

How Current Direct-Acting Antiviral and Novel Cell Culture Systems for HCV are Shaping Therapy and Molecular Diagnosis of Chronic HCV Infection

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1 **Abstract:** We have entered a new era of hepatitis C virus (HCV) therapy in which
2 elimination of infection and disease is a real possibility. HCV cell culture models were
3 instrumental for identification of therapeutic targets, testing candidate drugs, and
4 profiling of therapeutic strategies. Here we describe current and novel methods of cell
5 culture systems for HCV that are allowing investigation of HCV life cycle and virus-host
6 interaction required for replication and propagation. The development of protocols to
7 grow infectious virus in culture and generate hepatocyte cell lines from specific
8 individuals hold great promise to investigate the mechanisms exploited by the virus to
9 spread the infection and the host factors critical for HCV replication and propagation, or
10 resistance to infection. Since host factors are assumed to be conserved among HCV
11 isolates and genotypes and antagonizing molecules should possess high genetic barrier to
12 viral resistance, the development of drugs targeting host factors essential for virus
13 replication holds great promises in further increasing treatment efficacy. Refocusing of
14 therapeutic goals also impacted *in vitro* diagnosis. The primary goal of anti-HCV therapy
15 is to achieve a sustained virologic response (SVR) defined as “ undetectable” HCV RNA
16 genome in the serum or plasma at 12 to 24 weeks following the end of treatment. Use of
17 direct antiviral agents has substantially changed threshold of the viral load used to define
18 SVR and led to a reassessment, discussed herein, of result interpretation and requirements
19 of clinically-approved, quantitative molecular assays.

20

21 **Keywords:** Cell culture-derived HCV; Hepatitis C virus; HCV cell culture systems;
22 Induced pluripotent stem cells; Molecular diagnosis; Pseudoparticles; Quantitative
23 molecular assay; Replicon.

1 **1. INTRODUCTION**

2 As clearly evident from previous chapters of this issue, we are witnessing an
3 epochal change in the management and therapy of HCV infection. Up to four years ago
4 there were drug regimens modestly effective, which required prolonged treatment and
5 caused significant side effects. This gloomy picture dramatically changed with the advent
6 of novel molecular techniques and computer modeling, and the development of *in vitro*
7 systems to study replication, pathogenetic mechanisms, and, ultimately, Achilles' heel of
8 HCV life cycle. Intense use of these technologies had its payoff to the point that some
9 obscure points of viral replication and interaction with cell and host have been unveiled
10 [1] and novel antiviral molecules were developed. These directly target the virus and, in
11 preliminary clinical tests, proved easy-to-administer, well tolerated, and surprisingly
12 effective. These features and, by contrast, poor performances of conventional therapies
13 ensured fast lane for clinical approval to several drugs.

14 Novel direct antiviral agents (DAAs) completely changed scenario and expectance
15 of patients: some DAAs, either alone or in combination with interferon (IFN) and
16 ribavirin (RBV) - the mainstay of therapy until a few years ago - boast cure rates
17 exceeding 95% [2, 3], reduce liver damage even in long-term infected patients, and have
18 high genetic barriers, i.e. the virus can become resistant but at a great cost in terms of
19 reduction of fitness and virulence [4].

20 Here, we describe current and novel technologies being used to study HCV life
21 cycle, investigate viral-host interaction, and develop highly potent HCV antivirals. These
22 novel drugs changed expectations and management of patients, and, as discussed, caused
23 reassessment of molecular diagnosis of infection and interpretation of results.

1 **2. HCV REPLICATION IN HEPATOCYTES AND PATHWAYS TO**
2 **DISSEMINATION INTO OTHER TARGET CELLS**

3

4 HCV was the first human pathogen to be discovered by molecular methods and up
5 to few years ago most of which was known was, indeed, molecular. Since its discovery,
6 occurred in late eighties [5], we clearly understood that the virus has a high degree of
7 genetic heterogeneity [4], which helps the virus to resist immunological and other host
8 pressures, adapt to replicate in various body sites and tissues, and causing, ultimately,
9 rapid inter- and intra-host evolution of the viral genome [6]. This wealth of information
10 contrasted with poor knowledge about virus replication and persistence in infected cells.

