Hepatitis C Virus Infection and Hepatic Stellate Cell Activation Downregulate miR-29: miR-29 Overexpression Reduces Hepatitis C Viral Abundance in Culture

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Hepatitis C Virus Infection Down-regulates miR-29: Effects on Viral Fitness and Hepatic Stellate Cell Activation

Sarmistha Bandyopadhyay¹, Robin C. Friedman², Rebecca T. Marquez³, Kathy Keck¹, Benjamin Kong⁴, Michael S. Icardi⁵, Kyle E. Brown⁶, Christopher B. Burge², Warren N. Schmidt⁷, Yulei Wang⁴ and Anton P. McCaffrey¹,⁸

¹University of Iowa School of Medicine
Department of Internal Medicine
University of Iowa
Iowa City, IA 52242, USA

²Department of Biology,
Massachusetts Institute of Technology,
Cambridge, Massachusetts 02139, USA

³Department of Pharmacology
University of Kansas Medical Center
3901 Rainbow Blvd.
Kansas City, KS 66160, USA

⁴Life Technologies
850 Lincoln Centre Dr.
Foster City, CA 94404, USA.

5Department of Pathology and Research Service  
Veterans Administration Medical Center  
200 Hawkins Drive  
Iowa City, IA 52242, USA

6Iowa City Veterans Administration Medical Center, Iowa City, IA  
Division of Gastroenterology-Hepatology and Program in Free Radical and Radiation Biology,  
University of Iowa Roy J. and Lucille A. Carver College of Medicine,  
Iowa City, IA 52242, USA

7Department of Internal Medicine and Research Service  
Veterans Administration Medical Center  
200 Hawkins Drive  
Iowa City, IA 52246, USA

8Corresponding author

sarmistha-bhattacharya@uiowa.edu, robinf@mit.edu, rmarquez2@kumc.edu, kathy-keck@uiowa.edu, benjamin.kong@lifetech.com, michael-icardi@uiowa.edu, warren-schmidt@uiowa.edu, kyle-brown@uiowa.edu, cburge@mit.edu, yulei.wang@lifetech.com, antonmccaffrey@gmail.com
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Contact information:
Anton P, McCaffrey, Department of Internal Medicine, University of Iowa, 3166 MERF, Iowa City, IA 52242
antonmccaffrey@gmail.com
Phone: (319) 335-7862,
Fax: (319) 353-5905

Abbreviations:
HCV, Hepatitis C virus; HCC, hepatocellular carcinoma; miR/miRNA, microRNA; ECM, extracellular matrix; UTR, untranslated regions; qRT-PCR, quantitative reverse transcription polymerase chain reaction; C-HCV, Chronic HCV; PCT, probability of conserved targeting; PACT, Pathway Analysis of Conserved Targets; PDGF, platelet derived growth factors; PI3K, phosphatidylinositol 3 kinase; HSC, hepatic stellate cell; TGF-β, transforming growth factor-β
**ABSTRACT**

**Background.** Chronic Hepatitis C Virus (HCV) induced liver fibrosis is mediated by up-regulation of Transforming Growth Factor (TGF)-beta and subsequent hepatic stellate cell (HSC) activation. MicroRNAs (miRNAs) regulate HCV infection and HSC activation.

**Methods.** TaqMan miRNA profiling identified 12 miRNA families differentially expressed between chronically HCV-infected human livers and uninfected controls. To identify pathways affected by miRNAs, we developed a new algorithm (PACT, Pathway Analysis of Conserved Targets), based on the probability of conserved targeting.

**Results.** This analysis suggested a role for miR-29 during HCV infection. Intriguingly, miR-29 was down-regulated in most HCV-infected patients. miR-29 regulates expression of extracellular matrix (ECM) proteins.

In culture, HCV infection down-regulated miR-29 and miR-29 over-expression reduced HCV RNA abundance. miR-29 also appears to play a role in HSCs. Hepatocytes and HSCs contribute similar amounts of miR-29 to bulk liver. Primary HSC activation and TGF-β treatment of immortalized HSCs both down-regulated miR-29. miR-29 over-expression in LX-2 cells decreased proliferation and collagen expression. miR-29 down-regulation by HCV may de-repress ECM synthesis during HSC activation.

