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Hepatitis C virus RNA replication in human stellate cells regulates gene expression of extracellular matrix-related molecules

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ABSTRACT

Hepatitis C virus (HCV) infection is a major cause of chronic liver disease, including chronic hepatitis, fibrosis, and cirrhosis. Fibrosis often develops in HCV-infected livers and ultimately leads to cirrhosis and carcinoma. During fibrosis, hepatic stellate cells (HSC) play important roles in the control of extracellular matrix synthesis and degradation in fibrotic livers. In this study, we established a subgenomic replicon (SGR) cell line with human hepatic stellate cells to investigate the effect of HCV RNA replication on HSC. Isolated SGR clones contained HCV RNA copy numbers ranging from 10^4 to 10^7 per μg total RNA, and long-term culture of low-copy number SGR clones resulted in markedly increased HCV RNA copy numbers. Furthermore, HCV RNA replication affected gene expression of extracellular matrix-related molecules in both hepatic stellate cells and hepatic cells, suggesting that HCV RNA replication and/or HCV proteins directly contribute to liver fibrosis. The HCV RNA-replicating hepatic stellate cell line isolated in this study will be useful for investigating hepatic stellate cell functions and HCV replication machinery.

1. Introduction

Hepatitis C virus (HCV) infection is a major cause of chronic liver disease, including chronic hepatitis, fibrosis, and cirrhosis, with greater than 170 million individuals infected worldwide [1,2]. Presently, there is no preventive vaccine for HCV infection, and standard therapy involves the combination of pegylated interferon-alpha and ribavirin [3]. However, as the effects of this combination therapy approach are often insufficient to completely eliminate viruses from HCV carriers, novel antiviral therapies are desired to increase sustained virological response rates and reduce adverse effects.

Fibrosis is often observed in chronic HCV infections and is part of the dynamic process of extracellular matrix (ECM) remodeling that occurs continuously during chronic liver injury. Such remodeling results in excessive accumulation of ECM proteins, ultimately leading to cirrhosis and carcinoma [4,5]. Hepatic stellate cells are the main collagen- and ECM-producing cells and play a key role in liver fibrogenesis [6]. In liver tissue, the balance between ECM synthesis and degradation is regulated by gene expression of ECM-regulatory molecules, such as matrix metalloproteinases (MMP) and tissue inhibitors of matrix metalloproteinase (TIMP). On activation of hepatic stellate cells by inflammatory molecules, the balance of ECM regulation shifts towards progression of liver fibrosis. During fibrosis, however, little is known about the contribution of HCV to fibrogenesis.

HCV is a positive-strand RNA virus with a genome that encodes an approximately 3000-amino-acid (aa) polyprotein, which is co- and post-translationally processed by proteolysis into ten mature proteins, consisting of a capsid (core), two envelope (E1, E2), and seven non-structural (NS) proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) [1,7]. Past investigations of HCV have been limited because no suitable HCV culture systems were available to observe all steps of the viral life cycle, including entry, replication, translation, assembly, and secretion. In 1999, however, Lohmann et al. [8] developed a subgenomic replicon (SGR) system using replicon RNA encoding NS proteins (NS3-NS5B) for the analysis of RNA replication and translation [8]. In addition, an HCV pseudo-particle system (HCVpp) based on HIV and MMLV was developed for viral entry analysis [9,10].

Recently, HCV strain JFH-1, which was isolated from a Japanese patient with fulminant hepatitis, has permitted all steps of the HCV life cycle to be examined in a cultured cell line [11]. Replication of the transfected JFH-1 genome in host cells was restricted to the human hepatoma cell line Huh7 or its derivatives. Hepatocytes

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are thought to be permissive for HCV infection because liver tissue is a major target organ for HCV infection. However, virus replication levels in HCV patients are typically too low to detect the distribution of viral proteins in liver biopsies by antibodies directed against HCV proteins, and no clear evidence indicates which cell groups are the major target of HCV infection. Thus, it is unclear whether hepatic cells are the only target of HCV infection in liver tissue, and if other cells, particularly HSCs, can serve as hosts for HCV replication.

