Hepatitis C virus core protein, lipid droplets and steatosis

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REVIEW

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SUMMARY. Lipid droplets are intracellular organelles involved not only in lipid storage, but also in cell signalling and the regulation of intracellular vesicular trafficking. Recent basic studies have suggested that interactions between hepatitis C virus (HCV) core protein and lipid droplets are required for the HCV infection cycle. In infected cells, the HCV core protein is associated with the surface of lipid droplets and the endoplasmic reticulum membranes closely surrounding these droplets, and its self-assembly drives virion budding. This interaction also seems to be directly linked to a virus-induced steatosis, which involves the deposition of triglycerides in the liver and contributes to the progression of fibrosis in patients with chronic hepatitis C. Many clinical studies have reported that virus-induced steatosis is significantly more severe with HCV genotype 3 than with other genotypes, and this phenomenon has been modelled in recent basic studies based on the production of HCV core proteins of various genotypes *in vitro*. The association of HCV core protein with lipid droplets seems to play a central role in HCV pathogenesis and morphogenesis, suggesting that virus-induced steatosis may be essential for the viral life cycle.

INTRODUCTION

Hepatitis C virus (HCV) has a major impact on public health, with an estimated 170 million infected individuals worldwide [1]. HCV infection is a major cause of chronic liver disease: more than 50% of individuals exposed to HCV develop chronic hepatitis and 20 % of chronically infected individuals develop cirrhosis. HCV infection is also a major cause of hepatocellular carcinoma and the primary reason for liver transplantation in Western Europe and the United States. No vaccine protecting against HCV infection has yet been developed, and current antiviral strategies are of limited efficacy and have major side effects [2]. HCV is a small enveloped RNA virus belonging to the Flaviviridae family, genus *Hepacivirus*. This virus is highly heterogeneous and has been classified into six major genotypes and numerous subtypes [3]. Its has a single-stranded RNA genome of positive polarity encoding a single polyprotein precursor of just over 3000 amino-acid residues, flanked by structured 5' and 3' untranslated regions (UTRs) essential for RNA replication and translation (Figure 1) [4]. The HCV polyprotein is cleaved co- and post-translationally by cellular and viral proteases to yield 10 mature structural and nonstructural proteins (Figure 1). The structural proteins of HCV - the core (C) and the two envelope glycoproteins E1 and E2, released by the host cell signal peptidase and the signal peptide peptidase (SPP) – are the basic components of the viral particle. The non-structural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B) are involved in proteolytic processing of the residual polyprotein and HCV genome replication, and are not incorporated into the viral particle. The structural proteins are separated from the nonstructural proteins by the short membrane peptide p7, which is thought to be a viroporin.

MODEL SYSTEMS FOR STUDYING THE LIFE CYCLE OF HCV IN VITRO

Since the cloning of the viral genome in 1989, considerable progress has been made towards characterizing the viral genome and proteins, using heterologous expression systems. In sharp contrast, little is known about the life cycle of HCV because, until recently, no system supporting HCV replication and particle formation *in vitro* was available. Viruses are obligate intracellular parasites, so studies of their multiplication cycle require a permissive host cell. Many attempts were made to propagate HCV in cell culture, although these attempts were entirely unsuccessful for many years [5]. Alternative cellular models were therefore established for studies of individual steps in the HCV lifecycle. The first such model consisted of subgenomic HCV RNAs containing sequences encoding the non-structural proteins flanked by the 5' and 3' UTRs [6, 7]. These subgenomic RNAs, called replicons, were able to self-replicate in the human liver hepatoma cell line Huh7, and this replication was dependent on adaptative mutations affecting sites in the non-structural proteins. This system provided the first opportunity for studies of efficient HCV RNA replication *in vitro* and evaluations of the effect of antiviral drugs targeting the translation or replication of viral RNA [8, 9]. Ultrastructural studies of Huh7 cells harboring such HCV replicons demonstrated the presence in these cells of specific endoplasmic reticulum (ER) membrane alterations, resulting in a membranous web containing the viral replication complex [10]. Another model, based on the pseudotyping of retroviruses with the two HCV envelope glycoproteins E1 and E2, has proved a powerful tool for functional studies of HCV binding to and entry into Huh7 cells and other human liver cell lines [11, 12]. These pseudotyped retroviral particles differ from native serum-derived HCV virions, but studies with such particles identified both CD81 and SR-BI as potential high-affinity receptor candidates [13]. HCV-like particles (HCV-LPs) obtained by expressing genes encoding HCV structural proteins in mammalian cells have been used as an alternative model for studying HCV morphogenesis [14, 15]. Studies with

this HCV-LP model have demonstrated that HCV budding occurs at the ER membrane and is driven by the core protein [16, 17].

