miRNA-939 regulates human inducible nitric oxide synthase posttranscriptional gene expression in human hepatocytes

Zhong Guo^a, Lifang Shao^a, Liang Zheng^b, Qiang Du^a, Peiyuan Li^a, Bino John^b, and David A. Geller^{a,1}

Departments of ^aSurgery and ^bComputational Biology, University of Pittsburgh, Pittsburgh, PA 15261

Edited by Charles J. Lowenstein, University of Rochester School of Medicine and Dentistry, Rochester, NY, and accepted by the Editorial Board March 5, 2012 (received for review November 4, 2011)

Human inducible nitric oxide synthase (hiNOS) gene expression is regulated by transcriptional and posttranscriptional mechanisms. The purpose of this study was to determine whether specific microRNA (miRNA) directly regulate hiNOS gene expression. Sequence analysis of the 496-bp hiNOS 3'-untranslated region (3'-UTR) revealed five putative miR-939 binding sites. The hiNOS 3'-UTR conferred significant posttranscriptional blockade of luciferase activity in human A549, HCT8, and HeLa cells. The hiNOS 3'-UTR also exerted basal and cytokine-stimulated posttranscriptional repression in an orientation-dependent manner. Functional studies demonstrated that transfection of miR-939 into primary human hepatocytes (HCs) significantly inhibited cytokine-induced NO synthesis in a dose-dependent manner that was abrogated by a specific miR-939 inhibitor. MiR-939 (but not other miRNAs) abolished cytokine-stimulated hiNOS protein in human HC, but had no effect on hiNOS mRNA levels. Site-directed mutagenesis of miR-939 binding sites at +99 or +112 bp in the hiNOS 3'-UTR increased reporter gene expression. Furthermore, intact miR-939 binding sites at +99 or +112 positions were required for posttranscriptional suppression by miR-939. Cytokine stimulation directly increased miR-939 levels in human HC. Transfection of miR-939 inhibitor (antisense miR-939) enhanced cytokine-induced hiNOS protein and increased NO synthesis in vitro in human HC. Finally, cytokine or LPS injection in vivo in mice increased hepatic miR-939 levels. Taken together, these data identify that miR-939 directly regulates hiNOS gene expression by binding in the 3'-UTR to produce a translational blockade. These findings suggest dual regulation of iNOS gene expression where cytokines induce iNOS transcription and also increase miR-939, leading to translational inhibition in a check-and-balance system.

nitric oxide | miRNA regulation

Nitric oxide (NO) is an important cellular messenger molecule that has both beneficial and detrimental actions in liver function (1–3). During inflammatory conditions, the rodent (4–6) and human inducible nitric oxide synthase (hiNOS) genes (7, 8) are activated by LPS and cytokines. Transcriptional regulation of the hiNOS gene involves transcription factors NF- κ B, Stat-1, AP-1, C/EBP β , KLF6, and NRF (9–16). Important posttranscriptional mechanisms also regulate hiNOS mRNA stability through RNA binding proteins HuR, TTP, and KSRP (17–20).

In primary human hepatocytes (HCs), cytokine stimulation leads to rapid induction of human iNOS mRNA, protein, and induced NO synthesis. In contrast, primary human macrophages as well as certain human tumor cell lines demonstrate iNOS mRNA following inflammatory cytokine stimulation, yet human iNOS protein is difficult to detect (21). In human cardiac myocytes, human iNOS mRNA was readily seen after cytokine stimulation, but human iNOS protein and NO synthesis were not identified (22). These observations are consistent with a translational blockade and suggest the possible role of microRNA in exerting negative posttranscriptional regulation.

MicroRNAs (miRNA) are short (~21) nucleotides that are complementary to 3'-UTR mRNA sequences and have been recently shown to mediate negative posttranscriptional regulation by either facilitating mRNA degradation or exerting a translational blockade of protein synthesis (23, 24). Certain miRNAs are expressed in solid organ cancers and have been shown to correlate with survival (25–27). The hiNOS gene is expressed in many cancers, and NO has been shown to exert a complex role during inflammation-associated carcinogenesis (28–32). However, no information exists as to a direct role for miRNA in regulating hiNOS gene expression. Furthermore, the specific mechanisms for posttranscriptional silencing of the human iNOS gene are poorly defined.