Here, we established HCV-replicating hepatic stellate cells to address whether these cells can be a potential target of HCV infection. Furthermore, we analyzed gene expression profiles of ECM-related molecules in HCV-replicating stellate cell clones to investigate the effect of HCV RNA replication on hepatic stellate cell functions.

2. Materials and methods

2.1. Cells and reagents

Human hepatic stellate cells, TWNT-4 JP7 cells derived from the LI90 cell line [12,13], were maintained in D-MEM supplemented with 10% fetal bovine serum (FBS), 100 unit/ml penicillin, 100 μg/ml streptomycin, 10 mM Hepes (pH 7.4), 1 mM sodium pyruvate, and MEM Non-Essential Amino Acids. Electroporated TWNT-4 JP7 cells and isolated SGR cells were maintained in prepared D-MEM containing either 500 or 1000 μg/ml G418. SGR Huh7 cell lines (JFH-1/4-1, JFH-1/4-5, Con1 NK5.1/0-6, and Con1 NK5.1/0-11) were maintained in prepared D-MEM containing 500 μg/ml G418 [14]. Rabbit polyclonal antibodies against NS3 and NS5A proteins were raised by immunization with recombinant NS proteins (NS3, 1195–1661 aa; NS5A, 2001–2441 aa).

2.2. Transfection and isolation of SGR clones

JFH-1 SGR RNA was synthesized using a Megascript T7 Kit with linearized pSGR-JFH-1 and pSGR-JFH-1 GND plasmids as templates. Ten micrograms HCV RNA was electroporated into 2×10^6 TWNT-4 JP7 cells, as previously described [15], which were then cultured for 3 weeks under G418 selection (500 and 1000 μg/ml). Single colonies were isolated, and the selected SGR clones were expanded and stored at −80 °C until used for analysis.

2.3. Quantification of HCV RNA in SGR cells

Total RNA was purified from each SGR clone using ISOGEN (Nippon Gene), as directed by the manufacturer's protocol. The HCV RNA copy number of each SGR clone was analyzed by a real-time PCR method, as described previously [16].

2.4. Western blot analysis

Twenty micrograms of cell lysates were separated on 10% polyacrylamide gels and then transferred to nitrocellulose membranes. Membranes were first blocked with 2% skim milk in TBS-T (20 mM Tris–HCl [pH 7.2], 500 mM NaCl, and 0.01% Tween20) and then incubated with 2% skim milk in TBS-T containing primary antibody (α-NS3 or α-NS5A), followed by secondary antibody (α-rabbit IgG HRP-conjugated). After washing membranes, bands were detected by Enhanced Chemiluminescence (ECL) Plus reagent (GE Healthcare). Luminescence signals were analyzed using the LAS-3000 Mini image analysis system (Fujifilm).

2.5. Immuno-staining of non-structural HCV proteins

Isolated SGR cells were fixed with acetone-methanol. Fixed cells were dried and incubated with primary antibodies (rabbit polyclonal α-NS3 or α-NS5A), followed by secondary antibody (α-rabbit IgG-Alexa488). Both antibodies were diluted in Block Ace (DS Pharma Biomedical) prior to use. Fluorescence of NS proteins was visualized using a Biozero microscope (Keyence).

2.6. Gene expression analysis of ECM-related genes

Total RNA was purified from SGR clones in growth phase using ISOGEN-LS (Nippon Gene). cDNA was synthesized from total RNA using random primers and Superscript III (Invitrogen). TaqMan array plates (Custom Format) were purchased from Applied Biosystems. Information of ECM-related genes in the Custom Format plate is listed in Table 1S. cDNA (55 ng/reaction) was mixed with an equal volume of TaqMan Gene Expression Master Mix (Applied Biosystems) and real-time PCR was performed using a 7500 FAST Real-Time PCR System (Applied Biosystems).

