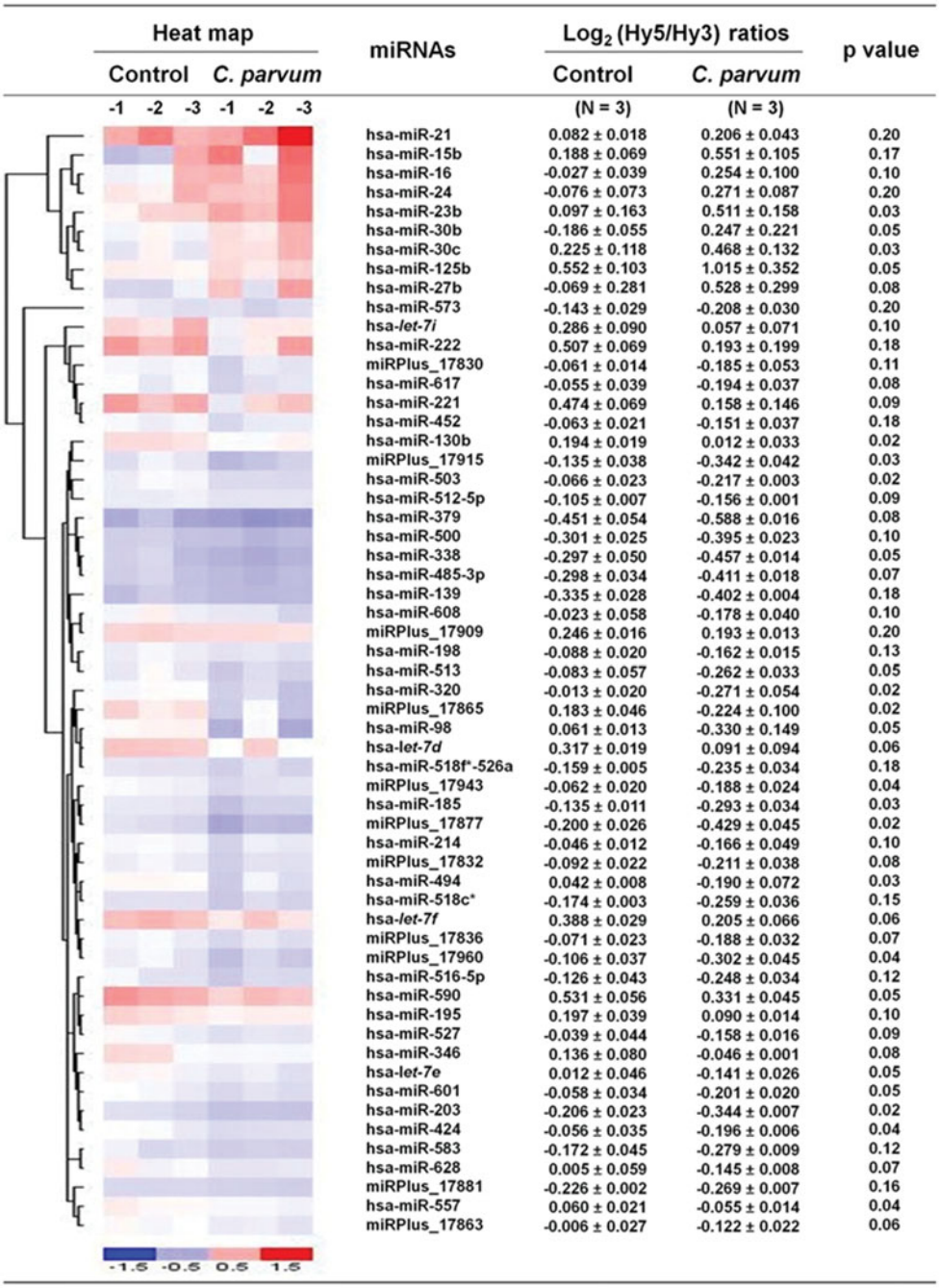

miRNAs	Log_2 (Hy5/Hy3) ratios Control (N = 3)	Log_2 (Hy5/Hy3) ratios *C. parvum* (N = 3)	p value
hsa-miR-21	0.082 ± 0.018	0.206 ± 0.043	0.20
hsa-miR-15b	0.188 ± 0.069	0.551 ± 0.105	0.17
hsa-miR-16	-0.027 ± 0.039	0.254 ± 0.100	0.10
hsa-miR-24	-0.076 ± 0.073	0.271 ± 0.087	0.20
hsa-miR-23b	0.097 ± 0.163	0.511 ± 0.158	0.03
hsa-miR-30b	-0.186 ± 0.055	0.247 ± 0.221	0.05
hsa-miR-30c	0.225 ± 0.118	0.468 ± 0.132	0.03
hsa-miR-125b	0.552 ± 0.103	1.015 ± 0.352	0.05
hsa-miR-27b	-0.069 ± 0.281	0.528 ± 0.299	0.08
hsa-miR-573	-0.143 ± 0.029	-0.208 ± 0.030	0.20
hsa-*let-7i*	0.286 ± 0.090	0.057 ± 0.071	0.10
hsa-miR-222	0.507 ± 0.069	0.193 ± 0.199	0.18
miRPlus_17830	-0.061 ± 0.014	-0.185 ± 0.053	0.11
hsa-miR-617	-0.055 ± 0.039	-0.194 ± 0.037	0.08
hsa-miR-221	0.474 ± 0.069	0.158 ± 0.146	0.09
hsa-miR-452	-0.063 ± 0.021	-0.151 ± 0.037	0.18
hsa-miR-130b	0.194 ± 0.019	0.012 ± 0.033	0.02
miRPlus_17915	-0.135 ± 0.038	-0.342 ± 0.042	0.03
hsa-miR-503	-0.066 ± 0.023	-0.217 ± 0.003	0.02
hsa-miR-512-5p	-0.105 ± 0.007	-0.156 ± 0.001	0.09
hsa-miR-379	-0.451 ± 0.054	-0.588 ± 0.016	0.08
hsa-miR-500	-0.301 ± 0.025	-0.395 ± 0.023	0.10
hsa-miR-338	-0.297 ± 0.050	-0.457 ± 0.014	0.05
hsa-miR-485-3p	-0.298 ± 0.034	-0.411 ± 0.018	0.07
hsa-miR-139	-0.335 ± 0.028	-0.402 ± 0.004	0.18
hsa-miR-608	-0.023 ± 0.058	-0.178 ± 0.040	0.10
miRPlus_17909	0.246 ± 0.016	0.193 ± 0.013	0.20
hsa-miR-198	-0.088 ± 0.020	-0.162 ± 0.015	0.13
