Kinetics of miR-122 Expression in the Liver during Acute HCV Infection

Youkyung Choi1, Hans-Peter Dienes2, Kris Krawczynski1*

1 Division of Viral Hepatitis, Centers for Disease Control and Prevention, Atlanta, Georgia, United States of America, 2 Institute of Pathology, University of Cologne, Cologne, Germany

Abstract

The relationships among micro RNA-122 (miR-122) expression in the liver, hepatitis C virus (HCV) replication and hepatic damage were analyzed in three chimpanzees observed for 180 days after inoculation with HCV genotype 1a. Levels of miR-122 in the liver and serum were measured by real-time RT PCR in serial liver biopsies and serum samples. Hepatic miR-122 levels were normalized separately for each of the three chimpanzees with small RNAs and microRNAs that are endogenous to the liver and are stably expressed. Two- to 4-fold rise in hepatic miR-122 levels was observed at the onset of HCV infection (the first 4 weeks) when HCV titers in the liver and serum increased rapidly in all three chimpanzees in concordance with in vitro data indicating the miR-122 significance for HCV replication. Between 10 to 14 weeks after inoculation, when hepatic and serum HCV RNA titers exceeded 3 logs and alanine aminotransferase (ALT) activity was elevated, hepatic miR-122 levels were in decline. Cumulative data derived from all three chimpanzees from 180 days of observation documented an inverse (negative) correlation between hepatic miR-122 and HCV RNA in the liver and serum and positive correlation between level of serum miR-122 and HCV replication. Subsequent rise of miR-122 level during HCV clearance and ALT normalization suggested a tri-phasic occurrence of the relationship among hepatic miR-122 expression, HCV replication and hepatic destruction, which was the most apparent in one chimpanzee but less evident in two other animals. In vivo kinetics of hepatic and serum miR-122, HCV replication and hepatic destruction reflects complexities of the virus-host interaction during the acute phase of HCV infection.

Citation: Choi Y, Dienes H-P, Krawczynski K () Kinetics of miR-122 Expression in the Liver during Acute HCV Infection. PLoS ONE 8(10): e76501. doi:10.1371/journal.pone.0076501

Received March 7, 2013; Accepted August 27, 2013; Published October 4, 2013

Editor: Birke Bartosch, Inserm, U1052, UMR 5286, France

This is an open-access article, free of all copyright, and may be freely reproduced, distributed, transmitted, modified, built upon, or otherwise used by anyone for any lawful purpose. The work is made available under the Creative Commons CC0 public domain dedication.

Funding: The study was funded from Centers for Disease Control budget. Financial Management Office of CDC had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: kzk1@cdc.gov

Introduction

More than 170 million people world-wide are estimated to be chronically infected with HCV, and in the USA, about 25,000 individuals every year are newly infected by HCV. Viral persistence commonly follows primary infection, which may result in liver fibrosis, cirrhosis, and hepatocellular carcinoma [1,2]. Among a variety of factors identified to influence HCV replication, microRNA (miR)-122 and miR-199 have been reported to be of particular significance [3–7]. MicroRNAs are small, noncoding RNA molecules of 19–22 nucleotides in length able to repress gene expression of a broad array of targeted transcripts by either RNA interference or impairment of translational initiation and elongation. MicroRNAs are implicated in a wide variety of cellular processes like cell differentiation, proliferation, and apoptosis [8,9]; miR-122 is specifically and abundantly expressed in hepatocytes, comprising approximately 70% of total miRNA in the liver [10].

In vitro studies showed high expression of miR-122 in Huh7 cells hosting HCV replication and documented interaction of miR-122 with the 5′-untranslated region (UTR) of the HCV RNA genome [4,11,12]. Other studies confirmed that miR-122 regulates the abundance and production of infectious HCV [13]. Although miR-122 does not directly stimulate HCV RNA synthesis [14], it facilitates HCV replication by recruiting an RNA-induced silencing complex (RISC) containing Ago2 (Argonaute-2) protein, mediating the stabilization of HCV, and slowing the 5′ decay of the viral genome in infected cells [15–17]. Furthermore, it was suggested that miR-122 triggers HCV replication by post-transcriptional repression of heme oxygenase enzyme synthesis [7].

