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TOPIC HIGHLIGHT

2015 Advances in Hepatitis C virus

Hepatitis C virus comes for dinner: How the hepatitis C virus interferes with autophagy

Daniela Ploen, Eberhard Hildt

Daniela Ploen, Eberhard Hildt, Paul-Ehrlich-Institut, Department of Virology, 63225 Langen, Germany

Eberhard Hildt, Deutsches Zentrum für Infektionsforschung, 38124 Braunschweig, Germany

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Correspondence to: Dr. Eberhard Hildt, Paul-Ehrlich-Institut, Department of Virology, Paul-Ehrlich-Street 51-59, 63225 Langen, Germany. eberhard.hildt@pei.de
Telephone: +49-6103-772140
Fax: +49-6103-771273

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Abstract

Autophagy is a highly-regulated, conserved cellular process for the degradation of intracellular components in lysosomes to maintain the energetic balance of the cell. It is a pro-survival mechanism that plays an important role during development, differentiation, apoptosis, ageing and innate and adaptive immune response. Besides, autophagy has been described to be involved in the development of various human diseases, *e.g.*, chronic liver diseases and the development of hepatocellular carcinoma. The hepatitis C virus (HCV) is a major cause of chronic liver diseases. It has recently been described that HCV, like other RNA viruses, hijacks the autophagic machinery to improve its replication. However, the mechanisms underlying its activation are conflicting. HCV replication and assembly occurs at the so-called membranous web that consists of lipid droplets and rearranged endoplasmic reticulum-derived membranes including single-, double- and multi-membrane vesicles. The double-membrane vesicles have been identified to contain NS3, NS5A, viral RNA and the autophagosomal marker microtubule-associated protein 1 light chain 3, corroborating the involvement of the autophagic pathway in the HCV life-cycle. In this review, we will highlight the crosstalk of the autophagosomal compartment with different steps of the HCV life-cycle and address its implications on favoring the survival of infected hepatocytes.

Key words: Hepatitis C virus; Hepatitis; Autophagy; Morphogenesis; Replication

Core tip: The hepatitis C virus (HCV) is the major cause of chronic liver disease worldwide. According to the world health organization, approximately 130-170 million people are chronically infected with HCV. It has recently been described that HCV hijacks the autophagosomal pathway. Autophagy is a conserved cellular process that catabolizes intracellular components to maintain cellular homeostasis. Besides, autophagy is involved in the development of human diseases. In this review,

we will depict the data known so far, corresponding the interplay between the autophagosomal pathway and the different steps of the HCV life-cycle.

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INTRODUCTION

Hepatitis C virus (HCV) is one of the major causes of chronic liver diseases worldwide including chronic hepatitis, fibrosis, cirrhosis and hepatocellular carcinoma (HCC). At present, more than 185 million people are persistently infected with HCV reflecting 3% of the world population[1]. Until now, there is no preventive vaccine available and the current standard therapy is a 24-48 wk course of pegylated interferon (IFN)-α and ribavirin (RBV) that is only effective in 40%-50% of the patients infected with genotype 1. Since 2013, two first-generation direct-acting antivirals (DAAs), telaprevir (TVR) and boceprevir (BOC), both NS3-4A protease inhibitors, are approved for therapy in combination with IFN-α and ribavirin in patients infected with the HCV genotype 1[2]. In 2015, new DAAs and host-targeted agents (HTAs) will be available to further improve the HCV treatment in IFN-free regimens. These second generation of antivirals, including NS3/4A protease inhibitors (Simeprevir, Faldaprevir, Asunaprevir), NS5A inhibitors (Daclatasavir) and NS5B inhibitors (Sofusbuvir), are currently under clinical evaluation and further target patients infected with the HCV genotypes 2-7 to induce an increased sustained virological response (SVR)[3-5]. However, despite the improvement of the HCV therapy, the number of patients chronically infected with HCV developing a liver cirrhosis or HCC will increase within the next years[1,6]. One important component of new HCV regimens remains the guanosine nucleoside analog ribavirin (RBV). RBV enters the cell by binding to nucleoside transporters like the equilibrative nucleoside transporter-1 (ENT1). Intracellularly, RBV is phosphorylated by cellular kinases to form the RBV-monophosphate (RMP), diphosphate (RDP), or triphosphate[7]. Its mode of action is versatile including the mutagenesis of viral RNA by incorporation of RBV-triphosphate, inhibition of IRES-RNA translation at the level of polyribosome formation, inhibition of the viral polymerase NS5B, inhibition of inosine monophosphate dehydrogenase (IMPDH), induction of T_H1 response resulting in increased clearance of the infected cells and interaction with the IFN signaling pathway[8]. However, the antiviral activity of RBV alone is impaired in chronically HCV-infected patients. In line with this, it has recently been described that the HCV-dependent induction of autophagy impairs the uptake of RBV in persistently infected Huh7.5 cells by reduced expression of ENT1. ENT1 is expressed on the plasma membrane of many cell types[9] *via* a clathrin-dependent mechanism. The HCV-induced autophagic response provokes the consequential degradation of the clathrin heavy chain and thereby prevents the clathrin-mediated recycling of ENT1 to the plasma membrane forcing ENT1 for lysosomal degradation[10]. Based on this, the development of antiviral drugs that target factors involved in the autophagosomal pathway will be attractive.

Autophagy has been described to be involved in several steps of the HCV life-cycle including entry[11], replication[12-16], assembly and release[17-19]. In addition, it has been reported that HCV-induced autophagy impairs innate antiviral activity[20,21] and plays a role in the development of HCV-related liver diseases[22-24].

Based on published data, in this review we will summarize the crosstalk between the autophagic pathway and the different steps of the HCV life-cycle and discuss the impact of innate immunity for HCV-induced autophagy.

