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Effect of ribavirin and interferon ß on miRNA profile in the hepatitis C virus subgenomic replicon-bearing Huh7 cells

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Abstract. Hepatitis C virus (HCV) infection is still a major global health issue despite decades of research. The liver-specific microRNA-122 (miR-122) can stimulate HCV replication/translation *in vitro*, indicating that miR-122 contributes to pathogenesis of HCV. However, it remains controversial whether interferon (IFN) inhibits HCV via modulating miR-122 expression. The underlying mechanism of ribavirin (RBV) in enhancing IFN treatment for HCV patients has yet to be explored. We investigated the relationship between miR-122 expression and anti-HCV activity of IFN ß in combination with RBV *in vitro*, due to difficulty accessing an HCV animal model. Upregulation of ISG54 mRNA or cytostatic effect was detected in Huh7 and HCV replicon cell lines in response to IFN ß or RBV stimulation, respectively. It was found that IFN ß and/or RBV suppressed miR-122 expression marginally, with a synergetic anti-HCV effect between IFN ß and RBV. Marginal modification of other miRNAs was also observed in these cell lines, using miRNA array following IFN ß and RBV treatment. Taken together, our data suggest that miRNAs are not crucial in anti-HCV action, following IFN ß and/or RBV stimulation *in vitro*.

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Abbreviations: miRNA, microRNA; RT-qPCR, RT-quantitative PCR; HCV, hepatitis C virus; IFN, interferon; RBV, ribavirin; GAPDH, glyceraldehydes 3-phosphate dehydrogenase; ISG, interferon stimulated gene; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; SVR, sustained virological response

Key words: miRNA, hepatitis C virus, interferon, ribavirin

Introduction

There are ~180 million people worldwide suffering from hepatitis C virus (HCV) infection chronically (1), characterized by chronic liver inflammation and fibrogenesis, leading to end-stage liver failure and hepatocarcinoma (2). The current standard therapy for chronic hepatitis C (CHC) consists of the pegylated interferon α (Peg-IFN α) in combination with ribavirin (RBV), but viral resistance still develops in a large fraction of patients. Improved outcomes may be achieved by modifying doses, dose regimen and also patient compliance in response to Peg-IFN α and RBV treatment. However, the underlying mechanism of host response to Peg-IFN α and RBV needs to be explored further. Understanding such mechanism may provide insight and substantially improve the outcomes.

MicroRNAs (miRNAs) are ~22 nucleotide-long RNAs that silence gene expression post-transcriptionally, and is suggested to play an important role in a broad range of biological processes, including intrinsic antiviral immunity (3). miR-122 is a liver-specific miRNA responsible for modulating HCV replication (4), translation (5) and lipid metabolism (6). miR-122, downregulated in hepatocellular carcinoma (7), suppresses intrahepatic metastasis (8). It is controversial whether miR-122 is profoundly downregulated, and the other five miRNAs (miR-196b, -296, -351, -431 and -448) are up-regulated following IFN ß treatment, subsequently inhibiting HCV replication *in vitro* (9,10).

Surprisingly the addition of RBV, a guanosine analogue and broad antiviral drug, to IFN α led to marked improvements in sustained virological response (SVR) rates, but the underlying mechanism remains to be explored (11). It is hypothesized that RBV modifies miRNA gene expression, based on reports suggesting that RBV inhibited cell proliferation and interfered with nucleic acid metabolism (12,13).

In the current study the effects of RBV and IFN ß on modifying miR-122 and other miRNAs, and subsequent antiviral response was investigated, using Huh7 and HCV replicon cell lines. Such data may provide useful information for both basic research and guidelines in clinical treatment.

Materials and methods

Cell culture. The human hepatoma cell line Huh7 and the HCV subgenomic replicon-bearing Huh7 were kindly provided by Professor Jin Zhong, Institut Pasteur of Shanghai (14). In the HCV subgenomic replicon, the neomycin phosphotransferase replaced the sequences of structural HCV proteins as a selectable marker gene, and a second internal ribosome entry site element of the encephalomyocarditis virus was inserted to allow translation of the nonstructural proteins NS3 to NS5B (Fig. 5A). Cells were cultured in Dulbecco's Modified Eagle Medium (Gibco) supplemented with 10% fetal calf serum (Gibco), 100 U/ml of penicillin and 100 μg/ml of streptomycin at 37˚C with 5.0% CO_2, as described previously (14). To select cell lines carrying the HCV subgenomic replicon, G418 (Sigma) was added to the culture medium (final concentration, 400 μg/ml). Selected cells were treated with RBV (Sigma) and/or recombinant human IFN ß-1a (PBL), without G418 for indicated time periods.

