Published in final edited form as:
J Infect Dis. 2010 July 1; 202(1): 125–135. doi:10.1086/653212.

MicroRNA-98 and *let-7* Regulate Expression of Suppressor of Cytokine Signaling-4 in Biliary Epithelial Cells in Response to *Cryptosporidium parvum* Infection

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Abstract

Expression of the cytokine-inducible Src homology 2 protein (CIS) and suppressors of cytokine signaling proteins (SOCS) represents an important element of host cell reactions in response to pathogen infection. We previously demonstrated that *Cryptosporidium parvum* infection downregulates miR-98 and *let-7* to induce CIS expression in biliary epithelial cells. We reported here that downregulation of miR-98 and *let-7* also coordinates epithelial expression of SOCS4 following *C. parvum* infection. Targeting of SOCS4 3'-untranslated region by miR-98 or *let-7* resulted in translational repression. Functional manipulation of miR-98 caused reciprocal alterations in SOCS4 protein expression. Transfection of miR-98 precursor abolished *C. parvum*-stimulated SOCS4 upregulation. Moreover, expression of SOCS4 in epithelial cells showed an inhibitory effect on phosphorylation of signal transducers and activators of transcription proteins induced by *C. parvum*. These data suggest an important role for miRNAs in the coordinated regulation of CIS/SOCS expression in epithelial cells in response to *C. parvum* infection.

Keywords

MicroRNAs; *Cryptosporidium parvum*; CIS/SOCS; Epithelium, biliary; Immune response; Parasites; STAT

Introduction

Epithelial cells play a critical role in the initiation, regulation, and resolution of both innate and adaptive immune reactions in response to microbial infection in the gastrointestinal tracts. Those epithelial cells express pathogen recognition receptors including Toll-like receptors (TLRs). TLRs recognize pathogens and activate a set of adaptor proteins (e.g., myeloid differentiation protein 88 [MyD88]) leading to activation of a variety of intracellular regulatory signals, including the nuclear factor-kappaB (NF-κB) and the Janus kinase (JAK) and signal transducers and activators of transcription (STAT) signaling pathways [1,2]. Activation of these signaling pathways initiates a series of host cell defense reactions against pathogens, including parasites [3]. Such epithelial defense responses are finely controlled and reflect a delicate balance between effector functions and their potential to cause damage to healthy tissues [4–6].

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The authors have no financial conflict of interest.

To carry out a fine-tuning of immune responses, epithelial cells have developed multiple strategies for the feedback regulation of intracellular signaling pathways. Several endogenous proteins have recently been identified to counter-regulate TLR and cytokine signaling cascades and promote resolution of inflammation, such as Toll-interacting protein and A20 [4–6]. The cytokine-inducible Src homology 2 protein (CIS) and suppressors of cytokine signaling (SOCS) proteins are a family of intracellular molecules which have emerged as key physiological regulators of cytokine responses in many cell types [7]. The best-characterized SOCS family members are CIS and SOCS1-3, which function in a classical, negative-feedback loop and inhibit cytokine signaling by interacting with JAK/STAT signaling cascades [7–10]. Pathogen recognition with TLRs can induce CIS/SOCS expression and manipulate cytokine receptor signaling in infected cells [11,12].

The protozoan parasite, *Cryptosporidium parvum*, is a causative agent of human gastrointestinal disease worldwide [13,14]. Humans are infected by ingesting *C. parvum* oocysts; oocysts then excyst in the gastrointestinal tract releasing infective sporozoites. The released sporozoite infects epithelial cells and forms a vacuole in which the organism remains intracellular but extracytoplasmic [5,15]. Because of the “minimally invasive” nature of *C. parvum* infection, innate immune responses are critical to the host’s defense to infection [16, 17]. *C. parvum* sporozoites can also travel up the biliary tract to infect biliary epithelial cells, particularly in patients with the acquired immunodeficiency syndrome (AIDS) [16]. Infection of human biliary epithelial cells by *C. parvum in vitro* mimics parasitial apical invasion and epithelial innate immune responses *in vivo* [18]. The invasion of epithelial cells *in vitro* by *C. parvum* results in the rapid expression of anti-microbial peptides (e.g., β-defensins) and the inflammatory chemokines [19,20]. How epithelial cells finely balance the inflammatory reactions and anti-microbial immune responses during *C. parvum* infection is still unclear.

MicroRNAs are a newly identified class of endogenous small regulatory RNAs that mediate either mRNA cleavage or translational suppression resulting in gene suppression [4–6]. Over 700 miRNAs have been identified in human and it has been predicted that miRNAs control 20–30% of human genes [4–6]. The importance of miRNA-mediated posttranscriptional mechanisms is beyond simply determining the rate of mRNA translation and degradation. Each miRNA has multiple predicted targets and many of the targets are functional related [21,22]. Various miRNAs can also target the same mRNA molecule resulting in coordinated expression outcome [23]. Thus, miRNAs can coordinately regulate multiple mRNAs and allow cells to respond with unusual ability to environmental cues. Studies have revealed key roles for miRNAs in diverse regulatory pathways, including timing control in development, cell differentiation, apoptosis, and cell proliferation and more recently, in immune regulation [10, 15,24–27]. Induction of miR-155 during the macrophage inflammatory response suggests its potential involvement in regulation of inflammation [15,25,28].

We recently identified that miR-98 and *let-7* regulate translation of CIS in human biliary epithelial cells in response to *C. parvum* infection or LPS stimulation [29]. We show here that *C. parvum* infection induces SOCS4 expression in biliary epithelial cells. *C. parvum*-induced expression of SOCS4 involves a relief of miRNA-mediated translation repression by miR-98 and *let-7*. In addition, *C. parvum* infection increases phosphorylation of STAT3 and STAT6 in epithelial cells and induction of SOCS4 appears to provide an inhibitory effect on *C. parvum*-stimulated STAT phosphorylation. Thus, a miRNA-coordinated regulation of CIS and SOCS4 expression has been identified in epithelial cells following *C. parvum* infection.

METHODS

C. parvum and infection model

C. parvum oocysts of the Iowa strain were purchased from a commercial source (Bunch Grass Farm, Deary, ID). H69 cells are SV40 transformed human biliary epithelial cells originally derived from normal liver harvested for transplant [29,30]. Non-immortalized normal human biliary epithelial cells (HIBEpiC) were obtained from ScienCell Research Laboratories. Before infecting cells, oocysts were treated with 1% sodium hypochlorite on ice for 20 min followed by extensive washing with DMEM-F12 medium. Infection was done in a culture medium (DMEM-F12) containing viable *C. parvum* oocysts (oocysts with host cells in a 5–10:1 ratio) as previously described [29,30].

Plasmids

The functionally defective dominant negative (DN) mutant of TLR4 was obtained from Dr. M. F. Smith (University of Virginia). MyD88-DN (a DN mutant of MyD88) was a gift from Prof. J. Tschopp (University of Lausanne). H69 cells stably transfected with TLR4-DN or MyD88-DN plasmid constructs were obtained as previously reported [18,31]. The HuSH 29mer shRNA-SOCS4 and control constructs were purchased from OriGene (Rockville).

