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Is miR-21 a potent target for liver fibrosis?

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MicroRNAs, small non-coding RNAs, regulate gene expression post-transcriptionally by binding to targeted sites within the 3'-UTR regions of mRNAs and play a role in a wide range of physiological and pathophysiological conditions including liver fibrosis. The microRNA miR-21 has been reported to be upregulated in patients (1, 2) and mouse models (1–5) of liver fibrosis, and previous results suggest that its deletion or suppression can reduce fibrosis severity (1–5).

In contrast to these studies (Table 1)(1–5), in the current issue of HEPATOLOGY, Caviglia et al. (6) showed that miR-21 was not crucial for the activation of hepatic stellate cells (HSCs) and the development of liver fibrosis. To reach this conclusion, they performed rigorous examinations with genetic and pharmacologic manipulation of miR-21 in multiple models of liver fibrosis. For example, three models of fibrogenesis [carbon tetrachloride (CCl₄) injection, multidrug-resistance (MDR)2 knockout (KO) mice and 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) diet] were applied to miR-21 null (miR-21 KO) and wildtype (WT) mice. None of these models demonstrated significant differences in liver fibrosis and/or HSC activation among these mice as assessed by Sirius red staining and hepatic hydroxyproline content as well as markers of HSC activation, including α -smooth muscle actin (SMA) protein, *Acta2*, *Colla1*, *Lox*, and *Timp1* mRNA. Further, to address a concern regarding the possible compensatory changes resulting from the absence of miR-21 throughout development and adulthood, they acutely suppressed miR-21 immediately prior to and after the onset of hepatic fibrosis with antisense DNA oligonucleotides [miR-21 knockdown (KD) antimirs] in four fibrotic models [the previous three models plus bile duct ligation (BDL)]. Furthermore, they examined CCl₄-induced fibrosis in a different line of miR-21 KO mice. All of these *in vivo* experiments and other studies including *in vitro* experiments and experiments using human HSC cells and liver slices provided consistent results that led to their conclusion.

Given their finding on miR-21, the authors proceeded to examine the importance of microRNAs in HSC activation and liver fibrosis. For this purpose, they generated mice with

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HSC-specific deletion of *Dicer1*. *Dicer1* is a key RNase that generates mature microRNAs. Although deletion of *Dicer1* decreased microRNAs including miR-21, miR-199a-3p and let-7i in HSCs by at least 50 percent, HSCs exhibited a normal phenotype and the effect of the knockout on HSC activation and liver fibrosis was minimal. Furthermore, given that miR-21 is known as an “oncomir”, the authors investigated the contribution of miR-21 to liver cancer with miR-21 KO and KD approaches, multiple liver cancer models, and human cancer cell lines. Although the experiments performed were not as rigorous as those studying liver fibrosis, miR-21 was shown to be dispensable for hepatic carcinogenesis.

What would account for these discrepancies among this study and others? A simple explanation points to differences in experimental design. For example, two previous studies that described a pro-fibrotic effect of miR-21 on the liver also used CCl₄ injection to induce fibrosis(1, 2), but their treatments were much more severe than the one used in Caviglia’s study. While Caviglia et al. administered CCl₄ by oral gavage or intraperitoneally every 3 days for a total of 8 doses, with a dose of 0.5 µl/g body weight in a 1:3 ratio of CCl₄ and corn oil, one prior study gave a total of 24 subcutaneous injections, twice a week for 12 weeks, with a dose of 2 µl/g body weight in a 2:3 ratio of CCl₄ and olive oil, and the other administered injections 8 times intraperitoneally, twice a week for 4 weeks, with a dose of 4 µl/g body weight in a 1:3 CCl₄ to olive oil ratio. Accordingly, HSC activation and liver fibrosis may be milder in Caviglia’s study than these two preceding studies [e.g., 5 percent fibrosis by Sirius Red positive areas in Caviglia’s study (6) vs. 16 percent fibrosis in Wu’s study(2)], possibly suggesting stage-dependent differential effects of miR-21 on liver fibrosis. Perhaps miR-21 does not play a role in early stage fibrosis but may gain importance with fibrosis progression. Therefore, it is interesting to know whether Caviglia et al. would come to the same conclusion if they apply antisense DNA oligonucleotides to more severe cases of liver fibrosis. Different methods used to knock down miR-21 may have also affected outcomes. While Caviglia et al. used inhibitory antisense DNA oligonucleotides, the two prior studies employed adenoviruses and antagomirs, respectively(1, 2). Experimental environments may also account for some differences. It is reported that the intestinal microbiome influences the development of liver fibrosis (7) and that different experimental environments generate different populations of the intestinal microbiome in mice(8), which may affect experimental outcomes in relation to fibrogenesis.