**Conclusions.** HCV infection down-regulates miR-29 in hepatocytes and may potenti ate collagen synthesis by reducing miR-29 levels in activated HSCs. Treatment with miR-29 mimics *in vivo* might inhibit HCV while reducing fibrosis.

**Key words:** Liver Fibrosis, Pathway Analysis, Hepatitis C, microRNA, Hepatic Stellate Cells, Extracellular Matrix
INTRODUCTION

Hepatitis C virus (HCV) is an RNA virus infecting 170 million people worldwide. Persistent infection causes fibrosis, cirrhosis, end-stage liver disease and ultimately liver cancer in up to 30% of patients. Fibrotic progression is highly variable, and patient prognosis correlates with fibrotic stage. Fibrosis is morphologically characterized by increased deposition of extracellular matrix (ECM) proteins, including collagen types I/III, fibronectin and laminin. Transforming growth factor-β (TGF-β), released in response to HCV-mediated damage, activates hepatic stellate cells (HSCs) and strongly up-regulates ECM protein production. Mechanisms regulating ECM gene expression in activated HSCs are of interest as potential therapeutic targets. Novel markers for fibrosis progression would also allow early treatment of rapid progressors.

MicroRNAs (miRNAs) are ~22 nucleotide single-stranded non-coding RNAs (guide strands) that silence endogenous mRNA transcripts. Complementarity between miRNAs and the 3' untranslated regions (UTRs) of mRNAs directs gene silencing by stimulation of target mRNA degradation and/or translational repression. Individual mRNAs can be targeted by multiple miRNAs, and individual miRNAs often have hundreds of mRNA targets. The “seed sequence” (nucleotides two through seven) of the miRNA is especially important for target site recognition, and miRNAs that share the same seed sequence are grouped into families [1, 2]. Target mRNAs containing complementary “seed matches” as short as six nucleotides in their 3' UTRs can be targeted for repression [3, 4]. Algorithms such as TargetScan (www.TargetScan.org) use seed matches in conjunction with additional sequence features as well as conservation to predict possible mRNA targets of miRNAs [1, 2].
HCV interacts with the miRNA machinery [reviewed in [5]]. The most abundant liver miRNA, miR-122, binds the HCV 5' UTR, which surprisingly leads to increased HCV RNA abundance [6]. Other miRNAs (miR-122, miR-199a*, miR-196, miR-296, miR-351, miR-431 and miR-448) can also regulate HCV genomic RNA abundance [7-10].

In this study, we used Multiplex RT and TaqMan miRNA Assays (Life Technologies) to profile 346 miRNAs by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) in control (uninfected) human liver and liver biopsies from patients with chronic HCV infection (C-HCV). Interestingly, levels of numerous miRNAs were dysregulated in C-HCV relative to uninfected controls. We utilized a novel strategy called Pathway Analysis of Conserved Targets (PACT) to conduct a pathway analysis of predicted targets with a 50% statistical chance of having a conserved miRNA/target interaction (see Materials and Methods). We identified three pathways associated with down-regulated miRNA families. Based on the pathway analysis and miRNA profiling data, we selected the mir-29 family, which consists of mir-29a, mir-29b and mir-29c, for further analysis. Hereafter, we will either indicate the individual miR-29 members or refer to the miR-29 family as miR-29.

Our measurements of miR-29 in purified primary rat hepatocytes and HSCs indicated that both of these cell types contribute roughly equivalent levels of miR-29 to bulk liver. We thus studied the role of miR-29 during HCV infection in hepatocytes and during HSC activation. This study expands the repertoire of miRNAs involved in the HCV lifecycle.
MATERIALS AND METHODS

Materials and Methods contained in Supplemental Web Materials.

RESULTS

C-HCV miRNA expression profiling

We analyzed the expression of 346 miRNAs in liver samples from 22 C-HCV patients and four uninfected controls using multiplex stem-loop RT primers and miRNA qRT-PCR. Patient data and miRNA levels are listed in Supplementary Tables 1 and 2, respectively. All patients gave informed consent. After normalizing expression values, grouping miRNAs with shared seed regions and filtering for broadly-conserved miRNA families [12], we selected miRNA families with ~1.4-fold change between HCV patients and the mean of the controls. We filtered for highly expressed miRNA families (top 50% of expression in controls) with consistent expression patterns (up- or down-regulated in >9 C-HCV patients). These relatively strict criteria yielded one consistently up-regulated miRNA family (miR-150) and 11 consistently down-regulated families (Table 2).

