Open Access

OBM Genetics

LIDSEN Publishing Inc.

Original Research

Potential CRISPR-Cas9-based Antiviral Activity Against Hepatitis C Virus in Liver Cancer

Majid Monajjemi [1,*], Fatemeh Mollaamin [2], Ahmad R. Alsayed [3]

1. Department of Chemical Engineering, Central Tehran Branch, Islamic Azad University, Tehran, Iran; E-Mails: maj.monajjemi@iauctb.ac.ir; m_monajjemi@srbiau.ac.ir
2. Department of Biomedical Engineering, Faculty of Engineering and Architecture, Kastamonu University, Kastamonu, Turkey; E-Mails: fmollaamin@kastamonu.edu.tr; smollaamin@gmail.com
3. Department of Clinical Pharmacy and Therapeutics, Faculty of Pharmacy, Applied Science Private University, Amman, Jordan; E-Mails: a_alsayed@asu.edu.jo; a.alsayed.phd@gmail.com

* **Correspondence:** Majid Monajjemi; E-Mails: maj.monajjemi@iauctb.ac.ir; m_monajjemi@srbiau.ac.ir

Academic Editor: Rouben Aroutiounian

Special Issue: Pharmacogenomics and Precision Medicine: Unraveling the Future of Personalized Therapy

OBM Genetics
2025, volume 9, issue 1
doi:10.21926/obm.genet.2501287

Received: July 04, 2024
Accepted: February 19, 2025
Published: February 28, 2025

Abstract

Hepatitis C virus (HCV) is a primary global health concern, and though therapeutic options have improved, no very effective vaccine is available despite decades of research. The health and vitality of the organism are related to the result of homeostatic regulation of the internal environment. In contrast, it follows that disruption of homeostatic mechanisms leads to disease, particularly in liver cancer. Since HCV can rapidly mutate to evade the immune response, an effective HCV vaccine must rely on the identification and characterization of sites critical for broad immune protection and viral neutralization. In this work, we discussed the Hepatitis C virus (HCV) from the viewpoint of homeostasis due to its heterogeneous structure with numerous genomes and different recognized subunits

containing discrete segments. However, the interplay between HCV proteins during genomic RNA replication and progeny virion assembly is not completely understood. Here, we studied the dynamics and intracellular localization of non-structural 5A protein (NS5A), a protein involved in genome replication. HCV genome can encode several proteins with about 4,000 amino acids, each containing glycoproteins (E1 and E2) and seven non-structural (NS) proteins. E1 and E2 form a heterodimer complex on the virus structure, where E2 contains a receptor binding domain (RBD) that affects entry receptors. Since DNA double-strand breaks (DSB) often involve breaks in either foreign DNA strand, it can be repaired using NHEJ or HDR pathways. By this method, through enzyme endonuclease, site-specific segments of DNA are inserted, and then DNA repair mechanisms are used to close the DNA breaks, known as genetic scissors (CRISPR/Cas9). CRISPR/Cas9 technology is precise and rapidly reprogrammable depending on the experimental setup. Therefore, specific genomic loci can be activated or interfered with by CRISPR/Cas9 ribonucleic-protein (CRISPR or CREPR) interactions. This NS5A-APEX2 protein was fully visualized by fast transmission electron microscopy (TEM). These results exhibited hepatitis virus (HCV) replication during conjugated membrane vesicles containing replication complexes. Most studies on NS5A dynamics highlight the increased movement of NS5A in the absence of the nucleus, which could be related to altered nucleus-dependent activity associated with microtubules and/or dynein.

Keywords
Hepatitis C virus; CRISPR/Cas9; liver cancer; oncogenic signaling

1. Introduction

1.1 Hepatitis C Virus (HCV)

Hepatitis C is an infectious problem caused by the hepatitis C virus (HCV) that mainly affects the liver. Although during the initial infection event, occasionally there are no symptoms, subsequently; symptoms start with dark urine, abdominal pain, and yellow-tinged skin [1]. The virus remains in the liver and converts to a chronic infection that generally has no symptoms; of course, after several years, it becomes cirrhosis. Despite the approval of direct-acting antiviral products for various treatments, hepatitis C remains a serious health problem and is the reason for approximately 400,000 deaths each year [1]. The hepatitis C virus can encode a single leader protein, which is capable of viral proteases producing 15 polypeptides [2, 3]. Hepatitis C virus (HCV) is structurally heterogeneous, with numerous genomes and different recognized subunits containing discrete segments. HCV genome can encode several proteins with about 4,000 amino acids, each containing glycoproteins (E1 and E2) and seven non-structural (NS) proteins. E1 and E2 form a heterodimer complex on the virus structure, where E2 contains a receptor binding domain (RBD) that affects entry receptors. Moreover, E2 is the primary goal of neutralizing antigens [4]. Other proteins are known as non-structural (NS) forms, including NS2, NS3, NS4A, NS4B, NS5A and NS5B, and ferroporin [4] (Figure 1). Many of these NS macromolecules consist of optimal subunits

that are needed for HCV RNA translation and also create the NS3-NS5B repeat [5]. Among these structures, NS5B is an RNA-dependent polymerase that straightly controls the synthesis of RNA [6].

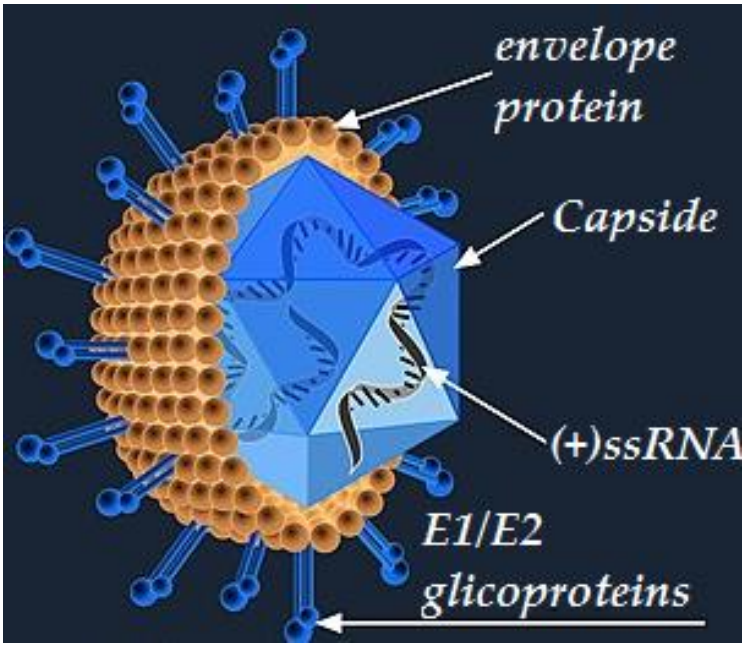

Figure 1 Structure of Hepatitis C.

Although any type of caring depends on genotype, it sometimes needs a combination therapy with direct-acting antivirals (DAAs), a safer and more effective treatment than previous interferon and ribavirin-based regimens [6-8]. HCV, like single-stranded RNA viruses, also affects Trans membrane sequences required for specialized translation by double and multi-membrane vesicles [9]. Although the advent of DAA treatment for HCV has led to a remarkable cure rate of over 75%, some challenges remain, such as the high cost of treatment and the potential for reactivation of hepatitis B virus (HBV) in people infected with HBV/HCV [7-9], as well as advanced liver diseases with a high rate of HCC recurrence [10, 11]. Nevertheless, its expression alone is insufficient to induce DMV formation [9] due to the necessity of other factors of the replication complex [12]. In addition, there is still no vaccine or preventive method to prevent hepatitis C virus infection, and at present, liver transplantation inevitably follows infection resulting from liver transplantation. Recent works on various aspects of HCV disease have been challenged by the lack of a robust HCV cell culture system. Therefore, recently, the expression of recombinant viral proteins and pseudo-lent viral particles was transcribed using glycoproteins. E2 systems have been studied [11] and similar systems [12].

Establishing a fully permissive HCV cell culture system [13] over the past decades following molecular cloning of the HCV genome has enabled new research and provided more data on the interactions between the virus and its host. NS5A is a poly-phospholipid protein for HCV genome replication [13-15], and this macromolecule is placed in the endoplasmic reticulum on the surface of the LD [14, 16]. Its presence at key sites of replication and synthesis, as well as its interaction with RNA, suggest an essential role for NS5A as the protein responsible for transporting newly synthesized RNA from its replication site (Figure 1).

1.2 Viral Glycan's Profiling

Viral envelope proteins of various pathogens are strongly glycosylated, and therefore, the viruses exploit the human cells to glycosylate their structures during translation and replication [17-20]. Glycan of HCV (E1 and E2) play various critical roles in viral RNA translation. [19] HCV proteins (E1 and E2) are highly N-glycosylated in their N-terminal domain for approximately one-third of the heterodimer mass. The N-glycosylation positions in many genotypes suggest that glycan plays an amazing role in HCV disease [18-23] (Figure 2). Moreover, of these common glycosylation positions, some more places have been discovered in this disease during the cell cultures [23-25], denoting that glycan permits the HCV to adapt more under this condition. In past decades, detailed evaluation of the HCV glycoproteins mechanism was complex due to the lack of an efficient cell culture method [25]. In past decades, recombinant expressed pocket glycoproteins or HCVpp were applied to study glycan and their function in HCV disease [26-30]. Although this knowledge considerably helped the understanding of HCV glycosylation, developing the accurate HCVcc system allowed for glycan analysis in HCV glycoproteins [28-34]. It should be noted that a significant difference in E2 glycol forms was observed in HCVpp and HCVcc systems.