In vivo experiments in chronically HCV-infected chimpanzees treated with antisense miR-122-locked nucleic acids revealed significant repression of HCV replication and further implicated miR-122 in virus replication [18]. In view of these findings, blockage of miR-122 has been considered as a therapeutic approach against chronic hepatitis C [19]. During acute and chronic HCV infections, responses from type 1 interferon [interferon alpha (IFN-α) and interferon beta (IFN-β)] produced in the liver are a part of the innate immune response against HCV infection inducing mediators of antiviral responses such as protein kinase K, the 2′ 5′- oligoadenylate synthetase, the adenosine deaminase ADR1, and the Mx GTPase [20]. IFN-β treatment can lead to a significant reduction in the expression of miR-122 levels in vivo [11], although no significant correlation has been observed between miR-122 levels and serum HCV RNA titer in chronic hepatitis C patients with IFN-α therapy [21].

Most of the studies outlined above were conducted using in vitro models. In this study, hepatic and circulating miR-122 levels were measured serially in chimpanzees undergoing acute HCV infection in order to determine the association between miR-122...
expression, HCV replication, and ensuing liver pathology. Critical to the investigation was the development and application of protocols for normalizing hepatic miR-122 expression levels using small RNAs and microRNAs expressed in the liver.

Materials and Methods

HCV infection in chimpanzees

All animal procedures were approved by the Institutional Animal Care and Use Committee at CDC and in accordance with the guidelines of the Guide for the Care and use of Laboratory Animals. CDC primate protocol number: 1363KRACHIC. The study was done between Sept.23, 2004 and March 22, 2005. The animals were single or pair housed in accordance with the Guide for the Care and Use of Laboratory Animals in an AAALAC accredited facility. Chimpanzees were housed individually for short periods of time due to the restrictions of the scientific protocol involving the infectious nature of hepatitis research. A 12:12:12 light:dark cycle was used at a room temperature of 17.8 to 28.9°C and a relative humidity of 30% to 70%. Water was provided ad libitum through an automatic watering system. The diet consisted of a nonhuman primate chow (Lab Diet High Protein Monkey Diet 5045, PMI Nutrition International, LLC, Saint Luis, MO), fruits, and treats (Bio-serv, Frentonch, NJ). Environmental enrichment plan for chimpanzees consisted of the following: social enrichment, structural enrichment, manipulanda, novel food items/facating, and sensory enrichment. Potential suffering during liver biopsy and bleeding procedures was alleviated using anesthetic medication using ketamine mixture and tilexamine (HCL/Zolazepam HCL (Telazol). None of chimpanzees included in the study was sacrificed. All chimpanzees (CH6413, CH256 and CH1541) were inoculated intravenously with HCV genotype 1a, varying between 10^4 to 10^5 chimpanzee infectious doses (CID) [22-24]. Serum samples were obtained every week at baseline and during 180 days after inoculation. HCV RNA in sera was tested and quantified by Cobas Amplicor HCV v. 2.0 (Roche Diagnostics Systems, Branchburg, NJ). The VITROS Anti-HCV assay (Ortho-Clinical Diagnostics, Rochester, NY) was used for detection of anti-HCV IgG, and serum ALT levels were quantified with a colorimetric assay (Drew Scientific, Huston, TX). Cut off for detection of anti-HCV IgG, and serum ALT levels was 100 international units/mL (IU/mL) and 30 arbitrary units/L, respectively.

