Review

JOURNAL OF HEPATOLOGY

Basics

The history of hepatitis C virus (HCV): Basic research reveals unique features in phylogeny, evolution and the viral life cycle with new perspectives for epidemic control

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Keywords: HCV; Phylogeny; Evolution; Antivirals; MicroRNA; Vaccine; Neutralizing antibodies; Receptors; Genotypes; Animal models.

Received 25 July 2016; accepted 29 July 2016

Abbreviations: HCV, hepatitis C virus; HTA, host targeting agents; DAA, direct acting antivirals; ORF, open reading frame; UTR, untranslated region; NS, non-structural; E, envelope; IFN, interferon; GBV-B, GB virus B; miR-122, microRNA-122; HBV, hepatitis B virus.

Summary

The discovery of hepatitis C virus (HCV) in 1989 permitted basic research to unravel critical components of a complex life cycle for this important human pathogen. HCV is a highly divergent group of viruses classified in 7 major genotypes and a great number of subtypes, and circulating in infected individuals as a continuously evolving quasispecies destined to escape host immune responses and applied antivirals. Despite the inability to culture patient viruses directly in the laboratory, efforts to define the infectious genome of HCV resulted in development of experimental recombinant *in vivo* and *in vitro* systems, including replicons and infectious cultures in human hepatoma cell lines. And HCV has become a model virus defining new paradigms in virology, immunology and biology. For example, HCV research discovered that a virus could be completely dependent on microRNA for its replication since microRNA-122 is critical for the HCV life cycle. A number of other host molecules critical for HCV entry and replication have been identified. Thus, basic HCV research revealed important molecules for development of host targeting agents (HTA). The identification and characterization of HCV encoded proteins and their functional units contributed to the development of highly effective direct acting antivirals (DAA) against the NS3 protease, NS5A and the NS5B polymerase. In combination, these inhibitors have since 2014 permitted interferon-free therapy with cure rates above 90% among patients with chronic HCV infection; however, viral resistance represents a challenge. Worldwide control of HCV will most likely require the development of a prophylactic vaccine, and numerous candidates have been pursued. Research characterizing features critical for antibody-based virus neutralization and T cell based virus elimination from infected cells is essential for this effort. If the world community promotes an ambitious approach by applying current DAA broadly, continues to develop alternative viral- and host-targeted antivirals to combat resistant variants, and invests in the development of a vaccine, it would be possible to eradicate HCV. This would prevent about 500 thousand deaths annually. However, given the nature of HCV, the millions of new infections annually, a high chronicity rate, and with over 150 million individuals with chronic infection (which are frequently unidentified), this effort remains a major challenge for basic researchers, clinicians and communities.

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Discovery and basic characterization of an important human pathogen

Hepatitis C virus (HCV) is a main contributor to chronic liver diseases worldwide. Its existence was first fully recognized in 1975 when Feinstone *et al.* found that most cases of transfusion-associated hepatitis were not associated with hepatitis A virus or hepatitis B virus (HBV) infections, and thus defined the disease non-A, non-B hepatitis [1]. Subsequent transmission studies in chimpanzees showed that non-A, non-B hepatitis was likely caused by a small enveloped agent [2,3]. In 1989, Houghton and colleagues cloned and sequenced the genome of HCV (strain HCV-1) using high-titer samples

collected from an experimentally infected chimpanzee, and developed diagnostic tests [4,5].

It was established that HCV infection is associated with acute and chronic hepatitis [6] and liver cancer [7–9]. During the next 25 years, despite numerous huge challenges including the inability to culture patient viruses in the laboratory, impressive advances have been made in strives to understand HCV's genetic heterogeneity and complex life cycle, and in developing experimental recombinant cell culture systems. These advances have permitted the development of blood screening programs to prevent transmission and of highly effective medical treatment regimens for patients with persistent HCV infection, progressing from interferon (IFN) monotherapy with low cure rates, to IFN combined with ribavirin with intermediate cure rates, and finally to IFN-free therapy using combined direct acting antivirals (DAA) with high cure rates [10]. Thus, current DAA-based therapy targeting key HCV encoded proteins, introduced from 2014, can cure over 90% of individuals for their infection, including patients with advanced HCV induced liver disease [11]. However, challenges remain in optimizing current drug regimens, limiting the problem of resistance mutations and providing individualized therapy [10,12–14]. Furthermore, there is still no vaccine against HCV and this effort represents a major challenge for worldwide control of HCV [15–22].

Despite the great advances in HCV research and treatment, over 150 million people (>2% of the world population) remain chronically infected with HCV [23]. The prevalence of HCV varies greatly in the different countries [24]. For example, in the Scandinavian countries <0.3% of the population is infected, whereas 1–3% are infected in Southern European countries and in the U.S.; in Egypt >20% is infected. There are still 3–4 million new cases of acute HCV infections worldwide annually. In the developed countries the main risk factor for acute HCV infection is injection drug abuse. Other risk factors include sexual activity, mother-to-child transmission, medical procedures and occupational and household contacts. Only about 25% of the patients acutely infected with HCV have clinical evidence of disease. However, 70–80% will develop a persistent infection. The majority of persistently infected individuals develop chronic hepatitis, and HCV is a main etiological agent of liver-cirrhosis and cancer, which causes about 500 thousand deaths yearly, worldwide. Although HCV-related end-stage liver diseases remain a leading cause of liver transplantation, DAA induced virus elimination in the late stages of the disease can now prevent many such transplantations [11,25]. However, it remains to be determined whether they also prevent the development of liver cancer in patients with advanced liver disease.

The HCV genome was found to consist of approximately 9,600 nucleotides [26–28]. It has a single open reading frame (ORF), about 9,000 nucleotides in length. The ORF is flanked at the termini by 5′ and 3′ untranslated regions (UTR) critical for viral replication and translation [29]. Translation of the HCV ORF produces a polyprotein of about 3,000 amino acids that is cleaved by a combination of host and viral proteases into three structural proteins involved with viral particle production (Core and envelope glycoproteins E1 and E2) and seven nonstructural proteins permitting viral processing and replication, as well as particle assembly (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) [29]. Thus, HCV was classified as the prototype member of genus *Hepacivirus* in the *Flaviviridae* family of viruses; this genus also includes GB virus B (GBV-B) of unknown origin [30]. Viruses belonging to this family have positive-sense single-stranded RNA genomes with a similar organization; in the host cell their genomes serves directly as messenger RNA and in association with modified cell membranes as template for replication through negative-strand full-length intermediates [30]. The *Flaviviridae* also include the genus *Pestivirus*, with members such as bovine viral diarrhea virus and classical swine fever virus that cause disease in cattle and pigs, respectively, genus *Flavivirus*, which includes members such as yellow fever-, zika-, West Nile-, and dengue- viruses, all important causes of arthropod-transmitted viral diseases in humans, and genus *Pegivirus*, including the human virus GBV-C [30,31].

HCV is an enveloped virus [30]. It replicates primarily, if not exclusively, in hepatocytes of infected patients [32], and its replication was found to be dependent on the liver-specific microRNA-122 (miR-122) [29,33]. The viral genomic RNA is associated with the capsid protein (Core) to form the nucleocapsid, which is spherical and about 30 nm in diameter. The infectious virion consists of the nucleocapsid surrounded by a lipid-containing envelope derived from host endoplasmatic reticulum (ER) membranes. The experimental inactivation of HCV by chloroform, followed by transmission to chimpanzees, first indicated its enveloped nature [2,3]. The envelope contains the two viral glycoproteins E1 and E2 involved with entry of HCV into hepatocytes through a number of host cell receptors [34]. *In vitro* and *in vivo* generated virus particles are 40–80 nm in size as observed by electron microscopy [35,36].