MTT assay. Cells (10,000) in 200 μl culture medium were plated in each well of a 96-well plate 1 day prior to treatment. Different doses of RBV and/or IFN ß were added for indicated time points. Cells were washed three times with PBS after removing culture medium. Then cells treated with 100 μl of culture medium, containing sterile 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) dye (0.5 mg/ml), were incubated for 4 h at 37˚C. Subsequently, MTT solution was replaced with 150 μl of dimethyl sulfoxide, and mixed thoroughly for 10 min. The intensity of each well was determined with a spectrometric absorbance at 490 nm on the microplate reader MPC-1 (VersaMax, USA).

RT-PCR detection of HCV and ISG54. Total cellular RNA was extracted from treated cells, as described above, using Trizol (Invitrogen). For RNA detection, HCV and ISG54 expressions were evaluated with the SYBR Green RT-qPCR (Applied Biosystems) on Prism 7500 system (Applied Biosystems Inc., Foster City, USA), and semi-quantitative RT-PCR, respectively, according to instructions from the manufacturers. GAPDH mRNA from each dish was used as a reference control. Primers are indicated in Table I. Comparison of RNA expression was based on a comparative C_T method ($\Delta\Delta C_T$), and relative RNA expression can be quantified according to the formula of $2^{-\Delta\Delta CT}$, where $\Delta\Delta C_T$ = (C_T HCV RNA in treatment group - C_T reference RNA in treatment group) - (C_T HCV RNA in mock group - C_T reference RNA in mock group).

Northern analysis. A total of 10 μg per sample was denatured at 80˚C for 5 min with deionized formamide, separated on 15% of urea-polyacrylamide gel electrophoresis, and transferred onto BrightStar-Plus positively charged nylon membranes (Ambion). Northern blot was probed with Dig-labeled miR-122 LNA probe (Exiqon) as described previously (15). Hybridized RNA was illustrated, using the DIG Luminescent Detection Kit for nucleic acids (Roche Diagnostics). Intensities of band signals were quantified, using the densitometric software Quantity One (Bio-Rad). The amount of miR-122 was normalized with the corresponding 5S RNA.

Table I. Primers used for RT or PCR of mRNA or miRNA.

Gene name	Primer sequences (5'-3')
ISG54	Sense: CCTTTAGTGGAGTA ATCTACTGGGC
	Antisense: TGCTACATTATG ACTATGAGGAGGG
GAPDH	Sense: GAAGGTGAAGGTCGGAGTC
	Antisense: AAAGATGGTGATGGGATTTC
HCV	Sense: TCTGCGGAACCGGTGAGTA
	Antisense: TCAGGCAGTACCACAAGGC
U6	Sense: CTCGCTTCGGCAGCACA
	Antisense: AACGCTTCACGAATTTGCGT
miR-122	RT primer: GTCGTATCCAGTGCAG GGTCCGAGGTATTCGCA CTGGATACGACCAAACA
	Sense: GCCCTGGAGTGTGACAATGG
miR-196b	RT primer: GTCGTATCCAGTGCAGGG TCCGAGGTATTCGCACTGGA TACGACCCCAACAACAG
	Sense: GCCCGCTAGGTAGTTTCC
miR-638	RT primer: GTCGTATCCAGTGCAGG GTCCGAGGTATTCGCACTG GATACGACAGGCCGCC
	Sense: AGGGATCGCGGGCGG
miR-1181	RT primer: GTCGTATCCAGTGCA GGGTCCGAGGTATTCG CACTGGATACGACCGGCTC
	Sense: CCGTCGCCGCCACC
miRNA	Universal antisense: GTGCAG GGTCCGAGGT

miRNA RT-qPCR. Stem-loop RT-PCR was performed to quantify the miRNAs (16). Briefly, 0.5 μg of total RNA was reverse transcribed with ImProm-II (Promega) in 10 μl of reverse transcriptase reaction to cDNA, using a stem-loop RT primer indicated in Supplementary Table I. Sybr Green qPCR was used to assay miRNA expression with the gene specific forward primers and the universal reverse primer specific to the stem-loop RT primer indicated in Table I. Relative quantification of miRNA expression levels was performed as described above. In addition, the quantity of miRNA relative to a reference gene, U6 small nuclear RNA (U6) can be calculated, using the formula $2^{-\Delta CT}$, where ΔC_T = (C_T miRNA - C_T U6) (17).