11 The lack of solid cell culture systems in which to replicate and produce infectious
12 HCV virions has represented a major hurdle to study this pathogen and develop strategies
13 for prophylactic and therapeutic interventions. First attempts to adapt patient-derived
14 HCV isolates failed to establish productive infection in cell culture and, as described
15 below, there were no progresses in this direction until 1997, when molecular clones
16 infectious in chimpanzees were developed. Unfortunately these viral genomes were able
17 to generate infectious particles *in vitro* under certain conditions but were unpractical to
18 study key steps of viral replication and antiviral potency of drug candidates [7, 8]. These
19 clones, however, were important landmarks as they paved the way to various surrogate
20 systems, described in the next paragraph and that, together with *in vivo* studies in
21 chimpanzees and recently developed small animal models, greatly contributed to shed
22 light on life cycle, virus–host interactions that mediate infection, establishment of
23 chronicity, and progression of HCV-mediated disease.

1 HCV exhibits several peculiar features that are still puzzling researchers and
2 hampered the development of drugs directly targeting the virus. As shown in Figure 1,
3 HCV circulates in the bloodstream as lipoviral particle (LPV), in which the virion is
4 surrounded and partly shielded to most immune effectors [9]. Once it reaches its primary
5 target organ, the liver, HCV crosses the fenestrated endothelium and interacts with an
6 ever-increasing complex network of putative receptors which roles and importance in
7 driving virus entry is still uncertain. According to recent molecular models, viral
8 absorption to cell surface is initiated by binding to heparan sulfate proteoglycans
9 (HSPGs) and receptors such as the tetraspanin CD81 on the basolateral side of
10 hepatocytes [10, 11]. The virus then, presumably facilitated by its circulation as LPV,
11 absorbs to two lipoprotein molecules: the scavenger receptor class B, type I (SR-BI) [12]
12 and the low-density lipoprotein receptor (LDLR) [13]. This list of putative receptors
13 increased of six other novel entities in the last eight years: the tight junction molecules
14 claudin-1 (CLDN1) [14] and occluding (OCLN) [15], the epidermal growth factor
15 receptor (EGFR) and the ephrin type-A receptor 2 (EphA2) [16], the cholesterol uptake
16 molecule Niemann-Pick C1-like 1 (NPC1L1) [17], and the transferrin receptor 1 (TFR1)
17 [18]. Since these molecules segregate into different subcellular domains, it is likely that
18 interaction with receptors occurs in a step-wise fashion that actively transports the virus
19 towards the apical side of the hepatocyte where the virus eventually enters by fusion and
20 is internalized by clathrin-mediated endocytosis. Low pH of the endocytic compartment
21 then disassemble the viral capsid that releases the positive single-strand HCV RNA
22 genome that is immediately translated by the host protein synthesis machinery to produce
23 a single HCV polyprotein precursor of about 3000 amino acids [19, 20]. The polyprotein

1 is then processed by both cellular and viral proteases to give rise to 10 viral proteins:
2 three are structural components of the virion - core, which forms the nucleocapsid, and
3 the viral envelope glycoproteins, E1 and E2 -, and seven are non-structural (NS) proteins
4 that have various functions [21, 22]. A few NS proteins organize in a replication complex
5 that produces multiple copies of the HCV RNA genome via a minus-strand replicative
6 intermediate [23]. Others cleave and mature viral proteins from the polyprotein precursor
7 and help assembly of nucleocapsid to bud into the endoplasmic reticulum from which it
8 eventually acquires the envelope and E1 and E2 glycoproteins. Along the secretory
9 pathway, virions undergo maturation, mainly operated by HCV protease, and associate
10 with endogenous lipoproteins to form LPVs [9, 24]. LPVs are eventually released from
11 the cell to start a new round of infection.