hsa-miR-513	-0.083 ± 0.057	-0.262 ± 0.033	0.05
hsa-miR-320	-0.013 ± 0.020	-0.271 ± 0.054	0.02
miRPlus_17865	0.183 ± 0.046	-0.224 ± 0.100	0.02
hsa-miR-98	0.061 ± 0.013	-0.330 ± 0.149	0.05
hsa-*let-7d*	0.317 ± 0.019	0.091 ± 0.094	0.06
hsa-miR-518f*-526a	-0.159 ± 0.005	-0.235 ± 0.034	0.18
miRPlus_17943	-0.062 ± 0.020	-0.188 ± 0.024	0.04
hsa-miR-185	-0.135 ± 0.011	-0.293 ± 0.034	0.03
miRPlus_17877	-0.200 ± 0.026	-0.429 ± 0.045	0.02
hsa-miR-214	-0.046 ± 0.012	-0.166 ± 0.049	0.10
miRPlus_17832	-0.092 ± 0.022	-0.211 ± 0.038	0.08
hsa-miR-494	0.042 ± 0.008	-0.190 ± 0.072	0.03
hsa-miR-518c*	-0.174 ± 0.003	-0.259 ± 0.036	0.15
hsa-*let-7f*	0.388 ± 0.029	0.205 ± 0.066	0.06
miRPlus_17836	-0.071 ± 0.023	-0.188 ± 0.032	0.07
miRPlus_17960	-0.106 ± 0.037	-0.302 ± 0.045	0.04
hsa-miR-516-5p	-0.126 ± 0.043	-0.248 ± 0.034	0.12
hsa-miR-590	0.531 ± 0.056	0.331 ± 0.045	0.05
hsa-miR-195	0.197 ± 0.039	0.090 ± 0.014	0.10
hsa-miR-527	-0.039 ± 0.044	-0.158 ± 0.016	0.09
hsa-miR-346	0.136 ± 0.080	-0.046 ± 0.001	0.08
hsa-*let-7e*	0.012 ± 0.046	-0.141 ± 0.026	0.05
hsa-miR-601	-0.058 ± 0.034	-0.201 ± 0.020	0.05
hsa-miR-203	-0.206 ± 0.023	-0.344 ± 0.007	0.02
hsa-miR-424	-0.056 ± 0.035	-0.196 ± 0.006	0.04
hsa-miR-583	-0.172 ± 0.045	-0.279 ± 0.009	0.12
hsa-miR-628	0.005 ± 0.059	-0.145 ± 0.008	0.07
miRPlus_17881	-0.226 ± 0.002	-0.269 ± 0.007	0.16
hsa-miR-557	0.060 ± 0.021	-0.055 ± 0.014	0.04
miRPlus_17863	-0.006 ± 0.027	-0.122 ± 0.022	0.06