Therefore, the purpose of this study was to determine whether specific miRNAs directly regulate hiNOS gene expression. We identify adjacent miR-939 binding sites in the hiNOS 3'-UTR, and we show that miR-939 binds in vitro and in vivo to exert a translational blockade of cytokine-stimulated hiNOS protein expression in primary human hepatocytes. This is a unique identification of a specific miRNA that directly down-regulates human iNOS expression.

Results

Cytokine Inducibility of the Human iNOS Gene Suggests Posttranscriptional Blockade. The human iNOS gene is highly inducible by cytokines. Previously we reported that the hiNOS gene was markedly induced by cytokine mixture (CM) of TNF- α , IL-1 β , and IFN- γ in human primary hepatocytes (7, 8). Using quantitative real-time PCR, human hepatocyte mRNA peaked at 6 h after stimulation and exhibited 2,587-fold induction over unstimulated (control) mRNA (Table 1). In contrast, the human iNOS protein determined by Western blot analysis showed a 265-fold increase over basal protein level, which was barely detectable in resting human hepatocytes. Induced NO synthesis showed a 72-fold increase over basal levels, determined by Griess assay for nitrite release into culture supernatants for 24 h. These results indicate that cytokines trigger a dramatic transcriptional induction of the human iNOS gene. The results also suggest the possibility of posttranscriptional regulation because the magnitude of human iNOS protein and NO levels were much less than the fold increase in iNOS mRNA. Whereas others have shown direct posttranscriptional regulation of the human iNOS gene by RNA

Author contributions: Z.G., L.Z., P.L., and D.A.G. designed research; Z.G. and L.S. performed research; B.J. contributed new reagents/analytic tools; Z.G., L.S., L.Z., Q.D., and D.A.G. analyzed data; and Z.G. and D.A.G. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. C.J.L. is a guest editor invited by the Editorial Board.

¹To whom correspondence should be addressed. E-mail: gellerda@upmc.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1118118109/-DCSupplemental.

Table 1. Inducibility of human iNOS gene expression in human hepatocytes

Human iNOS	Method	Fold ↑ (CM/basal)
mRNA	Real-time PCR	2,587 ± 336
Protein	Western blot	265 ± 43
NO (nitrite)	Griess assay	72 ± 9

binding proteins (17), nothing is known about miRNA binding to the human iNOS mRNA 3'-UTR.

Human iNOS 3'-UTR Mediates Translational Repression of hiNOS Expression. To determine the effect of the human iNOS gene 3'-UTR on transcription, we inserted the full-length 496-bp hiNOS 3'-UTR cDNA into the 3'-UTR of a luciferase reporter plasmid containing a constitutively active pCMV promoter (Fig. 1A). Transfection of pCMV-Luc-hiNOS 3'-UTR into human lung (A549), colon (HCT8), or uterine (HeLa) cells significantly inhibited basal transcription compared with control plasmid without exogenous 3'-UTR (Fig. 1A).

These results indicate that the presence of the hiNOS 3'-UTR confers posttranscriptional repression on a heterologous reporter system. Next, to show the effect on transcription driven by the native human iNOS promoter (rather than constitutive CMV promoter), we substituted in the -7.2 kb hiNOS promoter for the CMV promoter and ligated the hiNOS 3'-UTR downstream in forward or reverse orientation (Fig. 1B). The hiNOS 3'-UTR significantly decreased both basal and CM-induced luciferase activities driven by the endogenous hiNOS promoter, whereas hiNOS 3'-UTR (reverse) had no significant effect on basal or inducible reporter activity. These findings suggest that the hiNOS 3'-UTR confers posttranscriptional repression on hiNOS gene expression in an orientation-dependent manner.