miRNA expression profiling. Each sample was prepared according to the Agilent's miRNA Microarray System protocol, and total RNA (100 ng) was dephosphorylated and ligated with pCp-Cy3. Labeled RNA was purified and hybridized to Agilent human miRNA arrays for 20 h at 55˚C with rotation, with each array containing probes interrogating 721 human miRNAs from the Sanger miRBase, release 12.0. Images were scanned with an Agilent microarray scanner, using high dynamic range settings, as specified by the manufacturer, extracted using Agilent feature extraction software version 9.5.3, and analyzed using GeneSpring GX 10.0.5 software.

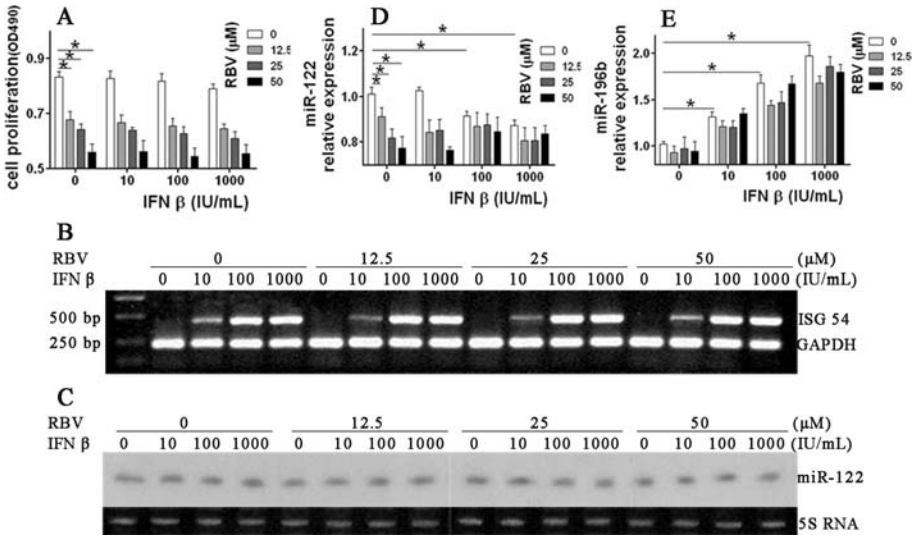

Figure 1. Dose effects of RBV and/or IFN ß on miR-122 and miR-196b in Huh7 cells. Huh7 cells were treated with RBV for 3 days, and with/without IFN ß for 4 h, as in the indicated concentrations. (A) Cell proliferation was measured in the different groups, using the MTT method. (B) Endogenous expression of ISG54 was detected, using semi-quantitative RT-PCR. (C) miR-122 was assayed using Northern blot, and 5S RNA was used as loading control. (D) miR-122 or (E) miR-196b was quantified with qPCR. The data are presented as means ± SD of three independent experiments. *P<0.05 vs. mock group.