Western blot

Whole cell lysates were obtained with the M-PER Mammalian Protein Extraction Reagent (Pierce) plus several protease inhibitors (1 mM PMSF; 10 μg/ml leupeptin, 2 μg/ml pepstatin). To detect STAT tyrosine phosphorylation, cells were harvested in the lysis buffer containing phosphatase inhibitor (1 mM Na_3VO_4, 1 mM NaF). Antibodies to SOCS4 (Invitrogen), phosphor-STAT1 (Tyr705), phosphor-STAT3 (Tyr705), phosphor-STAT6 (Tyr641), STAT1, STAT3, STAT6 (all from Cell Signaling) and actin (Sigma-Aldrich) were used. Densitometric levels of SOCS4 signals were quantified and normalized to actin and relative to uninfected cells.

Northern blot

Total cellular RNAs were obtained using the TRIzol reagent (Invitrogen) and 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). A LNA DIG-probe of miRNA-98 (Exiqon) was hybridized using UltraHyb reagents (Ambion) according to the manufacturer's instructions and snRNA RNU6B was blotted for control [24,29].

Real-time PCR

Comparative real-time PCR was performed using the SYBR Green PCR Master Mix (Applied Biosystems). The sequences for the amplification of human *SOCS4* were: 5′-GTTACTCGCACAACCCCAGTTAA-3′ (forward) and 5′-TTTCCAGCAGGAAGTTTCTTCTG-3′ (reverse). The primer sequences for the amplification of *IL-8* were as follows: 5′-GGGCCATCAGTTGCAAATC-3′ (forward); 5′-GGAAGAAACCACCGGAAGGAA-3′ (reverse). The primer sequences for the amplification of *GAPDH* were: 5′-TGCACCACCAACTGCTTAGC-3′ (forward); 5′-GGCATGGACTGTGGTCATGAG-3′ (reverse). The Ct values were analyzed using the comparative Ct (ΔΔCt) method and the amount of target was obtained by normalizing to the endogenous reference (GAPDH) and relative to the control (uninfected cells) [24].

miRNA precursors and anti-miRs

To manipulate cellular function of miR-98 and *let-7* in H69 cells, we utilized specific antisense oligonucleotides to miRNAs (anti-miRs) to inhibit miRNA function and specific miRNA

precursors to increase miRNA expression as previously reported [24,26]. Anti-miRs (anti-miR™ miRNA inhibitors) are commercially available, chemically modified single stranded nucleic acids designed to specifically bind to and inhibit endogenous miRNAs [32]. For experiments, H69 cells were grown to 90% confluent and treated with anti-miRs or precursors to miR-98 and *let-7* (0–30 nM, Ambion) using the lipofectamine™ 2000 reagent (Invitrogen). Nonspecific anti-miR (anti-miR-Ctrl) and precursor (precursor-Ctrl) (Ambion) were used as the controls.

Luciferase reporter constructs and luciferase assay

Complementary 37 bp DNA oligonucleotides containing the putative miR-98/*let-7* target site within 3' untranslated region (3′UTR) of human SOCS4 were synthesized with flanking *Spe*I and *Hind*III restriction enzyme digestion sites (Sense: 5′-ctagTACAAGGTTAGAAAAGTCTCATACTACCTCATCTTTA-3′; antisense: 5′-agctTAAAGATGAGGTAGTATGAGACTTTTCTAACCTTGTA-3′) and cloned into the multiple cloning site of the pMIR-REPORT Luciferase vector (Ambion). Another pMIR-REPORT Luciferase construct containing mutant 3′UTR (TACTACCTCATC to ATGATGGAGTAG) was also generated as a control. We then transfected cultured cells with each reporter construct, as well as anti-miR-98/*let-7* or miR-98/*let-7* precursor. Luciferase activity was measured and normalized to the control β-gal level as previously reported [29, 31].

RESULTS

C. parvum infection induces SOCS4 protein expression in biliary epithelial cells in a TLR4/MyD88-dependent manner

When H69 cells were exposed to *C. parvum* for up to 48h, a significant increase of SOCS4 protein content was detectable in cells from 12h to 48h following *C. parvum* infection (Figure 1*A*). Increase of SOCS4 protein levels was also detected in HIBEpiC cells at 24h and 48h after exposure to *C. parvum* (Figure 1*B*). Activation of TLR4/MyD88 signaling was previously demonstrated in biliary and intestinal epithelial cells during *C. parvum* infection [18, 29, 33]. To test whether TLR signals are involved in *C. parvum*-induced SOCS4 expression, we tested the expression of SOCS4 in H69 cells stably transfected with TLR4-DN or MyD88-DN [32]. No increase of SOCS4 protein was found in TLR4-DN or MyD88-DN cells following *C. parvum* infection compared with non-infected cells (Figure 1*C*).

C. parvum infection does not alter SOCS4 mRNA levels in biliary epithelial cells

When HIBEpiC and H69 cells were exposed to *C. parvum* for up to 12h, no significant change in SOCS4 mRNA levels was detected with the use of real-time PCR (Figure 2*A*). No change in SOCS4 mRNA was detected in TLR4-DN or MyD88-DN cells following *C. parvum* infection (Figure 2*B*). As a positive control, we analyzed *IL-8* transactivation, a TLR4/NF-κB-dependent process induced by *C. parvum* in epithelial cells [34]. A significant increase of IL-8 mRNA expression was confirmed in H69 cells at 8h and 12h after exposure to *C. parvum* (Figure 2*C*).

miR-98 and *let-7* target SOCS4 3'UTR resulting in translational suppression

The inconsistence of SOCS4 mRNA level with its protein content in H69 and HIBEpiC cells following *C. parvum* infection suggests the involvement of posttranscriptional regulation. To test whether miRNA-mediated posttranscriptional gene regulation is involved in this process, we used the MicroRNA.org (http://www.microrna.org) [35] to screen those miRNAs expressed in H69 cells based on our previous microarray analysis [36]. We found that miR-98 and *let-7* family have complementary to SOCS4 3′UTR. We identified one potential binding site

for miR-98 and *let-7* family in SOCS4 3'UTR, extending between 1825 and 1850 (Figure 3*A*).