Although the possibility of studying HCV in chimpanzees has been rather limited, such studies played an essential role in the original identification of HCV [2] and in defining its natural history and correlates of protective immunity [3,37]. Chimpanzees remain the only model that permit studies of HCV infection and related innate and adaptive host immune responses [37]. However, since 2011 their use has been highly restricted and in reality eliminated even in the U.S. Thus, HCV studies in animal models are now dependent on rodent models which all have severe shortcomings [37]. In these

Key point

Basic research of hepatitis C virus (HCV) defined critical components of a complex life-cycle for this important human pathogen, which contributed to the development of highly effective direct acting antivirals (DAA) curing over 90% of treated patients with chronic infection.

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Key point

Hepatitis C virus (HCV) exhibits extensive genetic heterogeneity, and has been classified into 7 major genotypes and over 75 subtypes with important implications for diagnosis and treatment. The major genotypes are found worldwide, but with distinct differences in their geographical distribution. Among individuals with persistent HCV genotypes 1 and 3 account for about 75% of all infections.

models, robust HCV infection can only be achieved in T and B cell deficient mice with human chimeric livers, thus preventing studies of adaptive immunity [38,39]. However, they have provided insights into innate host responses, receptor interactions and HCV neutralization [37]. More recently, Ploss and colleagues succeeded in developing genetically humanized mice with limited HCV replication, which might open up new possibilities for studies of HCV, such as studies of protective immunity in vaccine studies [40,41]. Experimental infection of New World monkeys with GBV-B causes acute hepatitis, and it was suggested as a potential surrogate animal model for HCV [42].

Since 2011, a diverse group of viruses infecting different mammalian species, including rodents, and found to be most closely related to viruses in the *Hepacivirus* and *Pegivirus* genera, were identified [43]. The virus most closely related to HCV was originally identified from dogs [44,45], but it is more prevalent in horses and might cause acute hepatitis in experimentally infected horses, thus representing another potential surrogate model for the study of HCV [46]. It would be desirable if the rodent HCV-like viruses could be adapted to infect regular laboratory mouse species, thus providing a readily available surrogate animal model for HCV.

Phylogenetic classification of HCV variants into highly diverse genotypes and subtypes

A distinctive characteristic of HCV is its extensive genetic heterogeneity, which exist at several levels among viral populations in individual infected patients at any given time and during evolution (quasispecies; see below), and worldwide among isolates from different patients (genotypes, subtypes, and isolates/strains). Thus, in 1993 phylogenetic analysis of partial HCV sequences recovered from a large number of patient isolates from around the world demonstrated that the virus could be classified into 6 major genotypes with important subtypes [47–49]. Genotypes 1–6 still contain all the identified epidemiologically important HCV variants. This classification was later confirmed based on analysis of full-length ORF sequences [50–53]. In addition, a seventh major genotype was reported [54]; this variant has only been found in a few individuals. With the advances in sequence analysis techniques there has been a dramatic increase in the number of published ORF sequenced HCV isolates, and phylogenetic analysis performed by Smith *et al.* in 2014 confirmed the existence of 7 major genotypes and 67 subtypes [55] (Fig. 1); subsequently a number of additional subtypes have been confirmed [56–58]. Subtypes 1a, 1b, 2a, 2b, 2c, 3a, 4a, 4d, 5a and 6a are well-defined worldwide or in specific population groups [59]. The genomes of the HCV isolates belonging to different major genotypes differ by about 30% at the nucleotide and deduced amino acid level; subtypes typically differ by >15%. Even within subtypes, different isolates can vary by up to 10%. Thus, HCV has a high level of genetic heterogeneity throughout the genome with important implications for diagnosis and treatment, as well as the possibility of developing an effective vaccine. Naturally occurring intra- or intergenotypic recombinants are rare, but a particular 1b/2k intergenotypic recombinant has spread to become of epidemiological importance [60,61].

The impact of HCV genotypes on the long-term outcome of HCV infection appears to be minimal. Thus, all genotypes are associated with severe liver diseases; genotype 3 patients might have an increased tendency to develop liver steatosis [62]. It is well established that HCV genotype was associated with response to IFN-based treatments; patients infected with genotype 1 and 4 responded poorly to treatment compared to patients infected with genotypes 2 or 3 [10]. For novel IFN-free DAA-based treatments, genotype 3 has become the more difficult to treat genotype [11,63].

Although the different major genotypes have all been found worldwide, there are clear differences in their geographical distribution (Fig. 1). A recent global survey found that genotypes 1 and 3 are the most prevalent, accounting for 46% and 30% of all infections, respectively; genotypes 2, 4, 5 and 6 accounted for 9%, 8%, 1% and 6%, respectively [64]. Furthermore, it is of importance that the distribution of genotypes and subtypes has changed over time in specific geographical regions due to elimination of transfusion-associated transmission, altered transmission routes with intravenous drug abuse having a major role, and with emigration from regions with a different genotype distribution. Some subtypes, such as 1b, 2a, and 2b, are typically found in elderly populations and is believed to have resulted from iatrogenic spread, including transfusions; other subtypes, such as 1a, 3a, 4d and 6a, are closely linked with widespread intravenous drug abuse [59].

In Europe, about 90% of all infections are genotypes 1, 2 and 3 [64,65]; genotype 1 is the most prevalent in most countries, but genotype 3 has a significant prevalence in many countries and genotype 2 is a prevalent genotype in Italy. Among genotype 1, subtype 1a is the most prevalent in Northern Europe, whereas 1b is most prevalent in Southern Europe. Among genotype 2, subtype 2b is most prevalent in Northern Europe, whereas 2c is prevalent in Southern Europe, in particular in Italy; 2c was originally identified in a patient from Sardinia [47]. Genotype 3 infections are represented almost exclusively by subtype 3a. Genotypes 4 and 5 have increased presence due to emigration from the Middle East and Africa and spread of specific subtypes in intravenous drug addict populations. Genotype 6 is