Frozen liver biopsy samples

Lever needle biopsy samples were obtained before inoculation and every week after inoculation. Samples were frozen in liquid nitrogen immediately after the biopsy and subsequently stored at -80°C until further use. For CH256, 6 liver biopsy samples was obtained before inoculation (DAI = 0 days after inoculation [DAI]) and 13 samples after inoculation (DAIs 4, 12, 32, 40, 53, 75, 82, 95, 103, 124, 145, 152, and 180). For CH6413, 5 liver samples obtained before inoculation (DAIs 0, 32, 40, 53, 75, 82, 95, 103, 124, 145, 152, and 180) were used. For CH1541, 5 samples were obtained before inoculation (DAIs 0, 32, 40, 53, 75, 82, 95, 103, 124, 145, 152, and 180). HCV RNA in snap-frozen liver biopsy specimens was quantified by Taqman real-time PCR using 7900 HT fast real-time PCR system (Applied Biosystems, Foster City, CA). Primers and probe sequences were derived from 5’ non-coding region of the HCV genome (forward primer: 5’-GTCTGCGGAACCggGTT-

GAG-3’), reverse primer: 5’- CGACCCAACRGCTACTCCGCG-

TAG-3’, probe: 5’-ACACCGGAATTTGGCAAGGACC-3’).
values from these selected control RNAs were calculated and then subtracted from the miR-122 \( \Delta \text{Ct} \) (miR-122). Liver samples obtained before HCV inoculation were used as calibrator samples, and the arithmetic mean Ct of each calibrator sample \( (n = 6 \) in CH256, \( n = 5 \) in CH6413 and \( n = 5 \) CH1541) was calculated. Fold change of miR-122 expression relative to the endogenous control genes was determined by the \( 2^{-\Delta \text{ΔCt}} \) method. The \( \Delta \text{ΔCt} \) was obtained by mean Ct of the calibrator samples \( (\text{Ct miR-122} - \text{Ct endogenous control genes}) \) subtracted from \( \Delta \text{Ct} \) miR-122 (Ct miR-122 – Ct endogenous control genes).

**Table 1. Selection of the most stable endogenous control genes for miR-122 results normalization in HCV-inoculated chimpanzees [25].**

| Rank | CH256  | CH6413  | CH1541  |
|------|--------|---------|---------|
|      | Gene name | Stability value | Gene name | Stability value | Gene name | Stability value |
| Best gene combination | SNORD95 and SNORD72 | 0.245 | SNORD95 and SNORD72 | 0.188 | SNORD95 and miR-191 | 0.112 |
| 1    | SNORD61 | 0.315 | SNORD96A | 0.390 | miR-191 | 0.131 |
| 2    | SNORD95 | 0.467 | SNORD72 | 0.412 | SNORD95 | 0.181 |
| 3    | let-7b  | 0.467 | let-7b  | 0.414 | miR-103 | 0.206 |
| 4    | miR-17  | 0.473 | SNORD61 | 0.417 | SNORD72 | 0.224 |
| 5    | RNU6    | 0.607 | SNORD95 | 0.426 | let-7b  | 0.241 |
| 6    | SNORD72 | 0.661 | RNU6    | 0.458 | SNORD68 | 0.261 |
| 7    | miR-15a | 0.694 | miR-15a | 0.550 | RNU6    | 0.273 |
| 8    | SNORD96A | 0.781 | miR-191 | 0.706 | SNORD96A | 0.292 |
| 9    | miR-39 | 0.819 | SNORD61 | 0.383 |
| 10   | SNORD68 | 0.832 | miR-17  | 0.443 |
| 11   | miR-39 | 0.700 | SNORD68 | 0.432 |

