國立臺灣大學醫學院微生物學研究所

碩士論文

Graduate Institute of Microbiology
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Master Thesis

LMBRD1 基因產物在 C 型肝炎病毒 RNA 複製及病毒組裝中扮演的角色

Roles of LMBRD1 gene products involved in the replication and assembly of hepatitis C virus

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中文摘要

C 型肝炎病毒 (hepatitis C virus, HCV) 主要經由血液和體液傳染，感染後會造成 C 型肝炎，屬於全球性的感染疾病。HCV 具有正向單股 RNA 基因體，在感染宿主細胞後,會以其正向單股 RNA 基因體進行轉譯,並在內質網的周邊形成適合病毒複製的特殊網狀結構，藉由細胞中油滴的幫助，進行病毒顆粒的組裝。本實驗室先前的研究發現，在細胞中表現 LMBD1 重組蛋白質時，此蛋白質與細胞中的油滴有明顯共位的現象。此外，在細胞中同時送入 LMBD1 以及 HCV core 蛋白質的表現質體後，也可觀察到 LMBD1 和 core 蛋白質的共位現象。利用共同免疫沉澱分析發現 LMBD1 以及 core 兩蛋白質在細胞中有交互作用。另外,在踢弱 (knockdown) LMBRD1 基因表現的細胞感染 HCV 後，其細胞中的 HCV 負向基因體 RNA 量有上升的趨勢。為了更深入的瞭解 LMBRD1 基因與 HCV 基因體複製與病毒增殖的相關性，本研究利用 HCVR 次基因體細胞以及在 HCV 細胞培養系統中所產生的病毒 (HCVcc) 進行研究。結果顯示，在 HCVR 細胞中 LMBRD1 基因表現缺失會造成 HCV 的 RNA 負向基因體顯著降低，而其基因產物 LMBD1 以及 NESI 對於 HCV 基因體 RNA 複製皆有正向的調控作用。此外，HCV core 蛋白質表現在踢弱 LMBRD1 基因的細胞中會使得 HCV 負向基因體量增加，推測 core 蛋白質可能會透過 LMBD1 蛋白質的幫助，穩定基因體 RNA 複製推進至病毒包裹的動態平衡。另一方面，在踢弱 LMBRD1 基因表現的情況下，被 HCVcc 感染的細胞所產生出來的病毒顆粒數量並無明顯差異，但其感染能力明顯降低。LMBRD1 基因的表現影響了 HCV 基因體的複製以及病毒顆粒的組裝，然而其中的機轉仍然有待進一步的研究。

關鍵字：C 型肝炎病毒、LMBRD1 基因、LMBD1 蛋白質、病毒基因體複製、病毒組裝

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Abstract

Hepatitis C virus (HCV) is mainly transmitted by blood and body fluids. It causes hepatitis C after infection, which is a worldwide infectious disease. HCV possesses positive single-strand RNA genome. Upon infection with a host cell, the positive single-strand RNA acts as a template and translates to viral proteins. In the HCV-infected cells, HCV induces formation of a special membrane structure around the endoplasmic reticulum suitable for the replication of viral genome. In addition, assembly of HCV particles is helped by lipid droplets. Previous studies from our laboratory demonstrated that the LMBD1 recombinant proteins colocalized with the lipid droplet marker ADRP in the cells. Co-localization of LMBD1 with the viral core protein was also detected when co-expression of LMBD1 and HCV core protein. Co-immunoprecipitation analysis revealed that LMBD1 protein interacts with core protein. In addition, the level of HCV antigenomic RNA tends to be higher in HCVcc-infected Huh7.5 cells to which LMBRD1 gene has been knocked-down (Huh-shLMBRD1) compared to the control Huh7.5 cells to which LUC gene has been knocked-down (Huh-shLUC). In this study, HCVR subgenomic replicon cells and HCVcc infection system were applied to further examine the roles of LMBRD1 gene on HCV replication and assembly. The results show a significant lower level of HCV antigenomic RNA in LMBRD1-knockdown HCVR cells compared to the control LUC-knockdown cells, both LMBRD1 gene products LMBD1 and NESI had positive effects on HCV replication. In addition, HCV core protein expression in LMBRD1-knockdown HCVR cells increased the levels of HCV antigenomic RNA, suggesting that core protein may interact with LMBD1 to stablize the turn over between viral replication and assembly. On the other hand, HCVcc produced from HCVcc-infected Huh-shLMBRD1 cells (HCVcc-shLMBRD1) had no significant

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difference in the number of virus particles compared to the control HCVcc produced from HCVcc-infected Huh-shLUC cells (HCVcc-shLUC) but significantly reduced the infectivity. Expression of LMBRD1 gene affects HCV replication and assembly, the detailed mechanism remains to be further elucidated.

Key words: Hepatitis C virus, LMBRD1 gene, LMBD1 protein, viral genome replication, viral assembly

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I. Introduction

A. Hepatitis C virus (HCV)

a. History

The existence of hepatitis C was suggested in the 1970s and proven in 1989. In 1975, researcher observed enveloped viral particles in serum of patients who had an episode of transfusion-associated hepatitis not positive for hepatitis A and B antigen. They named this hepatitis as non-A, non-B hepatitis (NANBH) [1]. In 1989, researchers injected the serum from NANBH patients to chimpanzee in order to increase viral titer, then isolated the virus from chimpanzee. They got cDNAs of virus genome through reverse transcription. A cDNA library derived from the infectious material was constructed. They confirmed that the isolated cDNAs belong to the genome of the virus that caused NANBH, this virus was named as hepatitis C virus (HCV) [2].

b. Virology

The HCV is a small, enveloped, single-stranded, positive-sense RNA virus. It is a member of the *hepacivirus* genus in the family *Flaviviridae* [2,3]. The genome of HCV is highly variable due to its non-proof reading RNA-dependent RNA polymerase. So far, there are seven major genotypes of HCV classified by the degree of genetic dissimilarity, which are known as genotypes 1 to 7 [4]. The genotypes are divided into several subtypes with the number of subtypes depending on the genotype. In the United States, about 70% of cases are caused by genotype 1, 20% by genotype 2 and about 1% by each of the other genotypes [5].

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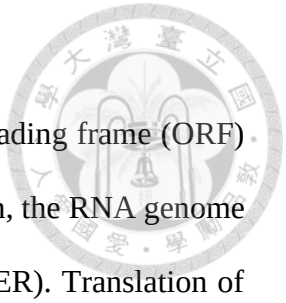

c. The structure and genome of HCV

The genome of HCV is about 9.6 kb and carries a long open reading frame (ORF) encoding a polyprotein precursor [6]. Upon release into the cytoplasm, the RNA genome is directly used for translation at the rough endoplasmic reticulum (ER). Translation of the HCV ORF is directed via 5' nontranslated region (NTR) functioning as an internal ribosome entry site; it permits the direct binding of ribosomes in close proximity to the start codon of the ORF [7]. The HCV polyprotein is cleaved co- and post-translationally by cellular and viral proteases into ten different products including the structural proteins (core, E1 and E2) and the nonstructural replicative proteins (NS2-5) (Appendix Fig. 1). Core and envelope glycoproteins E1 and E2 are main constituents of the virus particle, whereas the p7 viroporin and nonstructural protein 2 (NS2) participate in virus assembly. NS3, NS4A, NS4B, NS5A, and NS5B form the replication complex that is sufficient for viral RNA replication occurring via a negative-strand copy [8, 9].

Structural proteins

i. Core protein

HCV core protein is the viral nucleocapsid protein with numerous functionalities including RNA binding, immune modulation, cell signaling and oncogenic potential. HCV core protein also associates with the lipid droplets where HCV assembly takes place. HCV core protein is released as a 191 amino acid (aa) precursor. Although proteins of various sizes (17 to 23 kDa) were detectable, the 21-kDa core protein is the predominant form [10]. The HCV core protein contains three distinct parts: a N-terminal hydrophilic domain (domain D1), a C-terminus hydrophobic domain (domain D2), and the last part serving as a signal peptide for the downstream envelope protein E1. Domain D1 contains numerous positive charges. It is principally involved in RNA binding and nuclear

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localization, as suggested by the presence of three predicted nuclear localization signals (NLS) [11, 12]. Domain D2 possesses a helix-turn-helix structure that is responsible for core protein association with ER membranes and lipid droplets, avoiding degradation by protease [12].

In addition to its role in viral capsid formation, the core protein has been suggested to directly interact with a number of cellular proteins that may be important in the viral life cycle such as viral assembly. Through the protein-protein interactions between HCV core protein and host proteins such as DGAT1 (diacylglycerol acyltransferase-1), cPLA2 (cytosolic phospholipase A2) and AP2M1 (μ subunit of clathrin adaptor protein complex 2), core protein could be trafficked to lipid droplets [13, 14, 15]. HCV core protein also interacts with the C-terminus of the viral NS5A protein and brings it to the surface of lipid droplet to form a complex with the viral RNA [16].

ii. Envelope proteins

HCV envelope proteins E1 and E2 are type I transmembrane glycoproteins that can form non-covalent heterodimers within the infected cells or large covalently linked complexes on the viral particle. These two envelope glycoproteins are essential components of the HCV virion envelope and necessary for viral entry and fusion [17]. E2 protein plays a crucial role in the early steps of infection. Viral attachment is thought to be initiated via E2 interaction with scavenger receptor class B member 1 (SRB1) and CD81 [18].

Nonstructural proteins

i. p7 protein

p7 is a small, 63 aa polypeptide, that has been shown to be an integral membrane

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protein. It could act as an ion channel protein required for the viral assembly and release [19].

ii. NS2 protein

NS2 is a non-glycosylated transmembrane protein of 21–23 kDa, which together with the amino-terminal domain of the NS3 protein function as the NS2-3 protease that cleaves the site between NS2 and NS3 [20]. After self-cleavage from NS3, NS2 loses its protease activity and is degraded by the proteasome.

iii. NS3 protein

NS3 is a multi-functional viral protein containing a serine protease domain in its N-terminal and a helicase/NTPase domain in its C-terminal. NS3 protease plays a critical role in HCV processing by cleaving downstream of NS3 at 4 sites (between NS3/4A, NS4A/4B, NS4B/NS5A, NS5A/NS5B) through the help of cofactor, NS4A which forms a stable complex with NS3 [21].

iv. NS4A protein

NS4A is a cofactor of NS3 protease which forms a stable complex with NS3 and NS4A could enhance the helicase activity of NS3 as well [22].

v. NS4B protein

NS4B is an integral membrane protein of 27 kDa, with four transmembrane domains. It is known to anchor on ER or ER derived membrane and is responsible for membrane association to form the membranous web, offering an environment proper for HCV replication complex to go on replication [23].