12 In addition to dissemination and infection via the bloodstream, HCV actively
13 exploits the so called “cell-to-cell” spread [25, 26]. Here, the virus released by an
14 infected cell directly infects neighboring cells without diffusion through the extra-cellular
15 environment. Cell-to-cell transmission occurs through the engagement of two co-
16 receptors, CLDN1 and OCLN, at the intercellular interface (Figure 1). Compared to
17 “cell-free” spread, cell-to-cell transmission is thought to be an important mechanism of
18 HCV persistence as it provides several advantages as regards efficiency of infection, viral
19 dissemination, immunological escape, and resistance to DAAs [25, 27-31].

20 Even though hepatocytes are the main site for viral replication, a broad clinical
21 spectrum of extrahepatic complications and diseases are associated with chronic HCV
22 infection, which suggests the existence of some other target cells for viral replication. As
23 described in this thematic issue, B-cell abnormalities and lymphoproliferative disorders

1 are frequently observed in HCV infected patients [32-35], and, therefore, B-lymphocytes
2 as well as peripheral blood circulating cells are thought to be target of HCV replication.
3 Whereas there are many *in vitro* and *in vivo* studies supporting this hypothesis [36-42], it
4 is unclear what are the molecular pathways used by the virus to enter and replicate in
5 extrahepatic cells. None of these cells expresses all receptors required for viral entry in
6 hepatocytes. Since cell-to-cell transmission appears to be less dependent on virus-host
7 receptor interaction compared to cell-free transmission, it has been postulated that
8 dissemination to extrahepatic cells occurs via cell contiguity or that other viral proteins,
9 beside HCV envelope glycoprotein E2, interact with the target cells and anchor the virus
10 on the cell surface [28, 43, 44]. Evidences supporting cell-to-cell transmission are the
11 close anatomical vicinity of hepatocytes and lymphoid cells in the liver (especially during
12 chronic inflammation) and the presence of CD81 and SR-BI on the lymphoid cell
13 membrane [45, 46]. This finding is particularly relevant since among all above mentioned
14 putative receptors and coreceptors, CD81 and SR-BI are the only two molecules
15 demonstrated to bind the glycoprotein E2 [11, 12]. Evidences against this hypothesis are
16 the absence of CLDN1 and OCLN in lymphoid cells [47] and conflicting results with *in*
17 *vitro* models [48]. An alternative hypothesis is that HCV infects lymphoid and other non-
18 hepatic target cells using different receptor [28, 47, 49, 50]. Downstream viral entry, it is
19 very likely that HCV interacts with proteins that are absent in hepatocytes and that cells
20 themselves react to infection in different ways, implying once again the necessity for the
21 virus to adapt to the new environmental conditions. Here again there are many studies *in*
22 *vitro* and *in vivo* corroborating these considerations and ranging from genome evolution
23 following replication in lymphoid cells [39, 51, 52], engagement of specific molecular

1 pathways [28, 53, 54], different interferon production, cell responsiveness and
2 consequence to infection [34, 55]. Whereas there are many features that need to be
3 uncovered to understand the pathobiology of HCV replication and role of extrahepatic
4 sites in fueling the HCV syndrome [33, 56], non-hepatic cells are clearly important sites
5 of HCV persistence with a relevant impact to antiviral treatment and possibility to
6 eradicate the infection [57, 58].

7

8 **3. IN VITRO METHODS TO STUDY HCV LIFE CYCLE AND DEVELOP** 9 **ANTIVIRAL DRUGS**

10

11 Complicated interactions with putative cellular receptors, association of the virion
12 with endogenous lipoproteins, difficulties to propagate hepatocytes *in vitro*, and adapt the
13 virus to grow in non-hepatic cells, have insofar impeded the development of cell culture
14 systems in which the virus consistently establishes a truly productive infection.

15

16 **3.1. Conventional *in vitro* methods**

17 As shown in Figure 2, there are four standard methods currently used to study the
18 replication cycle and develop DDAs: 1. The HCV subgenomic replicon; 2. The HCV
19 pseudoparticles (HCVpp); 3. Cell culture derived HCV (HCVcc); 4. HCV trans-
20 complemented particles (HCV_{TCP}).

