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REVIEW

Hepatitis C virus cell culture models: an encomium on basic research paving the road to therapy development

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Abstract

Chronic hepatitis C virus (HCV) infections affect 71 million people worldwide, often resulting in severe liver damage. Since 2014 highly efficient therapies based on directly acting antivirals (DAAs) are available, offering cure rates of almost 100%, if the infection is diagnosed in time. It took more than a decade to discover HCV in 1989 and another decade to establish a cell culture model. This review provides a personal view on the importance of HCV cell culture models, particularly the replicon system, in the process of therapy development, from drug screening to understanding of mode of action and resistance, with a special emphasis on the contributions of Ralf Bartenschlager's group. It summarizes the tremendous efforts of scientists in academia and industry required to achieve efficient DAAs, focusing on the main targets, protease, polymerase and NS5A. It furthermore underpins the importance of strong basic research laying the ground for translational medicine.

Keywords Hepatitis C virus · HCV · Hepatocyte · DAA · Therapy · Antiviral · Cell culture · Replicon · Genotype · Bartenschlager · Adaptation

The history of HCV: from non-A, non-B hepatitis to discovery of the virus

Hepatitis C virus research, from identification of the virus to the development of efficient cell culture models, has been difficult from the start. The presence of a parenterally transmitted infectious agent causing hepatitis apart from hepatitis A and B viruses was postulated already in the 1970s after establishment of efficient hepatitis A and B virus diagnostics [1–3]. This assumption was based on many cases of post-transfusion hepatitis cases with unknown etiology designated non-A, non-B hepatitis [4]. The infectious agent was readily transmittable to chimpanzees and filtration experiments suggested a small, enveloped virus as responsible infectious agent. However, conventional cell culturing, and antibody- and antigen-detection technologies failed to identify the virus [5, 6]. After more than a decade of intense efforts by Michael Houghton and his team at Chiron, HCV was identified by probing a shotgun cDNA expression library obtained from the serum of an experimentally infected chimpanzee with patient antisera [7]. Later, it was verified that this agent indeed was associated with most cases of transfusion-associated non-A, non-B hepatitis and efficient diagnostic tools were established within relatively short time, preventing further iatrogenic spread of HCV by blood transfusions and blood products [7, 8]. Indeed, the availability of efficient diagnostic procedures dramatically decreased the rate of new infections and had a major impact on public health, particularly regarding the safety of transfusion and blood products [9]. The following phase of intense research was focused on optimizing diagnostics, studying disease in chimpanzees, attempts to establish replication of HCV in cell culture using patient specimens and development of tools to clarify molecular aspects of virus biology including the functions of distinct viral proteins, as described in the next section.

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HCV replication cycle, consequences for therapy development, and identification of targets

The discovery of HCV and the availability of its genomic sequence did initially not permit propagation of the virus in cell culture, which remained extremely inefficient, although now sensitive and specific means of detection were available (reviewed in Ref. [10]). Nevertheless, simple sequence comparison revealed some basic insights into certain aspects of virus biology, albeit not in the detail provided in Fig. 1: HCV was recognized as a positive-strand RNA virus, and it was grouped into the family of *Flaviviridae*, building its own genus named Hepacivirus. The closest known relatives at that time were Pestiviruses, animal pathogens including bovine viral diarrhea virus (BVDV) and classical swine fever virus [11]. The replication cycle of positive-strand RNA viruses is exclusively cytoplasmatic and RNA synthesis involves a negative-stranded RNA replication intermediate. However, it lacks any stable DNA forms in contrast to retroviruses

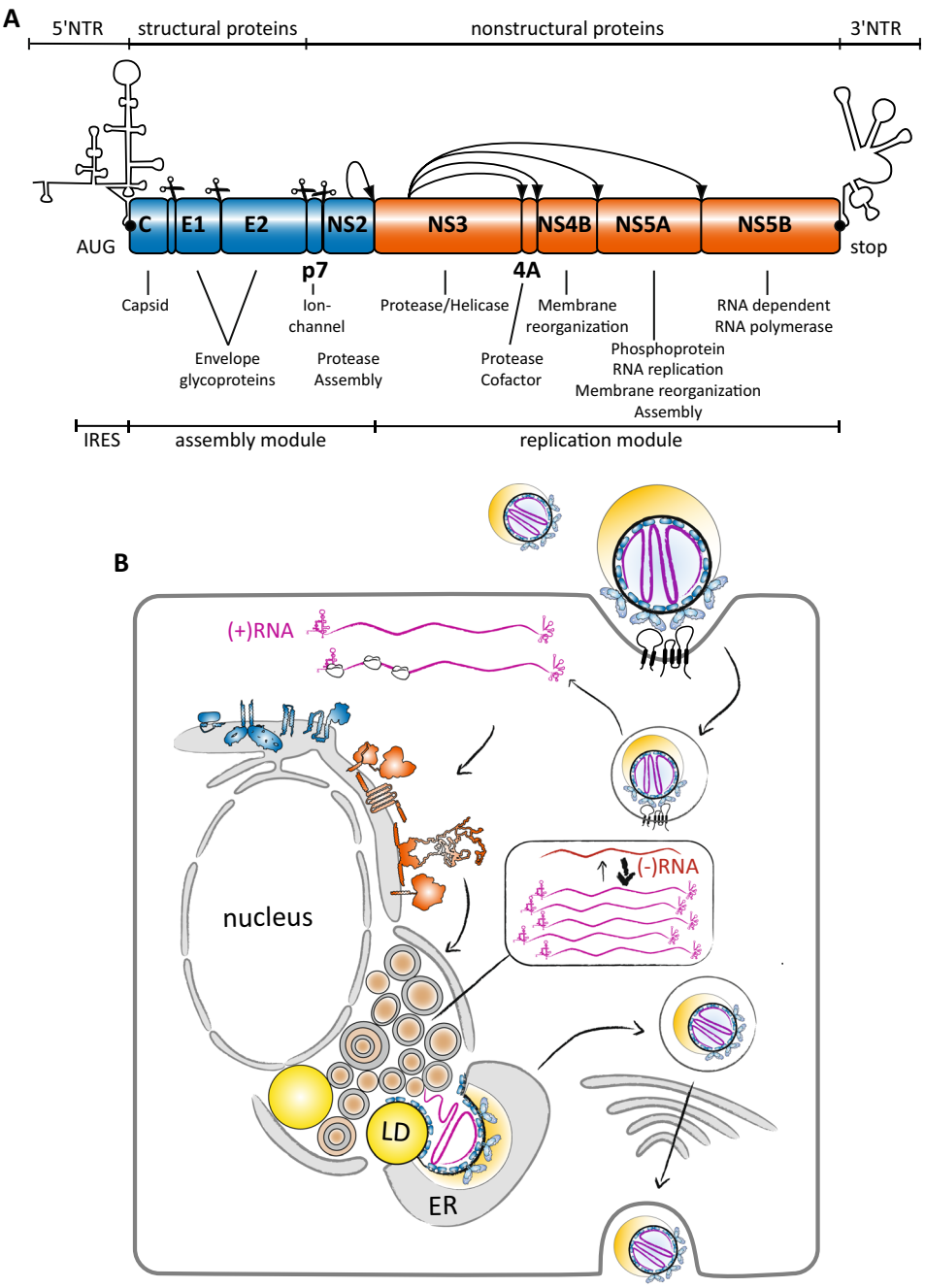

Fig. 1 HCV genome organization and a simplified view on the replication cycle. **A** HCV genome organization. The 5′ and 3′ NTRs are indicated by their proposed secondary structures, coding regions are given as boxes. The polyprotein, the cleavage sites for viral (arrows) and cellular (scissors) proteases and the functions of the cleavage products are indicated. **B** HCV replication cycle. Schematic representation of virus particles and an infected hepatocyte and with indication of nucleus, endoplasmic reticulum (ER) and Golgi apparatus. Virus entry by receptor-mediated endocytosis as well as the release of the viral positive-strand RNA genome (red line) into the cytoplasm is shown. The RNA is translated at the rough ER and viral proteins, indicated by their proposed structures, induce formation of membrane alterations, the so-called membranous web [234, 235], consisting mainly of double- and multi-membrane vesicles [236], associated with lipid droplets (LD). Packaging of viral genomes into virions in proximity to LDs is indicated as well as non-cytolytic release of virus particles by the secretory pathway. Synthesis of viral RNA takes place in association with the membranous web and results in a surplus of positive-strand genomic RNA, probably mediated by a double-stranded replication intermediate (not shown). Virions are shown as so-called lipo-viroparticles, in association with very low-density lipoproteins [155]

(integrated proviral DNA) or hepatitis B virus (cccDNA), which represents a major hurdle for curing infections by these latter pathogens. Positive-strand RNA viruses typically cause rather acute than persistent infections, since they have to continuously replicate to maintain themselves in an infected host, constantly generating antigens, thereby being an "easy" target for adaptive immunity. HCV is a major exception from this rule by causing persistent infections in 70% of infected individuals [12]. Still, it was clear based on this biology that HCV was a curable infection, if efficient directly acting antivirals (DAAs) would be developed. In addition, RNA synthesis of positive-strand RNA viruses essentially relies on a virus-encoded RNA-dependent RNA polymerase (RdRp), an enzymatic activity not found in host cells, therefore providing an obvious target for antiviral therapy, likely with sufficient specificity to avoid side effects. The bad news was that these enzymes lack proof-reading activity, rendering RNA replication an error-prone process, generating on average one mutation per replication cycle [13]. Indeed, HCV represents a very heterogeneous group of viruses, which is subdivided into seven or even eight genotypes and multiple subtypes, differing more than 30% on nucleotide level [14, 15]. The viral heterogeneity is particularly high in the envelope proteins, representing a major hurdle for vaccine development and in part explaining, why a protective vaccine still is not available. The high genetic variability also had several direct implications for drug discovery and development: (1) it would be challenging to get single drugs covering all genotypes with comparable efficiency; (2) cell culture models or protein models needed to cover the genetic variability as close as possible to obtain broadly acting drugs; (3) a single drug would most likely not be sufficient to cure HCV infections due to the risk of selecting for mutations conferring resistance. Another consequence of the discovery of HCV was substantial knowledge about the viral genome organization that could be directly deduced from the sequence or from comparison with related viruses: HCV contains one large open reading frame, flanked by short non-translated regions, encoding one polyprotein of 3000 amino acids in length. All related *Flaviviridae*, therefore, encode at least one protease required for cleavage of parts of this polyprotein, which was the second obvious and specific target for drug development.

