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Primary Human Trophoblasts Expressing Four Essential Receptors of Hepatitis C Virus Entry but Resistance to Infection *in Vitro*

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Abstract

Background: Vertical transmission (VT) is generally considered to be the leading route of pediatric acquisition of Hepatitis C Virus (HCV). However, the mechanism of HCV VT has never been satisfactorily illustrated. Moreover, CD81, scavenger receptor class B type I (SR-B1), claudin 1 (CLDN1) and occludin (OCLN) were four necessary receptors for HCV cell infection. The expression of the essential receptors may explain the reason of HCV VT.

Objective: To investigate the probable mechanism of HCV VT.

Methods: We isolated trophoblasts from human term placental samples with approval from the Institutional Review Board at the Tongji Hospital of Tongji University and cultured the cells in vitro. Flow cytometry and Western blotting were used to detect the expression of the essential receptors for HCV infection on trophoblasts. Then we generated HCV pseudoparticle (HCVpp) and cell culture-produced HCV (HCVcc) to infect HEK-293T cells and trophoblasts,.At 72 h post-infection, HCVpp and HCVcc were determined in the infected cells.

Results: Trophoblasts expressed CD81, SR-B1, CLDN1 and OCLN, which were essential for HCV cell entry. However, trophoblasts were not infected by HCVpp or HCVcc.

Conclusion: Primary human trophoblasts possess the receptors necessary for HCV infection but resistant to infection *in vitro*.

Keywords: Trophoblast; Receptor; Hepatitis C virus; Vertical transmission

Introduction

Hepatitis C virus (HCV) is an enveloped single-stranded RNA (ssRNA) virus. It is a well-known origin of adult liver morbidity and mortality, but its problem in children is often overlooked [1]. As we all know, the risk factors most frequently cited as accounting for the bulk of HCV transmission worldwide are blood transfusions from unscreened donors, injection drug use, unsafe therapeutic injections, and other health-care related procedures [2]. Although some studies suggested that many American children acquired HCV from infected blood or blood products, the leading route of pediatric acquisition of HCV in most parts of the world is now vertical transmission [1, 3-5]. And the prevalence of vertical transmission (VT) of HCV is estimated at approximately 5% (3%-10%) [5]. Despite an increased understanding of the risk factors involved in HCV transmission; thus far, little is known about the mechanism of HCV VT.

Studies have presented conclusive evidence indicating that HCV infection is initiated by interactions between the E2 glycoprotein in HCV and several cell surface molecules [6]. Furthermore, advances in the development of *in vitro* system to study the HCV life cycle have demonstrated an essential role for tetraspanin CD81 [7-11], scavenger receptor class B type I (SR-B1) [12-14], claudin 1 (CLDN1) [15] and tight-junction protein occludin (OCLN) [16] in virus entry. CD81 and SR-B1 bind HCV encoded E1E2 glycoproteins with high affinity. The CD81 tetraspanin is first identified as an HCV envelope glycoprotein E2-binding receptor and shown to be required for HCV entry into target cells [7]. SR-B1 acts at similar time points than CD81 forming part of the receptor complex required for HCV entry into the target cell [9]. And The HCV envelope glycoproteins do not directly interact with CLDN1, but CLDN1 interacts with CD81 and thereby plays an important role during post-binding steps of the HCV entry process [10,15,17]. OCLN have also been demonstrated to participate in post-binding steps of the HCV entry process [16].

The placenta is a hematopoietic organ mainly consisting of placental epithelium (trophoblasts), and it serves as a barrier against maternal-fetal transmission. Nutrients, certain drugs, and hormones, as well as viruses such as HCV, are some of the substances that can pass through the placental barrier to reach the fetus. Trophoblasts are the first line of defense against virus infection. Therefore, this study aimed to investigate the mechanism of HCV VT by isolation and *In vitro* culture of trophoblasts.