Finally, a milestone in HCV research was achieved with the recent development of a cell culture system for HCV propagation, resulting in the production of significant levels of infectious virus particles [18-20]. This development built on the initial finding that a unique clone derived from a genotype 2a viral isolate from a Japanese patient with fulminant hepatitis (JFH-1) replicated strongly in the absence of adaptative mutations in the replicon model [21]. Wakita *et al.* demonstrated that RNA transcripts from the full-length JFH-1 genome could generate viruses in Huh7 cells that were capable of infecting naive Huh7 cells [18]. Moreover, the viral particles produced by these cells were infectious in a chimpanzee, confirming the authenticity of the HCV particles generated during cell culture [18]. Studies of individual steps in the viral life cycle (entry, replication and morphogenesis) remain difficult and sometimes no easier with this model than with the other models described above [22, 23], but this model does provide the first opportunity to study the complete viral life cycle *in vitro.*

MODEL SYSTEMS FOR STUDYING HCV PATHOGENESIS IN VIVO

The restricted host range of HCV has hampered the development of a suitable small-animal model of viral replication and pathogenesis. Indeed, chimpanzees remain the only recognised animal model for studying HCV [24]. The main advantage of using the chimpanzee as an animal model for HCV infection is that disease progression follows a clinical course similar to that seen in human patients. Both HCV-infected chimpanzees and human patients display long-term liver damage, characterized by the formation of similar necroinflammatory hepatic lesions and changes in hepatocyte morphology [25]. However, this model is limited by its high cost and, above all, ethical considerations. Other primates do not appear to be reproducibly susceptible to HCV infection, but the related GB virus B (GBV-B), a more

recently discovered member of the Flaviviridae family, can be transmitted to tamarins and may represent a valuable surrogate model for HCV [26]. Attempts to develop a small-animal model have met with limited success, with only transient HCV replication being reported in the HCV-Trimera and chimeric SCID-Alb/uPA Hepatech mouse models [27-31]. Several transgenic mouse models have been developed for studies of the potentially pathogenic effect of HCV proteins on hepatocytes. However, these studies have yielded conflicting results. Some studies have shown that the core and envelope proteins have no pathogenic effect [32, 33], whereas others have shown that mice expressing the core protein develop hepatic steatosis, lymphocyte infiltration, hepatocyte necrosis and hepatocellular carcinoma [34, 35].

LIPID DROPLETS

Lipid droplets are intracellular storage organelles found in all eukaryotic organisms. They consist of a core of neutral lipid, comprising mainly triacylglycerols and/or cholesterol esters, surrounded by a monolayer of phospholipids [36]. The phospholipid layer is bounded by a proteinaceous coat. In mammalian cells, the principal lipid droplet-binding proteins identified to date are adipophilin, TIP47 and a family of related proteins, the perilipins. Adipophilin and TIP47 are present in a wide range of cell types, whereas perilipins are restricted to adipocytes and steroidogenic cells [37]. On electron microscopy (EM), lipid droplets appear as grey amorphous circular masses, usually surrounded by a darker rim (Figure 2). However, rather than inert inclusions, they should be considered as metabolically active and mobile organelles participating in a range of functions other than lipid homeostasis, including cell signaling, and the regulation of intracellular vesicular trafficking and membrane compartments [38]. All cell types have the ability to generate lipid droplets in response to high fatty acid levels, and to metabolise and disperse these lipid droplets when the conditions are reversed. However, their biogenesis is poorly understood. It has been suggested that neutral lipid accumulation within

the lipid bilayer of the ER membrane induces the budding of an independent organelle, surrounded by the phospholipid monolayer originating from the cytoplasmic leaflet of the ER membrane [39]. It remains unknown whether mature lipid droplets stay in physical contact with the ER, or bud to form a completely independent organelle [38]. Lipid droplet-associated proteins are thus thought to be at least partly derived from proteins resident in the ER membrane [36]. However, this model was recently called into question by a three-dimensional EM study which suggested that the biogenesis of lipid droplets occurred in specialised cup-shaped regions of the ER [40].