Functional roles of differentially expressed miRNA families

To identify potential roles for the down-regulated miRNA families, we developed a general algorithm called PACT (Pathway Analysis of Conserved Targets) to determine functional categories enriched in targets of co-expressed miRNAs. Recently, we (R. C. F. and C. B. B.) refined the sensitivity and statistical power of TargetScan prediction sufficiently to rank individual targets using a metric called the probability of conserved targeting ($P_{CT}$). The $P_{CT}$ controls for seed match type, dinucleotide content and local background conservation. For the set
of down-regulated miRNA families, we pooled all predicted target mRNAs with a $P_{CT}$ of at least 0.5, which corresponds to at least a 50% chance of the miRNA/mRNA target relationship being evolutionarily conserved. To correct for the biases caused by selecting predicted targets based on conservation, we generated 20 simulation cohorts of mock miRNAs, selecting mock targets using the same criteria as the true predicted targets (see Supplemental Materials). These background sets were used to estimate the number of predicted miRNA targets found in any particular pathway by chance. We ranked pathways using the Group Enrichment $p$-value, which is the probability that the given set of miRNAs (e.g., down-regulated miRNAs) have more conserved targets in a pathway than expected by chance, controlling for relevant sources of bias in the $P_{CT}$ where enrichment is the tendency of a set of miRNAs to target a particular biological pathway more than expected at random. Intriguingly, the predicted targets of down-regulated miRNAs were highly enriched in ECM-receptor interaction ($p=0.0046$) and focal adhesion pathways ($p=0.017$) (Table 1, Table 2). We next tested whether these enrichments could be due to a general propensity for miRNAs to target these pathways. We calculated a Random Enrichment $p$-value by repeating the analysis with randomly selected sets of actual miRNAs. We found that the enrichment for the ECM-receptor interaction and focal adhesion pathways was specific to the set of down-regulated miRNA families in Table 2 ($p=0.0031$ and $p=0.022$, respectively).

The focal adhesion category is composed mainly of integrins, laminins, platelet derived growth factor (PDGF) and phosphatidylinositol 3 kinase family (PI3K) members. Laminins mediate ECM attachment, and integrins mediate signaling between the ECM and cell signaling pathways. PDGF is a powerful HSC mitogen whose signaling is mediated by PI3K. The ECM-receptor
interaction category is overwhelmingly composed of ECM components, integrins and laminins. Thus, HCV infection is associated with a miRNA expression pattern in human liver that de-represses cellular adhesion and ECM genes, which could contribute to C-HCV-mediated fibrogenesis.

**Down-regulation of miR-29 in HCV-infected patients**

Among the down-regulated miRNA families, miR-29 stood out in several respects: For each miRNA family and significant pathway, we calculated an Individual Enrichment \( p \)-value representing the probability of a specific miRNA family having more conserved targets in the pathway than expected by chance. The two most significant pairs were miR-29 targeting ECM-receptor interactions and focal adhesion pathways (Individual Enrichment \( p=1 \times 10^{-32} \) and \( 2 \times 10^{-7} \), respectively). miR-29 also had the largest number of predicted targets in the three significant pathways (Table 2), all of which are relevant to HCV pathogenesis. Lastly, all three miR-29 family members were down-regulated in almost all C-HCV-infected patients (Figure 1A). Therefore, we chose to further analyze miR-29’s role in HCV pathogenesis.

Individual TaqMan miRNA assays (Figure 1B) validated the multiplex data (Figure 1A) showing miR-29 down-regulation of approximately two-fold in the majority of C-HCV samples irrespective of fibrotic stage. Previous results suggested that miRNA changes of this magnitude could be physiologically relevant; for example, deleting one of two copies of \( \text{mir-}1 \) in mice resulted in substantially increased fatality due to heart defects [13].

