Virology 398 (2010) 57–67

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Systematic identification of microRNA and messenger RNA profiles in hepatitis C virus-infected human hepatoma cells

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ARTICLE INFO

Article history:
Received 12 October 2009
Returned to author for revision
2 November 2009
Accepted 20 November 2009
Available online 14 December 2009

Keywords:
Hepatitis C virus
Acute infection *in vitro*
microRNA
mRNA
Systematic profiling

ABSTRACT

In order to investigate the global and dynamic host microRNAs (miRNAs)/messenger RNAs (mRNAs) expression alteration during *in vitro* acute HCV infection, a comprehensive microarray analysis was performed using human hepatoma cells. Totally, 108 human miRNAs and 1247 mRNAs were identified whose expression levels changed for more than 2.0-fold in response to HCV infection. Upon HCV infection, signature from the unique miRNA expression pattern reflected the involvement of miRNA-regulated host cellular physiology and antiviral mechanism, whereas a preponderance of differentially regulated genes associated with metabolism, cell growth, apoptosis and cytokine/chemokine pathways. Furthermore, a reverse regulatory association of differentially expressed miRNAs and their predicted targets was constructed. Finally, the differentially expressed miRNAs such as miR-24, miR-149*, miR-638 and miR-1181 were identified to be involved in HCV entry, replication and propagation. These results suggest that combined miRNA and mRNA profiling may have superior potential as a diagnostic and mechanistic feature in HCV infection.

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Introduction

Hepatitis C virus (HCV) is a major human blood-borne pathogen that has infected almost 170 million people worldwide and induces hepatic cirrhosis and hepatocellular carcinomas (HCC) (Choo et al., 1989; Shepard et al., 2005). Our understanding of the dynamic host response to HCV infection had been greatly hampered until the JFH-1-derived cell culture-grown HCV (HCVcc) system became available in 2005 (Cai et al., 2005; Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). This new HCVcc system now permits us to study the entire virus life cycle *in vitro* and to devise strategies for prophylactic and therapeutic interventions.

Up to date, JFH-1 or JFH-1-derived chimeras have been successfully utilized in studies such as co-receptor identification and virus transmission (Evans et al., 2007; Liu et al., 2009; Timpe et al., 2008), immune and chemokine pathways (Johansson et al., 2007; Uzri and Gehrke, 2009; Ye et al., 2009), fatty acid synthesis (Yang et al., 2008a), rRNA transcription (Raychaudhuri et al., 2009), lipid metabolism (Amemiya et al., 2008) and apoptosis (Zhu et al., 2007). Increasing evidence has demonstrated that microRNAs (miRNAs) have profound impact in modulating HCV infection and host defense (Jopling et al., 2005; Pedersen et al., 2007). Similar to small interference RNA (siRNA), miRNAs are small, endogenous non-coding RNAs that assemble into RISC complexes, guide them onto target transcripts and then bind mRNAs at their 3′ untranslated regions (UTR) and ultimately induce mRNA degradation or suppress protein translation (Bartel, 2004). Many miRNAs are regulated in response to cellular stress including infections and are known to modify essential cellular functions of antiviral effects, cell proliferation, differentiation and apoptosis (Marsit et al., 2006). For example, a recent study has implicated the liver-specific miR-122 as an essential regulator of HCV replication in cultured human hepatoma cell Huh7 (Jopling et al., 2005). Moreover, another study of chronic hepatitis C liver biopsies demonstrated that the miR-122 level was markedly lower in primary nonresponding subjects than in early virological responding subjects regardless of the HCV genotype examined (Sarasin-Filipowicz et al., 2009). Furthermore, researchers recently discovered that interferon beta (IFNβ) rapidly modulated host miRNAs, of which five showed significant anti-HCV effects in a viral sequence-specific manner (Pedersen et al., 2007). Above evidence suggests a critical role of host miRNAs in regulating HCV infection.

Although the miRNA expression profile was investigated recently in HCC to reveal the mechanisms of liver oncogenic progress (Varnholt et al., 2008), the dynamic and global changes of miRNA expressions in HCVcc-infected cell culture model has yet to be extensively elucidated. In this study, we performed comprehensive

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doi:10.1016/j.virol.2009.11.036

miRNA and mRNA profilings using HCVcc-infected Huh7.5.1 cells. The roles of several identified miRNAs were subsequently examined for their capabilities to regulate HCV infection and replication.

Results

HCV infection alters host miRNA expression in hepatoma cells

To profile global changes in miRNAs, naïve Huh7.5.1 cells were inoculated with relative high titer (MOI = 1.0) JFH-1 virus or same amount of UV-inactivated virus (HCVUV) at day 0 for overnight and then washed and allowed for further culture for 1, 2, 3 and 4 days, respectively, before total RNA preparation. Virus infection at various time points was confirmed by immunofluorescent staining of the cells with antibody against HCV structure protein Core. The percentage of Core-positive cells increased gradually over the course of infection, while the HCVUV-infected cells showed completely negative staining at all times (Supplemental Fig. 1A). To monitor the possible virus infection-caused cytotoxicity, the MTT assay was carried out. Upon inoculation of Huh7.5.1 cells with HCV or HCVUV, no significant cell death was observed except for a ~20% cell viability reduction in HCVcc-infected cells on the fourth day (Supplemental Fig. 1B). Together, we were able to establish a dynamic *in vitro* HCV infection system for further global gene expression profiling.

Subsequently, we examined the hepatocytes miRNA profiles in response to acute HCV infection with the CapitalBio multi-species miRNA microarray (version 4.0; for details, see Materials and methods), on which triplicate probes were included. After the filtration of the signals below the threshold level, 142 miRNAs (including human, non-human and predicted miRNAs) were identified as differentially expressed >2.0-fold in HCVcc-infected samples (Supplemental Tables 1–4). To confirm the microarray hybridization results, RT-qPCR was performed on five randomly selected differentially expressed miRNAs. All the selected miRNAs exhibited expressional patterns that are consistent with the microarray results (Fig. 1A), indicating the reliability of the microarray data. Unsupervised clustering (Fig. 2A) and scatter (Supplemental Fig. 2) analysis of the expression profiles revealed a distinct miRNA signature during acute HCV infection and demonstrated that the majority of the differentially expressed miRNAs were up-regulated in response to virus infection. In summary, 5 miRNAs from day 1 sample, 40 miRNAs from day 2, 63 miRNAs from day 3 and 108 miRNAs from day 4 were differentially expressed by more than two-fold after infection. In addition to day 1, the intersection of the number of co-regulated miRNAs on day 2, 3 and 4 are illustrated in Fig. 2B. The result suggests a continuous and well-regulated miRNA expression pattern through the course of infection. The most significant 22 up-regulated and 20 down-regulated miRNAs in response to HCV exposure are summarized in Table 1, in which 8 down-regulated and 10 up-regulated miRNAs with the highest fold of change (Figs. 2C and D) were selected for further functional study.

mRNA expression profile of HCV-infected hepatoma cells

A 35K human genome array was used to perform a comprehensive analysis of mRNA expression from HCV or HCVUV-infected Huh7.5.1 cells, on which we also had the miRNA expression profiles. Of 25,100 genes represented on the array, 1247 genes were differentially expressed >2.0-fold (with *P*-value <0.01, estimated false discovery rate 0.03) during various time courses between infected and control cells (Supplemental Tables 5–8). Unlike the miRNA profile, the clustering result demonstrated that the number of up-regulated and down-regulated mRNAs are almost identical between HCV-infected and control cells (Fig. 3A). Quantitative RT-PCR confirmation on randomly selected differentially expressed mRNAs from microarrays demonstrated a satisfying consistence (Fig. 1B). The overlaps of co-regulated genes on day 2, 3 and 4 showed similar pattern with miRNA