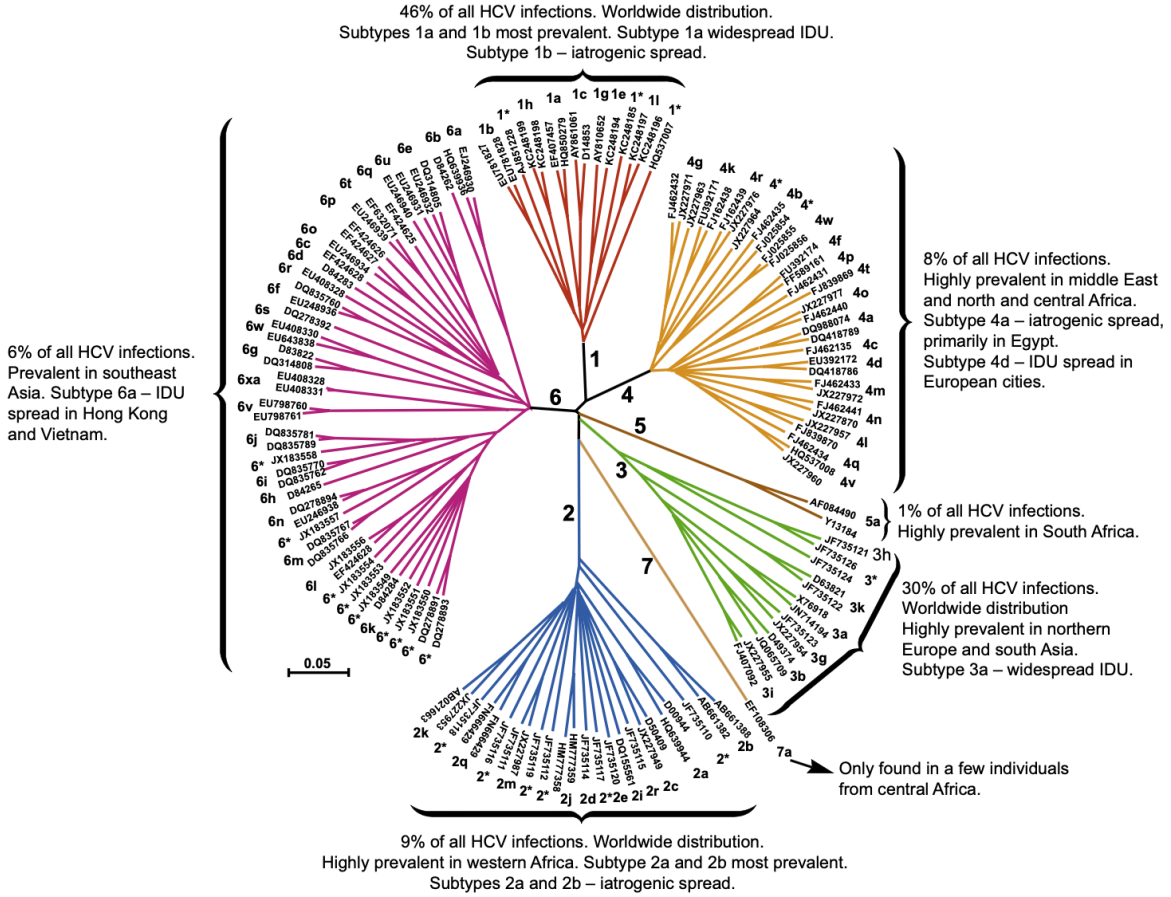

Fig. 1. Classification of hepatitis C virus (HCV) into 7 major genotypes and a large number of subtypes. The tree is based on phylogenetic analysis of the open reading frame (nucleotide) sequences. The overall prevalence and distribution is indicated for each major genotype. In part adapted from Smith *et al.* [55].

only found sporadically, mostly in emigrants from Southeast Asia. However, genotypes 3a and 4d for example, have had increasing importance in Europe because of transmission among intravenous drug addicts. Thus, in some Northern European countries genotype 3a constitute nearly half of all infections [66]. Furthermore, although genotype 4 is primarily found in the Middle East and Africa, 4d was originally identified in a Danish patient [47,67] and is an important genotype among intravenous drug addicts in Europe.

In most countries of the Americas the majority of infections are genotype 1 (subtypes 1a and 1b); the remaining infections are genotypes 2 (in particular 2a) and 3a [64]. Genotype 3 is found infrequently in Africa (and nearly exclusively in North and South Africa), where genotypes 1, 2 and 4 are the prevalent genotypes in North and Central Africa and genotypes 1 and 5 in South Africa; genotypes 4 is the most prevalent in North East and Central Africa and genotype 5 in South Africa. There are numerous subtypes of genotype 4, as originally identified among HIV infected individuals from the Democratic Republic of the Congo (Zaire at the time) [47,67]. In Egypt most infections are subtype 4a, resulting from extensive transmission during a national anti-schistosomiasis injection campaign [68,69]. Only a single subtype of genotype 5 was identified [47,67]. In the Middle East genotypes 1 and 4 predominates. Although genotype 1 is most prevalent in Asia, genotypes 2, 3 and 6 are other important genotypes. Most infections in India, Pakistan, Bangladesh, Myanmar, Nepal, and Thailand are genotype 3, including numerous subtypes as originally evidenced in samples from Nepal [70]. In Japan most infections are genotypes 1b and 2a. Genotype 6 is found in 10–20% of the population in many areas in East and South East Asia; the relative high prevalence of subtype 6a, originally identified in a patient from Hong Kong [47,67,71], is the result of spread among intravenous drug addicts in Vietnam and Hong Kong. Numerous subtypes of genotype 6 exist, as originally found in individuals from Vietnam [72]. Finally, in Russia and Australia/

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New Zealand genotypes 1 (subtypes 1a and 1b) are most prevalent, followed by 3a.

Quasispecies nature of HCV – a moving target for drug and vaccine development

Key point

In infected individuals, hepatitis C virus (HCV) circulates as a continuously evolving quasispecies destined to escape host immune responses, including neutralizing antibodies and activated T-cells, and antivirals. Evolution has grave implications for the effectiveness of vaccine candidates and contributes to viral escape from DAA, with variants evolving from preexisting resistant associated substitutions or developing *de novo* during treatment.

Within infected individuals, HCV circulates as a quasispecies, which is a mixture of closely related but distinctly different genomes. The viral genomes of a quasispecies typically differ by 1–3%. The quasispecies composition of HCV in an infected individual is the result of mutations that accumulate over time during infection or mutations that are present from the onset of the infection due to simultaneous transmission of multiple viral species. A new dominant HCV sequence can result from accumulation of mutations over time and/or from the selection of a preexisting minor viral species (evolution). Such mutations might enable HCV to replicate more efficiently or might help the virus evade host immune responses or antivirals. Although the genetic heterogeneity defining a quasispecies is found throughout the genome, certain regions are hypervariable, including hypervariable region 1 (HVR1) at the N-terminus of E2. The quasispecies nature of HCV might have implications for the natural history, for the response to antiviral therapy, and for the effectiveness of vaccine candidates [12,73,74].

The great potential for HCV to introduce functional genome changes have been experimentally shown to promote escape from neutralizing antibodies and cellular immune responses [75,76]. It is associated with the outcome of acute HCV infection [77]. Further, it affects the viral population following reinfection, for example, after liver transplantations [78–80]. Importantly, it was evidenced that this HCV heterogeneity found in the individual patient can contribute to viral escape from DAA, with variants evolving from preexisting resistant associated substitutions or developing *de novo* during treatment [12,81].

Recombinant systems to study HCV replication for the different genotypes: an arduous undertaking

Key point

Despite the inability to culture patient viruses, detailed characterization of the infectious genome of hepatitis C virus (HCV) resulted in the development of recombinant systems *in vivo* (infectious clones) and *in vitro* (replicons, pseudoviruses, and infectious cultures in human hepatoma cell lines). They have made it possible to discover key viral and host elements in the HCV life-cycle.

Since clinical isolates of HCV cannot be cultured efficiently *in vitro*, even in cells now known to be susceptible to functional recombinant viruses, it has been an enormous challenge to develop experimental systems required for true basic studies of the viral life cycle [82]. Infections were originally reported in continuous human T and B cell lines, but these replication systems are very inefficient and results have been difficult to reproduce. In addition, it turned out that the HCV genome originally described were missing the 3′ terminal sequence. Thus, the development of recombinant lab-generated culture systems only became possible after the identification, in pioneering studies in 1995–1996, of a structured sequence following the poly-pyrimidine tract at the 3′ terminus of HCV [27,28].

Infectious cDNA clones – in vivo studies

A major breakthrough in the development of recombinant systems to study HCV was the generation of infectious molecular cDNA clones in 1997 [83,84]. This was achieved by determining the consensus sequence of the prototype strain H77 (genotype 1a) of HCV, and thereafter generating molecular clones encompassing this sequence (Fig. 2). In the absence of cell culture systems, infectivity was demonstrated by inoculating RNA transcripts synthesized *in vitro* directly into the liver of chimpanzees. Subsequently, *in vivo* infectious cDNA clones have been developed for HCV strains of other genotypes, including 1b, 2a, 3a and 4a [85–87]. This effort was greatly aided by the availability of prototype strains characterized in chimpanzees [88]. The resulting monoclonal HCV infection in chimpanzees did not differ significantly from the polyclonal infection observed in animals infected intravenously with wild-type virus [89–92]. Furthermore, these studies formally proved that HCV causes liver disease, since chimpanzees transfected with HCV genomic RNA developed acute hepatitis (Fig. 2). Finally, the availability of infectious HCV clones permitted for the first time true reverse genetics studies of the importance of genetic components, including the 3′UTR, p7 and key viral enzymes, for virus infectivity [93–95].

The wild-type *in vivo* infectious HCV sequences turned out not to be replication competent in transfected continuous cell lines, including Huh7 derived hepatoma cell lines [85,96]. However, they facilitated development of *in vitro* systems for HCV, since subsequent studies could be performed with genomes known to have all genetic elements required for infection [82,97]. This research resulted in the development of HCV replicons in 1999 and in a true infectious culture system in 2005 [97,98].