**Contribution of hepatocytes and HSCs to miR-29 levels in bulk liver**
The liver is composed of many cell types that could serve as sources of miR-29 expression. Hepatocytes and HSCs are of special interest to HCV pathogenesis. Hepatocytes compose ~70% of the cells in the liver and ~78% of the liver mass. HSCs comprise ~8-14% of liver cells [14], but comprise only ~1.4% of liver mass because of their smaller size [15]. To determine the relative contribution of these two cell types to miR-29 levels in total liver, we measured miR-29 in purified primary rat hepatocytes and freshly isolated HSCs, compared to total liver (Table 3). HSCs expressed much higher levels of miR-29 than hepatocytes (~6.8- to 53-fold higher). Based on cell number or cell mass, hepatocytes outnumber HSCs by ~7-fold or ~55-fold, respectively. Using these corrections for abundance as well as our measurements of miR-29 in isolated hepatocytes and HSCs, we calculated cell number and cell mass adjusted expression ratios for comparison (Table 3, 1 = equal contribution). We conclude that both of these cellular compartments likely contribute to miR-29 levels measured in the needle biopsies from patients. We therefore addressed the role of miR-29 during HCV infection of hepatocytes in culture and during stellate cell activation.

**Down-regulation of miR-29 upon acute HCV infection in vitro**

First, we assessed the effects of HCV infection on miR-29 expression in hepatocytes using an HCV infectious cell culture model (HCVcc). HCVcc infects, replicates and produces infectious virus in Huh7.5 hepatoma cells [16]. Huh7.5 cells were infected with HCVcc, and ~40% and ~100% of cells were infected at 48 and 96 hours, respectively (data not shown). In agreement with recent reports [17-19], HCV infection of Huh7.5 cells down-regulated miR-29 by a factor similar to that observed in C-HCV patients (Figures 1C-E). Thus, miR-29 down-regulation in the livers of C-HCV patients may be, at least in part, mediated by HCV infection of hepatocytes.
Modulation of HCV RNA levels by miR-29

To assess whether miR-29 down-regulation is advantageous to the virus, we determined the effects of miR-29 over-expression on levels of HCV genomic RNA in Huh7.5 cells after HCVcc infection. Interestingly, miR-29 over-expression resulted in a ~3-fold decrease in HCV RNA (Figure 1F), indicating an anti-viral function. MiR-29 over-expression did not cause significant cytotoxicity (Figure 1G). These data expand the small repertoire of miRNAs known to regulate HCV [8-11] and raise the intriguing possibility that HCV may down-regulate miR-29 in order to enhance its fitness.

miR-29 down-regulation during HSC activation

As shown above, freshly isolated HSC express high levels of miR-29 family members, which target multiple transcripts relevant to HSC activation and fibrogenesis. We activated HSCs by plating them on plastic [14] and measured miR-29 expression. MiR-29 was rapidly and dramatically down-regulated upon primary rat HSC activation (Figures 2A-C). Thus, miR-29 down-regulation appears to be a consistent feature of early stellate cell activation. Up-regulated TGF-β by HCV is a primary inducer of HSC activation, so we assessed whether or not TGF-β modulates miR-29 expression in HSCs. Indeed, following TGF-β treatment of LX-2 immortalized human HSCs, miR-29 was down-regulated (Fig. 3A). In contrast, TGF-β stimulation of hepatocytes (Huh7.5 cells) did not alter miR-29 levels (Figure 3B). These results suggest that TGF-β signaling may be one mechanism by which miR-29 is down-regulated during C-HCV infection.
miR-29 over-expression alters HSC proliferation and ECM production

We next examined the effects of miR-29 restoration on features of the activated LX-2 phenotype by transfection of LX-2 cells with miR-29 mimics or negative control mimics. Transfection with Cy3-labeled siRNA indicated >90% transfection efficiency of LX-2 cells (data not shown). Restoration of miR-29 resulted in a modest (~20%) but statistically significant reduction in proliferation as determined by two separate assays (Figures 4A and B). Venugopal et al. observed similar decreases in HSC proliferation upon over-expression of miR-150 and miR-194 in LX-2 cells (28). miR-29 targets numerous collagen genes [20-22], including COL1A1 and COL3A1 but not the activation marker α-SMA. Consistent with these targeting relationships, miR-29 over-expression resulted in profound suppression of COL1A1 and COL3A1 expression in LX-2 cells with no change in α-SMA expression as determined by qRT-PCR (Fig. 4C). These results were not due to cell death, as neither cytotoxicity nor apoptosis were increased by miR-29 over-expression (Figures 4D and E, respectively). Thus, miR-29 over-expression alters two key features of HSC activation – proliferation and collagen expression – but not α-SMA expression.

