Decreased levels of microRNA miR-122 in individuals with hepatitis C responding poorly to interferon therapy

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Several microRNAs (miRNAs), including liver-specific miR-122, have been implicated in the control of hepatitis C virus (HCV) RNA replication and its response to interferon (IFN) in human hepatoma cells. Our analysis of liver biopsies from subjects with chronic hepatitis C (CHC) undergoing IFN therapy revealed no correlation of miR-122 expression with viral load and markedly decreased pretreatment miR-122 levels in subjects who had no virological response during later IFN therapy; other investigated miRNAs showed only limited changes. These data have implications for the prospect of targeting miRNAs for CHC therapy.

The abundant liver-specific miRNA miR-122 is crucial for efficient replication of HCV RNA in cultured human hepatoma Huh7 cells stably expressing an HCV replicon[1,2]. This observation raised much interest in the role of miR-122 in HCV infection and its potential as a therapeutic target. Current therapy of CHC consists of pegylated IFN-α (pegIFN-α) and ribavirin but achieves a sustained virological response in only ~55% of patients[3,4]. It was recently reported that the levels of miR-122 and several other miRNAs are regulated by IFN in Huh7 cells and primary mouse hepatocytes and that miRNAs might mediate at least some effects of IFN on HCV RNA replication *in vitro*[5]. All experiments performed to date have assessed the role of miR-122 in HCV replication in cultured cells. We now compared levels of miR-122 and other miRNAs in liver biopsies from 42 subjects with CHC undergoing treatment (**Supplementary Table 1** online) by quantitative PCR (qPCR) with chemically synthesized miRNAs as standards (**Supplementary Methods** online). Subjects who did not respond to therapy with a decrease in viral load of more than two orders of magnitude at week 12 (primary nonresponder (PNR) subjects) had significantly lower miR-122 levels than subjects with a strong response to IFN-α and undetectable HCV-RNA at week 12 (complete early virological responder (cEVR) subjects; **Fig. 1a**). This result was confirmed by an RNase protection assay (**Fig. 1b**). The difference in miR-122 levels between PNR subjects and cEVR subjects was also apparent when only subjects infected with difficult-to-treat HCV genotypes 1 and 4 were examined (**Supplementary Fig. 1a** online),

indicating that the difference is not due to the biased distribution of HCV genotypes between the two response groups (**Supplementary Table 1**). The decrease in miR-122 levels was independent of liver fibrosis (**Supplementary Fig. 1b,c**) and was not a result of a lower proportion of hepatocytes in the biopsy material of PNR subjects (**Fig. 1c**), further supporting a direct correlation between the pretreatment miR-122 level and the response to therapy.

Clinical studies have shown that subjects with a high HCV viral load respond less well to therapy than subjects with a low viral load[3,4]. In our subject cohort, the median viral load was indeed higher (though not significantly) in the PNR group when compared to the cEVR group (**Supplementary Fig. 1d**). Because miR-122 is required for efficient HCV replication in Huh7 cells[1], we were surprised by our finding of low miR-122 levels in nonresponders. Moreover, measurements of HCV RNA in liver and serum of the subjects showed no positive correlation between miR-122 abundance and viral load (**Fig. 1d,e**). It is possible that even the low miR-122 levels found in PNR subjects is not limiting for HCV replication or that measurements of total miR-122 in the biopsy extracts do not reflect the miR-122 levels in the fraction of hepatocytes that are infected with HCV. Alternatively, the role of miR-122 in HCV replication may be less pronounced *in vivo* than *in vitro*.