HCV CORE PROTEIN ASSOCIATION WITH LIPID DROPLETS

A link between lipid droplets and HCV was first suggested 10 years ago, when the HCV core protein was produced *in vitro* in various cell types, using heterologous expression systems. These studies showed that the HCV core protein was cytoplasmic, and either associated with the ER membrane or on the surface of lipid droplets [41, 42]. Subsequent studies have confirmed these observations [43], but some studies have also reported the presence of the HCV core protein in the nucleus [44] or mitochondria [45]. Interestingly, although the HCV core protein is very difficult to detect by immunocytochemistry in biopsy samples from infected patients, it seems to have a discrete granular distribution in the cytoplasm [46, 47], which may be explained by its association with lipid droplets.

The HCV core protein is produced at the N-terminal end of the polyprotein and is followed by the signal sequence of the E1 envelope glycoprotein. The signal sequence targets the nascent HCV polyprotein to the ER, allowing the translocation of E1 to the ER lumen, an essential step in the membrane-dependent processing of the core protein. Cleavage by a signal peptidase in the ER lumen releases the N-terminal end of E1, leaving the 191aa core protein anchored by the signal peptide [48]. This 191 aa polypeptide is an immature form of the core.

It is further processed by an intramembrane protease, the signal peptide peptidase (SPP), which cleaves within the C-terminal signal peptide and releases the N-terminal 173-179 aa of the core protein from the ER (Figure 1). This cleaved core protein remains associated with the cytoplasmic monolayer of the ER membrane via a hydrophobic domain located between aa 120 and aa 175 [17, 49]. This enables it to move along this monolayer towards the surface of the lipid droplets [48]. This hydrophobic domain, which mediates targeting to the lipid droplets, is not found in the core protein of related pesti- and flaviviruses, with the exception of GB virus B (GBV-B), the virus most closely related to HCV [17, 50]. The role of the association between HCV core and lipid droplets in the viral life cycle remains unknown. However, studies in the HCV-LP model have recently shown that SPP cleavage is required not only for the trafficking of the HCV core to the lipid droplet surface, but also for its self-assembly and budding at the ER membrane [51]. Studies in this model have shown that the HCV core protein self-assembles in HCV-LPs in ER membranes, which often appear to be located very close to the lipid droplets (Figure 3). Unfortunately, it was extremely difficult to document viral assembly and morphogenesis by similar EM studies in cell culture systems in which the JFH-1 virus is efficiently propagated [22]. However, early studies of cells infected with the JFH-1 virus showed that the core protein was detected at the surface of lipid droplets or in association with the ER membranes surrounding the lipid droplets [22]. In these cells, the HCV core protein was never detected in the nucleus or mitochondria [22]. More recently, it has been shown that the lipid droplets are directly involved in the production of infectious JFH-1 virus particles [52]. The authors demonstrated that the HCV core protein recruits non-structural proteins and replication complexes to lipid droplet-associated membranes, and that this recruitment is essential for producing infectious viruses. In another study, disrupting the association of HCV core protein with lipid droplets was correlated with a loss in production of the JFH-1 virus [53]. These observations suggest that the association between the HCV core

protein and lipid droplets plays a critical role at some stage of virion morphogenesis. It is possible that the ER membrane supporting lipid droplet formation provides a platform for nucleocapsid assembly, by concentrating the viral and/or cellular factors required for viral assembly, and/or by excluding those that inhibit this process.

HCV CORE PROTEIN AND STEATOSIS

Studies based on the use of heterologous expressions systems have shown that the HCV core protein interacts with a broad range of cellular proteins and influences numerous host cell functions including gene transcription, the inhibition or stimulation of apoptosis, cell signaling and the suppression of host immunity [54, 55]. This protein also plays a role in lipid metabolism, as it has been shown to bind and activate the DNA-binding domain of the retinoid receptor α (RxRα), a transcriptional regulator controlling many cellular functions, including cellular lipid synthesis [56, 57]. The HCV core protein also decreases expression of the peroxisome proliferators-activated receptor α (PPARα), a nuclear receptor regulating several genes responsible for fatty acid degradation [56]. HCV-infected patients have recently been shown to have lower than normal levels of PPARα mRNA in their liver [58, 59]. Nonetheless, the relevance of these *in vitro* experiments, mostly involving heterologous overexpression, to the natural course and pathogenesis of HCV infection, remains unclear.