Bioinformatic Analysis of Potential miRNA Binding Sites in the 3'-UTR of the hiNOS Gene. We next sought to identify specific miRNA binding sites in the hiNOS 3'-UTR. Eukaryotic miRNAs have been reported to functionally target endogenous mRNAs mostly through binding sites in the 3'-UTR (33). To investigate the human iNOS gene as a potential target of miRNA regulation, the 496-bp hiNOS 3'-UTR mRNA sequence was analyzed using MicroInspector, a Web-based tool for searching miRNA binding sites in a target mRNA sequence. By using this program with temperature setting at 37 °C and free energy at -20 kcal/mol, which characterizes the stability of the miRNA/mRNA interaction, we were able to identify about 100 potential binding sites for human iNOS 3'-UTR sequence. The top 20 putative miRNA binding sites with free energy range -27 to -35 kcal/mol are shown (Table S1). The strongest predicted miRNA binding site in the human iNOS mRNA 3'-UTR is miR-939, which has five predicted binding sites listed. Therefore, we focused on the role of miR-939 to determine whether there was direct regulation of human iNOS mRNA by miR-939 binding.

Exogenous miRNA-939 Inhibits Cytokine-Induced NO Synthesis. We examined the effect of miRNA transfection on CM-induced NO synthesis in primary human hepatocytes using exogenous miR-939 (24-nt sequence mimic). Twenty-four hours after miRNA transfection (5 pmol/mL), primary human HCs were stimulated with CM, and NO synthesis determined 24 h later. MiR-939 significantly inhibited induced NO synthesis, whereas miR-146b inhibited NO synthesis to a lesser extent (Fig. 2A). The other miRNA and negative control (miR-NC) had no effect on CM-induced NO synthesis. The inhibitory effect of miR-939 was exerted in a dose-dependent manner with a 1:100 dilution resulting in loss of inhibition (Fig. 2B). To show specificity for miR-939, we performed antisense miRNA inhibitor experiments.

Fig. 1. The human iNOS (hiNOS) 3'-UTR region confers translational gene repression. (A) Inhibitory effect of human iNOS 3'-UTR on CMV-luciferase reporter activity in human cells. The hiNOS 3'-UTR cDNA was inserted into the 3'-untranslated region of a luciferase reporter plasmid containing a constitutively active pCMV promoter to determine its effect on reporter expression. The control vector is the same vector without any 3'-UTR fragment insertion. DNA vectors were transfected in A549, HCT8, and HeLa cells. Relative luciferase activity (RLA) was normalized to the cotransfected β -galactosidase as an internal standard. Values shown are the means $\pm$ SD of at least three separate experiments performed in triplicate. White bars represent the expression of luciferase reporter gene from the cells transfected with control vector; gray bars represent the expression of luciferase reporter gene from the cells transfected with 3'-UTR. (B) The hiNOS 3'-UTR down-regulates basal and cytokine-induced hiNOS promoter activity in A549 cells in an orientation-dependent manner. The native -7.2 kb human iNOS promoter was used to drive luciferase reporter gene expression. The hiNOS 3'-UTR cDNA was cloned in forward and reverse orientation into the 3'-untranslated region of luciferase. White bars represent the expression of luciferase reporter gene in untreated cells as control; gray bars represent the expression of luciferase reporter gene with cytokine mixture treatment. * $P < 0.01$ vs. control.

