Micro-RNA Profiling Reveals a Role for miR-29 in Human and Murine Liver Fibrosis

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Liver fibrosis is orchestrated by a complex network of signaling pathways regulating the deposition of extracellular matrix proteins during fibrogenesis. MicroRNAs (miRNAs) represent a family of small noncoding RNAs controlling translation and transcription of many genes. Recently, miRNAs have been suggested to crucially modulate cellular processes in the liver such as hepatocarcinogenesis. However, their role in liver fibrosis is not well understood. We systematically analyzed the regulation of miRNAs in a mouse model of carbon tetrachloride–induced hepatic fibrogenesis (CCl_4) by gene array analysis, which revealed a panel of miRNA that were specifically regulated in livers of mice undergoing hepatic fibrosis. Within those, all three members of the miR-29-family were significantly down-regulated in livers of CCl_4-treated mice as well as in mice that underwent bile duct ligation. Specific regulation of miR-29 members in murine fibrosis models correlated with lower expression of miR-29 in livers from patients with advanced liver fibrosis. Moreover, patients with advanced liver cirrhosis showed significantly lower levels of miR-29a in their serum when compared with healthy controls or patients with early fibrosis. On a cellular level, down-regulation of miR-29 in murine hepatic stellate cells (HSCs) was mediated by transforming growth factor beta (TGF-β) as well as inflammatory signals, namely, lipopolysaccharide (LPS) and nuclear factor kappa B (NF-κB). Furthermore, overexpression of miR-29b in murine HSC resulted in down-regulation of collagen expression. *Conclusion:* Our data indicate that miR-29 mediates the regulation of liver fibrosis and is part of a signaling nexus involving TGF-β- and NF-κB–dependent down-regulation of miR-29 family members in HSC with subsequent up-regulation of extracellular matrix genes. Thus they may represent targets for novel therapeutic strategies against hepatic fibrogenesis and also might evolve as biomarkers in the diagnosis of liver fibrosis. (HEPATOLOGY 2011;53:209-218)

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The principal cells responsible for promoting the accumulation of extracellular matrix proteins during hepatic fibrogenesis are activated hepatic stellate cells (HSC). Although the cytokine transforming growth factor beta (TGF-β) has been shown to be a key regulator of this process, a variety of other cytokines and their downstream signaling pathways also have been identified as crucial actors in the context of fibrotic liver disease.[1] MicroRNAs (miRNAs) are small, noncoding, 21-nucleotide-long to 23-nucleotide-long RNAs that negatively regulate gene expression by base pairing with the 3-untranslated region of their target messenger RNAs (mRNAs).[2] If pairing is perfect or nearly perfect, target mRNAs are degraded (predominantly seen in plants). However, their pairing with most mammalian mRNAs is imperfect, resulting

Abbreviations: CCl_4, carbon tetrachloride; GRX-HSC, immortalized murine hepatic stellate cells; HSC, hepatic stellate cells; LPS, lipopolysaccharide; miRNA, microRNA; mRNA, messenger RNA; NF-κB, nuclear factor-κB; qPCR, quantitative polymerase chain reaction; TGF-β, transforming growth factor-β; TNF, tumor necrosis factor.

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in translational repression.[3] In the last years, the number of known miRNAs has grown exponentially, and currently more than 1000 miRNAs are known to be encoded by the human genome.[4] Recently, an involvement of miRNAs was demonstrated in highly regulated processes such as hepatocyte apoptosis and hepatocarcinogenesis.[5,6] Furthermore, expression of miR-122 correlates with response to interferon treatment of patients infected with hepatitis C virus.[7] However, the involvement of miRNAs in the development of liver fibrosis remains to be determined.

Here, we demonstrate that several miRNAs are specifically regulated in mouse models of liver fibrosis. Among those, the miR-29 family members showed a significant down-regulation in livers of mice developing liver fibrosis as well as in livers from patients with advanced hepatic fibrosis. We show that murine miR-29b inhibits the expression of collagen in HSCs and is down-regulated during the activation of HSCs in a TGF-β and lipopolysaccharide (LPS)/nuclear factor kappa B (NF-κB)–dependent manner. Finally, we confirm that the specific regulation of miR-29 family members in livers of fibrosis patients correlates with down-regulation of miR-29a in the serum of fibrosis patients, suggesting that miR-29 might not only be a candidate for novel treatment strategies but also might have potential as a biomarker to monitor liver fibrosis in humans.

Material and Methods

Array Analysis. Total RNA (3 μg) was labeled and hybridized to the array-system miCHIP as previously described.[8] MiCHIP is based on Tm- normalized capture probes (miRCURY; Exiqon, Copenhagen, Denmark). The miRCURY probes spotted on these arrays were designed to target approximately 500 (miRBase v9.2) unique mouse miRNAs. Array images were generated by using the Genepix 4200AL laser scanner (Molecular Devices, Sunnyvale, CA), miCHIP arrays were scanned in batches using the Genepix auto Photo Multiplayer algorithm, with pixel saturation tolerance set to 0.2%. Tiff images generated by the Genepix 4200AL laser scanner were processed by the Genepix 6 microarray analysis software (Molecular Devices). Gene array analysis was performed according to the manufacturer's instructions (GeneChip 430 2.0 and 230 2.0; Affymetrix, Santa Clara, CA), followed by data analysis as described.[9] Primers for quantitative polymerase chain reaction (qPCR) validation of array analysis are summarized in Supporting Table 1.