To test the potential targeting of SOCS4 mRNA by miR-98 and/or *let-7*, we generated pMIR-REPORT luciferase constructs containing the SOCS4 3'UTR with the putative *let-7* and miR-98 binding site (Figure 3*B*). In addition, constructs with the TACTACCTCATC to ATGATGGAGTAG mutation at the putative binding sites were also generated as controls (Figure 3*B*). We then transfected H69 cells with these reporter constructs followed by assessment of luciferase activity 24h after transfection. As shown in Figure 3*B*, a significant decrease of luciferase activity was detected in cells transfected with the SOCS4 3'UTR construct containing the potential binding site compared with mutant control vector. No change of luciferase activity was observed in cells transfected with the mutant SOCS4 3'UTR construct, suggesting endogenous translational repression of the construct with the SOCS4 3'UTR. In addition, anti-miR-98 and anti-*let-7i* markedly increased SOCS4 3'UTR-associated luciferase reporter translation (Figure 3*B*). In contrast, miR-98 and *let-7i* precursors significantly decreased the luciferase activity (Figure 3*B*). The above data suggest that miR-98 and *let-7* target SOCS4 3'UTR resulting in posttranscriptional suppression.

Manipulation of miR-98 function results in reciprocal alterations in SOCS4 protein expression

To test whether miRNA-mediated translational repression of SOCS4 is directly relevant to SOCS4 protein expression, we treated H69 cells with anti-miR-98 or miR-98 precursor for 72h and then measured SOCS4 protein expression by Western blot. Transfection of H69 cells with miR-98 precursor caused a dose-dependent decrease of SOCS4 protein content (Figure 4*A*). In contrast, a dose-dependent increase of SOCS4 protein level was identified in H69 cells treated by anti-miR-98 (Figure 4*B*). However, no significant change in SOCS4 mRNA levels was found between the control cells and cells treated with miR-98 precursor (Figure 4*C*) or anti-miR-98 (Figure 4*D*), suggesting that miR-98 does not induce SOCS4 mRNA degradation.

TLR4/MyD88-dependent downregulation of miR-98 and *let-7* is involved in *C. parvum*-induced SOCS4 protein expression

To further test whether miRNA-mediated SOCS4 translational repression is involved in *C. parvum*-induced SOCS4 expression, we measured expression of miR-98 and *let-7* in H69 cells after exposure to *C. parvum* for up to 48h. Consistent with results from our previous studies using a Northern blot probe that recognizes both miR-98 and *let-7* [29,31], decreased expression of miR-98/*let-7* was detected in cells at 8h to 48h following *C. parvum* infection (Figure 5*A*). *C. parvum*-repressed expression of miR-98/*let-7* was not identified in TLR4-DN or MyD88-DN stably transfected cells (Figure 5*B*), confirming the involvement of the TLR4/MyD88 signaling.

To further test whether miRNA-mediated SOCS4 translational repression is indeed involved in *C. parvum*-induced SOCS4 protein expression, we transfected H69 cells with various doses of miR-98 precursor for 48h and then exposed cells to *C. parvum* for 24h followed by Western blot for SOCS4. miR-98 precursor significantly inhibited *C. parvum*-induced upregulation of SOCS4 protein in H69 cells in a dose-dependent manner (Figure 6*A*). No significant change in SOCS4 mRNA levels was found in miR-98 precursor-treated cells at 24h following *C. parvum* infection compared with cells treated with the control precursor or with the non-treated control cells (Figure 6*B*). Additionally, cells treated with either miR-98 precursor or the control precursor followed by exposure to *C. parvum* for various periods of time displayed similar cellular levels of SOCS4 mRNA (Figure 6*C*). Taken together, the above data suggest that *C. parvum* infection decreases expression of miR-98- and *let-7* through activation of the TLR4/MyD88 signaling pathway to regulate SOCS4 protein expression in biliary epithelial cells.

Upregulation of SOCS4 may feedback affect *C. parvum*-induced STAT phosphorylation

Activation of STAT signaling has previously been reported in parasite infection [37]. Expression of SOCS4 and SOCS5 provides a feedback loop to the STAT signaling pathway during septic peritonitis [38]. We then tested whether expression of SOCS4 influences STAT phosphorylation in H69 cells following *C. parvum* infection. We first used a specific siRNA to knockdown SOCS4 expression in H69 cells and measured levels of phosphorylated STAT1, STAT3 and STAT6. While all three STATs showed a basal level of phosphorylation in cells transfected with a control siRNA, a significant increase of phosphorylation of STAT3 and STAT6, but not STAT1, was detected in cells treated with the SOCS4 siRNA by Western blot (Figure 7*A*). Knockdown of SOCS4 protein by the siRNA was confirmed by Western blot. No significant change in the total level of each tested STAT was detected following SOCS4 siRNA treatment. Moreover, increased phosphorylation of STAT3 and STAT6 was found in cells after exposure to *C. parvum* for 12h. In contrast, cells after exposure to *C. parvum* for 24h showed a significant decrease of STAT3 and STAT6 phosphorylation (Figure 7*B*). Accordingly, a higher level of SOCS4 protein was detected in cells after exposure to *C. parvum* for 24h compared with cells after exposure to *C. parvum* for 12h (Figure 7*B*).

DISCUSSION

Our results provide the first evidence suggesting an important role of miR-98 and *let-7* in *C. parvum*-induced expression of SOCS4 in host epithelial cells. Using an *in vitro* model of human cryptosporidiosis employing human biliary epithelial cells, we found that *C. parvum* infection induces SOCS4 expression through posttranscriptional gene regulation involving activation of the TLR/MyD88 pathway. Targeting of SOCS4 3'UTR by miR-98 and *let-7* results in translational suppression. *C. parvum*-induced SOCS4 expression in biliary epithelial cells involves relief of miR-98/*let-7*-mediated translational suppression. Moreover, SOCS4 may provide an inhibitory effect on *C. parvum*-induced STAT phosphorylation in host cells. Coupled with our recent studies demonstrating miR-98/*let-7*-mediated regulation of CIS [29], these data suggest that miRNAs coordinate CIS and SOCS4 expression in biliary epithelial cells following *C. parvum* infection, which may be relevant to the regulation of epithelial cell responses against microbial infection in general.

We previously demonstrated that miR-98 and *let-7* regulate CIS protein expression via translational repression in epithelial cells [29]. Several lines of evidence from this study support that miR-98 and *let-7* also target SOCS4 3'UTR resulting in translation suppression in biliary epithelial cells. First, complementarity of miR-98 and *let-7* to SOCS4 3'UTR was identified using the MicroRNA.org (http://www.microrna.org) for miRNA target prediction [35]. A significant decrease of luciferase activity was detected in cells transfected with the pMIR-REPORT luciferase construct containing SOCS4 3'UTR with the putative miR-98 and *let-7* binding site. This translational repression is likely mediated by miR-98 and *let-7*, because precursors and anti-miRs specific to miR-98 or *let-7* caused reciprocal alterations in SOCS4 3'UTR-associated luciferase activity. Second, transfection of cells with anti-miR-98 induced a significant expression of SOCS4 protein. In contrast, miR-98 precursor decreased cellular SOCS4 protein content. Finally, transfection of cells with miR-98 precursor did not induce SOCS4 mRNA degradation. Therefore, miR-98 and *let-7* regulate both SOCS4 and CIS protein expression via translational repression in epithelial cells, implicating that miR-98 and *let-7* can coordinately regulate expression of CIS and SOCS4.