Specific antisense miRNA to miR-939 (miR-939 inhibitor) abrogated the inhibitory effect of miR-939 on CM-induced NO synthesis in a dose-dependent manner (Fig. 2C). Moreover, nonspecific antisense miRNA to miR-939 was not able to attenuate miR-939 mediated inhibition of inducible NO synthesis in CM-stimulated human hepatocytes (Fig. S1).

miR-939 Decreases hiNOS Protein but Not mRNA Levels. To further investigate the mechanism of miR-939 mediated hiNOS suppression, we performed Western blot for hiNOS protein and RT-PCR for hiNOS mRNA. Transfection of miR-939 into human hepatocytes significantly inhibited cytokine-induced hiNOS pro-

Fig. 2. Effect of specific miRNA on nitric oxide (NO) synthesis in human hepatocytes. (A) miRNA mimics to miR-939 and to a lesser extent miR-146 decrease cytokine mix (CM)-stimulated NO synthesis in human hepatocytes. An aliquot 5×10^6 of human hepatocytes was first transfected with various miRNA mimics, then stimulated with CM (TNF α + IL-1 β + IFN γ) or left unstimulated for 24 h. The production of NO (nitrite) in culture supernatants was determined with a Griess assay. Measurement of nitrite value was normalized with its corresponding protein concentration. The graph shows means $\pm$ SD. (B) miR-939 inhibits NO synthesis in a dose-dependent manner. A total of 5×10^6 human hepatocytes were transfected with serial dilution of miR-939 mimic, then stimulated with CM or left unstimulated for 24 h. (C) miR-939 inhibitor attenuates the suppressive effect of miR-939 on CM-induced NO synthesis. A total of 5×10^6 human hepatocytes were transfected with miR-939 mimics and its inhibitor at different ratios, then stimulated with CM or left unstimulated for 24 h.

tein, but not hiNOS mRNA (Fig. 3). The Western blot shown in Fig. 3 is representative of three different experiments, and the average of these experiments is shown as Fig. S2. MiR-146b had a modest inhibitory effect on hiNOS protein, whereas the other miRNAs had no effect. To further examine whether miR-939 might alter hiNOS mRNA half-life, we transfected miR-939 into human hepatocytes and used real-time PCR to measure hiNOS mRNA expression at different time points after CM stimulation for 6 h followed by actinomycin-D chase. CM-stimulated hiNOS mRNA half-life was ~ 2.5 h and was not altered by miR-939 transfection (Fig. S3). Therefore, miR-939 does not affect hiNOS mRNA stability. These findings are consistent with a direct effect of miR-939 producing a translational blockade for CM-induced hiNOS protein and not by enhanced hiNOS mRNA degradation.

Functional Mutagenesis Analysis of miRNA Binding Sites in the hiNOS 3'-UTR. Because exogenous miR-939 (and to a lesser extent miR-146b) inhibited hiNOS protein, we next sought to demonstrate

Fig. 3. Exogenous miR-939 inhibits cytokine-induced hiNOS protein but not hiNOS mRNA levels. (A) Western blot analysis of iNOS protein expression in human hepatocytes. (B) RT-PCR analysis of iNOS mRNA expression in human hepatocytes. Hepatocytes were transfected with miRNA mimics, then treated with cytokine mixture (CM). Protein and mRNA were extracted from hepatocytes after CM treatment for 8 and 6 h, respectively. Blots shown are representative of three similar experiments, and the Western blots for hiNOS protein are quantified (Fig. S2).

a functional role for endogenous miR-939 and miR-146b binding in the hiNOS 3'-UTR. Site-directed mutagenesis was used to mutate the five putative miR-939 binding sites at +99, +112, +374, +375, and +395 bp in the hiNOS 3'-UTR, as well as the miR-146b site at +48 bp (Fig. 4A, Upper). Transfection of these mutant plasmids into primary human HCs showed that mutation of miR-939 at +99 or +112 bp positions significantly increased luciferase reporter activity driven by the heterologous CMV promoter compared with the wild-type CMV-Luc-hiNOS 3'-UTR. The increased reporter activity with +99 or +112 mutants indicates a loss of function of endogenous miR-939 for translational repression at these sites. In contrast, mutation of miR-146b binding site, or the remaining miR-939 sites had no effect.