These conflicting results, however, may reflect a more fundamental question regarding microRNA regulation. In general, individual microRNAs target many different mRNAs, while individual mRNAs are regulated by many different microRNAs. Thus, there is likely to be functional redundancy of many microRNAs. In many cases, therefore, the effect of a single microRNA on targeted mRNAs may not be decisively crucial. In addition, as Ebert and Sharp (and Caviglia et al. as well) suggested(9), the function of microRNAs may be mainly to contribute to robustness in biological processes, rather than to control biological processes as primary regulators. Alberti et al. also pointed out the moderate regulatory effects of microRNAs on gene expression, given that deletion of a wide range of specific microRNAs seldom causes major defects in the overall organism(10). They classified microRNAs into two categories: “core microRNAs” and “periphery microRNAs”. The core microRNAs are involved in the early stage of cellular differentiation and their loss can cause lethal effects on the whole organism. The periphery microRNAs are implicated in the late

stage of development with their comparatively subtle effects on the whole organism, and account for approximately 75 percent of microRNAs. Consistent with this concept, the anti-fibrotic effects of miR-21 blockade observed in several studies were mild, noted to be within a 2-fold change compared to control (3, 4). Conflicting results regarding miR-21 regulation have also been reported in mouse models of cardiac fibrosis, with one demonstrating a pro-fibrotic effect and another showing no effects. Most indices used for assessment of this pro-fibrotic effect were also within a 2-fold change. Furthermore, the effects of other microRNAs implicated in liver fibrosis were often moderate. For example, miR-29 was shown to be anti-fibrotic in mice and administration of miR-29a resulted in reduced liver fibrosis. However, most of the scores used to assess the improvement in liver fibrosis were within a 2-fold change. These observations, including the ones presented by Caviglia et al., may support the notion of moderate biological regulation by microRNAs.

Acknowledgments

The findings of Caviglia et al. strongly support the conclusion that miR-21 is not a significant regulator of hepatic fibrogenesis under the conditions of their study. As they also note, however, their study does not completely exclude the role of miR-21 in HSC activation and the development of liver fibrosis. Providing us with an opportunity to consider the nature of microRNA regulation, this study is timely and important.

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Table 1*In vivo* pre-clinical studies showing miR-21 profibrotic effects

Authors	<i>In vivo</i>	<i>In vitro</i>	miR-21 target genes
Zhang et al.(1)	Antagomir-21 in CCl ₄ model (4 µl/g body weight in 1:3 mixture of CCl ₄ and olive oil, 8 doses, twice a week for 4 weeks, intraperitoneally), TAA model (200 mg/kg body weight, 16 doses, twice a week for 8 weeks, intraperitoneally)	α -SMA and <i>Col1a1</i> mRNA expression were down-regulated in isolated primary HSCs after miR-21 knockdown.	Smad6/7
Wu et al.(2)	Adenovirus transfected miR-21 inhibition in CCl ₄ model (2 µl/g body weight in 2:3 mixture of CCl ₄ and olive oil, 24 doses, twice a week for 12 weeks, subcutaneously)	Hepatocyte apoptosis (no significant differences)	SPRY2
Zhang et al.(3)	Chemically modified antisense oligonucleotides specific to miR-21 in mice with hepatocyte-specific deletion of Pten.	Liver fibrosis-related <i>in vitro</i> assays were not performed.	Spry1/2
Rodrigues et al.(4)	MCD diet (2 and 8 weeks) to miR-21 global KO mice	Liver fibrosis-related <i>in vitro</i> assays were not performed.	PPAR α
Kennedy et al.(5)	miR-21 global KO mice with BDL (1 week)	Cholangiocyte proliferation, HSC proliferation/fibrotic reaction (miR-21 KD)	Smad7

Abbreviation: CCl₄: Carbon tetrachloride, TAA: Thioacetamide, KO: Knock out, BDL: Bile duct ligation, MCD: Methionine- and choline-deficient.