In the absence of a cell culture model, research efforts focused on the characterization of cloned viral sequences. The sequence of an almost complete genome of the initial isolate [7], termed HCV-1, as well as several other isolates with high homology (HC-J1) [16] followed shortly after the identification of HCV. A longitudinal study on patient H in Bob Purcell's group, spanning serum samples from 1977 to 1990, already revealed a high mutation rate of the virus [17]. The 1977 isolate of this patient, designated H77, later build the backbone of the first infectious clones [18, 19]. Around the same time, sequences with higher divergence were identified as well, particularly in Japanese patients (HC-J4, JH-1 [20, 21]). These initial subtypes represented the prototype sequences of viral genotypes 1a and 1b, respectively, which are worldwide most prevalent [11, 22]. By 1993, already six genotypes and multiple subtypes had been identified and a system for nomenclature was proposed, which is in principle still in place [23, 24]. Some of these genomes were regarded as full length and contained a polyU stretch of variable length at their 3′ end, not knowing that an essential sequence was missing [25, 26]. The first steps in understanding the biology of the virus and identifying distinct drug targets relied on sequence comparisons and protein expression, studying polyprotein processing and functions of individual cleavage products [27]. Thereby, the distinct functions of most polyprotein cleavage products could be proposed and verified, such as the protease activities of NS3 [28–30] and NS2 [31–33] in 1993, the NS3 co-factor function of NS4A in 1994 [34–36], the ATPase and helicase activity of NS3 in 1993 and 1995, respectively [37, 38], the phosphorylation of NS5A in 1995 [39, 40] and the polymerase activity of NS5B in 1996 [41, 42]. These discoveries allowed the setup of first high-throughput screening assays aiming to identify specific inhibitors of enzymatic functions, such as protease and polymerase activity [43]. In the late 1990s, pharmaceutical companies had already identified a number of candidate inhibitors. These molecules were discovered by in vitro assays using heterologously expressed and purified viral enzymes, and they targeted particularly the NS3-4A protease (first patents submitted 1996) and the NS5B polymerase. However, the next step in the drug development process essentially required a functional testing of antiviral efficiency in cell culture to ensure that a drug would indeed reach and inhibit its target in an authentic replication setting, before it could be moved forward to more expensive tests in animals. This hurdle could be partly overcome in case of NS3-4A by the use of cell-based assays analyzing polyprotein processing or by inserting the enzymatic activity into the genome of other viruses, e.g. poliovirus [44]. Such strategies were not possible for the NS5B polymerase, where no cell-based assays were available and only related viruses such as BVDV could be used as surrogates [45], bearing the risk to move forward with the wrong drugs. Therefore, any search for inhibitors of proteins with unknown functions or functions not accessible to in vitro assays were out of reach, thereby, e.g. missing NS5A inhibitors, which are today a key component of all HCV DAA-based therapies. In addition, the lack of a cell culture system allowing reverse genetics severely limited mechanistic studies; for example, assigning functions to proteins lacking enzymatic activities such as NS5A or proving functional roles of cellular proteins in viral replication was not possible at all.

The first infectious clones: trial and error

Initial efforts to establish cell culture models for HCV focused on experimental infection of all types of cultured cells including primary human hepatocytes using patient sera as a source of infectious virus (reviewed in Ref. [46]). All studies reporting successful replication had to rely on very sensitive RNA detection methods. "Replication" levels in these models never reached input amounts; therefore great care had to be taken to dissect RNA from input virus from newly replicated genomes. For this reason, detection of negative-strand RNA seemed the method of choice. This is the only molecule species exclusively produced during viral RNA replication, which cannot be provided just by entry and translation of incoming genomic RNA. However, it turned out that negative-strand detection by RT-PCR tended to false-positive results in the presence of high amounts of positive-strand RNA [46]. Several methods were developed to increase the specific detection window for negative-strand RNA [47, 48] and with the help of those replication of HCV could be shown convincingly at least in primary human and chimpanzee hepatocytes [47]. Another approach to demonstrate bona fide HCV RNA replication in cell culture relied on the use of inhibitors of viral replication, at that time primarily interferon alpha. Anyhow, in vitro infection of cells with patient specimens never provided a robust cell culture model for HCV research [46]. This was a major roadblock for many years, severely hampering basic research and drug development. Therefore, starting with the first cloned full-length genomes, tremendous efforts aimed to generate a reverse genetics model for HCV. The principal setup of such a model followed the strategies established for other positive-strand RNA viruses [49] (Fig. 2): a full-length viral genome is cloned under transcriptional control of a phage promoter. In vitro transcription should generate RNAs which are in principal identical to viral genomes, if transcription is directed to initiate and terminate at the appropriate sites. Transfection of the in vitro-transcribed viral genomes should initiate a regular replication cycle (compared to Fig. 1B, after release of viral RNA to the

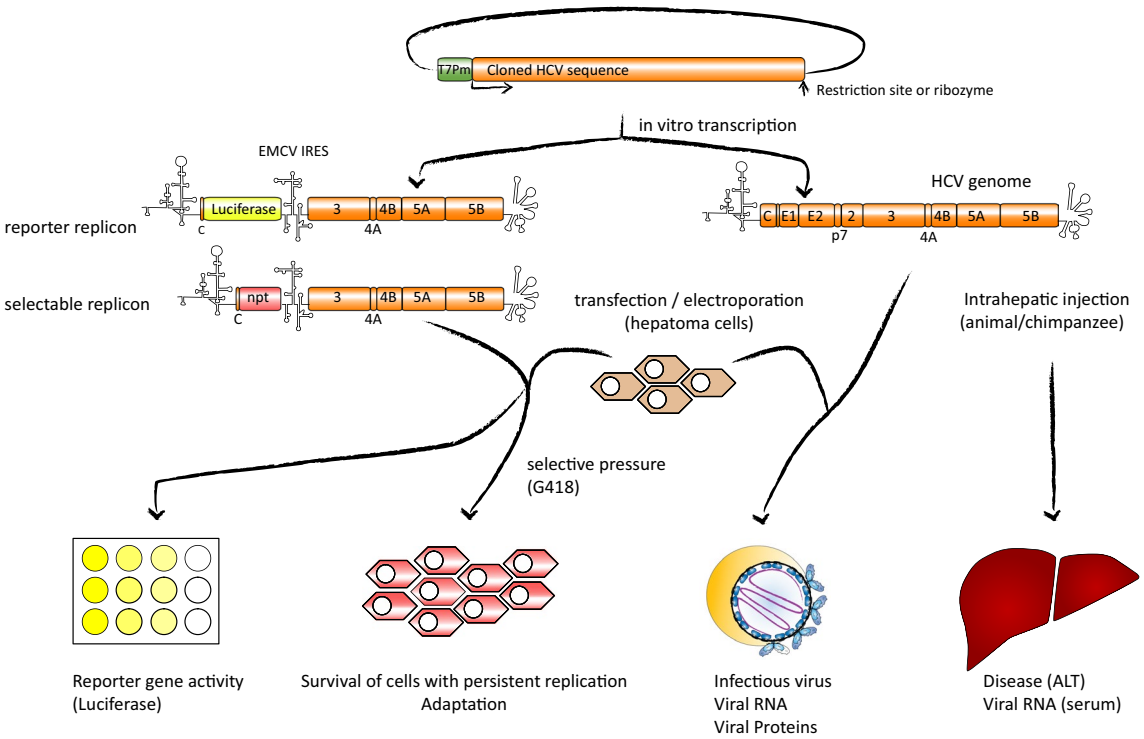

Fig. 2 Experimental strategies to establish cell culture models for HCV replication based on cloned viral isolates. Viral consensus genomes are cloned under transcriptional control of the T7-RNA polymerase promoter into a plasmid and amplified in bacteria. T7-RNA polymerase allows the generation of authentic viral genome copies by in vitro transcription. After transfection of in vitro-transcribed RNA into permissive cells or intrahepatically, full-length genomes should give rise to the generation of infectious virus or disease, respectively (right half). The viral genome can, furthermore, be manipulated by including reporter genes (e.g. firefly luciferase) or selection markers (e.g. neo), allowing detection of viral replication by reporter assays or selection, the latter resulting in cell clones with persistent viral replication (left half)

cytoplasm) and finally result in the release of infectious virus. The viral genome contained in a plasmid, furthermore, allows almost unlimited amplification of the viral sequence in bacteria, as well as any kind of modification, e.g. by inserting reporter genes or selection markers to facilitate detection.