Here, trophoblasts obtained from placentas after delivery via Caesarian section were cultured in vitro. Flow cytometry and Western blotting were used to detect the essential receptors of HCV infection on the trophoblasts. However, HCVpp and HCVcc infection of trophoblasts *in vitro* could not be observed and thus it could not be verified whether HCV is capable of entering and infecting primary human trophoblasts.

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Materials and Methods

Trophoblast isolation and culture

Trophoblasts were isolated from human term placental samples with approval from the Institutional Review Board at the Tongji Hospital (affiliated to the Tongji University). Human term placenta was obtained from normal pregnant women after they underwent Cesarean section. Trophoblasts were purified according to the method of Kliman et al. [18]. After delivery of the infant, the placenta was removed and placental lobules were cut, stored in an aseptic bottle containing 4°C normal saline, and immediately brought to the laboratory for the experiment. The placental lobules were transferred to a beaker and rinsed thoroughly in 4°C normal saline under aseptic conditions. The tissues were cut into 1-3 mm^3 sections and digested with 0.125% trypsin (Sigma) and 0.01% DNase I (Sigma) in warmed Dulbecco's modified Eagle's medium (DMEM) for 30 min at 37°C. After filtration through 0.01-mm stainless steel wire mesh, cell suspensions were centrifuged at 2,200 rpm for 10 min, and the supernatants were resuspended in 5 ml DMEM. The resulting cell suspension was placed in a 5-70% Percoll (GE) gradient made up in Hanks' solution. The gradients were centrifuged at 2,550 rpm for 20 min. The 30-45% band containing the trophoblasts were removed after centrifugation, washed with DMEM, and resuspended in DMEM. The viability of the trophoblasts was assessed by trypan blue exclusion. Live cells comprised more than 90% of the cytotrophoblast preparation. The purified trophoblasts were counted and then diluted with DMEM-HG containing 4 mM glutamine (Sigma), 100 U/ml penicillin, 0.1 mg/ml streptomycin and 10% heat-inactivated fetal calf serum (Gibco) to achieve a concentration of 10^6 cells/ml, seeded in 24- and 96-well plates, then plated in 35-mm culture dishes. Cells were incubated in 5% CO_2 at 37°C. Culture media were changed every 24 h.

Identification of trophoblasts

The Percoll gradient-purified cells cultured in the 96-well plate were fixed using methanol for 30 min at minus 20°C. The cells were washed twice with phosphate-buffered saline (PBS), blocked with 3% bovine serum albumin (BSA) at room temperature for 1 h, and incubated with anti-cytokeratin or anti-vimentin antibodies (Invitrogen). Then, the cells were incubated with anti-rabbit IgG (Invitrogen) and stained with 4′-6-diamidino-2-phenylindole (DAPI). The stained cells were then observed under a fluorescence microscope.

Detection of SR-B1, CLDN1 and OCLN in trophoblasts by Western blotting

Western blotting was used to analyze the expressions of SR-B1, CLDN1, and OCLN on the trophoblasts. Total protein was extracted from cells after they were cultured for 48 h. Huh7.5.1 cells were used as the positive control and processed using a similar protocol. The protein extract (50 μg) was loaded onto 12.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel and electrophoresed. After electrophoresis, the resolved proteins were transferred onto nitrocellulose membrane. Nonspecific binding sites were blocked using 5% non-fat milk in Tris Buffered Saline with Tween-20 (TBST) at room temperature for 2 h. Immunoblotting was carried out using monoclonal antibodies against SR-B1 (BD), CLDN1, and OCLN (Invitrogen). For the loading controls, the same membranes were immunoblotted using monoclonal antibodies against GAPDH (Sigma). The antibodies were detected using rabbit anti-mouse antibody (Invitrogen) and visualized using a highly sensitive chemiluminescence system (GE Healthcare).