3. Results

3.1. Isolation of SGR cells

To evaluate the potential of HCV to replicate in human hepatic stellate cells and examine the role of HCV replication and viral protein in liver fibrogenesis, we established HCV SGR cells from human hepatic stellate cells. We first examined the colony formation efficiency of HCV SGR transfected into hepatic stellate cells using JFH-1 SGR RNA (Fig. 1A), which was electroporated into the human hepatic stellate cell line, TWNT-4 JP7. The transfected cells were selected using G418 for 3 weeks, and the resulting colonies were stained by crystal violet. Interestingly, many colonies (~150) were formed on transfection of cells with 10 μg JFH-1 RNA; however, no colonies were detected among cells transfected with a HCV replication-defective mutant, JFH-1 GND (Fig. 1B). This result indicated that TWNT-4 JP7 cells can support HCV replication, although colony formation efficiency of TWNT-4 JP7 cells transfected with JFH-1 SGR was much lower than that in transfected Huh7 cells (data not shown).

Next, we selected colonies of JFH-1 SGR-transfected cells and established a number of SGR TWNT-4 JP7 clones; SGR clones #1–8 and clones #11–18 were isolated from 500 and 1000 μg/ml G418 selection, respectively. The HCV RNA copy number of each SGR clone was measured by real-time RT-PCR, which revealed that the clones contained RNA copy numbers ranging from 10^4 to 10^7 copies per μg total RNA (Table 1).

Among the isolated SGR clones, we focused on SGR #1 and #2 for further analyses, because these two clones allowed HCV RNA replication at a relatively low RNA copy number compared with the other examined SGR clones. SGR #1 and #2 were cultured for an additional 8 weeks in G418 selection medium, during which time the HCV RNA copy number in both clones increased from 10^4 to 10^7 copies, which represented increases of 3200- and 470-fold, respectively (Table 2). This observed increase suggested that the occurrence of viral adaptive mutations or modifications of cellular factors during cell passaging resulted in the increased efficiency of viral RNA replication in hepatic stellate cells. We thus determined the viral RNA sequences in clones SGR #1 and #2 and some other additional clones, and identified several synonymous and non-synonymous mutations in the HCV RNA sequences (data not shown). Although two common non-synonymous mutations were found in the NS4B and NS5A gene-encoding regions, these mutations did not increase HCV RNA replication in human

