Antiviral Research 86 (2010) 220–223

Contents lists available at ScienceDirect

Antiviral Research

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

Short communication

# Creation and characterization of a cell-death reporter cell line for hepatitis C virus infection

Zhilei Chen[a,b,*,1], Rudo L. Simeon[b], Karuppiah Chockalingam[b], Charles M. Rice[a,**]

[a] Center for the Study of Hepatitis C, The Rockefeller University, USA
[b] Artie McFerrin Department of Chemical Engineering, Texas A&M University, USA

ARTICLE INFO

*Article history:*
Received 1 December 2009
Received in revised form 17 January 2010
Accepted 19 February 2010

*Keywords:*
Cytopathic effect
Genetic selection
Enrichment
Plaque assay
n4mBid (hepatoma cell line)

ABSTRACT

The present study describes the creation and characterization of a hepatoma cell line, n4mBid, that supports all stages of the hepatitis C virus (HCV) life cycle and strongly reports HCV infection by a cell-death phenotype. The n4mBid cell line is derived from the highly HCV-permissive Huh-7.5 hepatoma cell line and contains a modified Bid protein (mBid) that is cleaved and activated by the HCV serine protease NS3-4A. N4mBid exhibited a 10–20-fold difference in cell viability between the HCV-infected and mock-infected states, while the parental Huh-7.5 cells showed <2-fold difference under the same conditions. The pronounced difference in n4mBid cell viability between the HCV- and mock-infected states in a 96-well plate format points to its usefulness in cell survival-based high-throughput screens for anti-HCV molecules. The degree of cell death was found to be proportional to the intracellular load of HCV. HCV-low n4mBid cells, expressing an anti-HCV short hairpin RNA, showed a significant growth advantage over naïve cells and could be rapidly enriched after HCV infection, suggesting the possibility of using n4mBid cells for the cell survival-based selection of genetic anti-HCV factors.

Published by Elsevier B.V.

Approximately 180 million people worldwide are infected with hepatitis C virus (HCV), with an incidence of 3–4 million each year (Alter and Seeff, 2000; Wasley and Alter, 2000). Hepatitis C virus is an enveloped, positive-sense RNA virus belonging to the *Flaviviridae* family. The 9.6-kb viral genome encodes a single large polyprotein that is processed by viral and cellular proteinases to produce the virion structural proteins (core and glycoproteins E1 and E2), P7, and nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B) (Bukh et al., 2002; Ikeda et al., 2002; Pietschmann et al., 2002). Although HCV was first discovered two decades ago (Choo et al., 1989), our knowledge of the virus remains very limited. The recent development of a HCV cell culture system (HCVcc) (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005) that supports the entire HCV life cycle in vitro opens new doors to HCV antiviral research. The HCVcc system is based on the genotype 2a JFH-1 strain of HCV that can replicate efficiently without adaptive mutations in human Huh7 cells and derived hepatocellular carcinoma cell lines, such as Huh-7.5 (Blight et al., 2002). Transfection of these hepatocytes with in vitro-transcribed JFH-1, or chimeric J6/JFH-1 genomic RNA, results in the secretion of infectious viral particles.

Unfortunately, cultured cells infected with HCVcc do not show a conveniently identifiable phenotype. Currently, antibody staining is the most widely used assay for detecting HCV infection. The amount of infectious HCV particles in a sample is typically expressed as 50% cell culture infectious dose/ml ($CCID_{50}$/ml) (Lindenbach and Rice, 2005) or focus forming units/ml (FFU/ml) (Zhong et al., 2005). Both methods for infectious virus titer determination entail immune staining of viral proteins by HCV-specific antibodies. Antibody-based detection methods are usually expensive, tedious and not easily adapted to high-throughput settings. The most easily observable cellular phenotype is cell death. The availability of many commercial assays for quantifying cell viability also makes the reporting of cell death easily adaptable to high-throughput applications. Although it has been reported that Huh-7.5 and derived cells infected with HCVcc demonstrate a cytopathic effect (Gottwein et al., 2007; Zhong et al., 2006), the amount of cell death is not sufficient for cell death-based in vitro assays.