21

22 **3.1.1. HCV subgenomic replicon**

1 This system was described in 1999 and is based on a bicistronic RNA construct
2 expressing the minimal set of HCV proteins required to initiate and maintain HCV
3 replication in human cells [59]. The first cistron encoded *neo*, a selection marker placed
4 under control for expression of the HCV internal ribosome entry site (IRES). The second
5 cistron, placed under control of an IRES from a picornavirus, contained the HCV NS3-
6 NS5B segment (Figure 2a). NS3-NS5B encodes the HCV proteins essential for
7 replication, namely: NS3/4A protease, NS4B participating in the replicase complex [60],
8 the NS5A protein, also an essential component of the replicase complex and exerting a
9 wide range of effects on cellular pathways and processes [61], and the RNA-dependent
10 RNA polymerase NS5B. The subgenomic construct was then used as template to
11 transcribe, *in vitro*, an RNA that was transfected in Huh-7 cells – a human continuous
12 cell line of well differentiated hepatocytes derived a hepatocellular carcinoma – or
13 equivalent cells. Cells were finally cultivated in the presence of G418, an antibiotic
14 blocking the polypeptide synthesis in both prokaryotic and eukaryotic cells and
15 inactivated by the *neo* coding protein. Transfection and antibiotic selection resulted in the
16 generation of cell lines stably reproducing some specific steps of viral life cycle.
17 Unfortunately, due to the lack of genes encoding for the structural proteins there was no
18 production of infectious particles (Figure 2a) [59]. This method was scarcely efficient
19 and viral “replication” occurred at low levels, most of the times unpractical for screening
20 of large number of candidate antiviral molecules. To obviate this limitation, the
21 subgenomic replicon underwent several modifications, mainly site-specific mutations that
22 were either naturally selected during the replication process or manually inserted into the
23 subgenomic replicon to produce viral variants with higher replication capability [62-70].

1 Of note, most of naturally occurring mutations were found localized in the N-terminus of
2 the NS3 helicase [66, 71], NS4B [72] and NS5A [66, 73, 74], thus showing us the protein
3 domains critical for *in vitro* replication and where to intervene for functional adjustments.
4 From the first subgenomic replicon produced, derived from a HCV isolate of genotype
5 1b, others were described deriving from either other isolates of genotype 1b or isolates
6 belonging to the 6 major genotypes described so far [65, 75-84]). More recently,
7 modified versions have been described, these include the presence of reporter genes such
8 as luciferase (Luc) or green fluorescent protein (GFP) that simplify measurement of
9 replication activity [66, 85], and a tricistronic version also encoding for a reporter gene
10 that was placed under control of an own and independent HCV IRES [86].

11

12 **3.1.2. HCV pseudoparticles (HCVpp)**

13 Together with a soluble form of the HCV glycoprotein E2 [87], not described here
14 for brevity, the HCVpp model was extensively used to identify viral receptors, study the
15 mechanism of entry, examine host immune response and, more recently, investigate for
16 potential entry inhibitors. HCVpp represented a great advance in HCV research and was
17 inspired by previous works with HIV and viral vectors. Similarly to lentiviral and
18 retroviral vectors, HCVpp was produced from defective retroviral particles that were
19 assembled in such a way to incorporate and express on their surface the HCV envelope
20 glycoproteins [88, 89]. This procedure, called pseudotypization, is extensively used in
21 viral vector production and is exploited, in essence, to alter host tropism of parental virus
22 and target specific tissues, increase/decrease stability of the virus particle, investigate for
23 viral receptors and antibody-mediated neutralization for those viruses for which an easy