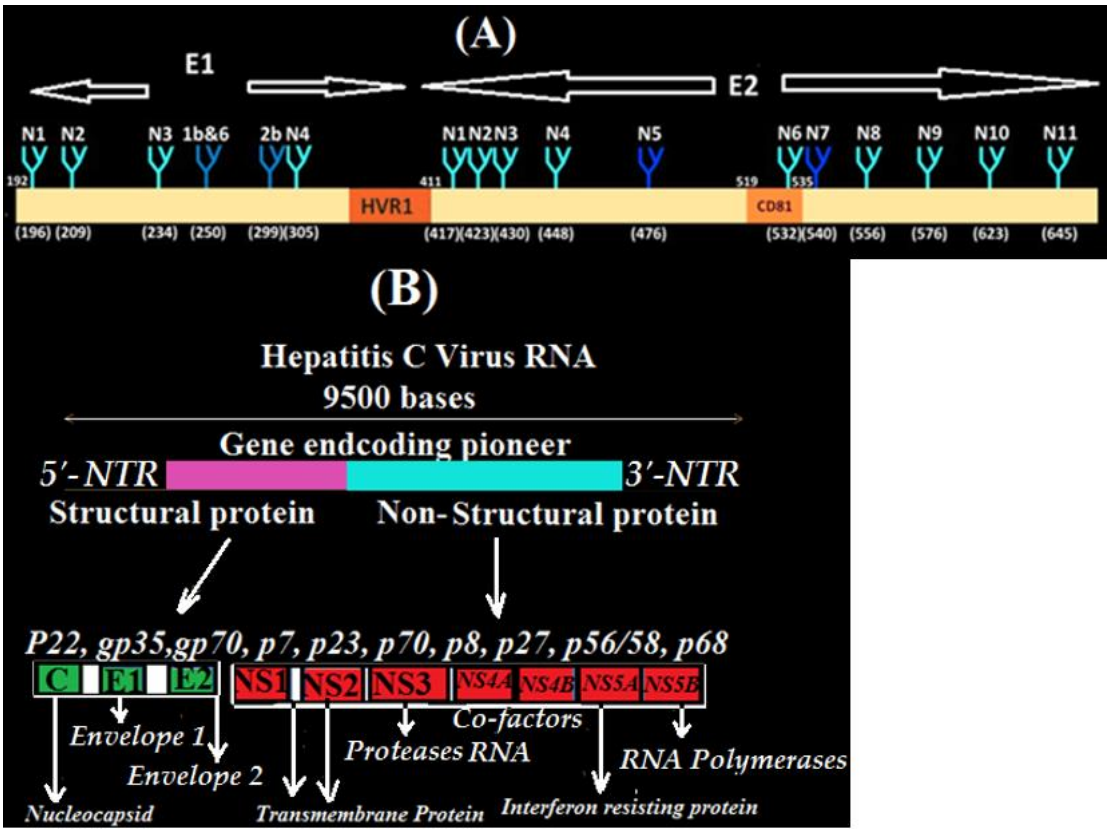

Figure 2 (A) Envelope protein including E1 and E2 according to various sites from sequences of PDB files. (B) Hepatitis C Virus RNA.

1.3 CRISPR/Cas9 Techniques

CRISPRs are found in many prokaryotes cells, containing most of the eubacteria. It consists of a series of repetitive sequences ranging from 24 to 47 bp, which are commonly called Direct repeats (DRs), divided by DNA sequences of equal length [35-38]. Although the root of the spacers is

unknown, several works have shown them as a part of foreign DNA, most of which are viral [39, 40]. CRISPR saves sequences of data about harmful mobile genetic parts in a unique queue and then uses this data to destroy the DNA or RNA of targeted cells, depending on the type of CRISPR [41, 42]. Each CRISPR row includes direct repeats by small sequences known as "spacers" that correspond to the DNA of early invaders [40]. Ishino et al. [43, 44] accomplished the first tests for 29-bp CRISPR that was repeated in 1987 in Escherichia bacteria [44-46]. A new pathway for gene rehabilitation was presented in biomedical research in 2013 for genome editing by CRISPR/Cas9 in cultured human cells successfully [47, 48]. By this method, through enzyme endonuclease, site-specific segments of DNA are inserted, and then DNA repair mechanisms are used to close the DNA breaks, known as genetic scissors [49]. Double-stranded DNA breaks can be created from different genetic engineering phenomena, containing meganucleases, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and CRISPR/Cas9 nuclease systems [50].

Consequently, DNA repairing rout with repair patterns or through the non-homologous joining (NHEJ) route are used to complement cellular DNA repairing in destroyed DNA [51]. Nowadays, the CRISPR/Cas9 genome editing methods have progressed as a strong genetic tool for human cell treatment [52] and also for killing disease viruses. Through growing scientific knowledge in gene editing, a new branch of medicine based on CRISPR/Cas9 editing technology is entering the clinical stage for treating virus disease [53]. The CRISPR/Cas9 system works sequence-specific by identifying and attaching foreign DNA or RNA. The defense mechanism is accomplished in three steps: The first step is adaptation, the second is crRNA synthesis, and the third step is targeted intervention. By this action, a primary sequence is segregated from the destroyed DNA from the CRISPR primary array, and then a new spacer is created for a proper gen [54]. This process demonstrates the adaptability of the immune system and allows the host organism to retain genetic material from invaders [55]. The CRISPR pattern is translated into long precursor cr-RNAs (pre-cr-RNAs) that are processed into mature guide crRNA that carry the stored sequences of the invaders to enable immunization. In the final step, i.e., the intervention stage, which is the immunity stage, Mature RNAs interfere with invading nucleic acids [56-70].

The history of the initial events of CRISPR/Cas9 is listed in (Table 1).

Table 1 History of revolutionary events of CRISPR/Cas9.

Year	Revolutionary Events	References
(1987)	It was in the Escherichia coli genome	[44]
(1989)	First application of gene transfer in humans	[71, 72]
(2002)	Identification of the Cas gene and proposed name for CRISPR. CRISPR version	[73]
(2007)	S. thermophilus provides the first test of adaptive CRISPR	[74]
(2008)	Identifying mature CRISPR RNAs (crRNAs) in E. coli	[75]
(2009)	The CRISPR/Cas9 systems are used to investigate the antiviral capabilities of Pyro coccus furiosus. Identification of the Cmr type III-B ssRNA complex	[76]
(2010)	Identification of a bacterial immune CRISPR/Cas cleavage at position 3 nucleotides upstream of the PAM sequence.	[77]
(2011)	Transient activation of CRISPR RNA (tracrRNA) was detected.	[78]
(2012)	The CRISPR operating system is compatible with sgRNA.	[79]

(2013)	Successfully alteration of the genome in eukaryotic cells using Cas9.	[80, 81]
(2015)	CMR complex crystal structure; Use of CRISPR/Cas9 in human embryos.	[82]
(2016)	The National Institutes of Health has approved the first human CRISPR gene editing trial.	[83]
(2017)	Discovery of a specific CRISPR protein (CRISPR/Cas13) that prefers to target RNA to DNA.	[84]
(2019)	The first in vivo clinical trials of CRISPR for managing blindness in human cells in the United States with Cas12a orthologs demonstrate editing capabilities.	[85]
(2020)	Nobel Prize for CRISPR/Cas9 genome editing.	[86, 87]

1.4 A CRISPR-Cas–based Platform for Detection of HCV

Various genomes of HCV are generated due to the viral resistance to antivirals and vaccines. Finding and diagnosing the virus is related to accurate therapeutic intervention. Since techniques known as immunoassay play a significant role in the diagnosis of HCV, quick testing is needed to identify individuals' HCV, especially in molecular testing. Therefore for removing this problem investigation of a new molecular docking approaches for the rapid detection of HCV RNA settings. Kham-Kjing et al. [88] studied a fast and sensitive method for the detection of HCV RNA, which is based on a reverse transcription loop-mediated isothermal amplification (RT-LAMP)-coupled CRISPR–Cas12 system for the detection of HCV genome sequences. Results after reactions can be seen and measured with a fluorescence detector. When tested on clinical samples from individuals infected with HCV from healthy donors, the CRISPR-Cas12 assay containing RT-LAMP compared to the reference method, Roche COBAS AmpliPrep/COBAS TaqMan HCV Test, showed 96% sensitivity. This test enables to detect HCV RNA concentrations as low as 10 ng/μL (Table 2) [88]. These overall studies proved the potential of the CRISPR/Cas system as an effective diagnostic tool for viral hepatitis infections.

Table 2 Applications of CRISPR/Cas system in viral hepatitis detection.

Types of Hepatitis	Platform Name	Cas Protein	Amplification Methods	Visualization	Sensitivity	Times	References
HBV	CRISPRHBV	Cas12b	MCDA	Fluorescence	10 copies/μL	60 min	[89]
HCV	Cas12a-based	Cas12a	LAMP	Lateral flow test	1 copy/μL	20 min	[90]
HCV	CRISPR–as12	Cas12a	RT-LAMP	Fluorescent/	10 ng/μL	20 min	[91]

2. Materials and Methods

2.1 Docking Simulation

BIOVIA-2020's docking software, Chem-3D, Hyper-Chem, Visual Molecular Dynamic (VMD), and Chemistry Harward molecular mechanic (Charmm) software have been used for all molecular minimization and Gibbs energies calculation from molecular docked. Molecular docking methods

have been accomplished to distinguish how the mutations bonded the several ligands to the Hepatitis C virus (HCV). We are looking to understand the mechanism of HCV, which may assist us in investigating more potent inhibitors against the Hepatitis C virus without destroying the structure. We designed and docked several ligands to the Hepatitis C virus through various mutations with 4xvj: pdb [88] Figure 3 and Table 3.