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vi. NS5A protein

NS5A is a membrane-associated phosphoprotein of unknown structure and function that probably plays an important role in virus replication and regulation of cellular pathways. It exists in a basally phosphorylated form of 56 kDa and in a hyperphosphorylated form of 58 kDa. The mechanisms by which NS5A regulates HCV replication are not entirely clear. A report suggested that the level of NS5A phosphorylation plays an important role in the viral life cycle by regulating a switch from replication to assembly, whereby hyperphosphorylated forms function to disrupt interaction with hVAP-A (human vesicle-associated membrane protein-associated protein A) and negatively regulate viral RNA replication [24].

vii. NS5B protein

NS5B is an RNA polymerase, having the key function of replicating HCV viral RNA by using the viral positive RNA strand as a template to catalyze the polymerization of ribonucleoside triphosphates (rNTP) during RNA replication.

Virion and lipoviroparticle (LVP)

The HCV particle consists of RNA genome, core and the envelope glycoproteins, E1 and E2. HCV RNA genome interacts with the core protein to form the viral nucleocapsid that is enveloped in a lipid-rich viral envelope with the E1 and E2 glycoproteins. Interestingly, HCV virion also associates with various lipoproteins such as apoE, apoB to form a complex lipoviroparticle (LVP). Various lipoprotein components can influence HCV entry [25].

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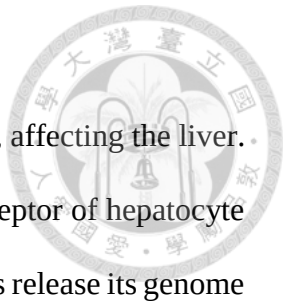

d. Life cycle of HCV

HCV has tissue tropism that primarily infects human hepatocyte, affecting the liver. During the viral infection, envelope proteins interact with surface receptor of hepatocyte and the virus entries cell through endocytosis. In the endosome, viruses release its genome into cytoplasm and move near to ER. Through the translation system of host, the genomic RNA translates to a polyprotein, controlling by its IRES. Cleavage of the signal sequence by the host signal peptidase and viral own peptidase yields the mature viral proteins. Non-structural proteins will form a replication complex and start to replicate viral RNA. Then through the help of core protein on the surface of lipid droplet, the viral genomic RNA is wrapped. After viral particle assembly at ER membrane, these particles will deliver to Golgi and release from cells through VLDL secretory pathway [Appendix Fig. 2; 26, 27, 28, 29].

i. Attachment and entry

The observation of the interaction between HCV particles and lipoproteins leads to the proposal that the LDL receptor (LDLR) plays a role in the early phase of HCV attachment. HCV-LDLR interaction can potentially lead viral particle close to the surface of hepatocytes [30, 31]. The initial attachment of HCV particles onto hepatocytes was also suggested to be mediated by scavenger receptor class B type I (SRB1). SRB1 involvement in HCV entry was indicated by its ability to interact with E2, the hypervariable region 1 (HVR1) of E2 is essential for this interaction [32]. Additionally, the interaction of SRB1 with HVR1 could also unmask the CD81 binding site of E2. Further interaction of CD81 with E2 leads viral particles to the tight junction of cells, and the virion entries into cells by clathrin-mediated endocytosis [33]. Two tight junction proteins (CLDN1 and OCLN) interact with CD81 as a part of the HCV receptor complex

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[34, 35]. HCV envelope glycoproteins promote coendocytosis of CD81 and CLDN1 in a clathrin- and dynamin-dependent process, followed by fusion between viral and endosomal membranes, which leads to the release of the nucleocapsid into the cytoplasm [36]. The E1 envelope glycoprotein is believed to be the fusogen (the glycoprotein that facilitates cell fusion) [37].

ii. Replication

Following target cell entry through receptor-mediated endocytosis, HCV particle undergoes pH-dependent membrane fusion within an acidic endosomal compartment to release its RNA genome into the cytoplasm [38, 39]. A single polyprotein of ~3,000 aa polyprotein is synthesized in rough ER and is further processed by cellular and viral proteases to generate individual viral proteins as mentioned above.

In the course of viral replication, HCV proteins are associated with a membranous web which includes double-membrane vesicles (DMVs) [23, 40]. The membranous web appears to be induced by HCV NS4B protein possibly in combination with NS5A protein [41]. These DMVs may sequester replication intermediates from cytosolic innate immunity sensors of non-self RNAs, such as retinoic acid-inducible gene I (RIG-I) and protein kinase R (PKR). In addition, DMVs are enriched in cholesterol and sphingolipids, being relatively resistant to detergents and protecting the vesicle contents from host ribonucleases and proteases [42]. Viral RNA replication is believed to occur in these webs with the positive strand RNA genome as a template for the NS5B RdRp to generate the negative strand replicative intermediate, to produce further positive sense genomes. Nascent positive strand RNA genomes can be further translated to produce new viral proteins, or serve as templates for further RNA replication, or be assembled to infectious virions.

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iii. Assembly and release

The HCV assembly process is not fully understood. However, it appears to be dependent on lipid droplets (LDs), which are cytosolic lipid storage organelles involved in many cellular processes [43]. Two HCV-encoded proteins, the nucleocapsid core and NS5A, associate with LDs during virion assembly. Core protein traffics to LDs and associates with LDs through its D2 domain, and then NS5A may bring the RNA genome out of DMVs to the LD surface by interacting with the core protein [44]. Given that preformed capsids have not been identified, nucleocapsid formation likely takes place in concert with budding. Furthermore, virus particle assembly may be intimately coordinated with RNA replication, as seen for other members of this virus family.

The VLDL secretion pathway is closely related to that of assembled virions. The virion is a lipoviroparticle with a lipid composition that resembles VLDL and LDL with associated apoE and/or apoB [45, 46].

B. Limb region 1 (LMBR1) domain containing 1 gene (LMBRD1)

The limb region 1 (LMBR1) domain containing 1 gene (LMBRD1) encodes proteins that belong to the membrane protein family of lipocalin-1-interacting membrane receptors. Two major proteins are encoded by the LMBRD1 gene, namely, the nuclear export signal-interacting protein (NESI, 467 aa) and the LMBD1 protein (540 aa). Previous studies have shown that NESI is a nuclear membrane protein that is critical for the assembly of the hepatitis δ virus (HDV) [47].

LMBD1 contains 73 additional aa at the N terminus of the NESI protein, with nine putative transmembrane domains and a cytoplasmic C-terminus. A previous study demonstrated that LMBD1 is localized in the lysosomal membrane, and the mutations of LMBRD1 gene are associated with the deficiency of lysosomal cobalamin export [48]. It

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was speculated that LMBD1 is a lysosomal Cbl transporter. Further study showed that LMBD1 interacts with ABCD4, which is also a putative Cbl transport protein and localizes on the lysosomal membrane [49]. In addition, LMBD1 has been found to localize in the plasma membrane [50]. It might interact with AP2 protein through its AP2 binding motif and participates in the internalization of insulin receptor through cathrin-mediated endocytosis [50].

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II. Specific aims

LMBD1 that anchors in cell membrane is an adaptor protein in clathrin-mediated endocytosis [49]. The entry of HCV is also considered to be via clathrin-mediated endocytosis that internalizes the surface receptor [51]. In our previous study, after HCVcc infection for 1 hour, no significant change in the level of HCV genomic RNA was detected in LMBRD1 knockdown cells compared to the control [52]. Interestingly, after HCVcc infection for 2 hours, a higher level of HCV genomic RNA was detected in LMBRD1 knockdown cells compared to the control. These suggest that the LMBD1 protein has no effect on the early stage of HCV infection but has an effect on the late stage of HCV infection.

The potential relationship between LMBRD1 gene products and HCV replication

LMBD1 protein is a transmembrane protein, expressing at plasma membrane, lysosome membrane, and ER membrane [49, 50, 53]. The ER membrane and ER membrane-derived structure, membranous web, are crucial for HCV RNA replication. The NS4B and NS5A proteins trigger the derivation of ER membrane to form the membranous web [23]. Due to the association of LMBD1 with membrane structure, we hypothesize that LMBD1 protein may participate in HCV replication.

The potential relationship between LMBRD1 gene products and HCV assembly

In our previous study, LMBD1 was demonstrated to express on the surface of LD and colocalize with lipid droplet marker ADRP in cells [54]. It's already known that LDs play a role in HCV assembly through interacting with the viral core protein [43]. Core protein associates with cytosolic LDs and packages viral RNA into ER lumen by the fusion of LDs and ER membrane [44]. Perhaps LMBD1 is one of membrane protein located at LDs and ER membrane that participate in HCV assembly. On the other hand, our previous study suggested an association of LMBD1 with the viral core protein since

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it colocalized with core protein and triggered core aggregation in the cytosol [54].

Since HCV life cycle highly relies on membrane structures and LMBD1 is a transmembrane protein located at ER membrane and LDs, we hypothesized that LMBD1 may play roles in HCV life cycle. Based on the previous studies mentioned above, this study will further elucidate the roles of LMBRD1 gene in HCV replication, assembly and release.

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III. Materials and Methods

A. Materials

a. Chemicals and reagents

Acrylamide	United State Biochemical
Ammonium persulfate (APS)	Bio-Rad
Ampicillin	United State Biochemical
Bis-acrylamide	Bio-Rad
β-mercaptoethanol (β-ME)	Merck
Boric acid	Merck
Bovine serum albumin (BSA)	Sigma
Bromophenol blue	Merck
Calcium chloride	Merck
Complete EDTA-free protease inhibitor	Roche
Chloroform	Merck
Dimethyl sulfoxide (DMSO)	Merck
Dulbecco's Modified Eagle medium (DMEM)	GIBCO
Ethanol (EtOH)	Merck
Ethyenediaminetetraacetic acid (EDTA)	Merck
Fetal bovine serum (FBS)	Hyclone
Geneticin (G418)	Biowest
Glycine	United State Biochemical
Hydrogen chloride (HCl)	Merck
Isopropanol	Merck