As reported above, the HCV core protein has been implicated in lipid accumulation in the liver of some lines of transgenic mice. The mechanism underlying this liver steatosis in transgenic mice remains a matter of speculation, but it has been suggested that HCV core protein inhibits microsomal triglyceride transfer protein (MTP) activity [60]. MTP plays a key role in very-low density lipoprotein (VLDL) assembly. The inhibition of this enzyme would therefore result in the accumulation of triglycerides. However, no interaction of the HCV core protein with MTP has been demonstrated, and this inhibition may be indirect. It has also been

suggested that HCV core protein may accumulate in the mitochondria and induce liver damages, by producing reactive oxygen species (ROS), these effects being prevented by a mitochondrial electron transport inhibitor [61]. An increase in the level of intrahepatic lipid peroxidation products was observed in response to carbon tetrachloride in one transgenic mouse model [62]. ROS production may result in the peroxidation of membrane lipids and proteins involved in trafficking and secretion, inhibiting VLDL secretion. In another transgenic mouse model, it was suggested that the effect of HCV core protein on steatogenesis might be related to its interaction with the nuclear proteasome activator PA28γ, leading to the up-regulation of genes encoding proteins involved in fatty acid biosynthesis [63]. However, the HCV core protein is not detected in the nucleus or mitochondria of cells supporting the JFH-1 virus life cycle, and the relevance of these particular localizations remains debated [22]. This raises the interesting possibility that the localization of HCV core protein on the surface of lipid droplets may be directly linked to liver steatosis. Although core protein is difficult to detect in biopsy samples from infected chimpanzees, as in those from humans, it was found to be associated with lipid droplets in the liver of chimpanzees displaying fatty change [42].

Most HCV-associated liver injury results from the host immune response [64], but liver steatosis, which involves the deposition of triglycerides, may contribute to the progression of fibrosis in patients with chronic hepatitis [65-70]. Liver steatosis occurs in more 50% of patients with chronic hepatitis C [71]. It may be related to host metabolic factors (excess weight, diabetes, hyperlipidaemia, excessive alcohol intake), but many studies have clearly demonstrated a significant association between HCV genotype 3 and the presence of steatosis [72-77]. In patients infected with viruses of genotypes other than 3, steatosis seems to be mostly metabolic in origin, with little effect of HCV viral load. In contrast, in cases of genotype 3 infection, the presence of steatosis is directly correlated with serum and

intrahepatic titers of HCV RNA. Interestingly, the outcome of steatosis matches the virological response to treatment in patients infected with HCV genotype 3 with purely virus-induced steatosis but not in patients with metabolic causes of steatosis, generally infected with other genotypes [78, 79]. However, both types of steatosis (viral and metabolic) may well co-exist in at least some chronic hepatitis C patients, although steatosis is more likely to be predominantly viral in origin in patients infected with genotype 3 viruses and predominantly metabolic in patients infected with viruses of other genotypes [80, 81].

The steatosis observed in HCV-positive chronic hepatitis has the potential to affect the natural course of the infection through different routes. One study based on the analysis of paired liver biopsies from patients with chronic hepatitis C clearly showed that worsening of steatosis was the only independent factor associated with hepatic fibrosis progression [66]. Recently, a meta-analysis of more than 3000 patients with chronic HCV infection in databases in Europe, Australia and the USA confirmed the role of steatosis as significantly and independently associated with fibrosis in these patients [82]. The role of liver steatosis in chronic hepatitis C is further extending into the area of the complications of chronic liver diseases. Recent evidence suggests that HCV-related steatosis may contribute to liver carcinogenesis. In one study investigating risk factors for the development of hepatocellular carcinoma (HCC) in HCV-positive patients, the presence of steatosis was significantly associated with the incidence of HCC in a multivariate analysis [83].