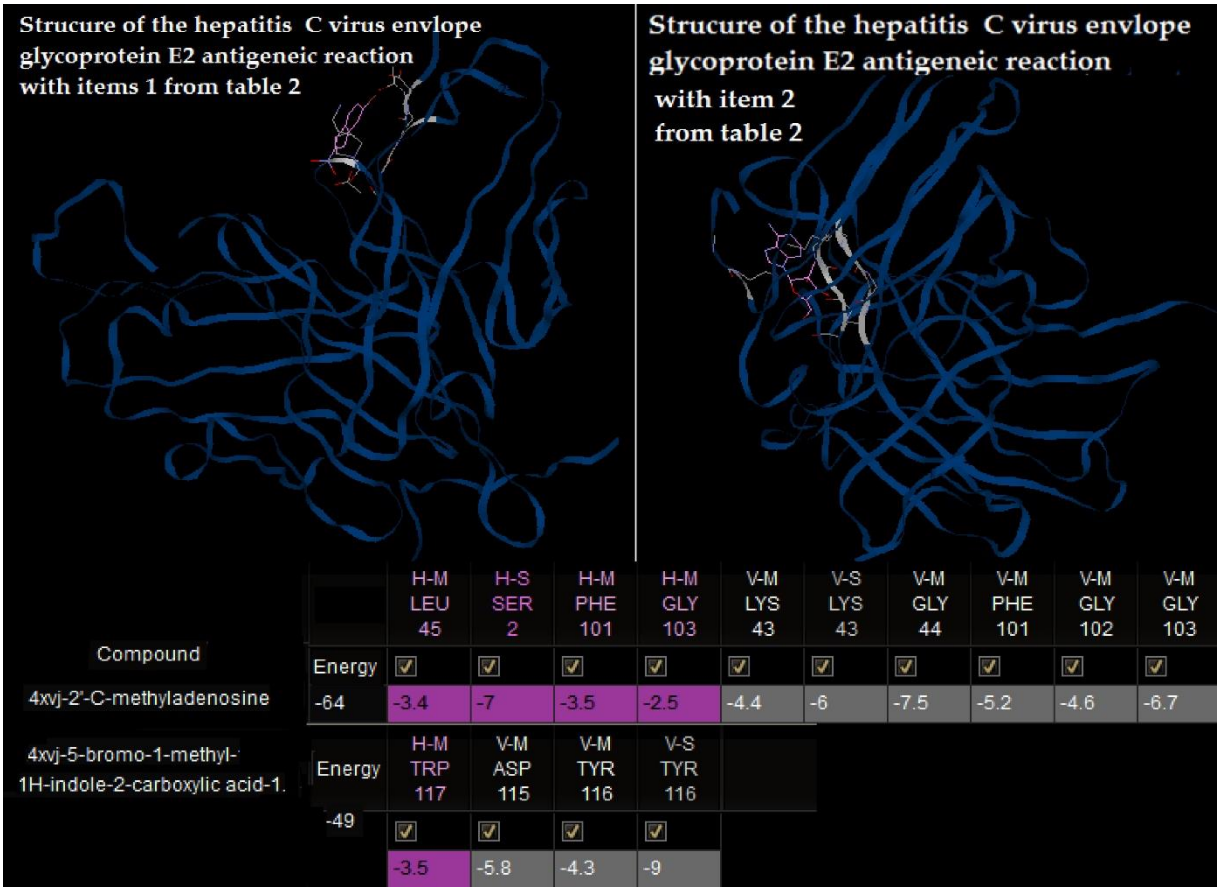

Figure 3 Docking image of 5-bromo-1-methyl-1H-indole-2-carboxylic acid and 2'-C-methyl adenosine molecules inhibitors with 4xvj.

Table 3 Docking calculation data of interaction of two small molecules inhibitors.

Items	ligands	Energy	VDW	H-bond	Docking fitness
1	5-bromo-1-methyl-1H-indole-2-carboxylic acid	-44.27	-37.76	-5.24	-44.12
2	2'-C-methyladenosine	--69.67	-51.08	-8.59	-55.67

2.2 RNA Transcription and Electrophoresis

After linearization of the plasmids described in the previous paragraph using XbaI and treatment with Mung Core as described in reference [8], the RNA can be transcribed in vitro using MEGA-script T7. Kit (Life Technologies, Carlsbad, CA, USA). Huh-7 cells were electrophoresed using a 0.2 cm gap with 10 µg of RNA as previously described [89-93]. Bowl (Biorad, Hercules, California, United States). Meanwhile, the antibodies and reagents Monoclonal antibody (mAb) A4 against

HCV E1 were produced in vitro using a Mini-Perm device (Heraeus, Hanau, Germany) according to the manufacturer's protocol.

2.3 Transcriptional Regulations

The two endonuclease domains of the Cas9 protein, HNH and RuvC, partition the target sequence site into DNA strands, respectively, after binding to foreign DNA [62]. DNA double-strand breaks (DSB). The resulting form of cleavage often involves breaks in either foreign DNA strands and can be repaired using NHEJ or HDR pathways [94, 95]. By adding a donor pattern, a second repair can be started to make the necessary insertions in the target gene. Therefore, theoretically, the CRISPR/Cas9 system can modify any DNA target site, including a PAM motif, by altering the guide RNA sequence [96]. CRISPR/Cas9 technology is precise and rapidly reprogrammable depending on the experimental setup. Genomic loci can be interfered with by CRISPR/Cas9 ribonucleic-protein (CRISPR or CREPR) interactions. CRISPR activation is a form of CRISPR in which the catalytic processing entity Cas9 is incorporated into a transcriptional effector molecule to alter the transcription of target genes. In one case, the g-RNA and the effector's arm are Trans located to a specific genomic site, dCas9 cannot leave, and the effector leads to transcription of downstream genes (Figure 4). Consequently, HCV E2 glycoprotein exhibits a fewer shielding phenomenon of the non-antigenic in E2 (Figure 5).

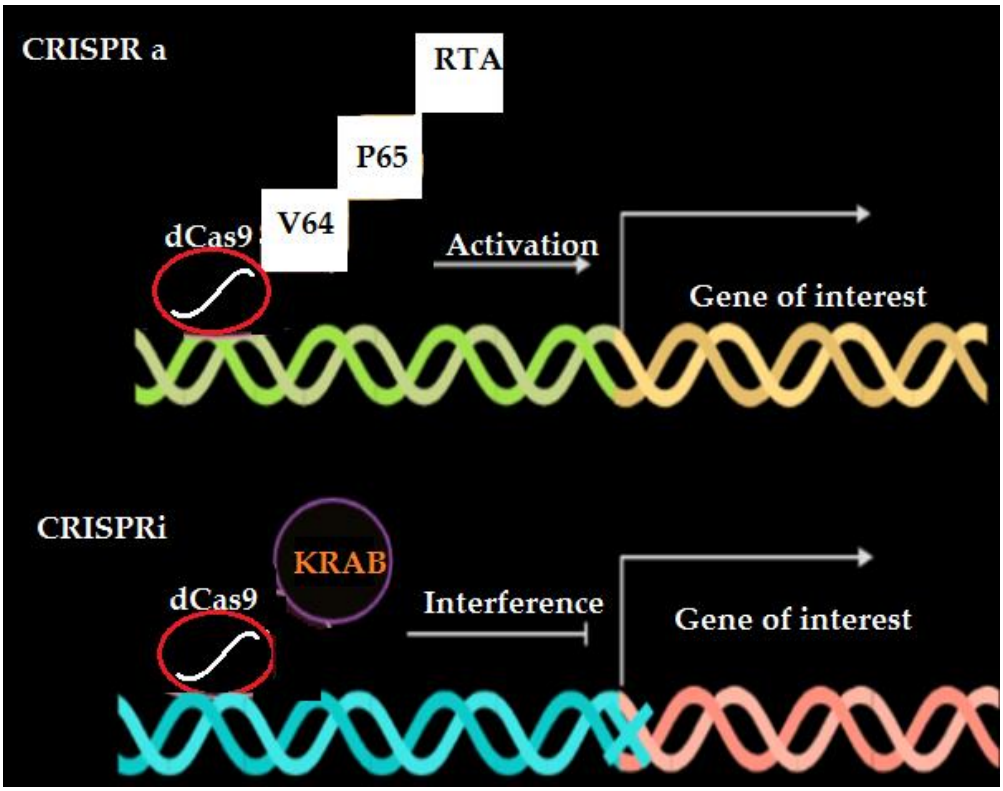

Figure 4 Crisper/Cas9 method for Hepatitis C Virus Infection.

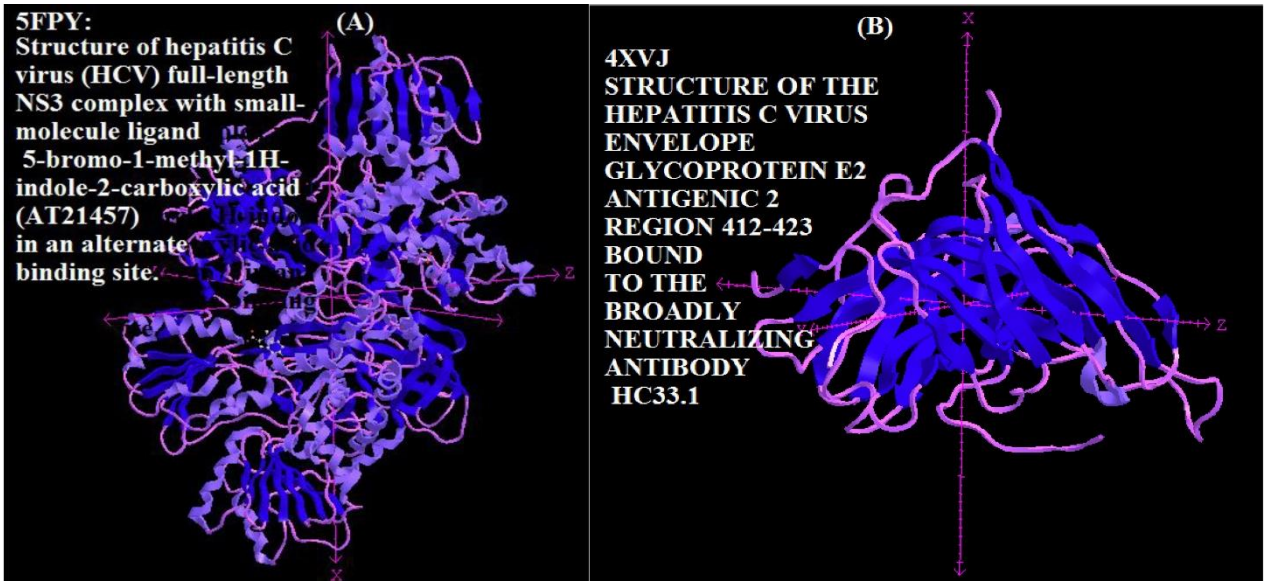

Figure 5 HCV E2 glycoprotein (left), there are fewer shielding phenomena of the non-antigenic in E2 (right).

This is ambiguous and needs further investigation. Another strategy for engineering glycan-linked epitopes is hyper glycosylation, which has been used to mask the epitopes involved in non-neutralizing antibodies and direct the immune response to the desired epitopes. Reduction of interfering antibodies can lead to the appearance of reciprocal genotype neutralizing activity, implying that hyper glycosylation may be an effective strategy to mask non-neutralizing/interfering antibody epitopes in HCV. In a recent study, hyper glycosylation was used to mask E2 antigen domains bound to non-neutralizing antibodies, but no increase in neutralizing antibody titer was observed due to the hyperglycosylation mutation. Altogether, targeting viral or cellular glycan offers new perspectives to prevent HCV infection through antiviral prophylaxis or vaccine development (Figure 6 and Table 4, Table 5) [97-99].