AUTOPHAGY

Autophagy ("self-eating") is a highly regulated and conserved cellular process that catabolizes intracellular components to maintain cellular homeostasis. It is initiated by the formation of membrane crescents known as phagophores or isolation membranes that increase to enclosed double-membrane vesicles, called autophagosomes. The autophagosome finally fuses with lysosomes to form autolysosomes where their cargo is digested by lysosomal proteases[25] (Figure 1). Autophagy is tightly regulated by more than 32 so-called autophagy-related genes (*Atg*) as a response to nutrient starvation, damaged organelles, protein-aggregates, ER-/oxidative-stress or infection with pathogens[26]. One important regulator represents the mammalian target of rapamycin (mTOR) that represses autophagy by phosphorylation of Unc-like kinase 1 and 2 (ULK1/2-complex). During starvation, mTOR kinase activity is inhibited by AMP-activated protein kinase (AMPK) resulting in the induction of autophagy[27]. As an initial step, Beclin1 forms a complex with the class Ⅲ phosphatidylinositol-3-kinase (PI3K3) Vps34, p150 and Atg14 (PI3K-complex). The activated ULK1/2- and PI3K-complexes further catalyze the formation of an autophagosome-specific phosphatidylinositol-3-phosphate (PI3P)-enriched environment[28]. The PI3P further recruits the effector DFCP1- (double FYVE-containing protein 1) and WIPI- (WD-repeat domain phosphoinositide-interacting) proteins, leading to phagophore nucleation by formation of an ER-associated Ω-structure ("omegasome"). Autophagosomal membranes can further originate from Golgi-[29], mitochondrial-[30], or plasma membrane (PM)-[31] derived membranes.

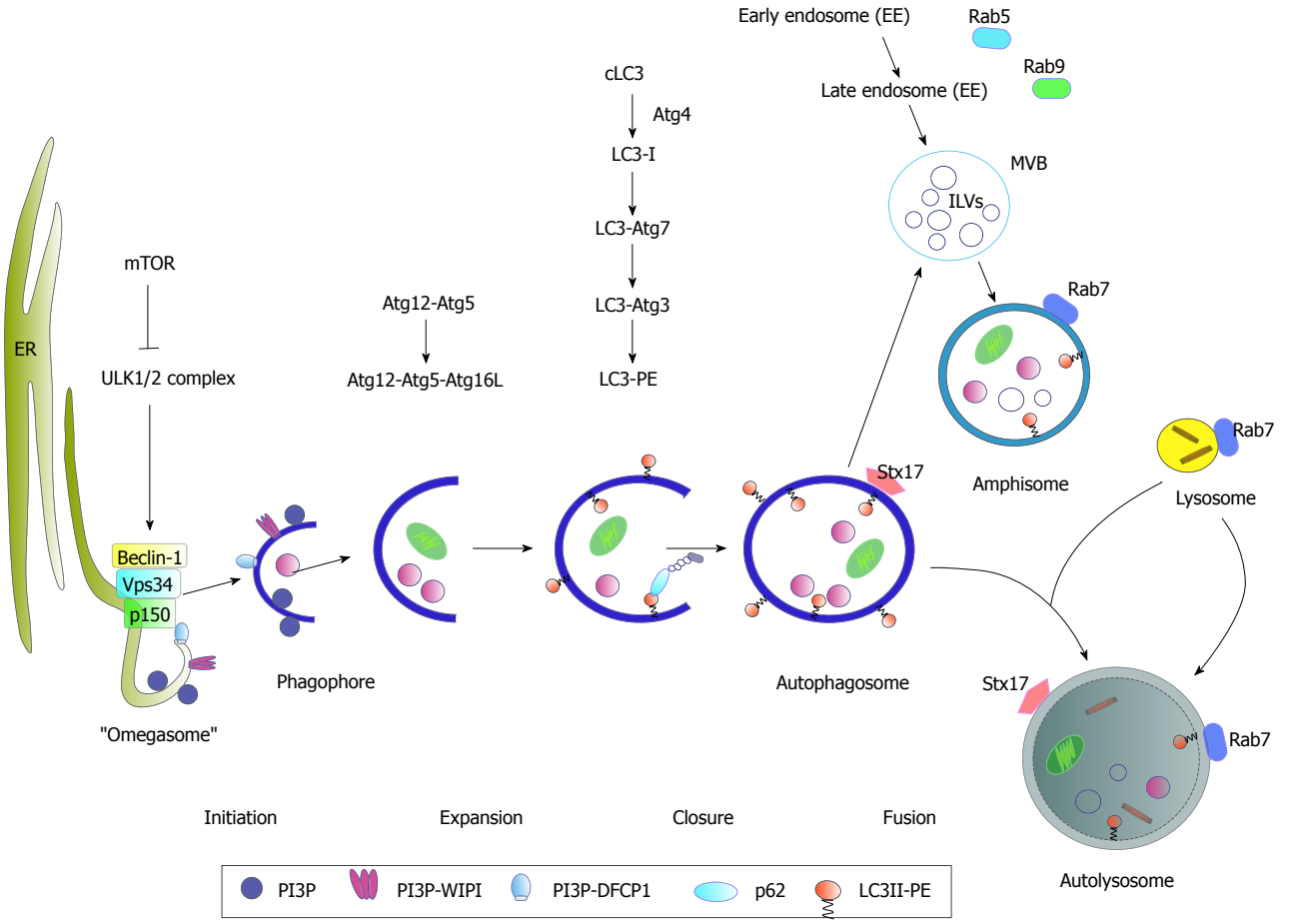

Figure 1 Schematic model of autophagy. Under nutrient-rich conditions mammalian target of rapamycin (mTOR) represses the ULK1/2 complex. During starvation, mTOR kinase is inhibited resulting in activation of autophagy. As an initial step, Beclin-1 forms a complex with Vps34 and p150 (PI3K complex). The activated ULK1/2 and PI3K complex catalyze the formation of a PI3P (phosphatidylinositol-3-phosphate)-enriched environment. The PI3P further recruits the effector double FYVE-containing protein 1 (DFCP1) and WD-repeat domain phosphoinositide-interacting (WIPI) proteins, leading to phagophore nucleation by formation of an ER-associated Ω-structure ("omegasome"). After the nucleation process the formation of the autophagosome occurs. Here, the Atg5-Atg12-Atg16L complex and LC3 Ⅱ, conjugated to phosphatidylethanolamine (PE) (LC3-PE), play a crucial role in the elongation and closure of the membrane. The autophagosomes can either directly fuse with lysosomes to form the autolysosome or fuse with late endosomes/multivesicular bodies (MVBs) forming an amphisome prior fusion with the lysosome where their cargo is digested. Rab7-GTPase activity triggers the fusion of the autophagosome with the lysosome. Another factor involved in autophagosome-lysosome fusion is the autophagosomal SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein) syntaxin 17 (Stx17) that can be found on the outer membrane of completed enclosed autophagosomes.