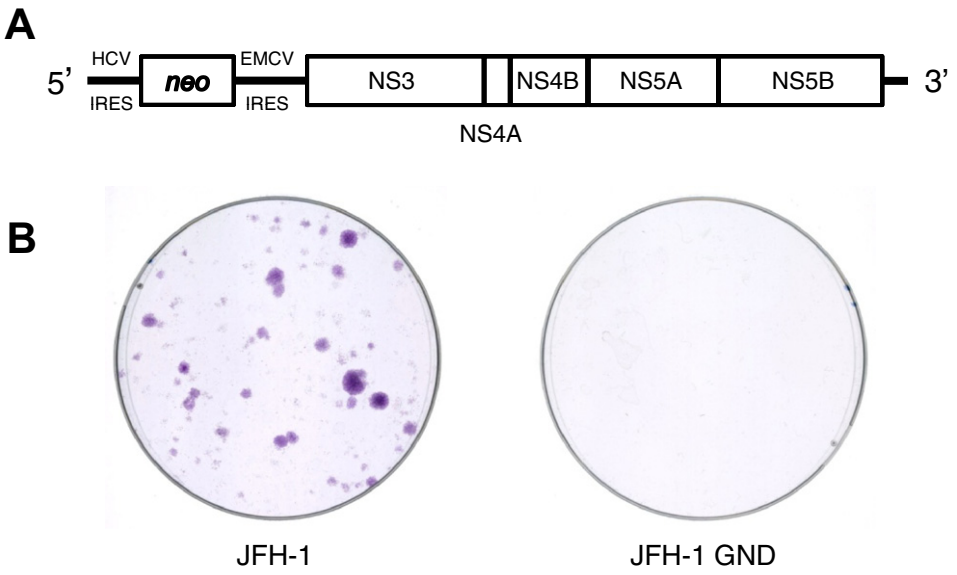

Fig. 1. Structure of the JFH-1 subgenomic replicon (SGR) and colony formation of SGR TWNT-4 JP7 cells. (A) Diagram of the JFH-1 SGR. (B) Colony formation of SGR TWNT-4 JP7 cells. TWNT-4 JP7 cells were transfected with either wild-type JFH-1 or replication-defective GND SGR RNA, and transfected cells were then cultured in G418 selection medium for 3 weeks. Cells were then fixed using formalin, and colonies were visualized by crystal violet staining. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
RNA copy number of isolated subgenomic replicon (SGR) clones.

SGR clone	HCV RNA copy number (copies/μg of total RNA)
#1	1.5×10^4
#2	5.3×10^4
#3	9.4×10^5
#4	2.3×10^5
#5	1.7×10^5
#6	1.5×10^4
#7	4.2×10^5
#8	1.3×10^6
Average	3.9×10^5
#11	3.8×10^5
#12	1.2×10^7
#13	4.8×10^5
#14	2.3×10^6
#15	4.7×10^5
#16	2.1×10^6
#17	5.1×10^5
#18	2.0×10^6
Average	2.5×10^6

Table 2
Change of RNA copy number in isolated subgenomic replicon (SGR) clones following long-term culture.

SGR clone	4 weeks*	12 weeks*
#1	1.5×10^4	4.9×10^7
#2	5.3×10^4	2.5×10^7

* Copies/μg of total RNA.

hepatic stellate cells when they were artificially introduced into the replicon genome (data not shown).

3.2. Expression of non-structural HCV proteins

The expression of several NS HCV proteins, from NS3 to NS5B, is required for HCV RNA replication in infected cells. It is reported that these NS proteins form complexes to replicate HCV RNA, with NS5A protein in particular localizing to specialized membranous web structures around lipid droplets, which are considered to serve as the scaffold for HCV RNA replication and assembly [17,18]. To confirm the expression of NS proteins in isolated SGR clones, we performed western blotting and immunostaining of HCV NS proteins (Fig. 2). As shown in Fig. 2A, NS3 and NS5A proteins of expected size were detected by western blotting in SGR #1 and #2 cells. The expression of both NS3 and NS5A in SGR #1 was higher than that in SGR #2, a result that is consistent with the determined viral RNA copy number of each clone (Table 2). Immunostaining of NS proteins clearly showed cytoplasmic distribution, similar to that observed in Huh7 cells (Fig. 2B). Specifically, the fluorescence of NS5A was distributed as a fine reticular pattern with occasional granular staining. These results demonstrated that HCV RNA replicated in hepatic stellate cells and that HCV NS proteins were expressed in hepatic stellate cells at identical levels as in Huh7 cells.

3.3. Gene expression profiles of ECM-related molecules in SGR cells

In liver tissue, once stellate cells are activated by inflammation mediators secreted from infected or injured cells, the activated cells differentiate into myoblast cells. In addition, ECM-related molecule gene expression is altered, which leads to the progression of liver fibrosis. Here, we examined if HCV RNA replication in stellate cells affects the gene expression of ECM-related molecules by measuring gene expression in both parental TWNT-4 JP7 and isolated SGR cells using a TaqMan array plate (Custom Format) consisting of 24 selected genes from the collagen (COL), MMP, and TIMP families (Table 1S) [19,20]. Among the 16 isolated SGR clones in this study, 10 in growth phase were examined for ECM-related molecule gene expression. All SGR clones exhibited a decrease of collagen gene expression compared with parental TWNT-4 JP7 cells. In particular, four collagen genes (COL1A1, COL4A2, COL5A1, and COL15A1) were expressed at levels approximately 0.5-fold lower than those of parental cells (Fig. 3A), whereas MMP1, MMP3, and MMP12 increased 4-, 5.6-, and 4.8-fold, respectively (Fig. 3B). These results suggest that HCV RNA replication or viral proteins in hepatic stellate cells down- and up-regulate COL and MMP gene expression, respectively.