Table 4 Experimentally determined structures of E1, E2, and monoclonal antibodies.

X-ray pdb	Glycoprotein	Antibody	Residue range	Ref.
4N0Y	E1	IGH526	314-324	[100]
4MWF	E2	AR3C	421–645	[101]
4web	E2	2A12	486-645	[97]
5FGB	E2	HC33.4	417-421	[45]
5KZP	E2	HCV1	412–423	[17]

Table 5 Gene of Interest for Cas9 and docking fitness.

ligands	X-ray pdb	Energy	VDW	H-bond	Docking Fitness	Gene of Interest for Cas9
propane,1,2,3 triol	4N0Y.pdb	-39.05	-19.32	-19.72	-38.50	5'-CGCCACATGCATGCAAGCTGAC-3'
(2R,3S,4R,5S)-5-((1-hydroxyethyl)amino)-2-(hydroxymethyl)tetrahydro-2H-pyran-3,4-diol	4MWF.pdb	-45.65	-16.34	-12.86	-44.76	5'-CCCCAGAGGAAGAGGAGAGACAGTGGGTCTGAGCGAGAGC-ACC-3'
(2S,3S,4R,5S)-5-(((R)-1-hydroxyethyl)amino)tetrahydro-2H-pyran-2,3,4-triol	4WEB	-51.65	-17.98	-15.94	-50.05	5'-TCAGAATCTGGCCGGCCGAACTACAAGAATGAACGA-3'
5-bromo-1-methyl-1H-indole-2-carboxylic acid	5FGB	-46.32	-33.75	-8.21	-45.11	5'-AGGTTTAAACTCATTGTTTCATTTGAGAACTCGC-3'
2'-C-methyladenosine	5KZP	--58.61	-50.18	-7.56	-56.63	5'-CTGTGAGGAACTACTGTCTT-3’

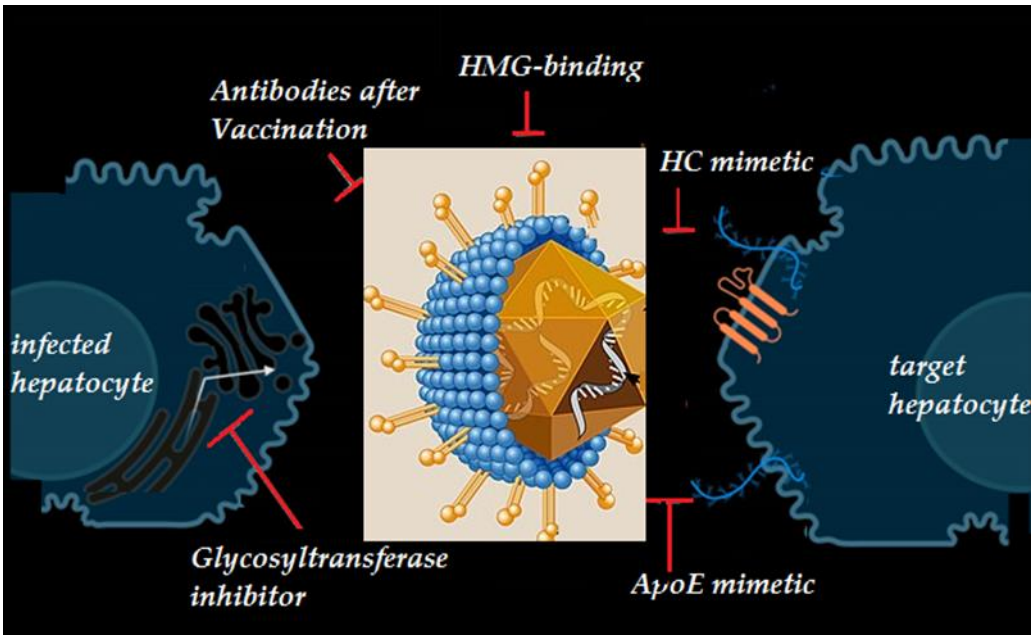

Figure 6 Inhibitors action versus HCV based on subunits challenging.

2.3 Experiments

The RNA oligonucleotides were from Dharmacon Research, Inc. (Boulder, CO). DNA primers for PCRs were from Integrated DNA Technologies. The genotype preparation was described based on reference 102 [102]. Most NS5A deletion mutants were cloned by PCR amplifying HCV-5A was used to clone NS5A by a sequence of 5'--GCG-GGT-ACC-AAG-CTT-CTA- TTA GCA GCA GAC GAC GTC CTC ACT-3. At first, the cells were grown at 37.8°C and then at 25°C for 24 h. The cells were harvested by centrifugation in a Beckman JLA-16.250 rotor at 6,000 rpm for 20 min, washed once in 300 ml T10E1 (10 mM Tris [pH 8.0], 1 mM EDTA), and centrifuged several times. The typical yield was 15 grams of cell paste per liter of culture. The cells were suspended in lysis buffer (100 mM potassium phosphate [pH 7.4], 400 mM NaCl, 12 mM -mercaptoethanol [BME], 15% glycerol, 10 mM imidazole, 2 μg/ml pepstatin A, and 0.5 μg/ml leupeptin) supplemented with protease inhibitor cocktail tablets (Roche). Phenyl methyl sulfonyl fluoride (PMSF) was added to a final concentration. The extract was centrifuged in a Beckman JA-30.50 rotor for half an hour at 30,000 rpm, and then the collected fractions were assayed for purity by SDS-PAGE. The cleaved NS5A protein was diluted to 100 mM NaCl and passed through a Q-Sepharose column. The protein was then loaded onto an S-Sepharose column and concentrated by stepping the protein off in 1-column-volume fractions of buffer B containing 1 M NaCl. The concentrations of NS5A proteins were determined using Sypro Ruby protein gel stain (Molecular Probes) and proteins were around 90% pure. NS5A-APEX2 protein was fully visualized by fast transmission electron microscopy (TEM), which sections were observed with a JEOL model TEM (TEM; JEOL, model no. 1200 TEM, operating at 80 keV).

3. Results

To evaluate the effect of the nucleus on NS5A dynamics, we produced a JFH-1 sub-genomic in which NS5A was tagged with e-GFP. According to published data, to transfer this marker to a wild-

type virus, domain II of NS5A was introduced, which allows HCV assembly [26]. Therefore, comparing the level of replication of this replicon with the corresponding uncharacterized level confirmed that the insertion of e-GFP in NS5A did not inhibit HCV replication (Figure 7 A &B). Then, the NS5A dynamic was analyzed with these two viruses by video microscopy. As shown in Figure 7-B, the absence of the nucleus strongly increased the mobility of NS5A. According to this information, we explained why the absence of a core allows NS5A to move faster. Notably, interaction with the nucleus affects NS5A recruitment to lipid droplets (LDs. Indeed, deletion of this protein by HCV shows a significant reduction in the presence of NS5A in LD [14].

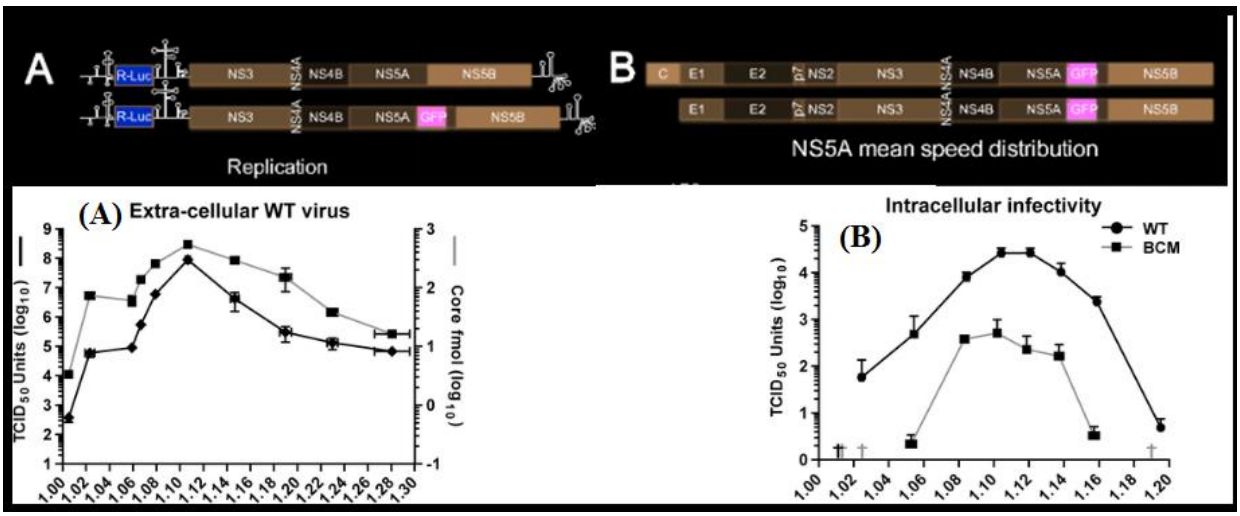

Figure 7 Analyze the effect of the nucleus on NS5A dynamics.

Due to less localization of NS5A in LDs, it was identified whether these polypeptides could be associated with replication mechanisms in the core virus context. Therefore, we immunologically demonstrated NS3, other marker replication positions [27], and measuring of the co-localization these polypeptides with NS5A, have been also estimated. Deleted viruses exhibited a fantastic reduction in NS5A-NS3 localization (Figure 8 A, B). To understand whether NS5A is less associated with replication systems in the core virus, we measured double-stranded RNA as an intermediate between DNA and NS5A. Consequently low Pearson coefficients were calculated, indicating that only a tiny fraction of NS5A signals overlapped with double-stranded RNA (ds-RNA). No statistical differences were yielded between wild-type (WT) and nuclear viruses (Figure 8 C, D). Transcription assays were accomplished using NS5A-like viruses. Labeled with Gaussian luciferase instead of e-GFP, there was no difference in amplification efficiency (Figure 8 E). Therefore, the decrease in localization between NS5A and NS3 indicates a reduction in subsets of these two proteins that are not co-expressed. It is involved in RNA replication. It should be noted that, unless otherwise stated, experiments were performed 48 hours after electroporation, a time frame that allows for more than one round of HCV to be replicated. Since nucleation is incompatible with the propagation of infectious viral strains [14], the total number of proliferating cells is equal. They are expected to be more abundant in the wild type than in the original virus, which generally leads to more significant aggregation of viral proteins, as documented.