After the nucleation process, the formation of the autophagosome occurs. For this, the two ubiquitin-like conjugation systems Atg5-Atg12-Atg16L and Atg4-Atg3-LC3-Ⅱ are required. Here, Atg12 is conjugated to Atg5 by ubiquitination-like reactions that require Atg7 (E1-like) and Atg10 (E2-like). The Atg12-Atg5 conjugate associates with Atg16L resulting in the formation of the Atg12-Atg5-Atg16L complex (E3-like). Subsequently, the microtubule-associated protein 1 light chain 3 (LC3) is cleaved at its carboxy terminus by Atg4. The cleaved cytosolic LC3-I is finally converted to the lipidated form LC3-Ⅱ by conjugation to phosphatidylethanolamine (PE) through Atg7 and Atg3 and localized to the autophagosomal membrane[32]. The autophagosomes can either directly fuse with lysosomes to form the autolysosome or fuse with late endosomes/MVBs (multivesicular bodies) forming an amphisome prior fusion with the lysosome[33]. The fusion of the autophagosome with the lysosome has been described to be stimulated by Rab7-GTPase activity[34]. In a recent study, it was reported that Rab7 is associated with lipid droplets (LDs) where it plays an important role during "lipophagy", as activated Rab7 promotes the interaction of LDs with MVBs as well with lysosomes[35]. Another factor involved in autophagosome-lysosome fusion is the autophagosomal SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein) syntaxin 17 (Stx17). Stx17 can be found on the outer membrane of enclosed completed autophagosomes where it promotes its fusion with lysosomes by interaction with SNAP29 and the autophagosomal SNARE VAMP8[36-38].

Taken together, autophagy is a highly regulated, conserved and complex intracellular mechanism that requires the interplay of various factors to maintain the cell homeostasis and to finally ensure the survival of the cell. Autophagosomes can either directly fuse with lysosomes to form the autolysosome or fuse with late

endosomes/MVBs forming an amphisome prior fusion with the lysosome.

ER STRESS AND THE UNFOLDED PROTEIN RESPONSE

Autophagy can be triggered by ER stress that results in the unfolded protein response (UPR). UPR activates three signaling pathways: the inositol-required protein 1 (IRE1), the activating transcription factor-6 (ATF6) and the protein kinase (PKR)-like ER kinase (PERK) pathway[39]. Upon activation of ER stress, IRE1 RNase catalyzes the removal of a small intron from the X-box binding protein 1 (XBP1) mRNA, resulting in spliced XBP1 (*sXBP1*) that encodes for an active transcription factor. XBP1 binds to the ER stress response element (ERSE) and the UPR element (UPRE), resulting in expression of more than 380 target genes, including the ER chaperone 78 kDa glucose-regulated protein (GRP78) and ER-associated degradation (ERAD) factors[40,41]. In response to ER stress, ATF6 is released from GRP78 and translocates to the Golgi where it undergoes protease cleavage to obtain an active transcription factor (cATF6). The cATF6 shuttles to the nucleus where it binds to ERSEs and UPREs, resulting in gene expression of mainly ER chaperones to remove misfolded proteins[42]. Activation of PERK results in phosphorylation of eukaryotic translation factor 2α (eIF2α) following the inhibition of translation, induction of ATF4 and expression of the CCAAT/enhancer-binding protein-homologous protein (CHOP)[43,44]. Induction of UPR after elevated intracellular reactive oxidant species (ROS) further results in a PERK-dependent activation of the transcription factor NF-E2 related factor 2 (Nrf2)[45]. PERK induces the phosphorylation of Nrf2 and dissociation from Keap1. Besides the PERK-Nrf2 pathway, indirect activation of Nrf2 *via* IRE1α-JNK-Nrf2 occurs[46]. Nrf2 belongs to the Cap´n´collar-bacic leucine zipper (CNC-bZIP) transcription factor that plays a crucial role in the defense against oxidative stress. One protection mechanism against oxidative stress is the expression of cytoprotective genes. Many of these genes harbor a short *cis* acting sequence in their promoter, the antioxidant response element (ARE, 5´-TGANNNNGC-3´), and encode for proteins involved in the detoxification of the cells, *e.g.,* NQO1, GCS, GPx or catalytic subunits of the proteasome PSMB5 and PSMB6[47-49]. In its inactive form, Nrf2 is localized in the cytoplasm of the cell, bound to the actin-binding protein Keap1 which mediates its ubiquitin-dependent degradation. Upon activation, Nrf2 dissociates from Keap1 and translocates to the nucleus where it binds to ARE sequences and together with other proteins (*e.g.,* sMaf proteins) activates the transcription of detoxifying genes[47,50]. Moreover, a crosstalk between Nrf2 and the autophagic pathway has been described[46,51]. It has recently been published that phosphorylation of the autophagy adaptor protein p62 activates the Keap1-Nrf2 pathway during selective autophagy as phosphorylated p62 (p-p62) binds to the Keap1 binding site of Nrf2 and therefore competitively inhibits the Keap1-Nrf2 interaction, followed by release of Nrf2 and increased expression of ARE-dependent cytoprotective genes[52]. p62, also known as sequestome-1, is a stress-inducible protein that regulates several transduction pathways involved in cell survival and cell death. In line with this, inhibition of autophagy results in the upregulation of p62 and activation of Nrf2[51].

The studies indicate that autophagy can be activated by endoplasmic reticulum (ER) stress-induced UPR resulting in activation of three signaling pathways.

HCV LIFE-CYCLE

HCV is an RNA virus that belongs to the *Hepacivirus* within the *Flaviviridae* family. Due to its high genetic variability, HCV can be divided into 7 genotypes (1-7) and several subtypes that differ 20%-30% in their sequence and display a different geographical distribution and treatment response[53].

The HCV genome is a single-stranded, positive orientated RNA genome with a size of 9600 bases length. The viral RNA encodes for a large polyprotein precursor of approximately 3100 amino acids that is co- and/or posttranslationally cleaved by viral or cellular proteases into the mature structural (core, E1, E2) and p7 protein and the nonstructural (NS) proteins (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B). The structural proteins form the viral particle, whereas the NS proteins are involved in viral replication and the assembly process[54,55].