Further description of Materials and Methods is included within the supporting data set (Supporting Materials and Methods)

Results

In Vivo Regulation of miRNAs in a Mouse Model of Liver Fibrosis. In a systematic approach to identify miRNAs involved in liver fibrosis, we applied the well-established model of carbon tetrachloride (CCl_4) treatment for hepatic fibrogenesis in mice (Supporting Fig. 1A-C). We compared miRNA expression profiles in fibrotic livers from mice treated for 6 weeks with CCl_4 to expression profiles in livers from control mice by performing microarray analysis on RNA extracts from these livers. MicroRNAs were considered as differentially expressed when differences in expression levels were significant both in unpaired Student t test ($P < 0.01$) and significance analysis of microarray test (q value $<5\%$). Among the individual miRNAs represented on the microarray, 31 miRNAs were differentially regulated on induction of liver fibrosis (Fig. 1A). As shown in Fig. 1B, 10 miRNAs were significantly overexpressed in fibrotic livers, whereas 21 miRNAs showed a significantly lower expression when compared with control animals. The regulation of exemplary miRNAs identified in the array analysis was confirmed by qPCR. As shown in Fig. 1C, up-regulation of miRNAs miR-125-5p, miR-199b*, miR-221, and miR-302c as well as down-regulation of miR-29 family members could be confirmed in this analysis. Thus, by applying a systematic array approach, we identified subsets of miRNAs that are differentially regulated during CCl_4-induced liver fibrosis.

miR-29 Family Members Are Down-regulated in Mouse Models of Liver Fibrosis and in Human Fibrotic Livers. Among the miRNAs that were shown to be differentially regulated during hepatic fibrogenesis, miR-29 family members showed a striking relationship to numerous genes encoding for collagen and other extracellular matrix proteins on an *in-silico* analysis on potential targets (Supporting Table 3). After 6

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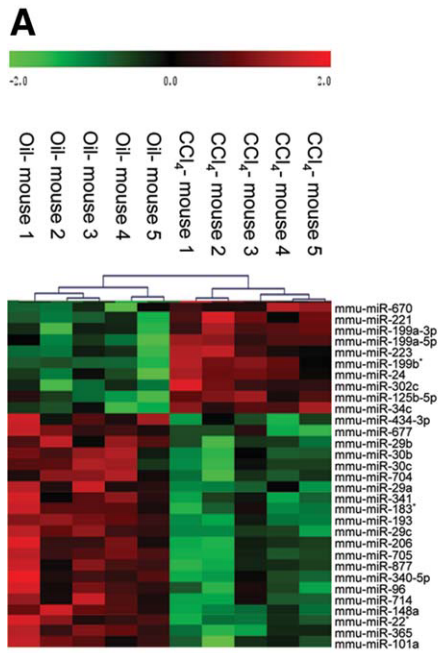

B Log_2 (CCl4/ Oil) Ratio of Differentially Expressed miRNAs That Reached Statistical Significance by T-test (P < 0.01) and Further Confirmed by SAM Test (q < 5%)

Up-regulated miRNAs (n=10)			Down-regulated miRNAs (n=21)		
miRNA	LOG_2(CCl4/Oil) ratio	P-value	miRNA	LOG_2(CCl4/Oil) ratio	P-value
miR-199a-5p	1.50	0.0043	miR-434-3p	-0.40	0.0086
miR-302c	1.23	0.0035	miR-183*	-0.45	0.0028
miR-199a-3p	1.18	0.0066	miR-22*	-0.45	0.0019
miR-223	0.88	0.0054	miR-340-5p	-0.47	0.0064
miR-199b*	0.85	0.0008	miR-206	-0.48	0.0051
miR-125b-5p	0.62	0.0067	miR-877	-0.50	0.0037
miR-221	0.61	0.0028	miR-29b	-0.51	0.0030
miR-34c	0.55	0.0029	miR-705	-0.51	0.0007
miR-24	0.43	0.0051	miR-29a	-0.52	0.0042
miR-670	0.36	0.0087	miR-714	-0.55	0.0053
			miR-341	-0.57	0.0034
			miR-29c	-0.62	0.0003
			miR-30b	-0.62	0.0040
			miR-101a	-0.62	0.0062
			miR-30c	-0.66	0.0089
			miR-365	-0.69	0.0010
			miR-677	-0.71	0.0087
			miR-96	-0.76	0.0075
			miR-148a	-0.79	0.0005
			miR-704	-0.94	0.0025
			miR-193	-1.16	0.0002

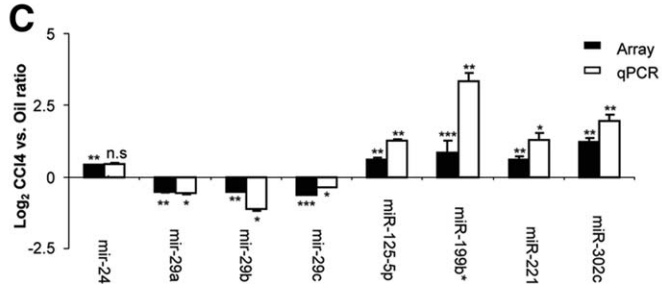

Fig. 1. Expression profiles of miRNAs on induction of liver fibrosis. (A) Microarray analysis for miRNA was performed with RNA extracts from livers of CCl_4-treated and vehicle-treated Balb/c-mice (6 weeks' treatment). Hierarchical cluster analysis of the significantly regulated miRNAs: bright green, underexpression; black, no change; bright red, overexpression. (B) Listing of differentially expressed miRNAs in the array analysis, including log_2 ratio and *P* values. (C) Differential expression of eight representative miRNAs was validated by qPCR. Results are indicated as mean of log_2 (CCl_4/oil) and are presented side-by-side with the respective array results. Error bars denote standard error of the mean. Asterixes indicate significant differences in miRNA expression between CCl_4-treated and oil-treated mice according to array or qPCR analysis, respectively. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; n = 5 per group. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

and 8 weeks of CCl_4 treatment, all miR-29 members were significantly down-regulated (Fig. 2A). Although down-regulation appeared most prominent for miR-29b, no significant differences between the individual members of the miR-29-family were detectable (Fig. 2A; data not shown). These results in Balb/c-mice were confirmed in mice with a C57BL/6 background (Fig. 2B; Supporting Fig. S2A, B). Interestingly, decreased expression of miR-29b in these animals was significantly correlated with the degree of liver fibrosis as determined by hydroxyproline assay (Fig. 2C). Furthermore, we measured expression of these miRNAs at 21 days after bile duct ligation as an additional model for liver fibrosis in mice. As seen in Fig. 2D, miR-29b/c were significantly down-regulated compared with sham-operated animals, whereas reduced expression of miR-29a did not reach statistical significance (Fig. 2D; Supporting Fig. S2C).