**Results**

Selection of the endogenous miRNA for miR-122 expression normalization

To identify the most stable endogenous RNA as normalizers in hepatic miR-122 expression, twelve small RNAs and miRNAs were selected as candidate endogenous control RNAs. Ct values of *C. elegans* miR-39, used as a spike-in RNA control to assess the quality control and confidence of the real-time PCR performance, were similar in all samples from all three chimpanzees included in the study (Table S1). The twelve endogenous control RNAs were expressed in all samples, with Ct values ranging from 22.36 to 36.74 in CH256, 18.08 to 44.88 in CH6413, and 22.24 to 35.42 in CH1541. miR-103 was not used for further calculation in CH6413 due to its low expression (from 32.62 to 44.88, average Ct 41.43) (Table S1). miR-122 Ct values were observed in range from 20.68 to 29.01 for CH256, 23.74 to 29.03 for CH6413, and 22.79 to 27.78 for CH1541, i.e., in range of Ct values of the endogenous controls. The ANOVA-based model to estimate intra- and inter-group variation was used to select endogenous control genes for unbiased normalization across all samples individually in each chimpanzee [25]. It determined a stability value for each endogenous control gene and indicated the single most stable endogenous control for each animal and endogenous control gene pair for which the stability of the latter was greater than that of the single endogenous control. Two Ct values of each endogenous control obtained from two separate reactions were combined and the mean Ct value was used for calculation. The combination of SNORD95 and SNORD72 was selected for two chimpanzees, CH256 and CH6413, and the combination of SNORD95 and miR-191 was selected for CH1541 (Table 1).

miR-122 expression and HCV RNA titer in the liver and serum

Normalized levels of miR-122 in the liver increased from 2.2 to 3.9 times those of pre-inoculation samples; from DAIs 4 to 32 for CH256, DAIs 4 to 19 for CH6413, and on DAI 4 for CH1541 (Fig. 1A). During the peak of HCV replication, miR-122 expression had either decreased or remained unchanged (DAIs 4 to 32 for CH256, DAIs 4 to 19 for CH6413, and on DAI 4 for CH1541).
in CH1541). In all three animals, hepatic miR-122 levels were on the rise coinciding with the period when HCV RNA titers were declining (Fig. 1A and B).

All three were HCV RNA-positive in serum from DAIs 4 (CH1541) or 7 (CH256 and CH6413) and viral titers were maintained until DAI 71 (CH1541) or DAI 95 (CH256 and CH6413) (Fig. 1B). A similar profile was observed for HCV RNA in the liver; about 2-log decline in liver viral RNA levels was detected at DAI 75 (CH1541), 131 (CH256) or 88 (CH6413) (Fig. 1B). ALT activity was significantly elevated at DAI 71 (CH1541), 95 (CH256) or 75 (CH6413) (Fig. 1C, Table S2). A statistically significant inverse correlation was found between hepatic miR-122 expression and liver HCV RNA titers in CH256 and with both liver and serum HCV RNA titers in CH1541 (Fig. 2A, B, and D, Table S3A). A statistically significant negative correlation between hepatic miR-122 expression and the levels of serum miR-122 was found in CH1541 (Fig. 2F, Table S3A). Serum miR-122 levels were positively correlated with HCV RNA titer in the liver and serum (Fig. 2I and J, Table S3C). Expression profiles of serum miR-122 detected in three chimpanzees were similar to those of ALT activity (Fig. 2C); the correlation between those two measures was statistically significant in CH256 (Fig. 2K, Table S3B).

**Discussion**

The kinetics of miR-122 in the liver and serum during the acute phase of HCV infection were determined in chimpanzees inoculated with HCV genotype 1a. Levels of hepatic miR-122 were positively correlated with HCV RNA titer in the liver and serum (Fig. 21 and J, Table S3C). Expression profiles of serum miR-122 detected in three chimpanzees were similar to those of ALT activity (Fig. 2C); the correlation between those two measures was statistically significant in CH256 (Fig. 2K, Table S3B).

**Type-1 interferon-induced mRNAs in the liver**

High levels of hepatic IFN-α and IFN-β mRNAs were detected during the period when levels of hepatic miR-122 expression were in decline (Fig. 1D, Table S2). Hepatic IFN-α and IFN-β mRNA expressions were correlated with hepatic miR-122 levels in CH256 (Fig. 3A and B, Table S3A). In CH6413, the levels of IFN-α and IFN-β mRNAs were correlated with serum miR-122 levels (Fig. 3E, F, and Table S3B). When all parameters from all three chimpanzees were included in one analysis, the moderate, but statistically significant positive correlation between hepatic IFN-α and IFN-β mRNA and hepatic miR-122 levels was observed (Fig. 3C and D, Table S3C).