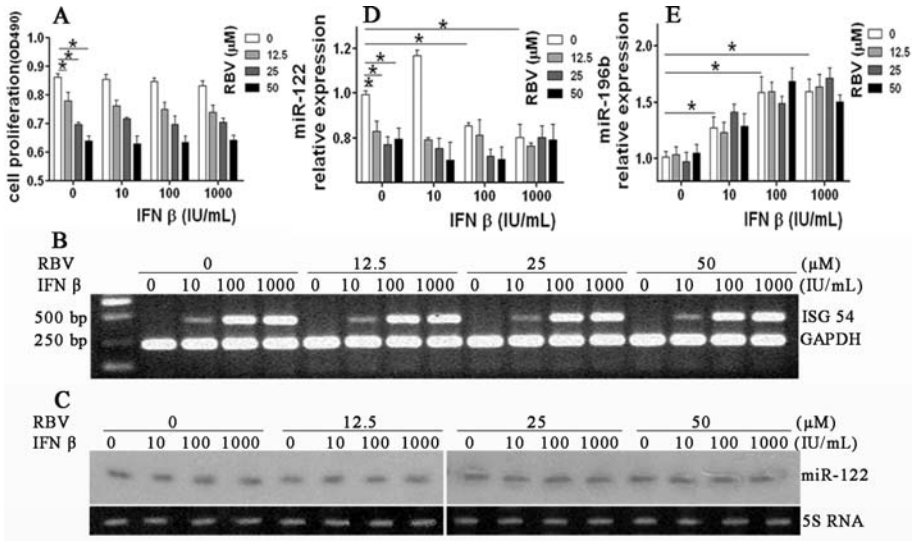

Figure 2. Dose effects of RBV and/or IFN ß on miR-122 and -196b in the HCV subgenomic replicon-bearing Huh7 cells. Cells were treated with RBV for 3 days, and with/without IFN ß for 4 h, as in the indicated concentrations. (A) Cell proliferation was measured in the different groups, using the MTT method. (B) The endogenous expression of ISG54 was detected, using semi-quantitative RT-PCR. (C) miR-122 was assayed using Northern blot, and 5S RNA was used as loading control. (D) miR-122 or (E) miR-196b was quantified with qPCR. The data are presented as means ± SD of three independent experiments. *P<0.05 vs. mock group.

Statistical analysis. Data are presented as means ± SEM calculated from three separate experiments. Statistical analyses of the data were performed using analysis of variance (ANOVA) and Duncan's multiple range test. P-values <0.05 were considered statistically significant.

Results

RBV monotherapy inhibits miR-122 expression and HCV subgenomic replicon in Huh7 cells. RBV (12.5, 25 or 50 μM) inhibited cell proliferation in a dose-dependent manner on the third day (Figs. 1A and 2A), but reduced HCV RNA expression in a dose-independent manner (Fig. 5C), compared to the mock group.

There was ~20% inhibition of miR-122 expression with statistical significance in the RBV treated group compared to the mock revealed by RT-PCR (Figs. 1D and 2D), but no significant difference was observed by Northern blot (Figs. 1C and 2C). No significant difference was detected in miR-196b expression in response to RBV treatment, using RT-PCR (Figs. 1E and 2E).

IFN ß monotherapy induces ISG54 and miR-196b, and reduces HCV subgenomic replicon, but has minimal suppression on miR-122 in Huh7 cells. The expression of ISG54 (a known IFN-stimulated gene) in both Huh7 cells was upregulated by ~80-fold in response to IFN ß stimulation in dose-dependent (Figs. 1B and 2B) as well as time-dependent