HCV replicons – in vitro studies

In a landmark study by Lohmann *et al.* that literally changed the perspective of developing drugs targeting HCV replication it was demonstrated that a subgenomic viral sequence consisting of the 5′ UTR, NS3-NS5B and the 3′ UTR from strain Con1 (genotype 1b) in a selectable bicistronic construct can function as a self-replicating autonomous unit in Huh7 hepatoma cell lines [99] (Fig. 3). This breakthrough finding permitted for the first time *in vitro* studies of HCV RNA replication. Subsequent analysis demonstrated that their replication capacity was determined by adaptive mutations of the replicating HCV RNA and by increased host cell permissiveness

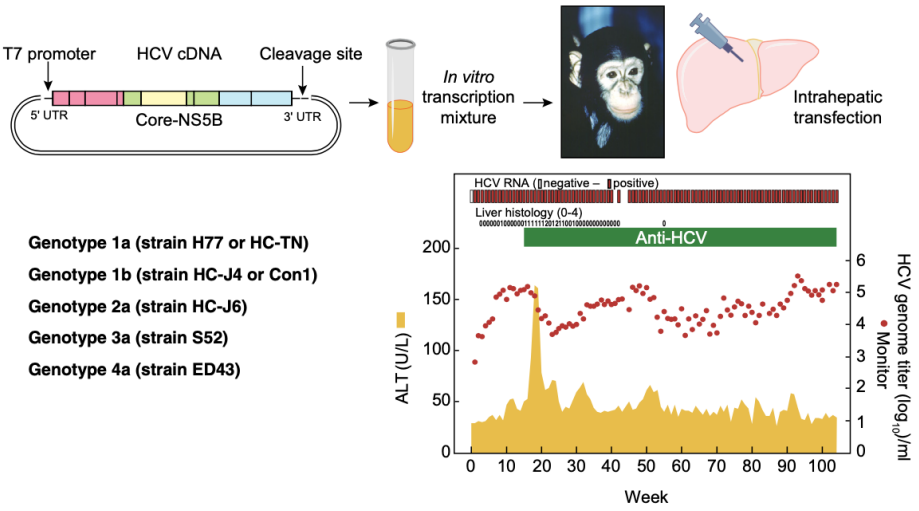

Fig. 2. The generation of infectious cDNA clones of HCV. The full-length consensus HCV sequence for selected HCV strains was engineered into plasmids. In the absence of cell culture systems, infectivity was demonstrated by inoculating *in vitro* generated RNA transcripts directly into the liver of chimpanzees. Such *in vivo* infectious cDNA clones have been developed for HCV strains of genotypes 1a, 1b, 2a, 3a and 4a. The resulting monoclonal HCV infection in chimpanzees did not differ significantly from the polyclonal infection observed in animals infected intravenously with wild-type virus; here is shown an example of the course of infection observed in a chimpanzee transfected with RNA transcripts from an infectious cDNA clone of HCV (pCV-H77C) from patient H. The chimpanzee developed acute hepatitis with elevated liver enzyme levels and necro-inflammatory changes in liver biopsies. Based on original findings by Kolykhalov *et al.* [83] and Yanagi *et al.* [84].

[100–103]. Numerous studies addressing the function of these adaptive mutations led to highly adapted subgenomic and full-genome length replicons, and by curing Huh7 cell lines with high level RNA replication highly permissive cell lines [104,105]. The improved permissiveness of Huh7.5 cells appears to be the result of a defect in the IFN signaling pathway of major importance for antiviral immunity. Subsequently, HCV replicons depending on signature adaptive mutations have been developed for other genotype 1 strains [106–109], as well as for selected strains of genotypes 2–6 [110–115]. An important finding was that a replicon of JFH1, a genotype 2a strain from a Japanese patient with fulminant hepatitis [116], could replicate in original Huh7 derived cell lines without the requirement for cell culture adaptive mutations [112], thus leading the way to the development of the first infectious cell culture system for HCV (see below).

It was unclear why the HCV RNA of most isolates required adaptive mutations to autonomously replicate in Huh7 hepatoma cells. However, in 2015 it was found that the host cells lack a key factor, SEC14L2, which permit replication of HCV sequences of different genotypes without the requirement for adaptive mutations [117]. The dramatic effect of SEC14L2 for HCV replication was confirmed for genotypes 1–4 replicons in a recent study that also showed that specific modifications of the non-HCV replicon sequences could enhance HCV replication in this system [118].

The development of full-length adapted replication competent HCV genomes provided hope that these systems would eventually yield cell culture derived infectious viruses. However, they turned out to be incapable or restricted in generating infectious HCV particles. Thus, it was originally found that an *in vitro* optimized combination of highly adaptive mutations into the Con1 full-length genome abrogated its ability to productively infect chimpanzees [119] (Fig. 4). Similarly, it was observed that the *in vivo* infectious Con1 full-length genome without adaptive mutations could produce virions in culture, albeit at very low levels, in contrast to the genome with adaptive mutations which did not [120]. Thus, cell culture adaptive mutations in the Con1 strain apparently prevented

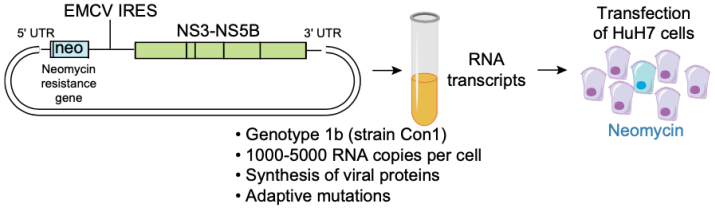

Fig. 3. Principle of the generation of HCV replicons. Diagram showing the composition of a subgenomic viral replicon sequence consisting of the 5′ UTR, NS3-NS5B and the 3′ UTR from strain Con1 (genotype 1b) in a selectable bicistronic construct. To demonstrate viral replication, RNA transcripts generated from this genome were transfected into human hepatoma derived cells and the cells with replicating RNA was selected following treatment with neomycin. Subsequent analysis demonstrated that their replication capacity was determined by adaptive mutations of the replicating HCV RNA and by increased host cell permissiveness. Based on findings by Lohmann *et al.* [99].

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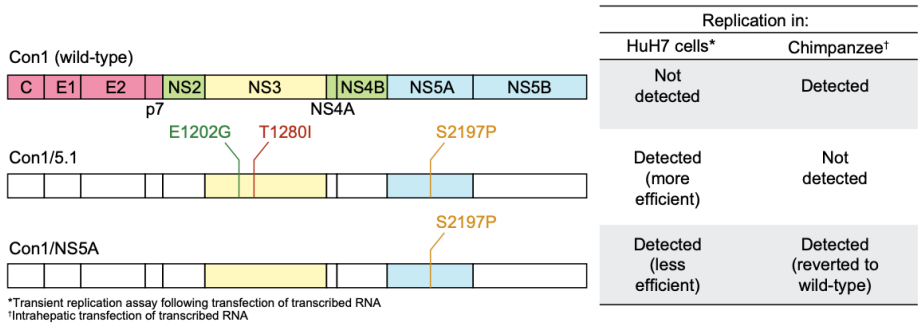

Fig. 4. Replication enhancing adaptive mutations renders full-length HCV clone non-viable *in vivo*. The wild-type Con1 genome was viable *in vivo*, but did not replicate in cell culture. Contrarily, genomes with mutations that permitted replication in cell culture were non-viable *in vivo*. Based on findings by Bukh *et al.* [119].

formation of infectious virions *in vitro* and *in vivo*. After the development of infectious JFH1-based culture systems, however, the use of replicon adaptive mutations yielded infectious cultures of isolates of genotypes 1a, 2a and 3a [121–123]. However, these systems are inefficient with low virus titers and poor capacity to infect naïve cells.

The described replicon systems proved extremely valuable for studies of the role of different HCV genome segments and proteins for HCV RNA replication, of the intracellular localization of HCV proteins, of virus-host interactions, and for testing of therapeutic compounds interfering with HCV replication [124]. Thus, these systems have had a major impact on identifying and advancing candidate DAA [105,125].

