Research Article

EASL EUROPEAN ASSOCIATION FOR THE STUDY OF THE LIVER | JOURNAL OF HEPATOLOGY

Human pluripotent stem cell-derived hepatocytes support complete replication of hepatitis C virus

Philip Roelandt[1,2], Susan Obeid[3], Jan Paeshuyse[3], Jolien Vanhove[1], Alfons Van Lommel[4], Yaakov Nahmias[5], Frederik Nevens[2,*], Johan Neyts[3,*], Catherine M Verfaillie[1,*]

[1]*Interdepartmental Stem Cell Institute Leuven, KU Leuven, Belgium;* [2]*Hepatology Department, University Hospitals Leuven, Belgium;* [3]*Department of Microbiology and Immunology, Rega Institute for Medical Research, Leuven, Belgium;* [4]*Pathology Department, KU Leuven, Belgium;* [5]*Center for Bioengineering, Hebrew University of Jerusalem, Israel*

Background & Aims: Worldwide, about 180 million people are chronically infected with the hepatitis C virus (HCV). Current *in vitro* culture systems for HCV depend chiefly on human hepatoma cell lines. Although primary human hepatocytes support HCV infection *in vitro*, and immunodeficient mice repopulated with human hepatocytes support HCV infection *in vivo*, these models are limited because of shortage of human livers to isolate hepatocytes. Therefore, there is significant interest in the establishment from of a HCV culture system in human stem cell-derived hepatocyte-like cells.

Methods: Human embryonic stem cell (hESC)-derived hepatocytes were infected with HCV in the presence or absence of direct acting antivirals. After inoculation, replication of HCV was analyzed extensively.

Results: We demonstrate that hESC-derived hepatocytes can be infected with the HCV JFH1 genotype 2a, resulting in the production of viral RNA in the stem cell progeny. Viral replication is inhibited by a non-nucleoside HCV polymerase-inhibitor (HCV-796), a cyclophilin binding molecule (Debio 025-Alisporivir) and the protease inhibitor VX-950 (Telaprevir). Stem cell-derived hepatocytes produced, for more than 10 days, the HCV core protein as well as virions that were capable of re-infecting hepatoma cells.

Conclusions: Hepatocytes derived from hESC support the complete HCV replication cycle (including the production of infectious virus), and viral replication in these cells is efficiently inhibited by selective inhibitors of HCV replication.

Keywords: Hepatitis C virus; Replication; Human embryonic stem cells; Differentiation.

Received 19 September 2011; received in revised form 26 March 2012; accepted 29 March 2012; available online 17 April 2012

*Corresponding authors. Addresses: Interdepartmental Stem Cell Institute Leuven, UZ Leuven Campus Gasthuisberg, Onderwijs & Navorsing 4, Herestraat 49 Box 804, 3000 Leuven, Belgium. Tel.: +32 16/33 02 95; fax: +32 16/33 02 94 (C. Verfaillie), Department of Microbiology and Immunology, Rega Institute for Medical Research, Minderbroedersstraat 10, 3000 Leuven, Belgium. Tel.: +32 16/33 73 53; fax: +32 16/33 73 40 (J. Neyts), Hepatology Department, UZ Leuven Campus Gasthuisberg, Herestraat 49, 3000 Leuven, Belgium. Tel.: +32 16/34 42 99; fax: +32 16/34 43 87 (F. Nevens).

E-mail addresses: Frederik.nevens@uzleuven.be (F. Nevens), Johan.neyts@rega.kuleuven.be (J. Neyts), Catherine.verfaillie@med.kuleuven.be (C.M Verfaillie).

Abbreviations: AFP, α-foetoprotein; ALB, albumin; ApoB100, apolipoprotein B100; CLDN1, claudin-1; CLDN6, claudin-6; CYP3A4/5, cytochrome P450 family 3 subfamily A polypeptide 4/5; HCV, hepatitis C virus; hESC, human embryonic stem cells; HNF4α, hepatocyte nuclear factor 4α; OCLN, occludin; SR-B1, scavenger receptor B1.

Introduction

About 180 million people worldwide are chronically infected with the hepatitis C virus (HCV), this virus is the leading cause of chronic liver disease in the Western world. When left untreated, patients have a high chance of developing cirrhosis and hepatocellular carcinoma. Treatment with pegylated interferon and ribavirin for 24–48 weeks, the current standard of care, clears the virus in about 50% of the patients but is most often associated with hematological, systemic and psychological side effects. The HCV NS3 protease inhibitors, boceprevir [1] and telaprevir [2,3], have recently been approved for use in combination with pegylated interferon and ribavirin. A number of other potent and selective antivirals are currently in clinical development [4–6].