We showed recently that PNR subjects have a preactivated IFN system in the liver already before treatment and show no significant changes in expression of IFN-regulated genes (IRGs) upon pegIFN-α administration[6]. The decrease in miR-122 level in nonresponders raised the possibility that *MIRN122* is a negatively regulated IFN target gene. Indeed, it has previously been reported that miR-122 is downregulated within 2–8 h by treatment with IFN-β and that this downregulation contributes to the antiviral effect of IFN in Huh7 cells[5]. To determine the effect of IFN on miR-122 in subjects with CHC, we compared levels of miR-122 in paired liver biopsies collected before and 4 h after administration of pegIFN-α. PegIFN-α did not decrease the level of miR-122 in either group of subjects (**Fig. 1f**), whereas five established IRGs were strongly upregulated in response to pegIFN-α in cEVR subjects[6] (**Supplementary Fig. 2** online). We used mice to investigate whether more prolonged IFN treatment influences miR-122 level but found no pronounced decrease in livers of mice treated with IFN-α for up to 49 h (**Fig. 2a,b** and **Supplementary Fig. 3** online). Hence, miR-122 is not an early IRG *in vivo*, but we cannot exclude that prolonged activation of the IFN system in PNR subjects contributes to miR-122 downregulation. Indeed, miR-122 levels negatively correlated with the expression of IRGs (**Supplementary Fig. 4a–d** online). However, this negative correlation might just indicate that later PNRs have both low pretreatment miR-122 levels and high pretreatment IRG levels without any causal relationship.

We also assessed the potential role of other miRNAs identified as IFN-β inducible and implicated in the control of HCV replication in

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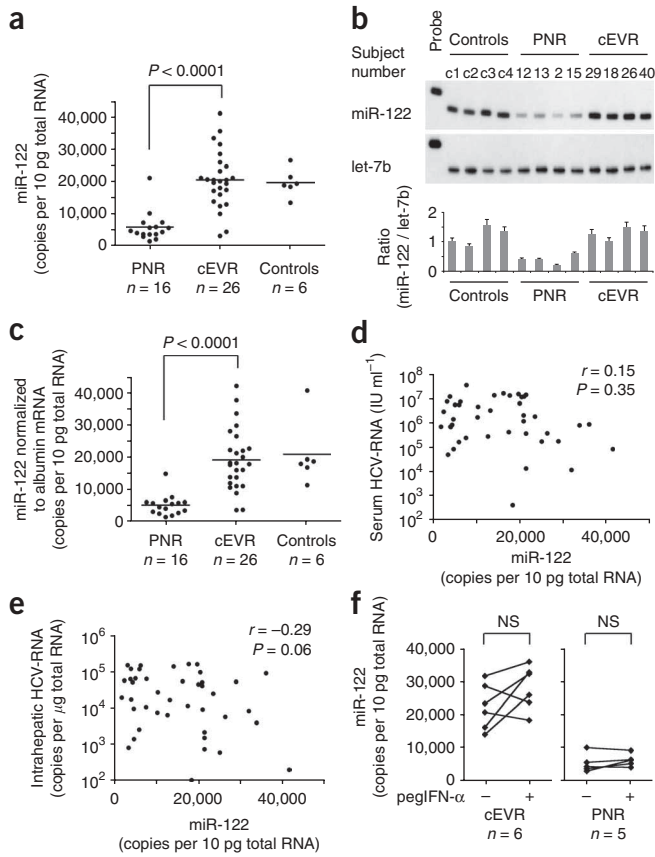
Figure 1 MiR-122 levels in livers of subjects with CHC. (**a**) MiR-122 levels in livers of 42 CHC subjects and six control subjects, as measured by RT-qPCR. MiR-122 levels are significantly ($P < 0.0001$; two-tailed Mann-Whitney test) lower in subjects with PNR to pegIFN-α therapy than in subjects with cEVR. There is no significant difference between cEVR subjects and controls. (**b**) Quantification of miR-122 levels by RNase protection assay. RNA originating from four control subjects (c1–c4) and eight CHC subjects (four PNR and four cEVR; numbers refer to subjects listed in **Supplementary Table 1**) was analyzed with probes specific for miR-122 and let-7b. The graph shows a quantification of the relative ratios between miR-122 and let-7b RNA (averages from two experiments). (**c**) Comparison of miR-122 levels between PNR and cEVR subjects, using normalization for both U6 RNA (as in panel **a** and in all other experiments) and hepatocyte-specific albumin mRNA ($P < 0.0001$; two-tailed Mann-Whitney test). (**d**) Serum HCV-RNA levels do not correlate with intrahepatic miR-122 expression. The Spearman correlation is not significant ($P = 0.35$, correlation coefficient $r = 0.15$). (**e**) Intrahepatic HCV-RNA levels do not positively correlate with miR-122 expression. The Spearman correlation is not significant ($P = 0.06$, correlation coefficient $r = -0.29$). (**f**) Effect of pegIFN-α administration on the miR-122 level in human liver. MiR-122 levels in paired liver biopsies from six cEVR and five PNR subjects were measured by RT-qPCR. The first liver biopsy was performed before treatment initiation (−), whereas the second was performed 4 h after the pegIFN-α injection (+). The values for each individual subject are connected by a line. There is no significant (NS) difference between the − and + samples (paired t-test; see **Supplementary Fig. 7**). The protocol was approved by the Ethics Committee of the University Hospital Basel, Switzerland, and written informed consent was obtained from all subjects.