Detection of CD81 by flow cytometric analysis

After the trophoblasts cultured for 48 h, they were digested with trypsin, centrifuged at 3,000 rpm for 10 min, resuspended in PBS, and centrifuged again. Then, the suspensions were incubated with anti-CD81 (Santa) antibody diluted in 1% BSA at room temperature for 80 min. After washing twice with PBS, goat anti-rabbit antibody (Invitrogen) was added, and the mixture was incubated in the dark at room temperature for 80 min. The cells were subjected to flow cytometric analysis.

Infection with HCVpp

HEK-293T cells were co-transfected with an HCV envelope protein expression vector and packaged plasmid based on the HIV-1 strain NL4-3 (Invitrogen) to generate HCVpp as described previously [19]. The co-transfected plasmids could encode HCV envelope glycoproteins, HIV gag/pol (pLP1), and HIV rev (pLP2), and pLenti 6 could encode enhanced green fluorescence protein. The HCV envelope-expressing plasmid used here was genotype 1b strain Con-1 (provided by CM Rice, Rockefeller University, NY, USA). At 66 h after transfection, the supernatants containing pseudotype particles were harvested by centrifugation and used for infection. Mock pseudoparticle was generated using a similar protocol, but in the absence of an HCV envelope protein expression vector.

The trophoblasts were infected with HCVpp after cultured for 24 h. At 24 h post-infection, the supernatants were removed and cells were incubated in normal culture media. At 72 h post-infection, cells were observed with a fluorescence microscope to determine the infection of HCVpp. Mock infections were performed in parallel to HCVpp infection.

Infection with HCVcc

Plasmid pJFH1, containing the full-length genomic cDNA for HCV JFH-1 (genotype 2a), kindly provided by Dr. Takaji Wakita, National Institute of Infectious Diseases, Tokyo Japan, was used to generate HCVcc as described previously [19]. Briefly, the plasmid was linearized and used as the template for transcription using an *In vitro* MEGAscript kit (Promega). The transcribed RNA was electroporated into Huh7.5.1 cells. Viral stocks were obtained by harvesting the culture supernatants of electroporated Huh 7.5.1 cells and virus titers were determined with Huh 7.5.1 cells [19,20]. Three days later, the infected cells were washed and fixed with ice-cold methanol for 20 min at minus 20°C, and stained for NS5A with monoclonal antibody (mAb) 9E10 and Alexa 488-conjugated anti-mouse IgG. Infection was quantified by enumerating NS5A-positive foci and the virus titer of focus-forming units (FFU)/ml was calculated.

Following an overnight culture, the trophoblasts, seeded in 24-well plate, were infected with HCVcc and Huh7.5.1 cells were used as positive controls, respectively. The medium was removed after 8 h, washed with 1ml PBS and 500 ul normal medium was added per well. The medium was changed every 24 h. At three days post infection, infected cells were identified by the expression of NS5A or HCV RNA. Purified cellular RNA samples (Qiagen, Hamburg, Germany) were amplified for HCV RNA (Primer Design Ltd, Southampton, UK) in a single-tube reverse-transcription polymerase chain reaction in accordance with manufacturer's guidelines (CellsDirect kit; Invitrogen) and fluorescence monitored in an MxPro 3000 real-time polymerase chain reaction machine (Stratagene, La Jolla, CA). In all reactions the house keeping gene glyceraldehyde-3-phosphate dehydrogenase was included as an internal endogenous control for amplification efficiency and for RNA quantification (primer-limited endogenous control; ABI, Carlsbad, CA).

Results

Trophoblast isolation and culture

Trypan blue staining of the gradient-purified cells showed that more than 90% of the cells were viable. Negative vimentin staining and positive cytokeratin staining were observed, verifying that the collected cells were human trophoblasts. The purified trophoblasts contained very little contamination of endothelial cells or fibroblasts, as evidenced by the anti-vimentin antibody staining (Figure 1).

Initially, trophoblasts showed individual growth when cultured, but they appeared to form aggregates consistently. After 5 days of culture, syncytia were formed.

Expression of CD81, SR-B1, CLDN1, and OCLN on trophoblasts

The expressions of CD81, SR-B1, CLDN1, and OCLN were detected with Western blotting (Figure 2) and flow cytometry analysis (Figure 3), suggesting the possibility of HCV infection of trophoblasts.