Establishment of a cell culture system based on a cloned viral isolate without having an in vitro infection model, as in case of HCV, was without precedence and finally took almost a decade. The major difficulty was the lack of knowledge of which cells would be capable of supporting HCV replication. However, none of the above-mentioned strategies could work before the very 3′ end of the viral genome was identified. The precise sequence of the 5′ end of the HCV genome was already identified in 1991 [50]. It took another 5 years to determine the very 3′ end of the genome [51, 52]. One year later, intrahepatic injection of RNA transcripts (Fig. 2) derived from two cloned viral genomes, both derived from isolate H77 (genotype 1a), indeed gave rise to an HCV infection in chimpanzees using standard diagnostic markers as readout [18, 19]. This was a clear proof that the cloned HCV sequence of isolate H77 was functional and capable of initiating the whole viral replication cycle in an animal. Another important conclusion from these studies was the need to generate a consensus sequence based on multiple clones from one patient, to avoid potentially deleterious mutations present in individual clones, a strategy which had been established first for other positive-strand RNA viruses such as classical swine fever virus [53]. After this initial breakthrough, a cell culture model based on these infectious clones seemed in near reach, but it turned out later that the H77 isolate was not capable of replicating in cell culture [54, 55].

The first replicon model: a long breath

It took until 1999 to establish the first robust cell culture model for HCV, based on so-called subgenomic replicons [56], substantially moving the field forward. The establishment of this system is tightly linked to Ralf Bartenschlager's name and required a combination of diligence, expertise, precision, endurance, persistence, frustration tolerance and luck.

Ralf started working on HCV already in 1991 as a postdoc at Hoffmann–LaRoche in Basel. Within 2 years, he managed to clone two almost complete genomes from the serum of HCV-infected patients by RT-PCR. This was a major challenge due to the limited availability of sequence data for primer design, the highly variable quasi-species nature of the virus, the lack of knowledge on the geographic distribution of HCV genotypes and the far less advanced PCR techniques (PCR was established only in 1988 [57]).

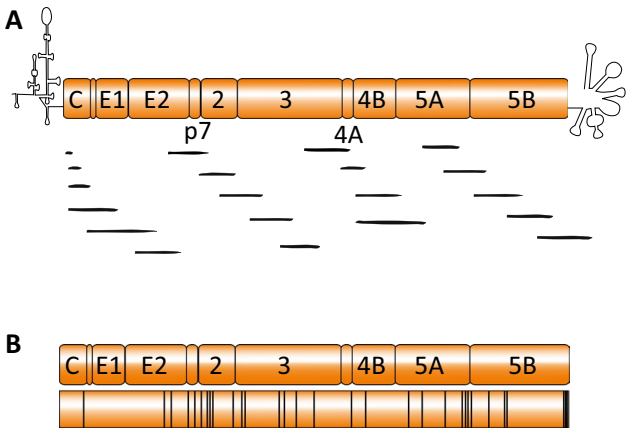

Fig. 3 Comparison of a non-consensus genome composed of multiple individual PCR fragments and deviations from the isolate-specific consensus sequence, which was determined later. **A** Short, overlapping fragments generated by RT-PCR from the serum of a patient were cloned and assembled to a non-consensus RB01 genome. **B** Determining the consensus sequence of the RB01 sequence from the liver of the same patient revealed a total of 30 amino acid deviations (modified from [64])

These genomes were assembled from short, individually cloned PCR fragments (Fig. 3A), a strategy bearing the risk of introducing mutations inactivating yet unknown protein functions. Later, one of the two genomes, termed RB01, was randomly chosen and built the ancestor of all subsequent studies, including the successful replicon approach. And here luck comes into place: the RB01/Con1 isolate belonged to genotype 1b, which is the most prevalent in central Europe and Japan, whereas genotype 1a is more prevalent in the US. We now know that most genotype 1b isolates can efficiently replicate in cell culture by acquiring only one replication-enhancing mutation, whereas genotype 1a isolates, including all original US prototype genomes, as well as all other HCV genotypes require several mutations (see next section on adaptation). Therefore, the replicon approach, albeit tried hard by several teams, could not succeed based on genotype 1a isolates as used by many US scientists, although these genomes worked in chimpanzees; so it was mere coincidence that Ralf started his work on HCV with sera from German patients, but this was an essential prerequisite for the later breakthrough.

After cloning of the genomes, Ralf spent most efforts on the characterization of polyprotein processing, which was accessible by protein expression systems. He was among the first to identify the NS3 protease [30] and NS4A [34] as a co-factor for efficient protease activity. He further characterized the kinetics of polyprotein cleavage and defined the sequence determinants of the NS3-4A cleavage sites [58, 59]. This work had a strong translational aspect, since inhibitors of NS3-4A are basically derived from peptides mimicking the cleavage site (reviewed in Ref. [60]).

In 1993, Ralf moved back to academia and was recruited as a junior group leader at the Institute for Virology in Mainz, where he setup a small team consisting of three Ph.D. students, including myself, and a technician. Besides his work on the protease, his main focus was the establishment of a cell culture model, which was my primary task. We exactly followed the approach depicted in Fig. 2 and first completed the genomes by adding NTR sequences taken from literature, verifying the sequence of the complete genome by conventional Sanger sequencing (this took already 3 months at that time), positioning the T7 promoter at the 5′ end and a restriction site at the 3′ end to ensure authentic genome termini. Since we aimed to determine bona fide replication by the detection of negative-strand RNA, which is the only product in the replication cycle that cannot be generated from the input RNA (in contrast to viral proteins and virions), most time was spent on the establishment of a robust detection method to discriminate positive- and negative-strand genomes. This was finally achieved by high RT-temperatures and a tagged primer approach [47]. In addition, we had to ensure that the in vitro transcripts transfected into target cells were free of contaminating plasmid DNA, which required repetitive cycles of DNase digests and phenol extractions. Despite these efforts, our first experiments failed to show any sign of HCV replication in a various hepatoma cell lines, which was judged by a direct comparison with a negative control harboring a deletion in the polymerase gene NS5B (ΔGDD). At that time, the HCV genome was supposed to end with an oligoU stretch [7]. However, all related viruses in the *Flaviviridae* family had a highly structured 3′ end essential for initiation of RNA synthesis. Therefore, we and many others assumed that an essential piece was missing and I spent a couple of months on the identification of additional sequences, by affinity purification of viral genomes from infected liver and ligation of a synthetic oligonucleotide to the 3′ end, followed by RT-PCR on this sequence. Indeed, we found a sequence of 37 nucleotides following a short polyU/C tract, similar to cellular Alu-elements [61]. However, ultimately we learned that our sequence was not correct and a non-specific amplification product when the true sequence was published at about the same time in late 1995 by Charlie Rice's and Kunitada Shimotohnoh's groups [51, 52]. But also addition of the newly identified, correct 3′ end did not render our isolate replication competent. We next envisaged potential errors in our cloned genome as the most likely problem and focused on the NS5B polymerase as the main driver of RNA synthesis. We realized that our NS5B sequence deviated at ten positions from a genotype 1b consensus sequence derived from a comparison of all published isolates. But again, mutating these positions to the consensus sequence did not result in a replication competent genome. In 1996 then the first successful purification of heterologously expressed NS5B polymerase was published by Raffaele De Francesco's group [41] and we were keen to test our sequence for enzymatic activity as a final quality control. We indeed found that our consensus NS5B was active and although this was a positive result, we had no idea how to continue with the replication model. Since we were the first academic group succeeding in purifying NS5B and the first at all to establish affinity purification of a tagged NS5B, we instead put more energy in the characterization of sequence determinants of polymerase activity, optimal reaction conditions, etc. [42, 62, 63], which was an important contribution to the development of NS5B inhibitors. The enthusiasm on the replication model only returned after Charlie Rice's and Jens Bukh's groups reported the successful infection of chimpanzees by intrahepatic injection of in vitro transcripts from cloned full-length genomes in 1997 [18, 19].

In contrast to us, both groups relied on isolate-specific consensus sequences, to exclude unwanted deleterious mutations. Since this was a possible cause explaining the failure of our experiments, we determined the consensus sequence of the RB01 genome by sequencing repetitively cloned long RT-PCR fragments amplified from total liver RNA of the respective patient. Sequencing and cloning of this consensus genome took another year. Compared to the previously used non-consensus isolate cloned from the serum of the same patient, this so-called Con1 (consensus 1) genome differed at roughly 30 amino positions, which indeed had an impact on NS5A phosphorylation as an indication for improved functionality of the consensus sequence [56, 64] (Fig. 3B). However, although it was shown later that this Con1 clone was indeed infectious in chimpanzees [65], we again could not find evidence for replication in cell culture using unmodified full-length transcripts and RT-PCR-based measurement of viral negative-strand RNA compared to a replication-deficient mutant. This method was and still is technically error prone, particularly due to possible plasmid DNA contaminations carried over from in vitro transcription [61] (Fig. 2). In addition, the input RNA was detectable for weeks in the transfected cells, thereby potentially masking low levels of replication. However, we were not the only group facing such negative results. I still remember quite well a general feeling of depression in the field following the HCV conference 1998 in Venice, when it became clear that nobody had success in setting up a cell culture model, despite the availability of cloned viral genomes with proven replication competence in animals.