DISCUSSION

miRNAs are known to play important roles in HCV infection and associated pathological processes. Using a quantitative miRNA profiling approach, we observed that miR-29 was the most consistently dysregulated miRNA in C-HCV-infected patient samples. Measurements of miR-29 in purified primary cell populations relevant to HCV pathogenesis indicated that both hepatocytes and HSCs contributed roughly equally to miR-29 expression in bulk liver. Together, our results identify miR-29 as a potential new HSC activation marker and as a possible anti-HCV and anti-fibrotic therapeutic target.
Pathway analysis can identify biologically relevant signaling pathways coordinately regulated by miRNAs. Two groups previously used miRNA profiling in conjunction with pathway analysis to assess functional relationships in the context of HCV infection in humans. These studies relied on the fact that miRNA targeting reduces target mRNA stability. Ura et al. and Peng et al. measured miRNA and mRNA levels in HCV-infected and normal tissues and identified pathways associated with predicted target mRNAs [18, 23]. Ura et al. predicted cell adhesion, cell cycle, protein folding and apoptosis pathways. Peng and colleagues used inverse expression between miRNAs and mRNAs to define targets as well as a graph theoretic approach to define miRNA/mRNA modules. They identified 38 miRNA/mRNA regulatory modules that were HCV-associated. Both of these approaches utilized inverse miRNA/mRNA relationships, which can be a powerful tool for uncovering biological relationships, although it does not necessarily identify direct targets. We chose a complementary pathway analysis approach that differed in several fundamental ways from these previous studies.

First, pathway analyses relying on miRNA/mRNA inverse relationships only interrogate interactions resulting in mRNA changes measurable by microarray. Given that miRNAs typically have small effects on individual target mRNAs (<25% change and often much less) [24], many bona fide miRNA/mRNA interactions may not be observable by inverse expression, i.e., the small direct effects are easily masked by microarray noise or other biological effects. Also, we (R. C. F. and C. B. B.) recently showed that 6-mer seed matches, which produce marginal mRNA abundance changes, were maintained in 3' UTRs in numbers similar to highly effective sites, such as 8-mer seed matches [12]. This observation suggests that many marginally effective
sites are nevertheless important enough to be maintained by natural selection. Thus, we analyzed our miRNA expression data using an approach that was independent of inverse miRNA/mRNA relationships. Second, we predicted targets using the $P_{CT}$ conservation metric [12], which scores sites based on likelihood of preferential conservation over evolutionary time, specifically due to miRNA targeting. By using highly conserved seed matches to predict targets rather than inverse mRNA correlations, we select a relatively small number of targets that have been preserved through millions of years of evolution and may provide a clearer opportunity for biological interpretability. Although species-specific targets cannot be identified using conservation, we can confidently detect targeting relationships of longer-term importance and hence identify pathways involved in miRNA function. Third, we proceed directly from predicted targets to pathway analysis without imposing a requirement on the network structure. Peng et al. explicitly selected bicliques, i.e., completely connected sets of miRNAs and mRNAs; this could preclude detection of regulatory subnetworks in which in aggregate (although perhaps not individually) a set of miRNAs targets many, but not all, members of a functional pathway. Finally, we rigorously controlled for biases in target prediction by explicitly controlling for UTR conservation, seed match number and type, seed match conservation, number of conserved targets specific to the particular miRNA family and implicitly controlling for UTR length and nucleotide composition [12]. Importantly, target predictions that select for genes with seed matches and expression changes select for biased sets of genes with long UTRs (which have more miRNA seed matches by chance) and particular expression levels in the tissue of interest that are more readily detectable by array analysis and therefore render hypergeometric tests for function statistically invalid. Importantly, miRNA pathway analyses have not, to date, accounted for this critical bias. Taken together, our complementary approach was designed to find a small
set of confident targets comprising conserved miRNA-pathway interactions in a rigorously controlled manner, leading to a clear biological interpretation and suggesting follow-up experiments.