Most *in vitro* studies using transfected cells and transgenic mouse models addressing the role of HCV core protein in lipid accumulation have been conducted with constructs derived from isolates of HCV genotype 1. Recent *in vitro* studies have therefore investigated whether an HCV core protein of genotype 3 increases the level of lipid accumulation [84-86]. One study demonstrated that lipid droplet accumulation occurred with core proteins from all viral genotypes, but the genotype 3 core protein resulted in the accumulation of larger

numbers of lipid droplets than the genotype 1 core protein [84]. In a subsequent study, a genotype 3-specific residue (phenylalanine 164, this residue being replaced by a tyrosine in all non-3 genotypes) present in the hydrophobic domain of the HCV core protein known to interact with lipid droplets, replaced the equivalent residue in the genotype 1 core protein [85]. This resulting Y164F mutant had a significant higher level of lipid droplet accumulation, consistent with the clinical evidence showing that viral core sequences have a direct effect on genotype 3-specific steatosis [85]. Structural studies on the HCV core protein have suggested that the residue in position 164 is part of an α-helix present at the interface of the two phospholipid layers of the ER membrane [49, 87], which is presumed to be the site of lipid droplet morphogenesis. Phenylalanine is more hydrophobic than tyrosine and may be involved in lipid interactions. The presence of this residue in the core protein of genotype 3 may therefore increase the affinity of the protein for the lipids present between the leaflets of the ER membrane, ultimately leading to higher levels of lipid droplet formation. However, other mechanisms may also be involved in this phenomenon. It has been suggested that the Y164F mutant up-regulate the promoter activity of the gene encoding fatty acid synthase (FAS), a major enzyme involved in *de novo* lipid synthesis, by interacting with the transcription factor sterol response element binding protein-1 (SREBP-1) [86].

CONCLUSION

The association of the HCV core protein with lipid droplets is probably involved in the virus-induced steatosis. This association is also shown to be essential for the virion production, using the recently developed cell culture system supporting HCV propagation, and this suggests that virus-induced steatosis may be essential for the viral life cycle. Alternatively, the interaction of the HCV core protein with lipid droplets may play a role in the formation and release of subviral or hybrid particles, which have been shown to circulate in the serum of

chronically infected patients, bound to lipoproteins in the form of lipo-viro-particles (LVPs) [88, 89]. These particles appear as large lipoprotein-like structures enriched in apoB, apoE and triglycerides, containing HCV core protein and carrying viral envelope proteins at their surface [90]. However, the role of these LVPs in HCV biology and their contribution to the viral spread remain largely unknown. It is expected that the recently developed cell culture system in which HCV propagation has been reported *in vitro* will help to resolve these major issues.