Sequence predictions of miR-939 miRNA binding to hiNOS mRNA at +99 and +112 bp in the 3'-UTR is shown in Fig. S4. MiR-939 binding at +99 bp or +112 bp would involve a single loop mRNA bend. To further examine the functional role of miR-939 binding at these sites, we generated +99- and +112-bp mutants (Fig. 4B, Upper), driven by the native hiNOS promoter to allow for cytokine stimulation. Then, we determined the ability of exogenous miR-939 to inhibit cytokine-induced reporter expression in human A549 cells under the influence of the hiNOS promoter and 3'-UTR compared with negative control scrambled miRNA. MiR-939 significantly inhibited CM-induced reporter activity and also slightly decreased basal activity. When the constructs containing mutants +99 or +112 were transfected, the reporter activity was restored. Our mutagenesis results suggest that both sites are functional because mutation of either miR-939 site prevents translational repression of luciferase activity.

In Vivo miRNA-939 Binding to hiNOS mRNA Using an Ago "Pull-Down" Assay. Recent studies demonstrated that mammalian mRNAs associate with members of the Argonaute (Ago) family of proteins, which facilitates sequence-specific miRNA targeting of mRNAs (34, 35). Therefore, we used anti-Ago-specific antibody to coimmunoprecipitate the Ago-bound miRNA/mRNA complex, whereas IgG served as negative control. mRNAs extracted after coimmunoprecipitation were used as templates for real-time qPCR for hiNOS mRNA, which would only be detected if bound with miRNA/Ago complex. Pull down with the anti-Ago antibody resulted in a fivefold increase in hiNOS mRNA/miRNA/Ago complex after cytokine stimulation of primary human HC compared with resting (basal) cells (Fig. S5A). As

activity in an orientation-dependent manner; (ii) miRNA-939 decreases cytokine-induced hiNOS protein expression, but does not affect hiNOS mRNA levels or hiNOS mRNA stability; (iii) hiNOS 3'-UTR contains two functional miR-939 binding sites at +99 and +112 that are critical for miR-939-mediated translational blockade; (iv) transfection of miR-939 inhibitor enhanced cytokine-induced hiNOS protein and NO synthesis in human HC; and (v) cytokines can induce endogenous hepatic miR-939 expression *in vitro* and *in vivo*.

Previously, our group identified that the nuclear protein NRF (NF- κ B repressing factor) functioned as a negative transcription factor for transcriptional repression of hiNOS gene expression. The hiNOS gene exhibited constitutive silencing by the trans-acting NRF protein binding to the *cis*-acting NRE at -6.7 kb in the hiNOS promoter (11). Whereas this is an example of direct negative transcriptional regulation of hiNOS gene expression, others have shown that posttranscriptional mechanisms also govern hiNOS expression by regulating mRNA stability, which involves by HuR, TTP, and KSRP RNA binding proteins to hiNOS 3'-UTR (17–20, 36). However, there have been no reports of direct miRNA regulation of rodent or human iNOS genes. In this study, we identified that cytokine-induced miR-939 functionally binds the hiNOS 3'-UTR to posttranscriptionally repress its protein expression, without affecting hiNOS mRNA stability.

MiR-939 mediated hiNOS translational suppression is an example of epigenetic gene silencing. There are three distinct epigenetic mechanisms that regulate gene expression: DNA methylation, histone modifications, and RNA-associated silencing, which includes miRNA regulation. Chan et al. found that the human iNOS gene is epigenetically repressed by DNA methylation and histone H3 lysine 9 methylation (21). In human cells resistant to iNOS induction such as endothelial and vascular smooth muscle cells, the human iNOS proximal promoter was densely methylated at CpG dinucleotides. In contrast, human hepatocytes and transformed cells such as A549 and DLD-1 capable of iNOS induction had a lower density of methylation at the proximal promoter. The upstream cytokine-responsive enhancer did not display important differences in DNA methylation between iNOS-inducible and noninducible cell types. Using chromatin immunoprecipitation, they also showed that the human iNOS promoter was basally enriched with di- and trimethylation of H3 lysine 9 in endothelial cells, and this did not change with cytokine addition.