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Luria Bertani (LB) agar	Lab M Limited
Luria Bertani (LB) broth	Lab M Limited
Magnesium chloride ($MgCl_2$)	Merck
Methanol	Merck
Nonidet P-40 (NP-40)	Sigma
Non-essential amino acids (NEAA)	GIBCO
Opti-MEM®	GIBCO
Penicillin-streptomycin	Corning
Potassium chloride (KCl)	Merck
Potassium dihydrogen phosphate (KH_2PO_4)	Merck
Puromycin	Sigma
REzol™ C&T	PROtech Technologies
Skim milk	安佳
Sodium chloride (NaCl)	Merck
Sodium deoxycholate	Sigma
Sodium dihydrogen phosphate (NaH_2PO_4)	Merck
Sodium dodecyl sulfate (SDS)	Merck
TEMED	Bio-Rad
Tris-base	United State Biochemical
Triton X-100	Sigma
TrypLE™ Express	GIBCO
T-pro Non-liposome Transfection Reagent II	Genestar Biotech
Tween 20	Sigma

b. Enzymes

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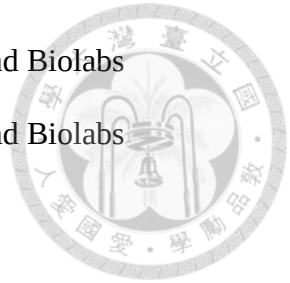
Restriction enzymes	New England Biolabs
T4 DNA ligase	New England Biolabs
Taq DNA polymerase	Gene Teks



c. Antibodies

HRP-conjugated goat-anti-mouse IgG (115-035-003)	Jackson
HRP-conjugated goat-anti-rabbit IgG (sc-2004)	Santa Cruz Biotech
Mouse anti-βactin monoclonal antibody (AC-74)	Sigma
Mouse anti-Flag M2 monoclonal antibody (F3165)	Sigma
Mouse anti-LMBD1	實驗室製備
Mouse anti-NS5A monoclonal antibody (MAB8694)	Chemicon
Mouse anti-NS5A monoclonal antibody	BioFront
Mouse anti-NS3 monoclonal antibody	Abcam
Mouse anti-core monoclonal antibody	BioFront
Mouse anti-E1 monoclonal antibody (sc-65459)	Santa Cruz Biotech
Rabbit anti-LMBRD1 polyclonal antibody (PAB21217)	Abnova

d. Kits

Bio-Rad Protein Assay Kit	Bio-Rad
DNA Clean/Extraction Kit	GeneMark
Plasmid Miniprep Purification Kit	GeneMark
Plasmid Midiprep Plus Purification Kit	GeneMark
Maxima SYBR Green	Thermo
PVDF membrane	millipore

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WesternBright™ ECL Kit	Advanta
X-Ray film	FUJI
ProtoScript® II First Strand cDNA Synthesis Kit	New England Biolabs

e. Cell lines

i. HEK 293T

HEK 293T cell line is derivative from human embryonic kidney 293 (HEK 293) cell line and contains SV40 T antigen. It is highly transfectable and is frequently used for protein expression. This cell line is cultured in DMEM supplied with 10% FBS, 1% NEAA, 100 U/ml penicillin and 100 μg/ml streptomycin under condition of 37 ℃ and 5% CO_2.

ii. Huh7.5 [55]

Huh7.5 (kindly provided by Dr. Rice CM at Rockefeller University, New York) is a type of differentiated hepatocyte-derived carcinoma cell line that originally taken from IFN-α cured Huh7 cell. Huh7.5 cells have a defect in the RIG-I pathway, making them less responsive to intracellular dsRNA, generated during virus replication and inducing an antiviral program. The reduced efficiency of the host cell's innate defenses could explain the higher permissiveness. This cell line is cultured in DMEM supplied with 10% FBS, 1% NEAA, 100 U/ml penicillin and 100 μg/ml streptomycin under condition of 37 ℃ and 5% CO_2.

iii. HCVR [56]

HCVR cell is a subgenomic replicon cell that contains HCV 1b strain subgenome, including 5’UTR, 3’UTR, neomycin selection gene and open reading frame of NS3 to

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NS5B. This cell line could stably express NS3, NS4A, NS4B, NS5A, and NS5B protein, and is an important tool for the research of HCV replication. It is cultured in DMEM supplied with 10% FBS, 1% NEAA, 100 U/ml penicillin, 100 μg/ml streptomycin and 1 mg/ml G418 under condition of 37 ℃ and 5% CO_2.

f. Plasmids

i. pCMV-JFH1-RZ-PA [57]

The expression plasmid, pCMV-JFH1-RZ-PA, contains a cytomegalovirus (CMV) immediate early gene promoter to regulate the transcription of HCV JFH1 viral genome. This RNA contains ribozyme and polyA tail of HDV in order to cut 3'UTR of JFH1 correctly, making virus could replicate in cell properly.

ii. pAdTrack-CMV (Addgene)

It is the expressing plasmid for mammalian cells and has size approximately 9220 bp. Vector pAdTrack-CMV is used for expression of transgenes under a CMV promoter when a GFP tracer is desired. It contains two CMV promoters to regulate target gene and GFP gene transcription.

iii. pAdTrack-CMV-flag-core [58]

This plasmid has size approximately 9800 bp. It has two CMV promoters to drive the flag- tagged core and GFP gene expression respectively.

iv. pLenti-C-LMBD1-mGFP (Origene)

This plasmid drives the expression of GFP-tagged wild type LMBD1 protein by using CMV promoter.

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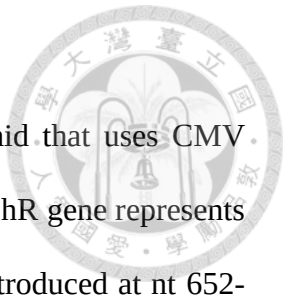

v. pLenti-C-LMBD1-shR-mGFP [52]

This plasmid is a GFP-tagged LMBD1-shR expression plasmid that uses CMV promoter to drive the transcription of LMBD1-shR gene. LMBRD1-shR gene represents LMBD1 coding sequences to which wobble mutations have been introduced at nt 652-672, so it could express LMBD1 in the presence of LMBD1-shRNA.

vi. pEGFP-N1 (Clontech)

This plasmid has size approximately 4700 bp. It uses the CMV promoter to drive the transcription of GFP gene.

vii. pMD.G (Academia Sinica RNAi core)

This plasmid has size 6246 bp. It uses the CMV promoter to drive the expression of the G protein (glycoprotein) of vesicular stomatitis virus (VSV). VSV-G expresses at the envelope of viral particle to infect most mammalian cells for its extensive tissue tropism.

viii. pCMVδR8.91 (Academia Sinica RNAi core)

This plasmid uses the CMV promoter to regulate the transcription of Gag, Pol, Tat and Rev gene from HIV-I. It offers the needed protein and reverse transcriptase for Lentivirus.

ix. pLKO.1-shLuc (Academia Sinica RNAi core, TRCN0000072243)

This plasmid has size approximately 7090 bp, and it uses the respiratory syncytial virus (RSV) promoter to drive the expression of shRNA for the firefly luciferase gene (target sequence: 5’ -CTTCGAAATGTCCGTTCGGTT- 3’).

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x. pLKO.1-shLMBD1 (Academia Sinica RNAi core, TRCN0000062280)

This plasmid has size approximately 7090 bp, and it uses the respiratory syncytial virus (RSV) promoter to drive the expression of shRNA for the LMBRD1 gene (target sequence: 5’ -GCGTTACCTTTAAATCTGATA- 3’).

B. Methods

a. Construction of plasmid pLenti-C-NESI-shR-mGFP

Plasmid pLenti-C-NESI-shR-mGFP represents the GFP-tagged NESI protein. The cDNA fragment of NESI protein were amplified by PCR using plasmid pLenti-C-LMBD1-shR-mGFP as the template and the primer sets: CS-LMBD1-3(SgfI)f: 5’-GGG CGA TCG CWT GGT TTC TTA CAT GA-3’ and CS-LMBD1-2(MluI)r: 5’-GAC GCG TCC AAG CAG AAT AGA CAG AGG-3’. The 1415 bp PCR product was digested with SgfI and MluI restriction endonucleases and the resultant cDNA fragment of NESI was used to replace the 1634 bp LMBD1 cDNA fragment following a treatment of plasmid pLenti-C-LMBD1-shR-mGFP with SgfI and MluI restriction endonucleases.

b. Transfection

One day before transfection, cells were seeded at about 50% confluency with complete medium. The next day, plasmid diluted in Opti-MEM was mixed with T-pro NTR II transfection reagent at a ratio of 1 μg plasmid to 2 μl T-pro NTR II transfection reagent. Following incubating at 25 ℃ for 20 min, the mixture of plasmid and T-pro NTRII was added into cultured medium and incubated under the condition of 37 ℃ and 5% CO_2 for about 48 hours.

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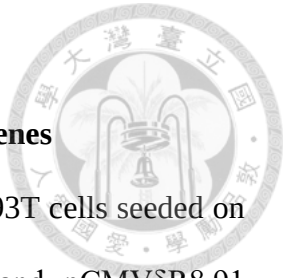

c. Lentiviral transduction

i. Packaging of recombinant lentivirus for knocking down genes

For preparation of lentivirus expressing shLMBRD1, 3 x 10^6 293T cells seeded on 10-cm dish were transfected with pLKO.1-shLMBD1, pMD.G and pCMVδR8.91 plasmid at a ratio of 10:1:9. Twenty hours posttransfection, the medium was replaced with 10 ml culture medium containing 1% BSA. The medium containing recombinant lentivirus was harvested after two days and 10 ml of fresh culture medium were added and again harvested on the next day. All medium harvested were pooled together and kept at -80 ℃. A similar approach was taken for preparation of lentivirus expressing shLUC expect pLKO.1-shLuc was used in the cotransfection.

ii. Transduction of lentiviral shLUC and shLMBRD1

For knocking down LMBRD1 gene in cells, 1 x 10^5 Huh7.5 cells or HCVR cells were seeded on 6-cm dish. The cells were infected with lentivirus containing shLMBRD1 or shLUC. Three days postinfection, cells were transferred to a 10-cm dish and then selected with 2 μg/μl puromycin. On the next day, cells were re-infected with the same recombinant lentivirus. After 2 days, total RNA was extracted from the cells and analyzed by RT-qPCR to confirm knocked-down of the LMBRD1 gene.