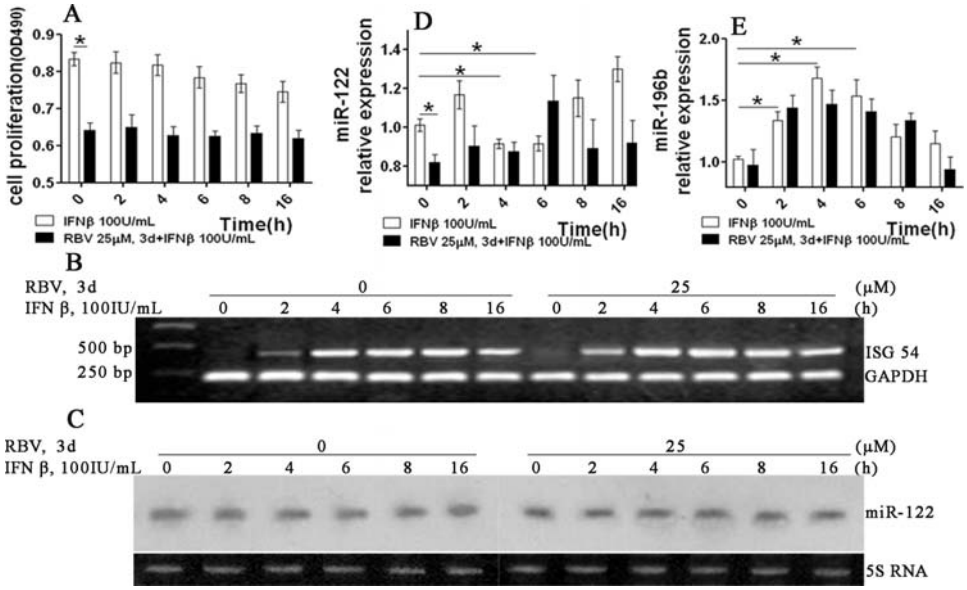

Figure 3. Time course of miR-122 and -196b in Huh7 cells to IFN ß and RBV stimulation. Huh7 cells were stimulated with RBV (25 μM) for 3 days with/without IFN ß (100 U/ml) for the indicated times. (A) Cell proliferation was evaulated, using the MTT. (B) The endogenous expression of ISG54 was detected, using semi-quantitative RT-PCR. (C) miR-122 was assayed using Northern blot, and 5S RNA was used as loading control. (D) miR-122 or (E) miR-196b was quantified with qPCR. The data are presented as means ± SD of three independent experiments. *P<0.05 vs. mock group.

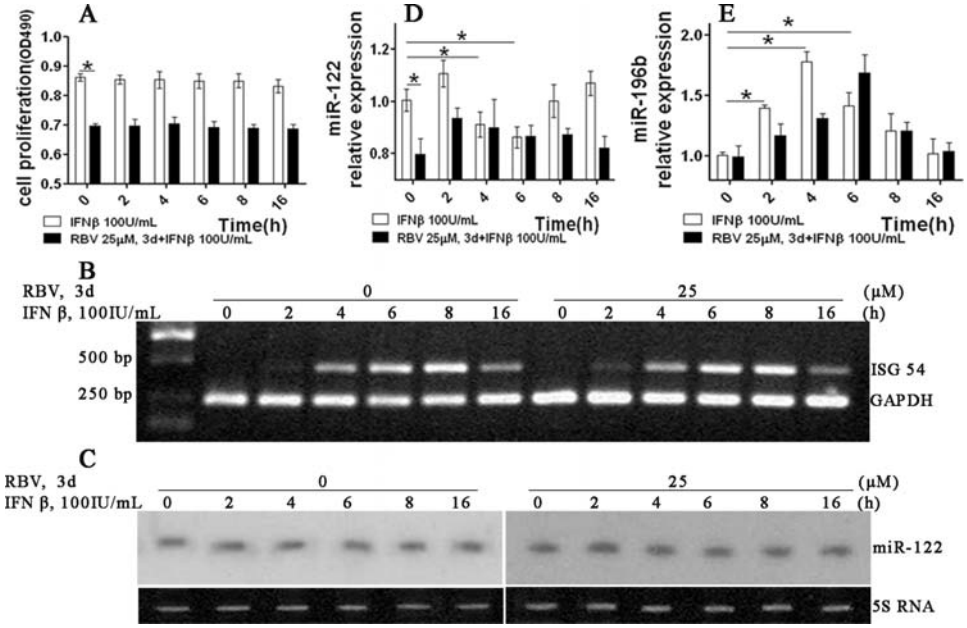

Figure 4. Time course of miR-122 and -196b in the HCV subgenomic replicon-bearing Huh7 cells to IFN ß and RBV stimulation. Cells were stimulated with RBV (25 μM) for 3 days with/without IFN ß (100 U/ml) for the indicated times. (A) Cell proliferation was evaulated, using the MTT. (B) Endogenous expression of ISG54 was detected, using semi-quantitative RT-PCR. (C) miR-122 was assayed using Northern blot, and 5S RNA was used as loading control. (D) miR-122 or (E) miR-196b was quantified with qPCR. The data are presented as means ± SD of three independent experiments. *P<0.05 vs. mock group.

(Figs. 3B and 4B) manner. Similar to ISG54 induction, miR-196b was increased by ~2-fold depending on dose-response (Figs. 1E and 2E) and time-response (Figs. 3E and 4E). There was ~10% transient reduction of miR-122 expression observed in the cell response to IFN ß stimulation at 4 and 6 h in RT-PCR (Figs. 1D, 2D, 3D and 4D), but not in Northern blot (Figs. 1C, 2C, 3C and 4C). IFN ß inhibited 80% HCV RNA at 16 h (Fig. 5D). Taken together, miR-122 might not play a key role in antiviral activity *in vitro* when stimulated with IFN ß.