The HCV life-cycle is closely linked to very low density (VLDL) synthesis, leading to the pleiomorphic, VLDL-like structure of the so-called lipoviroparticles (LVPs). LVPs display a heterogenity in their density ranging from 1.25 g/mL to 1.03 g/mL (human sera) or 1.10 g/mL to 1.05 g/mL (HCVcc)[56-61]. Their infectivity inversely correlates to their density, with low-densitiy particles being more infectious than high-density particles[62-64]. Thus, the presence of lipids and apolipoproteins such as apoE, apoB, apoC1, C2, C3, and apoA1 is important for the entry, assembly and release of viral particles.

ENTRY PROCESS

The entry process occurs in a coordinated way including several host factors located on the cell surface. The LVPs initially bind the LDL-receptor (LDLR) and glycosaminoglycans (GAG) *via* apoE[65-68] followed by subsequent interaction with the scavenger receptor class B type I (SR-BI)[68] and the tetraspanin CD81[69,70]. As reported previously, the viral particles primary bind the heparan sulfate proteoglycans (HSPGs) syndecan-1 and syndecan-4[71,72]. In line with

this, apoE has been described to interact with HSPGs to mediate the lipoprotein uptake. It has recently been reported that LVP-associated apoE and not the viral envelope glycoproteins mediate interaction with the HSPGs. For this, N- and 6-O-sulfation on the HS is essential and the minimal length of an HS decasaccharide is required[73,74]. The interaction of the viral particle with its major coreceptors SR-BI and CD81 occurs *via* E2[69,68]. Here, the highly conserved region of E2, spanning amino acids 502-520, has been proposed to act as a fusion peptide. The peptide consists of glycine and non-polar amino acids and non-charged residues, exhibiting a globular structure with no regular secondary structures, atypical for a fusion peptide[75]. However, the amino acids Y507, V514, and V515 have been identified to be involved in interaction with CD81 and SR-BI and neutralizing antibodies, thus promoting membrane fusion[76,77].

After the relocation of the LVPs to the tight junction proteins claudin-1 (CLDN-1)[78] and occludin (OCLN)[79-81], the virus becomes internalized by clathrin-mediated endocytosis[82,83] and is finally released into the cytosol in a pH-dependent manner[83,84]. It has recently been described that two receptor tyrosine kinases (RTK): epidermal growth factor receptor (EGFR) and ephidrin type A receptor 2 (EPHA2)[85,86] - and the Niemann-Pick C1-like 1 (NPC1L1) cholesterol uptake receptor[87] as well as the transferrin receptor[88] are additional cofactors involved in the entry process.

Taken together, the entry of the viral particles occurs in a highly organized way including the interaction of the viral particle with specific host factors as LDL-receptor, SR-BI, CD81, CLDN-1, OCLN, EGFR and EPHA2.

HCV REPLICATION

The uncoated positive-strand RNA genome is transported to the rough endoplasmatic reticulum (rER) where it serves as template for the synthesis of the HCV polyprotein precursor that is co- and post-translationally processed by cellular and viral proteases into the mature structural (core, E1, E2) and non-structural proteins (p7, NS2, NS3, NS4A-B, NS5A-B)[89]. HCV replication occurs in replication complexes (RCs) at the so-called membranous web (MW) that consists of lipid droplets (LDs) and rearranged ER-derived membranes including single-, double- and multi-membrane vesicles[90,91]. The double-membrane vesicles (DMVs) have been identified to contain NS3, NS5A, viral RNA and the autophagosome marker microtubule-associated protein 1 light chain 3 (LC3-Ⅱ), the lipidated form of LC3-Ⅰ, suggesting an involvement of the autophagic pathway in the HCV life-cycle[91]. In addition, the DMVs are highly enriched in cholesterol[92]. The formation of the MW is induced by the viral proteins NS4B[93] and NS5A as well as by cellular host factors such as the phosphatidylinositol 4-kinase Ⅲ α (PI4K-Ⅲα)[90,94]. Interactions of NS5A with PI4K-Ⅲα induces the accumulation of phosphatidylinositol 4-phospate (PI4P) in the MW. The PI4P-enriched environment is further stabilized by the interaction of NS5A with ARFGAP1, a GTPase-activating protein for ARF1, an adaptor protein involved in COPI trafficking[95]. Moreover, NS4B synergistically modulates core-mediated activity of sterol regulatory element-binding proteins (SREBP) and fatty acid synthetase (FAS), thus mediating lipogenesis and lipid accumulation in hepatoma cells[96]. Viral replication is catalyzed by the viral RNA-dependent RNA polymerase (RdRp) NS5B. NS5B presents the classical right-hand structure, consisting of finger, palm and thumb domains[97]. In addition, NS5B contains a β-loop insertion in the thumb domain and a C-terminal membrane-anchoring linker in its active site that are involved in the initiation of RNA synthesis[98]. The highly active site of NS5B further offers possible targets for antiviral strategies with higher barriers to the development of resistance[2]. In addition, host factors such as cyclophilin A (CypA) and microRNA (miR)-122 have been identified to participate in the viral replication[99]. MiR-122 is a small non-coding RNA that is highly expressed in hepatocytes and is involved in proliferation and differentiation of hepatocytes, lipid metabolism as well as in the development of liver disease. Besides, miR-122 stimulates HCV replication by binding to the 5′-UTR of the HCV genome[100,101]. CypA is a peptidyl-prolyl isomerase (PPIase) that catalyzes the *cis-trans* isomerization of peptide bonds at proline (Pro) residues and therefore assists in protein-folding[102,103]. The immunosuppressant CypA inhibitor, cyclosporine A (CsA), has been identified to inhibit HCV replication and translation in cell culture[104]. In line with this, it was recently reported that NS5A and CypA act in concert and are required for DMV formation. This suggests that CypA and NS5A inhibitors block HCV replication by inhibiting formation of the membranous web[105]. Due to their importance during the viral replication, the involved viral proteins (NS3/4A, NS5A, NS5B, p7) and cellular factors (miR-122, CypA, PI4K-Ⅲα) represent potential targets for antiviral therapy.

Summing up, HCV replication occurs in replication complexes at the so-called membranous web that consists of lipid droplets and rearranged ER-derived membranes including single-, double- and multi-membrane vesicles. This process is mainly regulated by the crosstalk between HCV NS-proteins and core and cellular host factors.