In this study, Huh-shLUC and HCVR-shLUC stand for Huh7.5 cells and HCVR cells to which LUC gene has been knocked-down by lentivirus infection, respectively. Huh-shLMBRD1 and HCVR-shLMBRD1 represent cells to which LMBRD1 gene has been knocked-down.

d. Western blot

The total protein was isolated from cells treated with RIPA lysis buffer (50 mM Tris-

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HCl, pH 7.5, 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, 1% SDS, 1% complete EDTA-free protease inhibitor). After preparing the protein samples by mixing 75-100 μg quantified proteins with optimal volume of protein sample dye (40% glycerol, 0.25 M Tris-HCl, pH 6.8, 8% SDS, 0.04% bromophenol blue, 20% β-mercaptoenthanol) and boiled for 5 mins, the protein samples were separated at 80 V by molecular weight using SDS poly-acrylamide-gel-electrophoresis (SDS-PAGE). The proteins were electro-transferred onto PVDF membranes at 250 mA for 80 min at 4 ℃. The membranes were then blocked by TBST buffer (Tris-buffered saline with 0.1% Tween 20) containing 4% non-fat milk at 25 ℃ for 1 hour and incubated with TBST buffer containing 4% non-fat milk diluted primary antibody at 4 ℃ overnight. Following washing with TBST buffer three times, the membranes were incubated with TBST buffer containing 4% non-fat milk diluted horseradish peroxidase (HRP)-conjugated secondary antibody at 25 ℃ for 1 hour. After washing with TBST three times, the bands were visualized by using enhanced chemiluminescence (ECL) reagent and the following autoradiography.

e. Quantification of viral RNA with reverse transcription-qPCR (RT-qPCR)

For analysis of HCV replication in cells, total RNA was extracted from cells using REzol. cDNA was synthesized using HCV specific primers and protoscript II according to the manufacturer's specifications. Quantitative PCR was performed using a SYBR green super mix. The following primer sets were used for PCR amplification of the HCV 5' -untranslated region (UTR): 5'-CTG GGT CCT TTC TTG GAT AA-3' (sense) and 5'-CCT ATC AGG CAG TAC CAC A-3' (antisense) for HCV-2a strain and the HCV NS3 coding region: 5'-CAT CAT CAC TAG CCT CAC AGG-3' (sense) and 5'-AGG TCC TGG TCT ACA TTG G-3' (antisense) for HCV-1b strain. Reaction mixtures were amplified for 40 cycles in a CFX Connect RT-qPCR machine thermocycler (Bio-Rad).

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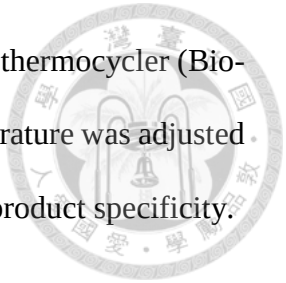
The results were analyzed with the CFX Connect RT-qPCR machine thermocycler (Bio-Rad), and normalized to GAPDH transcript levels. The melting temperature was adjusted to avoid quantification of nonspecific primer dimers and to enhance product specificity.



f. HCV cell culture system (HCVcc system)

For HCV propagation in cell culture, Huh7.5 cells were seeded at $1X10^6$ per 6cm dish. Then cells were transfected with plasmid pCMV-JFH1-RZ-PA. The next day changed the medium. Three days post-transfection, the culture medium was collected. The cells were all reseeded into a 10-cm dish, and the collected medium was added back into the dish. After 72 hours, all cells from the 10-cm dish were reseeded to three 6 cm dishes. To each dish, 3 ml culture medium were added. After 72 hours, medium containing HCV viral particles were collected and clarified by centrifugation for 5 min at 1000 rpm in an RS-240 rotor (Kubota). Meanwhile, the cell lysates were harvested to perform RT-qPCR and western blot analysis to confirm the expression of HCV genomic RNA and HCV NS5A protein, respectively.

g. Harvest and purification of HCVcc

The purification of HCVcc followed the procedure as previously described [59], with modifications. For intracellular HCVcc purification, the cells harvested from HCVcc-infected cells were resuspended in 1 ml PBS, frozen and thawed for three times and then was centrifugated at 1,000 rpm in an RS-240 rotor (Kubota) for 5 min. The viral-containing supernatant was collected and centrifuged at 36,000 rpm in an SW41 rotor (Beckman) for 6 hours at 4 °C. Total RNA of HCVcc was extracted from the pellet using Rezol for RT-qPCR. In order to purify extracellular HCVcc, the culture medium harvested from HCVcc-infected cells were centrifuged at 36,000 rpm in an SW41 rotor

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(Beckman) for 6 hours at 4 °C. Total RNA of HCVcc was extracted from the pellet using Rezol for RT-qPCR.

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IV. Results

A. Knocking down LMBRD1 gene reduced HCV replication.

Our previous study indicated that the level of HCV genomic RNA had no significant difference between Huh-shLMBRD1 and control Huh-shLUC cells after HCVcc infection for 1 hour [52]. It seemed that LMBRD1 gene might have no effect on the early stage of HCV infection. The potential effects of LMRBD1 gene on HCV replication were further investigated. LMBRD1 gene was knocked-down in HCV-1b subgenomic replicon cells (HCVR cells). The levels of LMBD1 RNA and the HCV anti-genomic RNA in HCVR were then measured by RT-qPCR. The data showed that the RNA level of LMBD1 was lower in LMBRD1-knockdown cells compared to the control HCVR cells (Fig. 1A). The level of anti-genomic RNA was significantly lower (Fig. 1B). The level of HCV NS5A protein was also lower in LMBRD1-knockdown HCVR. These suggested that LMBRD1 gene may participate in and positively regulate HCV replication.

B. Both LMBD1 and NESI expression in LMBRD1-knockdown cell rescue HCV replication.

There are two major proteins that are encoded by the LMBRD1 gene, namely, the nuclear export signal-interacting protein (NESI) and the LMBD1 protein. To figure out which gene product predominantly participates in HCV replication, the LMBD1 or NESI was over-expressed in HCVR cells individually to determine which protein could rescue the level of anti-genomic RNA in LMBRD1-knockdown HCVR cells. The RNA level of LMBD1 and the level of anti-genomic RNA in HCVR cells were analyzed by RT-qPCR. The data showed that the level of anti-genomic RNA was significantly increased when either the LMBD1 or NESI protein was over-expressed in LMBRD1- knockdown HCVR cells (Fig. 2). This indicated that both LMBD1 and NESI over-expression in LMBRD1

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knockdown cells could rescue HCV replication.

C. LMBRD1 gene expression might disrupt HCV replication in HCVcc-infected Huh7.5 cells.

To further investigate the effect of LMBRD1 gene in HCV life cycle, the LMBRD1-knockdown Huh7.5 cells were infected with HCVcc and then the level of HCV antigenomic RNA was detected to figure out whether LMBD1 and NESI play roles in HCV replication. In addition, to know the time course of HCV antigenome expression, total RNA was extracted from cells at different time points after HCVcc infection. The level of HCV anti-genomic RNA was measured. The data showed that HCV replication elevated at 6 hours after infection in HCVcc-infected Huh-shLUC cells (Fig. 3A). The elevation was also detected in HCVcc-infected Huh-shLMBRD1 cells but was much higher than the control HCVcc-infected Huh-shLUC cells (Fig. 3B). Because previous data already indicated that LMBRD1 gene has no effect on HCVcc attachment and entry [52], these results indicated that knocked-down LMBRD1 gene might help HCV replication in HCVcc-infected Huh7.5 cells.

D. Core expression in LMBRD1 knockdown cell increases the level of HCV antigenomic RNA.

The effect of LMBRD1 gene on HCV replication was different between HCV-1b subgenomic replicon cells and Huh7.5 cells infected with genotype 2a HCVcc. One possibility that caused the difference could be due to the lack of structural proteins in subgenomic replicon HCVR cells. To elucidate the possibility, core protein was overexpressed in HCVR-shLMBRD1 cells and then total cell lysate was harvested. Meanwhile, the level of HCV antigenomic RNA was measured. The data showed that the

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levels of HCV antigenomic RNA were significantly increased in both control HCVR-shLUC cells and HCVR-shLMBRD1 cells when core protein was overexpressed (Fig. 4A). It's worth to note that the level of HCV antigenomic RNA in HCVR-shLMBRD1 cells was much higher compared to the control. Moreover, the expression of viral non-structural protein was increased in the HCVR-shLMBRD1 cells when core protein was expressed (Fig. 4B). These suggested that expression of the viral core protein in LMBRD1 knockdown cell may have a temporal effect on replication and assembly. The observation may partially explain the finding of a higher levels of HCV RNA in HCVcc-infected Huh-shLMBRD1 cells when core protein is expressed.

E. LMBRD1 gene expression may contribute to the production of infectious HCVcc.

In order to figure out whether LMBRD1 gene participates in the late stage of HCV life cycle, the quantity of HCV particles was first investigated in HCVcc-infected Huh-shLMBRD1 cells. Here, the copy number of HCV RNA was used as a marker for the quantity of infectious HCVcc. LMBRD1-knockdown Huh7.5 cells infected with HCVcc-containing culture medium were harvested at 4 days post-infection. The genome copy numbers of intracellular particles from HCVcc-infected Huh- shLMBRD1 cells were analyzed by performing RT-qPCR. The data showed that there was no significant difference in HCV genome copy number between Huh-shLMBRD1 cells and control Huh-shLUC cells (Fig. 5A). In addition, the extracellular particles from Huh-shLMBRD1 cells infected with HCVcc-containing culture medium were also analyzed by performing RT-qPCR western blot analysis (Fig. 5B). The data showed that there was no significant difference in HCV genome copy number but the levels of E1 and core proteins of HCVcc-shLMBRD1 were higher in HCVcc-shLMBRD1 compared to the control HCVcc-LUC.

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These results indicated that LMBRD1 gene knockdown may have effect on HCV assembly, leading to an uncorrelation between the level of HCV genome copy number and the levels of HCV E1 and core protein in extracellular particles.

F. LMBRD1 gene might play a crucial role in HCVcc propagation.

To further investigate whether LMBRD1 gene affects HCV propagation without decreasing the virus titer, the HCVcc-shLMBRD1 from LMBRD1-knockdown HCVcc-infected Huh7.5 cells was collected for reinfection. After reinfection of naïve Huh7.5 cells, the total cell lysate was harvested at different time points for western blot analysis and the levels of HCV RNA were measured by RT-qPCR. The data showed a temporal change on the level of the HCV anti-genomic RNA (Fig. 6). In addition, genomic RNA increased in control cells, but not in the cells that were infected with HCVcc -shLMBRD1.

G. LMBRD1 gene knockdown might change the properties of HCVcc resulting in a reduced ability in cell-entry.

In order to figure out what makes the poor propagation of HCVcc-LMBRD1, the attachment ability and entry of HCVcc-shLMBRD1 were first investigated. For the study of viral attachment, naïve Huh7.5 cells were infected with the HCVcc- shLMBRD1-containing culture medium then incubated at 0 ℃. After the 15-min incubation at 0 ℃, the medium was washed away and the HCVcc-shLMBRD1 infected Huh7.5 cells were further incubated at 37 ℃ for 1 hour for viral entry. The levels of HCV genomic RNA were measured by performing RT-qPCR. The data showed that, at the attachment, the level of HCV genomic RNA harvested from the cells infected with HCVcc-shLMBRD1 was higher, but significantly reduced after incubation at 37 ℃ for 1 hour (Figure 7A). The levels of E1 protein, core protein, and NS5A protein were also examined by western

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blotting (Fig. 7B). The result showed that the non-structural protein NS5A expressed soon after viral entry in control cells, but not in cells that were infected with HCVcc-shLMBRD1. Furthermore, the level of core protein in HCVcc-shLMBRD1 infected Huh7.5 cells was significantly higher than the control cells at the stage of viral attachment. However, the level of E1 protein seemed to have no significant difference between control cells and cells infected with HCVcc-shLMBRD1.