RBV plus IFN ß has no synergistic effect on miR-122 and -196b, but affects the HCV subgenomic replicon in Huh7 cells. Although RBV inhibited cell proliferation and IFN ß upregulated ISG54, no synergistic effect was detected on either cell proliferation (Figs. 1A, 2A, 3A and 4A) or ISG54 expression (Figs. 1B, 2B, 3B and 4B). No additional effects of RBV plus IFN ß stimulation were observed in the down-regulation of miR-122, using either Northern blot (Figs. 1C, 2C, 3C and 4C) or RT-PCR (Figs. 1D, 2D, 3D and 4D), and in the upregulation of miR-196b using RT-PCR (Figs. 1E, 2E,

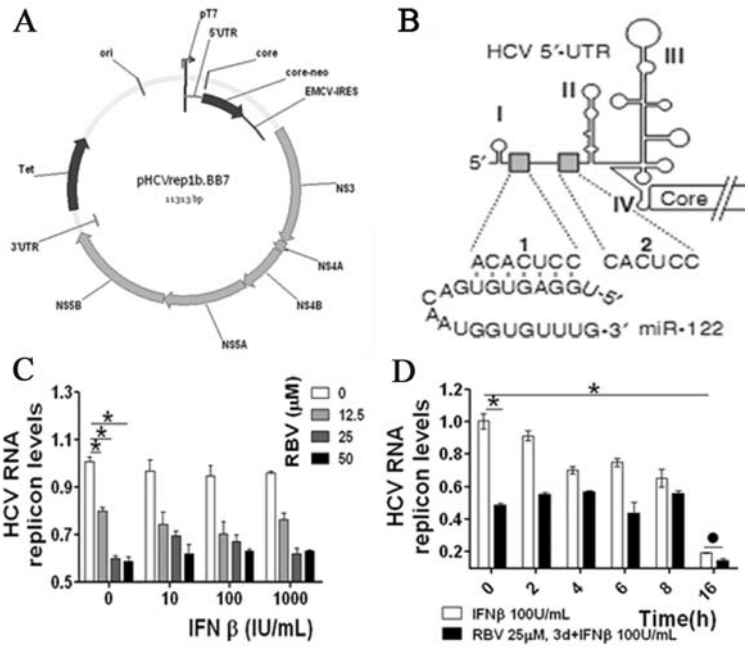

Figure 5. Effects of RBV and IFN ß on HCV RNA in the HCV subgenomic replicon-bearing Huh7 cells. (A) Structure of the HCV subgenomic replicon. (B) miR-122 and its target sequences. Two investigated miR-122 target sequences essential for HCV replication and translation were located between stem-loops I and II in the 5'-UTR. (C) Dose effects of RBV and IFN ß on HCV RNA in the HCV subgenomic replicon-bearing Huh7 cells. Cells were treated with RBV for 3 days, with/without IFN ß for 4 h, as in the indicated concentrations. qPCR was performed to detect HCV RNA. (D) Time course of HCV RNA in the HCV subgenomic replicon-bearing Huh7 cells treated by RBV and IFN ß. Cells were treated with RBV (25 μM) for 3 days with/without IFN ß (100 U/ml) for the indicated times. qPCR was performed to detect HCV RNA. The data are presented as means ± SD of three independent experiments. *P<0.05 vs. mock group; •P<0.05 vs. IFN ß monotherapy for 16 h.