In vitro HCVpp infection of trophoblasts

Huh7.5.1 cells and trophoblasts were infected with HCVpp. At 72 h after infection, Huh7.5.1 cells and trophoblasts had infections rates of 30% and 0%, respectively. The infection rates of trophoblasts were much lower than those of Huh7.5.1 cells, indicating that the trophoblasts cultured *In vitro* may be resistant to infection with HCVpp (Figure 4).

In vitro HCVcc infection of trophoblasts

Huh7.5.1 cells and trophoblasts were incubated with HCVcc for 8 h and 3 days after infection, NS5A and HCV RNA level were quantified (Figure 5A and B). There were no detectable NS5A and HCV RNA in trophoblasts three days post-infection, demonstrating trophoblasts resistance to HCVcc infection *in vitro*.

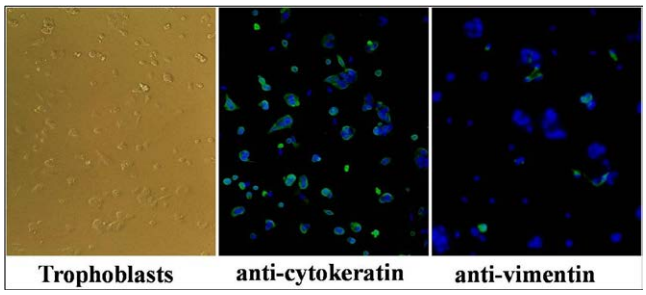

Figure 1: Percoll gradient-purified cells cultured for 48 h were verified to be trophoblasts by immunofluorescence staining. Negative vimentin staining and positive cytokeratin staining were observed, verifying that the collected cells were human trophoblasts. The purified trophoblasts contained very little contamination by endothelial cells and fibroblasts, as evidenced by the immunofluorescence staining with anti-vimentin antibodies.

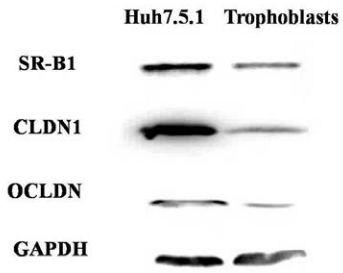

Figure 2: Expressions of scavenger receptor class B type I (SR-B1), claudin 1 (CLDN1) and occludin (OCLN) were detected with Western blotting after trophoblasts were cultured for 48 h *in vitro*.

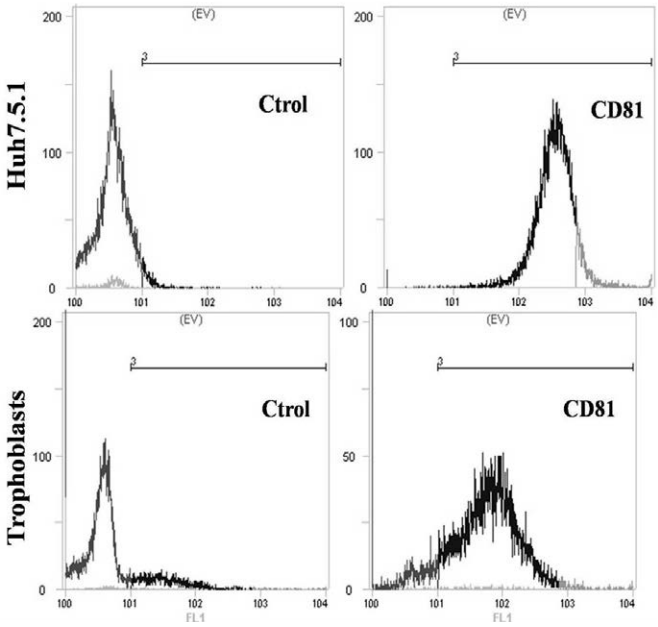

Figure 3: Expressions of CD81 was analyzed by flow cytometry after trophoblasts were cultured for 48 h *in vitro*.