Infectious HCV cell culture systems (HCVcc)

The development of a recombinant cell culture system resulting in production of significant levels of infectious virus particles has accelerated understanding of the complete HCV viral life cycle [126] (Fig. 5). In this breakthrough study, Wakita and Bartenschlager's research teams demonstrated that RNA transcripts from the full-length JFH1 genome (genotype 2a) could produce viruses in Huh7 cells [126], and subsequently it was found by Chisari and Wakita's research teams that this system could be adapted to generate relatively high titers of HCV [127]. In a different approach, Rice's research team generated a chimeric genome in which the structural genes (Core, E1 and E2), p7 and NS2 from an infectious clone of 2a strain J6 [87] were inserted into the subgenomic replicon sequence of the JFH1 strain [112], and demonstrated that RNA transcripts from this full-length chimeric genotype 2a genome could produce relatively high titers of HCV [128].

Whereas the J6/JFH1 genome can function without the requirement for adaptive mutations [129,130], the original JFH1 genome requires adaptive mutations for efficient virus production [131,132]. Both systems can be adapted to grow to high viral titers by continuous passage in culture [82]. In addition, culture derived viruses were viable also *in vivo*, as tested in chimpanzees and human liver chimeric mice [133–135] (Fig. 5). Therefore, these culture systems produce viruses that are biologically relevant, although the viruses recovered from animals had a specific infectivity, determined by comparing the infectivity titer with the HCV genome titer, that was 10–100 times greater than virus recovered from infected Huh7 cells. Overall, an important milestone was achieved in 2005 with the development of JFH1-based true cell culture systems that permits classical virological studies, but it was clear that new developments were required to expand the system beyond one virus strain and one type of cell line.

Initial research to expand the infectious culture system to other HCV variants and genotypes took advantage of the unique replication capacity of JFH1. Thus, JFH1-based recombinants comprising 5′UTR-NS2, Core-NS2, NS3P/NS4A, NS4A, NS5A, Core-NS2 plus NS5A, 5′UTR-NS3-protease plus NS4A-NS5A or most recently 5′UTR-NS5A of other HCV genotype strains have been developed; most of these systems depend on specific adaptive mutations [129,130,136–149]. The 5′UTR-NS2, Core-NS2, NS5A, Core-NS2 plus NS5A, and 5′UTR-NS5A systems have been developed for HCV strains of genotypes 1–6; the latter system depended on mutations identified in efforts to develop full-length culture systems (see below). Efforts have been made to adapt the cultures to grow to higher titers, in particular for the Core-NS2 systems, which makes them more relevant in particular for attempts to generate inactivated whole virus vaccine candidates [150]. The chimeric systems have permitted genotype-specific studies of novel antivirals, including human monoclonal antibodies (HMAb) and DAA [137,139,146,151–153]. However, systems for key viral enzymes, in particular the NS5B polymerase, were missing.

In breakthrough studies in 2012, it finally became possible to robustly culture other HCV strains independent of JFH1 elements. Li *et al.* iden-

tified LSG substitutions F1464L (NS3-helicase), A1672S (NS4A), and D2979G (NS5B) [154], which have permitted the development of robust full-length HCV genotype 1a, 2a, and 2b infectious cell cultures thus expanding efficient systems to other genotypes and subtypes [154–157]. The NS4A substitution might help overcome defects in oligomerization of the NS4A protein in non-replicative genomes [158]. Culture systems were developed for the prototype HCV isolates HCV-1, H77 and TN of genotype 1a, and prototype strains J6 and J8 of genotypes 2a and 2b, respectively. The adaptation and efficient growth in culture of the TN genome correlated with resistance to lipid peroxidation [159]. Most recently, Ramirez *et al.* succeeded in developing highly efficient adapted full-length genotype 3a culture systems [160]. Robust infectious HCV cell culture systems for isolates of different genotypes represent valuable tools for studies of the importance of genetic heterogeneity for antiviral therapy and vaccine development. It will therefore be important to also develop robust full-length culture systems of other important subtypes of genotypes 1, 2 and 3, and to succeed in developing such systems for genotypes 4, 5, 6 and 7.

A limitation of the current robust cell culture systems is their dependence on a single cell line, which is a hepatoma cell line known to have numerous genetic anomalies compared with hepatocytes. Such differences could provide data on virus-host interactions that might not be fully biologically relevant. Thus efforts have been undertaken to develop systems depending on cells more closely resembling the hepatocyte, including primary human hepatocyte cultures and hepatocyte-like cells derived from pluripotent stem cells [161–165]. It would be advantageous to identify cell lines approved for vaccine production, e.g., for an inactivated whole virus vaccine. Here, a recent report of Vero cell expressing critical HCV host factors with the capacity to complete the entire HCV life cycle has interest, since Vero cells have been used in vaccine production against other viruses [166].

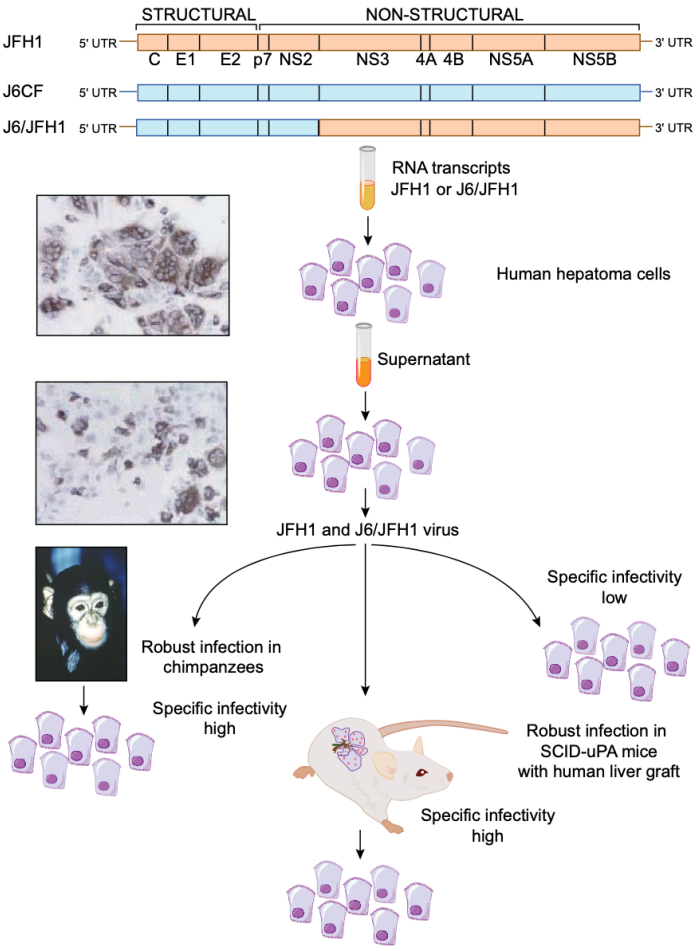

Fig. 5. Principle of the generation of infectious HCV in cell culture, and demonstration of *in vivo* infectivity. Diagram showing the composition of full-length JFH1 and J6/JFH1 genomes. To demonstrate infectivity RNA transcripts generated from these genomes were transfected into human hepatoma derived cells and collected supernatant viruses were used to infect naïve cells. The immuno-staining shown was performed with a mouse monoclonal NS5A antibody. When passaged viruses were transmitted to chimpanzees or SCID-uPA mice with a human liver graft (human liver chimeric mice), the animals developed a productive HCV infection. Compared to culture derived viruses, the *in vivo* derived viruses had a high specific infectivity (ratio of infectivity titer to RNA titer). In part adapted from Bukh and Purcell [135].

Discovery of unique features of the viral life cycle of HCV with relevance for development of host targeting agents (HTA)

An impressive amount of data has been generated on key viral and host elements in the HCV life cycle [124,167,168]. Here it is attempted to merely highlight basic research that has revealed unique features in the viral life cycle with new perspectives for epidemic control. Thus, basic research on HCV has revealed a number of potential molecules for HTA. An example is the novel finding, in 2005, demonstrating that a virus could be completely dependent of microRNA for its replication [33]. Thus, the essential role of miR-122 in HCV is a good example of how basic research revealed novel principles in biology, and thus identified drug targets. It is expected that future research on HCV RNA interactions could reveal other RNA-based drug targets.

