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1 **MicroRNAs contribute to the host response to *Coxiella burnetii***

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14

15 **Key words:** *Coxiella burnetii*, miRNA, macrophage, apoptosis, autophagy, infection

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21 **ABSTRACT**

22 MicroRNAs (miRNAs), a class of small non-coding RNAs, are critical to gene regulation in
23 eukaryotes. They are involved in modulating a variety of physiological processes, including the
24 host response to intracellular infections. Little is known about miRNA functions during infection
25 by *Coxiella burnetii*, the causative agent of human Q fever. This bacterial pathogen establishes a
26 large replicative vacuole within macrophages by manipulating host processes such as apoptosis
27 and autophagy. We investigated miRNA expression in *C. burnetii*-infected macrophages and
28 identified several miRNAs that were down- or up-regulated during infection. We further
29 explored the functions of miR-143-3p, an miRNA whose expression is down-regulated in
30 macrophages infected with *C. burnetii*, and show that increasing the abundance of this miRNA in
31 human cells results in increased apoptosis and reduced autophagy – conditions that are
32 unfavorable to *C. burnetii* intracellular growth. In sum, this study demonstrates that *C. burnetii*
33 infection elicits a robust miRNA-based host response, and because miR-143-3p promotes
34 apoptosis and inhibits autophagy, down-regulation of miR-143-3p expression during *C. burnetii*
35 infection likely benefits the pathogen.

36

37

38 INTRODUCTION

39 The highly infectious intracellular pathogen *Coxiella burnetii* is the etiological agent of Q fever
40 (1–3). After uptake by a host cell, typically an alveolar macrophage, *C. burnetii* establishes a
41 replicative niche termed the *Coxiella*-containing vacuole (CCV) that matures by fusing with
42 lysosomal, autophagic, and secretory vesicles (4–6). To facilitate intracellular replication, *C.*
43 *burnetii* secretes effector proteins into the host cytosol using a Dot/Icm Type IVB secretion
44 system (T4SS) that disrupt several host processes, including apoptosis, lipid metabolism,
45 inflammation, and vesicular trafficking (3, 7–12). For instance, *C. burnetii* inhibits host
46 apoptosis by recruiting anti-apoptotic Bcl-2 to the CCV, inactivating pro-apoptotic Bad, and
47 promoting a pro-survival response by activating Erk1/2, Akt, and PKA signaling (13–16).
48 Similarly, autophagy-related proteins such as LC3 and p62 are recruited to CCV in a T4SS-
49 dependent manner (10, 17), and inhibition of autophagy components results in reduced *C.*
50 *burnetii* replication (18, 19).

51 MicroRNAs (miRNAs) are a class of single-stranded, small (~22 nucleotides), non-
52 coding RNAs that orchestrate post-transcriptional gene regulation in eukaryotes and some
53 viruses (20). In humans, miRNAs regulate a large number of genes, primarily by inhibiting target
54 gene expression via translation repression and messenger RNA (mRNA) degradation (21–23).
55 Studies have shown that miRNAs are integral to the host response to bacterial, viral, and
56 parasitic infections (24–31); however, miRNAs could either promote or inhibit infection. For
57 example, induction of miR-142-3p expression in macrophages leads to down-regulation of N-
58 Wasp, an actin-binding protein, resulting in reduced uptake of *Mycobacterium tuberculosis* (32).
59 Conversely, induction of miR-125a-3p in macrophages inhibits autophagy and phagosomal

60 maturation, which favors intracellular survival of *M. tuberculosis* by promoting autophagosomal
61 escape (33).

62 Expression of miRNAs is perturbed in macrophages infected with *C. burnetii* (34), but
63 their potential roles are unknown. In this study, we investigated macrophage gene expression
64 across five time-points during *C. burnetii* infection and identified a large number of miRNAs and
65 protein-coding genes that were differentially expressed, suggesting their involvement in the host
66 response to infection. We also demonstrate that transfecting host cells with miR-143-3p results in
67 reduced *C. burnetii* growth, enhanced apoptosis and diminished autophagy. Collectively, our
68 data indicate that an intracellular environment with a low level of miR-143-3p is conducive to *C.*
69 *burnetii* growth.

70

71 **RESULTS**

72 **Host gene expression correlates with bacterial growth**

73 To identify infection-associated miRNAs and their potential mRNA targets, we measured
74 miRNA and protein-coding gene expression in THP-1 macrophages infected with *C. burnetii*
75 Nine Mile RSA439 Phase II (NMII). The total number of differentially expressed (\log_2 fold-
76 change ≥ 0.75 , $P_{adj} \leq 0.05$) miRNAs and mRNAs increased from 25 to 60 and 454 to 6,525,
77 respectively, from day 1 to day 3 (**Table 1 and S1**). By day 5, far fewer miRNAs and protein-
78 coding genes ((34, 211, respectively) were up- or down-regulated in *C. burnetii*-infected cells.
79 This pattern of gene expression suggests that the magnitude of the host cell response to *C.*
80 *burnetii* infection increases as the bacterium actively replicates (day 3), and as LCVs transition
81 to metabolically less active SCVs (day 5) (35), host response becomes muted in tandem.

82

83 **miRNAs potentially regulate multiple host signaling pathways during *C. burnetii* infection**

84 To identify genes and metabolic pathways targeted by miRNAs, we first performed inverse-
85 expression pairing using the Ingenuity Pathway Analysis (IPA) tool (36). We selected miRNAs
86 and their known or predicted target genes that showed an inverse pattern of expression in *C.*
87 *burnetii*-infected cells. For instance, if expression of an miRNA is up-regulated in *C. burnetii*-
88 infected cells, we chose targets that are down-regulated, and vice versa. This analysis identified
89 14, 18, 25, 51, and 23 miRNAs at 8 h, 24 h, 48 h, 72 h, and 120 h post-infection (hpi),
90 respectively, that were inversely paired with differentially-expressed target mRNAs (**Table S2**).
91 We then investigated biochemical pathways that were enriched for these proteins using the Core
92 Analysis function in IPA, which revealed 215 pathways, including apoptosis signaling,
93 PI3K/AKT, and autophagy that are likely regulated by miRNAs (**Figure 1, Table S3**). To
94 investigate the role of miRNAs in apoptosis, a process known to be important during *C. burnetii*
95 infection (13, 37–40), we measured expression of 84 miRNAs associated with apoptosis using a
96 targeted qPCR array (41). This assay showed that 12 miRNAs were up- or down-regulated in *C.*
97 *burnetii*-infected cells at 72 hpi (**Table 2**), suggesting their involvement in apoptosis regulation
98 as part of the host response to *C. burnetii*.