Microbe-induced upregulation of CIS/SOCS expression has predominantly been demonstrated in immune cells. Upregulation of CIS/SOCS proteins has previously been reported in epithelial cells with infections [39]. In this report, we found that *C. parvum* infection increased SOCS4 expression in human biliary epithelial cells via activation of the TLR4/MyD88 pathway. Interestingly, the cellular SOCS4 mRNA levels showed no significant change following *C.*

parvum infection. Instead, activation of TLR4/MyD88 signaling downregulates miR-98 and *let-7* expression and consequently, relieves miR-98/*let-7*-mediated translational suppression of SOCS4. Similarly, expression of CIS protein in biliary epithelial cells was upregulated by LPS stimulation or after exposure to *C. parvum* through downregulation of miR-98 and *let-7* [29]. A recent report by Lu *et al.* demonstrated that miR-155 maintains T_{reg} cell homeostasis by limiting SOCS1 protein expression [40]. Obviously, miRNA-mediated posttranscriptional gene suppression is an important mechanism for the regulation of CIS/SOCS protein expression in cells in response to microbial challenge.

Expression of SOCS4 in biliary epithelial cells may represent an important feedback mechanism to finely control the host cell reactions in response to *C. parvum* infection. CIS/SOCS proteins have classically been shown to be negative regulators of cytokine signaling. Each CIS/SOCS protein has two major domains: an SH2 domain and a SOCS box [7]. Whereas the SH2 domain binds to the phosphorylated tyrosine residues in the substrates, the E3 activity of CIS/SOCS proteins can cause ubiquitination of the substrates for proteasome degradation [41]. Thus, CIS/SOCS proteins function as E3 ubiquitin ligases and mediate the degradation of activated cytokine signaling complex resulting in negative feedback regulation [7]. We demonstrated, in this study, that SOCS4 may be involved in the regulation of *C. parvum*-induced STAT phosphorylation. Inhibition of SOCS4 by siRNA interference promoted phosphorylation of STAT3 and STAT6 in biliary epithelial cells. Decreased of STAT3 and STAT6 phosphorylation was detected in cells following *C. parvum* for 24h, correlated to a higher expression level of SOCS4 protein in the infected cells. SOCS4-mediated suppression of STAT phosphorylation may be associated with an increased degradation of phosphorylated STATs. A recent study by Watanabe *et al*, demonstrated that expression of SOCS4 and SOCS5 increases degradation of phosphorylated STATs providing a negative feedback loop to regulate STAT signaling during septic peritonitis [35]. Although the underlying molecular mechanisms are currently unclear, it is possible that SOCS4 may facilitate ubiquitination-associated degradation of phosphorylated STATs induced by *C. parvum*.

In conclusion, our data indicate that *C. parvum* induces SOCS4 expression in human biliary epithelial cells via relief of miRNA-mediated translational suppression of SOCS4. Moreover, expression of SOCS4 in biliary epithelial cells is involved in the regulation of STAT phosphorylation in response to *C. parvum* infection. Thus, miRNA-mediated posttranscriptional gene regulation coordinates CIS and SOCS4 expression in biliary epithelial cells in response to *C. parvum* infection and is involved in the regulation of epithelial anti-microbial responses. It will be of interest to extend these studies to determine the role of miRNAs in epithelial anti-*C. parvum* immunity *in vivo*.

Acknowledgments

We are grateful to Dr. Fang X (Creighton University) for help with statistical analysis, Dr. M. F. Smith (University of Virginia) for providing the TLR4-DN construct and Prof. J. Tschopp (University of Lausanne) for the MyD88-DN construct.

This work was supported by National Institutes of Health Grant R01 AI071321 and by the Nebraska Tobacco Settlement Biomedical Research Program (LB692) (to X-M.C).

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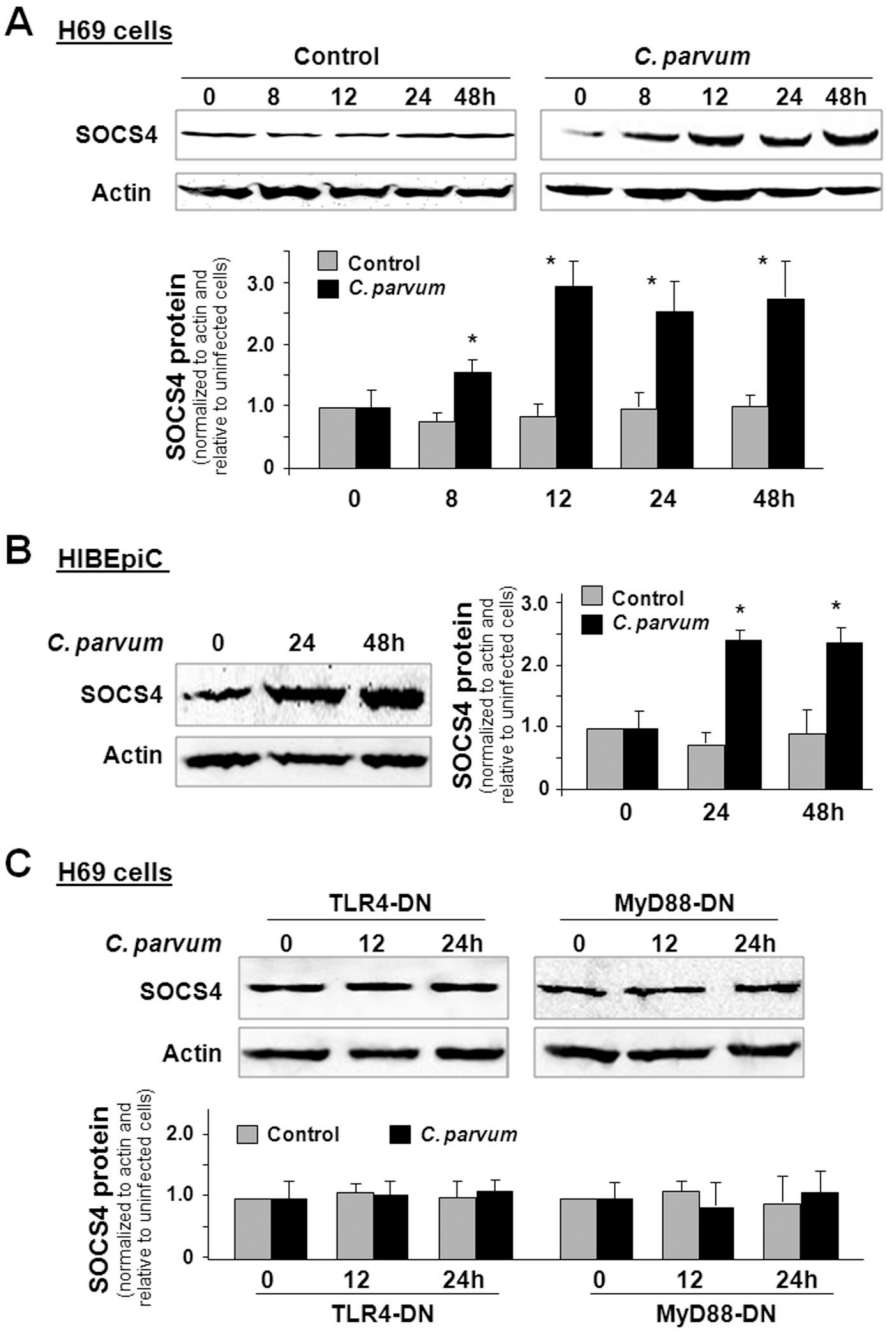

Figure 1.