Discovery of an essential role of microRNA 122 (miR-122) in HCV replication

The 5′UTR of HCV is a highly conserved region of about 340 nucleotides forming four major structured domains [71,169]. Three domains, including most of the 5′UTR sequence, create an internal ribosome entry site that controls translation of the HCV polyprotein. The fourth domain at the 5′ termini of the HCV genome contains a stem-loop structure

[169], followed by two miR-122 binding sites named S1 and S2 [33]. The stem-loop structure was found to be essential for the viability of infectious HCV genotype 1–6 5′UTR-NS2 recombinants [141]. In a landmark study, Jopling *et al.* discovered that the liver-abundant miR-122 is required for HCV RNA replication [33]. Subsequent studies have confirmed that this microRNA permits viral RNA replication and translation by binding to S1 and S2, as well as to upstream nucleotides at the 5′ end of the HCV genome, and apparently promote RNA production by controlling the relative amount of RNA involved with replication compared to that involved with translation [170–174]. miR-122 associates with host Argonaute 2 to bind the HCV RNA, and through this interaction stabilizes the viral RNA [175] and most likely protects its 5′ end from degradation [176]. In this fashion HCV also has a sponge effect in depleting the host cell for miR-122, which could impact the cell and perhaps even contribute to the oncogenic potential of HCV [177]. Overall, basic research demonstrated that miR-122 was essential for replication of the different HCV variants. Since it binds universally conserved HCV sequences, it was considered an exciting novel drug target.

The drug miravirsen is a locked nucleic acid antisense oligonucleotide that targets and inhibits miR-122 function in liver cells [178]. In infectious cell culture systems miravirsen inhibits HCV genotypes 1–6 [141], and *in vivo* it suppresses HCV genotype 1 chronic infection in experimentally infected chimpanzees and in patients with no or limited evidence of virus resistance [179–181], thus showing potential as a host targeting antiviral drug for HCV therapy. However, in recombinant culture systems it is possible to introduce mutations in the miR-122 binding sites or sequences in close proximity that confer virus resistance to miravirsen treatment [141,182,183]. Miravirsen has been tested in clinical trials with promising results, but at present it is unclear whether it will be developed further for HCV therapy. The finding, however, that a host targeting RNA drug can be developed as an effective antiviral, has wide reaching perspectives in medicine.

Key point

A number of host molecules critical for hepatitis C virus (HCV) entry and replication have been identified. Thus, basic HCV research revealed important molecules for development of host targeting agents (HTA), including microRNA-122, viral receptors and cyclophilins.

HCV co-receptors

The mechanism by which HCV enters the human hepatocyte to initiate infection is not fully known, but a number of molecules with important roles in a complex multistep entry process have been identified [34]. The E1 and E2 glycoproteins are involved in binding to receptors and subsequent fusion with the host cell. An essential receptor is CD81, and its discovery in 1998 represented a major breakthrough. By preparing a cDNA expression library from a cell line with a high capacity to bind recombinant HCV E2 protein, Pileri *et al.* identified the surface expressed CD81 as a binding partner [184]. *In vitro* studies using HCV pseudoparticles, developed in 2003 [185], and infectious HCVcc systems have confirmed an essential role of CD81 for viral entry [186,187].

The identification of the HCV-CD81 interaction led to searches for other putative receptors. Both the identified low density lipoprotein receptor (LDLr) [188–190] and scavenger receptor class B type I (SR-BI) [191] are believed to be involved in early interactions between the host cell and HCV, promoting interactions with late-stage receptor CD81 or identified tight-junction factors Claudin-I and Occludin [192–194]. Virus association with apolipoproteins most likely also has important roles in the entry process [34,195]. Several other molecules have been identified as important host HCV entry molecules, including most recently CD36 that apparently is a co-receptor for HCV E1 protein attachment [196–199]. A combination of multiple receptors is involved in both cell free and cell-to-cell transmission. However, despite these advances in the understanding of the HCV entry process it would be important to get a more complete insight including detailed data on the structural changes to the HCV envelope proteins and cell membranes during this multistep process.

Important proof-of-concept studies were published of the protective potential of antibodies against key host cell HCV receptors [37]. Monoclonal antibodies that block CD81 or SR-BI protected against subsequent HCV challenge with different genotypes in human liver chimeric mice [200–202]. The anti-SR-BI entry inhibitor ITX5061 has been tested in the clinic [203,204], and showed some evidence of reducing HCV RNA titers and viral evolution in patients undergoing liver transplantation [203]. An antibody against Claudin-1 can control HCV in human liver chimeric mice [205]. It is possible that these entry inhibitors will have a role in future HCV therapy [206].

Cyclophilin inhibitors against HCV

Basic research in replicon and infectious culture models revealed that host cell cyclophilins, which affects protein folding, stimulate HCV replication. In addition, inhibitors of this molecule reduce HCV replication [207,208]. Cyclophilins apparently affect several steps of the HCV life cycle including viral assembly; inhibitors thus have multiple modes of action [209–213]. Thus cyclophilin inhibitors have been advanced for testing in clinical trials, but it is unclear whether they will have a role in future HCV therapy.

Features of the viral life cycle of HCV, leading to development of direct acting antivirals (DAA)

The approval of numerous effective DAA for HCV treatment since 2011 represents a huge success for basic and translational research, and the interaction

with the pharmaceutical industry. These drugs are directed against the more classical viral targets, the NS3 protease and NS5B polymerase, as well as a novel viral target, the NS5A protein. Used in combination they can eradicate HCV from patients with chronic HCV in 8–24 weeks of oral treatment [11]. Many other viral targets in HCV has been pursued, including p7, the NS3 helicase and NS4B, but they have not lead to drugs introduced in the clinic for treatment of HCV [161].

Characterization of the NS3 protease and development of the first approved DAA

The amino-terminal part of the NS3 protein has serine-protease activity and has been shown to cleave NS3/4A, 4A/4B, 4B/5A, and 5A/5B junctions of the polyprotein [214]. The protease forms a stable complex with the NS4A protein, and NS4A functions as an essential cofactor in the processing of NS3/4A and NS4B/5A sites, and enhances cleavage at the other sites. In advances of great importance for development of DAA, the crystal structure of the NS3 protease domain and of the protease domain complexed with a synthetic NS4A cofactor peptide were determined [215–218]. Solving the crystal structure gave the possibility of designing specific inhibitors of the enzyme for therapeutic use as inhibitors of viral replication.

Several NS3 protease drugs have been developed and approved for HCV treatment, including telaprevir, boceprevir, simeprevir, paritaprevir and grazoprevir [11]. Basic studies in infectious cell culture systems have shown great variation in their potency against different HCV variants and genotypes, and in their pattern of resistance [137,219–221]; since they all target the protease active site substitutions conferring cross-resistance have been identified [12].

Identification of the HCV NS5A protein as a novel drug target

From the early time in HCV research NS5A has been of great interest for studies on therapy of HCV. The NS5A protein is phosphorylated; protein phosphorylation can regulate protein-protein interactions as well as protein-nucleic acid interactions. A short region of the NS5A protein has been implicated in the modulation of the host IFN-mediated antiviral response. Mutations in this region, called the IFN-sensitive determining region appeared to correlate with the sensitivity of HCV genotype 1b viruses to IFN treatment [222]; NS5A interacts with the IFN-induced cellular protein kinase R (PKR), which could represent mechanisms used by the virus to escape IFNs antiviral activity.