Although two groups have recently shown that specific miRNAs are *indirectly* involved in the regulation of iNOS gene expression, a direct role for RNA silencing by miRNA binding in the iNOS gene has not been reported. Dai et al. (37) reported that miR-146a, a negative regulator of Toll-like receptor (TLR) signaling, was decreased in freshly isolated splenic lymphocytes from estrogen-treated mice. Increasing the activity of miR-146a significantly inhibited LPS-induced IFN- γ and iNOS expression in mouse splenic lymphocytes. Enhancing the activity of miR-146a also inhibited the expression of LPS-induced iNOS and nitric oxide (37); however the mechanism of action was not determined. Additionally, a recent report indicates that miR-155 expression was increased in *MKP-1*-deficient macrophages compared with wild-type macrophages. Transfection of miR-155 attenuated the expression of suppressor of cytokine signal (SOCS)-1 and subsequently enhanced the expression of iNOS (38). These two studies indicate that specific miRNA (miR-155 or miR-146a) can indirectly up- or down-regulate iNOS expression by altering upstream signal transduction pathways that effect iNOS expression.

MicroRNAs regulate gene expression by either repressing protein translation or degrading messenger RNA (mRNA) molecules. It has been shown that mRNAs containing multiple, nonoverlapping miRNA binding sites are more responsive to

miRNA-induced translational repression than those containing a single miRNA binding site (39). We identified two adjacent, but not overlapping, miR-939 binding sites in the hiNOS 3'-UTR that are functionally active as transfection of miR-939 mimics into human HC significantly inhibited cytokine-induced hiNOS protein, but not hiNOS mRNA. MiR-939 was originally cloned from human cervical cancer cells (40), and another group reported that miR-939 regulates the replication of H1N1 influenza virus in Madin-Darby canine kidney cells (41).

Historically, whereas cytokine-induced iNOS expression was readily detected in many rodent cell types, human iNOS protein expression and subsequent NO synthesis was more restricted. Because miR-939 binding to the hiNOS 3'-UTR translationally represses cytokine-induced human iNOS protein, we also sought to determine whether the rodent iNOS genes contain a miR-939 binding site in their respective 3'-UTR regions. Interestingly, sequence analysis showed that neither the murine nor the rat iNOS 3'-UTR region contained any predicted miR-939 binding sites (Table S2). This was confirmed when overexpression of miR-939 significantly inhibited cytokine-induced human NO synthesis (nitrite) in primary human HCs, but not in rat or mouse HCs (Fig. S6). These findings provide additional insight into the molecular mechanisms that account for species-specific regulation of iNOS gene expression.

Here, we propose a model regarding the functional role of miR-939 in the posttranscriptional regulation of the iNOS gene in human hepatocytes (Fig. S7). Human primary hepatocytes can be stimulated by a combination of cytokines (TNF- α , IL-1 β , and IFN- γ) to strongly express hiNOS mRNA. The cytokines activate specific transcription factors NF- κ B, Stat-1, AP1, and C/EBP β , which functionally interact with their corresponding *cis*-acting DNA binding sites to drive hiNOS transcription. Meanwhile, the same cytokines also increase miR-939 levels, which bind to two specific miR-939 binding sites in the hiNOS 3'-UTR, leading to translational inhibition of CM-induced hiNOS protein expression. Binding to both sites is likely required for maximal translational repression, because mutation of either binding site partially abrogates the inhibitory effect of exogenous miR-939 on luciferase activity (Fig. 4B). Teleologically, cytokine induction of a negative miRNA regulator of hiNOS expression would serve to protect the host against untoward consequences of prolonged human iNOS overexpression in a check-and-balance system.

Experimental Procedures

Materials. Human recombinant TNF- α and IFN- γ were obtained from R&D Systems, and IL-1 β was provided by Pierce Biotechnology. Lipofectamine was purchased from Invitrogen. MiRNA mimics and anti-miRNA inhibitors were purchased from Ambion. Antibodies used in immunoprecipitation of Ago-RNA complexes were obtained from BD Biosciences Pharmingen. Antibodies against hiNOS and IgG were obtained from Santa Cruz Biotechnology. All other reagents were obtained from Sigma.