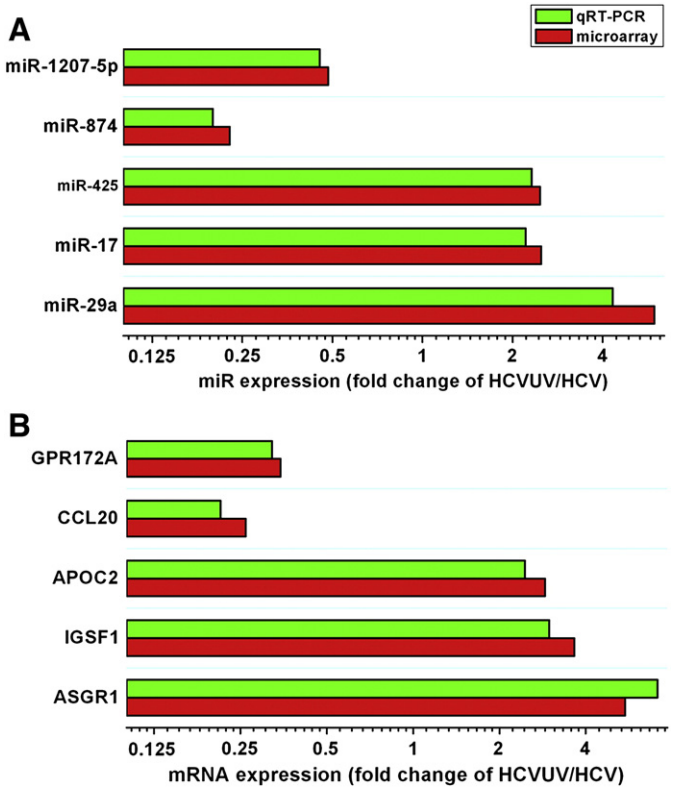

Fig. 1. qRT-PCR confirmation of randomly selected miRNAs and mRNAs from microarray. Expression comparison of randomly selected 5 miRNAs (A) and 5 genes (B) from microarray data was performed using quantitative real-time RT-PCR. Results were shown in fold change pattern of HCVUV/HCV.

profile (Fig. 3B), and the co-regulated genes among all the 3 days are summarized in Table 2. These consistently co-regulated genes may play critical roles in infection-induced cytopathogenesis and virus replication itself. Similarly, the top 25 up- and 25 down-regulated genes and their average fold of change during 4 days are showed in Figs. 3C and D. To elucidate the correlation between gene expression pattern and HCV infection-induced biological processes, functional classification of mRNA transcripts by Gene Ontology terms and pathway analysis were performed (Fig. 4). Upon *in vitro* HCV infection of hepatoma cells, serine protease inhibitor family members (SERPIN-C1, D1, F2), chemokines (CCL20, CXCL5), TGF-β superfamily members (BMP2, GDF15) and various tRNA synthetases (CARS, MARS, WARS etc.) may play central roles for HCV pathogenesis (Fig. 4B).

Correlation of miR and mRNA expression alteration during HCV infection

To determine whether miRNAs might be modulators of those mRNAs that were differentially expressed, we investigated whether those differentially expressed mRNAs are enriched for predicted targets of each of the human miRNAs in MSigDb database (http://www.broad.mit.edu/gsea/msigdb/annotate.jsp), in which gene sets contain genes sharing a 3′-UTR miRNA binding motif. To our surprise, targets for the top 10 miRNA or miRNA families account for 17% of the differentially expressed mRNAs in HCV-infected hepatocytes, whereas targets for the top 20 account for 23%, and top 50 for 31% of the differentially expressed mRNAs. Seventeen HCV-affected miRNAs or miRNA families with at lease 4 HCV-affected mRNA targets are listed in Supplementary Table 9. Although the total number of miRNAs listed in MSigDB is only 222, far less than the miRNA probes represented on the microarray, this preliminary analysis still support the notion that a relatively small number of miRNAs alteration in response to HCV infection could potentially contribute to a large number of changes in mRNA expression. Modulation of specific miRNAs might represent a

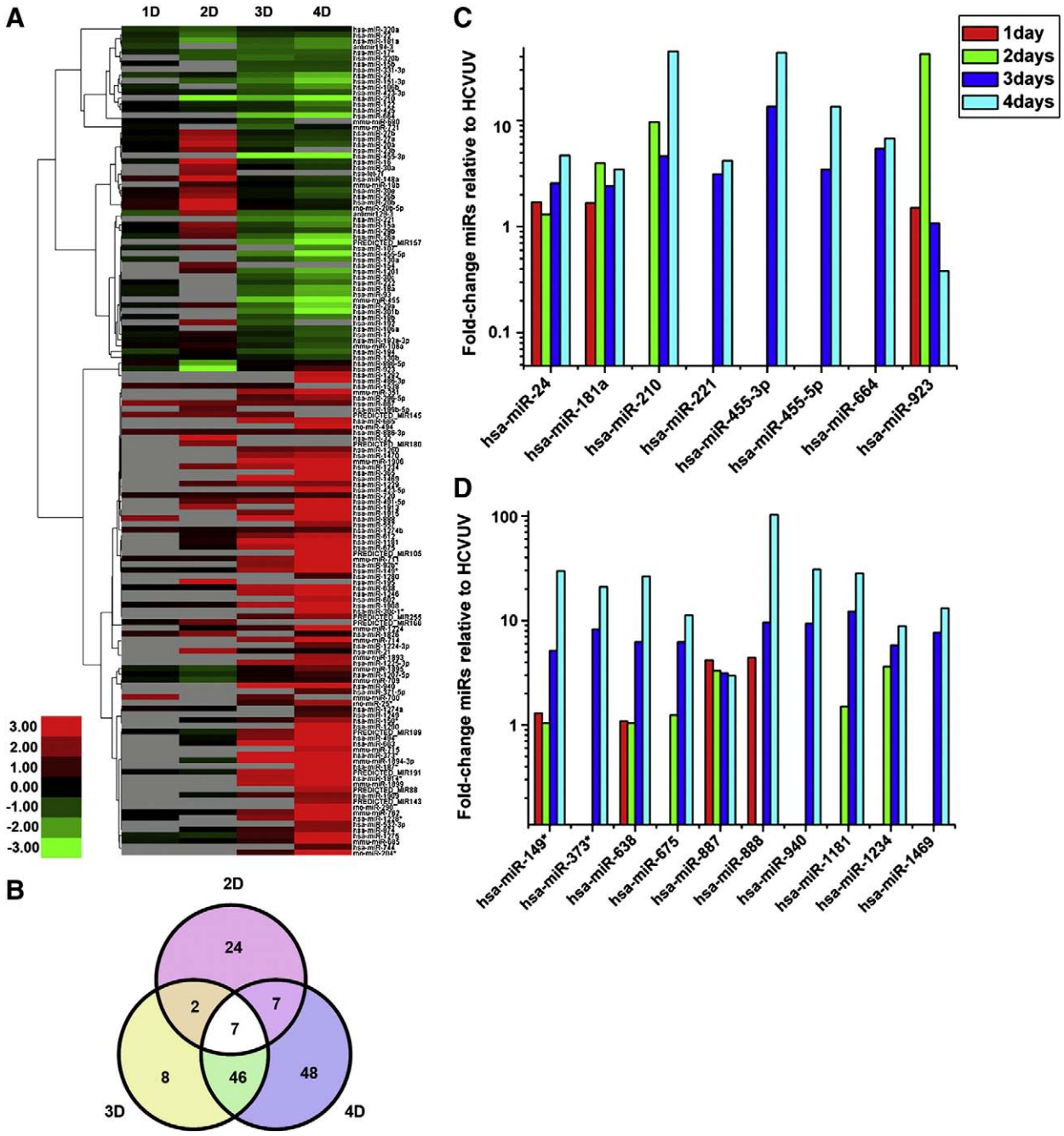

Fig. 2. miRNA expression in HCV-infected hepatocytes. (A) Unsupervised hierarchical clustering of differentially expressed miRNAs in HCV-infected Huh7.5.1 cells at different infection time points. Red indicates higher expression and green indicates lower expression in HCV-infected cells. Black means no expression difference; gray indicates signals under detection level. The small figure represents color scales used in the cluster map. (B) Overlaps of co-regulated miRNAs (>2.0-fold) across various infection time courses. Data from day 1 was omitted. Eight down-regulated (C) and 10 up-regulated (D) miRNAs with the highest expression difference ($P<0.01$) in HCV-infected Huh7.5.1 cells are demonstrated.

mechanism that contributes to a major host response to HCV propagation. To this end, we compared the reverse correlation for some of the differentially expressed miRNAs with some of their putative mRNA targets. With the use of either the PicTar or the Targetscan databases, results in Table 3 demonstrated that many differentially expressed miRNAs were inversely correlated with their predicted targets.