Fig. 3. The miRNA expression profile in human epithelial cells in response to *C. parvum* infection. The left panel shows a heat-map of miRNAs that showed changes in expression in H69 cells following *C. parvum* infection. The horizontal axis indicates samples of non-infected cells ($n = 3$; Control-1, -2, and -3) and cells after exposure to live *C. parvum* for 12 h ($n = 3$, *C. parvum*-1, -2, and -3). The right panel shows expression of miRNAs in H69 cells following *C. parvum* infection. Cellular levels of miRNAs were presented as the log2 (Hy5/Hy3) ratios that passed the filtering criteria variation across the samples. *P* values are from the *t*' test. hsa = *Homo sapiens* (Zhou *et al.* 2009).

AREs within the 3′UTR of iNOS mRNA (Linker *et al.* 2005). Interestingly, KSRP is a target for miR-27b and, thus, transactivation of the *mir-23b-27b-24-1* gene in *C. parvum*-infected epithelial cells through the NF-κB pathway causes translational suppression of KSRP (Zhou *et al.* 2009, 2012). Therefore, NO production in epithelial cells in response to *C. parvum* infection involves miR-27b-mediated stabilization of iNOS mRNA. Indeed, functional manipulation of KSRP or miR-27b caused reciprocal alterations in iNOS mRNA stability and NO production in infected cells. Forced expression of KSRP and inhibition of miR-27b resulted in an increased burden of *C. parvum* infection (Zhou *et al.* 2012) (Table 1; Fig. 4).

It is still unclear whether synthesis of other antimicrobial molecules is regulated by miRNAs. The 3′UTRs of human β-defensin 2 and LL-37

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Table 1. Validated targets of miRNAs relevant to innate immunity following *C. parvum* infection

miRNAs	Alteration	Transcription factors/other co-factors	Targets	Function relevant to innate immunity following *C. parvum* infection	References
let-7i	Down	P50; C/EBP*β*; HDAC3	TLR4	Regulation of epithelial defence responses against *C. parvum*	(Chen *et al*. 2007)
			SNAP23	Promotion of exosomal luminal release from infected epithelium	(Hu *et al*. 2013)
miR-98	Down	ND	CIS	Modulation NF-kB activity	(Hu *et al*. 2009)
			SOCS4	An inhibitory effect on phosphorylation of signal transducers and activators of transcription proteins	(Hu *et al*. 2010)
miR-513	Down	ND	B7-H1	Regulation of cholangiocyte inflammatory response	(Gong *et al*. 2010)
mir-424–503	Down	P50; HDACs	CX3CL1	Regulation of cholangiocyte inflammatory response	(Zhou *et al*. 2013)
miR-221	Down	ND	ICAM-1	Regulation of the interaction between epithelial cells and T cells	(Gong *et al*. 2011)
miR-27b	Up	P65	KSRP	Regulation of the RNA stability of iNOS and IL8 mRNA	(Zhou *et al*. 2012)

Abbreviations: *C. parvum*, *Cryptosporidium parvum*; HDAC, Histone deacetylase; C/EBP*β*, CCAAT/enhancer-binding protein beta; TLR4, Toll-like receptor 4; SNAP23, snaptosome-associated protein of 23 KD; CIS, cytokine-inducible Src homology 2; SOCS4, suppressors of cytokine signalling 4; iNOS, inducible nitric oxide synthase; B7-H1, B7 homologue 1; CX3CL1, chemokine (C-X3-C motif) ligand 1; KSRP, the KH-type splicing regulatory protein; ICAM-1, Intercellular cell adhesion molecule 1; ND, not determined.