To correlate these findings with data from human patients, we used samples from explanted livers of patients with grade 2 to 4 liver fibrosis/cirrhosis (Desmet score) based on different disease entities (Supporting Table S2) as well as liver samples from nonfibrotic livers. Quantitative polymerase chain reaction analysis of whole miRNA extracts showed significant down-regulation of miR-29a, miR-29b, and miR-29c in fibrotic/cirrhotic compared with nonfibrotic livers (Fig. 3E). Collectively, these data demonstrate that miR-29 family members are down-regulated during liver fibrogenesis in mice independent of the genetic background, in different models of liver fibrosis as well as in human livers with advanced liver fibrosis.

miR-29 Family Members Are Expressed in Hepatic Stellate Cells and Are Down-regulated in Response to TGF-β Stimulation. Based on the regulation of miR-29 members during liver fibrogenesis, we further

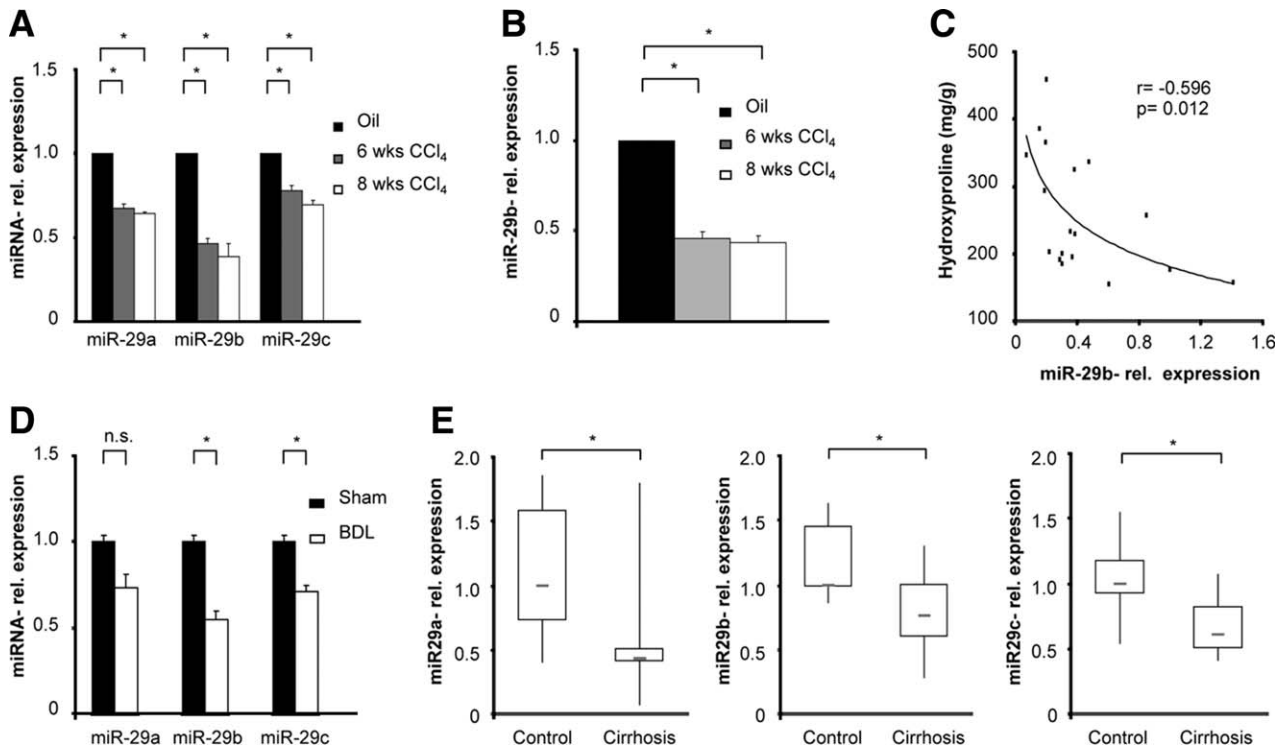

Fig. 2. Down-regulation of miR-29 during human and murine liver fibrosis. (A) Expression of miR-29 in livers from 6 and 8 weeks' CCl_4-treated or oil-treated Balb/c mice was analyzed by qPCR; n = 5 per group. (B) Expression of miR-29b in livers from 6 and 8 weeks' CCl_4-treated or oil-treated C57BL/6 mice was analyzed by qPCR; n = 4-6 mice per group. (C) Analysis of the Pearson correlation coefficient between miR-29b gene expression and collagen content in livers of CCl_4-treated and oil-treated C57BL/6-mice. (D) Expression of miR-29 in livers from C57BL/6 mice at 21 days after bile duct ligation or sham operation was analyzed by qPCR; n= 4 per group. (E) Representative hematoxylin-eosin staining of liver samples from donors without liver disease (control) and patients with liver fibrosis. (F) Determination of miR-29a/b/c expression by qPCR in livers of patients with liver fibrosis compared with healthy livers. Results are expressed as mean; error bars denote standard error of the mean. $*P < 0.05$.

characterized the mechanisms involved in this regulation. We performed qPCR analysis on RNA extracts to evaluate miR-29 expression in different mouse tissues. Comparable expression patterns for all three miR-29 family members were detected in liver, heart, spleen, and lung, whereas in brain and kidney miR-29b was expressed at higher levels compared with miR-29a/c (Supporting Fig. S3A). To characterize expression of miR-29 in different hepatic cell compartments, we isolated primary HSCs, hepatocytes, endothelial cells, and Kupffer cells from livers of control mice: miR-29b showed high expression in HSCs when compared with the other cell types, suggesting a specific function in these cells (Fig. 3A). To test whether activation of HSCs might be linked with dysregulation of miR-29 members, we cultured primary HSCs from C57BL/6 mice and measured their expression at different time points after isolation. *In vitro* activation of HSC led to a down-regulation of all miR-29-members during 8 days of culturing (Fig. 3B).