Potential effects of differentially expressed miRNAs on HCV entry, replication and propagation

MiRNA could exert regulatory effects on both host and the pathogen. To further investigate whether HCV life cycle is potentially affected by the selected miRNAs, 8 mimics for down-regulated miRNAs and 10 inhibitors for up-regulated miRNAs were synthesized, transfected into cells. Virus entry assay demonstrated that over-expression of hsa-miR-923 or inhibition of hsa-miR-149*, 373*, 638, 888, 940, 1181, 1234 slightly increased HCVpp entry (~1.4- to 1.6-fold) (Fig. 5A). By contrast, none of the miRNA mimics or inhibitors suppressed virus entry. When using genotype 1b replication system, gain of miR-221, 455-3p and loss of miR-887, 940 expression enhanced, but gain of miR-24 and loss of miR-149*, 373* expression suppressed HCV RNA abundance (Fig. 5B). Finally, HCVcc system was assessed. Results in Fig. 5C demonstrated that transfections of a few miRNA mimics or inhibitors into JFH1-infected Huh7.5.1 cells indeed altered HCV RNA abundance.

Discussion

Almost 30% of protein-coding genes are predicted to be regulated by miRNAs. Currently, nearly a thousand human miRNAs have been experimentally or *in silica* cloned, with each of them potentially regulating hundreds of genes by complementary binding to the 3′-UTR of target mRNAs. A number of miRNAs have been shown to play important roles in tumorigenesis, developmental timing and cell death as well as pathogen infection. The liver-specific miR-122 is the first host miRNA that exerts a positive effect on HCV replication in cell culture by binding in the viral 5′-UTR (Jopling et al., 2005). Recent report further demonstrated that the *in vivo* steady level of miR-122 correlates with the hepatitis C responsiveness to Interferon therapy (Sarasin-Filipowicz et al., 2009). Moreover, several interferon-modulated cellular miRNAs were characterized to target HCV genome and show significant antiviral effects in a sequence-specific manner (Pedersen et al., 2007). Collectively, these findings highlight the important role of host miRNAs in regulating the liver-specific HCV replication, and suggest that miRNAs might be potential targets for hepatitis C therapy. For that reason, the systematic discovery of miRNAs involved in HCV life cycle is urgently needed. Recently, several studies reported the global miRNA modulation in HCV or HCC-related liver biopsy samples (Peng et al., 2009; Ura et al., 2009; Varnholt et al., 2008) as well as the identification of 188 candidate miRNA expression modification in JFH-1 RNA transfected cells (Ura et al., 2009). However, the dynamic and high-throughput miRNA profiling during *in vitro* HCV acute infection and the potential functional significance of the differentially expressed miRNAs on HCV life cycle are still left unknown.

In this study, based on a comprehensive examination of miRNA expression from JFH-1 HCV-infected and parallel UV-inactivated virus-infected hepatocytes, we identified 142 miRNAs that are differentially expressed, with most (60%) up-regulated in HCV-infected cells. This finding is not similar to prior miRNAs study in HCV-associated HCC (Varnholt et al., 2008), in which 60% differentially expressed miRNAs were down-regulated. Also, only 4 miRNAs (miR-15a, miR-16, miR-106a and miR-199b) are overlapped between two studies. This may be because miRNA response in acute infection cell model is tightly linked to cell stress or survival and different from that in chronic infection and cancer that are more relevant to differentiation, fibrosis and tumorigenesis. Recent reports documented that host miRNAs (miR-122, miR-199a*, miR-196, miR-296,

Table 1
List of top differentially expressed miRNAs of hepatocytes in response to HCV acute infection.

microRNA	HCVUV/HCVcc (mean)				Chromosome location	Potential targets
	1D	2D	3D	4D		
Up-regulated						
hsa-miR-888	0.2262	UD	0.10435	0.00975	Xq27.3	NFAT5,NFYB
hsa-miR-940	UD	UD	0.107	0.03245	16P13.3	PDS5A, SSH2
hsa-miR-149*	0.76985	0.9603	0.19395	0.0337	2q37.7	AAK1, PHLPPL
hsa-miR-1181	UD	0.66655	0.0819	0.0355	19p13.2	NEUROD2,H1FX
hsa-miR-486-3p	UD	UD	UD	0.03645	8p11.21	FJL14154,RGAG4
hsa-miR-638	0.9198	0.96025	0.16065	0.038	19p13.2	STARD10, NPAS4
hsa-miR-92b*	UD	UD	0.2298	0.04415	1q22	P13, ARHGAP8
hsa-miR-1246	UD	UD	0.17975	0.0462	2q31.1	FAM53C,CREBL2
hsa-miR-373*	UD	UD	0.12125	0.0479	19q13.41	CROT, ZKSCAN1(373)
hsa-miR-663	UD	1.0001	0.1069	0.04915	20p11.21	GRIN2D,HSPG2
hsa-miR-1908	0.83315	0.73085	0.16485	0.05015	11q12.2	Unknown
hsa-miR-1914*	UD	UD	0.16145	0.05765	20q13.3	Unknown
hsa-miR-1228*	1.0193	UD	0.22815	0.06635	12q13.3	Unknown
hsa-miR-365	UD	UD	UD	0.0744	16p13.12	LPAR5,NRIB
hsa-miR-1469	UD	UD	0.1297	0.0763	15q26.2	Unknown
hsa-miR-1915	UD	UD	0.27235	0.07645	10p12.31	Unknown
hsa-miR-30c-1*	UD	UD	UD	0.07865	1p34.2	TREX2,HGS
hsa-miR-491-5p	UD	0.3943	0.238	0.0806	9p21.3	GATAD2B,FOXP4
hsa-miR-1290	UD	UD	UD	0.086	1p36.13	EHHADH,RTKN2
hsa-miR-675	UD	0.80215	0.1605	0.089	11p15.5	CALN1, MARK4
hsa-miR-1913	UD	0.18725	UD	0.09045	6q27	Unknown
hsa-miR-187*	UD	UD	UD	0.0953	18q12.2	ANKMY1, TRPC4
Down-regulated						
hsa-miR-18b	1.1149	UD	1.9325	3.24375	Xq26.2	NEDD9,GLRB
hsa-miR-107	1.34205	0.3951	UD	3.32925	10q23.31	DICER1,TMEM16C
hsa-miR-15a	1.08065	0.2441	1.8799	3.42875	13q14.3	AFF4,E2F3
hsa-miR-181a	1.67335	3.96905	2.41205	3.4518	1q31.3/9q33.3	AFF4,DDX3X
hsa-miR-30c	UD	UD	2.1891	3.47325	1p34.2/6q13	E2F3,MMD
hsa-miR-130a	1.4615	UD	1.91365	3.58475	11q12.1	ACSL1,CLCN5
hsa-miR-106b	1.4583	UD	2.28845	3.79565	7q22.1	JUB,EIF4G2
hsa-miR-221	UD	UD	3.1364	4.18645	Xp11.3	HRB,EIF3S1
hsa-miR-93	1.2671	UD	2.3475	4.4694	7q22.1	MAPK9,KLHL18
hsa-miR-24	1.6991	1.30595	2.5707	4.70065	9q22.32/19p13.12	AMOTL2,CCT3
hsa-miR-18a	1.3149	UD	2.39025	4.76715	13q31.3	NEDD9,GLRB
hsa-miR-1201	UD	0.46245	2.7384	4.95205	14q11.2	SAMD12,AAK1
hsa-miR-29a	1.2034	0.5192	2.5445	5.95215	7q32.3	TRAF4,RND3
hsa-miR-151-3p	UD	2.34315	3.0507	6.00525	8q24.3	RGS6,UPP2
hsa-miR-26a	1.4445	0.3561	2.40945	6.17725	3p22.2/12q14.1	CHORDC1,POLR3G
hsa-miR-664	UD	UD	5.44245	6.7645	1q41	HS6ST3,AHCTF1
hsa-miR-301b	UD	UD	3.23955	9.4938	22q11.21	MIER1,SLAIN1
hsa-miR-455-5p	UD	UD	3.45375	13.52905	9q32	PPP1R12A,GDAP2
hsa-miR-455-3p	UD	UD	13.59225	43.88985	9q32	NFIB,BRWD1
hsa-miR-210	UD	9.717	4.6329	45.1445	11p15.5	ISCU,GIT2