99

100 **miR-143-3p is down-regulated in *C. burnetii*-infected human alveolar macrophages**

101 Among apoptosis-related miRNAs, we focused on miR-143-3p, which was significantly down-
102 regulated in NMII-infected THP-1 cells (**Table 2, Figure 2A**). Because NMII is an avirulent
103 laboratory strain and THP-1 cells are not natural host cells for *C. burnetii*, we measured miR-
104 143-3p expression using primary human alveolar macrophages (hAMs) infected with the fully
105 virulent *C. burnetii* Nine Mile RSA493 Phase I (NMI) strain. At 72 hpi, expression of miR-143-

106 3p was significantly down-regulated in hAMs infected with *C. burnetii* (**Figure 2B**).
107 Intriguingly, expression of miR-143-3p was significantly lower in NMI-infected than in NMII-
108 infected hAMs. While the cause for this disparity is currently unknown, the full-length
109 lipopolysaccharide (LPS) present in NMI might play a role because LPS is known to repress
110 transcription of the miR-143/145 gene cluster (42).

111

112 **Increased miR-143-3p expression inhibits *C. burnetii* growth**

113 Because expression of miR-143-3p is down-regulated during *C. burnetii* infection, we tested how
114 higher levels of miR-143-3p in host cells would impact intracellular growth. We first transfected
115 HeLa cells with either miR-143-3p or a non-specific control miRNA (miR-control) and then
116 infected cells with NMII and measured bacterial growth at 48 hpi. As shown in **Figure 3, C**,
117 *burnetii* grew significantly better in untransfected and miR-control-transfected cells compared to
118 miR-143-3p-transfected cells, indicating that an intracellular environment with low levels of
119 miR-143-3p could be advantageous to the pathogen.

120

121 **Early apoptosis is enhanced in cells transfected with miR-143-3p**

122 To test the impact of miR-143-3p on apoptosis, we transfected HeLa cells with either miR-143-
123 3p or miR-control and assessed early- and late-stage apoptosis using annexin V-PE and
124 eFluor780 staining followed by flow cytometry (43). We observed that the percentage of early,
125 but not late, apoptotic cells in the miR-143-3p-transfected population was significantly higher
126 than in cells transfected with miR-control (**Figure 4**). To begin to understand miR-143-3p's
127 regulatory circuit, we assessed expression of *akt1* (AKT Serine/Threonine Kinase 1) and *bcl2* (B-
128 cell lymphoma 2), two genes targeted by miR-143-3p that are central to apoptosis regulation in

129 human macrophages (**Figure S1**) (44–47). Expression of *akt1* and *bcl2* genes and levels of
130 activated Akt and Bcl-2 proteins were significantly reduced in miR-143-3p-expressing cells
131 compared to cells transfected with miR-control (**Figure 5**). Together, our data suggest that lower
132 levels of miR-143-3p present in *C. burnetii*-infected cells increases expression of *akt1* and *bcl2*,
133 which likely stalls apoptosis induction, thereby supporting the pathogen's intracellular growth.

134

135 **miR-143-3p has potential roles in autophagy**

136 Analysis of miRNA-targeted pathways indicated that miRNAs could also be involved in
137 autophagy (**Table S3**), a process that is interconnected with apoptosis and is involved in the host
138 response to *C. burnetii* (18, 48–50). We measured rapamycin-induced autophagic flux in HeLa
139 cells transfected with either miR-143-3p or miR-control and observed that autophagic flux was
140 slightly, but significantly, lower in miR-143-3p-transfected cells compared to miR-control-
141 transfected cells (**Figure 6**). Several genes involved in autophagy, including *atp6v1a* (V-type
142 proton ATPase catalytic subunit A) and *slc7a11* (Solute Carrier Family 7 Member 11) are
143 controlled by miR-143-3p (24, 25). We observed significantly reduced expression of *atp6v1a*
144 and *slc7a11* and concordant reduction in their encoded proteins (VATA and xCT, respectively)
145 in miR-143-3p-transfected cells compared to control cells (**Figure 7, Table S4**), indicating a role
146 for these genes in reduced autophagic flux in miR-143-3p-expressing cells.

147

148 **DISCUSSION**

149 In this study, we show, for the first time, that *C. burnetii* infection elicits a robust miRNA-based
150 host response, and closer examination of miR-143-3p function revealed that the miRNA
151 promotes apoptosis and inhibits autophagy. This combination of phenotypes potentially produces

152 an intracellular environment that is not conducive to *C. burnetii* growth, likely explaining down-
153 regulation of miR-143-3p expression during *C. burnetii* infection. miR-143-3p presumably
154 antagonizes *C. burnetii* growth by modulating components of the PI3K-Akt signaling network,
155 which controls diverse host cell functions (**Figure S1**) (51). Activation of Akt by P13K induces
156 expression of pro-survival Bcl-2 and inhibits pro-apoptotic proteins such as Bad (52), leading to
157 decreased caspase-3 activation and subsequent inhibition of intrinsic apoptosis. During *C.*
158 *burnetii* infection, Akt is activated, Bcl-2 is recruited to CCVs, and the pathogen secretes
159 effector proteins that inhibit apoptosis (15, 16, 39, 53). Our results add to this knowledge by
160 demonstrating that levels of Akt and Bcl-2 are significantly reduced and early apoptosis is
161 significantly enhanced in cells transfected with miR-143-3p. No significant difference is evident
162 in late apoptosis, a process characterized by DNA fragmentation, suggesting that miR-143-3p
163 does not affect this process.