Down-regulated miRNA families were significantly associated with focal adhesion and ECM receptor interaction pathways (Tables 1 and 2); this is intriguing, since these pathways couple intracellular signaling pathways with the ECM. Excess ECM deposition is central to the pathogenesis of HCV. The miR-29 family had the highest enrichment for these pathways with the most significant Individual Enrichment p-value for any pathway of the up- or down-regulated miRNA families \( p=1 \times 10^{-32} \). Previous reports in other organs implicated miR-29 in numerous processes of potential relevance to C-HCV pathogenesis. miR-29 was previously shown to target ECM components [20-22]. Van Rooji and coworkers recently showed that miR-29 was down-regulated during cardiac fibrosis [20]. Two other reports showed that miR-29b regulates collagen production during osteoblast differentiation [22] and in immortalized LX-2 stellate cells [25]. Down-regulated miRNAs were also associated with a cancer-related pathway (Tables 1 and 2). miR-29 is dysregulated in a variety of cancers and targets important mediators of metastasis [reviewed in [26]]. C-HCV can lead to fibrosis and hepatocellular carcinoma (HCC). Indeed, miR-29 is down-regulated in HCC [19] and is negatively correlated with patient survival [27].

Our data support a role for HCV in down-regulating miR-29 in hepatocytes. C-HCV and HCVcc infection in culture resulted in roughly similar miR-29 reductions. miR-29 was previously shown to have antiviral activity against human immunodeficiency virus [28, 29], raising the possibility that HCV might down-regulate miR-29 to its advantage. Remarkably, miR-29 over-expression
decreased HCVcc genomic RNA abundance. Thus, miR-29 can be deleterious to HCV, providing a possible motivation for the virus to down-regulate miR-29 to enhance viral fitness. Several imperfect binding sites for miR-29 can be found in coding regions of the HCV genome. However, bulges in the central portion of putative miR-29/HCV duplexes make cleavage of the HCV genome by the RISC complex unlikely (data not shown).

In agreement with our observation, recent studies have established a role for miR-29 and other miRNAs in fibrogenesis of various organs, including heart [20, 30, 31], kidney [21, 32, 33], bone [22] and lung [34]; however, the role of miRNAs in liver fibrosis is only beginning to be explored [reviewed in [35]]. HSC activation by enhanced TGF-beta and subsequent fibrosis is a major consequence of C-HCV infection. Our pathway analysis pointed to a role for miR-29 in HSCs. A role for miRNAs during HSC activation is not without precedent. Several studies have implicated miRNAs in regulation of HSC activation, proliferation and apoptosis [36-39]. We observe up-regulation of miR-150 during C-HCV; this is interesting, since miR-150 was reported to negatively regulate HSC activation [40]. We also observed profound suppression of miR-29 levels upon HSC activation (Figures 2A-C).

Major aspects of HSC activation include cytoskeletal rearrangements (e.g., α-SMA expression and stress fiber formation), increased proliferation and increased ECM production (e.g., COL1A1 and COL3A1). Venugopal et al. showed that miR-150 and miR-194 were reduced in bile duct-ligated rats, and over-expression of these miRNAs in LX-2 cells reduced proliferation (by ~25%), increased apoptosis and reduced α-SMA and collagen I levels [40]. Another report showed that miR-27 was up-regulated upon cell culture activation of rat HSCs [39]. miR-27
inhibition increased lipid droplet formation and decreased HSC proliferation. In contrast to Venugopal’s results, miR-27 inhibition did not alter type I collagen or α-SMA expression. These published results demonstrate that miRNAs can influence global stellate cell activation generally or affect only subsets of stellate cell activation markers.

Restoration of miR-29 expression in LX-2 HSCs reversed some but not all markers of HSC activation. miR-29 over-expression did not alter α-SMA levels. In contrast, while miR-29 over-expression had a modest effect on proliferation (Figures 4A, 4B), miR-29 over-expression dramatically reduced COL1A1 and COL3A1 expression (Figure 4C). These data were not surprising, since COL1A1, COL3A1 and most other collagens expressed by HSCs are known miR-29 targets [references [20, 22] and Table 2]. Recent studies [ ] also support independent expression of collagen and SMA in stellate cells.