UD indicates undetected.

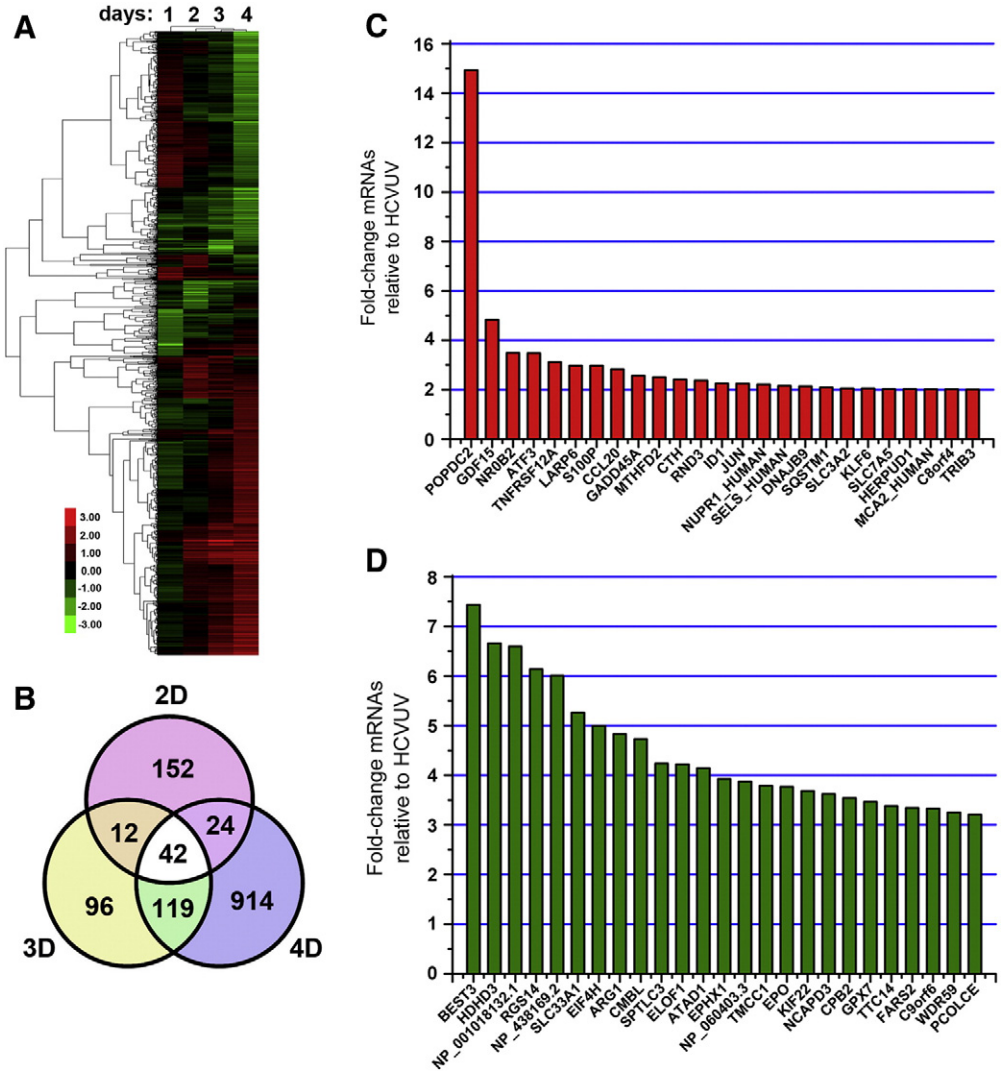

Fig. 3. mRNA expression in HCV-infected hepatocytes. (A) Unsupervised hierarchical clustering of differentially expressed mRNAs in HCV-infected Huh7.5.1 cells at different infection time points. Red indicates higher expression and green indicates lower expression in HCV-infected cells versus control. Black indicates no expression difference. (B) Overlaps of co-regulated mRNAs (>2.0-fold) across various infection time courses. Data from day 1 was omitted. The 25 most up-regulated (C) and 25 most down-regulated (D) mRNAs in HCV-infected Huh7.5.1 cells are shown in average fold change across all 4 infection days.

miR-351, miR-431 and miR-448) were able to regulate HCV replication by targeting viral genome (Jopling et al., 2005; Murakami et al., 2009; Pedersen et al., 2007). Thus, we analyzed whether these miRNAs are differentially expressed in response to virus infection as well. Interestingly, upon HCV acute infection in hepatocytes, miR-122 was down-regulated (2.8-fold), whereas miR-296 (4.9-fold) and miR-351 (15.2-fold) were up-regulated significantly at day 4 post-infection. Such results suggest a host defense response is perhaps mounted to elevate the levels of those antiviral miRNAs and suppress those are supportive to virus infection. This might be also caused by the indirect effects of HCV-induced interferon production.

Because we are unclear how many differentially expressed miRNAs in this study are truly regulators of HCV infection, 18 miRNAs with the highest expression variation and without any previous clue to HCV infection were selected for further functional study, in which 8 miRNAs are down-regulated and 10 up-regulated (Fig. 5). Several tested miRNAs indeed showed regulatory effects on HCV entry or replication, although results obtained from three experimental systems are hardly consistent. Especially, the lack of concordance of the effects of miRNAs between replicon system and HCVcc system is unexpected (Figs. 5B and C). On one hand, this may be explained by the characteristics of multiple targets of individual miRNA and these different gene targets exert distinct biological function on HCVcc life cycle. On the other hand, different genotypes, adaptive mutations, with or without an entry step between HCVcc and replicon systems might all potentially make those differences. In general, results from Fig. 5 suggest us that host miRNAs could be efficient therapeutic targets for HCV infection by modulating their expression. Here we take miR-24 for instance, it belongs to miR-23b cluster that targets Smads and suppresses TGF-β/BMP signaling in liver cells (Rogler et al., 2009). In this study, miR-24 was down-regulated post-HCV infection (Table 1), and we calculated the fold change of the intracellular HCV RNA abundance is ~2-fold decreased after the transfection of miR-24 mimic into HCVcc-infected Huh7.5.1 cells (Fig. 5B). These results suggest that host TGF-β signal pathway might be potentially activated upon HCV infection and thereafter a liver fibrosis progress was accelerated. Thus, systematic administration of miR-24 might not only decrease virus replication level but also inhibit hepatic fibrosis.