For *in vitro* studies on HCV infection and replication, human hepatoma cell lines are being employed [7]. However, because metabolic differences exist between rapidly proliferating cancer cell lines on the one hand and quiescent primary hepatocytes on the other hand, and the fact that hepatoma cells such as the HuH7.5.1 line carry mutations in the dsRNA sensor retinoic acid-inducible gene-I (*RIG-I*)[8], these hepatoma cells do not allow recapitulating all aspects of HCV replication in the human liver. Primary human hepatocytes were shown by several groups to support HCV infection *in vitro* [9,10] and in mouse models [11]. The scarcity of primary human hepatocytes and the fact that mature functioning hepatocytes cannot be readily expanded in culture, have generated a significant interest in the production of functionally active hepatocyte-like cells from stem cells, including human embryonic stem cells (hESC).

We here demonstrate that hepatocytes derived from hESC support the complete HCV replication cycle (including the

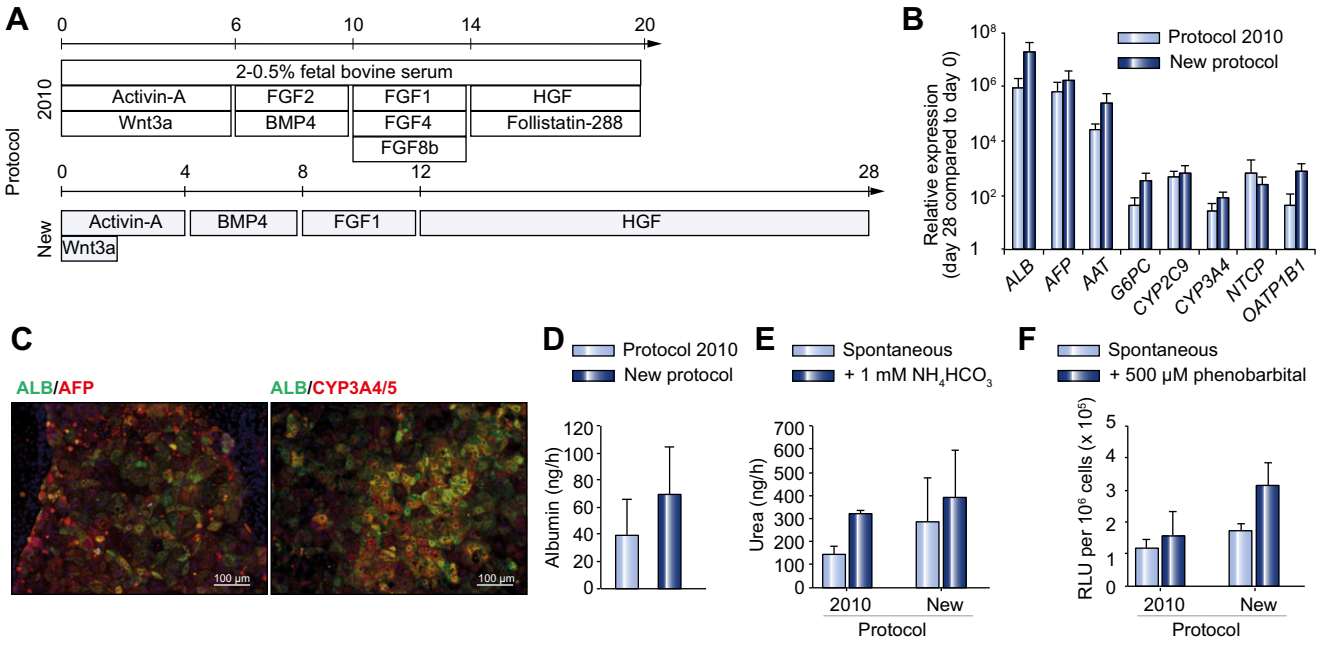

Fig. 1. Hepatocyte differentiation from human embryonic stem cells with adapted protocol. (A) Growth factor combinations used [12] compared with adapted protocol used in the current study. Final concentrations were Activin-A 100 ng/ml, Wnt3a 50 ng/ml, BMP4 50 ng/ml, FGF1 50 ng/ml, and HGF 20 ng/ml. (B) Differentiated progeny of the hESC cell line was harvested on day 28 of differentiation using the published protocol or the new protocol. Expression of hepatoblast/hepatocyte marker genes was evaluated by RT-qPCR. Data are provided as fold change compared with levels on day 0. Data are shown as mean ± SD (n >3). (C) After 28 days, cells that had been differentiated using the new protocol were fixed and immunostaining was performed for ALB/AFP and ALB/CYP3A4/5. (Representative example of n = 3). (D-F) After 28 days of differentiation, the secretion of albumin (D), the production of urea after addition of ammonia (E), and the CYP3A4 activity, un-induced and after induction with phenobarbital (F) were compared using cultures obtained using the published protocol and the new protocol (n = 3).

production of infectious virus) that can be inhibited by selective inhibitors of viral replication.