C. parvum infection induces expression of SOCS4 protein in biliary epithelial cells in a TLR4/MyD88-dependent manner. *A* and *B*, H69 and HIBEpiC cells were exposed to *C. parvum* oocysts for up to 48h followed by Western blot for SOCS4. Upregulation of SOCS4 protein was detected in both H69 (*A*) and HIBEpiC (*B*) following *C. parvum* infection. *C*, H69 cells stably transfected with TLR4-DN or MyD88-DN were exposed to *C. parvum* for 24h. *C. parvum*-stimulated expression of SOCS4 protein was not detected in cells transfected with either TLR4-DN or MyD88-DN. Western blots shown in *A–C* are representative from three independent experiments and densitometric levels of SOCS4 signals were quantified and

normalized to actin and relative to uninfected cells. *, $p < 0.05$ *t*' test *versus* non-treated controls.

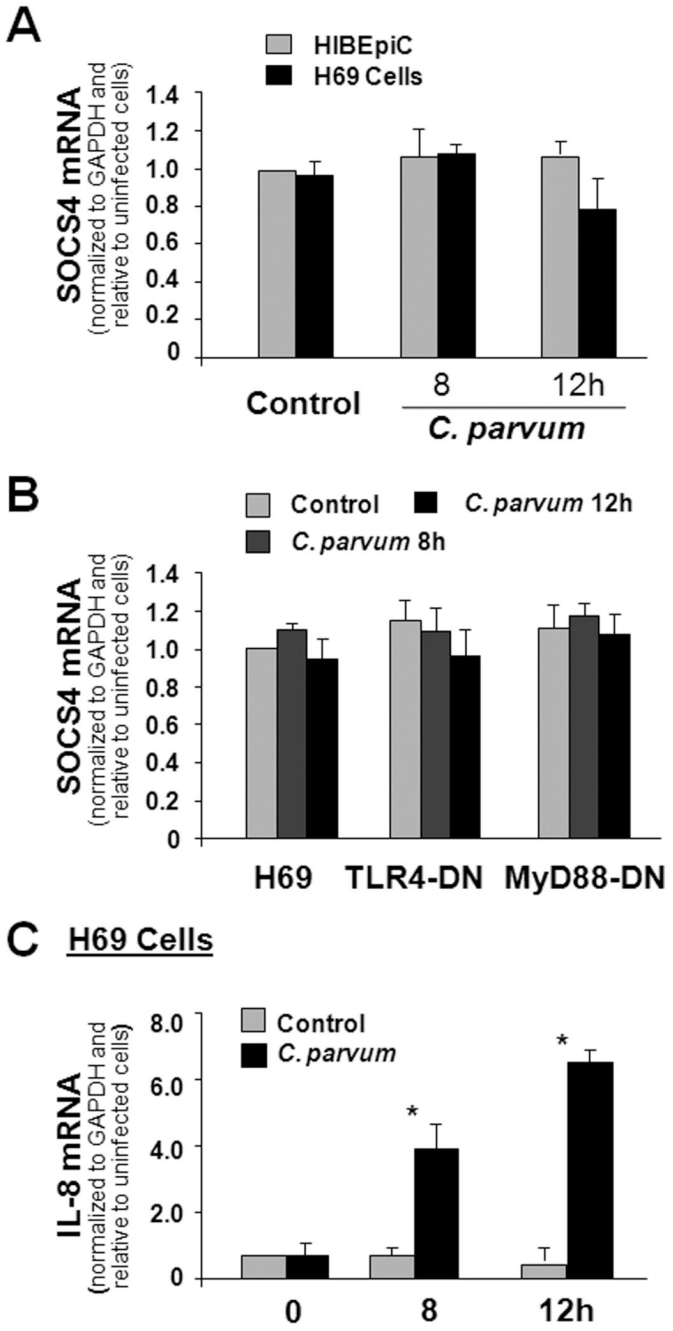

Figure 2.

C. parvum infection does not affect SOCS4 mRNA level in biliary epithelial cells. *A*, H69 and HIBEpiC cells were exposed to *C. parvum* oocysts for up to 12h followed by real-time PCR analysis for SOCS4 mRNA. No significant change in SOCS4 mRNA expression in human biliary epithelial cells in response to *C. parvum* infection. *B*, Expression of SOCS4 mRNA in TLR4-DN and MyD88-DN H69 cells in response to *C. parvum* infection. *C*, *C. parvum*-induced *e*xpression of IL-8 mRNA in H69 cells. IL-8 mRNA levels were presented as the Ct values normalized to GAPDH and relative to uninfected cells. *, $p < 0.05$ *t* test *versus* non-treated controls.