Several methods and studies have been established to address the regulatory relationships between miRNA and mRNA expression (Hon and Zhang, 2007; Marcucci et al., 2008). Our simultaneous collecting miRNA and mRNA data from the same samples allowed unveiling correlations of two sets expression profile and their potential

Table 2
List of most consistently modified genes of hepatocytes in response to HCV acute infection.

Gene symbol	Gene name	HCVUV/HCVcc (mean)				Molecular function	Cellular component
		1D	2D	3D	4D		
Up-regulated							
POPDC2	Popeye domain containing 2	0.0189	0.083	0.0794	0.0866	Unknown	Membrane; integral to membrane;
GDF15	Growth differentiation factor 15	0.605	0.0977	0.0601	0.0658	Cytokine activity; growth factor activity	Extracellular space
NR0B2	Nuclear receptor subfamily 0, group B, member 2	0.5356	0.1903	0.1504	0.2712	Transcription corepressor activity; steroid hormone receptor activity; transcription factor activity	Nucleus
ATF3	Activating transcription factor 3	0.873	0.14	0.0681	0.0674	Sequence-specific DNA binding; transcription corepressor activity; protein dimerization activity; transcription factor activity	Nucleus
TNFRSF12A	Tumor necrosis factor receptor superfamily, member 12A	0.5961	0.3159	0.1509	0.2217	Protein binding; receptor activity	Membrane; integral to membrane
LARP6	La ribonucleoprotein domain family, member 6	0.1035	0.4878	0.3878	0.3633	RNA binding; nucleotide binding	Cytoplasm; nucleus; ribonucleoprotein complex
S100P	S100 calcium binding protein P	0.711	0.2976	0.1297	0.2045	Magnesium ion binding; calcium-dependent protein binding; calcium ion binding	Cytoplasm; nucleus
CCL20	Chemokine (C-C motif) ligand 20	0.6872	0.3037	0.1598	0.261	Chemokine activity	Extracellular space
GADD45A	Growth arrest and DNA-damage-inducible, alpha	0.9292	0.2692	0.1776	0.1808	Unknown	Nucleus
MTHFD2	Methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2, methenyltetrahydrofolate cyclohydrolase	1.1215	0.2254	0.1257	0.1206	Hydrolase activity; methylenetetrahydrofolate dehydrogenase (NADP+) activity; oxidoreductase activity; magnesium ion binding; methylenetetrahydrofolate dehydrogenase (NAD+) activity; phosphate binding; methenyltetrahydrofolate cyclohydrolase activity	Mitochondrion
CTH	Cystathionase (cystathionine gamma-lyase)	0.85	0.2407	0.2955	0.2733	Cystathionine gamma-lyase activity; lyase activity	Unknown
RND3	Rho family GTPase 3	1.2063	0.2058	0.1855	0.088	Nucleotide binding; GTP binding	Golgi stack; intracellular
ID1	Inhibitor of DNA binding 1, dominant-negative helix–loop–helix protein	1.2371	0.4633	0.8375	0.9334	Protein binding; transcriptional repressor activity	Nucleus
JUN	jun oncogene	1.0542	0.1612	0.1988	0.3727	RNA polymerase II transcription factor activity; sequence-specific DNA binding; protein dimerization activity; transcription factor activity; transcription factor binding	Nuclear chromosome
DNAJB9	DnaJ (Hsp40) homolog, subfamily B, member 9	1.0705	0.3515	0.189	0.2561	Chaperone regulator activity; unfolded protein binding; heat shock protein binding	Nucleolus; cytoplasm; nucleus
SQSTM1	Sequestosome 1	1.1484	0.3813	0.2433	0.1389	Ubiquitin binding; metal ion binding; SH2 domain binding; protein kinase binding; zinc ion binding	Cytosol; nucleus
SLC3A2	Solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2	1.0499	0.5901	0.1981	0.11	Calcium:sodium antiporter activity; alpha-amylase activity	Cell surface; membrane; integral to membrane
KLF6	Kruppel-like factor 6	1.1845	0.3343	0.1756	0.2575	Metal ion binding; zinc ion binding; DNA binding; transcriptional activator activity	Nucleus
SLC7A5	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 5	1.1196	0.5201	0.2184	0.1145	Amino acid permease activity; neutral amino acid transporter activity	Plasma membrane; integral to membrane
HERPUD1	Homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	1.0333	0.4744	0.2692	0.3896	Unknown	Endoplasmic reticulum membrane; membrane; integral to membrane
TRIB3	Tribbles homolog 3 (Drosophila)	1.4303	0.2994	0.1254	0.1423	Protein kinase inhibitor activity; ATP binding; protein binding; protein kinase binding; protein kinase activity; transcription corepressor activity	Nucleus
Down-regulated							
HDHD3	Haloacid dehalogenase-like hydrolase domain containing 3	1.7177	7.1833	15.8024	1.9188	Hydrolase activity; phosphoglycolate phosphatase activity	Unknown
RGS14	Regulator of G-protein signaling 14	4.0125	2.6803	4.8661	12.9926	Receptor signaling protein activity; GTPase activator activity	Unknown
EIF4H	Eukaryotic translation initiation factor 4H	1.6982	1.5803	15.4493	1.257	Translation initiation factor activity; RNA binding; nucleotide binding	Eukaryotic translation initiation factor 4F complex
ARG1	Arginase, liver	1.0522	1.378	1.5378	15.3488	Extracellular matrix structural constituent; cysteine-type endopeptidase activity	Extracellular region
ELOF1	Elongation factor 1 homolog (S. cerevisiae)	1.2793	6.7626	6.5184	2.3069	Metal ion binding; zinc ion binding	Nucleus
ATAD1	ATPase family, AAA domain containing 1	1.5024	0.8722	13.5673	0.6332	ATP binding; nucleotide binding; nucleoside-triphosphatase activity	Unknown
EPHX1	Epoxide hydrolase 1, microsomal (xenobiotic)	1.209	2.5857	10.2859	1.6187	Hydrolase activity; epoxide hydrolase activity	Endoplasmic reticulum; microsome; membrane; integral to membrane
TMCC1	Transmembrane and coiled coil domains 1	0.8573	1.1731	1.4995	11.6291	Unknown	Membrane; integral to membrane

Table 2 (*continued*)

Gene symbol	Gene name	HCVUV/HCVcc (mean)				Molecular function	Cellular component
		1D	2D	3D	4D		
EPO	Erythropoietin	1.271	1.3359	1.444	11.0151	Hormone activity; erythropoietin receptor binding	Extracellular space
KIF22	Kinesin family member 22	0.9736	10.7807	1.5804	1.3917	ATP binding; nucleotide binding; microtubule motor activity; sequence-specific DNA binding	Microtubule associated complex; kinetochore; nucleus; microtubule
CPB2	Carboxypeptidase B2 (plasma)	1.5843	2.1498	2.9224	7.5	Metal ion binding; zinc ion binding; metallopeptidase activity; carboxypeptidase activity; carboxypeptidase A activity	Unknown
GPX7	Glutathione peroxidase 7	3.8581	7.3974	1.6027	1.0054	Oxidoreductase activity; glutathione peroxidase activity	Unknown
TTC14	Tetratricopeptide repeat domain 14	1.2936	9.5299	1.8073	0.889	RNA binding;	Unknown
FARS2	Phenylalanyl-tRNA synthetase 2, mitochondrial	6.474	1.0593	3.332	2.5128	Phenylalanine-tRNA ligase activity; ATP binding; tRNA binding; ligase activity; nucleotide binding	Soluble fraction; mitochondrion
PCOLCE	Procollagen C-endopeptidase enhancer	2.7612	1.8774	3.2183	4.9729	Collagen binding	Unknown

regulatory network during HCV infection. Within the top 10 differentially expressed miRNAs that correlate with differentially expressed mRNAs discovered in this study (Table 3), several of them have been implicated in other diseases and biological process. For instance, miRNAs encoded by the miR-15/16 cluster are known to be involved in apoptosis (Cimmino et al., 2005; Guo et al., 2009), Nodal signaling (Martello et al., 2007) and tumorigenesis (Bonci et al., 2008). miRNAs encoded by the miR-17/miR-106b cluster are important for epithelial morphogenesis (Carraro et al., 2009), hepatocellular carcinoma (Li et al., 2009) and transforming growth factor beta signaling (Petrocca et al., 2008). miR-29a/b are involved in host-HIV-1 interaction (Nathans et al., 2009), p53 pathway (Park et al., 2009) and skeletal myogenesis (Wang et al., 2008). These findings suggest that the expression modification of host miRNAs during acute HCV infection could be related to a number of cellular physiological processes that eventually control the cell fate after combating with virus.