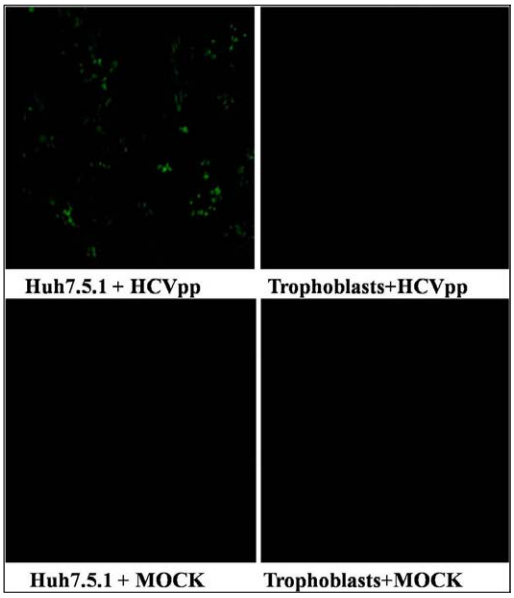

Figure 4: Trophoblasts and Huh 7.5.1 cells were infected with HCVpp after cultured for 24 h. At 72 h post-infection, luciferase activity was observed under a fluorescence microscope.

Discussion

HCV infection is recognized as a disease of global importance. It is considered to be a major health and economic burden in adults and in children in both developing and developed countries. And infection in the uterus is a significant route of vertical HCV transmission [1, 3-5]. However, the exact mechanism of HCV VT is not very clear.

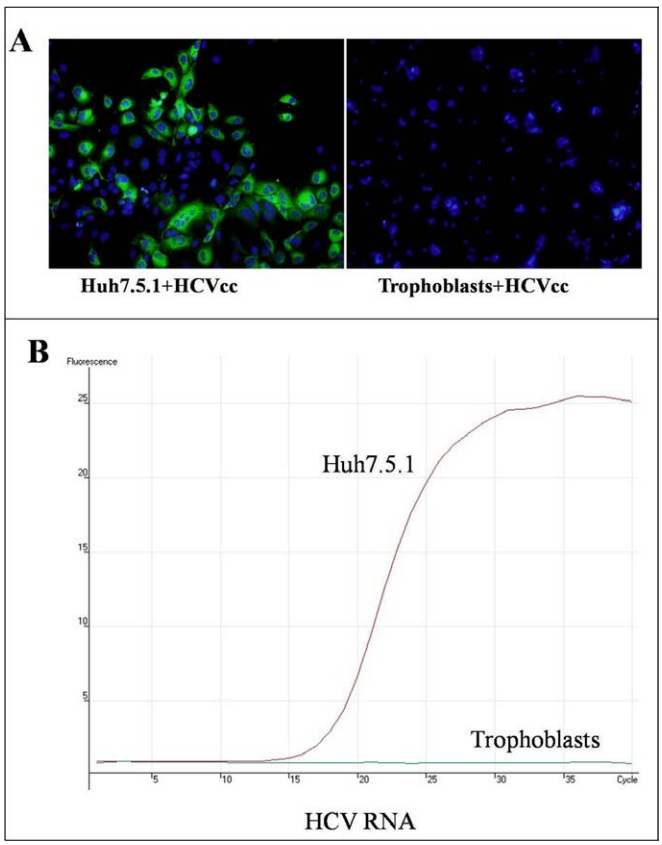

Figure 5: Huh7.5.1 cells and trophoblasts were incubated with HCVcc for 8 h and 3 days after infection, NS5A and HCV RNA level were quantified. (A) Infected cells stained for NS5A. (B) Amplification curve of HCV RNA from infected cells in real-time PCR.