Huh7 cells[5]. In two different Huh7 cell lines, we measured IFN-β–mediated induction of miR-1, miR-196b, miR-296-5p, miR-351 and miR-448, all reported to be IFN-β inducible in Huh7 cells[5] (**Supplementary Methods**). Whereas miR-448 expression remained unchanged, the other miRNAs were induced two- to sixfold; known IRGs were upregulated up to 400-fold (**Fig. 2c** and **Supplementary Fig. 5** online). The miR-122 level decreased in response to IFN-β, but only by 20–40%, rather than 75% as previously reported[5]. Treatment of Huh7 cells with IFN-α instead of IFN-β yielded similar results (data not shown).

Whereas other researchers have reported only fold changes in miRNA level[5], we also determined the number of miRNA copies per cell (**Fig. 2d**, **Supplementary Fig. 5b** and **Supplementary Methods**). MiR-1, miR-351 and miR-448 were present at very low levels (fewer than ten molecules per cell) even after IFN treatment, making it unlikely that they have a substantial biological effect, as it has been reported that a minimum threshold of ∼100 molecules per pg of small RNA (approximately the equivalent of one cell; see **Supplementary Methods**) must be reached for the miRNA to repress target messenger RNAs[7]. Notably, miR-351 is established as expressed in mice but not in humans[8].

The levels of miR-1, miR-196b, miR-296-5p, miR-351 and miR-448 were also analyzed in mouse liver. MiR-448 was not detected (data not shown), but the levels of the remaining miRNAs increased two- to eightfold after IFN-α administration (**Fig. 2a**). However, apart from miR-296-5p, their absolute levels were low (**Fig. 2b**). Examination of perfused mouse livers indicated that miR-196b, and probably miR-1, originate from blood cells rather than from hepatocytes, a conclusion supported by analysis of mouse blood; moreover, miR-296-5p showed no response to IFN in perfused livers but was stimulated by IFN in
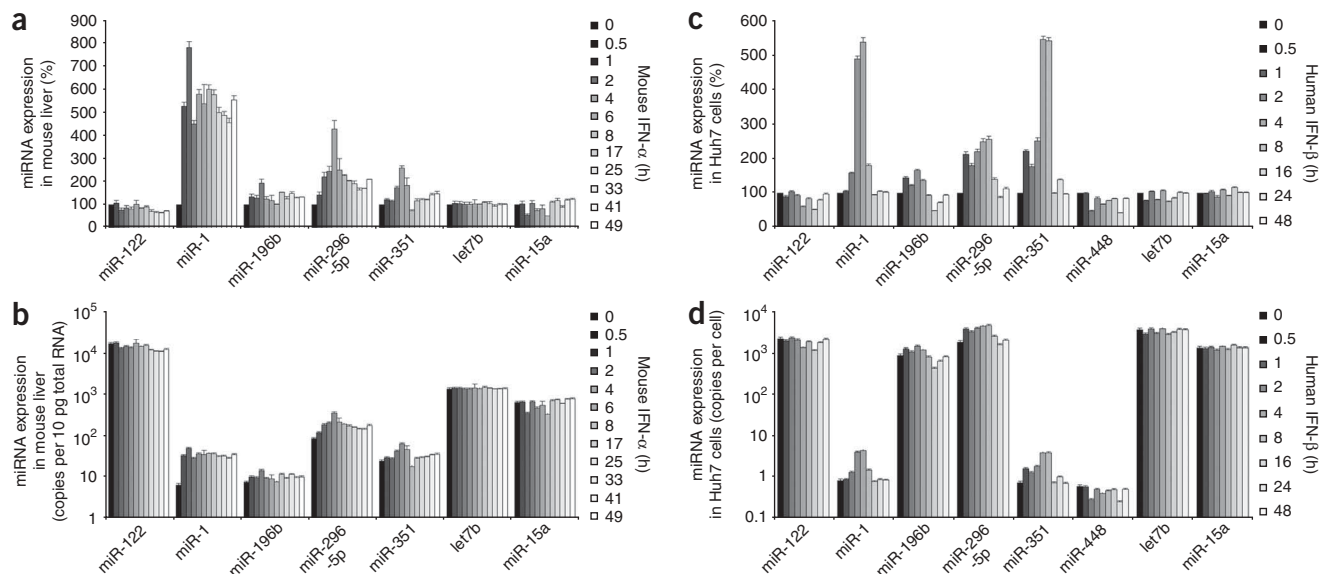
blood cells (**Supplementary Fig. 6** online). Finally, we analyzed the amounts of miR-1, miR-196b, miR-296-5p, miR-351 and miR-448 in human biopsies. Apart from miR-296-5p, which was moderately abundant and, notably, expressed to a significantly higher degree in PNR subjects than in cEVR subjects, these miRNAs were either absent (miR-448; data not shown) or present at only very low levels (fewer than ten copies per 10 pg of total RNA; **Supplementary Fig. 7** online). Except for miR-296-5p, no significant effect of pegIFN-α administration on the levels of these miRNAs was apparent in either cEVR or PNR subjects (**Supplementary Fig. 8** online). On the basis of the analysis in mice, it is likely that the observed stimulation of miR-296-5p by IFN originates from blood cells in the liver. Taken together, these findings argue against a role for most of the investigated miRNAs as mediators of IFN effects on HCV RNA replication *in vitro* and during CHC therapy.