ASSEMBLY AND RELEASE

The assembly of the virions occurs on the surface of core-associated cytosolic lipid droplets (cLDs) in close proximity to the RC at the membranous web[106] *via* budding at the ER membrane and seems to depend on VLDL-assembly[107]. cLDs are

organelles for the storage of neutral lipids and consist of a hydrophobic core of triacylglycerol (TAG) and cholesterol ester (CE) surrounded by a phospholipid monolayer that is surrounded by a coat of proteins, the so-called PAT-proteins including perilipin, adipose differentiation-related protein (ADRP) and tail interacting protein of 47 kDa (TIP47)[108]. During HCV infection, the core protein induces the formation of the LDs[109] and recruits NS proteins and the RC to LD-associated membranes[106]. The recruitment of core and NS5A to the LDs is mediated by the diacylglycerolacyltransferase-1 (DGAT-1)[110], an enzyme involved in triglyceride synthesis and LD maturation[111] and the mitogen-activated protein kinase (MAPK)-regulated protein cytosolic phospholipase A2 (cPLA2)[112]. In addition, NS5A interacts with LD-associated Rab18 and thereby promotes the interaction between the sites of viral replication and the LDs[113]. Proteomic analyses of LD-proteins in core-expressing cells revealed a higher content of the lipid droplet associated protein TIP47, whereas the amount of ADRP was significantly decreased[114]. Besides its role in the biogenesis of LDs[115], TIP47 has been described to act as an intracellular sorting factor that is involved in the retrograde trafficking of the mannose-6-phosphate receptor (M6PR) from late endosomes to the *trans*-Golgi network (TGN)[116]. Structural analysis of TIP47 revealed a high structural homology to apoE[117]. ApoE plays an important role in the VLDL pathway and has been described to directly interact with the viral protein NS5A[118,119]. This interaction is crucial for the virus assembly and the release of infectious viral particles[120,61]. It was recently observed that TIP47 binds the viral protein NS5A and targets the *de novo* synthesized viral RNA that is bound to NS5A from the replicon complex to the LDs, the assembly platform[121,122].

The initial step of virion assembly represents the interaction of NS5A with the cLD-bound core protein[106,123,124]. For this process, the phosphorylation of specific serine residues of NS5A-domain Ⅲ (DⅢ) by casein kinase Ⅱα (CKⅡα) is essential[125]. In addition, the viral proteins p7 and NS2 play a crucial role in the organization of the assembly complex. NS2 interacts with the viroporin p7. The p7-NS2-complex further interacts with the viral proteins NS3-4A and promotes the disruption of the core protein from the cLDs to the nascent viral particle[126,127]. The viral budding has been described to require late components of the ESCRT (endosomal sorting complex required for transport) pathway[128,129].

Maturation of viral particles is closely linked to VLDL synthesis as they are highly enriched in apoE, apoB and the microsomal transfer protein (MTP), proteins that are involved in VLDL-assembly[107]. The virions are packaged as LVPs that have similar densities to VLDLs and envelopment and maturation are thought to proceed at the luminal LD (luLD), the VLDL precursors[130].

The mature viral particles are finally released through the secretory pathway. During this process, the surface proteins E1 and E2 are modified by glycosylation and their disulfide bonds are reorganized[131-133].

For this, the interaction of TIP47 with activated GTP-bound Rab9 is essential. It has previously been described that the TIP47/GTP-Rab9 complex is important for the release of the viral particles and TIP47 becomes part of the viral particle. The intracellular trafficking of the HCV particles loaded with TIP47 that lacks the Rab9 binding site ends in the autolysosomal compartment with degradation of these viral particles[134]. Besides, heterogeneous nuclear ribonucleoprotein K (HNRNPK) has been identified as a host restriction factor that regulates the viral assembly. In HCV-infected cells, HNRNPK interacts with the viral plus-strand RNA and the core protein in close proximity of the LDs. The proposed mechanism indicates that HNRNPK binds the viral RNA to regulate its availability for packaging. Bound RNA cannot be incorporated into newly synthesized viral particles and thereby enters a new replication cycle[135].

These data so far suggest that the release of the viral particles is linked to the VLDL pathway. However, the exact mechanism remains enigmatic and needs to be further investigated.

AUTOPHAGY AND HCV

Activation of autophagy

The data concerning HCV and its role during UPR/autophagy are conflicting, and the mechanisms underlying its activation are not completely understood. As HCV replication occurs at the membranous web, it is believed that HCV induces ER stress following the induction of UPR *in vitro*[14,21,136,137] and *in vivo*[138]. For instance, the viral glycoproteins E1-E2 form a complex retained in the ER membrane that can form aggregates resulting in ER stress[139,140] (Table 1 and Figure 2). Furthermore, HCV infection is associated with oxidative stress in liver cells that results in elevated ROS[141]. As a response to the increased ER-/oxidative stress, the direct PERK-Nrf2 or indirect IRE1α-JNK-Nrf2 pathways trigger the expression of detoxifying genes[46]. In addition, the elevated ROS levels trigger phosphorylation of the autophagy adaptor protein p62 on Ser351 by the mTOR kinase. Phosphorylation of p62 results in the release of Nrf2 from Keap1 followed by increased expression of cytoprotective genes[52] (Figure 2). In a recent study of our group, we observed that HCV interferes with the production of cytoprotective genes by an impaired Nrf2/ARE-signaling. Here, the core protein triggers the delocalization of sMaf proteins to the cytosol where sMaf proteins bind to NS3 and prevent the entry of Nrf2 into the nucleus resulting in elevated ROS levels. Therefore, the increased ROS levels in HCV replicating