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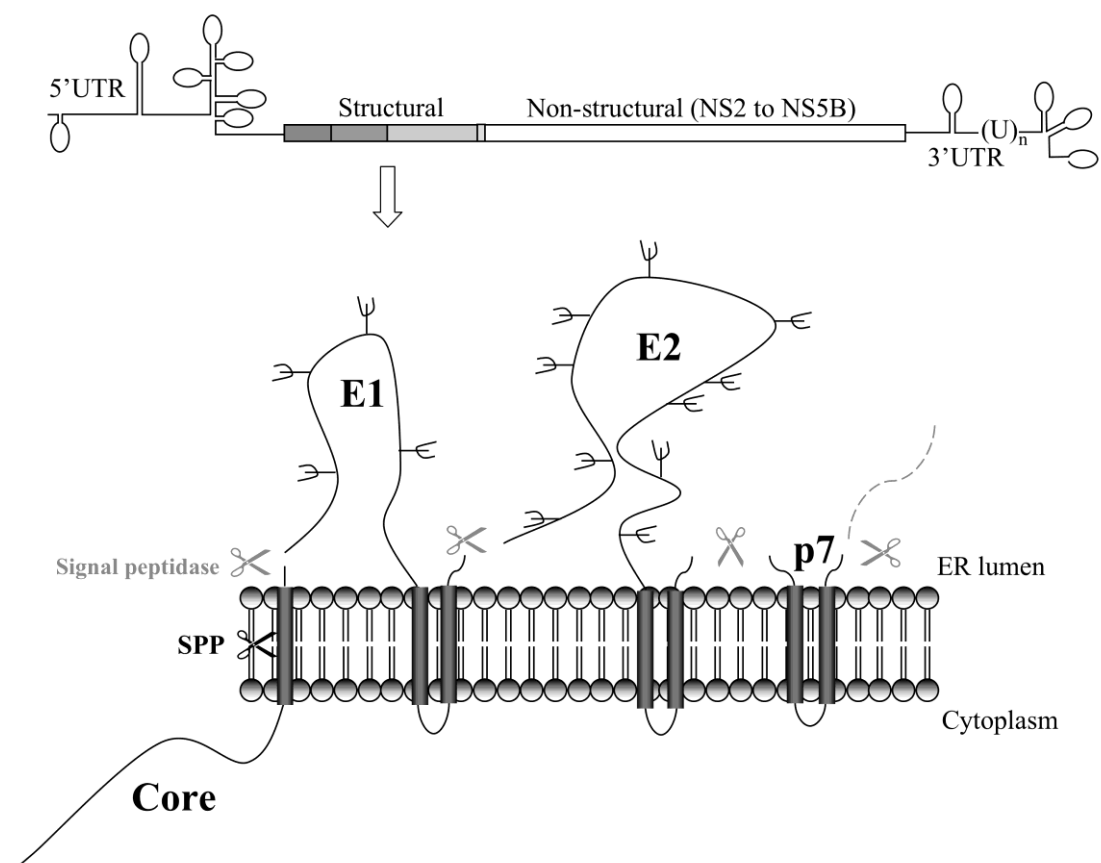
Fig.1 Hepatitis C virus (HCV) genome organization and schematic representation of the processing of the HCV structural proteins.
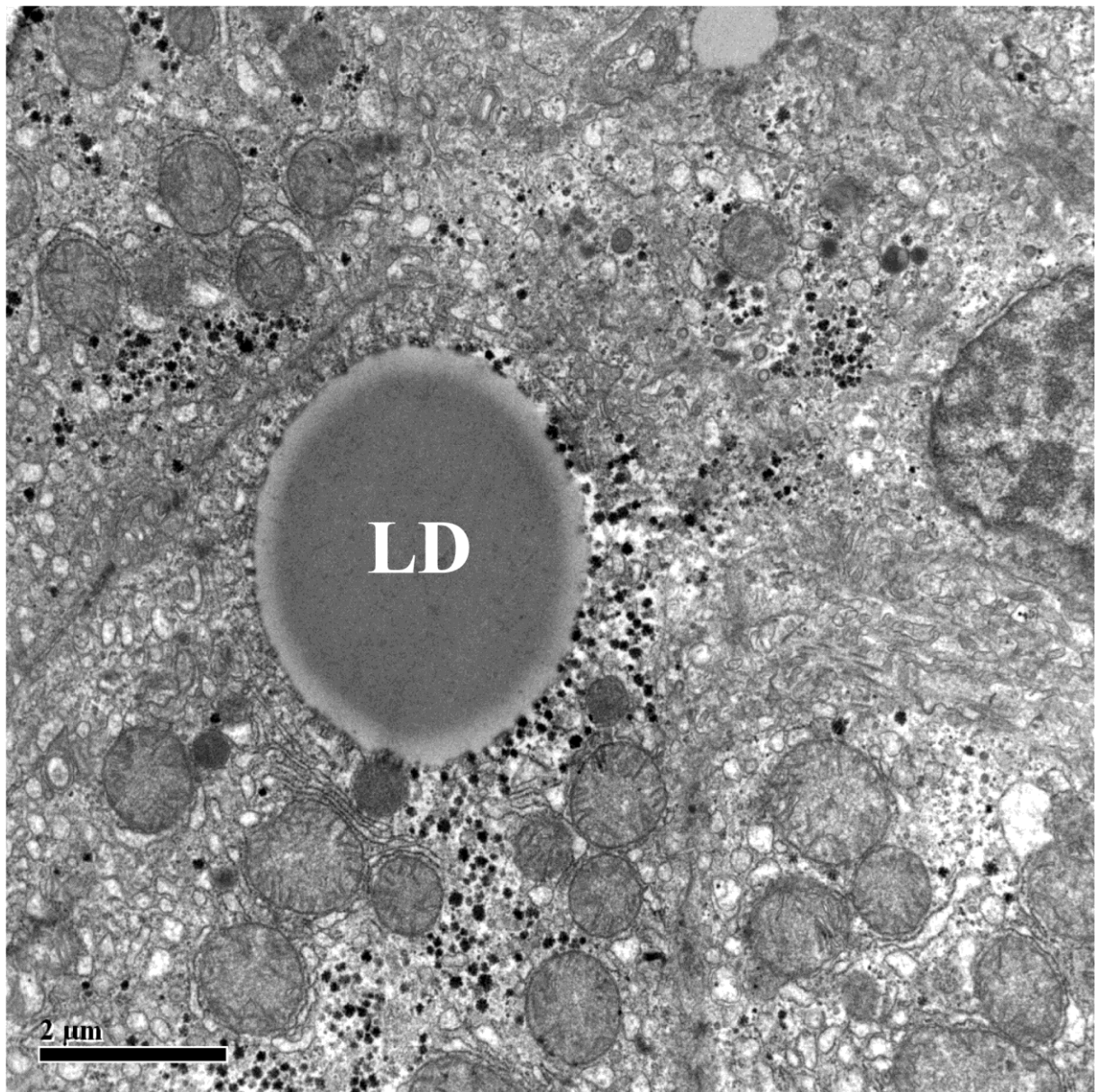
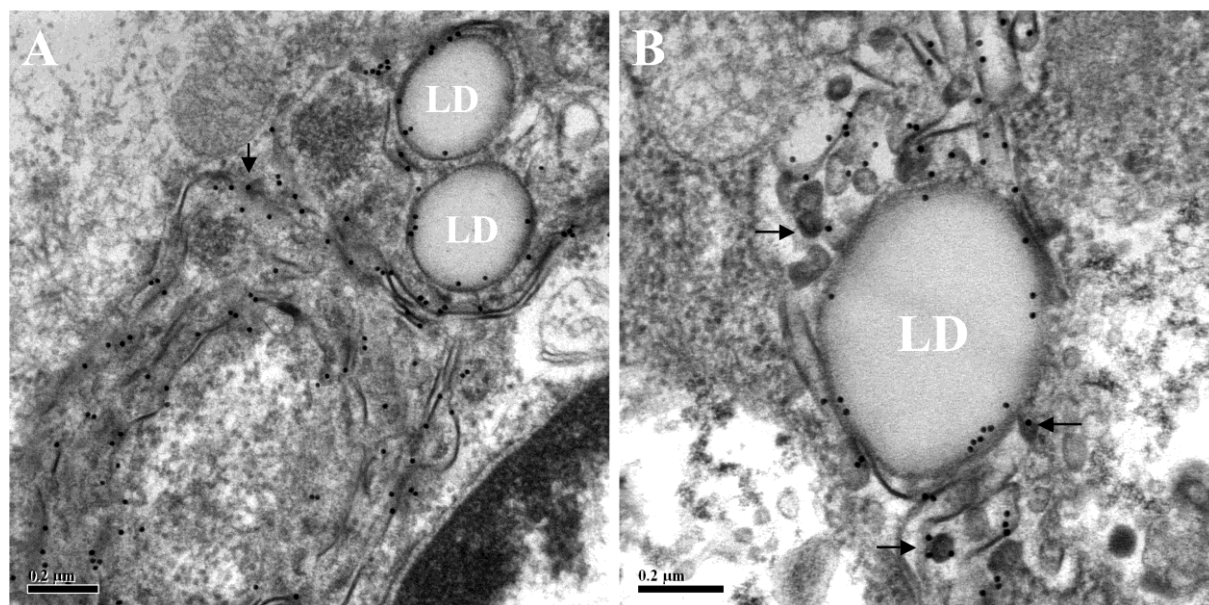**Fig.2** Electron micrograph of a liver biopsy showing a large lipid droplet (LD).

Fig.3 Electron micrographs of BHK-21 (baby hamster kidney) cells producing HCV-like particles (HCV-LPs) at low (A) and high (B) magnifications. The HCV core protein, visualised here by immunolabelling with gold particles, is present at the surface of the lipid droplets (LD) and in the convoluted ER membranes surrounding these lipid droplets. HCV-LP budding (arrows) occurs at the ER membrane surrounding these lipid droplets.