In this study, obtaining mRNA expression profile together with miRNA profiling cannot only provide a systematic data for miRNA regulatory network construction but also implicate the more accurate biological significance for HCV acute infection based on our existing knowledge. Recently, Walters and colleagues (2009) reported J6/JFH chimeric HCV infection-induced hepatocytes mRNA profiling using similar strategy, and suggested the potential regulation of cell cycle and apoptosis-related genes. By comparing these two sets data of mRNA profiling, a significant coherence was observed. Among 42 differentially expressed genes identified in this study across 2–4 days post-infection, total 32 (76%) of them were also reported to be regulated in Walters' study (data not shown). The consistent results from two independent experiments suggest the conserved HCV infection-stimulated cellular responses and cytopathic mechanisms existed. After carefully analyzing those differentially expressed mRNAs, we are interested in a number of genes that may deserve for further characterization. Among them, the expression of several circulating factors, including lipoprotein metabolism components (APOA1, APOC1, APOC2, APOC3, APOH, APOM), bone morphogenetic protein 2 (BMP2), erythropoietin (EPO), transforming growth factor, beta-induced (TGFBI) and growth and differentiation factor 15 (GDF15), was significantly regulated, which suggests that these factors might be prognostic biomarkers and dysregulate host homeostasis after HCV infection. The notable suppression of tight junction protein Claudin-14 (CLDN14) after infection implicated the potential role of CLDN14 on HCV entry (Evans et al., 2007; Yang et al., 2008b). The mechanisms of chronic hepatitis C-related hepatocellular carcinoma are still under debating between pathogen and immunological stress-induced tumorigenesis. Interestingly, our mRNA profiling results demonstrated that among differentially expressed genes, all 7 oncogenes (MYC, DEK, ELF3, JAK1, KLF6, KRAS and PTPN11) were up-regulated, but the only tumor suppressor fumarate hydratase (FH) was down-regulated. This result provided evidence that virus propagation stimulated liver gene modification might exert important function on HCV-induced hepatocarcinogenesis.

In summary, these findings suggest that host miRNAs are an important category of targets and may play a role in regulating the gene expression response to HCV infection in liver cells. miRNA and mRNA profiles obtained from these cells might therefore serve as biomarkers for HCV-related liver diseases and help elucidate the regulatory mechanisms that mediate the host response to HCV exposure and other pathogens.

Materials and methods

Cells, HCVcc production and infection

The human hepatoma cell line Huh7.5.1 was provided by Dr. Francis V. Chisari (The Scripps Research Institute, La Jolla, CA). The HCV genotype 1b-replicon-containing cell line ($2^{-}3^{+}$) was a generous gift from Dr. Stanley Lemon (University of Texas Medical Branch, Galveston, TX). HEK293T cells were obtained from ATCC. All cell lines were maintained in DMEM (Gibco/Invitrogen, Carlsbad, CA) supplemented with 1% Penicillin and streptomycin (Gibco), 1% NEAA (Gibco) and 10% fetal bovine serum (Gibco) in a 37 °C 5% CO_2 atmosphere. G418 (500 μg/ml) was routinely added into the $2^{-}3^{+}$ cells to maintain the viral RNA replication. The production of HCVcc (JFH-1) was performed as described (Wakita et al., 2005). Briefly, JFH-1 genome RNA was *in vitro* transcribed with RiboMAX Large Scale RNA Production Systems (Promega, Madison, WI), purified with MEGAclear Kit (Ambion) and transfected into Naïve Huh7.5.1 cells with TransMessenger Transfection Reagent (Qiagen). The transfected cells were cultured and routinely expanded for 10 days and the collected virus containing supernatants were pooled and further used to inoculate naïve Huh7.5.1 cells. This supernatant collection/naïve cells inoculation process was repeated at lease for 5 cycles with 7 days each cycle to produce large volume HCVcc stock with relatively high viral titer. For infections, HCVcc (MOI = 1.0) and same amount of parallel ultra-violet inactivated virus (HCVUV) were used to inoculate 5×10^{6} naïve Huh7.5.1 cells and incubate overnight. Inactivation of HCV by UV was achieved by exposing virus-containing supernatant in Petri dish (0.5 cm depth) to 254 nm UV source at 1.8 Jm^{-2} per second for 30 min. The infected cells were washed and maintained in fresh medium for 1, 2, 3 and 4 days, respectively, prior to microarray analysis.

Microarrays and bioinformatics

Total RNA was isolated from HCV or HCVUV-infected Huh7.5.1 cells with Trizol reagent (Invitrogen). miRNA and mRNA hybridization was performed by CapitalBio Corporation (Beijing, China) with