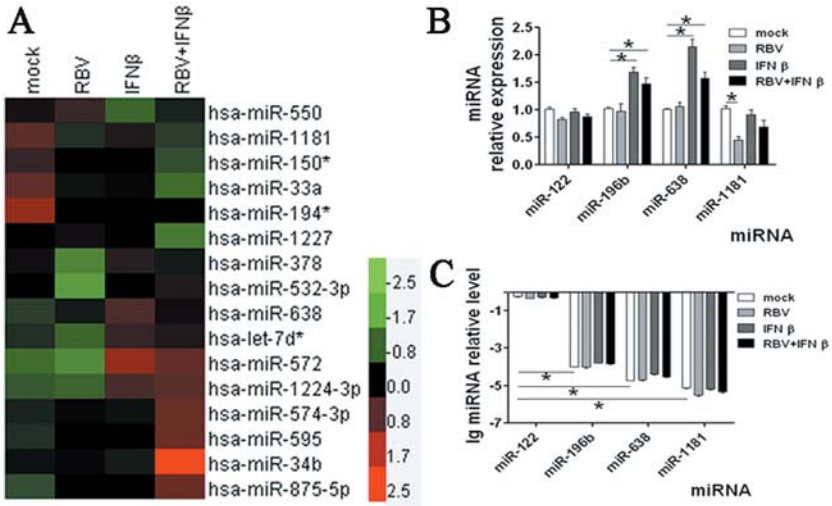

Figure 6. Effects of RBV and IFN ß on miRNA profile in the HCV subgenomic replicon-bearing Huh7 cells. The cells were stimulated with RBV (25 μM) for 3 days with/without IFN ß (100 U/ml) for 4 h. (A) miRNA profiles with >2-fold changes using miRNA array. (B) RT-qPCR validation of miR-122, -196b, -638 and -1181. (C) Relative levels of miR-122, -196b, -638 and -1181 to reference gene, U6 small nuclear RNA(U6). The data are presented as means ± SD of three independent experiments. *P<0.05 vs. mock group.

3E and 4E). A synergistic suppression of HCV RNA in response of IFN ß (100 U/ml for 16 h) and RBV (25 μM for 3 days) stimulation was observed in HCV subgenomic replicon-bearing Huh7 cells (Fig. 5C and D).

A marginal effect of IFN ß and/or RBV on miRNA profile in the HCV subgenomic replicon-bearing Huh7 cells. Using miRNA array processing, 16 miRNAs were up- or down-regulated 2 to 5-fold in response to IFN ß and/or RBV stimulation, among 173 detected miRNAs in the HCV subgenomic replicon-bearing Huh7 cells (Fig. 6A). Furthermore, the other four reported miRNAs (miR-196b, -296-5p, -431 and -448) could be modified >2-fold changes (9,10), but our results showed there was <2-fold changes (data not shown).

To validate the miRNA array data, RT-PCR was performed for all miRNA expression levels in each group. Among four selected miRNAs (miR-122, -196b, -638, and -1181), miR-122 was ~15% reduced in response to IFN ß and/or RBV.

Table II. Log_2NormSignal of miRNAs in the HCV sub-genomic replicon-bearing Huh7 cells.

Log_2norm signal	Systematic name
12 to 13	miR-720, 21
11 to 12	miR-1274b
9 to 10	miR-16, **122**, 923, 20a, 130a
8 to 9	miR-1260, 15a, 15b, 19b, 1274a, 17, 25, 103
7 to 8	miR-1246, 130b, 22, 192, 92a, 19a, 107, 27b, 320d, 93, 106b, 320b
6 to 7	miR-196a, 24, 320c, 23b, 215,23a, 26a, 331-3p, 151-5p, 483-5p, 320a, 148a, 483-3p, 194, 20b
5 to 6	let-7e, miR-28-5p, 27a, 125b, 125a-5p, 99a, 210, 30b, 1268, 29a, 455-3p, 18a, 26b, 1280, 885-5p, 197, 99b, 1202, 324-3p, 494, 185, 7, 181a, 200b, 574-5p, 193b
4 to 5	let-7a, miR-122*, 1207-5p, 181b, 365, 1290, 30d, 424, 1915, 425, **574-3p**, 1225-5p, 96, 224, 940, **638**, 590-5p, 193a-3p, 221, 128, 34a, 30c, 29b, 1234, 151-3p, 100, 1275, 1308, 629*
3 to 4	let-7f, miR-1281, 1228, 551b, 1305, 625, 146a, 624*, 140-5p, 21*, 18b, 1825, 582-5p, 766, 29c, 1249, 30e, 17*, 324-5p, 342-3p, 374a, 1225-3p, 140-3p, 126, 939, 374b, 301a, 148b, 362-5p, 183, **296-5p**, 1238, 191*, **1181**, 532-5p, 30e*, 575
2 to 3	let-7c, 7b*, 7f-1*, miR-1539, 149, 423-5p, 95, 33b*, 484, 218, **196b**, 425*, **378**, 877*, 1914*, 634, 548c-5p, 660, 1237, 933, 186, **550**, 328, 181d, 222, 301b
1 to 2	miR-500*, **150***, 193a-5p, **33a**, 1826, **1227**, 454, 19b-1*, 16-2*, **194***, 503, 615-3p
<1	**let-7d*, miR-431, 448, 532-3p, 1224-3p, 595, 34b, 875-5p**

miRNA with bold face indicates >2-fold changes in response to IFN ß and/or RBV in miRNA array.