164 In addition to inhibiting apoptosis, *C. burnetii* manipulates autophagy to generate CCVs
165 (10, 18, 49, 50). Interestingly, miR-143-3p appears to inhibit autophagy, and several genes
166 involved in this process, including *atp6v1a* and *slc7a11*, are regulated by miR-143-3p (23–25,
167 54–57). Expression of both genes is significantly reduced in miR-143-3p-transfected cells,
168 indicating that the miRNA's effect on autophagy involves regulation of v-ATPase and
169 cystine/glutamate antiporter xCT. In accordance with our results, inhibition of autophagy causes
170 improper CCV maturation and reduces intracellular *C. burnetii* growth (17, 58, 59). Inhibition of
171 ATP6V1A, a catalytic subunit of the lysosomal v-ATPase, likely disrupts autophagic flux by
172 inhibiting V-ATPase-dependent acidification of developing CCV, thereby reducing *C. burnetii*
173 growth (56). Furthermore, inhibition of xCT (encoded by *slc7a11*), a cystine/glutamate antiporter
174 that relies on autophagy machinery to import cystine, could deprive the pathogen of cysteine, an

175 amino acid essential to its growth (55, 60, 61). Finally, although this needs to be confirmed in
176 macrophages, overexpression of miR-143-3p in human endothelial progenitor cells decreased
177 LC3-II and increased p62 levels, two phenotypes that strongly imply a role for miR-143-3p in
178 dampening autophagic flux (62). Cumulatively, our data suggest that down-regulation of miR-
179 143-3p during *C. burnetii* infection promotes intracellular growth of the pathogen by delaying
180 apoptosis and promoting autophagy. Further studies are required to determine if this is a *C.*
181 *burnetii*-driven process and if LPS and/or T4SS effectors promote pathogen growth by down-
182 regulating miR-143-3p expression.

183

184 **MATERIALS AND METHODS**

185 **Bacterial strains and growth conditions**

186 *Coxiella burnetii* Nine Mile RSA439 (Phase II, Clone 4) isolate (NMII) was cultured in acidified
187 citrate cysteine medium-2 (ACCM-2) for 7 days at 37°C, 5% CO₂, 2.5% O₂ (63). Bacteria were
188 quantified using PicoGreen (64, 65), collected by centrifugation (3000 x g, 10 min, 4°C),
189 resuspended in PBS containing 0.25 M sucrose (PBSS), and stored at -80°C until further use.
190 Before infection, THP-1 cells (American Type Culture Collection, TIB-202) were differentiated
191 into adherent, macrophage-like cells in RPMI-1640 supplemented with 1 mM sodium pyruvate,
192 0.05 mM beta-mercaptoethanol, 4500 mg/L glucose, and 10% heat-inactivated fetal bovine
193 serum (FBS) at 37°C under 5% CO₂ for 24 h using 30 nM phorbol 12-myristate 13-acetate
194 (PMA), followed by 24 h of rest in PMA-free medium. Cells were infected with NMII at a
195 multiplicity of infection (MOI) of 25 in serum-free growth medium for two hours and this time-
196 point was considered to be 0 hpi. To remove extracellular bacteria, cells were washed three
197 times with PBS followed by replacement with complete growth medium, which was replaced

198 with fresh medium at 72 hpi. Primary human alveolar macrophages (hAMs) were harvested by
199 bronchoalveolar lavage (BAL) from postmortem human lung donors and infected with either
200 NMI (*C. burnetii* Nine Mile Phase I RSA493) or NMII at 25 MOI. hAMs were cultured at 37°C
201 under 5% CO₂ in Dulbecco's modified Eagle/F-12 (DMEM/F12) medium (Gibco) containing
202 10% FBS for 72 hpi, as described previously (66).

203

204 **Transfection of HeLa cells**

205 miRCURY LNA hsa-miR-143-3p and miRNA negative control were purchased from Qiagen.
206 The negative control (miR-control) is a non-specific miRNA that shows no homology to any
207 known miRNA or mRNA sequences annotated in mouse, rat, or human genomes. HeLa cells
208 were reverse transfected with miRNAs as previously described (67). Briefly, 25 nM (final
209 concentration) of either miR-143-3p mimic or miR-control was incubated in HiPerFect
210 Transfection Reagent (Qiagen) for 10 min at room temperature to allow formation of transfection
211 complexes. Transfection complexes were uniformly spotted at the bottom of each well of a 24-
212 well tissue culture plate and 3.5×10^4 HeLa cells were added to each well and incubated in
213 OptiMEM medium. After 24 h, the medium was removed, and cells were washed twice with
214 PBS before infection with NMII at an MOI of 100 in serum-free DMEM medium for two hours
215 followed by washing and replenishing cells with serum-containing DMEM medium for
216 experiments at 48 hpi.

217

218 **Intracellular growth assay**

219 *C. burnetii* growth was measured at 48 hpi in HeLa cells transfected 24 h prior to infection with
220 either miR-143-3p or miR-control. To quantify intracellular bacteria using qPCR, total DNA was

221 extracted using QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions
222 and SYBR Green-based qPCR was performed using *C. burnetii*-specific primers (**Table S5**), as
223 described previously (65). Quantification cycle (Cq) values were converted to bacterial genome
224 equivalents (GE) using a standard curve, as described previously (64). Since qPCR-based
225 quantitation does not differentiate between live and dead cells, we independently quantified
226 viable intracellular bacteria by enumerating colony forming units (CFUs), as described
227 previously (68, 69). Briefly, infected host cells were lysed in ice-cold water for 40 min at 4°C
228 followed by repeated pipetting with a syringe and 25G needle to lyse remaining cells,
229 centrifuged for 10 min at 70 x g (4°C) followed by centrifugation of supernatants for 1 min at
230 13,500 x g (4°C). Pellets were resuspended in ACCM-2, serially diluted, and spot-plated on
231 ACCM-2 containing 0.5 mM tryptophan and 0.5% agarose. Plates were incubated for 10 days at
232 37°C, 5% CO₂, and 2.5% O₂ before enumerating CFUs.