mRNAs are relatively short (105 nt for *β*-defensin 2 and 67 nt for LL-37) and, therefore, may not be directly targeted by miRNAs. Secretion of mucins in the gastrointestinal tract is an important element of mucosal defence and could protect the host against infection by extracellular stages of *C. parvum*. Additional epithelial cell-derived molecules, such as serum amyloid A 3 (Saa3) and regenerating islet-derived 3 gamma (Reg3g), have also recently been demonstrated to display antimicrobial activity (Reigstad *et al*. 2009; Choi *et al*. 2013). Their involvement in *Cryptosporidium* infection of gastrointestinal epithelium and potential association with miRNA-mediated post-transcriptional suppression has yet to be investigated.

MIRNAS IN PRODUCTION OF CYTOKINES/CHEMOKINES FROM EPITHELIAL CELLS AND MUCOSAL INFILTRATION DURING *C. PARVUM* INFECTION

Release of inflammatory cytokines and chemokines from epithelial cells following *C. parvum* infection will trigger adaptive immunity and attract inflammatory infiltration, including NK cells, macrophages and lymphocytes. Based on bioinformatics analysis, approximately 29% of cytokine/chemokine mRNAs have potential target sites for miRNAs (Asirvatham *et al*. 2008). Human gastrointestinal epithelial cell lines increase the production of IL-8, chemokine (C-X3-C motif) ligand 1 (CX3CL1, also known as fractalkine), chemokine (C-X-C motif) ligand 1 (CXCL1), prostaglandin E2 (PGE_2), and C-X-C motif chemokine 10 (CXCL10) following *C. parvum* infection. PGE_2 is catalysed by cyclooxygenase-2 (Cox-2). Notably, many of the mRNAs of these cytokines/chemokines, such as IL-8 and Cox-2, contain AREs in their 3′UTRs (Winzen *et al*. 2007). As discussed above, binding of the ARE-containing mRNAs by KSRP causes mRNA degradation, and KSRP is a target of miR-27b. Therefore, translational suppression of KSRP through induction of miR-27b in infected cells should influence the synthesis of these ARE-associated cytokines/chemokines. Indeed, stabilization of IL8 and Cox-2 mRNAs was confirmed to be associated with suppression of KSRP and miR-27b induction in cells following *C. parvum* infection (Zhou *et al*. 2012) (Table 1; Fig. 4).

mRNAs of other cytokines and chemokines may directly be targeted by *C. parvum*-responsive miRNAs in infected epithelial cells. CX3CL1 is a unique member of the CX3C family, and it binds only to and is the unique ligand of its receptor, CX3CR1 (Stievano *et al*. 2004). CX3CL1 is known to function as an adhesion molecule to interact with immune cells that express CX3CR1, including $CD4^+$ and $CD8^+$ *T*-cells, NK cells and monocytes (Chakravorty *et al*. 2002). Increased expression and

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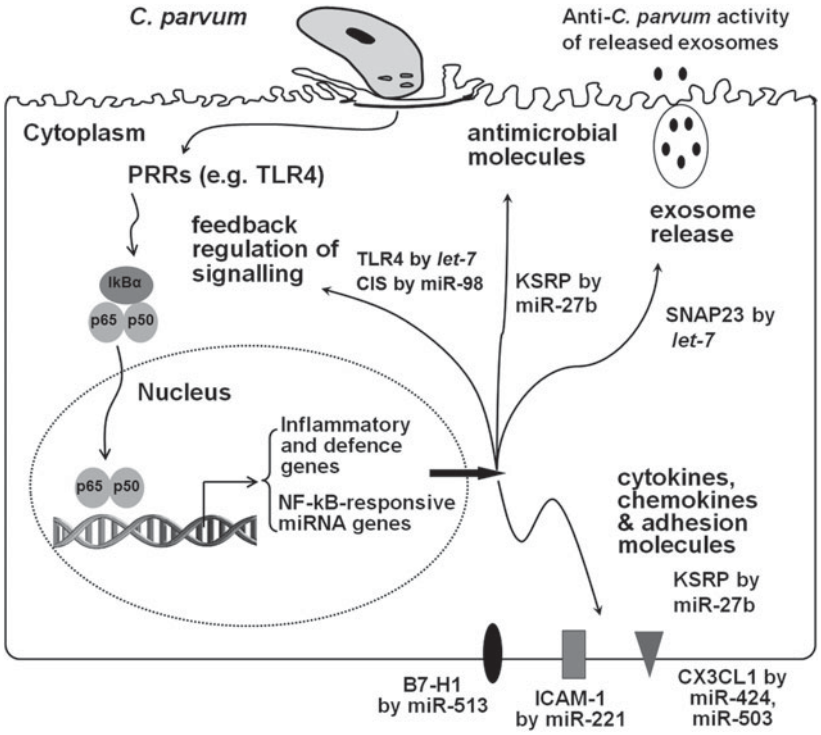