To determine if gene expression changes of ECM-related molecules in isolated SGR clones were specific to hepatic stellate cells,

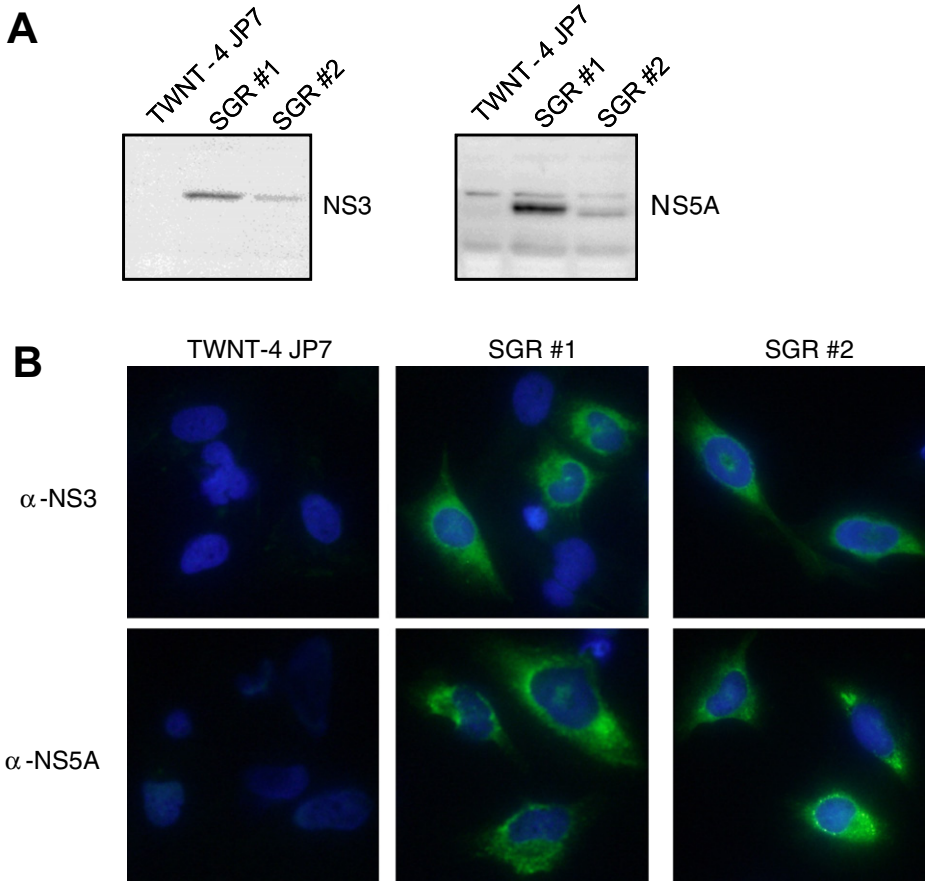

Fig. 2. Expression of non-structural (NS) NS3 and NS5A proteins in isolated SGR clones. (A) Total cell lysates of TWNT-4 JP7 and SGR clones were separated by SDS–PAGE and transferred to a nitrocellulose membrane. HCV NS proteins NS3 and NS5A were detected by western blot analysis with rabbit polyclonal α-NS3 and α-NS5A antibodies. (B) The expression of HCV NS proteins were also observed by immuno-staining with rabbit polyclonal α-NS3 and α-NS5A antibodies (blue, nuclear; green, NS proteins). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