233

234 **RNA sequencing, miRNA-target interactions and pathway analysis**

235 THP-1 macrophages infected with NMII (MOI of 25) and uninfected controls were analyzed at
236 8, 24, 48, 72, and 120 hpi for miRNA and mRNA expression. At each time point, growth
237 medium was replaced with 1 ml of TRI reagent (Life Technologies) and total RNA was extracted
238 and treated with DNase (Invitrogen) per the manufacturer's instructions. Samples were
239 sequenced using Illumina NovaSeq 6000 for mRNAs and Illumina HiSeq 2500 for miRNAs at
240 the Yale Center for Genome Analysis or Novogene Corporation, Sacramento, CA. Sequencing
241 reads were mapped to the reference human miRNA (miRbase 22.1) or genome (GRCh38)
242 databases using CLC Genomics Workbench v6.5 (Qiagen) (70, 71). Differential gene expression
243 (\log_2 fold-change ≥ 0.75 ; adjusted p-value ≤ 0.05 , Wald test) between NMII-infected and

244 uninfected cells was calculated using DESeq2 (72). Inverse-expression pairings of differentially-
245 expressed miRNAs and mRNAs and IPA core analysis of differentially-expressed genes were
246 performed using Ingenuity Pathway Analysis (IPA, Qiagen) (36).

247

248 **Quantitative Reverse Transcription PCR (qRT-PCR)**

249 Expression of 84 apoptosis-related miRNAs was measured using a miScript miRNA PCR Array
250 Human Apoptosis kit (Qiagen). Briefly, total RNA extracted from infected or uninfected
251 macrophages at 72 h pi (n = 3) were reverse transcribed using a miScript II RT kit and HiSpec
252 buffer (Qiagen) and qPCR reactions were performed on a Stratagene Mx3005P Real-Time PCR
253 system. miRNA expression data were normalized using the global quantitation cycle (Cq) mean
254 of expressed miRNAs, and relative miRNA expression levels were calculated using the delta-
255 delta Cq method (73). Expression of *akt1*, *bcl2*, *atp6v1a*, and *slc7a11* was quantified using qRT-
256 PCR using *gapdh* primers as an endogenous reference control, as described previously (65).
257 Briefly, total RNA was extracted, DNase-treated, and cDNA generated using RevertAid First
258 Strand cDNA Synthesis Kit (Thermo Scientific). To perform qPCR, cDNA templates were
259 diluted and mixed with gene-specific primers and SYBR Green (Applied Biosystems) in a 20 μ l
260 reaction according to the recommended protocol (Applied Biosystems). Primers used in this
261 study are listed in **Table S5**. To assay miR-143-3p expression in hAMs, PCR primers for miR-
262 143-3p and the endogenous reference *rnuc6* (small nuclear ribonucleic acid) were procured from
263 Qiagen and qRT-PCR reactions were performed as described above.

264

265

266

267 **Apoptosis assay**

268 Apoptosis was quantified in HeLa cells transfected with miR-143-3p or miR-control using
269 Annexin V-PE (Invitrogen) and Fixable Viability Dye eFluor780™ (Invitrogen) (43). At 72 h
270 post-transfection, cells were treated with trypsin, stained with eFluor780 Fixable Viability Dye
271 for 30 min in dark (4°C), followed by staining with Annexin V-PE for 15 min per the
272 recommended protocol (Invitrogen). Cells were immediately assayed for early apoptosis
273 (Annexin V-PE positive and eFluor780 negative) and late apoptosis/necrosis (Annexin V-PE
274 positive and eFluor780 positive) markers using a FACSAria Fusion flow cytometer (BD
275 Biosciences). Fluorescence parameters were gated using unstained and single-stained cells and a
276 total of 10,000 events were counted for each sample using FACSDiva Software (BD
277 Biosciences).

278

279 **Autophagic flux assay**

280 CYTO-ID Autophagy Detection Kit 2.0 (ENZ-KIT175, Enzo Life Sciences) was used to
281 quantify autophagic flux at 72 h post transfection on HeLa cells transfected with either miR-143-
282 3p or miR-control. Cells were treated with media containing 200 nM rapamycin for 16 h to
283 induce detectable levels of autophagy (74, 75), washed, and incubated for 30 min at 37°C in
284 Microscopy Dual Detection Reagent containing CYTO-ID green detection reagent and Hoechst
285 33342 nuclear stain in 1X assay buffer. Three sets of at least 200 cells per well were immediately
286 analyzed using a fluorescence microscope (Keyence Corporation) and levels of autophagic flux
287 were measured as average CYTO-ID green brightness per cell calculated using Keyence BZ-
288 X700 software (76, 77).

289

290 **Multiplex immunoassay**

291 Activation (phosphorylation) of proteins involved in early apoptosis, including Akt (pS473) and
292 Bcl-2 (Ser70), were quantified using 7-Plex Early Apoptosis Magnetic Bead Kit (EMD
293 Millipore). Total cell lysate from HeLa cells transfected with miR-143-3p or miR-control was
294 prepared using Lysis Buffer containing protease inhibitors, followed by bicinchoninic acid
295 (BCA) quantitation at 72 h post-transfection. Briefly, 17.5 µg/well of diluted cell lysate was
296 added to 1X magnetic beads at 1:1 ratio in a 96-well plate. The plate was incubated on a plate
297 shaker (4°C, 700 rpm, dark) for 18 h, followed by washing and incubation with 1X Detection
298 Antibody for 60 min at room temperature (RT) with shaking (700 rpm, dark). The detection
299 antibody was then removed, and samples were incubated for 15 min at RT in the dark with 1X
300 Streptavidin-PE (SAPE) followed by 15 min incubation (RT, dark) with the amplification buffer.
301 SAPE and amplification buffer were removed, and beads were resuspended in 150 µl of assay
302 buffer to analyze median fluorescence intensity (MFI) using a Luminex 200 system.