Table 1 Crosstalk of hepatitis C virus proteins with the autophagosomal pathway

Viral proteins	Cellular interacting partner/ pathway	Function	Ref.
Core	-	-	-
E1-E2	UPR-pathway	ER-retention of heterodimeric E1-E2-complexes Induction of UPR	Cocquerel *et al*[139], 1998
E1-E2	UPR-pathway	Formation of E1-E2 aggregates at ER Induction of UPR	Choukhi *et al*[140], 1998
E2	-	-	-
P7	-	-	-
NS2	-	-	-
NS3	sMafs	Entry of Nrf2 into nucleus prohibited Elevated ROS-levels Induction of autophagy	Carvajal-Yepes *et al*[142], 2011
NS3/NS4A	IRGM	Activation of autophagy	Grégoire *et al*[18], 2011
NS3/4A		Interaction of Atg5-Atg12 conjugate with RIGI/Mda5 and IPS-1/MAVS Cleavage of these helicases Inhibition of IFN-signaling	Shrivastava *et al*[20], 2012
NS4B	UPR-pathway	Interference with Ca^{2+}-homeostasis resulting in elevated ROS Induction of UPR *via* activation of ATF6- or Xbp1-pathway Activation of NfκB *via* Ca^{2+}-homeostasis and elevated ROS	Li *et al*[144], 2009
NS4B	Rab5 + Vps34	Inhibition of mTOR kinase activity and interaction with Vps34 and Beclin1 Activation of autophagy	Su *et al*[16], 2011 Stone *et al*[147], 2007 Simonsen *et al*[146], 2009 Li *et al*[145], 2010
NS4B	Atg5	Possible involvement in formation of membranous web	Guévin *et al*[152], 2010
NS5A	-	Autolysosome formation	Shrivastava *et al*[20], 2012
NS5A	TIP47/Rab9 PKR	Release of viral particles Particles loaded with TIP47 lacking Rab9 are targeted towards the autolysosome	Ploen *et al*[121], 2013
NS5B	Atg5	Possible role in establishment of infection	Guévin *et al*[152], 2010

UPR: Unfolded protein response; PKR: Protein kinase R; ROS: Reactive oxidant species; mTOR: Mammalian target of rapamycin; ER: Endoplasmic reticulum.

cells cannot be efficiently compensated due to the impaired Nrf2 activity resulting in the induction of autophagy[142] (Table 1). Moreover, it has been reported that liver regeneration is impaired in mice lacking Nrf2 due to increased ROS levels that negatively affect insulin signaling[143]. UPR can be further activated by HCV NS4B through the activation of the ATF6- or IRE1-pathway as NS4B interferes with Ca^{2+} homeostasis resulting in elevated ROS[141,144] (Table 1). In addition, NS4B forms a complex with Rab5 and the PI3 kinase Vps34, triggering the induction of autophagy[16]. Active Rab5 activates autophagy by inhibition of mTOR kinase activity[145] and interaction with Beclin1 and Vps34[146]. Hence, interaction between Rab5 and NS4B have been described to be crucial for HCV replication[16,147] (Table 1). Grégoire *et al*[148] further identified the human immunity-associated GTPase family M (IRGM) protein to be involved in the autophagy modulation of different RNA-viruses including HCV. IRGM interacts with HCV NS3/4A and different autophagosomal proteins, *e.g.*, Atg5, Atg10, LC3[18,148,149] and thereby triggers autophagy (Table 1 and Figure 2). These studies so far indicate that HCV can induce autophagy by elevated ROS levels *via* UPR or by direct interference with the autophagic pathway.

Translation of incoming RNA and membranous web formation

Autophagy has been described to be involved in different steps of the HCV life-cycle. HCV replication occurs in "replication factories" at the so-called *membranous web* (MW)[93] that consists of LDs and rearranged ER-derived membranes including single-, double- and multi-membrane vesicles. The double membrane vesicles (DMVs) display the main component of the MW and are thought to be induced by NS5A during the early stage of infection[90,92,150]. The single membrane vesicles (SMVs) are induced by NS4B and have been reported to contain NS3 and NS5A. Multi-membrane vesicles (MMVs) can only be detected in the late stage of infection and are thought to occur as a consequence to the HCV-induced autophagic response[90]. Indeed, the DMVs have been identified to contain NS3, NS5A, viral RNA and the autophagosomal marker LC3-Ⅱ suggesting a link to the autophagosomal compartment[91]. Using