1 and reliable cultivation system is not at hand. Since pseudotyped particles do not carry
2 the structural genes, i.e. genes encoding capsid and foreign viral envelope glycoproteins,
3 they do not generate progeny viral particles [90]. HCVpp are generated by co-
4 transfection of packaging cells, i.e. human cell lines, usually the epithelial 293T cells,
5 easy-to-transfect and expressing the heterologous genes at high levels. Transfection is
6 performed with three plasmids encoding the HCV glycoproteins E1 and E2, the capsid
7 and enzyme proteins of HIV or murine leukemia virus (MLV) necessary for assembly
8 and processing the viral nucleic acid, and a defective HIV or MLV genome containing
9 Luc or GFP as reporter gene. Following transfection, the packaging cells start to produce
10 and release into the supernatant the vector particles that are harvested from the culture
11 and used to transduce the target cells. HCVpp entry into the target cells is determined by
12 measuring Luc or GFP activity one-two days after transduction. Since Luc and GFP
13 signals increase almost proportionally to the number of transduced cells, quantitative
14 evaluation of reporter genes permits precise measurement of HCV entry into target cells
15 (Figure 2b). HCVpp was extensively used to study the role of E1 and E2 glycoproteins in
16 HCV entry, to identify HCV receptors and other factors contributing to cell absorption
17 and to shed light on the mechanisms of virus internalization [91, 92]). This system was
18 also instrumental to elucidate protective role, kinetic of appearance, affinity, and breadth
19 of neutralizing antibodies elicited in infected individuals or immunized animals [93]. In
20 fact, HCVpp became the reference method to analyze the humoral response in acutely
21 and chronically infected individuals. These studies demonstrated that neutralizing
22 antibodies exert marginal role in controlling chronic infection and disease progression
23 [27, 94]. Nevertheless, patients who promptly developed neutralizing antibodies in the

1 early stage of infection were more likely to eradicate the infection [95, 96]. Finally,
2 HCVpp also played major role to assist in the design of anti-HCV vaccines [97].
3 Unfortunately, this model has some limitations. First of all, pseudotyped virions are more
4 susceptible to antibody-mediated neutralization compared to naïve isolates; this is likely
5 due to suboptimal organization and/or localization of envelope glycoproteins [98].
6 Furthermore, at a difference with HCV virions produce in hepatocytes, the HCVpp
7 generated in epithelial HEK-293T cells are not associated with lipoproteins (Figure 2b)
8 and may therefore exhibit different properties concerning interaction with putative
9 receptors and susceptibility to antibody-mediated neutralization [99, 100].

10

11 **3.1.3. Cell culture derived HCV (HCVcc)**

12 The above two methods neither yield infectious virus nor establish a truly
13 productive infection, which is essential to studying late stages of viral replication, e.g.
14 virion assembly, maturation and release. Further, a productive cell culture would simplify
15 definition and assessment of therapeutic targets. The first experiments to obviate to these
16 limitations were based on adaptation of patient-derived isolates to *in vitro* cell cultures.
17 Unfortunately, and despite countless attempts, this approach was unsuccessful and
18 demonstrated that HCV, like HIV and other patient-isolated viruses, requires adaptation
19 of some molecular and phenotypical properties to *in vitro* culture conditions [101].
20 Exception to this rule was JFH1, a HCV strain of genotype 2a isolated from a Japanese
21 patient with fulminant HCV-associated hepatitis and found able to replicate at low-levels
22 in Huh-7 (Figure 2c) [102, 103]. The HCVcc system derived from JFH1 was extensively
23 used to study HCV life cycle [104-106], identify novel entry factors, e.g. NPC1L1, EGFR

1 and EphA2 [16, 17], define morphological and biochemical features of the virion [107-
2 109], and characterize virion association to lipoproteins [110]. Unfortunately no HCVcc
3 systems were available for the clinically more relevant genotypes 1, 3 and 4.

4 With the idea to establish HCVcc from various genotypes and with higher
5 replication capacity, chimeric viruses were produced using JFH1 as backbone in which
6 were inserted the structural protein coding regions of other isolates. After first successful
7 attempt to produce a chimeric clone from J6 strain (also of genotype 2a) [111]
8 replication-competent chimeric clones were generated from genotype 1 through 7 [112].
9 Interestingly, propagation of these chimeric clones resulted in the accumulation of
10 scattered mutations, mainly in NS2-NS3 coding region. Some of these were deleterious
11 [104, 113], others progressively adapted the chimeric viruses to *in vitro* conditions and,
12 particularly, hepatoma cells [114-116].