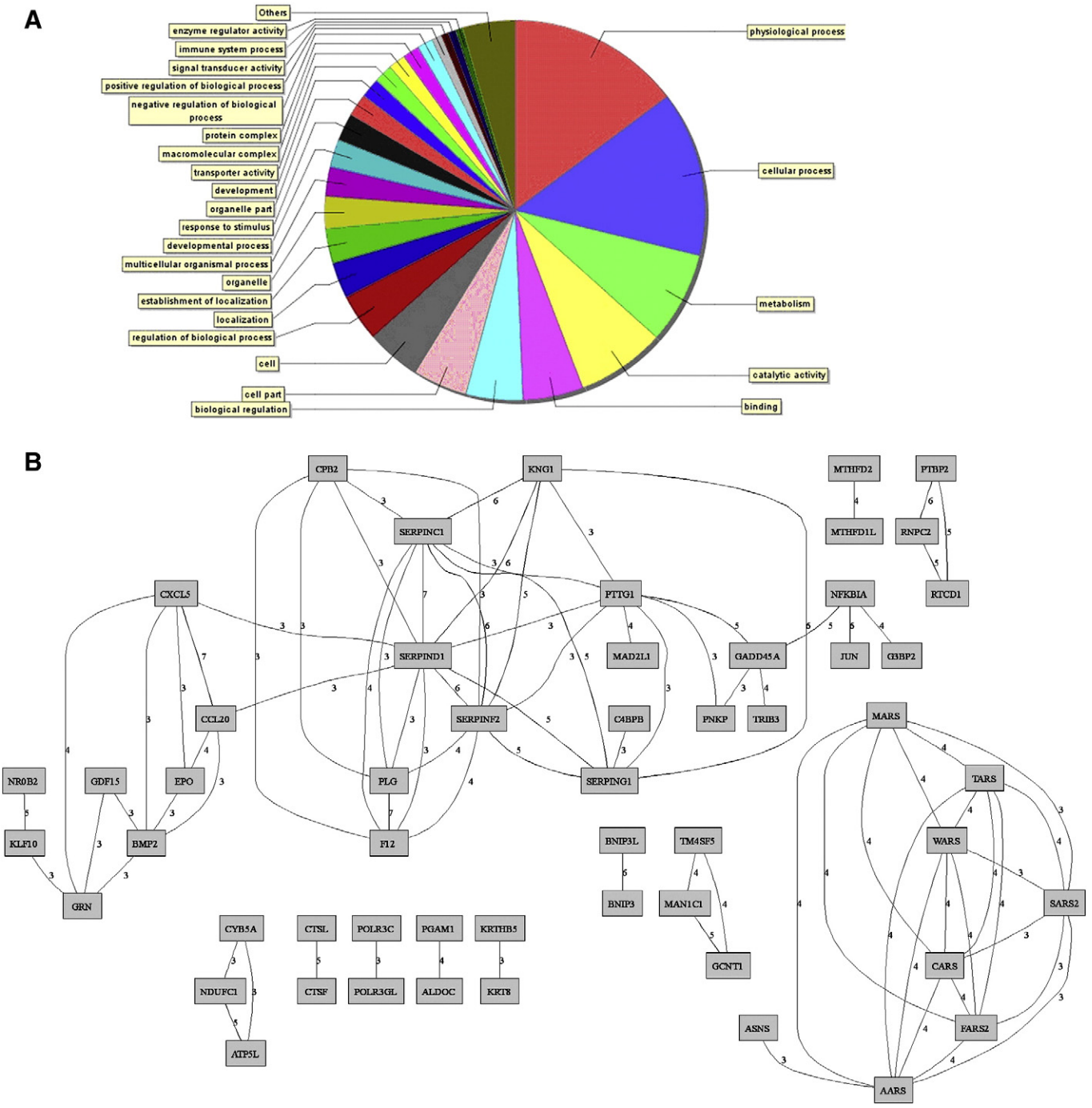

Fig. 4. (A) Significantly enriched Gene Ontology terms in the biological process category among differentially expressed genes. (B) Gene and pathway association map of differentially expressed mRNAs in Huh7.5.1 cells in response to HCV infection.

the use of mammalian multi-species miRNA microarray version 4.0 (total 1,320 oligo probes recognizing 988 human, 350 rat and 627 mouse mature miRNAs) and 35K Human Genome Array (total 35,035 70-mer oligo probes from Operon, representing 25100 human genes). miRNA nomenclature in this study is based on miRBase 12.0, September 2008. A two-channel microarray technology was employed. For each sample pair, the experiments were done with two independent hybridizations (Cy3 and Cy5 interchanging labeling). Raw data were extracted from the TIFF images using LuxScan 3.0 software (CapitalBio). Hierarchical cluster, gene ontology and pathway analysis were carried out with Gene Cluster 3.0, Eisen's Treeview (Stanford University, Palo Alto, CA), as well as Molecule Annotation System V4.0 (MAS: http://bioinfo.capitalbio.com/mas/).

Quantitative real-time RT-PCR (RT-qPCR)

For selected mRNA RT-qPCR, total RNA from the same samples used in microarray analysis was tested using the ABI Prism 7500 System. Real-time quantification was employed using SYBR Green PCR Master Mix (ABI). PCR primers were designed with AB PRISM Primer Express 2.0 software. Results are shown as fold change. For miRNA RT-qPCR, experiments were carried out with the miRNA Reverse Transcription kit (Applied Biosystems) and miRNA Taqman Expression Assays (Applied Biosystems) according to manufacture's instruction. Quantification of HCV genomic RNA was performed with real-time RT-PCR as described in reference (Komurian-Pradel et al., 2001).

Table 3
Reverse regulatory association of most differentially expressed miRNAs and their predicted mRNA targets.

miRNA (or family)	miRNA change	Target mRNAs	Target change
hsa-miR-15a, 15b	Down	AFF4, DDX3X, E2F3, ENAH, PITPNA, KLHL18, KIF21A, MAPRE1, ACSL1, ARHGAP5, SPRYD3, CAB39, FAM60A, KIAA0182, CLCN5, MMD, CDV3, ARL8B, TMEM87A, SPSB4, TRAM1, CHORDC1, KIF5B, HIRA, SUPT16H, HIGD1A	Up
hsa-miR-17,106a, 106b	Down	AFF4, E2F3, MAPRE1, FAM60A, PLCB1, M6PR, ARID4B, SOX4, BAMBI, RNF6, CAMTA2, SUV420H1, JOSD1, AKAP13, EIF4G2, SFRS2, PAPOLA, ADAM9, CRK, MAPK9, MKRN1, TMEM16F, DNAJB6, LPGAT1, BTG3, DDX5, DNAJB9, GRAMD1A, CALD1, NKIRAS1, JUB, NEDD4L, WAC, SQSTM1	Up
hsa-miR-181a	Down	AFF4, DDX3X, ENAH, ACSL1, KIAA0182, PALLD, AP1G1, G3BP2, ZFP36L1, MAT2A, HRB, TULP4, TGFBI, PALM2, IVNS1ABP, RNF6, CAMTA2, DUSP5, TARDBP, PHF3, SLC38A2, SFRS7, DOCK7, KPNB1, RNF34, RAN, GPD1L, KLF6	Up
hsa-miR-29a, 29b	Down	AFF4, DDX3X, PITPNA, GPAM, ZFP36L1, MAT2A, CTNND1, TDG, RCC2, CHIC2, WDR26, SUV420H1, JOSD1, AKAP13, RND3, GNG12, SMARCC1, COL2A1, SPAST, ARVCF, MGAT4B, ABCE1, TXNDC4, DNAJB11, RND1, TNFRSF1A, TRAF4, TUBB2B	Up
hsa-let-7f	Up	TRAPPC1, DMD, MYCBP, PIGA, RNF5, GNG5, IGSF1	Down
hsa-miR-93	Down	KLHL18, EPHA2, ARID4B, BAMBI, RNF6, SUV420H1, PAPOLA, ADAM9, CRK, MAPK9, MKRN1, TMEM16F, TARDBP, MTUS1, YWHAZ, TIPARP	Up
hsa-miR-130a, 130b, 310	Down	ACSL1, KIAA0182, CLCN5, PLCB1, AP1G1, G3BP2, KIAA0152, ANKRD12, M6PR, HRB, ARID4B, SOX4, RNF38, NRBF2, GJA1, RBBP8, CLTC, SUV420H1, TARDBP, PHF3, GADD45A, CANX, VPS24	Up
hsa-miR-30a, 30c, 30e	Down	E2F3, MMD, ARL8B, AFF4, TMEM87A, SEMA6A, MAT2A, TDG, TULP4, PALM2, ARID4B, SOX4, GJA1, KLF10, JOSD1, PAPOLA, ADAM9, LPGAT1, NEDD4L, SLC38A2, SFRS7, SPAST, YWHAZ, AMOTL2, KRAS, VAPA, EXTL2, GTF2H1, BECN1, RGS2, LRRC8D, HSPA5, CARS	Up
hsa-miR-24	Down	AMOTL2, KIAA0152, CDV3, JUB, CCT3, DHX30, FST, AARS	Up
hsa-miR-221, 222	Down	HRB, CDV3, EIF3S1	Up

Immunostaining and confocal microscope

HCV or HCVUV-infected Huh7.5.1 cells were fixed in 2% paraformaldehyde (dissolved in PBS, pH7.2) for 10 min at room temperature (RT). Cells were then permeabilized with 0.2% (v/v) Triton X-100 in PBS three times (PBST; 10 min each time, RT) and incubated in PBS (overnight at 4°C) with 1:200 diluted antibody against HCV Core protein (Affinity BioReagents, Golden, CO). Samples were washed in