To further specify the cell-specific regulation of miR-29b during liver fibrosis *in vivo*, we isolated different hepatic cell compartments from livers of mice treated with Oil and CCl_4 for 6 weeks. In HSCs from fibrotic livers, miR-29b showed a dramatic (>2000-fold) down-regulation compared with oil treatment, correlating with up-regulation of *Col1a1* and α-*Sma* (Fig. 3C). Down-Regulation also was detected in hepatocytes, whereas Kupffer cells and LSEC interestingly showed a miR-29b up-regulation (Fig. 3D). Thus, miR-29b is differentially regulated in hepatic cell compartments, but regulation in HSC and hepatocytes mainly contributes to the overall expression in the liver.

Because TGF-β represents a major cytokine driving the activation of HSC during liver fibrosis *in vivo*, we tested whether TGF-β might regulate the expression of miR-29. Stimulation of primary murine HSC (Fig. 3E) as well as immortalized murine HSC (GRX-HSC; Supporting Fig. S3B) with recombinant TGF-β led to a significant decrease of miR-29 expression, correlating with an increase in collagen expression in these cells. Interestingly, a similar regulation was detected in primary murine hepatocytes (Supporting Fig. S3C).

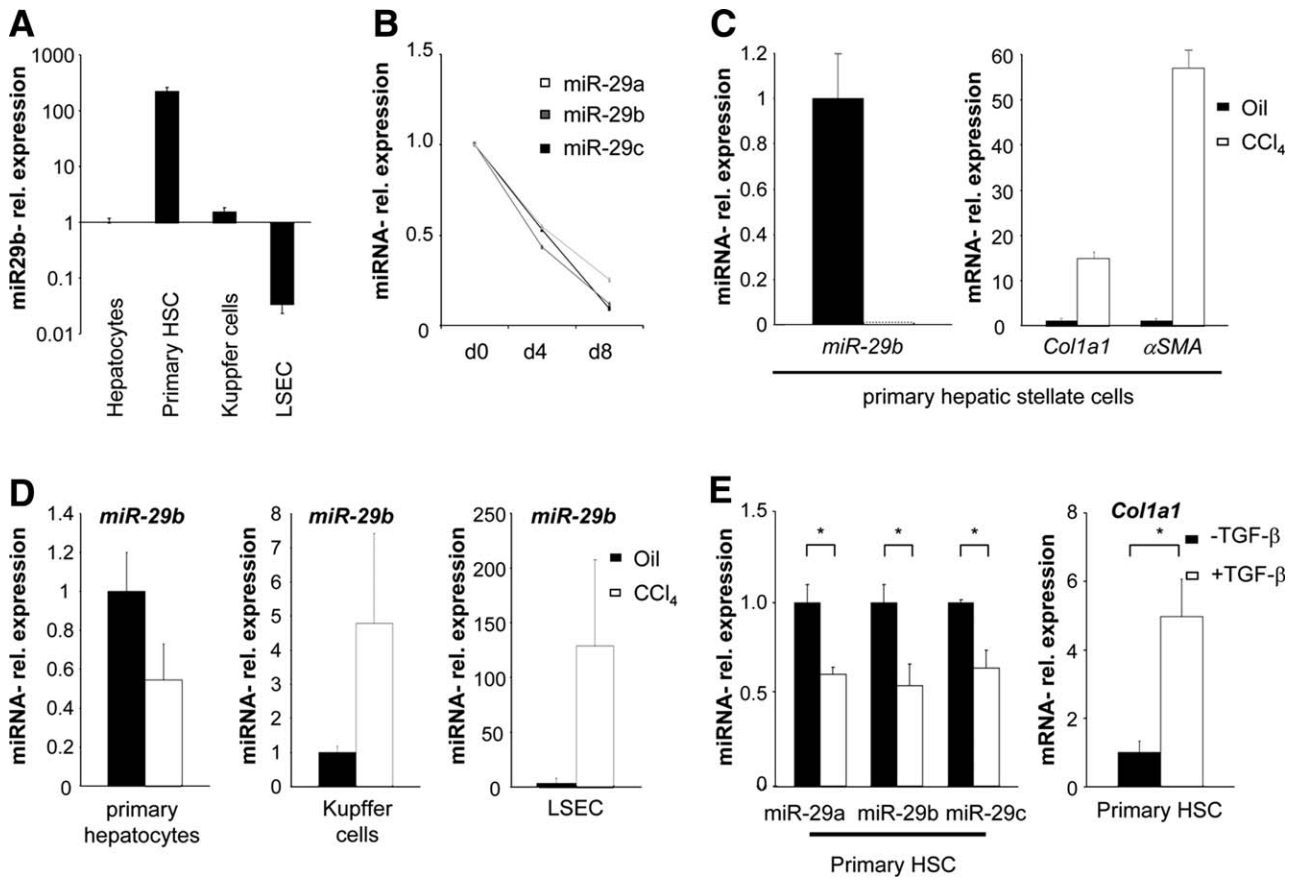

Fig. 3. The miR-29-family members are expressed in HSCs and are down-regulated during their activation and in response to TGF-β stimulation *in vitro*. (A) Hepatocytes, HSCs, Kupffer cells, and liver sinusoidal endothelial cells were isolated from livers of C57BL/6 mice, and relative miR-29 expression in comparison with hepatocytes was determined by qPCR. RNA extracts were pooled from 2-5 mice. (B) HSCs were isolated from C57BL/6 mice. The miR-29 expression during *in vitro* activation was determined by qPCR. (C) Primary HSCs were isolated by fluorescence-activated cell sorting from livers of C57BL/6 mice treated for 6 weeks with CCl_4 or oil, and expression of miR-29b, *Col1a1,* and *αSma* were determined by qPCR. HSCs were isolated from five mice, and cells were pooled immediately after isolation. (D) Hepatocytes, Kupffer cells, and liver sinusoidal endothelial cells were isolated from livers of C57BL/6 mice treated for 6 weeks with CCl_4 or oil as control and expression of miR-29b was determined by qPCR. Cells were isolated from 2-4 mice and pooled after isolation. (E) Primary HSCs were stimulated for 48 hours with TGF-β, and expression of miR-29 and *Col1a1* was measured by qPCR. Results are expressed as means; error bars denote standard error of the mean. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