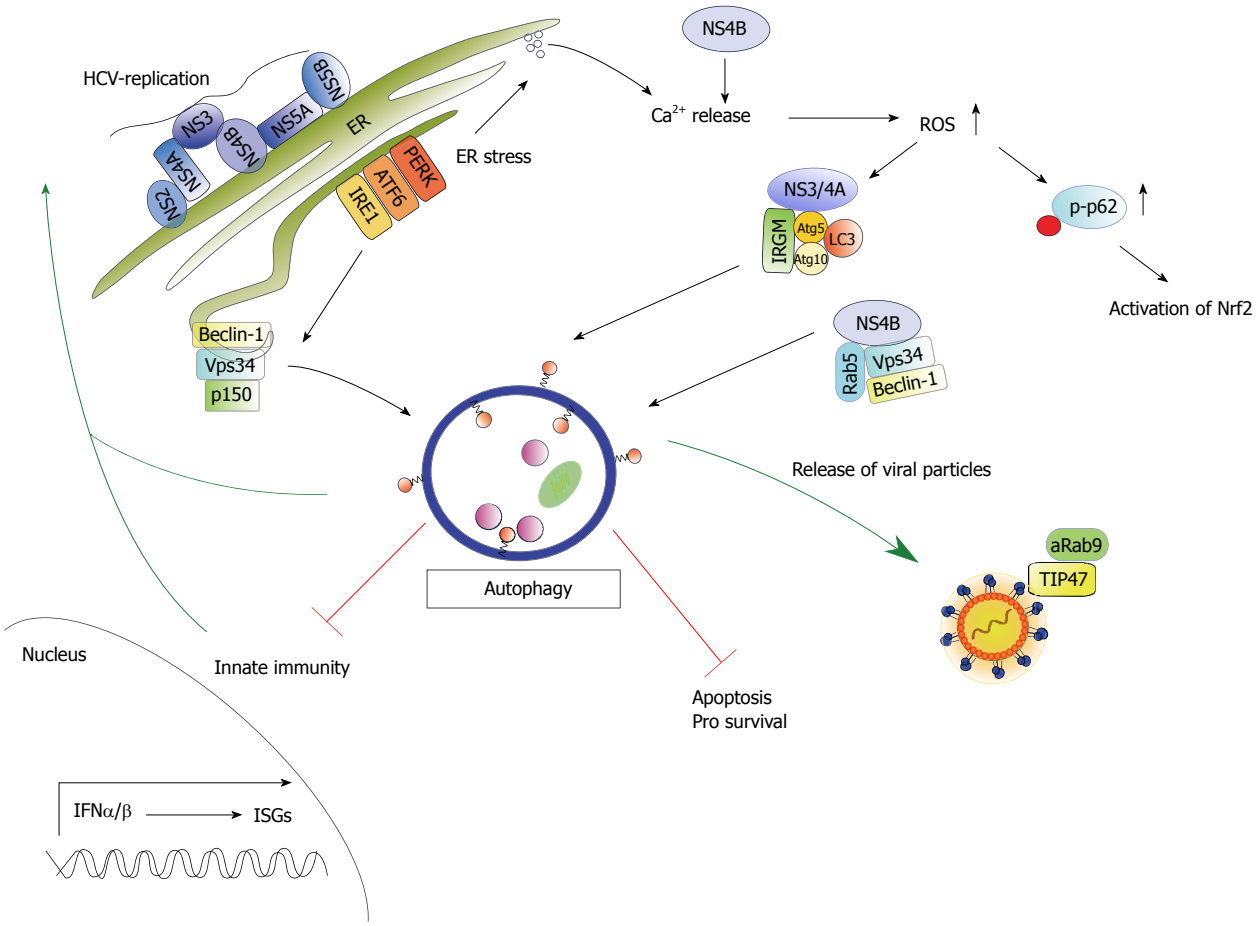

Figure 2 Schematic model of the crosstalk between hepatitis C virus and autophagy. Hepatitis C virus (HCV) replication occurs in replication complexes that assemble at the ER resulting in ER stress and induction of an unfolded protein response (UPR). The UPR activates three signaling pathways (ATF6, IRE1, PERK) and finally autophagy. In addition the UPR can be triggered by NS4B through activation of the IRE1 and ATF6 pathway as NS4B interferes with Ca^{2+} homeostasis resulting in elevated reactive oxidant species (ROS). Elevated ROS levels trigger phosphorylation of p62 on Ser351 (p-p62) following subsequent activation of Nrf2. NS4B further forms a complex with Rab5, Vps34 and Beclin-1 stimulating an autophagic response. In addition the human immunity-associated GTPase family M (IRGM) protein interacts with NS3/4A and the autophagosomal protein Atg5, Atg10 and LC3 that trigger autophagy. Activation of autophagy favors HCV replication and plays a crucial role in release of viral particles. Here, the interaction of TIP47 and activated Rab9 (aRab9) to the particles is essential. Moreover, HCV-induced autophagy impairs innate immunity and favors cell survival *via* inhibition of apoptosis.

immunofluorescence-analysis, Romero-Brey *et al*[90] could identify markers of early endosomes (EE), late endosomes (LE), COP vesicles, mitochondria, LDs and Rab proteins that are associated to the MW. Thus, a crosstalk between factors involved in HCV replication with individual autophagosomal factors may contribute to the formation of the MW. During the early steps of the HCV life-cycle, Atg5 has been described as a proviral factor required for translation and/or delivery of incoming viral RNA. Atg5 induces formation of DMVs in embryonic stem cells[151] and was found to be associated with the membranous web in Huh7 cells and further colocalizes with NS4B (Table 1). Besides, Atg5 colocalizes with the viral polymerase NS5B during the onset of infection. This interaction is abrogated during the course of infection[152]. In another study, Beclin1, Atg4B and Atg12 were identified as additional proviral factors required for establishment of viral replication[11,153].

Furthermore, the EE marker Rab5 has been identified to interact with NS4B and the autophagosomal regulators Beclin1 and Vps34, resulting in the activation of autophagy[16] (Table 1 and Figure 2). NS5A was described to induce the formation of DMVs and MMVs[90,150] and has been recently identified to induce autophagy as it triggers fusion of the autophagosome with the lysosome[15]. Moreover, NS5A interacts with the adaptor protein ARFGAP1, involved in COPI trafficking, to stabilize the PI4P enriched environment in the MW[95]. In addition, Sir *et al*[13] could detect NS5A, NS5B and viral RNA on autophagosomal structures.

Taken together, induction of autophagy is crucial for the onset of the infection process. Interaction between HCV NS proteins and molecules of the autophagic pathway is required for the reorganization of intracellular membranes resulting in the formation of the MW.

Replication

Ait-Goughoulte *et al*[12] indicated that serial passages

of an HCV genotype 1a (clone H77) isolated in immortalized human hepatocytes (IHH) resulted in the accumulation of LC3B-positive autophagic vesicles. This could be confirmed by other groups using different cells types and full-length as well as subgenomic replicons of different virus strains[14,17,91,154]. Ke *et al*[21,137] further demonstrated that HCV induces complete autophagy *via* the unfolded protein response (UPR) to promote viral replication. In contrast to this, using subgenomic replicons, Sir *et al*[14] claimed that maturation of autophagic vesicles seems to be incomplete in HCV replicating cells, since degradation of the long lived protein p62, a polyubiquitin binding protein that is known to be degraded during autophagy, is not enhanced. Subgenomic replicons enable the analysis of the crosstalk between HCV genome replication and autophagy in the absence of processes that are triggered by virus morphogenesis or release. Based on this, they further demonstrated that down-regulation of Atg7 and LC3 that are crucial for autophagosome formation reduced HCV replication. Moreover, they were able to co-preciptate the HCV replication complex with autophagosomes indicating that the membranous structure of virus-induced autophagosomes may serve as site of RNA replication in the early state of infection[13]. However, inhibition of PI3K3, involved in the initial step of autophagy, did not affect HCV replication. The data regarding the involvement of the autophagic pathway during HCV replication are conflicting and not completely understood. The inconsistent results might be explained by the use of different cell culture systems. *In vitro* studies of HCV are based on human hepatoma cell lines (Huh7). Huh7 clones behave differently and vary in their ability to induce an innate immune response. In line with this, it has been reported that HCV-induced autophagic response represses the innate antiviral immunity[11,153-155]. Based on this, one could argue that the contradictory results can be in part deduced from the different cellular background. In addition, the conflicting results can be referred to different experimental settings using either full length or subgenomic HCV replicons of different virus strains. Nevertheless the published data indicate that autophagy favors HCV replication.