303

304 **Quantitative mass spectrometry**

305 Total cell lysate from HeLa cells transfected with either miR-143-3p or miR-control were
306 collected at 48h post-transfection in triplicates. TMT labeling and mass spectrometry were
307 performed at the Proteomics Shared Resource facility at Oregon Health & Science University, as
308 described previously (78). Briefly, samples were lysed, sonicated, and heated at 90°C for 10 min
309 followed by overnight micro-digestion of each sample using an S-trap micro protocol. Peptides
310 were labelled with TMT6-plex reagents, and multiplexed TMT-labeled samples were separated
311 by two-dimensional reversed-phase-reversed-phase (2DRPRP) liquid chromatography on a
312 Orbitrap Fusion Tribrid instrument (Thermo Scientific). Proteins were identified by searching

313 against the human proteome in UniProt, and TMT reporter ion intensities were processed with
314 in-house scripts. Differential protein abundance was determined by the Bioconductor package
315 edgeR.

316

317 **Data availability**

318 Sequencing reads from this study have been deposited on NCBI Sequence Read Archive (SRA)
319 under the BioProject accession PRJNA679931.

320

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324

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533

534 **FIGURE LEGENDS**

535 **Figure 1. Host pathways potentially regulated by miRNAs during *C. burnetii* infection.** Top
536 20 miRNA-targeted pathways significantly impacted in THP-1 macrophages infected with NMII.
537 Orange bars show pathways that are putatively activated ($z\text{-score} \geq 1.5$), and blue bars
538 correspond to pathways that were predicted to be inhibited ($z\text{-score} \leq 1.5$) using ingenuity
539 pathway analysis (IPA). See full list in **Table S3**.

540

541 **Figure 2. miR-143-3p expression is down-regulated in *C. burnetii*-infected macrophages. (A)**
542 Expression of miR-143-3p measured via RNA-seq ($n = 3$; 72 hpi) in NMII-infected THP-1 cells
543 compared to uninfected cells. **(B)** Primary human alveolar macrophages (hAMs) infected with
544 NMI or NMII or uninfected controls were analyzed for miR-143-3p expression using qRT-PCR at
545 72 hpi. Statistical significance in (A) was calculated using two-tailed paired Student's t-test
546 followed by Welch's correction, and in (B) using one-way ANOVA followed by Tukey's multiple
547 comparison test ($n = 3$).

548

549 **Figure 3. Intracellular growth of *C. burnetii* is inhibited in the presence of excessive miR-**
550 **143-3p.** Quantification of intracellular *C. burnetii* at 48 hpi using qPCR **(A)** or CFU assay **(B)** in
551 miR-143-3p-transfected HeLa cells compared to untransfected cells (Mock) and cells transfected
552 with non-specific control miRNA (miR-control). Statistical significance was determined using
553 one-way ANOVA followed by Tukey's multiple comparison test (ns: non-significant, $n = 3$).

554

555 **Figure 4. miR-143-3p transfection promotes early apoptosis.** Percentage of early **(A)** or late
556 **(B)** apoptotic HeLa cells transfected with either miR-143-3p or a non-specific control miRNA

557 (miR-control). Early apoptosis (Annexin V-PE positive and eFluor780 negative) and late
558 apoptosis/necrosis (Annexin V-PE positive and eFluor780 positive) were quantified by flow
559 cytometry. Statistical significance was determined using two-tailed paired Student's t-test
560 followed by Welch's correction (n = 3).

561
562 **Figure 5. Increased miR-143-3p expression leads to reduced expression of apoptosis-related**

563 **genes.** (A) Expression (fold change) of *akt1* and *bcl2* genes in HeLa cells transfected with miR-
564 143-3p compared to cells transfected with control miRNA (miR-control) measured using qRT-
565 PCR. Statistical significance was determined using two-tailed paired t-test followed by Welch's
566 correction (n = 3). (B) Fold change in phosphorylated Akt (pSer473) and Bcl-2 (pSer70) proteins
567 in HeLa cells transfected with miR-143-3p compared to cells transfected with miR-control
568 measured using a multiplex immunoassay. Activated proteins were measured as median
569 fluorescence intensity and statistical significance was determined using two-tailed paired
570 Student's t-test followed by Welch's correction (n = 3).

571
572 **Figure 6. miR-143-3p inhibits autophagic flux.** Y-axis shows relative autophagy flux reported
573 as average brightness of CYTO-ID green (a cationic tracer that selectively labels autophagic
574 compartments) per cell in miR-143-3p-transfected HeLa cells compared to cells transfected with
575 control miRNA (miR-control). Average CYTO-ID green brightness values were calculated from
576 three sets of at least 200 cells per well and statistical significance was determined using two-
577 tailed paired Student's t-test followed by Welch's correction (n = 3).

578

579 **Figure 7. Transfection of HeLa cells with miR-143-3p decreased *atp6v1a*/VATA and**
580 ***slc7a11*/xCT expression. (A) *atp6v1a* and *slc7a11* gene expression (fold change) in HeLa cells**
581 **transfected with miR-143-3p compared to cells transfected with control miRNA (miR-control)**
582 **measured using qRT-PCR. (B) VATA (encoded by *atp6v1a*) and xCT (encoded by *slc7a11*)**
583 **proteins (fold change) in HeLa cells transfected with miR-143-3p compared to cells transfected**
584 **with miR-control measured using quantitative mass-spectrometry. Statistical significance was**
585 **determined using two-tailed paired Student's t-test followed by Welch's correction (n = 3).**
586
587
588

589 **Table 1.** Number of differentially-expressed miRNAs and mRNAs in *C. burnetii*-infected THP-1
590 macrophages.

Time	miRNA ^a			mRNA ^a		
	Total ^b	Down	Up	Total ^b	Down	Up
8 hpi	25	12	13	454	220	234
24 hpi	25	16	9	1,160	665	495
48 hpi	35	23	12	1,742	445	1,297
72 hpi	60	43	17	6,525	3,236	3,289
120 hpi	34	18	16	211	80	131

591

592 ^aThe current build of the human genome (GRCh38) has 2,654 mature miRNA sequences and
593 19,231 protein-coding genes.