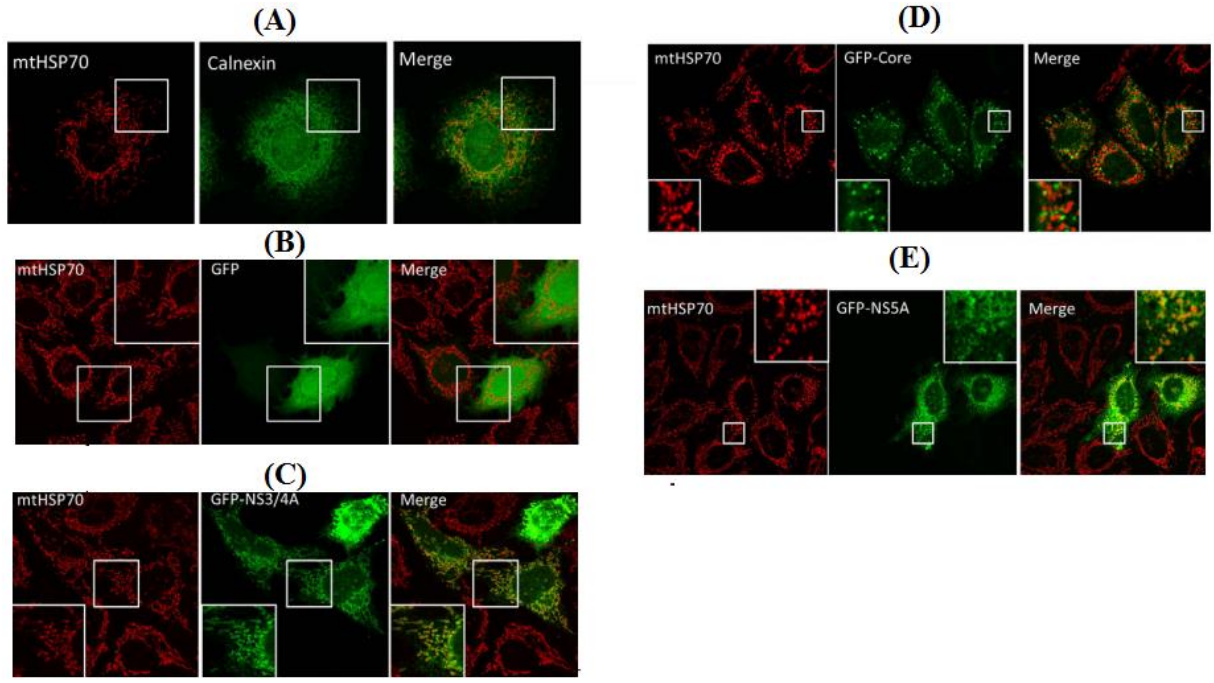

Figure 8 Localization of NS5A in LDs and characterized NS5A in JFH-1-derived wild-type virus and in JFH-1 virus with deleted core protein.

A is not the only protein involved in proliferation and aggregation; it is located in membranes derived from the endoplasmic reticulum (ER) and near the surface of the LD. Indeed, NS4B has been described to participate in membrane formation web [12, 28, 29], but should also localize in membranes associated with lipid droplets [30] and affect virus aggregation or release [28, 31, 32]. To determine whether nuclease deficiency affects NS4B in addition to NS5A, we labeled and quantified both LD and NS4B. Similar to what was reported by Mianari et al. [14], NS4B was less present on the surface of LDs when nuclei were absent (Figure 9 A). This is a fundamental concept in understanding how the NS5A interacts with NS protein, as well as how this interaction affects the localization of NS5A. For viral replication in the endoplasmic reticulum [16], we investigated whether the fact that both are less recruited in LDs could increase co-localization. As previously seen in NS3, it was well observed in the NS5A-NS4B-like locus in viruses (Figure 9 B). High-resolution microscopic motion images are needed to promote localized proteins at the surface of the LD or sites not present near the LD. To further this, LD sequences are definitively transferred from shedding to HCV Viral proteins and were immobilized using a standard system. As expected, this increased significantly because both LDs bound to ADFP (Figure 9 C), for which we generated more proteins with bound LDs. Unlike NS4B, NS5A and, to a lesser extent, NS3 is enriched in LDeenriched and other Schnauzer sections containing calnexin, a type of ER inclusion. This is the recommendation of the father; therefore, NS protein because the LD in moving fluorescent microscopes is located close to the LD surface; they are not directly attached to the desktop. Accordingly, there are further differences between NS and LD proteins. The difference in the association of NS perturbations with these lipoproteins is likely a replacement change of NS proteins originating from standard ER membranes. To get a better overview of the techniques, let's pick two previously described NS5A viruses whose defects were shown, detailed, and corrected during the installation phase: Good String (SC) [20] and Good Screen (Bc) [19, 20]. In the

BCM virus, it is the best central part 352-355 NS5A's DIII switch to glutamate and ssRNA interaction disruption of NS5AHCV and subsequent deficiencies of several ssRNAs in the previous lineage [19]. To detail and customize a local NS5A promoter, these devices were constructed at higher resolution by creating WT and Jeft viruses and included eGFP with APEX2 [33], allowing rapid processing. This NS5A-APEX2 protein was fully visualized by fast transmission electron microscopy (TEM), in which sections were observed with a Hitachi H7500 TEM (Milexia, Verri ères-le-buisson, France). APEX2 signaling function is highlighted in S1 and S2 modules (Figure 9 D). As previously described [34], EMT was detailed and corrected using the NS5A-APEX-2 viral program that recruits NS5A to LDs. (Figure 9 D). It should be noted that the level of LD was not well known. NS5A in intermediate LD neighbors is likely to show odd selections located near the corresponding LDs, and the use of the proposed LD qualifier has also previously been considered. NS5A in BCM virus is located near LD (Chcl 3C), while almost none was used for Cor hosts and SC scenes. Over the past year, NS5A has been used in some membrane connectors that are not directly compatible with LD connectors. (Figure 9 E). These localizations were activated in whole LDs for NS5A WT and BCM viruses and were not associated with LDs for NS5A Δcore and SC viruses. This was modeled and imaged by confocal microscopy using eGFP software. (Figure 9 F, G).

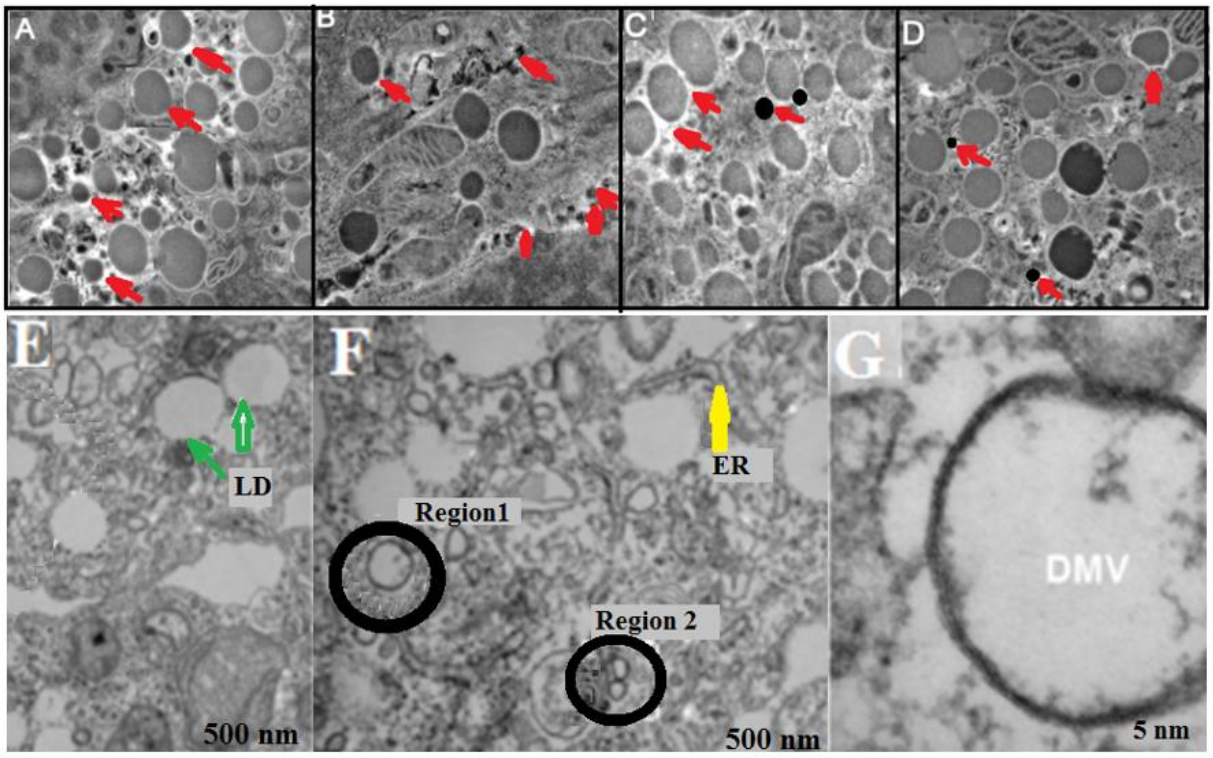

Figure 9 TEM images of A (WT), B (core), C (BCM), D (SC), E (LD), F(ER), G(DMV).