The interaction of HCV with its cellular receptor initiates a chain of dynamic events that enables its entry into the trophoblasts, then to the fetus. CD81, SR-B1, CLDN1, and OCLN are considered essential for HCV cell entry [6]. CD81 and SR-B1 are the classical receptors of HCV and work together in the initial stage of HCV infection. They are considered vital for the infection of HCV [7,11]. OCLN and CLDN1 are tight junction proteins, and a previous study has demonstrated that the expression of these proteins is indispensable for the invasion of HCV [16]. This study focused on these four HCV receptors and attempted to investigate the mechanism of HCV VT.

The placenta serves as a barrier between the mother and fetus, and it prevents the transmission of certain drugs and infectious agents. The placental epithelium mainly consists of trophoblasts; therefore, isolating and culturing trophoblasts *in vitro* is vital for researching the mechanism through which infectious agents crossing the placenta barrier infect the fetus [21]. Here, trophoblasts were isolated successfully according to the method of Kliman et al. as described previously [18].

Trophoblasts have always been the focus for the study of HCV infection *in vitro* to investigate VT. Nevertheless, little evidence has been reported about the possibility of HCV infecting trophoblasts through receptors or about the mechanism by which this occurs. Our study is the first to detect the expressions of the HCV receptors CD81, SR-BI, CLDN1, and OCLN on trophoblasts.

Through a series of experiments, the expressions of four essential receptors in primary human trophoblasts were verified, suggesting the possibility of HCV infection of trophoblasts. However, green fluorescence was not observed in trophoblasts infected with HCVpp and NS5A and HCV RNA were not detectable in trophoblasts infected with HCVcc. A possible explanation is that the expressions of the four receptors on trophoblasts cultured *in vitro* were considerably less than those *in vivo*, as a result of which the trophoblasts were not susceptible to HCV infection in vitro. Moreover, it is possible that other factors involved in HCV entry were not expressed by primary trophoblasts in vitro. In addition, the exact timing of vertical HCV transmission is unknown. Both intrauterine and intrapartum infections are possible [22-24]. The intrauterine infection maybe happens at an early or middle or late stage of pregnancy [25, 26] to date little is known about that. Consequently, there is no enough evidence to explain the exact mechanism of HCV VT. Further experiment and inquiry is required.

In this study, HCV infection of primary human trophoblasts could not be simulated *in vitro* using HCVpp and HCVcc. Nevertheless, our study is the first to verify that primary human trophoblasts possess the receptors necessary for HCV infection. Further studies should be performed to obtain additional insights into HCV VT.

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References

1. Jhaveri R, Hashem M, El-Kamary SS, Saleh DA, Sharaf SA4, et al. (2015) Hepatitis C Virus (HCV) Vertical Transmission in 12-Month-Old Infants Born to HCV-Infected Women and Assessment of Maternal Risk Factors. Open Forum Infect Dis 2: ofv089.
2. Shepard CW, Finelli L, Alter MJ (2005) Global epidemiology of hepatitis C virus infection. Lancet Infect Dis 5: 558-567.
3. Raynes-Greenow C, Polis S, Elliott E, Hardikar W, Kesson A, et al. (2015) Childhood hepatitis C virus infection: An Australian national surveillance study of incident cases over five years. J Paediatr Child Health.
4. Benova L, Awad SF, Miller FD, Abu-Raddad LJ (2015) Estimation of hepatitis C virus infections resulting from vertical transmission in Egypt. Hepatology 61: 834-842.
5. Pawlowska M, Domagalski K, Pniewska A, Smok B, Halota W, et al. (2015) What's new in hepatitis C virus infections in children? World J Gastroenterol 21: 10783-10789.
6. Zeisel MB, Felmlee DJ, Baumert TF (2013) Hepatitis C virus entry. Curr Top Microbiol Immunol 369: 87-112.
7. Pileri P, Uematsu Y, Campagnoli S, Galli G, Falugi F, et al. (1998) Binding of hepatitis C virus to CD81. Science 282: 938-941.
8. Khan AG, Whidby J, Miller MT, Scarborough H, Zatorski AV, et al. (2014) Structure of the core ectodomain of the hepatitis C virus envelope glycoprotein 2. Nature 509: 381-384.
9. Kapadia SB, Barth H, Baumert T, McKeating JA, Chisari FV (2007) Initiation of hepatitis C virus infection is dependent on cholesterol and cooperativity between CD81 and scavenger receptor B type I. J Virol 81: 374-383.
10. Krieger SE, Zeisel MB, Davis C, Thumann C, Harris HJ, et al. (2010) Inhibition of hepatitis C virus infection by anti-claudin-1 antibodies is mediated by neutralization of E2-CD81-claudin-1 associations. Hepatology 51: 1144-1157.
11. Lavie M, Sarrazin S, Montserret R, Descamps V, Baumert TF, Duverlie G, et al. Identification of conserved residues in hepatitis C virus envelope glycoprotein E2 that modulate virus dependence on CD81 and SRB1 entry factors. J Virol 88: 10584-1097.
12. Scarselli E, Ansuini H, Cerino R, Roccasecca RM, Acali S, et al. (2002) The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. EMBO J 21: 5017-5025.
13. Bartosch B, Vitelli A, Granier C, Goujon C, Dubuisson J, et al. (2003) Cell entry of hepatitis C virus requires a set of co-receptors that include the CD81 tetraspanin and the SR-B1 scavenger receptor. J Biol Chem 278: 41624-41630.
14. Catanese MT, Graziani R, Hahn TV, Moreau M, Huby T, et al. (2007) High-