**Discussion**

The kinetics of miR-122 in the liver and serum during the acute phase of HCV infection were determined in chimpanzees inoculated with HCV genotype 1a. Levels of hepatic miR-122
genomic expression were evaluated by quantitative real-time RT-PCR (qRT-PCR) with the values stringently normalized using selected host genes to correct for biological and experimental bias and for inter-assay variations in each of these chimpanzees included in the study [25,29]. The combination of SNORD95/SNORD72 was selected for CH256 and CH6413, and SNORD95 and miR-191 for CH1541 as the most stable endogenous control genes (Table 1). The RNU6B (U6) gene used in other studies for evaluation of miR-122 expression [21,30,31] has proven to be an unreliable normalizer [26,32,33].

Up-regulated expression of miR-122 in the liver was observed during the first four weeks after HCV inoculation in all three chimpanzees, at the beginning of HCV viremia and before any evidence of liver injury as measured by serum ALT elevation (Fig. 1A, B, C, and Table S2). Temporal concordance between increasing levels of miR-122 and rapidly increasing HCV RNA titer in the liver and blood has been consistent with data obtained in vitro which showed augmentation of HCV RNA accumulation in Huh7 cells consequential to expression of miR-122 [3,4]. The kinetics of miR-122 expression during the acute phase of HCV infection further may validate positive impact of miR-122 on viral abundance and its role in the enhancement of HCV replication and stability. The positive influence of miR-122 on HCV replication has also been suggested by a study of chronically HCV-infected chimpanzees who were treated with antisense miR-122 and then achieved long-lasting suppression of HCV viremia and chronic hepatitis C patients treated with DNA antisense nucleotide that sequesters mature miR-122 [18,19].

During the course of acute HCV infection in our chimpanzees, when hepatic and serum HCV RNA titers had increased 4 to 5 logs, a trend was observed for hepatic miR-122 values either to have fallen or remain unchanged; for CH1541, the phase of decline in hepatic levels of miR-122 preceded quite distinctly the appearance of miR-122 in serum. The inverse relationship between hepatic miR-122 and hepatic HCV RNA titer was statistically significant in CH256 and CH1541, and was even more statistically apparent when data from all three animals were evaluated in a single analysis (Fig.2 and Table S3C). In addition, the statistically significant negative correlation between hepatic miR-122 expression and serum level of HCV RNA was observed in CH1541. It is conceivable that the decrease of hepatic miR-122 levels could be due to release of proteins from degraded hepatocytes upon injury as such decrease also coincided with the rise of ALT activity. An inverse correlation between hepatic miR-122 and HCV RNA level in serum has previously been reported in patients with chronic HCV infection [31]. Although a previous in vitro study documented suppression of miR-122 expression by IFN-β [11], and interferon repression of miR-122 in the liver was
suggested from clinical observations of interferon-treated patients with chronic hepatitis C [21], the association between decreased miR-122 and increased genomic expression of IFN-α and IFN-β in the studied chimpanzees was not found. To the contrary, there was a statistically significant positive correlation between hepatic miR-122 and type 1 interferon in CH256 and when all chimpanzee data were tested in a single analysis (Fig. 3 and Table S3A, C).

Towards the later phase of the acute HCV infection, hepatic levels of miR-122 in the chimpanzees were observed to have risen again, corresponding to the phase of declining HCV RNA titers in the liver and clearance of HCV RNA from serum (Fig. 1A). The mechanism underlying the miR-122 kinetics in this phase of acute infection is unclear. It is also unknown for how long this upsurge of miR-122 expression in the liver could have been maintained, or whether these rises predict the development of chronic HCV infection or resolution of acute infection, as the chimpanzees in our study were not tested beyond 180 days after HCV inoculation.