4. Discussion

4.1 Research Hypothesis and Specific Objectives

Since HCV can rapidly mutate to evade the immune response, an effective HCV vaccine must rely on the identification and characterization of sites critical for broad immune protection and viral neutralization. In this work, we discussed the Hepatitis C virus (HCV) from the viewpoint of

homeostasis due to its heterogeneous structure with numerous genomes as well as different recognized subunits containing discrete segments. Therefore, this work contributes to a better understanding of the interplay between HCV proteins during the viral life cycle understanding of the interplay between HCV proteins during the viral life cycle. As HCV can rapidly mutate to evade the immune response, an effective HCV vaccine must rely on the identification and characterization of sites critical for broad immune protection and viral neutralization. This knowledge depends on structural and mechanistic insights of the E1 and E2 envelope glycoproteins, which assemble as a heterodimer on the surface of the virion, engage co-receptors during host cell entry, and are the primary targets of antibodies. Due to the challenges in determining experimental structures, structural information on E1 and E2 and their interaction is relatively limited, providing opportunities to model the structures, interactions, and dynamics of these proteins. Due to the activity version of the eGFP chip to remove the BCM and SC files, they are the NS5A-NS3 solutions that have a different type and are a wild-type virus test. This hypothesis can be used to treat NS5A in order to end it by attracting it to LDs. Examination of NS5A dynamics on the SC mutant supports our idea and documents kinetics similar to those of the wide-type (WT) virus. As part of this mutation, NS5A is no longer able to interact with its nucleus and is therefore no longer used in LD. Therefore, the interaction of NS5A with the core of LDs is likely responsible for the reduced movement of NS5A. On the other hand, BCM also shows a significant increase in NS5A movement. However, it is a lower extent than the core, without changing NS5A localization, indicating that the core is not the only limiting factor affecting the dynamics of NS5A. Although abrogation of NS5A interaction with HCV RNA does not appear to affect NS5A localization, its kinetics are curious. A previous study described the co-occurrence of large static and small dynamic NS5A-containing structures in replicon-containing cells [38]. Localization of HCV RNA and NS5A in static and dynamic structures has been reported [42]. Since a subclass of mobile NS5A structures has been described as absent Viral RNA [42], the enhanced mobility of NS5A in BCM could be due to an enhanced population of NS5A that are free and are not bound to HCV RNA. Nevertheless, as no changes in the BCM repeat have been described [19], this increase in NS5A motion likely depends on the greater presence in the system. Alternatively, a stable population of NS5A-positive structures could be involved in HCV assembly. It was recently demonstrated that NS5A is located around the LD at the E2-positive center [43]. Additionally, peripheral NS5A was more mobile than NS5A-positive LD [40]. On the other hand, nuclear deletion also altered NS5A localization, leading to reduced NS5A recruitment to LDs ([14]), but also reduced NS3-NS5A localization as well as NS4B localization of NS5A. The interaction between NS3 and NS5A is responsible for hepatitis virus replication [27, 44]. In this study, NS5A and ds-RNA, an intermediate of RNA replication, were not significantly localized, indicating a very low proportion of NS5A in replication complexes. Additionally, NS4B was previously described as a negative regulator of the NS3-NS5B repeat complex [29], as it interacts with both proteins [29, 45]. We observed no differences in viral replication or NS5A phosphorylation, although NS4B did not appear to be at least partially affected. On the contrary, an increase in NS5A activity was observed. However, given the level of evolution of the SC It is similar to the expression of NS4B-mutated NS5A. Still, there is no change in the movement of NS5A, and we conclude that the phenotype observed in the nucleus, in the case of NS5A dynamics, should not be assigned to NS4B. Considering that NS5A and NS4B do not appear to exist directly on the surface of LDs, but in membrane structures adjacent to LDs, it is safe to assume that they are part of the replication

complexes. However, few of them are biochemists. Analyzes have also shown that most of the non-structural proteins of the hepatitis virus (HCV) are not integrated into the copy of the hepatitis virus [47]. Most studies on NS5A dynamics highlight the increased movement of NS5A in the absence of the nucleus, which could be related to altered nucleus-dependent activity associated with microtubules and/or dynein. These results support hepatitis virus (HCV) replication in conjugated membrane vesicles containing replication complexes. RNA is bidirectional. This program led to the creation of a deep bridge adventure and a mountain near Sharp (developed by Lee [43]. The membrane was developed by the dictionary NS5A, and its essential functions in the dynamic microbial machinery develop through HCV-positive and are newer than those of NS5A and NS5A-RNA. Viruses are currently transmitted to viruses. The treatment of the hepatitis C virus (HCV) is essential. Additional guidance is needed to describe, mechanize, and visualize positive NS5A motion selectors in HCV assembly and updating. Additionally, some proteins such as NS3, NS4B and NS5A stopped working. Most humans have no explicit signs and cannot be recognized before liver cirrhosis or hepatocellular carcinoma (HCC), because the effect of early clinical screening is negligible. During changing NAFLD to cirrhosis, the probability of a reverse situation has a high risk that HCC should be considered as a dangerous position. Consequently, by HCC, multiple systemic diseases appear in humans, such as cardiovascular disease, chronic kidney disease, and colorectal tumors, which all threaten human health. Finally by growing the scientific research in gene editing, a new branch of medicine based on CRISPR/Cas9 editing technology is entering the clinical stage for the treatment of virus disease, as well as most genes encoding CPT-II are known to be recessive genetic defects and the clinical manifestations of the related diseases could be listed as hypoglycemia, cardiomyopathy, arrhythmias and rhabdomyolysis

5. Conclusion and Future Perspectives

Most studies on NS5A dynamics highlight the increased movement of NS5A in the absence of the nucleus, which could be related to altered nucleus-dependent activity associated with microtubules and/or dynein, and at least BCM also showed swelling of NS5A. These results support hepatitis virus (HCV) replication in conjugated membrane vesicles containing replication complexes. RNA is bidirectional. In summary, these results constitute the importance of CRISPR/Cas9 systems directly targeting an authentic pathogenic hepatitis virus (HCV) with DNA and NS5A, and demonstrate the potential for antiviral therapy using Cas9, which may represent a significant step towards the cure of chronic HCV. The results shown here may also be used to inform the development of CRISPR/Cas9-based therapeutics for other DNA viruses, such as HBV and papillomaviruses, that use episomal DNA as a template for their gene expression and replication. Obviously, in future perspectives of CRISPR/Cas9 technologies, The CRISPR/Cas9 technology has enormously advanced our capability to edit and provide the new era of gene therapies for treating diseases. Although it is still in the clinical trial stage, the CRISPR/Cas9 system is used both in vitro and in vivo to inhibit HCV/HBV replication and gene expression. It may establish a new beneficial strategy for HCV/HBV infection. In the future, using combined CRISPR/Cas9 therapy help us to attain the maximum efficiency of viral treatment. Although many difficulties remain to be evaluated, including the safety and effective delivery of the system, CRISPR/Cas9 strategies provide immense potential to cure chronic HCV/HBV infection. Any problems should be resolved, and significant attempts are needed to achieve effective and safer

therapeutic techniques to use CRISPR/Cas9 technology to inhibit HCV/HBV. Despite the remarkable advances in CRISPR, several limitations and concerns still exist, which need to be addressed and solved for the optimized Cas systems development. One significant restriction can be mentioned as technical limitations and advances in the field of CRISPR technologies raise concerns for immunogenic toxicity. Recently, a study has shown that human subjects included, possessed pre-existing antibodies against Cas9. In addition, CRISPR has been extensively applied in clinical trials to modify somatic cells ex vivo, to reduce risk, and, subsequently, to transfer them for in vivo gene therapy applications. However, germ-line gene editing studies for therapeutic purposes still face ethical challenges. In this regard, the ongoing and near-future clinical trials on somatic CRISPR therapy need to be evaluated for the long term to check the system's efficacy and safety.

Abbreviations

BCM	Basic cluster mutant
HCV	Hepatitis C virus
NS5A	Non-structural 5A protein
DAAs	Direct-acting antivirals
TEM	Transmission electron microscopy
LDs	lipid droplets
DMV and MMV	Double and multi-membrane vesicles
SC	cluster mutant

Author Contributions

Conceptualization, M.M.; methodology, M.M.; software, F.M. and A.A; validation, A.A and M.M.; formal analysis, A.A.; investigation, M.M; resources, A.A and M.M.; data curation, A.A.; writing original draft preparation, F.M.; writing review and editing, A.A and F.M.; visualization, M.M.; supervision, M.M.; project administration, M.M.

Competing Interests

The authors have declared that no competing interests exist.

References

1. World Health Organization. Global Hepatitis Programme Global Hepatitis Report. Geneva, Switzerland: World Health Organization; 2017.
2. Choo QL, Richman KH, Han JH, Berger K, Lee C, Dong C, et al. Genetic organization and diversity of the hepatitis C virus. Proc Natl Acad Sci USA. 1991; 88: 2451-2455.
3. Neddermann P, Tomei L, Steinkühler C, Gallinari P, Tramontano A, De Francesco R. The nonstructural proteins of the hepatitis C virus: Structure and functions. Biol Chem. 1997; 378: 469-476.
4. Suzuki T, Ishii K, Aizaki H, Wakita T. Hepatitis C viral life cycle. Adv Drug Deliv Rev. 2007; 59: 1200-1212.
5. Lohmann V, Korner F, Koch JO, Herian U, Theilmann L, Bartenschlager R. Replication of

subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science. 1999; 285: 110-113.
6. Lohmann V, Körner F, Herian U, Bartenschlager R. Biochemical properties of hepatitis C virus NS5B RNA-dependent RNA polymerase and identification of amino acid sequence motifs essential for enzymatic activity. J Virol. 1997; 71: 8416-8428.
7. Jones CT, Murray CL, Eastman DK, Tassello J, Rice CM. Hepatitis C virus p7 and NS2 proteins are essential for production of infectious virus. J Virol. 2007; 81: 8374-8383.
8. Popescu CI, Callens N, Trinel D, Roingeard P, Moradpour D, Descamps V, et al. NS2 protein of hepatitis C virus interacts with structural and non-structural proteins towards virus assembly. PLoS Pathog. 2011; 7: e1001278.
9. Romero-Brey I, Merz A, Chiramel A, Lee JY, Chlanda P, Haselman U, et al. Three-dimensional architecture and biogenesis of membrane structures associated with hepatitis C virus replication. PLoS Pathog. 2012; 8: e1003056.
10. Gao L, Aizaki H, He JW, Lai MM. Interactions between viral nonstructural proteins and host protein hVAP-33 mediate the formation of hepatitis C virus RNA replication complex on lipid raft. J Virol. 2004; 78: 3480-3488.
11. Egger D, Wölk B, Gosert R, Bianchi L, Blum HE, Moradpour D, et al. Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex. J Virol. 2002; 76: 5974-5984.
12. Paul D, Romero-Brey I, Gouttenoire J, Stoitsova S, Krijnse-Locker J, Moradpour D, et al. NS4B self-interaction through conserved C-terminal elements is required for the establishment of functional hepatitis C virus replication complexes. J Virol. 2011; 85: 6963-6976.
13. Popescu CI, Riva L, Vlaicu O, Farhat R, Rouillé Y, Dubuisson J. Hepatitis C virus life cycle and lipid metabolism. Biology. 2014; 3: 892-921.
14. Miyanari Y, Atsuzawa K, Usuda N, Watashi K, Hishiki T, Zayas M, et al. The lipid droplet is an important organelle for hepatitis C virus production. Nat Cell Biol. 2007; 9: 1089-1097.
15. Ross-Thriepland D, Harris M. Hepatitis C virus NS5A: Enigmatic but still promiscuous 10 years on! J Gen Virol. 2015; 96: 727-738.
16. Biswas A, Treadaway J, Tellinghuisen TL. Interaction between nonstructural proteins NS4B and NS5A is essential for proper NS5A localization and hepatitis C virus RNA replication. J Virol. 2016; 90: 7205-7218.
17. Watanabe Y, Bowden TA, Wilson IA, Crispin M. Exploitation of glycosylation in enveloped virus pathobiology. Biochim Biophys Acta Gen Subj. 2019; 1863: 1480-1497.
18. Vigerust DJ, Shepherd VL. Virus glycosylation: Role in virulence and immune interactions. Trends Microbiol. 2007; 15: 211-218.
19. Goffard A, Dubuisson J. Glycosylation of hepatitis C virus envelope proteins. Biochimie. 2003; 85: 295-301.
20. Helle F, Goffard A, Morel V, Duverlie G, McKeating J, Keck ZY, et al. The neutralizing activity of anti-hepatitis C virus antibodies is modulated by specific glycans on the E2 envelope protein. J Virol. 2007; 81: 8101-8111.
21. Meunier JC, Fournillier A, Choukhi A, Cahour A, Cocquerel L, Dubuisson J, et al. Analysis of the glycosylation sites of hepatitis C virus (HCV) glycoprotein E1 and the influence of E1 glycans on the formation of the HCV glycoprotein complex. J Gen Virol. 1999; 80: 887-896.
22. Goffard A, Callens N, Bartosch B, Wychowski C, Cosset FL, Montpellier C, et al. Role of N-linked glycans in the functions of hepatitis C virus envelope glycoproteins. J Virol. 2005; 79:

8400-8409.
23. Helle F, Vieyres G, Elkrief L, Popescu CI, Wychowski C, Descamps V, et al. Role of N-linked glycans in the functions of hepatitis C virus envelope proteins incorporated into infectious virions. J Virol. 2010; 84: 11905-11915.
24. Anjum S, Wahid A, Afzal MS, Albecka A, Alsaleh K, Ahmad T, et al. Additional glycosylation within a specific hypervariable region of subtype 3a of hepatitis C virus protects against virus neutralization. J Infect Dis. 2013; 208: 1888-1897.
25. Fénéant L, Potel J, François C, Sané F, Douam F, Belouzard S, et al. New insights into the understanding of hepatitis C virus entry and cell-to-cell transmission by using the ionophore monensin A. J Virol. 2015; 89: 8346-8364.
26. Steinmann E, Pietschmann T. Cell culture systems for hepatitis C virus. In: Hepatitis C virus: From molecular virology to antiviral therapy. Berlin/Heidelberg, Germany: Springer; 2013. pp. 17-48.
27. Op De Beeck A, Voisset C, Bartosch B, Ciczora Y, Cocquerel L, Keck Z, et al. Characterization of functional hepatitis C virus envelope glycoproteins. J Virol. 2004; 78: 2994-3002.
28. Beyene A, Basu A, Meyer K, Ray R. Influence of N-linked glycans on intracellular transport of hepatitis C virus E1 chimeric glycoprotein and its role in pseudotype virus infectivity. Virology. 2004; 324: 273-285.
29. Flint M, Logvinoff C, Rice CM, McKeating JA. Characterization of infectious retroviral pseudotype particles bearing hepatitis C virus glycoproteins. J Virol. 2004; 78: 6875-6882.
30. Iacob RE, Perdivara I, Przybylski M, Tomer KB. Mass spectrometric characterization of glycosylation of hepatitis C virus E2 envelope glycoprotein reveals extended microheterogeneity of N-glycans. J Am Soc Mass Spectrom. 2011; 19: 428-444.
31. Bräutigam J, Scheidig AJ, Egge-Jacobsen W. Mass spectrometric analysis of hepatitis C viral envelope protein E2 reveals extended microheterogeneity of mucin-type O-linked glycosylation. Glycobiology. 2013; 23: 453-474.
32. Li Y, Pierce BG, Wang Q, Keck ZY, Fuerst TR, Foung SK, et al. Structural basis for penetration of the glycan shield of hepatitis C virus E2 glycoprotein by a broadly neutralizing human antibody. J Biol Chem. 2015; 290: 10117-10125.
33. Sandrin V, Boulanger P, Penin F, Granier C, Cosset FL, Bartosch B. Assembly of functional hepatitis C virus glycoproteins on infectious pseudoparticles occurs intracellularly and requires concomitant incorporation of E1 and E2 glycoproteins. J Gen Virol. 2005; 86: 3189-3199.
34. Goueslain L, Alsaleh K, Horellou P, Roingeard P, Descamps V, Duverlie G, et al. Identification of GBF1 as a cellular factor required for hepatitis C virus RNA replication. J Virol. 2010; 84: 773-787.
35. Nakata AT, Amemura MI, Makino KO. Unusual nucleotide arrangement with repeated sequences in the Escherichia coli K-12 chromosome. J Bacteriol. 1989; 171: 3553-3556.
36. Mojica FJ, Ferrer C, Juez G, Rodríguez-Valera F. Long stretches of short tandem repeats are present in the largest replicons of the *Archaea Haloferax mediterranei* and *Haloferax volcanii* and could be involved in replicon partitioning. Mol Microbiol. 1995; 17: 85-93.
37. Groenen PM, Bunschoten AE, Soolingen DV, Errtbden JD. Nature of DNA polymorphism in the direct repeat cluster of Mycobacterium tuberculosis; Application for strain differentiation by a novel typing method. Mol Microbiol. 1993; 10: 1057-1065.

38. Mojica FJ, Díez-Villaseñor C, Soria E, Juez G. Biological significance of a family of regularly spaced repeats in the genomes of Archaea, Bacteria and mitochondria. Mol Microbiol. 2000; 36: 244-246.
39. Grissa I, Vergnaud G, Pourcel C. The CRISPRdb database and tools to display CRISPRs and to generate dictionaries of spacers and repeats. BMC Bioinform. 2007; 8: 172.
40. Bolotin A, Quinquis B, Sorokin A, Ehrlich SD. Clustered regularly interspaced short palindrome repeats (CRISPRs) have spacers of extrachromosomal origin. Microbiology. 2005; 151: 2551-2561.
41. Barrangou R, Fremaux C, Deveau H, Richards M, Boyaval P, Moineau S, et al. CRISPR provides acquired resistance against viruses in prokaryotes. Science. 2007; 315: 1709-1712.
42. Makarova KS, Wolf YI, Iranzo J, Shmakov SA, Alkhnbashi OS, Brouns SJ, et al. Evolutionary classification of CRISPR–Cas systems: A burst of class 2 and derived variants. Nat Rev Microbiol. 2020; 18: 67-83.
43. Ishino Y, Krupovic M, Forterre P. History of CRISPR-Cas from encounter with a mysterious repeated sequence to genome editing technology. J Bacteriol. 2018; 200. doi: 10.1128/jb.00580-17.
44. Ishino Y, Shinagawa H, Makino K, Amemura M, Nakata A. Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in Escherichia coli, and identification of the gene product. J Bacteriol. 1987; 169: 5429-5433.
45. Sorek R, Kunin V, Hugenholtz P. CRISPR—A widespread system that provides acquired resistance against phages in bacteria and archaea. Nat Rev Microbiol. 2008; 6: 181-186.
46. Cho SW, Kim S, Kim JM, Kim JS. Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease. Nat Biotechnol. 2013; 31: 230-232.
47. Jinek M, East A, Cheng A, Lin S, Ma E, Doudna J. RNA-programmed genome editing in human cells. eLife. 2013; 2: e00471.
48. Lin H, Li G, Peng X, Deng A, Ye L, Shi L, et al. The use of CRISPR/Cas9 as a tool to study human infectious viruses. Front Cell Infect Microbiol. 2021; 11: 590989.
49. Lu XJ, Ji LJ, Torres-Ruiz R, Rodriguez-Perales S. CRISPR-Cas9 technology: Applications and human disease modelling. Brief Funct Genom. 2017; 16: 4-12.
50. Maeder ML, Gersbach CA. Genome-editing technologies for gene and cell therapy. Mol Ther. 2016; 24: 430-446.
51. Hryhorowicz M, Lipiński D, Zeyland J, Słomski R. CRISPR/Cas9 immune system as a tool for genome engineering. Arch Immunol Ther Exp. 2017; 65: 233-240.
52. Citorik RJ, Mimee M, Lu TK. Sequence-specific antimicrobials using efficiently delivered RNA-guided nucleases. Nat Biotechnol. 2014; 32: 1141-1145.
53. Hirakawa MP, Krishnakumar R, Timlin JA, Carney JP, Butler KS. Gene editing and CRISPR in the clinic: Current and future perspectives. Biosci Rep. 2020; 40: BSR20200127.
54. Richter H, Zoephel J, Schermuly J, Maticzka D, Backofen R, Randau L. Characterization of CRISPR RNA processing in *Clostridium thermocellum* and *Methanococcus maripaludis*. Nucleic Acids Res. 2012; 40: 9887-9896.
55. Haurwitz RE, Jinek M, Wiedenheft B, Zhou K, Doudna JA. Sequence-and structure-specific RNA processing by a CRISPR endonuclease. Science. 2010; 329: 1355-1358.
56. Zetsche B, Gootenberg JS, Abudayyeh OO, Slaymaker IM, Makarova KS, Essletzbichler P, et al. Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. Cell. 2015; 163: 759-