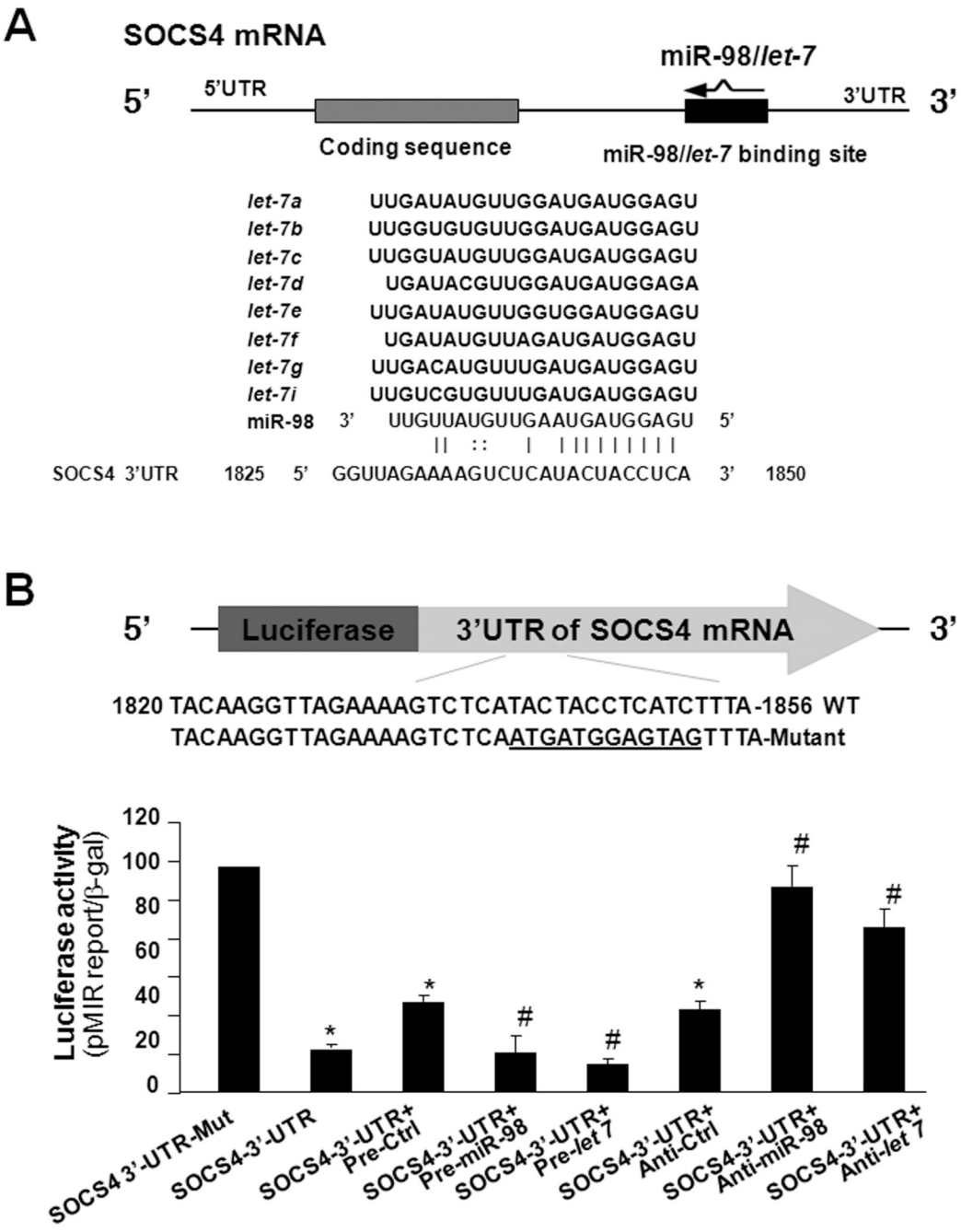

Figure 3.

MicroRNA-98 and *let-7* target SOCS4 3'UTR causing translational suppression. *A*, The schematic of SOCS4 mRNA shows one potential binding site in the 3'UTR for miR-98 and *let-7*. *B*, Binding of miR-98 and *let-7* to the potential binding site in the SOCS4 3'UTR results in translational suppression. Reporter constructs containing the potential binding site of miR-98 and *let-7* within SOCS4 3′UTR or the mutant sequence were generated respectively. H69 cells were transiently co-transfected with the reporter construct and either *let-7* or miR-98 precursor oligonucleotide or their antisense for 24h. Luciferase activities were then measured and normalized to the control β-Gal level. Bars represent the mean ± S.D. from three independent

experiments. Mut = mutant; *, $p < 0.05$ *t* test *versus* the controls; #, $p < 0.05$ *t* test *versus* SOCS4 3'UTR transfected cells.

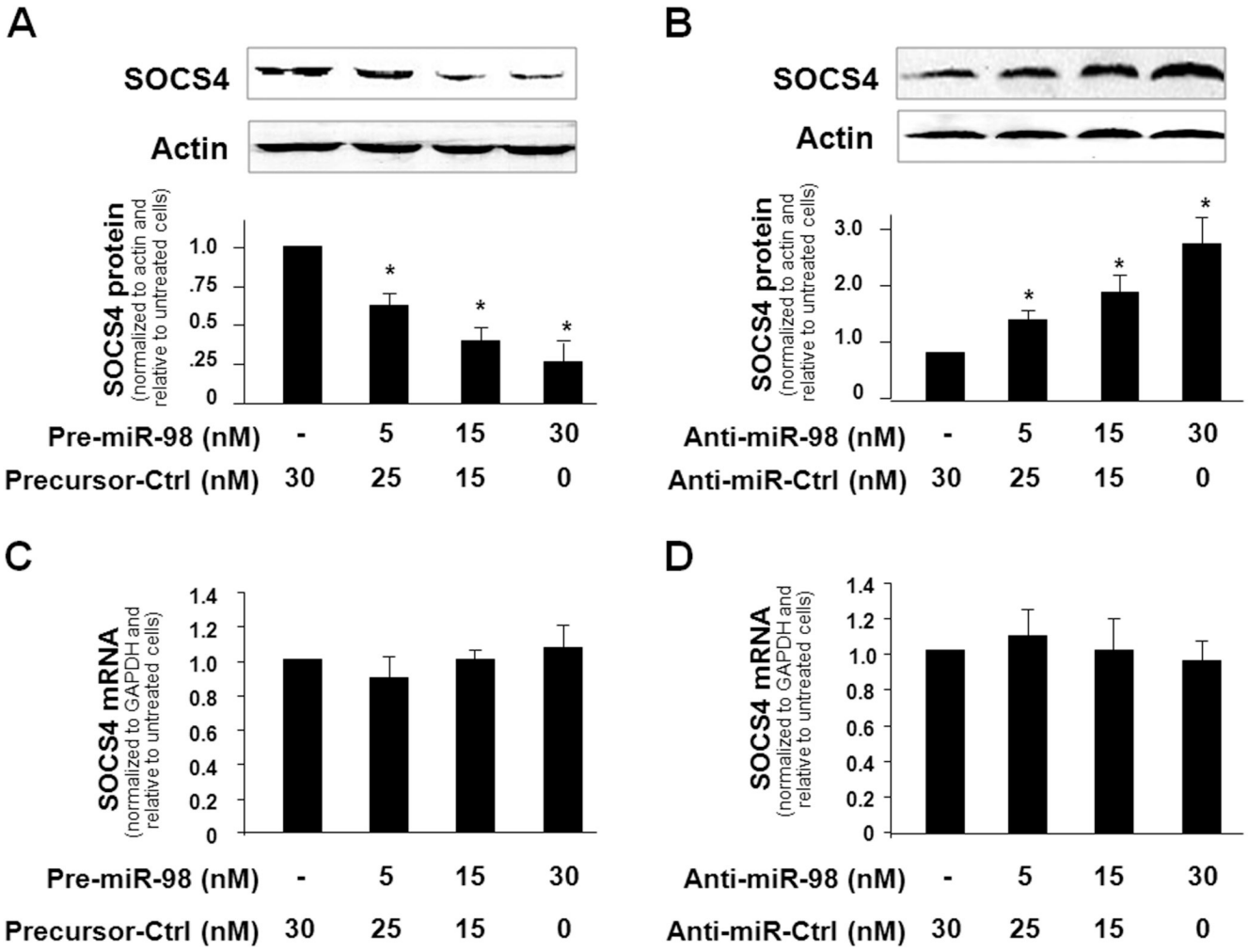

Figure 4.