In summary, a tri-phasic change in miR-122 levels in the liver was observed in CH256 and less prominently in two other chimpanzees during acute HCV infection. During the initial phase of the infection, rising hepatic miR-122 levels and HCV RNA in liver and serum likely reflects the phase of infection when miR-122 is participating in the enhancement of HCV replication, as reported from in vitro studies [4,7,13,15–17]. The second phase, when miR-122 levels in the liver are in a decline, may correspond to the phase of destruction of HCV-infected hepatocytes and release or transport of miR-122 into the plasma. The mechanisms underlying the third phase, of a renewed upsurge in miR-122 levels in the liver, remain unclear, although it may be related to the restoration of morphologic integrity of the liver during the HCV clearance and liver lesions healing. Kinetics of changes in miR-122 expression in the liver observed in vivo document the complexities of the virus-host interaction during the acute phase of HCV infection.

Supporting Information
Table S1 (XLSX)
Table S2 (DOCX)
Table S3 (DOCX)

Acknowledgments
The authors would like to thank Dr. Gary J. Latham, Asuragen, Inc., Austin, TX for his critical insights in real-time PCR data normalization and Dr. Chong-Gee Teo for a critical review of the manuscript.

Author Contributions
Conceived and designed the experiments: YC HPD KK. Performed the experiments: YC. Analyzed the data: YC HPD KK. Contributed reagents/materials/analysis tools: YC KK. Wrote the paper: YC KK.

References
1. Houghton M (2009) Discovery of the hepatitis C virus. Liver Int 29 Suppl 1: 82–88.
2. Schuppan D, Krebs A, Bauer M, Hahn EG (2003) Hepatitis C and liver fibrosis. Cell Death Differ 10 Suppl 1: S39–67.
3. Henke JI, Goergen D, Zheng J, Song Y, Schutler CG, et al. (2008) microRNA-122 stimulates translation of hepatitis C virus RNA. EMBO J 27: 3300–3310.
4. Jopling CL, Yi M, Lancaster AM, Lemon SM, Samrow P (2003) Modulation of hepatitis C virus RNA abundance by a liver-specific microRNA. Science 300: 1577–1581.

5. Murakami Y, Aly HH, Tajima A, Shimotohno K (2009) Regulation of the hepatitis C virus genome replication by miR-191a. J Hepatol 50: 455–460.

6. Niepmann M (2009) Activation of hepatitis C virus translation by a liver-specific microRNA. Cell Cycle 8: 1473–1477.

7. Shan Y, Zheng J, Lambrecht RW, Bonkovsky HL (2007) Reciprocal effects of micro-RNA-122 on expression of heme oxygenase-1 and hepatitis C virus genes in human hepatocytes. Gastroenterology 133: 1166–1174.

8. Cowland JB, Hother C, Gronbaek K (2007) MicroRNAs and cancer. APMIS 115: 1090–1106.

9. Croce CM, Calin GA (2005) miRNAs, cancer, and stem cell division. Cell 122: 6–7.

10. Lagos-Quintana M, Rauhut R, Yalcin A, Meyer J, Lendeckel W, et al. (2002) Identification of tissue-specific microRNAs from mouse. Curr Biol 12: 735–739.

11. Pedersen IM, Cheng C, Wieland S, Volinia S, Croce CM, et al. (2007) Identification of tissue-specific microRNAs from mouse. Curr Biol 12: 735–739.

12. Randall G, Panis M, Cooper JD, Tellinghuisen TL, Sukhodolets KE, et al. (2010) Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection. Science 327: 198–201.

13. Jangra RK, Yi M, Lemon SM (2010) Regulation of hepatitis C virus translation and infectious virus production by the microRNA miR-122. J Virol 84: 6615–6625.

14. Villanueva RA, Jangra RK, Yi M, Pyles R, Bourne N, et al. (2010) miR-122 does not modulate the elongation phase of hepatitis C virus RNA synthesis in infected replicase complexes. Antiviral Res 88: 119–123.

15. Shinakami T, Yamane D, Jangra RK, Kempf BJ, Spaniel C, et al. (2012) Stabilization of hepatitis C virus RNA by an Ago2-miR-122 complex. Proc Natl Acad Sci U S A 109: 941–946.