Fig. 4. Post-transcriptional gene suppression mediated by miRNAs is involved in the fine-tuning of epithelial anti-*Cryptosporidium* defence. *Cryptosporidium parvum* infection alters expression profile of miRNA genes in infected epithelial cells. A panel of miRNA genes is transactivated or transsuppressed through the activation of the NF-κB pathway. These NF-κB-responsive miRNAs may modulate epithelial anti-parasite responses at every step of the innate immune network, including feedback regulation to TLR/NF-κB signalling, post-transcriptional regulation of many NF-κB-regulated immune or inflammatory genes, release of epithelial exosomes and production of antimicrobial effector molecules.

release of CX3CL1 was demonstrated in cultured human biliary epithelial cells after *C. parvum* infection (Zhou *et al*. 2013). Such induction of CX3CL1 in infected cells depends, at least partially, on the transrepression of the *mir-424-503* gene. Both miR-424 and miR-503 can bind to the 3′UTR of CX3CL1 and cause translational suppression. Consequently, transrepression of the *mir-424-503* gene in infected cells may facilitate the synthesis of CX3CL1 protein and promote infiltration of $CX3CR1^{+}$ immune cells to infection sites (Zhou *et al*. 2013) (Table 1; Fig. 4).

Besides cytokine/chemokine release, *C. parvum* infection also induces expression of adhesion and costimulatory molecules in host epithelial cells (Zhou *et al*. 2011). Intercellular adhesion molecule 1 (ICAM-1) is well known for its importance in stabilizing cell-cell interactions and is critical for the firm arrest and transmigration of leucocytes out of blood vessels into tissues (Muller, 2009). Upregulation of ICAM-1 transcription and protein expression was detected in human gastrointestinal epithelial cells following *C. parvum* infection at both the transcriptional and posttranscriptional levels *in vitro* (Gong *et al*. 2011). At the posttranscriptional level, miR-221 regulates ICAM-1 translation through targeting the ICAM-1 3′UTR (Gong *et al*. 2011). Significant down-regulation of miR-221 has been identified in cultured human biliary epithelial cells following *C. parvum* infection. Functionally, down-regulation of miR-221 results in the relief of miR-221-mediated translational suppression, and, thus, facilitates ICAM-1 synthesis in epithelial cells and inflammatory infiltration of leucocytes during *C. parvum* infection (Gong *et al*. 2011) (Table 1; Fig. 4). Moreover, mucosal epithelial cells can express several co-stimulatory molecules, thus modulating the function of infiltrated immune cells at the mucosal site during infection. B7-H1 is a key member of the B7 family of costimulatory molecules important in regulation of immune response, in particular T cell homoeostasis (Dong and Chen, 2006). Expression of B7-H1 by epithelial cells in response to *C. parvum* infection is tightly regulated to ensure an appropriate antimicrobial immune response. Many researchers have indicated that B7-H1 protein is usually undetectable, although expression of B7-H1 mRNA is common in many cells, suggesting post-transcriptional suppression. B7-H1 is a target of miR-513; miR-513 targeting may account for the absence of B7-H1 protein in cells under a non-stimulation condition (Gong *et al*. 2010). In addition, down-regulation of miR-513 is required for up-regulation of B7H1

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protein levels in human biliary epithelial cells following *C. parvum* infection, suggesting a role of miR-513 in regulating inflammatory responses through targeting of B7-H1 (Gong *et al*. 2010) (Table 1; Fig. 4).