Materials and methods

Cell populations

Human embryonic stem cells (WA09) were purchased from WiCell, Madison, WI. Ethical approval was obtained for these studies from the Ethics Committee on Use of Human Subjects in Research, at the UZ Gasthuisberg, KU Leuven.

hESC were differentiated towards liver-specific progeny using a protocol described earlier [12] with minor modifications. Specifically, differentiations were done in 12-well plates (Corning 3513) pre-coated with 1.6% Matrigel (BD 356231) diluted in PBS (Gibco 10010) or DMEM-F12 (Gibco 21331) for 1–2 h at 37 °C, in a 21% O_2 – 5.8% CO_2 – 37 °C incubator. hESC were allowed to grow in mTeSR medium (Stem Cell Technologies) until 70–80% confluence was obtained. To induce differentiation, expansion medium was switched to basal differentiation medium, supplemented with the sequential growth factor cocktails, as described in Fig. 1, for 20 days. On day 23, cultures were infected with HCV virus (see below), and cells subsequently maintained in hepatocyte growth factor (HGF) supplemented medium. Differentiation of hESC under the new differentiation protocol was assessed by RT-qPCR for gene transcripts expressed in immature and mature hepatocytes; immunostaining for albumin (ALB), α-foetoprotein (AFP) and cytochrome 3A4/5 (CYP3A4/5); as well as functional tests including albumin secretion, ureagenesis (with and without ammonia), and CYP3A4 activity (with and without induction with phenobarbital) (see below).

The highly permissive Huh-7.5.1 cells (a kind gift of Prof. F. Chisari) were maintained in Dulbecco's modified Eagle's Medium (DMEM, Gibco, Merelbeke, Belgium) supplemented with 10% heat-inactivated fetal bovine serum (Integro, Zaandam, The Netherlands), 1× non-essential amino acids, 100 IU/ml penicillin (Gibco), 100 µg/ml streptomycin (Gibco) and maintained at 37 °C with 5% CO_2.

Hepatitis C virus

HCV cell-culture mutant virus (JFH1 genotype 2a containing 3 mutations N534K (N6), F172C and P173S (FP→CS)) was described by Delgrange *et al.* [13] A virus stock with a titer of 10^7 FFU/ml was used [14]. At the time of infection (day 23 of differentiation), 0.5 ml of HCV virus stock was used to inoculate the cell cultures. Approximately 10^6 cells/well were incubated in 1 ml (0.5 ml virus stock + 0.5 ml fresh medium) for 72 h at 37 °C. Next, supernatants were removed, cells were extensively washed (2 times 7 ml) with phosphate buffered saline (PBS), and incubated further in 1 ml fresh differentiation medium supplemented with HGF. Supernatants were subsequently collected on day 4, 8 followed by a wash step and culture medium replenishment until day 11 after infection. For the inhibition experiments, 10 µM HCV-796 [4], 5 µM Debio 025 [5] or 1 µM VX-950 [3] was added to the supernatants at day 29 of differentiation (n = 2–4). At the concentrations used, none of these molecules resulted in an adverse effect on the host cells (as assessed microscopically), which is also in line with absence of toxicity (as also documented earlier by us and others) in hepatoma cells. The inhibitory effect of VX-950 and HCV-796 on HCV-infected Huh7.5.1. cells is shown is Supplementary Fig. S2.

Immunocytochemistry

hESC progeny was fixed using 4% Neutral Buffered Formalin (NBF) for 15 min at room temperature. Permeabilization and blocking were done for 15 min using PBS containing 0.2% Triton X-100 (Acros Organics) and 3% donkey serum (Jackson). Cells were then incubated with a mixture of primary antibodies diluted in PBS overnight at 4 °C. After three washes in PBS, cells were incubated with a mixture of secondary antibodies and Hoechst (Sigma) for 30 min at room temperature. Primary antibodies and dilutions were mouse anti-CD81 (BD Biosciences 555675, 1:100), rabbit anti-SR-B1 (Abcam AB3, 1:1000), mouse anti-HNF4α (Abcam AB41898, 1:200), rabbit anti-ALB (Dako A0001, 1:4000), mouse anti-CYP3A4/5 (BD Biosciences WB-MAB-3A, 1:250) and rabbit anti-HCV NS5A (a kind gift of Prof. R. Bartenschlager, 1:2000). Isotypes were mouse IgG_1 (BD Biosciences 550878), mouse IgG_{2A} (Sigma M9144), rabbit serum (Dako X0902). All isotype control stains were negative a well as NS5A staining in mock infected cells