MiR-1181 was 2-fold down-modulation to RBV-stimulation only, whereas the expression of miR-196b and -638 was induced by ~2-fold by IFN ß treatment (Fig. 6B). All RT-PCR data were consistent with that of the miRNA array, suggesting that the data obtained from the miRNA array accurately reflected miRNA expression.

The relative contents of these miRNAs per cell were identified, since 16 miRNAs showed >2-fold changes to IFN ß and/or RBV stimulation in the miRNA array analysis. Low levels of these 16 miRNAs, as well as miR-196b, -296-5p, -431 and -448 in the miRNA array were determined, with their intensity (Log_2norm signal) <5/100 ng total RNA; but with the miR-122 intensity (Log_2 normsignal) >9/100 ng total RNA (Table II). RT-PCR was performed to confirm the relative low content of miR-196b, -638, and -1181, with (lg miRNA relative content) <-4; while (lg miR-122 relative content) was -0.3 (Fig. 6C). These data indicate that all miRNA tested above are less likely to contribute to antiviral activity in response to IFN ß and/or RBV.

Discussion

In the current study we found IFN ß upregulated ISG54 mRNA, and RBV had cytostatic effect in Huh7 and HCV replicon cell lines, meanwhile inhibiting HCV RNA production synergistically *in vitro*. MiR-122 could be suppressed marginally in response to IFN ß and/or RBV stimulation, while the changes of other miRNAs also were not significant.

Peg-IFN α and RBV are the most common combined anti-HCV treatment to date, because IFN α and RBV combination treatment can improve outcomes substantially in HCV patients, but ~50% HCV patients can not achieve SVR. Thus, it is necessary to understand the mechanism of IFN and RBV in anti-HCV treatment.

Recently Pederson *et al* (9) revealed that IFN ß could stimulate the expression of five miRNAs including miR-196b, which inhibited HCV replication via targeting the HCV genome. IFN ß suppressed ~80% of the expression of miR-122, which promoted HCV replication and translation. It is conceivable to envisage that IFN-induced miRNAs represent the first line of defense against HCV infection and subsequently upregulation of ISG to complete anti-viral activity.

However, Sarasin-Filipowicz *et al* (10) reported that there was 20-40% reduction of miR-122 in Huh7 cells without HCV replicon *in vitro*. Our data, in line with the previous finding, showed that there was ~10% suppression of miR-122 expression in response to IFN ß stimulation in both Huh7 cells with or without HCV replicon, the most common HCV model *in vitro*. Our results indicated that miR-122 does not play an important role in IFN ß antiviral activity. Furthermore, our novel findings showed that 16 out of 721 miRNAs were modified up to 2-fold in the miRNA assay, which might have a negligible effect due to very low basal expression. Interestingly the 16 miRNAs modified in our experiment, did not include the four reported miRNAs (miR-196b, -296-5p, -431 and -448), induced by IFN ß (9,10). Such a difference might be due to different sourcs of IFN ß and/or a difference between cell lines, although these two are of the same original. Importantly, significant upregulation of ISG54 and reduction of HCV RNA are observed in response to IFN ß stimulation *in vitro*. Therefore, these results might support the notion that the IFN system in the human liver uses the more robust JAK/STAT pathway (ISG54), instead of cellular miRNAs to combat HCV infection (18).

Low level and delayed upregulation of miR-155, was detected in primary murine macrophages with IFN ß stimulation in autocrine/paracrine fashion with close relation to TNF (19), but we were unable to detect miR-155 expression in Huh7 cells (data not shown). Additionally, some literature reports demonstrated that miRNAs in mammals were regulated by cytokines/chemokines. The first miRNA (lin-4) was reported in 1993 (20). Our results showed that there was no significant modification of miRNAs in response to IFN ß stimulation, indicating miRNAs are rarely responsible for hosting anti-viral action in mammals, but mainly in plants and insects (21).

The precise mechanism used when RBV augments the host immune response to IFN is unknown, but multiple mechanisms

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In summary, IFN ß in the human liver might not use cellular miRNAs, but a more robust JAK/STAT pathway (ISG54) to combat HCV infection. miRNAs might not be key players in IFN ß and RBV combine therapy *in vitro*.

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