In the present study, we describe the creation and characterization of a reporter cell line, n4mBid, that effectively reports HCV infection through a cell-death phenotype. N4mBid cells were generated from the highly HCV-permissive hepatoma cell line Huh-7.5 (Blight et al., 2002). To enhance the cytopathic effect of HCV, a mod-

*Abbreviations:* HCV, hepatitis C virus; HCVcc, HCV cell culture; shRNA, small hairpin RNA; Gluc, *Gaussia* luciferase; 2′-CMA, 2′-C-methyladenosine.

* Corresponding author at: 3122 TAMU, Texas A&M University, College Station, TX 77843, USA. Tel.: +1 979 862 1610; fax: +1 979 845 6446.
** Corresponding author at: The Rockefeller University, 1230 York Avenue, New York, NY 10065, USA. Tel.: +1 979 327 7046; fax: +1 212 327 7048.
*E-mail addresses:* zhilei.chen@chemail.tamu.edu (Z. Chen), ricec@mail.rockefeller.edu (C.M. Rice).
[1] Current address: Artie McFerrin Department of Chemical Engineering, Texas A&M University, USA.

0166-3542/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.antiviral.2010.02.321

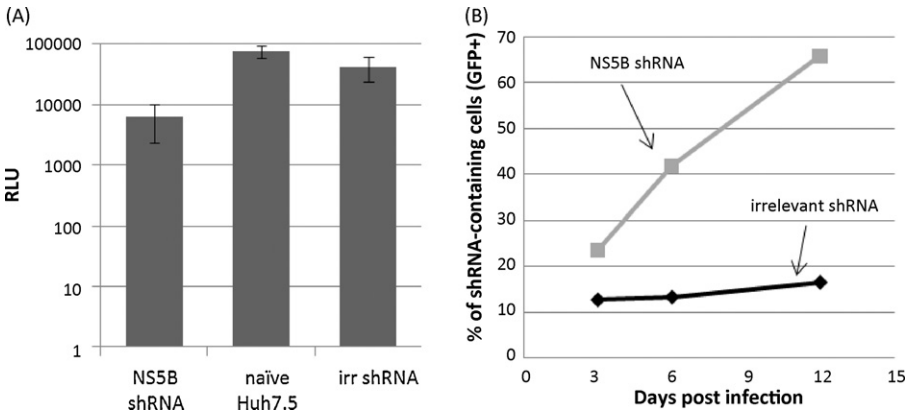


**Fig. 1.** NS5B shRNA expression provides a survival advantage to host Huh-7.5 cells. (A) HCV replication at 36 h after infection. Expression of NS5B shRNA resulted in a ~10-fold decrease in HCV replication levels, as reported by Gluc activity of Jc1FLAG(p7-nsGluc2A) HCVcc (Marukian et al., 2008). RLU, relative luciferase units. (B) NS5B shRNA-expressing cells (squares), but not irrelevant shRNA-expressing cells (diamonds), were quickly enriched from naïve Huh-7.5 cells after HCVcc infection. This experiment was repeated once with similar results.

ified version of the pro-apoptotic protein, Bid (Hsu et al., 2003), was introduced into Huh-7.5 cells. Apoptosis or programmed cell death is a highly regulated process triggered by genetically programmed signaling pathways, and is essential for normal cellular homeostasis and development (Thornberry and Lazebnik, 1998; Zimmermann et al., 2001). Bid is a member of the BH3-only family of apoptosis inducers (Strasser, 2005). Wild-type Bid is activated in the cell by cleavage of its leader peptide by caspases or granzyme B, exposing the BH3 domain. The exposed BH3 domain allows Bid to interact with the Bax protein, setting in motion an apoptotic cascade (Cory and Adams, 2002; Strasser, 2005). In the modified Bid (mBid) system, the endogenous cleavage site of Bid is replaced by the HCV NS5A/NS5B cleavage junction sequence (AEDVVCCSMSYS), making it susceptible to the HCV serine protease NS3-4A (Hsu et al., 2003). The level of mBid-mediated apoptosis is proportional to intracellular expression levels of NS3-4A. The mBid construct has been shown to induce apoptosis in rat fibroblasts in the presence of the HCV serine protease, and in Huh7 cells transfected with a HCV subgenomic replicon (Hsu et al., 2003).