In summary, our results show that subjects who will later not respond to IFN therapy generally have pretreatment levels of miR-122 several times lower than those in responders and that pegIFN-α has no significant effect on the level of miR-122 or other investigated miRNAs (with the possible exception of miR-296-5p) in livers of subjects assayed 4 h after administration. Moreover, no positive correlation between intrahepatic miR-122 and HCV RNA levels was observed. These findings are unexpected, as, on the basis of HCV replicon studies in Huh7 cells, a reduction in miR-122 should decrease viral yield and facilitate the therapy. Hence, caution should be exerted in extrapolating *in vitro* observations to subjects with CHC. In the case that miR-122 is indeed required for HCV persistence in all CHC subjects, its low level in nonresponders to IFN therapy might potentially benefit future therapeutic interventions involving the use of miR-122 antagomirs[9]. The finding that miR-122 expression is significantly lower in PNR subjects than in cEVR subjects makes it also a convenient marker that, together with preactivated IRGs[6,10,11], is suitable for predicting the outcome of IFN therapy.

Note: Supplementary information is available on the Nature Medicine website.

Figure 2 Effect of IFN on levels of miRNAs in mouse liver and human hepatoma Huh7 cells. (**a,b**) Levels of miR-122, miR-1, miR-196b, miR-296-5p, miR-351, let-7b and miR-15a (measured by RT-qPCR) in mouse liver in response to either single injection (time points 0.5−8 h) or multiple injections at 8 h intervals (time points 17−49 h) of mouse IFN-α. Six mice were used for time point 0, one mouse was used for each time point from 0.5 h to 8 h, and two mice were used for each time point from 17 h to 49 h. The levels are shown in **a** as percentage of pretreatment levels and in **b** as copies per 10 pg total RNA (equivalent of approximately 0.5 cell; see **Supplementary Methods**). All measurements for each mouse were done in triplicate. Data are means ± s.e.m. Procedures with the mice were conducted with the approval of the Animal Care Committee of the Kanton Basel-Stadt, Switzerland. (**c,d**) Huh7 cells were incubated with IFN-β for the indicated time points, and the levels of indicated miRNAs were determined by RT-qPCR. The results are shown as either percentage of pretreatment levels (**c**) or copies per cell (**d**). All measurements were done in triplicate. Data are means ± s.e.m.

AUTHOR CONTRIBUTIONS
M.S.-F., J.K., M.H.H. and W.F. conceived and designed the experiments; M.S.-F., J.K. and I.M. performed the experiments; M.S.-F. and J.K. analyzed the data and contributed to writing and editing the manuscript; M.H.H. and W.F. supervised the project and wrote the manuscript.

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