In search for strategies possibly enhancing replication efficiency and facilitating detection of replication, which we finally thought was the major problem, we came across with the idea to construct subgenomic HCV replicons for two reasons. First, naturally existing deletion variants of related pestiviruses replicated much more efficiently than their full-length counterparts due to the reduced genome

size [66]. Second, work with the flavivirus Kunjin demonstrated the feasibility of bicistronic selectable replicons in case of a related flavivirus [67]. By taking these lessons from other members of the *Flaviviridae* into account, we generated selectable subgenomic replicons based on the Con1 sequence in early 1998 (Fig. 4A). We deleted all genes most likely not involved in RNA replication (Core-NS2), with exception of the N-terminal coding sequence of core, which we found necessary for efficient translation of foreign genes [56]. This region was replaced by a reporter gene, e.g. firefly luciferase, or a selectable marker, e.g. the gene encoding neomycin phosphotransferase, conferring resistance to the cytotoxic drug G418, thereby allowing identification of HCV replication events by the appearance of G418 resistant cells. Translation of the non-structural proteins NS3–NS5B was mediated by insertion of a heterologous EMCV IRES. An alternative replicon variant was generated starting with NS2, in case a proteolytically processed N-terminus of NS3 was required (Fig. 4A), as it was shown for the polymerase of Sindbis virus [68]. Replication of these RNAs was tested by electroporation of in vitro transcripts into different cultured cell lines followed by measuring reporter gene activity or selection with G418 (Fig. 2), using a replication-deficient mutant as negative control. We first played around with the luciferase replicons for a couple of months, providing some promising results in Huh-7 hepatoma cells. However, the replication levels were not robust enough to be fully convincing. We, therefore, continued with the selectable marker gene and again only in case of the human hepatoma cell line Huh-7 the strategy indeed succeeded. In late 1998, we transfected Huh-7 cells with our selectable replicon and upon drug selection obtained a low number of surviving cell colonies, which could be expanded to cell clones [56]. Albeit clone formation efficiency was extremely low (1 colony/10^{11} in vitro transcripts, Fig. 4B), the HCV RNA and antigen levels in the surviving cells were surprisingly high and well detectable by standard methods such as northern blot (Fig. 4C). HCV RNA was generated in the presence of actinomycin D and no HCV encoding DNA (e.g. from residual cotransfected plasmids) could be detected in these cell clones, assuring that RNA synthesis was indeed due to autonomous replication and not due to cellular transcription [56].

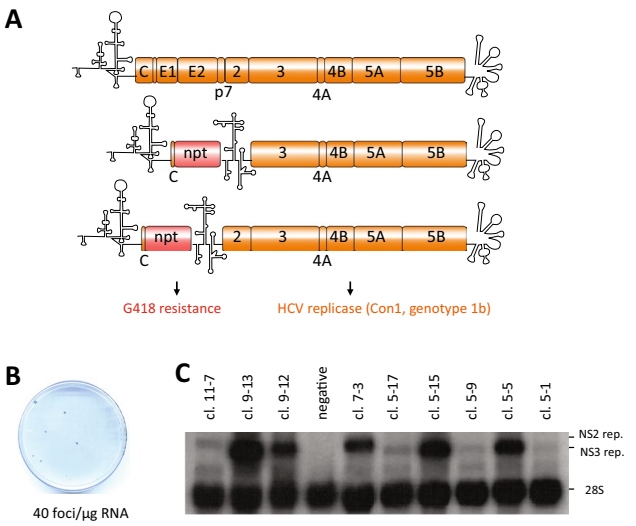

Fig. 4 Structure of selectable subgenomic replicons and properties of Huh-7-based replicon cells [56]. **A** Structure of the HCV genome and subgenomic replicons derived thereof. C, aminoterminal core-coding sequence required for optimal function of the HCV IRES, EMCV IRES, internal ribosome entry site of the Enteromyocarditis virus; *npt* gene encoding neomycin phosphotransferase. **B** Cell culture dish with colonies obtained after transfection of 10 µg of replicon RNA into Huh-7 cells, corresponding to 10^{12} RNA molecules, and selection with G418. Colonies are stained with Coomassie brilliant blue, a typical colony number after G418 selection is given relative to 1 µg transfected replicon RNA. **C** First northern blot analysis of total RNA from persistent replicon clones compared to a Huh-7 cell clone based on a replication-deficient replicon (negative). The position of the replicon and 28S RNA is indicated on the right. Note the slightly increased apparent molecular weights of clones 11-7 and 7-3, based on NS2-3′ replicons

Selected replicon cell clones showed high level, persistent HCV replication in almost all cells of a given population, which was kept for years of culturing by constant selection with G418 [69]. Selected replicon cell clones were not applicable to reverse genetic studies because this required the possibility to alter the HCV sequence, which was not possible after establishment of a cell clone. However, replicon cell clones were the optimal tool whenever a constantly high level of replication was required without the need to manipulate the HCV sequence. This held true for studies on viral replication (e.g. [70–74]) and virus–host interactions (e.g. [75–77]). In particular, the replicon system was the long sought robust cell culture model for screening and evaluation of antiviral drugs (Fig. 7A) (e.g. [78–81]). The suitability of this system for drug development was evident from the beginning and indicated by the fact that HCV replication in replicon clones was readily inhibited by interferons [54, 82, 83]. Furthermore, subgenomic replicons contained all major viral drug targets and seemed also suitable for the validation of host targets (Fig. 5). The replicon model was the first cell culture model for HCV allowing a stringent evaluation of candidate compounds obtained from in vitro assays against the viral protease NS3 and polymerase NS5B, which were already available at that time [43, 84]. Based on encouraging inhibition efficiencies obtained in replicon cells, many of these compounds were further developed to be tested in clinical studies [85].

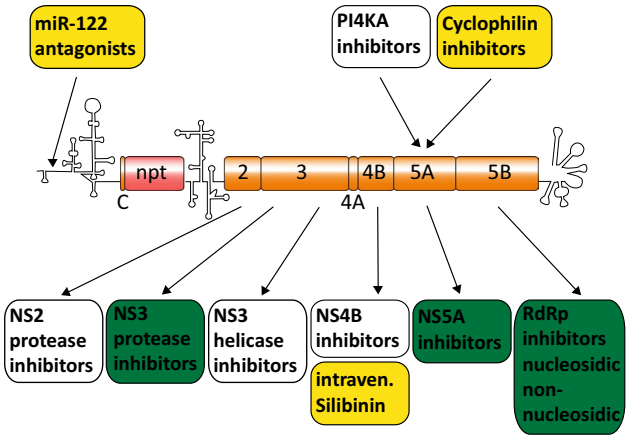

Fig. 5 Schematic representation of a subgenomic selectable replicon and targets for antiviral therapy. Classes of drugs approved for therapy are given in green boxes, classes of drugs with clinical proof of concept are in yellow boxes and compound classes with only preclinical candidates in white boxes. Above the replicon are host targeting antivirals, below the replicon direct acting antivirals. For closer explanation regarding the mode of action of the antiviral drugs refer to the text or Ref. [166]

Understanding the system: adaptation and host factors

In contrast to the high HCV replication rate in selected replicon cell clones, the efficiency with which these clones were established was originally extremely low (Fig. 4B) and it took again years to understand the underlying mechanisms. Two determinants were envisaged that could account for this striking discrepancy: (1) the Con1 wildtype isolate might replicate poorly in Huh-7 cells and had to acquire adaptive mutations that enhanced replication efficiency to a level that was sufficient to confer resistance to G418 selection. (2) Only a minor subpopulation of the Huh-7 cells supported persistent high-level HCV replication which was robust enough to permit cell growth under the applied selective pressure. Finally, it became clear that both mechanisms contributed to the efficiency of colony formation and HCV RNA replication [86].