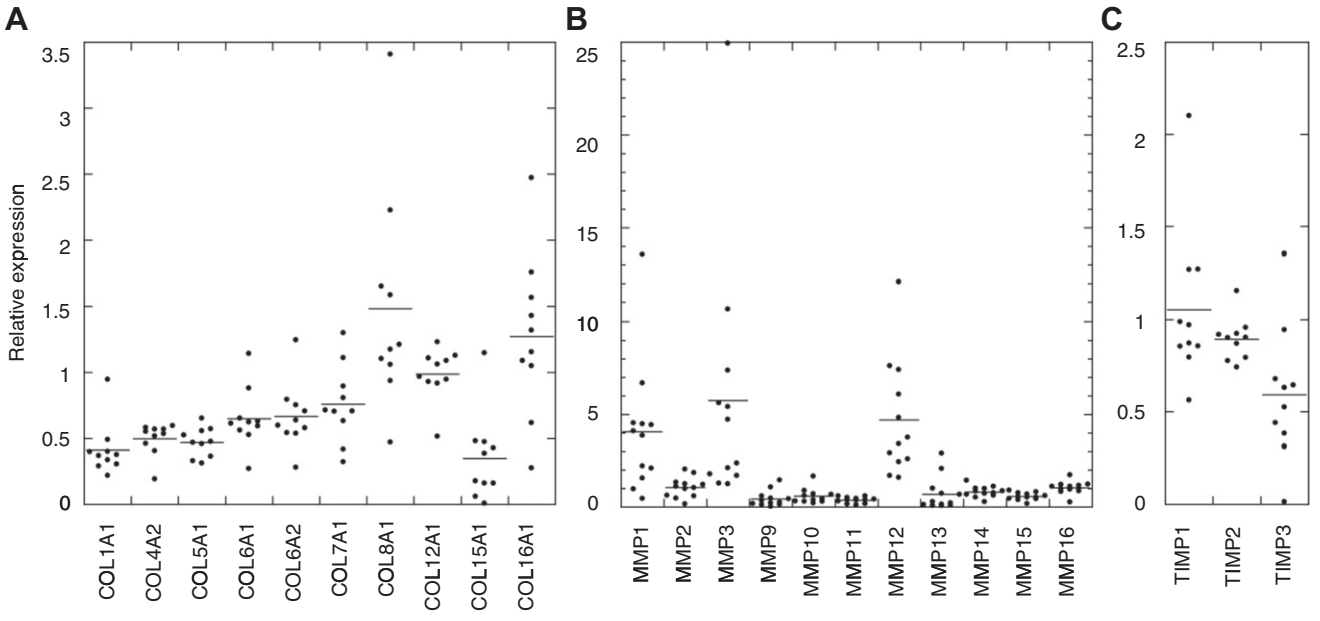

Fig. 3. Relative expression levels of extracellular matrix (ECM)-related genes in isolated TWNT-4 SGR clones. Gene expression levels of collagen (A), matrix metalloproteinase (B), and tissue inhibitor of metalloproteinase genes (C) were measured by TaqMan PCR. The expression levels of the indicated ECM-related genes were normalized to those of parental TWNT-4 JP7 cells. Values of gene expression level are summarized in Supplementary Table 2S. The bar in each column indicates the average.