The NS5A protein, consisting of three defined domains, is an essential component of the viral replication complex [223,224]. Another major achievement was the identification of NS5A as a regulator of replication and viral assembly. Reverse genetic studies demonstrated that the NS5A N-terminal amphipathic domain, which anchors this protein to ER membranes [225,226], as well as four conserved cysteine residues, localizing to the NS5A zinc binding site [224], were critical for replication. In addition, crystal structures were determined for the N-terminal domain I [227,228], which has RNA binding capacity [229]. Overall, it has been found that the amphipathic alpha-helix and domains I and II are essential for HCV RNA replication [224,225,230,231]; a recent study showed a critical role for generation of double-membrane vesicles associated with replication [232]. In addition, domain III has a primary role in production of infectious particles with a direct role in coordinating viral assembly [233–235].

Gao *et al.* developed a highly efficient HCV NS5A inhibitor daclatasvir that was found to have high potency against the different HCV genotypes *in vitro* although genotype 3 was less sensitive [146,236]. In addition, great differences in sensitivity were observed even within a single subtype due to differences at individual amino acid residues [146]. This discovery of NS5A as a DAA target paved the way for the development of several other similar NS5A inhibitors, including clinically approved elbasvir, ledipasvir, obitasvir, and velpatasvir [11,160]. They all target domain 1 of NS5A and although their exact mechanism of action has not been determined, it appears that their effect goes beyond merely inhibiting viral replication [237]. They have become a central part of current DAA therapy combinations, but they have a relatively low barrier of resistance [12,13].

Key point

The identification and characterization of HCV encoded proteins and their functional units led to effective antivirals against the NS3 protease, NS5A and the NS5B polymerase. In combination, these inhibitors permitted interferon-free therapy with high cure rates and minimal side effects. However, viral resistance represents a challenge for the continued success of these drugs.

Development of a blockbuster drug against the NS5B polymerase

The NS5B protein of HCV is an RNA-dependent RNA polymerase that has provided important targets for inhibition of viral replication [11,30]. The solving of the crystal structure of the NS5B protein provided critical data of relevance for DAA development [238–241]. Basic research on the NS5B polymerase has thus contributed to the development of highly effective nucleos(t)ide analogs against HCV that have pangenotypic activity, and most importantly with a high genetic barrier to resistance [12,160]. The drug sofosbuvir is the backbone of key approved drug regimens for HCV therapy of different genotypes; the combination of sofosbuvir with the NS5A inhibitor velpatasvir have been reported in clinical trials to be effective against genotypes 1–6 of HCV [242,243]. However, a recent study using culture derived HCV genotype 3a indicated that highly fit sofosbuvir resistant viruses could potentially develop during treatment, however, it remains to be seen whether such escape variants will

develop and spread in humans [160]. Potent nonnucleoside analogs of the NS5B polymerase have also been developed, including beclabuvir and dasabuvir, but they are not active against all genotypes and have a comparatively low genetic barrier to resistance [12].

Unique features of HCV of high relevance for vaccine development, and thus the possibility for worldwide control

Natural infection with HCV does not elicit protection against reinfection, thus posing a great challenge for development of a preventive vaccine. The chronicity rate however is lower after re-exposure, indicating some degree of protective immunity. Experimental *in vivo* studies demonstrated that chimpanzees that resolved their acute HCV infection could again develop hepatitis when re-challenged with the homologous strain [244,245]. However, in most cases rechallenged animals had protection against developing a chronic infection [246–248]. Also, by repeated challenge of a chimpanzee with homologous monoclonal recombinant virus, it was possible to generate sterilizing immunity against homologous challenge with a quasispecies of the parent virus [249]. However, this chimpanzee became infected when challenged with a heterologous virus (a different subtype of the same genotype), and eventually became persistently infected after multiple challenges with heterologous viruses. Furthermore, in an animal with viral clearance after an acute infection even challenge with the identical monoclonal virus resulted in chronic infection [249]. The immunity in challenged chimpanzees was based primarily on host T cell responses since neutralizing antibodies could not be detected [249]. Thus, a protective vaccine need to provide immune responses that exceeds those seen in a natural acute resolving infection, although it would be an acceptable original goal to approve vaccines that will significantly lower the chronicity rate.

Protective immunity of neutralizing antibodies against HCV

Studies using novel HCV *in vitro* systems, including pseudotyped virus particles or cultured infectious viruses, confirmed the existence of neutralizing HCV antibodies with cross-neutralization potential in acute- and chronic- phase patient samples [130,195,250,251], but with differences in the neutralization capacity against viral variants of the same genotype [75,252]. Further, the clearance of HCV during acute infection was found to be associated with the development of neutralizing antibodies [253–255]. During chronic HCV infection, the virus persists despite the presence of high-titer neutralizing antibodies, perhaps because it is shielded from neutralization [143,256–259] or neutralization resistant mutants are continuously developing [73,77,253,260]. *In vitro* viral escape from neutralizing antibodies was demonstrated [261], and studies by von Hahn *et al.* it demonstrated that chronic-phase sera neutralized only variants from time points early in infection, but not later variants [260]. Overall, the data suggested that antibodies from chronic-phase patient samples might have broad protection against hepatitis C, and thus could be used to define critical conserved epitopes for vaccine development [262–265].

It was previously demonstrated in human liver chimeric mice that chronic-phase patient immunoglobulin infused pre-challenge could prevent homologous infection in most animals [266,267]. Subsequently, it was shown that immunoglobulin infused pre-challenge and during weeks 3 and 4 post-challenge could suppress the homologous infection for at least 4 months in the chimpanzee model [268]. Thus, for the homologous strain polyclonal immunoglobulin with *in vitro* neutralizing activity could control viremia *in vivo*. However, the homologous challenge virus was not eliminated, since the virus reappeared when the neutralizing antibodies had disappeared, and then persisted. Thus, as reported [269], polyclonal antibodies given post-challenge could only control HCV infection temporarily in the chimpanzee model.

In contrast, human liver chimeric mice loaded with the immunoglobulin proven to protect against homologous challenge were only partially protected against heterologous viruses [266]. This failure to consistently prevent infection with heterologous challenge viruses was confirmed in the chimpanzee study [268]. Thus, although a chronic-phase antibody sample has similar *in vitro* neutralization titers against homologous and heterologous strains [130,195] there is incomplete *in vivo* protection against the heterologous strains. Thus it will be a challenge to develop an antibody-based vaccine protecting against a wide spectrum of HCV isolates.

The envelope proteins, including the E1/E2 heterodimer, as vaccine antigens

A main objective for developing vaccines expressing the HCV envelope proteins is to generate neutralizing antibodies. The E1 and E2 proteins of different HCV isolates exhibit a very high degree of genetic heterogeneity, in some cases varying by over 40% at the amino acid level. In particular, the N-terminus of the E2 protein is extremely variable and has been designated “hypervariable region 1” (HVR1). The envelope proteins, and in particular HVR1, changes rapidly in HCV-infected patients [73]. The first neutralization epitope was identified in HVR1 [270]. Subsequently, it has become clear that the E1 and E2 glycoproteins contain several linear epitopes and that the E1/E2 complex forms conformational neutralization epitopes [75,259].

The E1 and E2 envelope proteins of HCV are N-glycosylated proteins, and both contain C-terminal hydrophobic domains which function as membrane anchors. E1 and E2 glycoproteins apparently function as heterodimers on the virion surface. Only structures of the central domain of E2 have been determined [271]. Basic studies of the HCV envelope proteins using HCV pseudo-particles and HCVcc have revealed detailed information about their function, which is outside the scope of this review. Importantly, an experimental vaccine produced from expressed envelope glycoproteins of HCV protected chimpanzees from a low dose challenge with the homologous strain, but not from a low dose challenge with a closely related heterologous strain [272]. However, subsequent studies showed that this vaccine lowered the chronicity rate in vaccinated chimpanzees [18,19,273]. This vaccine has been tested in humans and it is safe; it induces neutralizing antibodies with cross-neutralizing *in vitro* potential [262,263,274].