First it was shown by us and Charlie Rice's group, also using the sequence of our isolate, that selected replicon cell clones had acquired conserved mutations not present in the

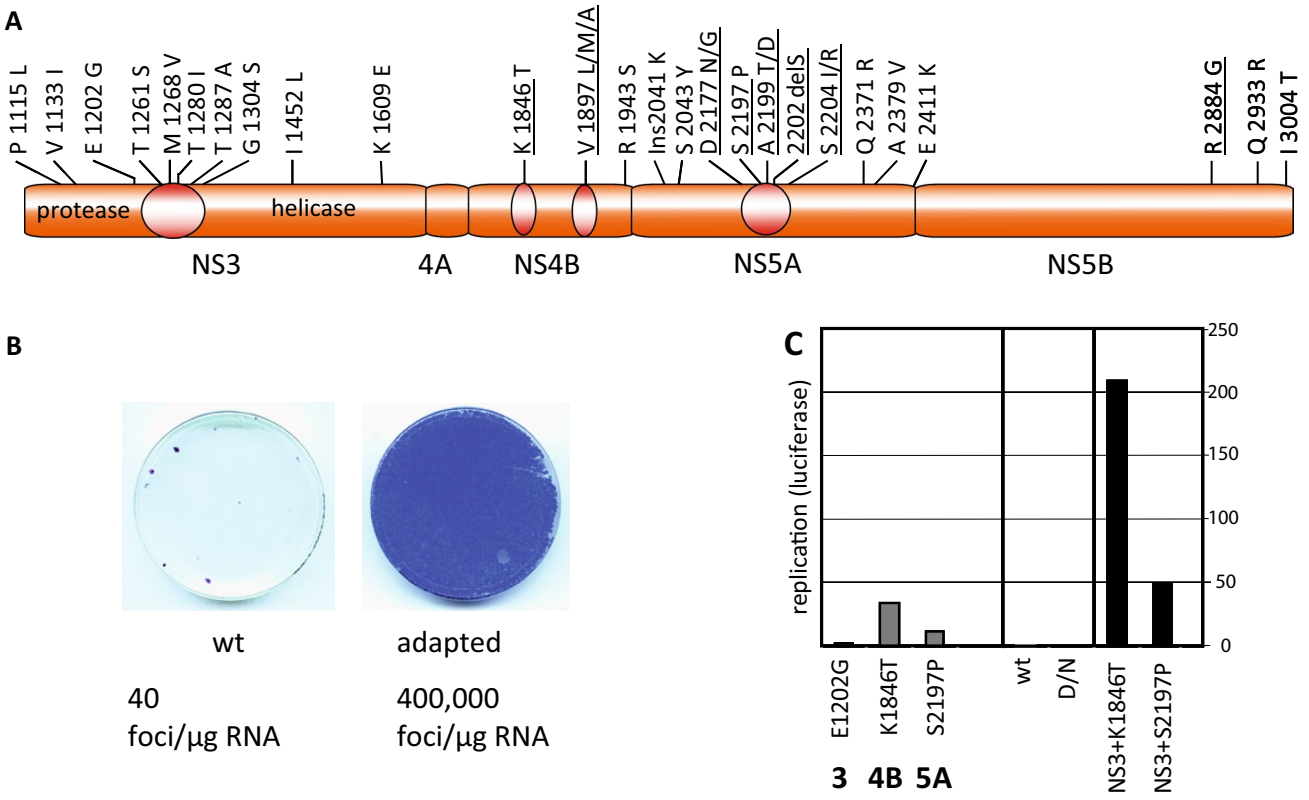

Fig. 6 Position and function of replication-enhancing mutations. **A** Schematic representation of conserved mutations identified by sequencing of viral genomes in 26 independent replicon cell clones relative to their position in the NS3 to NS5B polyprotein [86]. **B, C** Impact of individual and combined adaptive mutations on **B** colony formation efficiency, given as the number of G418 resistant foci per µg transfected replicon RNA [88] or **C** luciferase expression 48 h relative to 4 h after transfection of corresponding replicons [86]. Numbers refer to amino acid positions in the Con1 polyprotein. The initial letter refers to the amino acid of the wildtype sequence; the letter following the number is the replication-enhancing variant. *D/N* replication-deficient point mutant

original replicon sequence (Fig. 6A). When these mutations were transferred back into the wt Con1 replicon, the efficiency of colony formation increased dramatically (Fig. 6B; [54, 86–88]). Such adaptive mutations were identified in almost all non-structural proteins of the Con1 isolate, with particular clustering at the N-terminus of the NS3-helicase, within NS4B, in the center of NS5A and at the C-terminus of NS5B (Fig. 6A). NS5A at that time was a cryptic phosphoprotein with unknown function, which existed in a basal and a hyperphosphorylated variant. The adaptive phenotype correlated to some extent with reduced amounts of hyperphosphorylated NS5A [54, 76, 89]; therefore, it was speculated that basally phosphorylated NS5A was required for replication. However, we needed more than 15 additional years to understand the true and complicated mode of action of these mutations in detail: HCV has evolved a mechanism to recruit and activate a cellular lipid kinase, phosphatidylinositol 4-kinase IIIα (PI4KA), to produce sufficient amounts of phosphatidylinositol 4-phosphate (PI4P) for the biogenesis of the membranous viral replication organelle [90]. In Huh-7 hepatoma cells, PI4KA is expressed at far higher levels than in hepatocytes; therefore, activation of the enzyme results in an overproduction of PI4P, which is deleterious for HCV replication [91]. Thereby, we could show that adaptive mutations in NS5A and NS5B are in fact loss of function mutations that prevent activation of PI4KA to avoid the production of unfavorably high PI4P concentrations in hepatoma cells [91]. This knowledge enabled us recently to develop a drug-based regimen mainly based on the inhibition of PI4KA allowing now the replication of non-adapted wildtype gt1b isolates in cell culture [91].

Unfortunately, cell culture adaptation came with one important collateral damage: when we generated adapted selectable replicons containing full-length HCV genomes, we achieved high levels of RNA replication, but no bona fide virus production [92]. In vivo studies, furthermore, suggested that the presence of adaptive mutations indeed interfered with the production of infectious particles [65]. The Con1 wildtype isolate replicated well after intrahepatic inoculation of chimpanzees, whereas a highly adapted variant, containing three mutations, was unable to establish a productive infection. If only one mutation was included the whole virus population in the blood was reverted to the wildtype sequence, arguing for an inverse correlation between replication in Huh-7 cells and in vivo [65]. Cells containing full-length, adapted Con1 genomes indeed released substantial amounts of nuclease-resistant HCV RNA-containing structures with buoyant densities expected for viral particles; however, comparable amounts of such RNA-containing structures were found in the supernatant of cells carrying subgenomic replicons, work which was mainly done by Thomas Pietschmann in Ralf Bartenschlager's group [92]. We recently found that these secreted RNAs are in fact exosome-like structures, mainly containing double-stranded RNA [93], representing an immune escape mechanism. A more detailed study revealed that adaptive, replication-enhancing mutations indeed impair virion morphogenesis by an unknown mechanism [94], likely also causing the attenuated phenotype in vivo. This lack of virion production was a major impairment for basic science and restricted the use of the replicon model to studies on RNA replication, which could only be overcome by the JFH1 isolate in 2005 [95] (see next section), but it was only a minor problem for drug development, since the replicon contained the most promising targets for antiviral therapy (Fig. 5).

Another important factor determining the efficiency of HCV replication was the host cell. From the start, we encountered problems to reproducibly generate selected replicon cell clones from non-adapted variants, which we tried to solve using early passages of Huh-7 cells. We now know that this was the worst thing to do: even within different stocks and passages of Huh-7 cells, we later found more than 100-fold differences in permissiveness to support RNA replication and the efficiency constantly changed upon passaging [86]. By and large, early passages worked worse than late passages [96]. The system only got consistently more robust by the observation that curing cell clones by treatment from the replicon—with interferons or selective drugs—sometimes resulted in Huh-7 variants more permissive for HCV replication compared to naive cells [97, 98]. Some of these Huh-7 clones are now widely used in the field, e.g. Huh7.5 [97] or Huh7-Lunet [99], the latter identified by our lab. Therefore, establishment of replicon cell clones included selection not only for adaptive mutations but also for permissive host cells. However, the mechanisms underlying these substantial fluctuations are still poorly understood. Some studies reported that permissiveness is linked to the lack of distinct innate immune responses, namely the RIG-I pathway [100]. However, we did not find any evidence for the RIG-I pathway playing a limiting role for the permissiveness of Huh-7 and not even in Huh7.5, which harbor a mutated RIG-I variant [96]. Up to now numerous individual host factors are reported in literature to be required for HCV replication (e.g. CypA and PI4KA [90, 101]), most of them are ubiquitously expressed in sufficient amounts and seem not to limit the permissiveness of cells. The only exception reported so far is the liver-specific micro-RNA miR-122, which is essential for HCV replication [102] and indeed has been identified as a limiting factor in some hepatoma cell lines such as HepG2 [103] and Hep3B [104], but not HuH-7 [105]. Therefore, the host factors determining permissiveness in Huh-7 remain unclear [106].

Compared to Huh-7, RNA replication is less efficient in all other cell lines reported so far. In several cases, cell clones generated with selectable replicons could be generated with low efficiency, but after curing, these cell clones

seemed not to be dramatically more permissive [107–114]. Still some of these cell lines exhibited interesting phenotypes. In case of HuH6, a cell line derived from a human hepatoblastoma, we could show that HCV replication was independent of host cell proliferation and that HCV replication was resistant to IFNγ [107]. Recent progress in culturing HCV wt in hepatocyte-like differentiated stem cells (reviewed in Ref. [115]) and in 2D or 3D liver organoids (reviewed in Ref. [116]) will hopefully allow to study certain aspects in a more physiologically relevant setting in cell culture, e.g. pathogenesis or entry in polarized environments [117]. The main challenge of these systems is a strong induction of innate immune responses, limiting viral replication, as also observed in PHH [118]. The antiviral state induced by HCV infection in primary cells explains some of the difficulties to grow serum-derived HCV in culture from the start. This somehow reflects the situation in the infected liver, which is characterized by a strong innate immune response in infected hepatocytes [119–121] and difficult to detect amounts of viral antigen [90, 122]. Innate immunity-mediated dampening of viral replication might be an important mechanism of HCV to establish and maintain persistence, but also represents a major hurdle to study virus replication in a physiological environment.