16. Shinakami T, Yamane D, Welch C, Hensley L, Jangra RK, et al. (2012) Base pairing between hepatitis C virus RNA and microRNA 122 3′ of its seed sequence is essential for genome stabilization and production of infectious virus. J Virol 86: 7372–7383.

17. Li Y, Masaki T, Yamane D, McGovern DR, Lemon SM (2012) Competing and noncompeting activities of miR-122 and the 5′ exonuclease Xrn1 in regulation of hepatitis C virus replication. Proc Natl Acad Sci U S A 109: 941–946.

18. Lundord RE, Hildebrandt-Eriksen ES, Petri A, Persson R, Lindow M, et al. (2010) Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection. Science 327: 198–201.

19. Jansen HL, Reesink HW, Lawitz EJ, Zeuzem S, Rodriguez-Torres M, et al. (2013) Treatment of HCV infection by targeting microRNA. N Engl J Med 368: 1685–1694.

20. He YP, Katze MG (2002) To interfere and to anti-interfer: The interplay between hepatitis C virus and interferon. Viral Immunol 15: 95–119.

21. Sarasin-Filipowicz M, Krol J, Markiewicz I, Heim MH, Filipowicz W (2009) Decreased levels of microRNA miR-122 in individuals with hepatitis C responding poorly to interferon therapy. Nat Med 15: 31–33.

22. Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, et al. (1989) Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science 244: 359–362.

23. Major ME, Mihalik K, Fernandez J, Seidman J, Kleiner D, et al. (1999) Long-term follow-up of chimpanzees inoculated with the first infectious clone for hepatitis C virus. J Virol 73: 3317–3325.

24. Ogata N, Alter HJ, Miller RH, Purcell RH (1991) Nucleotide sequence and mutation rate of the H strain of hepatitis C virus. Proc Natl Acad Sci U S A 88: 3392–3396.

25. Andersen GL, Jensen JL, Orntoft TF (2004) Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res 64: 5245–5250.

26. Pehl HJ, Latham GJ (2008) Normalization of microRNA expression levels in quantitative RT-PCR assays: identification of suitable reference RNA targets in normal and cancerous human solid tissues. RNA 14: 844–852.

27. Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29: e45.

28. Roderburg C, Luedde M, Vargas Cardenas D, Vescur M, Scholten D, et al. (2013) Circulating MicroRNA-150 Serum Levels Predict Survival in Patients with Critical Illness and Septis. PLoS One 8: e56132.

29. Latham GJ (2010) Normalization of microRNA quantitative RT-PCR data in reduced scale experimental designs. Methods Mol Biol 667: 19–31.

30. Marquez RT, Bandyopadhyay S, Wendland EB, Keck K, Hoffer BA, et al. (2010) Correlation between microRNA expression levels and clinical parameters associated with chronic hepatitis C viral infection in humans. Lab Invest 90: 1727–1736.

31. Morita K, Taketomi A, Shirabe K, Umeda K, Kayashima H, et al. (2011) Clinical significance and potential of hepatic microRNA-122 expression in hepatitis C liver Int 31: 474–484.

32. Qi R, Weiland M, Gao XH, Zhou L, Mi QS (2012) Identification of endogenous micro-RNA-122 on expression of heme oxygenase-1 and hepatitis C virus genes in human hepatocytes. Cell Cycle 11: 1090–1106.

33. Zhu HT, Dong QZ, Wang G, Zhou HJ, Ren N, et al. (2012) Identification of tissue-specific microRNAs from mouse. Curr Biol 12: 735–739.

34. Marquez RT, Bandyopadhyay S, Wendland EB, Keck K, Hoffer BA, et al. (2010) Correlation between microRNA expression levels and clinical parameters associated with chronic hepatitis C viral infection in humans. Lab Invest 90: 1727–1736.

35. Zhou HT, Dong QZ, Wang G, Zhou HJ, Ren N, et al. (2012) Identification of suitable reference genes for qRT-PCR analysis of circulating microRNAs in hepatitis B virus-infected patients. Mol Biotechnol 50: 49–56.