miR-29 Regulates the Expression of Extracellular Matrix Genes in Hepatic Stellate Cells. Next we tested whether miR-29 members are able to modulate the expression of extracellular matrix genes during hepatic fibrogenesis. Possible miR-29 target genes were identified by three different miRNA target prediction algorithms (see Materials and Methods). We identified a high number of fibrosis-related mRNAs, including collagens, integrins, and metallopeptidases as possible targets for the miR-29 family (Supporting Table S3B). Expression of exemplary *in silico* identified targets was analyzed in liver samples from CCl_4-treated and oil-treated Balb/c mice (Fig. 4A), which confirmed up-regulation of the potential target genes *Col1a1*, *Col1a2*, *Col4a5*, and *Col5a3* on CCl_4 treatment.

We next transfected miR-29b at different concentrations into immortalized murine HSC. As shown in Fig. 4B and Fig. 4C, overexpression of miR-29b resulted in a dose-dependent and significant decrease in expression of *Col1a1*, *Col4a5,* and *Col5a3,* whereas down-regulation of *Col1a2* failed statistical significance. Transfected scrambled miRNA had no effect on the expression of the respective genes (Fig. 4B, C, and Supporting Fig. S4). Moreover, expression of other fibrosis-related genes (*Ctgf*, *Timp-1,* and *αSma*) was not affected by transfection of miR-29 (Fig. 4D and Supporting Fig. S4). Collectively, these experiments suggest a direct link between the TGF-β–dependent miR-29 down-regulation and collagen up-regulation in HSC during liver fibrosis.

LPS/NF-κB Dependent Down-regulation of miR-29 During Liver Fibrosis. Micro RNAs normally do not act in linear signaling cascades but are able to integrate signals from distinct upstream signaling