Numerous other envelope based vaccine candidates have been studied, including those using proteins expressed *in vivo* with DNA vaccine approaches [20,275]. Approaches with expressed envelope proteins require modification of the amino acid sequence either for secretion or for surface expression. Whether these modified envelope proteins have the confirmation characteristic of the natural infection remains to be determined. Also, purified HCV-like particles synthesized in insect cells (from a recombinant baculovirus expressing Core, E1 and E2 proteins) have been evaluated as a potential immunogen for vaccine development [20,276]. Finally, the success during the last 10 years in generating culture viruses expressing the envelope proteins of the different genotypes provide the opportunity of evaluating whole virus inactivated HCV vaccine candidates [277,278]. The difficulty in defining universally conserved epitopes for HCV neutralization also highlights the difficulties in developing a broadly protective HCV vaccine. A vaccine might require a combination of critical epitopes/antigens and removal of inhibitory epitopes [279], or it might require induction of antibodies that can overcome the inherent shielding of HCV neutralization epitopes (see below).

Broadly reactive human monoclonal antibodies

The identification and production of novel human monoclonal antibodies (HMAbs) with broadly neutralizing capabilities targeting conserved viral epitopes is highly relevant for HCV vaccine development. In a recent study, it was shown that HCV isolates that were resistant to polyclonal antibodies derived from patients with chronic HCV were sensitive to neutralization by HMAbs, thus indicating that it is possible to generate antibodies with higher efficiency than naturally occurring antibodies [143]. Several HCV-specific HMAbs with clinical potential have been developed. These HMAbs demonstrate neutralizing capabilities *in vitro* and *in vivo*, with the most efficient candidates targeting epitopes on E2 or E1/E2 [151,153]. Synergy was demonstrated among efficient HMAbs targeting different epitopes [280]. It is noteworthy that a humanized monoclonal anti-E2 prevented HCV infection in a virus-challenged chimpanzee [281]. Also HMAbs delayed HCV rebound following liver transplantation [282]. Thus, it is possible that a combination of monoclonal antibodies to several key HCV epitopes might give sustained protection. Recently, it was demonstrated that three broadly reactive HMAbs that was delivered with adeno-associated viral vectors yielded protection against HCV in humanized mice. They also controlled an established HCV infection in human liver chimeric mice [283]. Knowledge about the specificities of neutralization epitopes provided by the use of HMAbs could reveal novel targets for developing HCV vaccines.

Shielding of HCV from neutralization

HCV has a number of unique features that apparently limit the effect of neutralizing antibodies. These include cell-to-cell transmission [150,284], interfering antibodies [279], apolipoprotein E [285], N-linked glycosylation [256,257,286], and a broad neutralization protection mechanism involving shielding by HVR1 [143,259,287–289]. The latter research was spurred by the earlier finding that an HVR1 deleted genotype 1a recombinant was viable *in vivo* [290], an observation later expanded to different genotype recombinants in cell culture [288]. Furthermore, it could be demonstrated that recombinant viruses without HVR1 have markedly increased susceptibility to HCV-specific neutralizing antibodies both *in vitro*, *ex vivo* and *in vivo* [287–289]; this feature does not extend to HVR1 targeting antibodies or to antibodies against virus associated apolipoprotein E [190,259]. This protection by HVR1 was recently found to involve most, if not all, E1, E2 and E1/E2, including conformational, neutralization epitopes [259]. In fact, the large differences in neutralization susceptibility of different HCV variants can in most cases be explained by this HVR1-mediated shielding. Although the various features mediating protection against neutralizing antibodies might cooperate in shielding neutralization epitopes, the possible mechanism needs to be studied further. In order to develop neutralization-based vaccines against HCV it will be important to generate immune responses that can overcome viral shielding [143].

Protective cellular immunity and vaccines

It has been shown in re-challenge studies in chimpanzees that reinfection can be prevented in the

Key point

Worldwide control of HCV will require the development of a prophylactic vaccine, but this effort is a major challenge due the nature of HCV that has unique capability to elude host immune responses. Research characterizing features critical for antibody-based virus neutralization and T-cell based virus elimination from infected cells is essential for this effort.

absence of neutralizing antibodies [248,249,291], thus raising the possibility that a T cell based vaccine can constitute a prophylactic HCV vaccine. Yet it is equally clear that HCV can readily escape strong cellular immune responses [90,249]. An impressive array of studies have defined the components of cellular host responses involved with HCV infections [76,292], including groundbreaking $CD4^+$ and $CD8^+$ T cell depletion studies in chimpanzees that confirmed a critical role of these T cell responses in the control of HCV [293,294]. Thus, the goal of any T cell based vaccine is to induce responses with desirable phenotypes of immune cells. Numerous vaccine approaches to generate T cell responses have been tested for immunogenicity, and in some cases also in available challenge models [20,295]. The most advanced T cell based vaccine candidate, based on adenovirus expressed HCV proteins, is being tested in clinical trials [296,297]. In preclinical studies in chimpanzees using a prime boost regimen to deliver a T cell HCV vaccine encoding NS3-NS5B of an HCV genotype 1b strain, the virological and clinical courses of infection were markedly different between the vaccinated and control animals, following challenge with a genotype 1a virus, with lower HCV RNA plasma levels and lower liver enzyme levels in the vaccinated animals [298]. This observation corresponded with a potent and cross-reactive T cell response in the vaccinated group.

Key point

Hepatitis C virus (HCV) has become a model virus defining new paradigms in virology, immunology and biology. The discovery in HCV research that a virus could be completely dependent on microRNA is a good example of this development.

Perspectives for the future basic research on HCV

Despite the many difficulties in performing experimental studies of HCV it is apparent that since 1989 when the virus was first cloned and sequenced significant progress has been made toward defining the detailed molecular biology of this important human pathogen. The primary objectives for future basic research on HCV are to fully unravel the viral and host factors important for the viral life cycle, thus generating knowledge of interest for further advance in control of HCV, but also identifying novel viral mechanisms of potential importance for advancing other research fields. As such HCV has become an important model virus defining new paradigms in virology, immunology and biology. Future advances would require also the development of efficient full-length recombinant cell culture systems to propagate HCV genotypes 4–7, so the full extent of viral heterogeneity can be appreciated. Long-term it would be important to develop adaptation processes or cell lines that would permit efficient culture of patient isolates; a first step in this development might be the identification of a key host factor, SEC14L2, which when introduced in human hepatoma cell lines permit RNA replication of HCV sequences of different genotypes without the requirement for adaptive mutations.

Basic HCV culture studies of approved DAA's and viral escape are essential in efforts to fully appreciate their potential fitness in patients and thus promote measures to limit spread of resistant variants in the clinic. It would be prudent to continue the basic research to define novel antiviral targets, including DAA and HTA, and to develop antivirals against these targets to be able in the future to combat potential emerging HCV variants resistant to the currently licensed DAA. The identification of a robust immuno-competent small animal model would be of great importance, in particular for studies of protective immunity of vaccine candidates. Given the huge number of new infections annually and the high prevalence of HCV, it will be critical to continue basic research to permit development of an effective vaccine; this research involves further characterization of the virus particle, and expressed surface antigens, as well as knowledge about how to overcome shielding of neutralization epitopes, and further studies of host T cell responses against HCV, including knowledge about how to overcome T cell exhaustion.

With an ambitious approach applying current DAA effectively and broadly, introduction of additional viral- and host- targeted antivirals to combat resistant variants, and with the addition of a prophylactic vaccine, it should be possible to eradicate HCV. But this would require a more determined effort from the world community than has been the case for HBV, where an effective vaccine generated by basic research more than 30 years ago still has not lead to worldwide prevention of transmission, and HBV remain a major contributor to death by chronic liver diseases. In part modelled on the success with HCV, researchers and companies are now pursuing drugs that could cure HBV patients.

Financial support

The author's research is supported by grants from The Region H Foundation, The Lundbeck Foundation, The Novo Nordisk Foundation, The Danish Council for Independent Research (DFF), Medical Sciences and Innovation Fund Denmark (Infect-ERA EU). The author is the 2014 recipient of an advanced top researcher grant from DFF and the 2015 recipient of the Novo Nordisk Prize.

Conflict of interest

The author declared that he does not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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