avidity monoclonal antibodies against the human scavenger class B type I receptor efficiently block hepatitis C virus infection in the presence of high-density lipoprotein. J Virol 81: 8063-8071.

15. Evans MJ, von Hahn T, Tscherne DM, Syder AJ, Panis M, et al. (2007) Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. Nature 446: 801-805.
16. Ploss A, Evans MJ, Gaysinskaya VA, Panis M, You H, et al. (2009) Human occludin is a hepatitis C virus entry factor required for infection of mouse cells. Nature 457: 882-886.
17. Harris HJ, Davis C, Mullins JG, Hu K, Goodall M, et al. (2010) Claudin association with CD81 defines hepatitis C virus entry. J Biol Chem 285: 21092-21102.
18. Kliman HJ, Nestler JE, Sermasi E, Sanger JM, Strauss JF 3rd (1986) Purification, characterization, and in vitro differentiation of cytotrophoblasts from human term placentae. Endocrinology 118: 1567-1582.
19. Tong Y, Zhu Y, Xia X, Liu Y, Feng Y, et al. (2011) Tupaia CD8, SR-BI, claudin-, and occludin support hepatitis C virus infection. J Virol 85: 2793-2802.
20. Kato T, Date T, Murayama A, Morikawa K, Akazawa D, et al. (2006) Cell culture and infection system for hepatitis C virus. Nat Protoc 1: 2334-2339.
21. Yeung LT, Roberts EA (2010) Current issues in the management of paediatric viral hepatitis. Liver Int 30: 5-18.
22. Indolfi G, Resti M (2009) Perinatal transmission of hepatitis C virus infection. J Med Virol 81: 836-843.
23. Babik JM, Cohan D, Monto A, Hartigan-O'Connor DJ, McCune JM (2011) The human fetal immune response to hepatitis C virus exposure in utero. J Infect Dis 203: 196-206.
24. Tosone G, Maraolo AE, Mascolo S, Palmiero G, Tambaro O, et al. (2014) Vertical hepatitis C virus transmission: Main questions and answers. World J Hepatol 6: 538-548.
25. Davison SM, Mieli-Vergani G, Sira J, Kelly DA (2006) Perinatal hepatitis C virus infection: diagnosis and management. Arch Dis Child 91: 781-785.
26. Arshad M, El-Kamary SS, Jhaveri R (2011) Hepatitis C virus infection during pregnancy and the newborn period--are they opportunities for treatment? J Viral Hepat 18: 229-236.