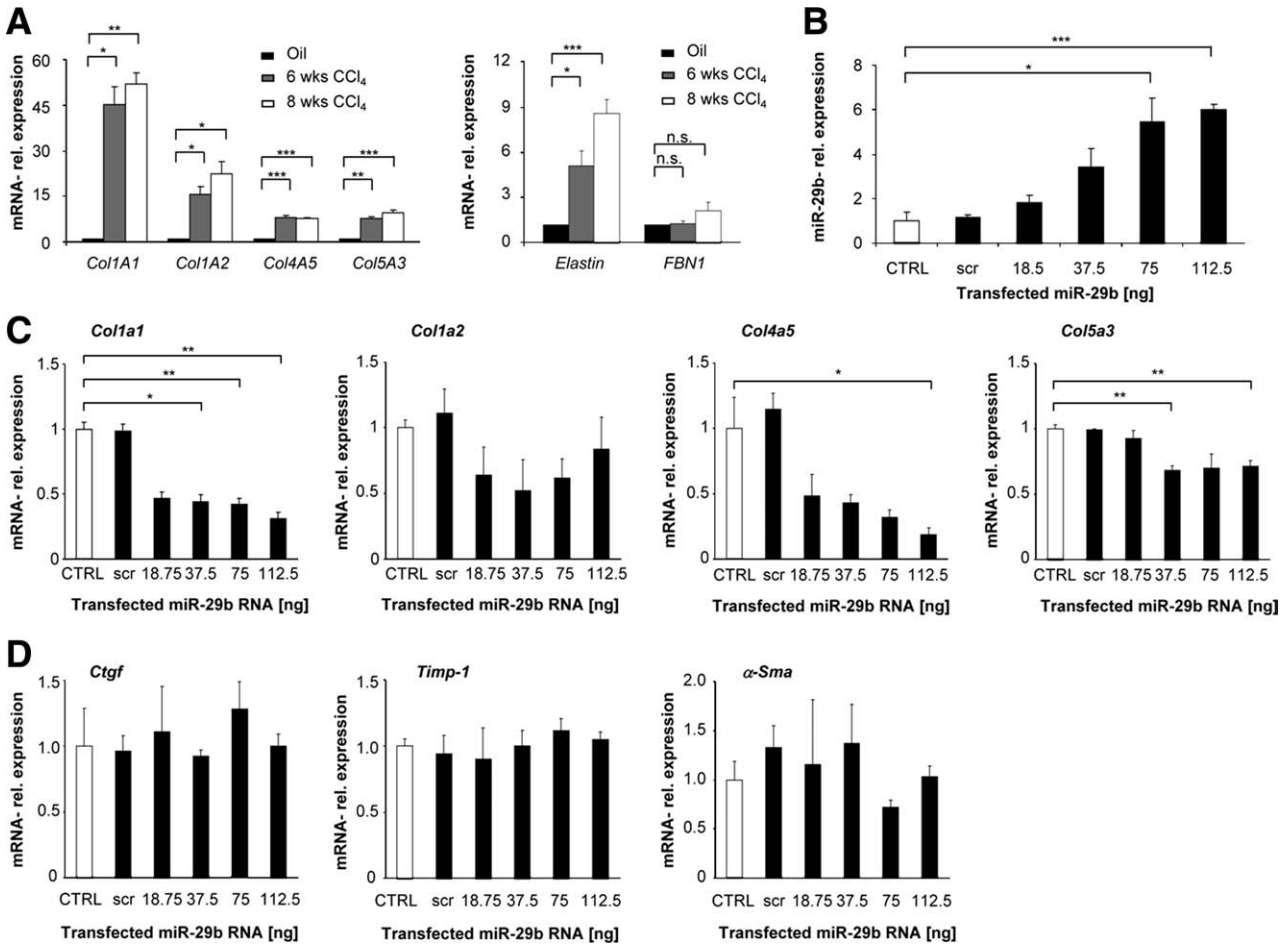

Fig. 4. The miR-29 regulates expression of extracellular matrix genes in HSCs. (A) Relative expression of miR-29 target genes in livers of Balb/c mice after treatment with oil or 6 and 8 weeks' CCl_4 was determined by qPCR. (B) GRX-HSCs were either transfected with indicated amounts of miR-29b mimic or 112.5 ng scrambled miRNA as control. Expression of miR-29b was determined by qPCR after 3 days of transfection. (C, D) GRX-HSC cells were transfected with either indicated amounts of miR-29b mimic or 112.5 ng scrambled miRNA. Expression of (C) *Col1a1*, *Col1aA2*, *Col4a5*, *Col5a3,* and (D) *Ctgf, Timp-1,* and *αSma* was measured by qPCR after 3 days of transfection. Results are expressed as means; error bars denote standard error of the mean.*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

pathways,[10] suggesting that regulation of miR-29 during liver fibrosis is not only regulated by TGF-β. Recently, it was shown that miR-29 can be regulated by the transcription factor NF-κB during myogenic cancer via recruitment of histone deacetylase 1 to the miR-29 promoter region.[11] We therefore examined a possible role of this pathway in the regulation of miR-29 in HSCs. Indeed, intraperitoneal injection of LPS into mice resulted in significant down-regulation of all miR-29 members in whole liver RNA extracts (Fig. 5A). Moreover, stimulation of primary HSC as well as primary hepatocytes with LPS resulted in down-regulation of miR-29a/b/c (Fig. 5B,C). However, LPS stimulation did not significantly enhance collagen expression in these cells (Fig. 5B). Stimulation with tumor necrosis factor (TNF) but not interleukin-1 led to a strong decrease in miR-29b expression (Fig. 5D). Finally, we treated GRX-HSCs and primary HSCs with a chemical inhibitor of NF-κB activation, pyrrolidine dithiocarbamate.[12] Although this treatment resulted in early cytotoxicity in primary HSCs (data not shown), GRX-HSCs—which were probably more resistant to NF-κB inhibition—showed a significantly higher expression of miR-29 compared with untreated cells (Fig. 5E). Collectively these data suggest that, next to TGF-β, inflammatory signaling pathways also might contribute to the regulation of miR-29 levels in HSCs.

Low Circulating Serum Levels of miR-29a Are Characteristic for Advanced Liver Fibrosis in Humans. Recently, plasma levels of miRNAs have emerged as potential biomarkers for various pathological conditions such as cancers.[13,14] We therefore hypothesized that dysregulation of members of the miR-29 family in fibrotic livers might be associated