RELEASE OF EPITHELIAL CELL-DERIVED EXOSOMES: ANTI-*CRYPTOSPORIDIUM* ACTIVITY AND RELEASE REGULATION

Exosomes represent a specific subtype of secreted membrane vesicles that are ~30–100 nm in size and are formed inside secreting cells in endosomal compartments called multi-vesicular bodies (MVBs) (Théry, 2011). Exosomes are produced by a variety of cells, including reticulocytes, epithelial cells, neurons and tumour cells (Théry, 2011). Exosomal vesicles shuttle a wide variety of bioactive molecules, such as proteins, lipids, mRNAs and miRNAs, and thereby traffic molecules from the cytoplasm and membranes of one cell to other cells or extracellular spaces (Smalheiser, 2007; Valadi *et al*. 2007). There is increasing evidence that exosomes play an important role in normal physiological processes, development, viral infection and other human diseases (Yu *et al*. 2006; Théry, 2011). We recently demonstrated that luminal release of exosomes from the biliary and intestinal epithelium is increased following infection by *C. parvum* (Hu *et al*. 2013). Intriguingly, released exosomes contain antimicrobial peptides with anti-*C. parvum* activity, including *β*-defensin-2 and LL-37. Exposure of *C. parvum* sporozoites to released exosomes decreases their viability and infectivity both *in vitro* and *ex vivo*. A direct binding of exosomes to the parasite surface was observed in cell cultures after exposure to freshly excysted *C. parvum* sporozoites by scanning and transmission EM. These parasites directly bound by exosomes showed a decrease in viability, suggesting the anti-*C. parvum* activity of exosomes at physiological conditions (Hu *et al*. 2013). Of note, the life cycle of *C. parvum*, both *in vitro* and *in vivo*, has extracellular stages (i.e. sporozoites, merozoites and microgametocytes), and they are likely vulnerable to exosomal binding/targeting (Table 1; Fig. 4).

Interestingly, release of exosomes from infected epithelium following *C. parvum* infection involves a miRNA-mediated exocytic mechanism (Hu *et al*. 2013). Secretion of exosomes is regulated by various stimuli, including the activation of P2X receptor by ATP on monocytes and neutrophils, thrombin receptor on platelets, and TLR4 by LPS on dendritic cells (Bhatnagar and Schorey, 2007). Formation of exosomes within MVBs and targeting of tran-membrane proteins involve a complex intracellular sorting network, including the endosomal sorting complex required for transport (ESCRT) machinery (van Niel *et al*. 2006). Fusion of MVBs with plasma membrane is an exocytic process that requires the association of v-SNAREs (from the vesicles) and t-SNAREs (at the membrane) to form a ternary SNARE (SNAP receptor) complex. The SNARE complex brings the two membranes in opposition, a necessary step in overcoming the energy barrier required for membrane fusion (Südhof and Rothman, 2009). *Cryptosporidium parvum*-stimulated release of exosomes involves TLR4/IKK2 activation and the SNAP23-associated vesicular exocytic process (Hu *et al*. 2013). Whereas a basal level of exosomal luminal release exists in cultured biliary epithelial monolayers and in the murine biliary tract, a TLR4-dependent increase in luminal release of epithelial exosomes was detected following *C. parvum* infection. Activation of TLR4 signalling increases SNAP23 expression and enhances phosphorylation of SNAP23 in infected cells. SNAP23 is a target of the *let-7* family of miRNAs. Since TLR4 signalling mediates transrepression of the *let-7* miRNA genes in *C. parvum*-infected epithelial cells (Hu *et al*. 2013), release of *let-7*-mediated SNAP23 translational repression facilitates SNAP23 protein synthesis in infected cells, promoting exosomal luminal release from infected epithelium (Hu *et al*. 2013) (Table 1; Fig. 4).

In addition, more recent studies have shown that miRNAs are also important components of exosomes. Intriguingly, exosome-shuttled miRNA molecules can be delivered to other cell types through exosomal uptake (Valadi *et al*. 2007). Given the importance of miRNAs in epithelial innate immune responses following *C. parvum* infection, it would be interesting to determine whether exosomes from epithelial cells also carry miRNAs and thus modulate epithelial-immune cell interactions and epithelial anti-*C. parvum* defence, via exosomal delivery of miRNAs. Because *Cryptosporidium* spp. does not have the siRNA machinery, delivery of exosomal-shuttled miRNAs to the parasite may not directly influence parasite biology. Nevertheless, these miRNAs shuttled in epithelial cell-derived exosomes released to the basolateral domain during *C. parvum* infection may modulate host anti-*C. parvum* immunity, a process that has been demonstrated in the intestinal epithelium during other mucosal infections (Mallegol *et al*. 2007). Given the evidence that exosomes from both immune and non-immune cells positively and negatively modulate the immune response (Robbins and Morelli, 2014), the role for basolateral exosomes from epithelial cells in host anti-*C. parvum* immunity needs further experimental elucidation.