13 HCVcc systems using genotype 1 subgenomic replicons were extensively used to
14 test efficacy of most DDAs. These HCVcc systems were also used as platform to develop
15 intergenotypic chimeras recombinant in DDA target regions (e.g. NS2, NS3/4a, NS5a
16 and NS5b alone or in combination) were used to assess the antiviral activity against other
17 genotypes. To this purpose and to permit simple and dynamic measurements of viral
18 replication, reporter genes were incorporated into the molecular clones as either fused
19 proteins [117] or additional proteins that were cleaved off by the HCV protease during
20 processing of the polyprotein precursor [118-120]).

21 JFH1 and HCVcc have been one of the biggest achievements in HCV research.
22 This system opened the way to the development of other full-length, replication-
23 competent molecular clones of genotype 1 and 2 [121, 122]. Nonetheless, we still lack

1 HCVcc systems for complete genotypes 3, 4, 5 and 6 and only a few isolates of genotype
2 1 and 2 proliferate in culture. Thus a broad and patient isolate-specific analysis of
3 particular life cycle steps is currently not possible.

4

5 **3.1.4. HCV trans-complemented particles (HCV_{TCP})**

6 An alternative system to study the whole life cycle of HCV is provided by the
7 trans-complemented JFH1 particles (HCV_{TCP}) and derives from the observation that HCV
8 subgenomic replicons can be packaged when structural proteins are supplied in *trans*
9 [123, 124]. HCV_{TCP} are infectious, relatively easy to produce, thus overcoming the hurdle
10 of isolate specificity, but support only single-round infection and are unable to spread
11 [125]. HCV_{TCP} is generated by cotransfection of a JFH1 subgenomic replicon, which
12 provides the replication machinery of the virus and most non-structural proteins, and the
13 coding RNA of all structural proteins and NS2 cloned from another isolate (Figure 2d). In
14 theory, structural proteins and NS2 can be derived from any patient isolate.
15 Unfortunately, genetic incompatibility between the various elements has been observed
16 and heavily reduce efficacy of virus production. This phenomenon is mostly linked to
17 non-structural proteins and particularly important when the genetic elements are derived
18 from distinct genotypes [113]. This hurdle can be overcome, with some difficulty, by
19 introducing specific adaptive mutations, deleting sequences in the subgenomic replicon
20 dispensable for replication, and/or using custom-designed packaging systems for
21 individual HCV genotypes [113, 125]. Efficient and easy production of HCV_{TCP} should
22 contribute to studying HCV in entry and assembly steps of the HCV life cycles, in
23 particular encapsidation of the viral genome.

1

2 **3.2. Limitations of standard *in vitro* methods**

3 All these methods use Huh-7 cells or derivative clones as substrate for HCV
4 replication. These cells are by far the most permissive to HCV replication and, depending
5 on mutations in genes involved in antiviral signaling, sustain high viral replication levels
6 [126, 127]. Unfortunately, these cells are highly divergent from primary cells from which
7 were derived, have different restriction mechanisms, lack of the cell polarity typically
8 observed in hepatic tissue and primary hepatocytes, and do not exhibit same
9 compartmentalization of HCV receptors. As a result, the mechanism of entry, assembly,
10 release, and cell-to-cell spread observed *in vivo* is not completely reproduced *in vitro*
11 [128].

12 Alternative cell substrates for HCV replication *in vitro* are HepG2 cell line and
13 hepatoma cells. HepG2 derive from primary hepatocytes and polarize in culture.
14 Unfortunately, HepG2 cells do not express CD81 and miR-122 - a microRNA highly
15 expressed in the adult liver [129] and essential host factor for HCV replication (reviewed
16 in chapter 4; [130]) - and are thus inherently resistant to HCV infection. To allow viral
17 entry and replication, HepG2 cells have been engineered to express human CD81 and
18 miR-122 [131, 132]. Hepatoma cells, in contrast, are CD81 and miR-122 positive and,
19 depending on malignant and differentiation features, retain their ability to polarize in
20 culture. Unfortunately, these cells are difficult to grow *in vitro* and often necessitate of
21 specific medium supplements [133] and gel substrate in which to adhere [134]. On this
22 regard, however, significant advancements have been obtained with hepatoma cell lines

1 capable of supporting HCV and hepatitis B virus replication and obtained from a liver
2 cells of an HCV-infected patients that were further selected in nude mice [135].