H. The HCVcc-shLMBRD1 has poor replication and protein expression in naïve Huh7.5 cells.

In order to investigate the replication of HCV after viral attachment and entry, the naïve Huh7.5 cells were infected with HCVcc-shLMBRD1. A similar approach was taken but instead, viral RNA was extracted from the cells infected with HCVcc-shLMBRD1 incubated 37 ℃ for different times. The levels of HCV genomic and antigenomic RNA were detected at different time points (Fig. 8A). The data showed that the levels of HCV genomic and antigenomic RNA were both lower in cells infected with HCVcc-shLMBRD1. These data were similar to those shown in Fig. 6. The levels of HCV NS5A protein were also analyzed at different time points (Fig. 8B). The data showed that the expression of viral NS5A protein in cells infected with HCVcc-shLMBRD1 were constantly lower than the control. All the data suggested that knocking-down LMBRD1 gene might reduce the replication of HCVcc after viral attachment, having an effect on HCV propagation.

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V. Discussion

The life cycle of HCV is linked to the machinery of host cells, growing evidence supports the involvement of host proteins in the viral entry, assembly, and release, emphasizing the importance of targeting host proteins as a broad spectrum antiviral approach. Although models about the regulation of host factors in HCV life cycle have been proposed, the detailed mechanism remains to be defined.

The classical link between HCV lipo-viral particles and clathrin-mediated endocytosis has been demonstrated previously [33]. During clathrin-mediated endocytosis, adaptor and accessory proteins help to package transmembrane receptors and their bound ligands into clathrin-coated vesicles. These adaptor and accessory proteins mediate the formation of a curved clathrin lattice at the site of the membrane to be internalized [60]. An earlier study demonstrated that HCV entry may occur via clathrin-mediated endocytosis through the help of Eps15 and the large GTPase dynamin [61]. In addition, it's known that AP-2-clathrin-mediated receptors require specific trafficking proteins for endocytosis [62]. We demonstrated that LMBD1 protein serves as a specific trafficking protein of the insulin receptor and participates in clathrin-mediated endocytosis through its interaction with AP-2 [50]. By analyzing HCV antigenomic RNA level in LMBRD1-knockdown cells, we found that LMBRD1 gene products has little effect on the endocytosis of HCV [52]. Nevertheless, potential relationships between LMBRD1 gene products and the HCV life cycle after entrying host cells have not been defined.

In this study, we identified LMBD1 as a key molecule in regulating HCV replication and assembly and its association with viral infectivity. Two systems were applied to examine the effect of LMBRD1 gene expression in HCV life cycle. By using HCVR subgenomic replicon cells carrying HCV cDNA (1b strain) from NS3 to NS5B, positive

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regulatory effect of LMBD1 and NESI proteins in HCV replication were observed (Fig. 2). In addition, the level of HCV antigenomic RNA was higher when HCV core protein was overexpressed in HCVR-shLMBRD1 (Fig. 4), indicating that core protein expression may stabilize HCV replication. On the other hand, when HCVcc (2a strain) infection system was used, LMBRD1 gene knockdown showed a little effect on HCV replication but still produced an equivalent amount of HCVcc after initial HCVcc infection (Figs. 3 and 5). Nevertheless, the HCVcc produced from Huh-shLMBRD1 cells has poor infectivity, as evidenced by lower levels of genomic and antigenomic RNA detected in the viral entry study (Figs. 7 and 8). Taken together, these results emphasized the importance of LMBRD1 gene expression on HCV replication and assembly.

A. LMBRD1 gene knockdown inhibits HCV replication in HCVR cells.

In this study, we found that the level of HCV antigenomic RNA was much lower in LMBRD1-knockdown HCVR cells compared to the control cells (Fig. 1). The inhibition could be rescued both by overexpression of LMBD1 and NESI (Fig. 2). This indicates that LMBRD1 gene product LMBD1 and NESI might participate in HCV replication, having a positive effect. It has been known that HCV proteins are associated with a membranous web. The membranous web includes double-membrane vesicles (DMVs) appearing to be induced by HCV NS4B possibly in combination with NS5A [41]. Previous study suggested that NS5A recruits PI4KIIIa to the ER, where PI4P accumulates to enhance proper DMV formation and HCV replication [63]. It is possible that LMBD1 or NESI participates in the formation of DMV to help HCV replication. In order to demonstrate this hypothesis, further experiments such as the detection of interaction between LMBD1/NESI and replication complex by co-immunoprecipitation is required. In addition, the detection of DMVs formation in HCVcc-infected Huh-shLMBRD1 cells

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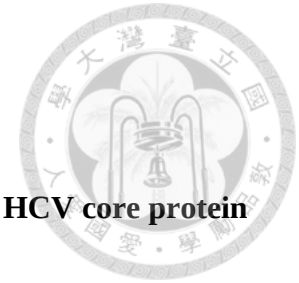
is also required.



B. LMBRD1 gene knockdown may have impact on the effect of HCV core protein in HCV replication.

In a previous study, we observed a higher level of HCV genomic RNA in HCVcc-infected Huh-shLMBRD1 cells compared to the control Huh-shLUC cells following an incubation with HCVcc for 2 hours [52]. This indicated that LMBRD1 gene expression may have effects on late stage of HCV infection. In this study, a slightly higher level of HCV antigenomic RNA in Huh-shLMBRD1 cells as compared to the control Huh-shLUC cells was detected over a period of 72 hours (Fig. 3). The phenomenon seems to conflict with the result shown in Fig. 1. There are two differences between the HCVcc infection system and HCVR subgenomic replicon cell system. First, HCVR subgenomic replicon cells could only express HCV non-structural proteins but not structural proteins including the core protein, E1 and E2 protein. In contrast, HCVcc carries HCV whole genome that expresses all viral proteins upon entrying cells. Previous studies suggested that structural protein expression may have effect on HCV replication [64, 65]. Indeed, in this study, an increased level of HCV antigenomic RNA was detected when the viral core protein was expressed in HCVR cells (Fig. 4). Interestingly, the increase was even more significant in HCVR-shLMBRD1 cells. In addition, our previous study demonstrated that the level of HCV antigenomic RNA was higher in Huh-shLMBRD1 cells compared to the control Huh-shLUC cells when transfected with a RNA representing HCV genome, but reduced while LMBD1 protein and HCV genome were coexpressed in Huh-shLMBRD1 cells [54]. These results may partially explain the conflict phenomenon between Fig. 1 and Fig. 3. It is possible that LMBD1 protein has a temporal effect on replication and assembly through the interaction with core protein. In order to demonstrate this hypothesis, further

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experiments are required. Another possibility that caused the difference could be due to the genotype. HCVcc infection system was established by using HCV-2a JFH1 strain whereas HCVR subgenomic replicon was established by using HCV-1b strain. It's known that genetic heterogeneity of HCV could account for some of the differences in disease outcome and response to treatment observed in HCV-infected persons [9, 66]. It's possible that the genetic heterogeneity causes the different phenomenon between Fig. 1 and Fig. 3. To elucidate this possibility, further experiments are required.

C. LMBRD1 gene expression may have no effect on viral release but impact on viral assembly, leading to a poor infectivity.

In this study, we used HCV genomic RNA copy number as an indication of the quantity of HCV. There were no significant differences on the copy number of intracellular and extracellular HCV genomic RNA between HCVcc-infected Huh-shLMBRD1 and the HCVcc-infected Huh-shLUC control cells (Fig. 5). Although LMBRD1 gene expression may help HCV replication (Fig. 1), the results shown here indicate that LMBRD1 gene expression may have no effect on the quantity of HCVcc produced in Huh7.5 cells. The quality of HCVcc-shLMBRD1 produced from Huh-shLMBRD1 cells was examined by analyzing the level of viral proteins in HCVcc-shLMBRD1. Data showed that the E1 and core protein were higher in HCVcc-shLMBRD1 compared to the control HCVcc-shLUC (Fig. 5B). It indicated that LMBRD1 gene expression may contribute to the production of infectious HCVcc. In addition, the quality of HCVcc-shLMBRD1 was further examined by analyzing the genomic RNA postinfection of naïve Huh7.5 cells. The result from viral entry study showed that when infected with HCVcc-shLMBRD1 for 1 hour, the level of HCV genomic RNA was lower compared to the control that infected with HCVcc-shLUC (Fig.

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7). Moreover, the level of E1 protein had significant difference between HCVcc-shLMBRD1-infected and control HCVcc-shLUC-infected cells. It's already known that E1 and E2 protein form heterodimer and are essential components for viral entry and fusion [17]. In addition, studies point out that E2 is the crucial protein for viral attachment and entry through the interaction with host cell receptor CD81 and SRB1 and its ability to the fusion of viral and cellular membranes [18, 67]. Our results suggested that LMBRD1 gene expression may have effects on viral assembly, resulting in a reduced ability in cell-entry. We hypothesize that LMBRD1 gene expression may impact on HCV assembly, changing the distribution of E1 and E2 protein on HCVcc. To elucidate this hypothesis, further experiments, including detection of E2 protein and investigation of the E2 protein movement during HCV assembly in Huh-shLMBRD1 cells, are required.

D. The replication of HCVcc-shLMBRD1 in cells is significantly less efficient.

Knowing that the efficiency of HCVcc-shLMBRD1 infectivity is much lower than the control HCVcc-shLUC, the following steps of HCVcc-LMBRD1 entry in naïve Huh7.5 cells were further investigated. The results showed that the levels of genomic and antigenomic RNA at different time points after HCVcc-shLMBRD1 infection were all lower (Fig. 6, Fig. 8A). The results also showed increases of viral protein NS5A and E1 in cells after 3 hours post-infection of control HCVcc-shLUC. This indicates a functional translation leading to the elevation on the levels of HCV genomic and antigenomic RNA at 6 hours and 12 hours post-infection. On the other hand, the levels of the viral protein and viral genomic and antigenomic RNA were all much lower in HCVcc-shLMBRD1 infected cells compared to control HCVcc-shLUC infected cells. We hypothesize that knocking-down LMBRD1 gene may have impact on the production of defective HCVcc that may have reduced ability of uncoating or replication.

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Taken together, this study discovered a novel function of LMBD1 in HCV life cycle. LMBD1 protein may help HCV replication and modulate the viral protein during viral assembly, leading to the production of HCV with efficient infectivity.