Based on genotype 1b replicons, harboring replication-enhancing mutations and permissive Huh-7 cells, it was possible to develop transient replication assays, allowing the quantitation of the replication efficiency of given replicon RNAs. The simplest "transient" replication assay used selectable replicons and quantified the efficiency of colony formation after transfection and selection by counting the number of selected colonies per µg of transfected RNA or relative to the amount of transfected cells (Fig. 6B) [87]. The easiest and fastest approach to measure HCV replication early after transfection was based on replicons with reporter genes instead of selectable markers (Fig. 2). The first reporter replicons contained the firefly luciferase, which provided a very low cellular background, a high sensitivity and a short half-life; therefore, luciferase activity in cell lysates well reflected RNA replication (Fig. 6C) [88]. Transient HCV replication assays using subgenomic replicons enabled us for the first time to apply reverse genetics to HCV research. Besides quantifying the impact of adaptive mutations, one of the first studies in this area mapped the role of particular regions in the 5′UTR and 3′UTR in RNA replication [123, 124]. Since then, a plethora of studies engaged transient replication assays to address functional aspects of HCV coding and noncoding sequences by mutational analysis. Adaptive mutations further allowed the establishment of more advanced tools for drug screening, particularly replicons with a combined reporter and selection marker, enabling high-throughput screenings of compounds based on simple luciferase assays (reviewed in Ref. [125]).

Broadening the scope: other genotypes and the full replication cycle

The replicon model based on the genotype 1b isolate Con1 and the identification of replication-enhancing mutations paved the road to the establishment of replicons from other isolates, starting in 2001 [126, 127]. Replicons from other genotype 1b isolates successfully tested so far either had acquired adaptive mutations after selection of replicon cell clones (reviewed in Ref. [128]) or contained a replication-enhancing insertion in NS5A [129]; the latter was based on the infectious isolate HCV-N established in Stanley Lemon's group [130]. However, it took until 2003 to establish replicons based on genotype 1a isolates, again starting with isolate H77, since those essentially required more than one mutation to replicate in cell culture and, therefore, could only be established by the introduction of an adaptive mutation based on the sites identified in gt1b [55, 131]. In essence, these results were consistent with all genotypes and isolates analyzed so far [128]: wildtype consensus genomes are infectious in vivo but barely replicate in cell culture. Adaptive mutations are required to allow robust RNA replication in cell culture. Only very recently, it has become possible to stimulate replication of gt1b wt by a drug-based regimen [91] or even isolates from various genotypes, albeit some to a limited extent, by overexpression of Sec14-like protein 2 (Sec14L2), a lipid transporter protein expressed in the liver but not in Huh-7 cells [132]. Fortunately, the major adaptive mutations identified in gt1b, particularly those in NS5A and NS5B, could be transferred to all other genotypes, due to the conserved mechanism of action [91]. Still, it was extremely difficult to cover all genotypes by this approach due to the necessity of more than two replication-enhancing mutations and special subclones of Huh-7 cells. Due to these difficulties, the establishment of gt3 and 4 replicons was only published in 2012/2013 [133–135], gt5 and gt6 in 2014 [136, 137] and a few more subtypes even in 2018 [138].

There is only one exception from this rule: motivated by the success of our replicon approach, Takaji Wakita's group cloned several gt2 isolates. He focused on patients with fulminant hepatitis, reasoning that such isolates might have particularly high replication capacity. While this was not generally true, he succeeded 2003 in generating a very efficient replicon based on the isolate Japanese fulminant hepatitis 1 (JFH1) [139]. Selectable JFH1 replicons generated colonies with yet unmet efficiency and were even capable of high-level transient replication without any need for adaptive mutations, which is due an exceptionally efficient polymerase and helicase [140–142]. Importantly, this isolate allowed for the first time the production of

infectious virus in cell culture in 2005, which was achieved by Takaji Wakita's group in cooperation with Ralf Bartenschlager [95] and first presented on the HCV meeting in 2004 in Heidelberg. The discovery of JFH1 thereby removed the final, central roadblock from HCV research, allowing now studies on the entire viral replication cycle. While the JFH1 replicase was extremely efficient and did not require any adaptation, virus production was not [95]. Efficiency strongly increased upon passaging of the virus [143], later again resulting in the identification of mutations, this time enhancing virus titers. Those were found in virtually all viral proteins and the underlying mechanisms are still not understood (reviewed in Ref. [144]). Another way to improve the model was the use of alternative structural protein genes. While the general possibility of using structural proteins of other genotypes fused to the replicase of JFH1 was already demonstrated for Con1 in the original publication [95], such intergenotypic chimeras were generally even less efficient and required further adaptation due to genetic compatibility issues between the viral proteins from different genotypes [145–147]. There was only one striking exception, stressing the importance of productive competition in basic research in the HCV field: the core-NS2 region of gt2a isolate J6, when fused to the JFH1 replicase, increased viral titers by several orders of magnitude, which was found in Charlie Rice's lab [148]. Interestingly, isolate J6 was established as an infectious clone in chimpanzees by Jens Bukh [149], but followed the general rule of not replicating at all in cell culture [141, 142]. The efficiency of this J6/JFH1 chimera was further improved in Ralf Bartenschlager's lab by optimizing the junction within NS2 to obtain the so-called chimera JC1 [145], which is now widely used in the field. Ralf's lab further contributed to the optimization of the HCV infectious model, which is now referred to as HCVcc (cell culture) by characterizing adapted JFH1 variants and identifying polyprotein processing kinetics as an important determinant for assembly efficiency [101, 150], by establishing various reporter viruses encoding luciferase genes or fluorescent tags [90, 151, 152], and by recognizing CD81 as a limiting factor for entry in some Huh7 populations [153].

The impact of JFH1 on HCV basic research was similarly dramatic as the earlier development of subgenomic replicons and a detailed description of all scientific progress based on JFH1 goes far beyond the scope of this review. It allowed for the first time studies on viral entry and assembly, thereby identifying numerous essential host factors (reviewed in Refs. [154–157]). The high efficiency of the JFH1 replicase further highly facilitated studies on viral RNA replication and established a wider range of host cell lines beyond Huh-7 (reviewed in Refs. [144, 158]). It further allowed parallel studies in vitro and in vivo, using humanized mouse models [159]. Particularly, Jens Bukh's laboratory widely expanded the availability of infectious virus models starting with JFH1 chimeras harboring the structural proteins of all major genotypes and later almost the entire replicase except NS5B, requiring extensive and cumbersome adaptation strategies [146, 147, 160]. Jens Bukh and his team as well as Stanley Lemon's group finally also succeeded in generating infectious virus from other genotypes, now including gt1a, 2b, 3a and 6a, again containing numerous adaptive mutations [161–165], but allowing now studies on the whole viral life cycle on a broader genetic landscape, albeit still far off representing the huge variety of HCV isolates found in vivo.

In contrast to the tremendous importance of the HCVcc models based on JFH1 for basic research, their impact on drug development was relatively moderate for several reasons. First, JFH1 is a gt2a isolate, which was not regarded as the major focus for drug development. gt1 infections were more widespread and compared to gt2 and 3 particularly difficult to treat with the available interferon-based regimens. Second, there was already a pipeline of specific compounds to be tested upon availability of the replicon model in 1999. In addition, replicon cell lines allowed from the start efficient screening of compound libraries, which was done immediately. Therefore, upon publication of the HCVcc model in 2005 most companies had already finished their screening approaches and the drug development programs had already moved forward to the next steps. Third, the HCVcc model indeed opened a number of new targets for antiviral therapies, e.g. entry and assembly inhibitors. However, the replicon model already offered the majority of classical targets (protease, polymerase, Fig. 5) and high-throughput screens on replicons already revealed numerous hits on unexpected viral and cellular targets, including NS5A, PI4KA, Cyclophilin and miR-122 (reviewed in Ref. [166]) that kept the companies busy enough. Although indeed some screening approaches based on the HCVcc model have been initiated (e.g. [167]), none of those hits made it to an approved drug, due to the (unexpected) efficiency of replicase inhibitors (NS3, NS5A, NS5B) in clinical trials. The major impact of JHF1 on drug development was already in 2003, when JFH1 replicons became available [139], together with gt1a [55], thereby allowing to validate drugs on a broader genotype coverage than just the original gt1b/Con1 isolate. Still, the first generation of therapies indeed had a bias towards gt1(b) and the delayed availability of efficient pan-genotypic inhibitors in part reflects the later development of cell culture models covering gt3–6 described above. Nowadays, powerful tools covering many viral genotypes are available to accompany the global distribution of drugs to vigilantly select for potential emergence of resistance in all possible drug combinations (reviewed in Ref. [168]). However, despite the previously mentioned recent progress based on Sec14L2 and PI4KA inhibition, it remains extremely difficult to efficiently propagate wildtype viruses from patient

serum in cell culture. Therefore, assays to monitor phenotypic drug resistance of field isolates are still challenging and require cloning of the protein domains of interest (e.g. protease) into existing cloned viral genomes (e.g. [169]).