Release

Particle assembly and release occurs in the MW and is closely linked to the VLDL synthesis. The mature viral particles are finally released *via* the secretory pathway. However, the detailed mechanism still remains to be elucidated. Initial experiments of our group suggested an involvement of the autophagic pathway in the release of the LVPs. Here, the TIP47/Rab9 complex plays an important role (Table 1 and Figure 2) as particles loaded with TIP47 lacking Rab9 are targeted towards the autophagosome[134]. Moreover, the inhibition of autophagolysosome formation resulted in a decrease of released infectious viral particles (not published data). Additionally, Tanida *et al*[17] recently observed that the silencing of Beclin-1 and Atg7 reduces the release of infectious viral particles, but did not affect the intracellular amount of viral RNA. Besides, they could only detect a poor colocalization of LC3-Ⅱ with LDs, NS5A and core. Moreover, Grégoire et *al*[18,148] observed that immunity-associated GTPase family M (IRGM) interacts with the autophagy-associated proteins Atg5, Atg10, MAP1CLC3C and SH3GBL1 and is involved in the activation of HCV-induced autophagy and release of viral particles (Figure 2).

These studies indicate that autophagy participates in the release of the viral particles. However, the detailed mechanisms are not understood. Further studies are required to elucidate the detailed role of autophagy for the release of HCV.

Interplay of innate immunity and autophagy in HCV infection

Besides its role in maintaining cell homeostasis, autophagy is involved in the defense against pathogens[156]. Autophagosomes hereby deliver intracellular pathogen-associated molecular patterns (PAMPs) to endosomal pattern recognition receptors (PRRs) or MHC-specific compartments in order to provoke an innate or adaptive immune response[157,158]. The innate immune response represents the first barrier after viral infection and can be activated by specific immune sensors (toll-like receptors (TLRs), RIG-I-like receptors (RLRs), and the nucleotide oligomerization domain (NOD)-like receptors) which detect PAMPs or intracellular viral nucleic acids. It has been described that autophagy can be directly activated by PAMP-dependent stimulation of TLRs resulting in the induction of an interferon (IFN)-response[159,160]. Furthermore, the autolysosomal degradation of viral particles or proteins leads to MHC class Ⅱ-dependent antigen presentation and subsequent activation of the adaptive immune response[161]. Many viruses have developed strategies to escape the innate immune response. Like other viruses, HCV infection triggers an innate immune response through RIG-I/Mda5-mediated IFN signaling in order to establish an antiviral state[162,163]. However, to escape the induction of an IFN-response, HCV proteins interfere with different steps of the IFN signaling pathway. The HCV NS3/4A protease *e.g.,* inhibits IFN signaling *via* blocking the RIG-I and TLR3 pathway[20,149]. In addition, HCV interferes with the activation of the double-stranded viral RNA-sensing kinase protein kinase R (PKR). HCV triggers the phosphorylation of PKR resulting in the phosphorylation of eIF2α and the consequential inhibition of translation including translation of ISGs. PKR is targeted by HCV core, E2 and NS5A that modulate the kinase activity by different mechanisms. In hepatocarcinoma (HCC) positive cells, PKR/core interaction results in the cell cycle arrest in the G2/M phase due to PKR phosphorylation on Thr446.

Overexpression of the core protein further leads to increased PKR activity that does not correlate with enhanced eIF2α phosphorylation[164]. Moreover, the core protein activates the expression of OAS-1[165] and PKR[166] in the HCV genotype 1b. Further inactivation of PKR by NS5A has been described for the HCV genotype 1a and 1b[167-169]. *In vitro* studies indicate a PKR/E2 interaction and inhibition of PKR. As E2 accumulates in the ER and PKR can be found in the cytosol of the cell, *in vivo* studies suggest a PKR interaction with cytosolic unglycosylated E2[170] resulting in the inactivation of PKR *via* inhibition of PKR-like ER-resident kinase (PERK)[171]. The paradoxon is that the ability of HCV to partially activate PKR may be an advantage for the virus during an IFN response since it preferentially suppresses the translation of ISGs[172]. Moreover, the activation of PKR results in the activation of the autophagic response[157,173,174]. Recent studies indicate that HCV-induced autophagy represses the innate immune response[10,11,21,137,175]. Silencing of Beclin-1 and Atg7 in immortalized human hepatocytes (IHHs) infected with HCV restored the IFN signaling pathway by expression of OAS-1, IFN-α, IFN-β, IFI-27 and induced apoptosis[20]. In line with this, HCV-induced autophagy augments the PAMP-mediated innate immune response (Figure 2). The silencing of autophagy or autolysosome formation by treatment with chloroquine (CQ) and bafilomycin (BFLA) resulted in the upregulation of the innate immune response[21,137]. Moreover, recent studies demonstrate that persistent HCV infection results in an impaired immune response after treatment with IFNα and Ribavirin, whereas treatment with IFNλ provokes a strong sustained virological response[175]. As a result, persistent HCV infection leads to chronic ER stress and an autophagic response followed by impaired type Ⅰ, but not type Ⅲ IFN signaling and impaired JAK-STAT signaling. Moreover, the uptake of the antiviral drug RBV is impaired in persistently HCV-infected Huh7.5 cells due to a decreased surface expression of the equilibrative nucleoside transporter-1(ENT1) as described above[10]. Besides its role in controlling HCV-dependent innate immunity, autophagy is involved in the survival of the HCV-infected host cell to promote viral replication (Figure 2). Taguwa et *al*[155] observed that cells infected with HCV genotype 1b prevented vacuole formation by incomplete autolysosome formation to maintain persistent infection. Besides, it was reported that HCV induces autophagy by the upregulation of Beclin-1 and activation of mTOR signaling, thereby promoting hepatocyte cell growth[15].

Based on this, the induction of autophagy is part of the viral escape strategy to counteract viral infection.

CONCLUSION

Autophagy is a highly regulated and conserved cellular mechanism to maintain cellular homeostasis and to promote cell survival. As described in this review, autophagy interferes with different steps of the HCV life cycle to establish a persistent viral infection. However, the detailed mechanisms are not completely understood and remain to be elucidated. Moreover, HCV-induced autophagy represses apoptosis to promote cell survival. In addition, recent studies indicate that the HCV-induced autophagic response interferes with antiviral immunity as it reduces the innate immune response in HCV-infected cells. Hence, the disruption of autophagy restores IFN signaling and could restore the defective RBV-dependent antiviral response *in vitro*. Based on this, the improvement of existing antiviral therapies and development of new antiviral strategies targeting the autophagosomal response will be promising. Moreover, the development of vaccines to eradicate HCV infection and the linked HCV-associated pathogenesis depict an auspicious future perspective.

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