Manipulation of miR-98 function results in reciprocal alterations in SOCS4 protein expression. *A* and *B*, Transfection of miR-98 precursor or anti-miR-98 induces a dose-dependent decrease or increase, respectively, in SOCS4 protein expression in H69 cells. H69 cells were treated with various dose of miR-98 precursor (*A*) or anti-miR-98 (*B*) followed by Western blot for SOCS4. A non-specific precursor (precursor-Ctrl) and a nonspecific anti-miR (anti-miR-Ctrl) were used as controls. A representative Western blot from three independent experiments is shown for *A* and *B*. Actin was also blotted to ensure equal loading. Densitometric levels of SOCS4 signals were quantified and expressed as their ratio normalized to actin and relative to non-anti-miR treated cells. *C* and *D*, miR-98 precursor or anti-miR-98 transfection does not affect SOCS4 mRNA levels. H69 cells were exposed to miR-98 precursor or anti-miR-98 followed by real-time PCR analysis for SOCS4 mRNA. *, $p < 0.05$ *t* test *versus* the non-treated cells.

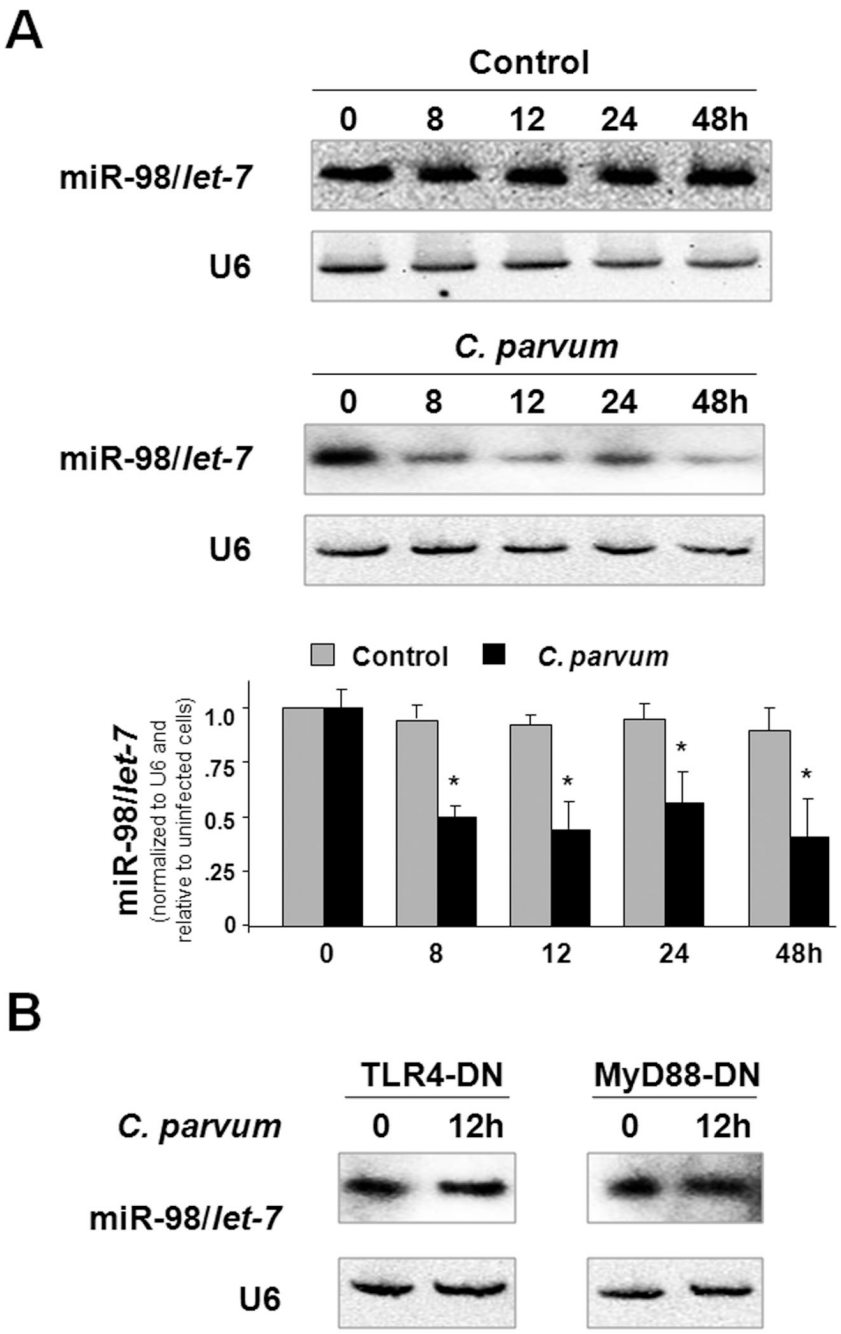

Figure 5.

C. parvum infection decrease expression of miR-98 and *let-7* in a TLR4/MyD88-dependent manner. *A*, *C. parvum* infection decrease miR-98/*let-7* expression in H69 cells by Northern blot analysis. RNU6B (U6) was used as a loading control. Densitometric levels of miR-98/*let-7* signals were quantified and expressed as their ratio normalized to U6 and relative to uninfected cells. *B*, *C. parvum*-induced down-regulation of miR-98/*let-7* expression is TLR4/MyD88-dependent. No decrease of miR-98/*let-7* expression was detected in H69 cells stably transfected with TLR4-DN or MyD88-DN after exposure to *C. parvum* as assessed by Northern blotting.