The history of HCV therapy: from interferon to highly efficient DAAs

Interestingly, first attempts to treat HCV infections were initiated even before the virus was discovered, a first report on an interferon-based therapy of ten nonA-, non-B hepatitis patients was published already in 1986 [170], using serum ALT and liver histology as readout with promising results. The therapy was approved in 1990, but it turned out that interferon monotherapy was not very efficient, with cure rates of 15–40% after 48 weeks of therapy, depending on the viral genotype. Interferon-based therapies could be improved by adding ribavirin, a broadly acting antiviral nucleoside analogue with still ill-defined mode of action, in 1998 and using pegylated interferon with improved pharmacokinetic profile in 2002 (reviewed in Ref. [171]). These optimized therapy regimens were capable of curing approximately 50% of gt1, 4, 5, 6 and 90% of gt2, 3 infected patients. However, treatment duration ranged between 24 and 48 weeks, and patients encountered severe side effects, including flu-like symptoms, depression and anemia, in many cases resulting in a discontinuation of therapy [171]. Therefore, right after the discovery of HCV in 1989 intense efforts were initiated by basic researchers and pharmaceutical industry to develop more specific and more efficient DAAs with fewer side effects. As already pointed out before, it was clear from the biology of the virus that HCV is a curable infection, and this was further proven by the principal, albeit limited, success of interferon-based therapies. While the classical targets polymerase and protease were defined and pursued from the start, the availability of the replicon model for compound screening identified a number of entirely novel and unexpected drug targets and inhibitor classes, driven by efforts in industry and basic research in academia. The principle applicability of persistent replicon lines for drug screening was clear from the start, since HCV replication was efficiently inhibited by interferons in this system [54, 82, 83]. Among the newly identified classes were extremely potent inhibitors targeting NS5A [172]. In addition, cyclosporin A and non-immunosuppressive derivatives thereof were shown to efficiently inhibit HCV replication in cell culture [79, 173] by targeting cyclophilins, in particular cyclophilin A [101, 174]. SiRNA screens aiming to identify host factors of HCV replication further brought up a large number of additional promising cellular targets for antiviral therapy, among them PI4KA (see Refs. [90, 175–178).

The first class of DAAs approved for HCV was inhibitors of the viral protease NS3-4A (termed -previr) in 2011, as add on to interferon-based therapies. Later, inhibitors of the viral polymerase followed in 2013 (termed -buvir). The first inhibitors of NS5A (termed -asvir) were approved in 2015. All current therapies are based on various combinations of these three inhibitor classes, due to the tremendous efficiency of these regimens. This became clear already in 2014 with phase 2 and 3 clinical trials showing cure rates above 90% after 12 weeks of therapy for genotype 1 (comments in Refs. [179, 180]). Initially, nobody even dared to dream of such a fast development of all-oral, interferon free treatments with such an outstanding efficiency. Ironically, those genotypes with highest response rates for interferon-based therapies, particularly gt3, presented the biggest challenge in the DAA era, mainly because of delayed accessibility of cell culture models for gt3. Therefore, the first-line inhibitors were mainly optimized towards the available gt1 models. Still, the first pan-genotypic regimens with comparable efficiency became available in 2016. After these breakthroughs, further promising candidates were not further pursued, despite in vivo proof of concept (Fig. 5), including miR-122 antagonists such as miravirsen, non-immunosuppressive cyclophilin inhibitors such as Alisporivir or intravenous Silibinin (reviewed in Ref. [166]). I will therefore focus on a short summary, how cell culture models were involved in the development of the three main inhibitor classes.

NS3-4A protease

Research on the enzymatic functions in the early years after discovery of HCV was driven by shared interests of basic researchers, who aimed to understand the biology of the virus, and groups in industry focusing on drug development. Protease inhibitors had a head start due to the early availability of in vitro models and clearly defined peptidomimetic lead structures based on the amino acid sequence of the cleavage site (reviewed in Ref. [60]), strategies successfully established also in case of HIV before. After identification of the NS3-4A protease activity and the characterization of the cleavage site by Ralf Bartenschlager and other teams in 1993–1995 [28–30, 34–36, 58, 59], a cell-based screening assay was readily available. Therefore, an authentic replication model was not as essential for inhibitor development as for other targets, but only required at a later stage to prove the true antiviral activity in cell culture and to obtain information about barriers to resistance, fitness of resistant variants, etc. The first clinical proof of concept for antiviral activity of a protease inhibitor was already published in 2003 by a group from Boehringer Ingelheim, revealing exciting results [181]. While this drug, BILN2061, later failed due to side effects [182], two other compounds, telaprevir [183] and boceprevir [184], developed by Vertex and Shering-Plough,

respectively, went further and were finally approved in 2012 for anti-HCV therapy. This first wave of protease inhibitors had a low barrier to resistance and a narrow genotype spectrum, mainly covering gt1. Due to lack of other DAAs, they could only be applied in combination with pegylated interferon and ribavirin. Still, addition of a protease inhibitor strongly improved cure rates, particularly for gt1 patients to approximately 70% [185]. Currently, paritaprevir and grazoprevir are included in DAA-based combination therapies for gt1,4 patients, and voxilaprevir and glecaprevir in pangenotypic treatment regimens [15], the latter showing far broader genotype coverage and high barriers to resistance.

NS5B polymerase

The development of NS5B inhibitors was more dependent on the availability of a cell culture model than protease inhibitors. In principle, there are two classes of polymerase inhibitors: non-nucleoside inhibitors binding outside the active center, allosterically blocking the enzyme, and nucleoside/nucleotide inhibitors, which are incorporated in the nascent RNA, resulting in chain termination (reviewed in Ref. [166]). The latter class is in principle preferable, since it acts on one of the most conserved structures of the virus, the enzymatic core of the polymerase. The disadvantage is that the active compounds are nucleotide triphosphates, and phosphorylation needs to happen inside the cells by cellular kinases. Nucleoside inhibitors in addition act competitively to the cellular NTP pools. The first heterologously expressed active NS5B polymerase was described in 1996 [41], we followed in 1997 with a purification protocol for a tagged enzyme and performed some basic studies characterizing the enzymatic activity [42, 62, 63, 186]. Upon availability of purified active NS5B, in vitro high-throughput screenings of compound libraries became possible. However, such assays would only identify non-nucleoside inhibitors, since nucleotide triphosphates are hardly contained in compound libraries and are chemically unstable. Nucleotide triphosphates of candidate nucleoside inhibitors, therefore, need to be individually synthesized, which is not suitable for thousands of compounds. Intense screening assays for nucleotides could only start after establishment of the replicon in 1999. Some screenings were done with BVDV as a surrogate model [45], bearing the risk of missing important candidates. In fact, 2′deoxy-2′-fluoro-2′-C-methylcytidine, which was a lead structure later resulting in the discovery of sofosbuvir, did not act against BVDV at all [187]. Sofosbuvir, the nucleotide inhibitor that finally helped to revolutionize HCV therapy is basically the uridine variant of this nucleoside, which also is barely active in cell culture due to inefficient intracellular synthesis of the monophosphate [187, 188]. Therefore, sofosbuvir (PSI-7977) is a monophosphate of this compound, which is further modified to facilitate hepatic uptake [189]. Many other promising nucleoside analogs were halted in clinical trials, mainly due to adverse effects (reviewed in Ref. [60]); therefore, sofosbuvir is currently the only nucleotide inhibitor approved for HCV therapy [15]. In case of non-nucleoside inhibitors, literally hundreds of candidates were identified by in vitro and replicon-based screening assays, binding to various regions of NS5B and exhibiting multiple modes of action (reviewed in Ref. [60]). Almost all of them failed at different stages of the development process, mainly due to very narrow genotype coverage and a very low barrier to resistance, since their binding sites were far less conserved than the active center of the polymerase, targeted by nucleosides. From all the non-nucleoside inhibitors only dasabuvir was finally approved in a combination therapy for gt1 [190–192]. In contrast, sofosbuvir was a cornerstone for the success of interferon-free DAA therapies in 2014 [193–199]. This was mainly for two reasons: sofosbuvir acts against virtually all HCV genotypes and has a very high barrier to resistance. While a resistance-associated mutation could be selected in the replicon model [188], this mutation was associated with such high fitness costs, that it was barely found in vivo [200]. Still, with addition of a broadly active NS5A inhibitor, the first pan-genotypic, one pill a day, all oral treatment regimen could be established in 2016 [201–204]. This was an exciting and surprising development in the light of the broad genetic diversity of HCV genotypes. The importance of sofosbuvir for HCV therapy, as well as the substantial contribution of the replicon model for the development of HCV therapies was broadly recognized by honoring Ralf Bartenschlager, Charles Rice and Michael Sofia with the Lasker-DeBakey Clinical Medical Research Award in 2016 [205].