594 ^bDifferential expression (\log_2 fold change ≥ 0.75 ; $P_{adj} \leq 0.05$, Wald test) at respective hours
595 post-infection (hpi) was calculated using DESeq2 (72).

596

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600

601 **Table 2.** Differentially-expressed ($p \leq 0.05$, $n = 3$) miRNAs in NMII-infected THP-1 cells
602 compared to uninfected controls.

603

miRNA	Fold Change	p-value
hsa-miR-708-5p	0.6225	0.037471
hsa-miR-145-5p	0.6312	0.006722
hsa-miR-143-3p	0.6535	0.007199
hsa-miR-106b-5p	0.7386	0.032799
hsa-miR-181d-5p	0.7611	0.028265
hsa-miR-16-5p	0.8026	0.024211
hsa-miR-222-3p	0.8804	0.014724
hsa-miR-365b-3p	1.1015	0.016939
hsa-miR-218-5p	1.5832	0.013546
hsa-miR-125a-5p	1.5868	0.010438
hsa-miR-192-5p	1.7689	0.03232
hsa-miR-146a-5p	5.9635	0.000006

604

605

606

607 **SUPPLEMENTARY FIGURE**

608 **Figure S1. PI3K/Akt signaling network.** Activation of PI3K by pro-survival stimuli leads to
609 phosphorylation/activation of Akt. Akt in turn activates anti-apoptotic proteins and pro-survival
610 transcription factor NF- κ B, which leads to the induction of pro-survival proteins Bcl-2 and Bcl-
611 xL, and activation of XIAP. Bcl-2 prevents the release of cytochrome c from mitochondria,
612 thereby preventing apoptosis. Inhibition of *akt1* and *bcl2* expression by miR-143-3p (44–47),
613 shown in red, could reverse this process to promote apoptosis.

614

615 **SUPPLEMENTARY TABLES**

616 **Table S1.** Differentially expressed miRNAs and mRNAs in NMII-infected THP-1 cells.

617 **Table S2.** Inverse expression pairs of differentially expressed miRNAs and target mRNAs.

618 **Table S3.** List of pathways enriched for miRNA-regulated genes.

619 **Table S4.** List of downregulated proteins in miR-143-3p-transfected HeLa cells identified using
620 mass spectrometry.

621 **Table S5.** List of primers used in this study.