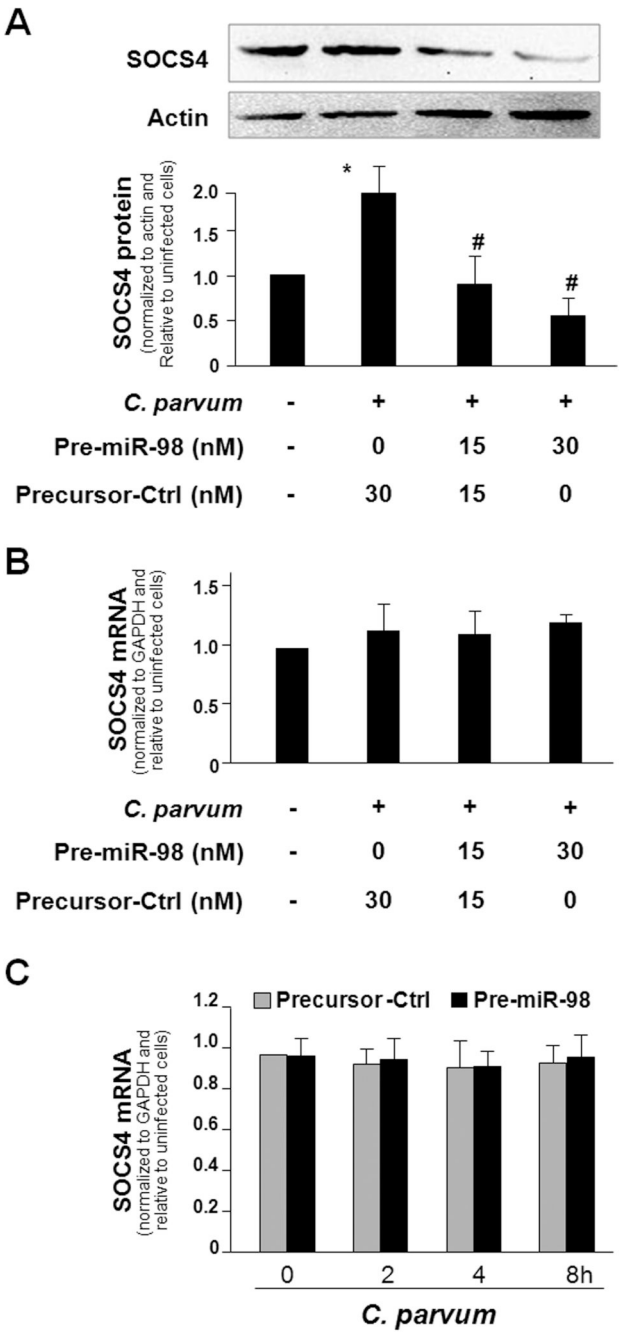

Figure 6.

Transfection of miR-98 precursor abolishes *C. parvum*-stimulated SOCS4 protein expression. *A*, Transfection of miR-98 precursor inhibits *C. parvum*-induced expression of SOCS4 proteins in H69 cells. Cells were transfected with the miR-98 precursor and then exposed to *C. parvum* followed by Western blotting for SOCS4. A representative Western blot from three independent experiments is shown in *A*. Densitometric levels of SOCS4 signals were quantified and expressed as their ratio normalized to actin and relative to uninfected cells. *B* and *C*, miR-98 precursor transfection does not affect SOCS4 mRNA levels in cells following *C. parvum* infection. H69 cells were exposed to miR-98 precursor for 48h and then exposed to *C. parvum* for 24h followed by real-time PCR analysis for SOCS4 mRNA (*B*). Similar SOCS4

mRNA levels were also detected in cells treated with miR-98 precursor following exposure to *C. parvum* for 2h to 8h. *$p < 0.05$ *t* test *versus* the uninfected cells; #, $p < 0.05$ *t* test *versus* infected cells.

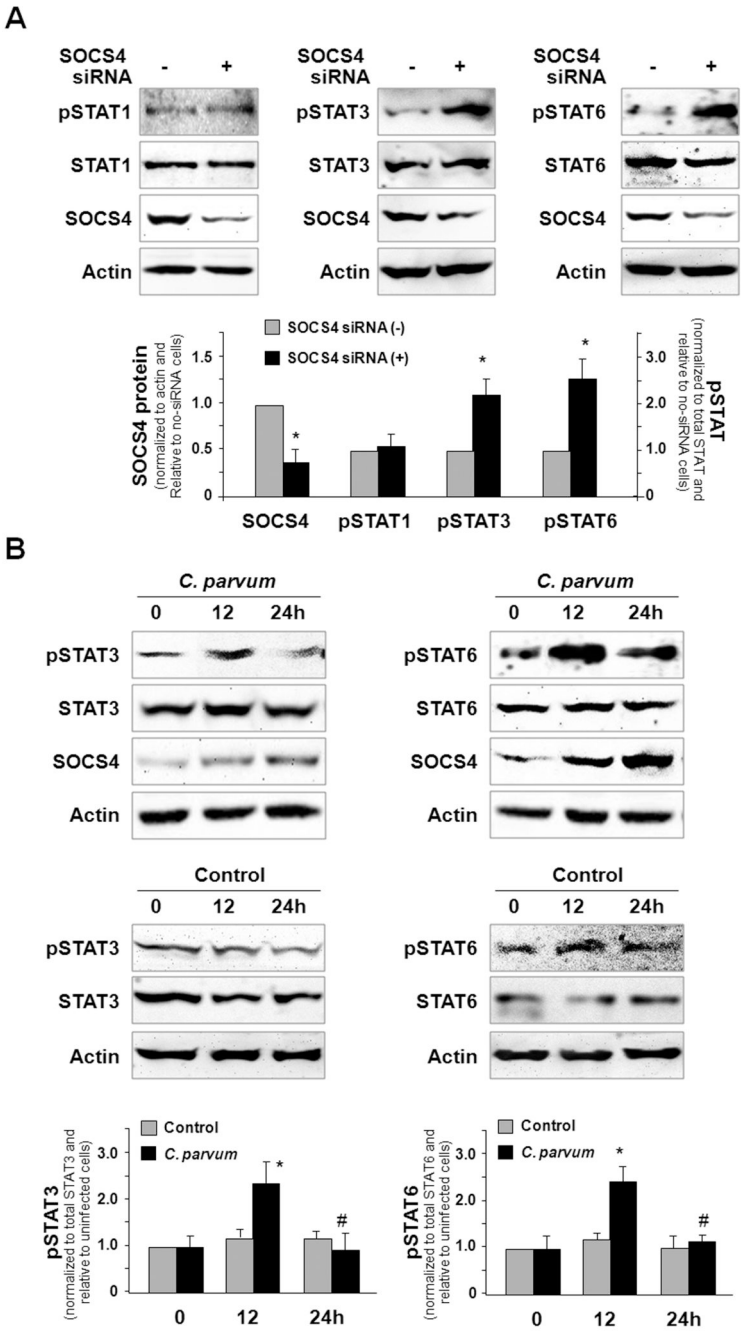

Figure 7.

SOCS4 suppresses STAT phosphorylation induced by *C. parvum*. *A*, Knockdown of SOCS4 by siRNA increases phosphorylation of STAT3 and STAT6, but not STAT1, in H69 cells. Cells were transfected with the shRNA against SOCS4 for 72h followed by Western blotting for tyrosine-phosphorylated STATs, total STATs, SOCS4 and actin. *B*, Correlation of STAT3 and STAT6 phosphorylation with levels of SOCS4 expression in H69 cells following *C. parvum* infection. H69 cells were exposed to *C. parvum* oocysts for 12h and 24h followed by Western blot for tyrosine-phosphorylated STATs, non-phosphorylated STATs, SOCS4 and actin. Densitometric levels of SOCS4 signals were quantified and normalized to actin and relative to SOCS4 siRNA (−) cells. Densitometric levels of phosphorylated STAT3 and STAT6

signals were quantified and normalized to total STAT3 and STAT6 and relative to SOCS4 siRNA (−) cells (*A*) or uninfected cells (*B*). *$p < 0.05$ *t* test *versus* the SOCS4 siRNA (−) cells (*A*) or uninfected cells (*B*); #, $p < 0.05$ *t* test *versus* 12h infected cells (*B*).