NS5A

From a basic science point of view, NS5A inhibitors are even more exciting than sofosbuvir. Their discovery marks a magic moment of drug development and further emphasizes the role of the replicon and other cell culture models established in basic science on different aspects of the drug discovery process. It also shows the power of cell-based, unbiased screening approaches over target-based strategies. NS5A is a nonstructural protein without known enzymatic function which is modified by phosphorylation and essential for viral RNA synthesis and assembly of viral particles (reviewed in Refs. [156, 158, 206]. A detailed description of our knowledge on NS5A goes far beyond the scope of this section, but to cut a very long story short, its detailed mechanisms of action are widely enigmatic, but likely mediated by multiple interactions with other viral proteins and multiple host factors. And again, Ralf Bartenschlager's group provided some seminal contributions to our basic understanding of NS5A [64, 65, 88–91, 101, 207–218]. A rationale-based

screening of NS5A inhibitors in vitro was not possible, but such inhibitors were indeed identified in high-throughput screenings of compound libraries using replicon cell lines by Min Gao's group at Bristol-Myers Squibb [219, 220]. While the initial compounds had only moderate antiviral activity, they were further optimized by medical chemistry and brought up an extremely potent class of inhibitors, acting with IC50 values in the low picomolar range [221] and a broad genotype coverage [222]. The most studied compound of this class was daclatasvir (BMS-790052), which also demonstrated an incredible antiviral activity in pilot clinical trials, with a single dose dropping viral titers by four orders of magnitude within 48 h for several days [172]. The most direct evidence that these drugs targeted NS5A again came from the replicon: co-selection of replicon cells with a compound of interest and the cytostatic drug which is detoxified by the replicon will typically result in killing of the cells, due to inhibition of viral replication (Fig. 7B). This process efficiently selects for mutations conferring drug resistance, resulting in surviving cell clones. The resistance-associated mutations are then easily identified, since they are conserved in the viral sequence. This strategy was applied to virtually all HCV inhibitors, to get a view on their mode of action, identify their binding sites, characterize their barrier to resistance, etc. (reviewed in Ref. [60]). Using this approach, we, e.g., found that intravenous Silibinin likely targets NS4B, due to the selection of resistance-associated mutations [223]. In case of daclatasvir, all resistance-associated mutations grouped to the N-terminus of NS5A, thereby revealing the most likely target and introducing the term "NS5A inhibitors" [81, 219, 221]. Daclatasvir was approved for HCV therapy already in 2015, and in combination with sofosbuvir, provided the first treatment regimen with reasonable efficiency against all genotypes including gt3 [197]. The major problem associated with this first line of NS5A inhibitors was a relatively low barrier to resistance and the high fitness of resistant variants, which are also found in some patients prior to treatment. This issue has been improved with following inhibitor generations, which also show a broad genotype coverage (reviewed in Refs. [15, 60]). Today, NS5A inhibitors are the only class of HCV inhibitors that are included in all treatment regimens, either in combination with an NS5B inhibitor and/or with a protease inhibitor [15].

Given their unprecedented target and their picomolar potency, NS5A inhibitors mark a triumph of drug discovery. It is still not clear, how NS5A inhibitors act at the molecular level, but one reason for their remarkable potency relies on their simultaneous activity on RNA replication and virus assembly. Ralf Bartenschlager's group recently showed that daclatasvir-like inhibitors block the formation of new membranous viral RNA replication organelles, probably by affecting dimerization or multimerization of NS5A [224]. Simultaneously, Stanley Lemon's team demonstrated that NS5A inhibitors block the assembly and release of infectious

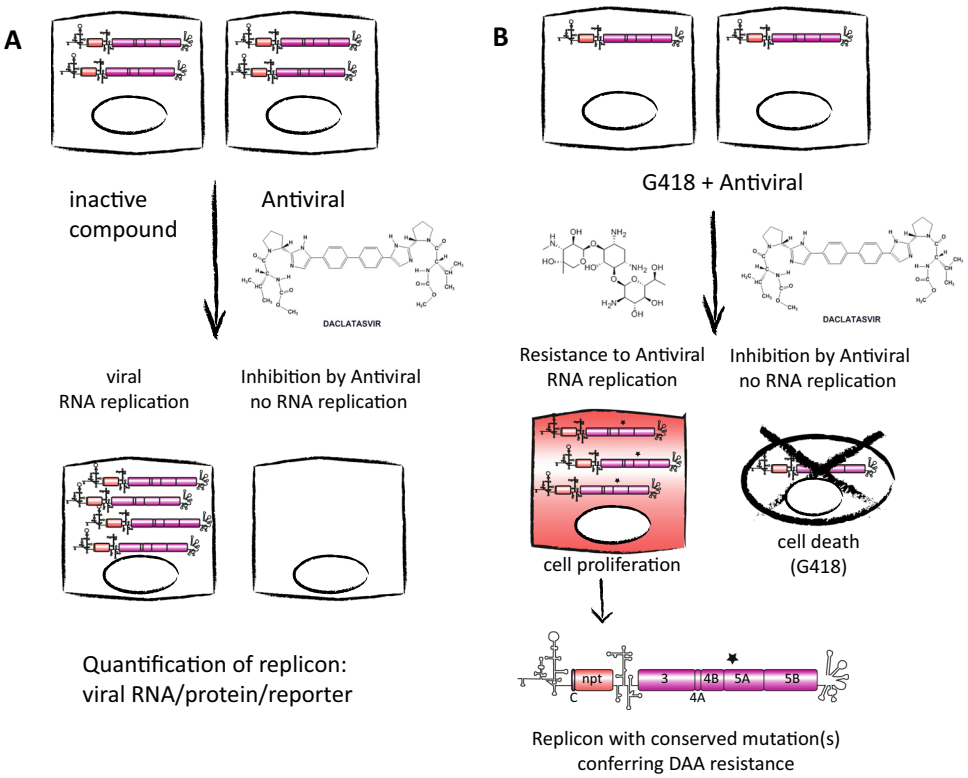

Fig. 7 Selection for antiviral drugs and drug-resistant replicon variants using replicon cells. **A** Huh-7 cells containing a persistent replicon are seeded in multiwell formats and treated with test compounds (here: daclatasvir). After several days of incubation, cells are harvested and replicon levels are quantified by RT-PCR (RNA), detection of viral proteins or reporter activity (if possible). Candidate HCV inhibitors result in reduced replicon levels. **B** Cell lines containing persistent selectable replicons are treated with G418 and a DAA (here: daclatasvir). Inhibition of HCV replication by the DAA will result in loss of G418 resistance and cell death. Only replicons acquiring mutations conferring drug resistance will replicate and give rise to surviving cell clones. These mutations are conserved in the surviving cell clones and point to the mode of action of the drug

virus, thereby explaining the rapid decrease in HCV serum titers after onset of therapy [225]. However, more efforts will be required to understand the precise mode of action of these remarkable compounds, which goes along with a better basic understanding of the still enigmatic functions of NS5A in viral replication.

Conclusions and future perspectives

Within the last years, chronic HCV infections, affecting an estimated 71 million people worldwide [226], have become a curable disease. We now have several all-oral, one-pill-a-day treatment regimens based on combinations of 2–3 inhibitors, offering cure rates far above 90% and covering all genotypes [15, 227].

One of the keys to this success story was the concerted efforts, in part competitive but importantly highly synergistic, of numerous research groups in academia and industry to understand the functions of viral proteins, to define targets for therapy, to develop cell culture models and to identify and optimize antiviral drugs in preclinical and clinical settings. The development of HCV cell culture models is an excellent example for the importance of complementing, competitive basic science in translational medical research: the first infectious clones were derived from genotype 1a, but did not replicate in cell culture. Genotype 1b replicons were essential to generate the first replication model, but did not produce viral particles. A very peculiar genotype 2a isolate was then required to generate infectious virus in cell culture. From the start, it was absolutely not clear what turns would be required to reach this point, and these efforts were not in first place driven by the need for drugs, but to gain a basic understanding of viral biology, which was, however, an essential prerequisite for drug development.

Some important clinical issues remain: treatment of patients with advanced liver disease, affordability of drugs in low income countries, a probably still increased risk of liver cancer after cure [228], diagnosing of yet asymptomatic infections, re-infections after cure, etc. (reviewed in Refs. [15, 229]). However, the mission of pharmaceutical industry seems accomplished and there is currently no obvious need to develop further drugs against HCV. Still, it has to be considered that 71 million people are globally infected and the drugs have been launched only recently; therefore, the virus might still hold back some clinical surprises, e.g. cases of resistance. For example, Jens Bukh's Team could demonstrate selection of efficiently replicating gt6a variants with resistance to sofosbuvir in cell culture [164] and very recently, a sofosbuvir resistant gt4d strain with high fitness was found in a patient after therapy failure [230]. On the contrary, due to the unexpectedly early development of one-pill-a-day regimens, only those companies remained on the market which could provide such an option early enough and many candidates were stopped even late in clinical development, in case this was not in reach. Whether the ambitious goal of eradicating HCV by 2030, recently announced by the WHO [231], can be reached based only on therapy still remains open. Eradication of a virus without a vaccine is without precedence. At this point, the majority of pharmaceutical industry seems not interested in developing a vaccine and some clinical virologists tend to regard any kind of HCV research unnecessary. In my view, this is very shortsighted (see also a recent standpoint paper by Ralf Bartenschlager and colleagues [229]). Due to the tremendous efforts over the last two decades HCV has become one of the best studied models for virus host interactions and we do now have an excellent toolbox. It would be a waste not to further continue basic research and use this precious model to gain further understanding on mechanisms driving hepatocellular carcinoma, virus-induced changes in lipid metabolism, etc. (see for recent review [229]).

Major fundamental breakthroughs rarely emerge from direct translational research, but are derived from new findings in basic research: the principle of gene silencing by siRNA was initially found in the nematode *Caenorhabditis elegans* [232] and Crispr Cas9 was originally identified as an adaptive immune defense mechanism in bacteria [233]. Research on these topics was unlikely driven by any medical implication, but still had a dramatic translational impact. These are just two examples demonstrating the need for unintentional, curiosity-driven basic research and, in my view, may important discoveries are still out there in case of HCV.

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Compliance with ethical standards

Conflict of interest Volker Lohmann is co-owner of Reblikon GmbH, a company licensing the replicon system for commercial purposes.

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