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V. Figures

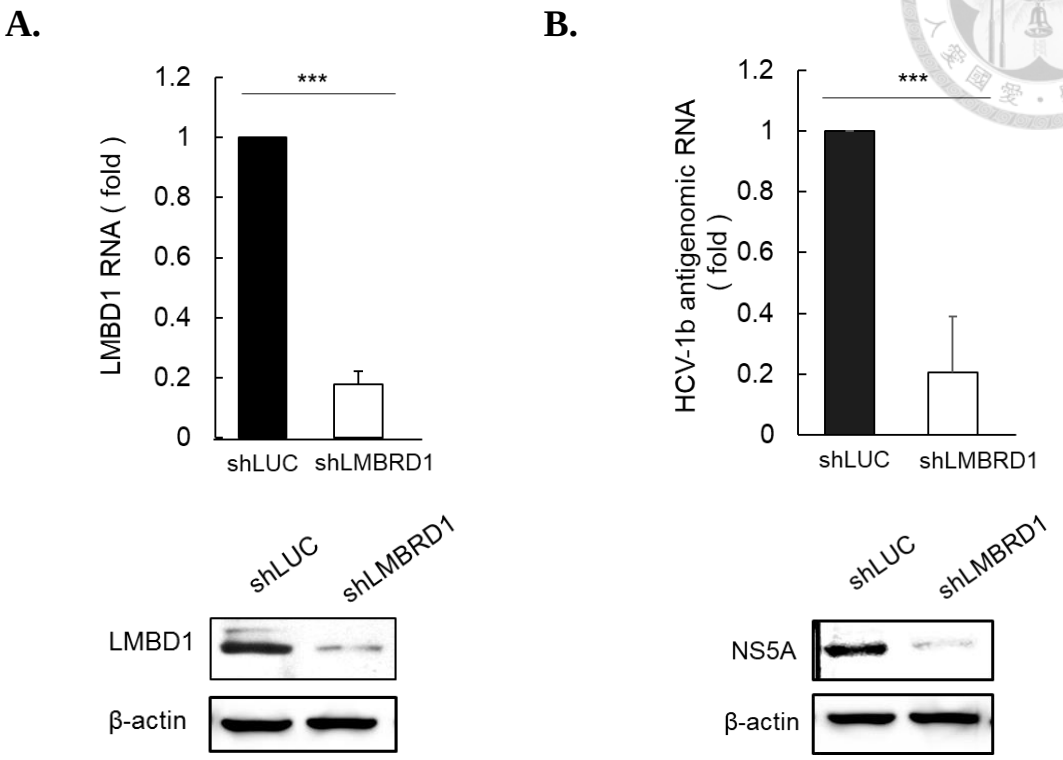

Figure 1. Knockdown LMBRD1 gene reduced HCV RNA replication and protein expression in the viral subgenomic replicon HCVR cells.

Cell lysate was harvested from HCV 1b subgenomic replicon HCVR cells 5 days after LMBRD1 gene knockdown for RT-qPCR and western blot analysis to detect the expression levels of LMBD1 RNA (panel A) and HCV antigenomic RNA (panel B) and the protein levels of LMBD1 (panal A) and NS5A (panal B), respectivly. Data shown represent the means plus standard deviations (error bars) from 3 independent experiments. P values define significant difference between the control HCVR-shLUC cells and HCVR-shLMBRD1 cells. ****P*<0.001.

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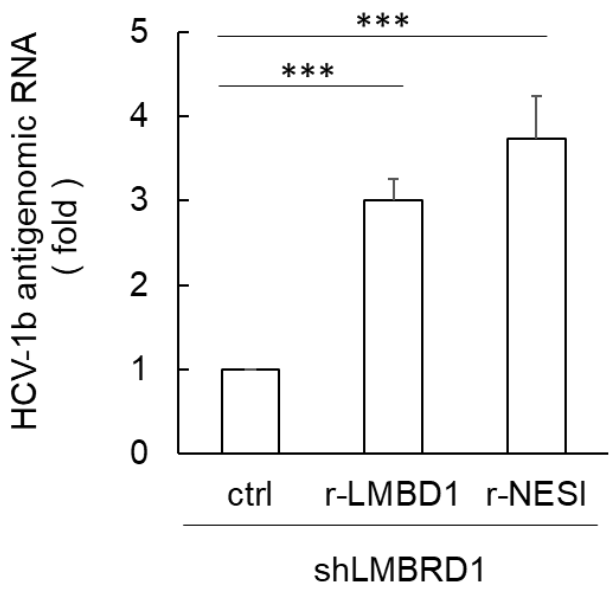

Figure 2. Both LMBD1 and NESI shRNA-resistant plasmid rescued the levels of HCV antigenomic RNA in HCVR-shLMBRD1 cells.

Seven microgrom of the plasmids representing control vector (ctrl), shRNA-resistant LMBD1 (r-LMBD1) and shRNA-resistant NESI (r-NESI) were independently transfected into HCVR-shLMBRD1 cells. After 72 hours, total RNA was extracted for detection of HCV-1b antigenomic RNA by RT-qPCR. Data shown represent the means plus standard deviations (error bars) from 3 independent experiments. P values define significant differences between the control cells and LMBD1-rescued cells, and between the control cells and NESI-rescued cells. ****P*<0.001.

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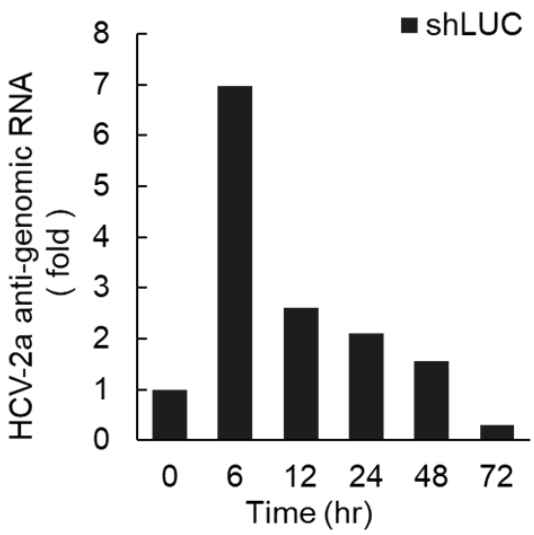

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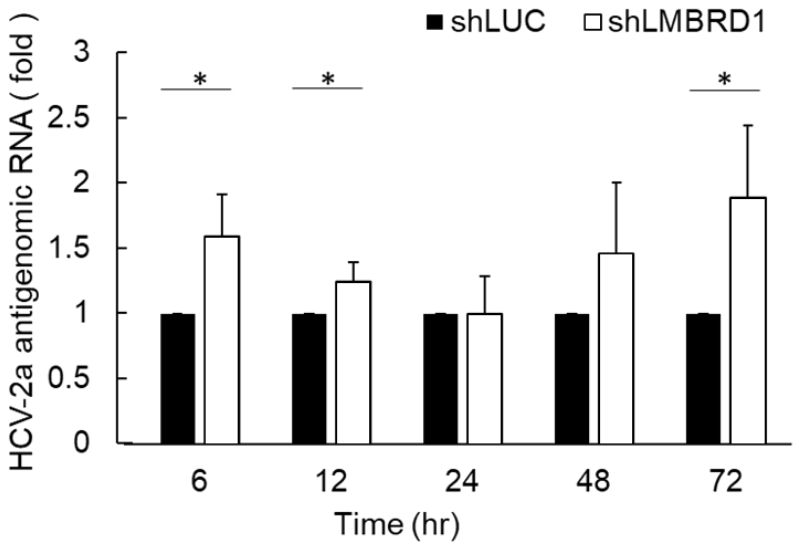

Figure 3. LMBRD1 gene expression might disrupt HCV replication in HCVcc-infected Huh7.5 cells.

A. The time course of HCV antigenomic RNA expression in HCVcc-infected Huh shLUC cells. Huh-shLUC cells were infected with HCVcc for 6 hours. After replacing with fresh medium, total RNA was extracted from the cells at indicated time points. The level of HCV antigenomic RNA were analyzed by RT-qPCR.

B. Knockdown LMBRD1 gene increased antigenomic RNA level of HCV in HCV-

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infected cells. The total RNA was extracted at indicated time points in HCVcc-infected Huh-shLMBRD1 cells. The level of HCV-2a antigenomic RNA was analyzed by RT-qPCR. Data shown represent the means plus standard deviations (error bars) from 3 independent experiments. P values define significant differences between the control cells (shLUC) and LMBD1-knockdown cells (shLMBRD1). *P<0.05.

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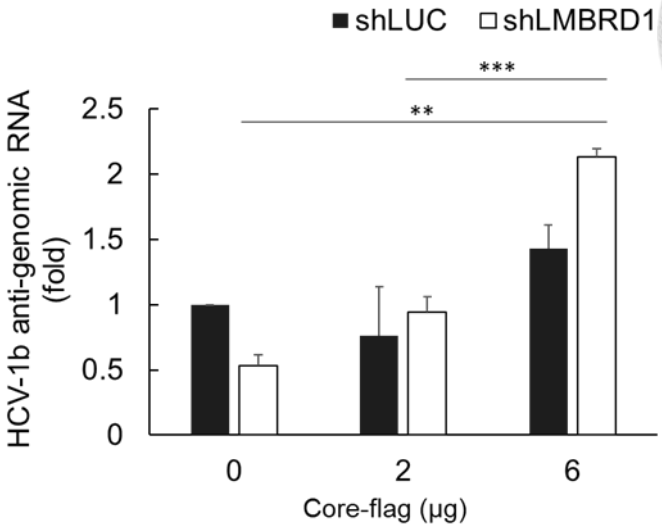

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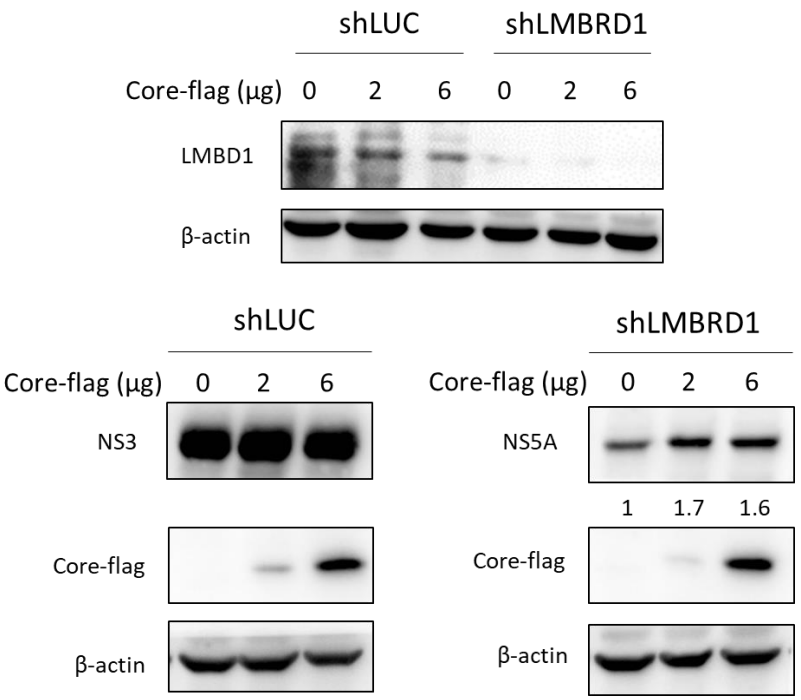

Figure 4. Core protein expression significantly enhances HCV replication in HCVR-shLMBRD1 cells.