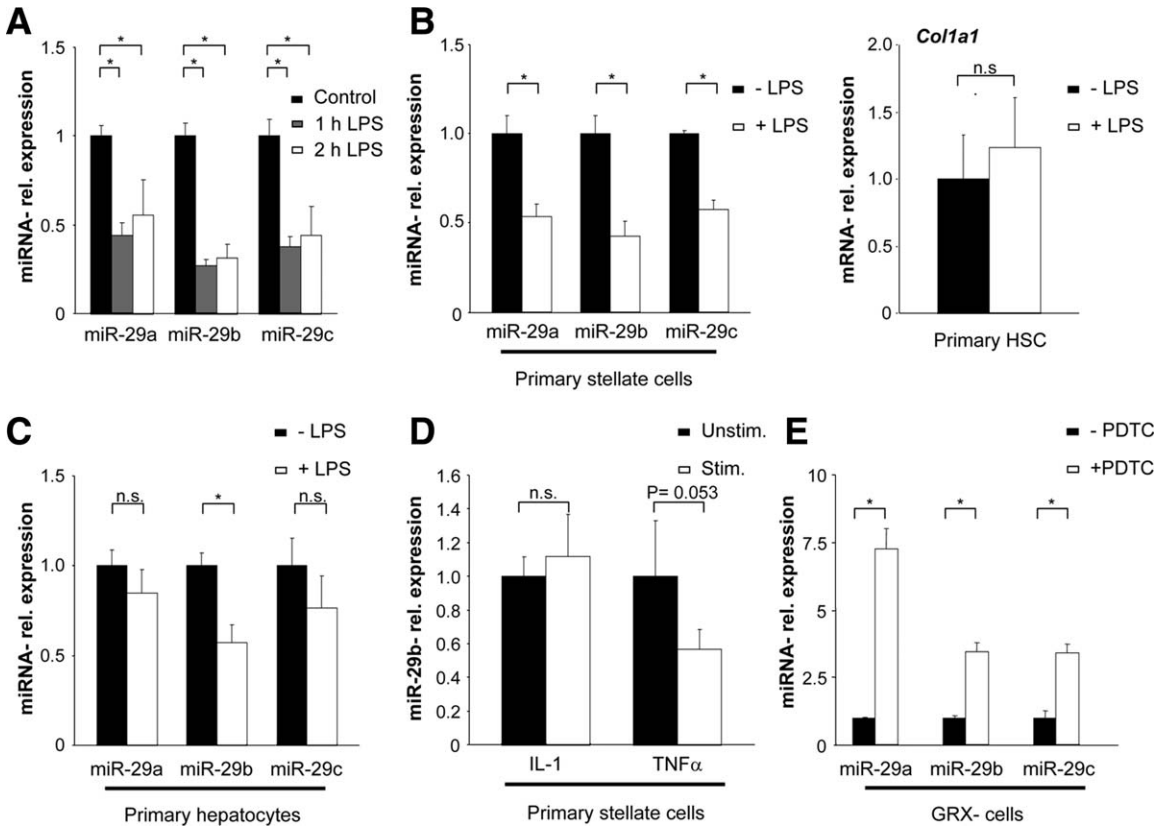

Fig. 5. LPS and NF-κB mediate down-regulation of miR-29 in HSCs. (A) Expression of miR-29 in the livers of wild-type mice on treatment with LPS was measured by qPCR; n = 3 per group. (B) Expression of miR-29 and *Col1a1* in primary HSCs in response to stimulation with LPS for 48 hours was measured by qPCR. (C) Regulation of miR-29 in primary hepatocytes was analyzed in response to stimulation with LPS for 48 hours by qPCR. (D) Induction of miR-29b in primary HSCs on treatment with TNFα or interleukin-1 for 48 hours was determined by qPCR. (E) Induction of miR-29 in GRX-HSC on treatment with the chemical NF-κB- inhibitor pyrrolidine dithiocarbamate for 24 hours was determined by qPCR. Results are expressed as means; error bars denote standard error of the mean. $*P < 0.05$.

with a significant change in miR-29 serum levels. To test this hypothesis, we isolated miRNAs from the serum of 67 patients with chronic liver disease at different stages and compared levels of miR-29a (which had shown the strongest regulation in human fibrotic livers; see Fig. 2F) in these patients to serum levels from 17 healthy volunteers. The miR-29a serum levels were significantly down-regulated in fibrosis patients compared with healthy controls (Fig. 6A). Strikingly, patients with advanced liver cirrhosis (Child stages B and C) displayed significantly lower miR-29 levels than patients with early cirrhosis (Child A, Fig. 6B). Furthermore, Model for End-Stage Liver Disease score inversely correlated with miR-29a serum level (Fig. 6C). In addition, the underlying cause of liver disease also influenced miR-29 serum levels; patients with alcoholic cirrhosis showed much stronger down-regulation of miR-29a, regardless of the Child-Pugh score of the individual patient, in comparison with patients with viral hepatitis (Fig. 6D, Supporting Fig. S5). Finally, low serum miR-29 levels predicted the presence of liver fibrosis, as shown by a c-statistic of 0.838 in receiver operating characteristic curve analysis (Fig. 6E).

Discussion

In the current study, we provided evidence for a functional role of miR-29 in murine and human liver fibrosis. Dysregulation of certain miRNAs and specifically miR-29c was previously shown in human liver specimens from patients with chronic viral hepatitis and liver fibrosis,[15,16] whereas miR-29 was not significantly dysregulated in another study that analyzed miRNA expression patterns in primary biliary cirrhosis samples.[17] These studies support our functional data on the role of miR-29 in HSC and liver fibrosis but also suggest that the regulation of miRNAs might vary with the distinct pathogeneses of liver diseases.

It has been previously suggested that the regulation and function of miRNAs is highly organ specific and cell-type specific.[18] However, because it was recently