771.
57. Rath D, Amlinger L, Rath A, Lundgren M. The CRISPR-Cas immune system: Biology, mechanisms and applications. Biochimie. 2015; 117: 119-128.
58. Babu M, Beloglazova N, Flick R, Graham C, Skarina T, Nocek B, et al. A dual function of the CRISPR–Cas system in bacterial antivirus immunity and DNA repair. Mol Microbiol. 2011; 79: 484-502.
59. Levy A, Goren MG, Yosef I, Auster O, Manor M, Amitai G, et al. CRISPR adaptation biases explain preference for acquisition of foreign DNA. Nature. 2015; 520: 505-510.
60. Perez-Rodriguez R, Haitjema C, Huang Q, Nam KH, Bernardis S, Ke A, et al. Envelope stress is a trigger of CRISPR RNA-mediated DNA silencing in *Escherichia coli*. Mol Microbiol. 2011; 79: 584-599.
61. Riva L, Spriet C, Barois N, Popescu CI, Dubuisson J, Rouillé Y. Comparative analysis of Hepatitis C virus NS5A dynamics and localization in assembly-deficient mutants. Pathogens. 2021; 10: 172.
62. Doerflinger M, Forsyth W, Ebert G, Pellegrini M, Herold MJ. CRISPR/Cas9—The ultimate weapon to battle infectious diseases? Cell Microbiol. 2017; 19: e12693.
63. White MK, Hu W, Khalili K. The CRISPR/Cas9 genome editing methodology as a weapon against human viruses. Discov Med. 2015; 19: 255-262.
64. Parrish CR, Holmes EC, Morens DM, Park EC, Burke DS, Calisher CH, et al. Cross-species virus transmission and the emergence of new epidemic diseases. Microbiol Mol Biol Rev. 2008; 72: 457-470.
65. Villa TG, Feijoo-Siota L, Rama JL, Ageitos JM. Antivirals against animal viruses. Biochem Pharmacol. 2017; 133: 97-116.
66. Castilla V, Ramirez J, E Coto C. Plant and animal steroids a new hope to search for antiviral agents. Curr Med Chem. 2010; 17: 1858-1873.
67. Nair DM, Vajravelu LK, Thulukanam J, Paneerselvam V, Vimala PB, Lathakumari RH. Tackling hepatitis B Virus with CRISPR/Cas9: Advances, challenges, and delivery strategies. Virus Genes. 2024; 60: 592-602.
68. Xiao Q, Guo D, Chen S. Application of CRISPR/Cas9-based gene editing in HIV-1/AIDS therapy. Front Cell Infect Microbiol. 2019; 9: 69.
69. Mohammadzadeh I, Qujeq D, Yousefi T, Ferns GA, Maniati M, Vaghari-Tabari M. CRISPR/Cas9 gene editing: A new therapeutic approach in the treatment of infection and autoimmunity. IUBMB Life. 2020; 72: 1603-1621.
70. Guo N, Liu JB, Li W, Ma YS, Fu D. The power and the promise of CRISPR/Cas9 genome editing for clinical application with gene therapy. J Adv Res. 2022; 40: 135-152.
71. Nidhi S, Anand U, Oleksak P, Tripathi P, Lal JA, Thomas G, et al. Novel CRISPR–Cas systems: An updated review of the current achievements, applications, and future research perspectives. Int J Mol Sci. 2021; 22: 3327.
72. Scheller EL, Krebsbach PH. Gene therapy: Design and prospects for craniofacial regeneration. J Dent Res. 2009; 88: 585-596.
73. Mojica FJ, Díez-Villaseñor CS, García-Martínez J, Soria E. Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. J Mol Evol. 2005; 60: 174-182.
74. Makarova KS, Haft DH, Barrangou R, Brouns SJ, Charpentier E, Horvath P, et al. Evolution and

classification of the CRISPR–Cas systems. Nat Rev Microbiol. 2011; 9: 467-477.
75. Marraffini LA, Sontheimer EJ. CRISPR interference limits horizontal gene transfer in staphylococci by targeting DNA. Science. 2008; 322: 1843-1845.
76. Hale CR, Zhao P, Olson S, Duff MO, Graveley BR, Wells L, et al. RNA-guided RNA cleavage by a CRISPR RNA-Cas protein complex. Cell. 2009; 139: 945-956.
77. Carte J, Christopher RT, Smith JT, Olson S, Barrangou R, Moineau S, et al. The three major types of CRISPR-Cas systems function independently in CRISPR RNA biogenesis in *Streptococcus thermophilus*. Mol Microbiol. 2014; 93: 98-112.
78. Marraffini LA. The CRISPR-Cas system of Streptococcus pyogenes: Function and applications. In: Streptococcus pyogenes: Basic biology to clinical manifestations. Oklahoma City, OK: The University of Oklahoma Health Sciences Center; 2016. pp. 1-14.
79. Simonson RE. Variations on the theme mental health in nursing. Public Health Rep. 1956; 71: 700-704.
80. Osawa T, Inanaga H, Sato C, Numata T. Crystal structure of the CRISPR-Cas RNA silencing Cmr complex bound to a target analog. Mol Cell. 2015; 58: 418-430.
81. Uddin F, Rudin CM, Sen T. CRISPR gene therapy: Applications, limitations, and implications for the future. Front Oncol. 2020; 10: 1387.
82. Pavani G, Fabiano A, Laurent M, Amor F, Cantelli E, Chalumeau A, et al. Correction of β-thalassemia by CRISPR/Cas9 editing of the α-globin locus in human hematopoietic stem cells. Blood Adv. 2021; 5: 1137-1153.
83. Vlaicu O, Selescu T, Pastrama F, Munteanu C, Riva L, Dubuisson J, et al. Novel replicons and trans-encapsidation systems for Hepatitis C Virus proteins live imaging and virus-host interaction proteomics. J Virol Methods. 2017; 246: 42-50.
84. Rose BI, Brown S. Genetically modified babies and a first application of clustered regularly interspaced short palindromic repeats (CRISPR-Cas9). Obstet Gynecol. 2019; 134: 157-162.
85. Kato T, Date T, Miyamoto M, Furusaka A, Tokushige K, Mizokami M, et al. Efficient replication of the genotype 2a hepatitis C virus subgenomic replicon. Gastroenterology. 2003; 125: 1808-1817.
86. Ledford H. CRISPR treatment inserted directly into the body for first time. Nature. 2020; 579: 185-186.
87. Westermann L, Neubauer B, Köttgen M. Nobel Prize 2020 in Chemistry honors CRISPR: A tool for rewriting the code of life. Pflügers Arch. 2021; 473: 1-2.
88. Kham-Kjing N, Ngo-Giang-Huong N, Tragoolpua K, Khamduang W, Hongjaisee S. Highly specific and rapid detection of hepatitis C virus using RT-LAMP-coupled CRISPR–Cas12 assay. Diagnostics. 2022; 12: 1524.
89. Yin L, Man S, Ye S, Liu G, Ma L. CRISPR-Cas based virus detection: Recent advances and perspectives. Biosens Bioelectron. 2021; 193: 113541.
90. Wang S, Li H, Kou Z, Ren F, Jin Y, Yang L, et al. Highly sensitive and specific detection of hepatitis B virus DNA and drug resistance mutations utilizing the PCR-based CRISPR-Cas13a system. Clin Microbiol Infect. 2021; 27: 443-450.
91. Wang H, Zhang Y, Zhou J, Li M, Chen Y, Liu Y, et al. Rapid visual detection of hepatitis C virus using reverse transcription recombinase-aided amplification–Lateral flow dipstick. Front Cell Infect Microbiol. 2022; 12: 816238.
92. Alsaleh K, Delavalle PY, Pillez A, Duverlie G, Descamps V, Rouillé Y, et al. Identification of basic

amino acids at the N-terminal end of the core protein that are crucial for hepatitis C virus infectivity. J Virol. 2010; 84: 12515-12528.
93. Macdonald A, Crowder K, Street A, McCormick C, Saksela K, Harris M. The hepatitis C virus non-structural NS5A protein inhibits activating protein–1 function by perturbing Ras-ERK pathway signaling. J Biol Chem. 2003; 278: 17775-17784.
94. Kweon J, Yoon JK, Jang AH, Shin HR, See JE, Jang G, et al. Engineered prime editors with PAM flexibility. Mol Ther. 2021; 29: 2001-2007.
95. Fu YW, Dai XY, Wang WT, Yang ZX, Zhao JJ, Zhang JP, et al. Dynamics and competition of CRISPR–Cas9 ribonucleoproteins and AAV donor-mediated NHEJ, MMEJ and HDR editing. Nucleic Acids Res. 2021; 49: 969-985.
96. Barman A, Deb B, Chakraborty S. A glance at genome editing with CRISPR–Cas9 technology. Curr Genet. 2020; 66: 447-462.
97. Khan AG, Whidby J, Miller MT, Scarborough H, Zatorski AV, Cygan A, et al. Structure of the core ectodomain of the hepatitis C virus envelope glycoprotein 2. Nature. 2014; 509: 381-384.
98. Keck ZY, Girard-Blanc C, Wang W, Lau P, Zuiani A, Rey FA, et al. Antibody response to hypervariable region 1 interferes with broadly neutralizing antibodies to hepatitis C virus. J Virol. 2016; 90: 3112-3122.
99. Pierce BG, Boucher EN, Piepenbrink KH, Ejemel M, Rapp CA, Thomas Jr WD, et al. Structure-based design of hepatitis C virus vaccines that elicit neutralizing antibody responses to a conserved epitope. J Virol. 2017; 91. doi: 10.1128/jvi.01032-17.
100. Kong L, Kadam RU, Giang E, Ruwona TB, Nieusma T, Culhane JC, et al. Structure of hepatitis C virus envelope glycoprotein E1 antigenic site 314–324 in complex with antibody IGH526. J Mol Biol. 2015; 427: 2617-2628.
101. Kong L, Giang E, Nieusma T, Kadam RU, Cogburn KE, Hua Y, et al. Hepatitis C virus E2 envelope glycoprotein core structure. Science. 2013; 342: 1090-1094.
102. Huang L, Sineva EV, Hargittai MR, Sharma SD, Suthar M, Raney KD, et al. Purification and characterization of hepatitis C virus non-structural protein 5A expressed in Escherichia coli. Protein Expr Purif. 2004; 37: 144-153.