Plasmids. The pMIR-REPORT (Ambion) luciferase miRNA expression reporter vector is used to evaluate miRNA regulation. We inserted the intact 496-bp 3'-UTR of human iNOS gene into the miRNA cloning site. A second vector, pMIR-REPORT beta-galactosidase reporter control vector, is used for normalization of transfection efficiency. The human iNOS promoter reporter plasmid vector pINOS (7.2)luc contains -7.2 kb of upstream 5'-flanking DNA linked to the luciferase reporter gene and has been described previously (8). This 7.2-kb intact human iNOS promoter is subcloned in pMIR-REPORT vector to substitute the native 2-kb pCMV promoter. The mutated constructs corresponding to each potential miRNA binding site were generated by using the QuikChange mutagenesis kit from Stratagene in vectors with both CMV and native human iNOS promoters.

Cell Culture. The A549, HCT8, and HeLa human cancer cell lines were obtained from the ATCC and cultured in medium as recommended. Primary human hepatocytes were obtained from freshly resected human liver specimens under institutional-review-board-approved protocol as previously described (7). A total of 3×10^6 human hepatocytes were plated onto six-well cell

culture plates (Corning) and stimulated with a CM of TNF- α (1,000 units/mL) + IL-1 β (100 units/mL) + IFN- γ (250 units/mL).

Transfections and Reporter Gene Assays. DNA, miRNA mimics and anti-miRNA inhibitors were transfected into cells in six-well plates using Lipofectamine for human cells. The procedure was followed as previously described (12).

Western Blot and qPCR. Protein extraction and Western blot analysis were performed as previously described (8). Quantitative RT-PCR was carried on the StepOne Plus real-time PCR system (Applied Biosystems) by using the threshold cycle method of calculating relative mRNA expression. The $2^{-\Delta\Delta CT}$ method was used to quantify human iNOS and β -actin gene mRNA in triplicate by SYBR green two-step, real-time PCR. The following primers were used: human iNOS primers, 5'-CAGCGGGATGACTTCCAA-3' (forward) and 5'-AGGCAAGATTTGGACCTGCA-3' (reverse); human β -actin iNOS primers, 5'-AGGCATCCTCACCTGAAGTA-3' (forward) and 5'-CACACGCAGCTCATTGTA-3' (reverse). Quantitative analysis of miRNA expression was also performed on the StepOne Plus real-time PCR system by using miRCURY LNA PCR system (Exigon).

Immunoprecipitation and qPCR Analysis of Ago-RNA Complexes. Human hepatocytes were transfected with miR-939 and miR-146b as described above. On the following day, cells were induced by CM for 6 h and then lysed after 48 h, using buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.25% Nonidet P-40, and 1.5 mM MgCl₂. The antibodies used were anti-Ago1 and irrelevant antibody IgG 2b (BD Biosciences Pharmingen; 1 μ g/mL). A total of 10 μ g antibody was mixed with 50 μ L protein G Dynabeads (Invitrogen) and incubated at room temperature for 10 min to form the Dynabead-Ab complex. The complex was further added to the cell lysate for 12 h of incubation at 4 °C, beads were pelleted, washed four times with the washing buffer, and dissolved in the elution buffer. RNA was extracted from beads by TRIzol (Invitrogen) and then detected by qPCR.

Animals and in Vivo Treatment. Male C57BL/6 mice were purchased from The Jackson Laboratory. To study the effect of CM or LPS on miR-939 expression, mice were treated by i.p. injection with a CM of TNF- α (5×10^5 units) + IL-1 β (2×10^5 units) + IFN- γ (1×10^5 units) per mouse, or LPS 100 μ g per mouse. I.p. injection of 1 $\times$ PBS served as control group. All mice were killed 0 h or 3 h after the injection and their liver samples were collected for RNA extraction.

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