MIRNAS AND FEEDBACK REGULATION OF EPITHELIAL ANTI-*C. PARVUM* IMMUNE RESPONSES

To carry out a fine-tuning of immune responses in response to infection, epithelial cells have developed

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multiple strategies for the feedback regulation of intracellular signalling pathways. Several endogenous proteins have recently been identified to counter-regulate intracellular signalling cascades and promote resolution of inflammation, such as Toll-interacting protein and A20 to the TLR and NF-κB signalling (Hayden and Ghosh, 2008). The cytokine-inducible Src homology 2 protein (CIS) and suppressors of cytokine signalling (SOCS) proteins are a family of intracellular molecules that have emerged as key physiological regulators of cytokine responses in many cell types (Yoshimura *et al*. 2007). The best-characterized SOCS family members are CIS and SOCS1-3, which function in a classical, negative-feedback loop and inhibit cytokine signalling by interacting with JAK/STAT signalling cascades (Mansell *et al*. 2006; Yoshimura *et al*. 2007).

These effector molecules of various intracellular signalling cascades can be targets of miRNAs. Targets of miR-146 include IL-1 receptor-associated kinase 1 (IRAK1) and TNF receptor-associated factor 6 (TRAF6) (Taganov *et al*. 2006). MyD88 has been identified as a novel target of miR-155 (Tang *et al*. 2010). Therefore, up-regulation of miR-146 and miR-155 has been implicated as a negative feedback to inhibit TLR/NF-κB signalling in cells following microbial challenge (Taganov *et al*. 2006; Tang *et al*. 2010). However, expression of miR-146 and miR-155 remains unchanged in biliary epithelial cells following *C. parvum* infection (Zhou *et al*. 2009). On the other hand, miRNAs down-regulated in cells following *C. parvum* infection may exert a positive feedback regulation to epithelial cell immune response. Specifically, TLR4 is a target of *let-7*; thus, suppression of *let-7* in cells following *C. parvum* infection contributes to an enhanced level of TLR4 protein in infected cells, facilitating epithelial anti-microbial defence (Chen *et al*. 2007) (Table 1; Fig. 4). In addition, it has been reported that miR-98 and *let-7* target CIS (Hu *et al*. 2009). *Cryptosporidium parvum* infection induces CIS expression in infected epithelial cells through TLR/NF-κB-suppressed expression of miR-98 and *let-7* (Hu *et al*. 2010). Induction of CIS expression enhances IκB degradation, promoting NF-κB activation (Hu *et al*. 2010) (Table 1; Fig. 4).

CONCLUDING REMARKS

While we have begun to recognize the critical role for ncRNAs in mucosal epithelial cells in the innate immunity against *Cryptosporidium* infection, our current understanding has been limited to miRNAs. A potential role for other ncRNAs, in particular lincRNAs, is yet to be investigated. The interactions of ncRNAs and their targets are complex. It has been proposed that a single miRNA can repress hundreds of target transcripts, and multiple miRNAs may target the same transcript. New technologies will help to identify ncRNA targeting globally, such as cross-linking-argonaute/RNA immunoprecipitation, proteomic approaches and high-throughput sequencing assays (Ørom and Lund, 2010; Leung *et al*. 2011). The development of ncRNA knockouts could greatly advance our understanding of ncRNA-mediated anti-*C. parvum* immune responses *in vivo*. ncRNAs may also be critical players in other pathogenic aspects of *C. parvum* infection, such as the lasting effects in intestinal epithelium. Unravelling the regulatory functions of ncRNAs in antiparasite immunity is still in its infancy, but will likely yield new insights into our understanding of the immunobiology and immunopathology of cryptosporidiosis. Given the importance of adaptive immunity to clear infection, the role for ncRNAs in adaptive immunity may be critical for anti-*Cryptosporidium* immunity, an under-studied area of *Cryptosporidium* research.

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