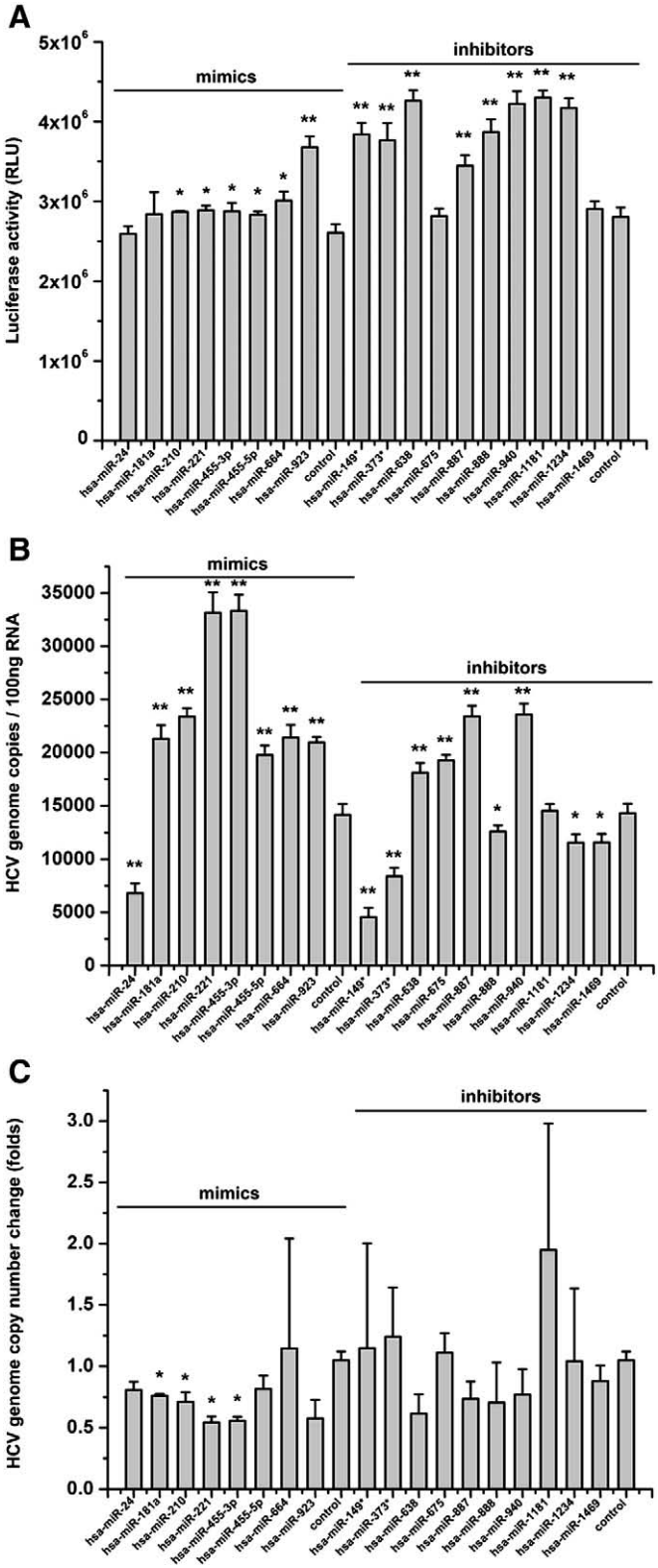

Fig. 5. Potential function of differentially expressed miRNAs on HCV life cycle. Eight synthetic mimics for down-regulated miRNAs, 10 inhibitors for up-regulated miRNAs and the miRNA mimic or inhibitor controls with no specific targets were transfected into naïve Huh7.5.1 cells respectively for determination of the function of selected miRNAs on HCV entry (A) and HCVcc propagation (C). Similar strategy was employed on $2^{-}3^{+}$ HCV replicon cells harboring genotype 1b full-length genome for determination of miRNA effects on HCV RNA abundance (B). Two-sample independent *t*-test statistics was performed between the individual transfection and correspondent control. One asterisk (*) stands for $P<0.05$ and double asterisk (**) for $P<0.01$.

PBS and sequentially incubated with FITC-conjugated secondary antibody (Santa Cruz) diluted in PBS (1:100). Samples were finally washed three times in PBS and mounted on a slide. Nucleus was visualized with DAPI staining during the second wash. Images were captured on a Leica TCS SP5 confocal microscope.

Cytotoxicity assays

Naïve Huh7.5.1 cells were plated in quadruplicate in 96-well plates at a density of 1×10^4 cells/well 1 day before infection, followed with HCV or HCVUV infection at MOI = 1.0 for various days. Cell viability and cytotoxicity were examined with MTT assay kit (Promega) according to manufacture's instruction.

MiRNA transfection

Selected miRNA mimics and inhibitors were synthesized by Qiagen. MiRNA mimics are chemically synthesized double stranded miRNAs which mimic naturally occurring miRNAs after transfection into the cell. miRNA inhibitors are single-stranded, modified RNAs which, after transfection, specifically inhibit miRNA function. These miRNA mimics and inhibitors were transfected into naïve Huh7.5.1 cells at a final concentration of 50 nM with HiPerFect Transfection Reagent according to Qiagen recommended protocol. Twenty-four hours post-transfection, the cells were spin infected with lentivirus particles pseudotyped with HCV E1 and E2 glycoproteins (HCVpp) to evaluate the effects of miRNAs on HCV entry. For 2^-3^+ replicon cells, total RNA was isolated 24 h post-transfection with miRNA mimics and inhibitors for HCV replication quantification by real-time RT-PCR. For the assessment of miRNA effects on HCVcc infection, naïve Huh7.5.1 cells were infected with HCVcc at low MOI (= 0.1) on the first day and followed with miRNA mimics and inhibitors transfection on the second day. HCV genome quantification by qRT-PCR was performed on the fourth day post-infection. All experiments were carried out at triplicates. To confirm the efficiency of transfection, the same amount of Cy3-labelled negative control (Ambion) was also transfected and followed by fluorescent microscope observation.

Production of HCVpp and the entry assay

HCVpp packaging was performed as described elsewhere (Yang et al., 2008b). Briefly, HEK293T cells were seeded 1 day before transfection at 2.5×10^6 cells in a 10-cm plate. Cells were transfected using TransMax Transfection Reagent (Giantagen, Beijing, China). The transfecting DNA mixture (1 ml) was composed of 15 μg of pNL-4.3-Luc-E^-R^-, either 15 μg of phCMV-HCV E1E2 (genotype 1a) or 3 μg of pHEF-VSV-G. Culture supernatants containing HCVpp or VSVpp (lentivirus particles pseudotyped with VSV-G glycoprotein) were collected at 48, 60 and 72 h post-transfection and filtered through 0.22-μm syringe filter. For virus entry assay, Huh7.5.1 cells were seeded in a 24-well plate at the density of 1×10^5/well on the day before transduction. On the following day, the cells will be supplemented with 500 μl of HCVpp or VSVpp containing supernatants in the presence of 8 μg/ml of polybrene and 5 μl of 2 M HEPES (pH 7.55) and spin infected for 1.5 h in a table-top centrifuge (2500 rpm, 30 °C) and followed by another 1.5 h incubation in a CO_2 cell incubator. Cells were lysed at 48 h post-spin infection and assayed with Luciferase Assay System (Promega) in a Modulus Microplate Luminometer (Turner BioSystems). All experiments were performed in triplicates. To obtain the specific HCV envelope proteins (E1/E2) mediated cell entry, normalization of HCV entry assay was carried out by dividing of HCVpp relative luciferase units (RLU) by relative VSVpp variation folds.

Acknowledgments

This work was supported by Chinese National Key Project (2008ZX10002-014, 2009ZX10603), National Natural Science Foundation of China (30970156), and an intramural grant from the Institute of Pathogen Biology, Chinese Academy of Medical Sciences (2008IPB107) to W. Y.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2009.11.036.

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