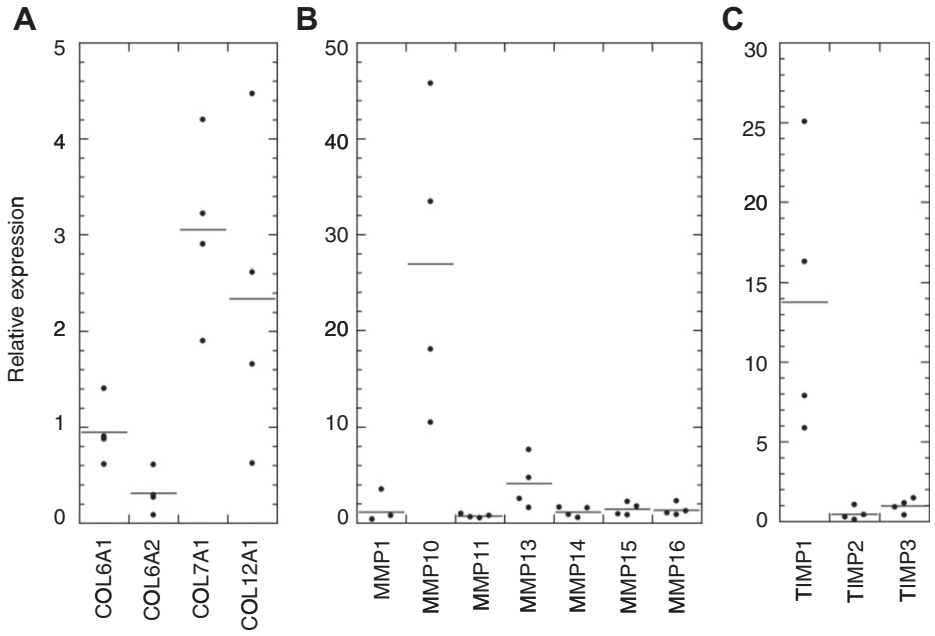

Fig. 4. Relative expression levels of extracellular matrix (ECM)-related genes in subgenomic replicon Huh7 clones. Gene expression levels of collagen (A), matrix metalloproteinase (B), and tissue inhibitor of metalloproteinase genes (C) were measured by TaqMan PCR. The expression levels of the indicated ECM-related genes were normalized to those of parental Huh7 cells. Values of gene expression levels are summarized in Table 3S. The bar in each column indicates the average.

we analyzed the expression of the identical 24 genes in SGR hepatic cells. The gene expression levels of four SGR hepatic cell clones derived from Huh7 cells (JFH-1/4-1, JFH-1/4-5, Con1 NK5.1/0-6, and Con1 NK5.1/0-11) were also measured by TaqMan array analysis (Fig. 4). Distinct differences in gene expression profiles were observed between hepatic and hepatic stellate cells, and the expression of several genes were not detected in Huh7 cells. In SGR Huh7 cells, COL7A1 and COL12A1 gene expression increased greater than 2-fold (Fig. 4A), while the expression of the COL1A1 gene increased from undetectable to detectable levels (data not shown). In addition, MMP10, MMP13, and TIMP1 gene expression was markedly increased in hepatic replicon cells compared with the levels in Huh7 cells (Fig. 4B and C).

4. Discussion

In previous studies, a few SGR cell lines were established from different HCV strains (JFH-1, H77, and Con-1) and several human cell lines (Huh7, HepG2, IMY-N9, HeLa, and 293 cells) [8,21,22]. The combination of JFH-1 SGR and Huh7 cells exhibited increased RNA replication without adaptive mutations compared with H77 and Con-1 strains. Furthermore, full genomic JFH-1 and its chimera virus have been shown to infect Huh7 and derivatives. Although we did not observe HCV infection using the combination of JFH-1 and the TWNT-4 JP7 cell line (data not shown), this is the first study to isolate SGR clones using hepatic stellate cells. Our findings indicate that hepatic stellate cells potentially support HCV replication in infected livers.