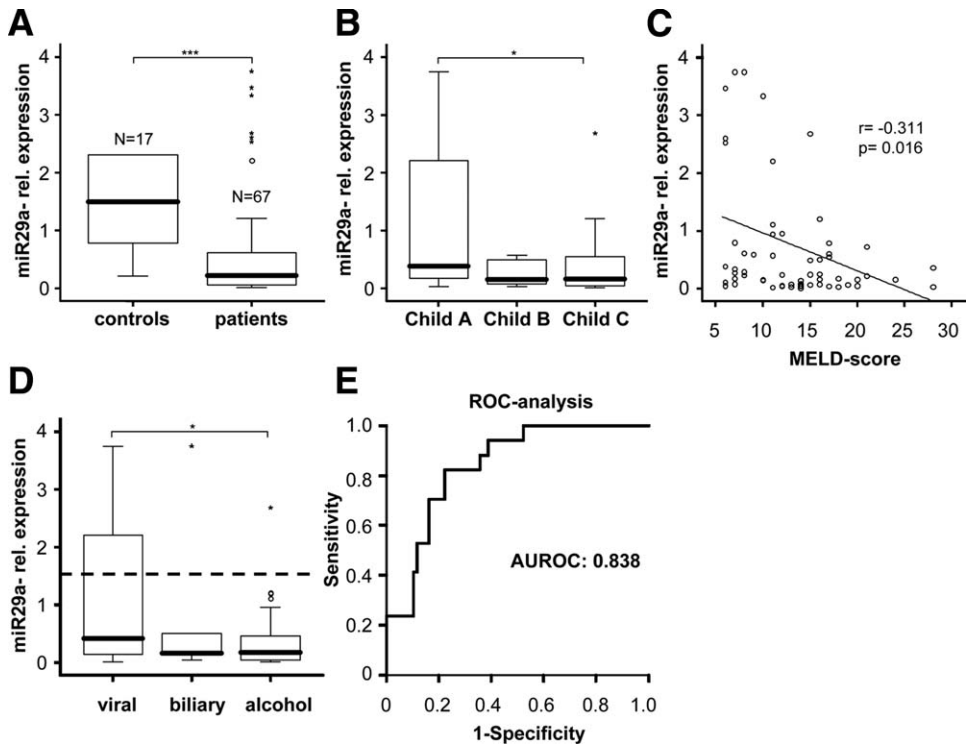

Fig. 6. Low circulating serum miR-29a levels are a characteristic of advanced liver fibrosis in patients. (A) Box-whisker-plot analysis indicated a significant down-regulation of miR-29a level in serum samples of patients with chronic liver diseases compared with healthy controls. Serum levels of miR-29a were determined by qPCR. Box plots are displayed; the bold line indicates the median per group, the box represents 50% of the values, and horizontal lines show minimum and maximum values of the calculated nonoutlier values; asterixes and open circles indicate outlier values. ***$P < 0.001$. (B) Plasma levels of miR-29a were determined by qPCR in samples of patients suffering from liver cirrhosis and revealed a stage-dependent down-regulation of miR-29a in plasma of fibrosis patients. Results are depicted as box-whisker-plot as indicated. *$P < 0.05$. (C) Correlation analysis of plasma levels of miR-29a and Model for End-Stage Liver Disease score revealed a correlation between these two parameters. R, correlation coefficient; Spearman rank correlation test. (D) Plasma levels of miR-29a were determined by qPCR in samples of patients suffering from liver cirrhosis. Results are depicted as box-whisker-plot. The dotted line represents the mean of miR-29a expression in the control group. (E) Receiver operating characteristic curve analysis displaying the diagnostic power in predicting fibrotic disease of the miR-29a level (area under the curve/AUC 0.838)

demonstrated that miR-29 belongs to a subset of miRNAs down-regulated in the lungs of cystic fibrosis patients or during fibrotic remodeling of the heart,[19,20] our data shed new light on a possible common paradigm regarding how miR-29 regulates fibrosis in different organs. Furthermore, down-regulation of miR-29 is found in various types of cancers, such as hepatocellular carcinoma.[21,22] Therefore, miR-29 also might play a crucial role in the transition from liver cirrhosis to the development of hepatocellular carcinoma. In line with this hypothesis, we found significantly decreased levels of miR-29 family members in hepatocellular carcinoma samples from mice treated with the genotoxic agent diethylnitrosamine (data not shown).

Strikingly, miR-29a serum levels were significantly down-regulated in patients with liver fibrosis/cirrhosis compared with healthy controls, and low serum miR-29a levels were associated with advanced cirrhosis stages. The molecular process that leads to lower serum levels of miR-29a in patients with cirrhosis is not clear. It was previously demonstrated that miRNAs are packed into exosomes, which can be exchanged between cells without loss of function of the included miRNA.[23] This raises the question whether miRNAs may play a role as extracellular messengers mediating intercellular communication. Despite the currently unknown mechanism of miRNA regulation in the serum, the striking regulation of miR-29a in the serum of cirrhosis patients might have implications for clinical aspects of liver cirrhosis. Therefore, larger patient cohorts with distinct hepatic disease-causes and differential fibrosis states will have to be analyzed to further test the potential of miR-29 levels in the serum as biomarkers for detection or monitoring of liver fibrosis. Because serum-miR29a levels were significantly different but still showed some overlap between fibrosis and control patients, it is likely that not one miRNA but detection of a whole panel might provide the necessary

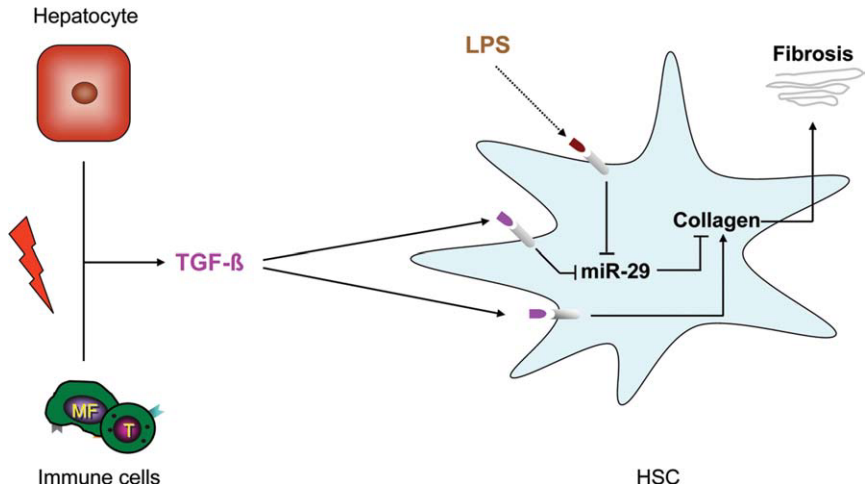

Fig. 7. A model for the role of miR-29 in hepatic fibrogenesis. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

sensitivity and specificity for diagnosis and monitoring of chronic liver diseases.

In the current study, we provide evidence for the hypothesis that different upstream signals regulate the expression levels of miR-29 during liver fibrosis *in vivo* and in HSCs *in vitro* (Fig. 7A). Although TGF-β–dependent down-regulation of miR-29 correlated with increased collagen expression, this was not the case for LPS-dependent miR regulation. The reason for this discrepancy is currently unknown. In line with our findings, it was previously shown that LPS stimulation alone does not lead to increased collagen production in HSCs.[24] It is possible that miR-29–dependent effects on their target mRNAs require a previous strong induction of the respective extracellular matrix genes by TGF-β. Conversely, interleukin-1, which normally also activates NF-κB, did not have a similar effect on miR-29 as LPS or TNF. Thus, it is possible that the regulatory network downstream of inflammatory signals is more complex than the more linear TGF-β/miR-29/collagen cascade. Stress-related signaling cascades other than NF-κB might influence miR-29 expression downstream of inflammatory receptors such as toll-like receptors or TNF. In line with this hypothesis, it was recently demonstrated that oxidative stress leads to a down-regulation of miR-29 in human trabecular meshwork cells.[25] Conversely, these various signals might "neutralize" the effects of miR-29 on collagen mRNA levels on LPS stimulation. Further biochemical experiments are needed to specify the interactions of miR-29 with inflammatory signaling pathways.[26]

The data presented here place miR-29 into a crucial position in the regulation of liver fibrosis. The distinct signals that influence its expression suggests that miR-29 might be an interesting candidate to develop future therapeutic tools to prevent or treat hepatic fibrosis, because it might be more efficient than targeting a single pathway or target gene. However, further studies are needed to evaluate the specificity of modulating this miRNA in various disease conditions.

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