We first devised a competitive enrichment assay for evaluating cells for their ability to both support productive virus infection (virus spread) and undergo a significant cytopathic effect. We reasoned that, upon HCV infection, cells with a lower load of intracellular HCV, via expression of an anti-HCV factor for example, should experience weaker apoptosis than cells with a high HCV load. The survival advantage of HCV-low cells in the midst of a HCV challenge should in turn lead to their enrichment within a cell population subjected to HCV infection over time. In addition, when such a population of cells is infected with HCV at a multiplicity of infection (MOI) less than 1, cells supporting faster virus spread (i.e. able to produce more viruses) should generate a higher selection pressure for the survival of HCV-low cells. To probe the survival advantage experienced by HCV-low cells present in a population of cells infected with HCVcc, we first created a HCV-low sub-population using a shRNA targeting the HCV genome. An anti-NS5B shRNA harboring the targeting sequence CATTATGACTCAGTCTTAA was inserted into the lentiviral vector pLVTHM (Wiznerowicz and Trono, 2003) and introduced into Huh-7.5 cells via lentiviral transduction. The shRNA-bearing lentiviral pseudoparticles contain an independent EGFP reporter gene under the control of a EF1a promoter, allowing the shRNA-expressing cells to be monitored by flow cytometry. After infection with a *Gaussia* luciferase (Gluc) reporter HCVcc, Jc1FLAG(p7-nsGluc2A) (Marukian et al., 2008), Huh-7.5 cells expressing NS5B shRNA showed a ~10-fold lower supernatant Gluc activity relative to naïve Huh-7.5 cells and cells expressing an irrelevant shRNA, demonstrating the ability of the selected shRNA to suppress HCV replication inside a host cell (Fig. 1A). To verify the growth advantage of the NS5B shRNA-expressing HCV-low cells over naïve cells after HCV infection, the shRNA-expressing Huh-7.5 cells were mixed with naïve Huh-7.5 cells at a ratio of ~1:10. This cell population was subsequently challenged with Jc1 HCVcc (Pietschmann et al., 2006) at a MOI of ~1 and the proportion of $GFP^+$ cells was monitored using flow cytometry. The percentage of NS5B shRNA-expressing ($GFP^+$) cells increased over time after infection, suggesting that HCV exerts a selective pressure for the survival and enrichment of HCV-low cells from within a cell population containing an excess of naïve cells (Fig. 1B). The percentage of irrelevant shRNA-expressing cells remained relatively constant under the same conditions. The enrichment of NS5B shRNA-expressing cells continued for ~12 days post-infection, resulting in a peak representation of ~66%. A decline in the percentage of NS5B shRNA-expressing cells was observed after 12 days (data not shown), perhaps due to the emergence over time of non-shRNA-containing cell populations resistant to HCV infection. Although these results demonstrate the growth advantage experienced by the HCV-low, NS5B shRNA-expressing Huh-7.5 cells over naïve cells in response to HCV infection, this growth advantage is only minor as the NS5B shRNA-expressing cells require 12 days to be significantly enriched for a cell population.

To generate a cell line that is able to more efficiently report HCV infection via cell death and thus able to support the more rapid enrichment of HCV-low cells from within a HCV-infected cell population, we introduced the mBid construct into Huh-7.5 cells. For generating a HCV-permissive cell line harboring mBid, HIV lentiviral particles harboring the mBid gene and pseudotyped with the envelope glycoprotein from vesicular stomatitis virus were produced from 293T cells using the pTRIP expression vector (Sirven et al., 2001; Zennou et al., 2000) and used to transduce Huh-7.5 cells to obtain a population of mBid-expressing cells. Ten different clones from this population were isolated and evaluated using our competitive enrichment assay, as described below.

A fraction (~0.5%) of each of the 10 clonally isolated mBid-expressing, Huh-7.5-derived cell lines was transduced with the above-described NS5B shRNA. These cell populations were subsequently challenged with Jc1 HCVcc at MOI <1. Naïve mBid-expressing Huh-7.5 cells underwent more pronounced apoptosis than cells expressing the NS5B shRNA, leading to a rapid increase in the percentage of NS5B shRNA-expressing ($GFP^+$) cells over time. Different rates of enrichment were obtained for different mBid clones after HCV infection. Data obtained from 3 representative mBid clones and naïve Huh-7.5 cells are shown in Fig. 2. The observed difference in enrichment rates is likely due to differ-