TGF-β secreted by hepatocytes, Kupffer cells and sinusoidal endothelial cells [41, 42] causes HSCs to activate, transdifferentiate and secrete ECM [42]. TGF-β has been shown to down-regulate miR-29 expression in cardiac fibroblasts [20]. Here, we show that TGF-β treatment of cultured HSCs resulted in miR-29 down-regulation; this effect appears to be specific to HSCs, since TGF-β treatment of Huh-7.5 cells did not down-regulate miR-29. Recently, Roderburg et. al. [ ] reported TGF-beta mediated down-regulation of miR-29 in HSCs. Although it remains to be determined whether TGF-β is the sole factor leading to down-regulation of miR-29 in HSCs of C-HCV patients, these data raise the possibility that TGF-β-mediated suppression of miR-29 is a common feature of fibrotic liver injury. If so, miR-29 may be an important therapeutic target in chronic liver disease.
There is significant interest in pharmacological manipulation of miRNAs in humans. Van Rooji and coworkers showed that inhibition of miR-29 in mice caused an up-regulation of collagen expression in liver [20]. Since fibrosis is a balance between ECM deposition and ECM degradation, miR-29-mediated suppression of ECM synthesis in HSCs could hopefully tip this balance toward reduced fibrosis in vivo. Our results clearly support further studies in animal models of HCV and fibrosis to determine the therapeutic utility of treatment with miR-29 mimics or small molecule drugs regulating miR-29 expression; this could have the dual benefit of inhibiting HCV while decreasing fibrosis. Notably, inhibition of miR-122 in primates has been shown to be safe [43], and clinical trials are currently underway to determine whether modulation of miR-122 levels in humans inhibits HCV.

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FIGURE LEGENDS

Figure 1. Down-regulation of miR-29 in C-HCV. Fold change in mature miR-29 levels relative to control uninfected livers. (A) miR-29 levels in C-HCV patients measured by multiplex qRT TaqMan miRNA profiling. (B) Individual qRT-PCR validated miR-29 down-regulation in HCV-infected needle biopsies (Pearson’s correlation between A and B: miR-29a, r=0.653 p=0.0010; miR-29b, r=0.623 p=0.0019; miR-29c, r=0.752 p<0.0001). Mean ± standard error of the mean (s.e.m.); three independent measurements. (C-G) HCVcc infection reduces miR-29,
and miR-29 inhibits HCV. Fold change of (C) miR-29a, (D) miR-29b and (E) miR-29c in HCVcc-infected cells relative to mock-infected cells. Average of two independent experiments in triplicate. Mean ± s.e.m. (F) Huh7.5 cells were treated with miR-29 mimic or negative control mimic (control-pre-miR) and infected with HCVcc. Fold change in HCV RNA relative to control-pre-miR. Representative experiment from three independent experiments in triplicate. Mean ± s.e.m. (G) Cytotox-Fluor cytotoxicity assay. Positive control, 30 µg/ml digitonin. Average of three independent experiments in triplicate. Mean ± s.e.m. * indicates a p-value < 0.05 for all panels.

**Figure 2. miR-29 down-regulation in activated HSCs.** Primary rat HSCs were isolated and activated in culture by plating on plastic. Data from three independent HSC isolations are shown (Time courses # 1, 2 and 3). Levels of (A) miR-29a, (B) miR-29b and (C) miR-29c.

**Figure 3. miR-29 down-regulation in TGF-β-treated HSCs but not in hepatocytes.** miR-29 levels in (A) LX-2 HSC cell line or (B) Huh7.5 cells treated with TGF-β (10 ng/ml) for 24 hours. Representative experiment from two independent experiments in quadruplicate. Mean ± s.e.m. * indicates a p-value < 0.05 for all panels.

**Figure 4. miR-29 overexpression in LX-2 cells inhibits proliferation and collagen expression.** LX-2 cells were transfected with 25 nM miR-29 mimic or negative control mimic (control-pre-miR). (A) WST-1 cell proliferation assay. Average of three independent experiments in quadruplicates. (B) Ki67 mRNA levels. (C) Col1A1, Col3A1 and α-SMA mRNA levels. Representative experiment from three independent experiments in triplicate. Mean ±
s.e.m. (D) Cytotox-Fluor cytotoxicity assay. Positive control, 30 µg/ml digitonin (E) Caspase-glow 3/7 apoptosis assay. Positive control, 2 µg/ml doxorubicin. Average of three independent experiments in quadruplicate. Mean ± s.e.m. * indicates a p-value < 0.05 for all panels.
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