A total of 16 SGR clones were isolated from SGR RNA-transfected TWNT-4 JP7 cells in this study. The HCV RNA copy number in the selected clones clearly differed between the two concentrations of G418 used for selection, with 500 and 1000 μg/ml G418 yielding an average RNA copy numbers of 3.9×10^5 and 2.5×10^6, respectively (Table 1). Although RNA replication in SGR clones was lower than that observed in hepatic replicon cells, such as Huh7 and IMY-N9 cells, the RNA copy number of SGR clones selected using 1000 μg/ml G418 was comparable to that of non-hepatic replicon cells derived from HeLa and 293 cell lines [21,22]. Since hepatic stellate cells localize in liver tissue where HCV replicates, hepatic stellate cells are likely to be exposed to HCV from both the circulation system and neighboring hepatocytes. Cell-to-cell HCV transmission was also reported in recent studies [23,24], further supporting our results that hepatic stellate cells are a possible target of HCV infection.

It was reported that a number of adaptive mutations in the HCV genomic sequence increase viral production in infected cells and RNA replication in SGR cells [25–27]. Here, isolated replicon cells exhibited increased RNA replication during an additional 8 weeks of culture, and several mutations were identified in the sequenced HCV RNA of SGR cells. Clones SGR #1 and #2 contained four synonymous and three non-synonymous mutations, respectively. Although we performed a colony forming assay by transfection of SGR RNA containing these mutations into TWNT-4 JP7 cells, no differences in colony formation were observed between wild-type and mutant RNA-transfected cells (data not shown). This finding suggests that modification of certain cellular factors may have occurred in the SGR clones during cell passage, rather than the appearance of adaptive mutations in the replicon genome.

From the results of our TaqMan analysis, the expression of several collagen genes were significantly down-regulated and three MMP genes (MMP1, MMP3, and MMP12) were up-regulated in isolated SGR clones compared with parental TWNT-4 JP7 cells (Fig. 3). Type I and IV collagen are important for liver fibrogenesis, and MMP-1 degrades type I collagen to regulate ECM regeneration in liver tissue [19]. Furthermore, MMP-3 regulates MMP-1 activation [28,29], while overproduction of MMP-12 causes ECM protein breakdown and excessive remodeling, which has been implicated in a range of respiratory diseases [30–32]. Although no relationship between HCV RNA copy number and the expression of these genes in SGR cells was detected, a certain degree of HCV RNA replication may be sufficient to influence the regulation of ECM-related genes. Taken together, these findings suggest that HCV RNA-replicating hepatic stellate cells may suppress ECM production and negatively regulate hepatic fibrosis. In contrast, increased expression of type I collagen and TIMP-1 genes, an inhibitor of MMP-1, was observed in hepatic replicon cells, suggesting that hepatic cells up-regulate

ECM accumulation through collagen production and the inhibition of MMP activity. As hepatic cells are the major cell group in liver tissue, HCV-infected hepatic cells may be one of the factors promoting fibrosis by producing ECM proteins such as type I collagen and TIMP-1. Our results indicate that although HCV RNA replication and/or HCV NS proteins affect gene expression of EMC-related molecules in both hepatic stellate cells and hepatic cells, the gene expression profiles differ between these cell types. Further studies are necessary to analyze the mechanisms of ECM-related gene expression in HCV-infected livers.

Primary hepatic stellate cells isolated from liver tissue typically transform into myofibroblastic cells (active formation) during subsequent passages. As the TWNT-4 JP7 cells used in this study also exhibited an activated phenotype [13,33], the results of our gene expression analyses may reflect the phenotype of activated hepatic stellate cells. In fact, it is presently difficult to isolate and maintain quiescent hepatic stellate cell populations. Although *in vitro* culture systems involving primary cells and hepatic stellate cell lines are valuable tools for studying liver fibrosis, SGR cells generated from quiescent hepatic stellate cells are required for the detailed analysis of hepatic stellate cell functions in fibrosis.

In conclusion, our study has shown that HCV RNA replicates in human hepatic stellate cells and affects the gene expression of ECM-related molecules, suggesting the potential of HCV to infect and directly influence ECM-related gene regulation in hepatic stellate cells. The HCV RNA-replicating hepatic stellate cell line isolated in this study is useful not only for investigating hepatic stellate cell functions, but also for studying HCV replication machinery.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.02.125.

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