622

623

624

Figure 1

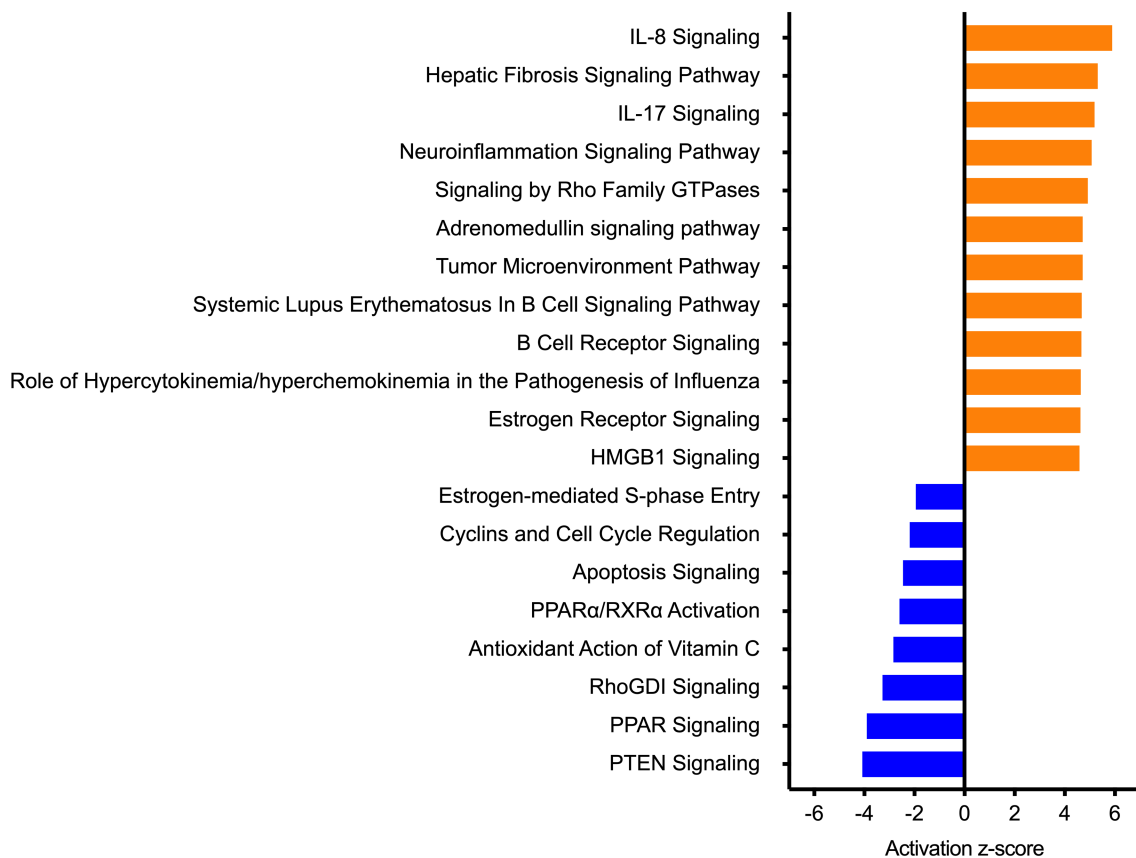


Figure 2

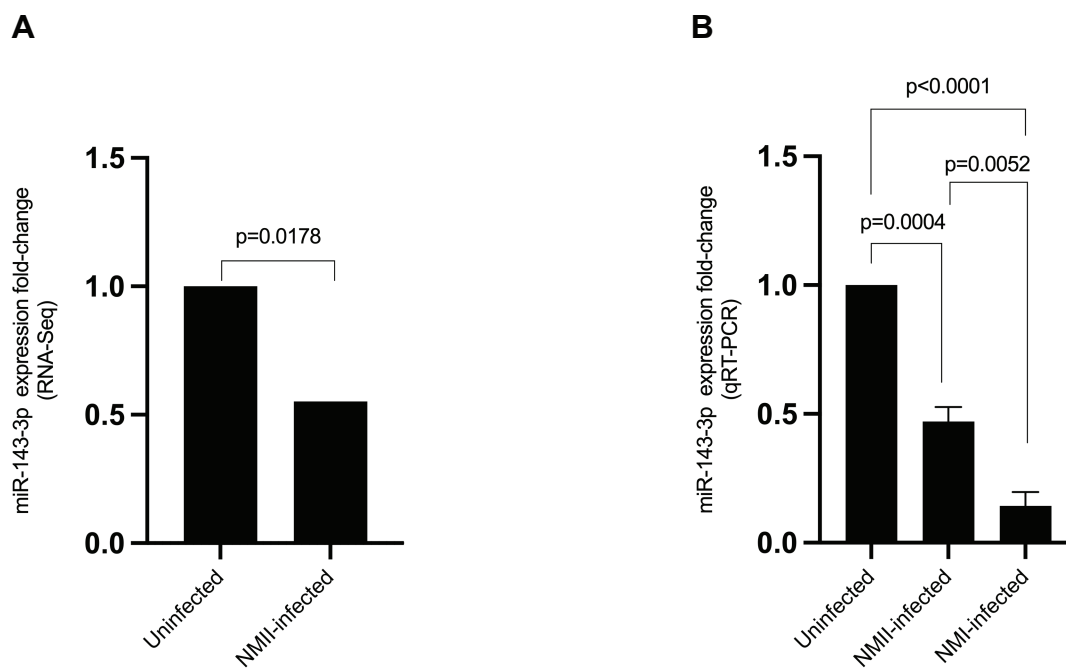


Figure 3

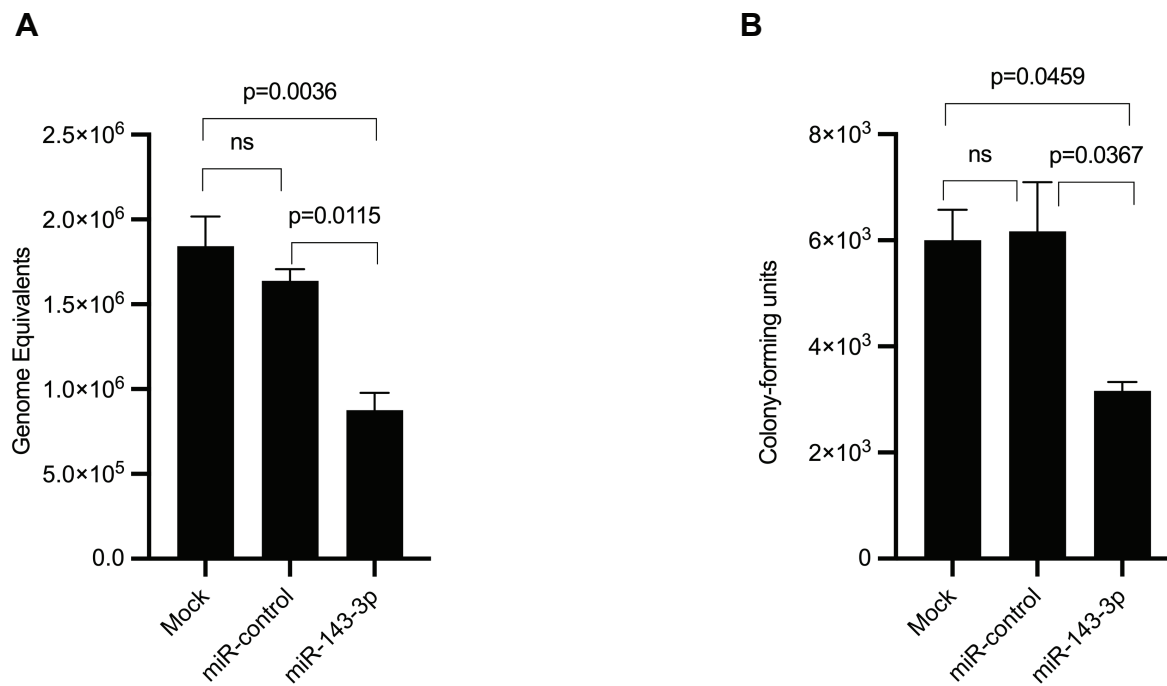


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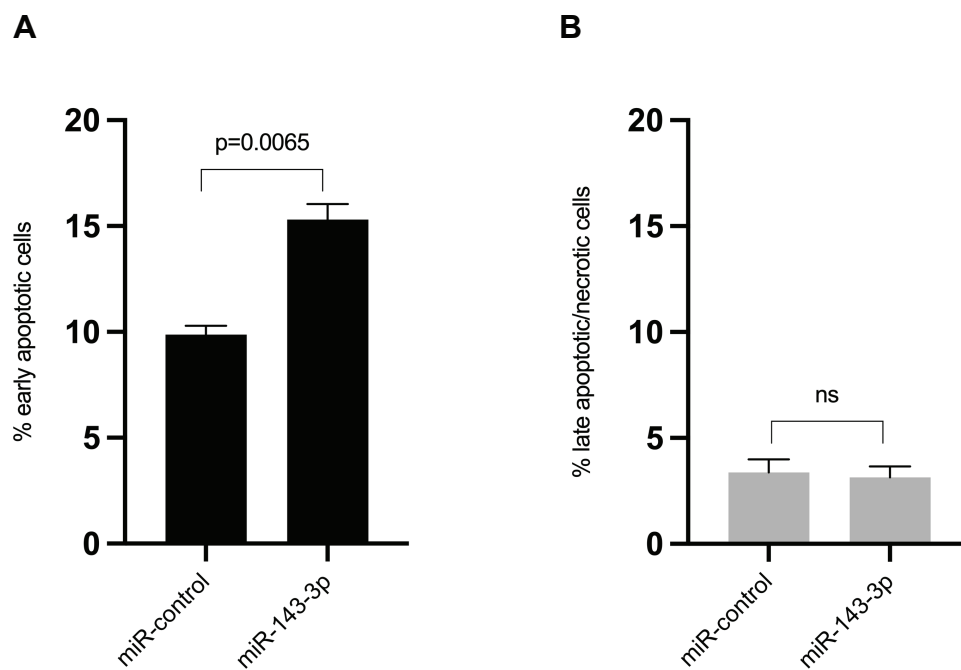