The HCVR-shLMBRD1 cells were transfected with various amounts of the plasmid encoding the flag-tagged core protein for 48 hours. The total RNA was extracted for RT-qPCR to measure the level of HCV antigenomic RNA (panel A) and the total cell lysate

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was harvested for western blot analysis to detect the expression of HCV proteins (panel B). Data shown represent the means plus standard deviations (error bars) from 3 independent experiments. P values define significant differences between the control and core protein expressing in Huh-shLMBRD1 cells. $^{**}P<0.01$, $^{***}P<0.001$.

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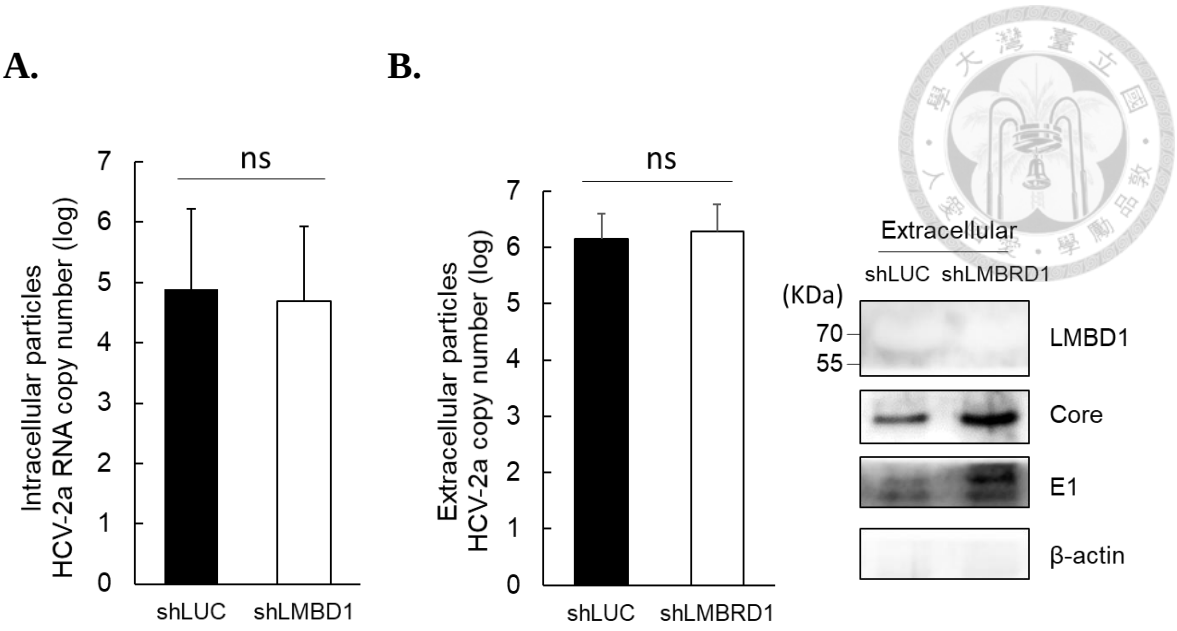

Figure 5. LMBRD1 gene expression may contribute to the production of infectious HCVcc.

The Huh-shLMBRD1 cells were infected with HCVcc for 4 days. The extracellular and intracellular HCVcc particles were collected from culture medium and cell lysate, respectively, by centrifuging at 36,000 rpm for 6 hours at 4 °C. The RNA was extracted from the intracellular particles for RT-qPCR and the copy number of RNA genome was calculated (Panel A). The extracellular particles for RT-qPCR and the copy number of RNA genome was calculated and proteins of particles were analyzed by western blotting analysis (Panel B). Data shown represent the means plus standard deviations (error bars) from 3 independent experiments.

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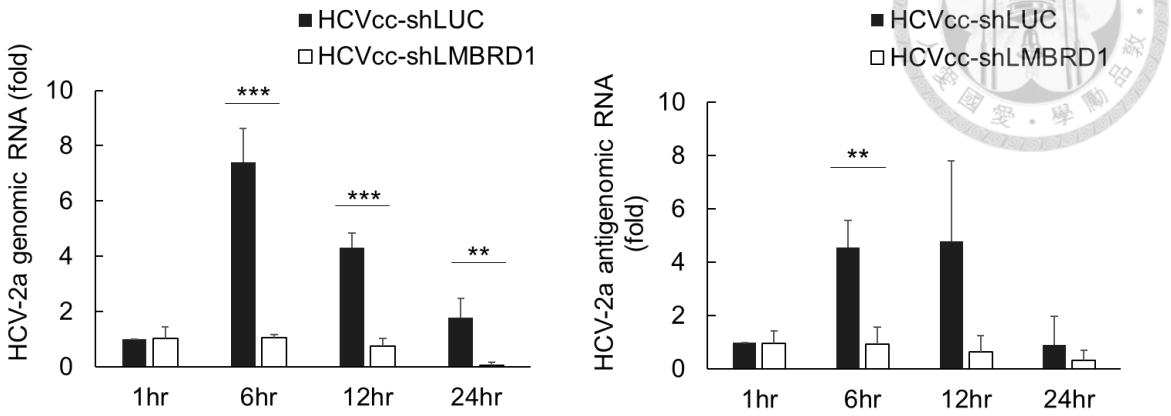

Figure 6. HCVcc produced in Huh-shLMBRD1 cells lost the ability to replicate in naïve Huh7.5 cells.

Culture medium collected from HCVcc-infected Huh-shLMBRD1 cells was used for reinfecting naïve Huh7.5 cells. Six hours postinfection, the medium was removed and total RNA was extracted at indicated time points. The HCV genomic and antigenomic RNA levels were analyzed by RT-qPCR. Data shown represent the means plus standard deviations (error bars) from 3 independent experiments. P values define significant differences between the HCVcc-shLMBRD1 infection and control HCVcc-shLUC infection. $^{**}P<0.01$, $^{***}P<0.001$.

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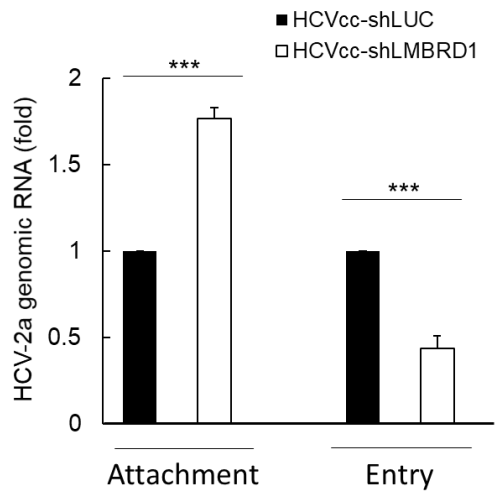

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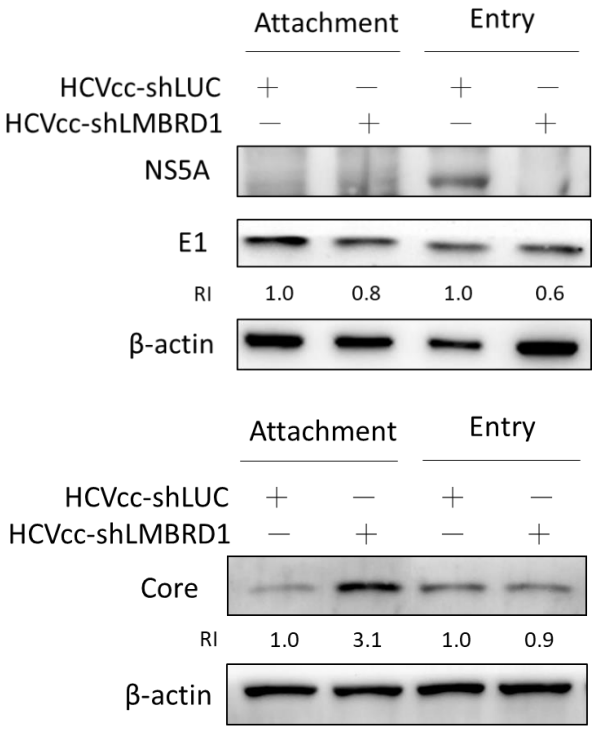

Figure 7. LMBRD1 gene knockdown reduced the ability of HCVcc in cell-entry.

Culture medium collected from HCVcc-infected Huh-shLMBRD1 cells was used for reinfecting naïve Huh7.5 cells. Naïve Huh7.5 cells were infected with indicated medium on ice for 15 min, then the medium was removed. The cells of the attachment group were

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harvested immediately. The cells of the entry group were harvested after a further incubation at 37 ℃ for 1 hour. The level of HCV genomic RNA was analyzed by RT-qPCR (panel A) and the HCV proteins NS5A, core and E1 were detected by western blot analysis (panel B). Data shown represent the means plus standard deviations (error bars) from 3 independent experiments. P values define significant differences between the HCVcc-shLMBRD1 infection and control HCVcc-shLUC infection. ***P<0.001. RI, relative intensity.

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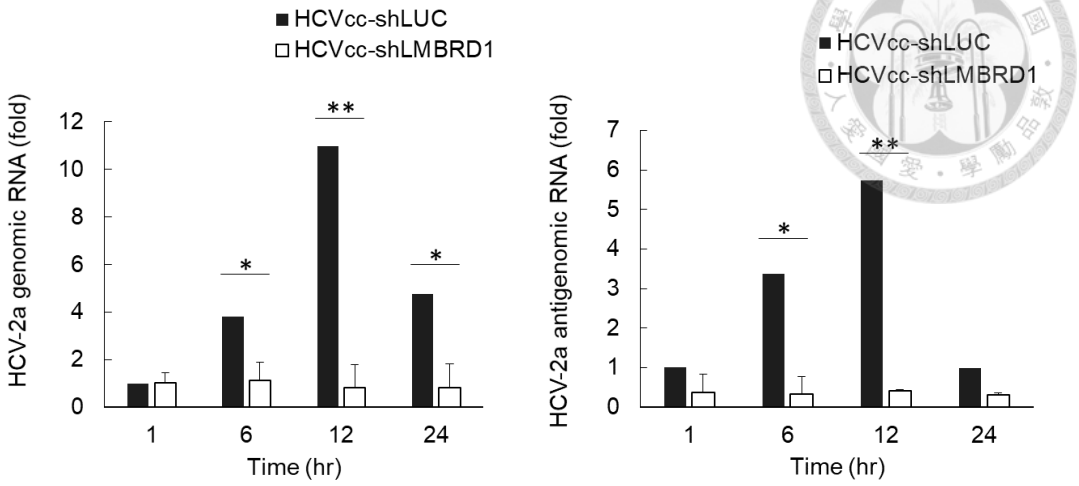
A.