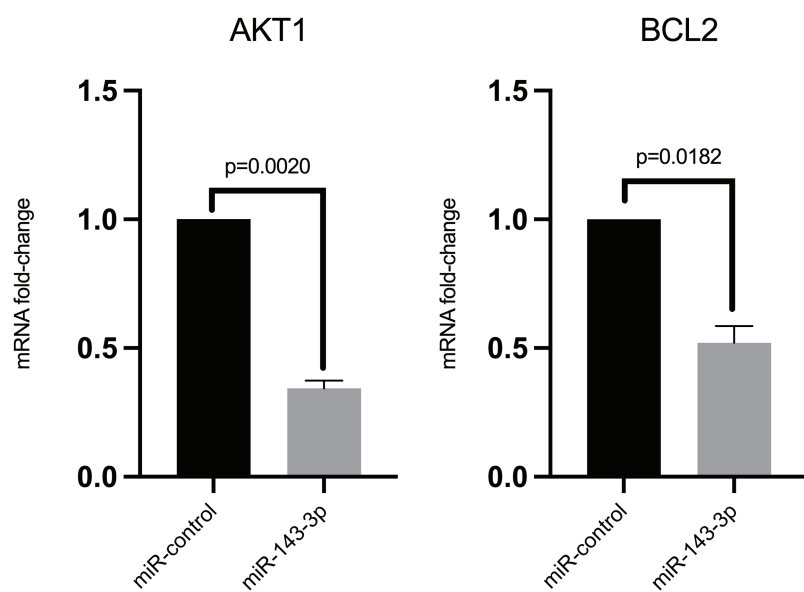


Figure 5

A



B

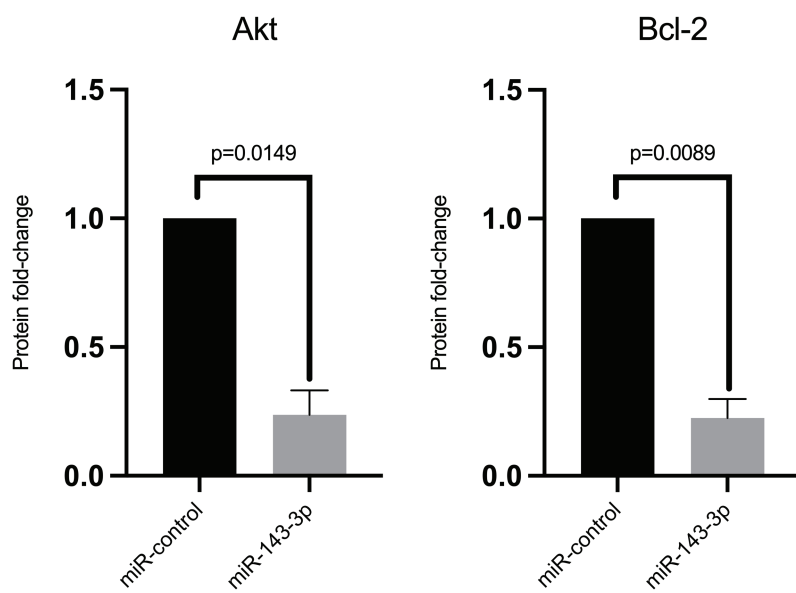


Figure 6

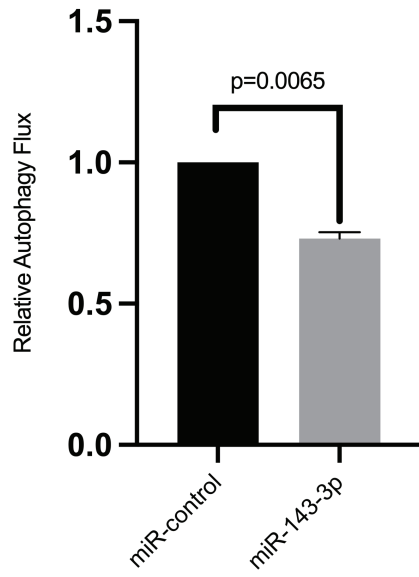
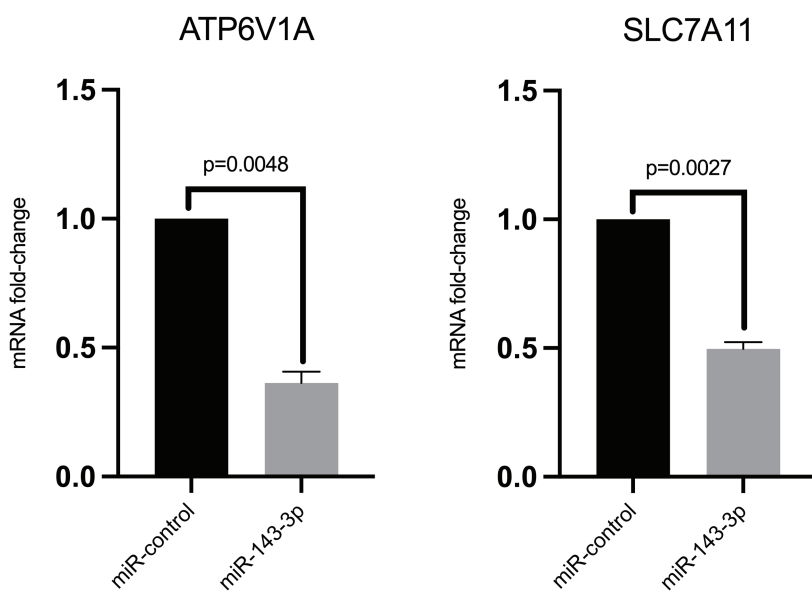


Figure 7

A



B