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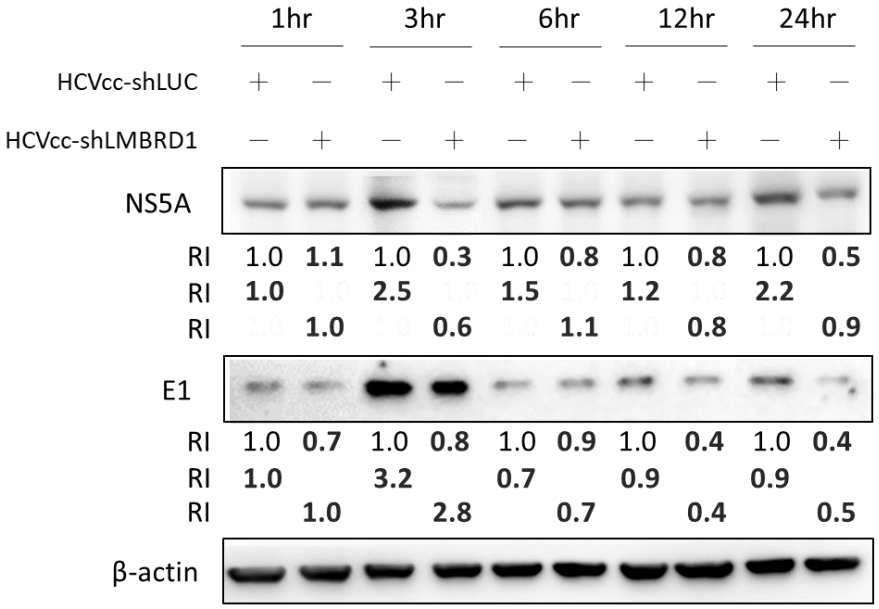

Figure 8. The HCVcc-shLMBRD1 has poor activity on genome replication and protein expression following incubating with naïve Huh7.5 cells.

Culture medium was collected from HCVcc-infected Huh-shLMBRD1 cells. Naïve Huh7.5 cells were exposed to the culture medium containing HCVcc-shLMBRD1 on ice for 15 min, then the medium was removed. After incubating at 37 ℃ for different time period, cells were harvested at indicated time point. The levels of HCV genomic and

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antigenomic RNA were analyzed by RT-qPCR (panel A) and the expression of HCV NS5A and E1 protein were detected by western blot analysis (panel B). Data shown represent the means plus standard deviations (error bars) from 3 independent experiments. P values define significant differences between the HCVcc-shLMBRD1 infection and control HCVcc-shLUC infection. *P<0.05, **P<0.01. RI, relative intensity.

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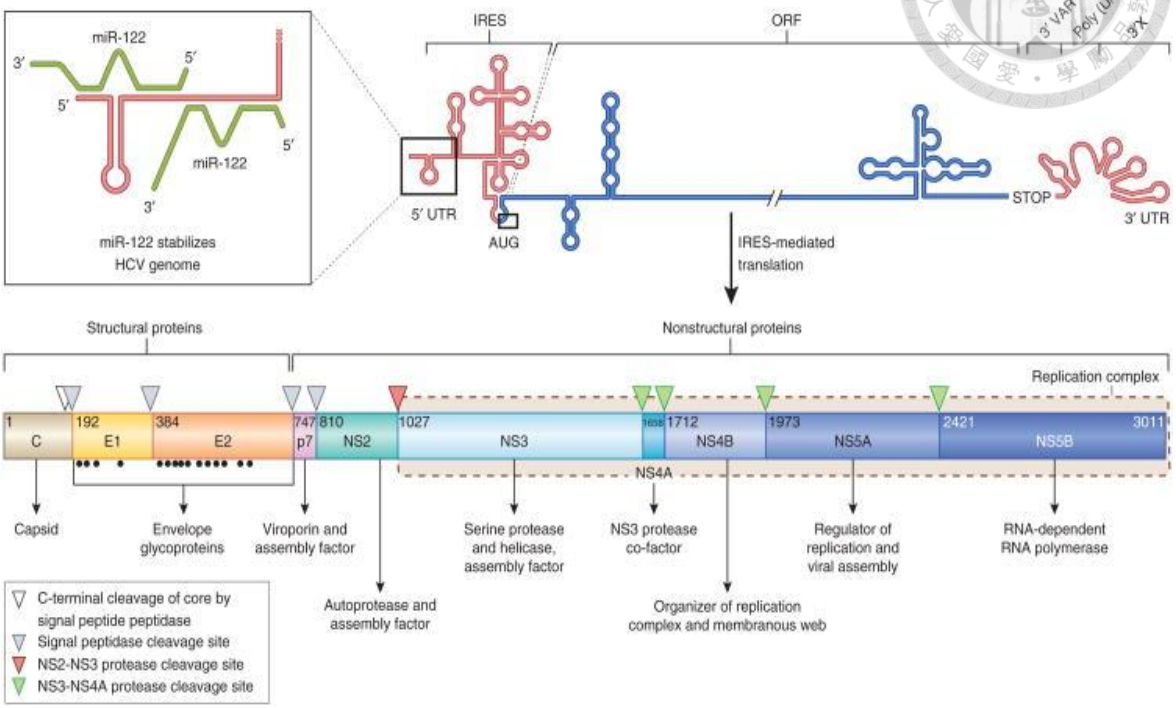
VII. Appendix Figures



Appendix Fig. 1 Genome organization of hepatitis C virus [9].

The HCV RNA genome (top) contains one long ORF (blue) flanked by 5′ and 3′ UTRs (red). The maturation process of the core protein involves a cellular signal peptide peptidase cleavage of a C-terminal signal peptide (white triangle) and cleavage from E1 by the cellular signal peptidase, which also cleaves E1, E2 and p7 from the polyprotein (gray triangles). In an autocleavage mechanism requiring two identical molecules to make up the composite active site, the NS2-NS3 protease cleaves itself (red triangle). The NS3 protease located in the first one-third of NS3, assisted by its membrane-bound cofactor, NS4A, cleaves the remaining proteins NS3, NS4A, NS4B, NS5A and NS5B (green triangles). Glycosylation of the envelope proteins (black dots) and the functions of the individual HCV proteins are indicated.

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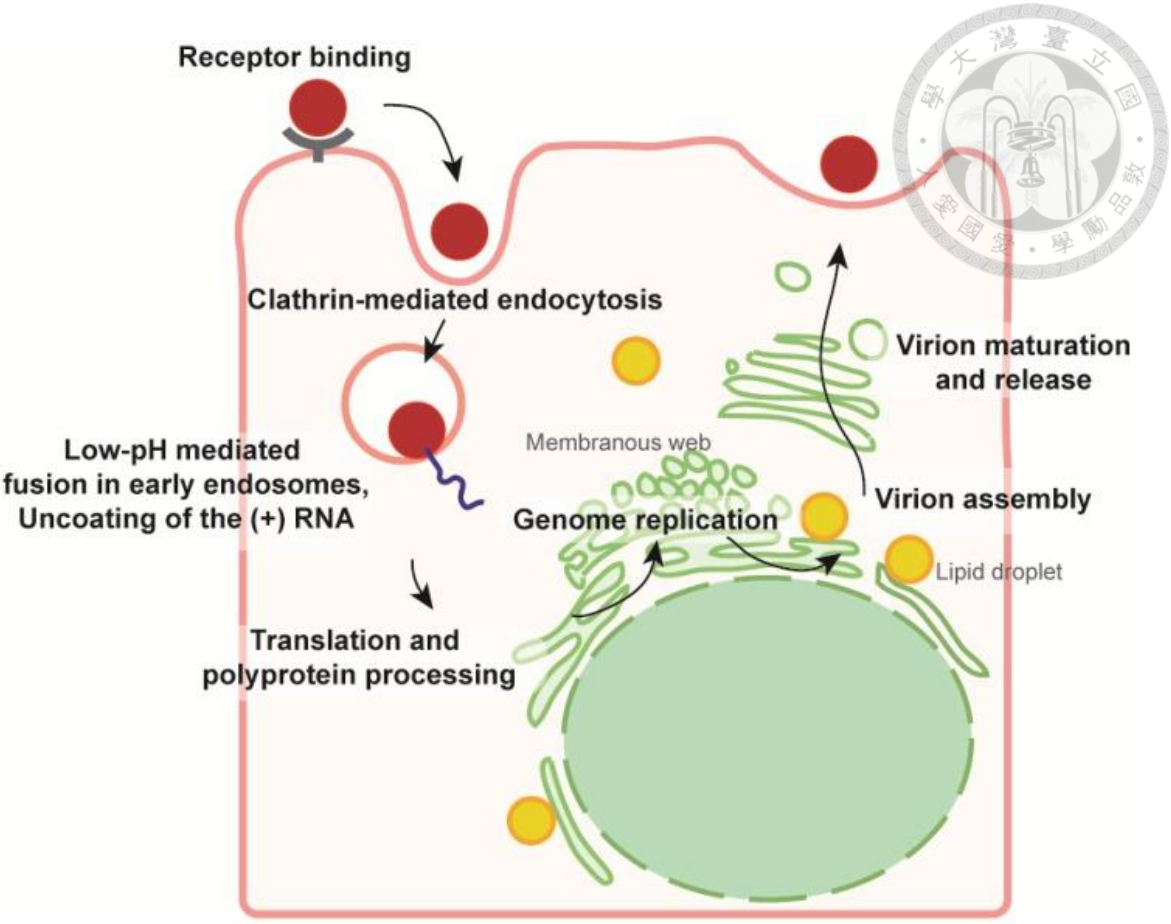

Appendix Fig. 2 The life cycle of hepatitis C virus [29].

The overview of HCV replication cycle in hepatocytes. The viral particle is represented by a red disk. Viral entry involves lots of receptors, occurring by clathrin-mediated endocytosis and terminates with fusion between viral and endosomal membranes. The released genome is translated into a single polyprotein, which is processed by host and viral proteases. Replication of the viral RNA occurs in membranous web. Assembly of new virions occurs at the ER membrane, but in close proximity to lipid droplets. Viral capsids bud within the ER lumen, travel through the Golgi apparatus and are released via the VLDL secretory pathway.

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