3 HCV replication and persistence greatly depends on host factors and genetic
4 background. It became clear in recent years, for instance, that host genetics influences
5 disease progression, host immune response, and response to antiviral therapy [136, 137]).
6 A small nucleotide polymorphism upstream of a λ -IFN gene, for instance, strongly
7 correlates with disease and therapy outcome, and host-targeting antivirals (HTAs) have
8 become an attracting field of drug research [138]. If hepatoma cell lines are the obvious
9 choice, they do not fully recapitulate all aspects of HCV replication in the liver, let alone
10 the extrahepatic target sites. Thus, HCVcc or equivalent systems based only on liver cells
11 might provide partial and biased information on type and extent of host factors playing
12 key roles in pathogenicity, and viral clearance or persistence.

13

14 **3.3. Novel *in vitro* methods**

15 Alternatives to the above-described methods are under investigation to achieve
16 productive HCV infection in more physiologic conditions. Primary human hepatocytes
17 from genetically diverse donors are difficult to obtain and can be propagated for short
18 periods during which cells progressively loss differentiation. Human fetal liver cells, also
19 difficult to achieve and exhibiting wide levels of permissiveness to HCV replication, can
20 be cultivated longer and are generally more susceptible to HCV infection compared to
21 adult human hepatocytes [139, 140].

1 In the last years the development of three-dimensional culture systems, natural and
2 synthetic anchoring gels, co-culture methods, and cell reprogramming techniques have
3 led to the establishment of novel cell substrates for HCV replication *in vitro*.

4 5 **3.3.1. Semi-continuous cell lines of primary human hepatocytes**

6 Co-cultivation with feeder cells prolong survival and have beneficial effects on
7 biological functions of primary human hepatocytes [141]. Micropatterned co-cultures of
8 primary human hepatocytes, usually seeded in small clusters and surrounded by
9 fibroblasts, consent cell polarization and periods of cultivation compatible with HCV
10 replication that however takes place at low efficiency and causes limited spread to
11 contiguous cells due to innate immune response and other intrinsic resistant factors [142,
12 143]. To increase permissiveness, good results have been achieved by combining co-
13 cultivation with strategies aimed to inhibit antiviral responses by engineering cells to
14 express antiviral proteins that antagonize IFN-antagonists, treating cells with inhibitors of
15 pathogen-associated pattern recognition receptors, reducing expression of genes involved
16 in host defense by RNA interference, etc. [107, 144, 145].

17 18 **3.3.2. Three-dimensional, tissue-engineered cultures**

19 Compared to conventional cultures, three-dimensional cultures more accurately
20 preserve multicellular architecture, viability, morphology, differentiation status,
21 proliferation capacity, and gene expression profiles of cell populations. In virology, three-
22 dimensional cultures are considered very important as they should maintain tissue-
23 specific host factors, innate immune function, cell cycle status, and polarity resembling

1 analogous cells *in vivo* and thus better supporting the natural course of viral infection
2 [141]. Another benefit of three-dimensional cultures is the possible combination of
3 multiple cell types involved by viral infection *in vivo*. This approach has been used to
4 demonstrate the dependence of hepatocyte function and growth on endothelial, Kupffer,
5 fibroblasts, and other cells part of liver architecture [146, 147]. As described above, these
6 interactions are also important in HCV infection, cell-to-cell transmission and, as
7 demonstrated for Kupffer cells and liver sinusoidal endothelium [148, 149], modulate
8 HCV replication and disease progression. Successful attempts to create three-dimensional
9 cultures of primary hepatocytes have been reported in recent years and are emergent *in*
10 *vitro* models of studying hepatotropic viruses.

11 The models developed insofar use different scaffolding biomaterials and cell types
12 and are supplemented with various growth factors or other biomolecules. Full description
13 of technological platforms, advantages, and limitations is provided elsewhere [141, 150].
14 Three-dimensional cultures of hepatic tissue should enable the study of HCV infection in
15 more physiological contexts, investigate virus spread in multiorgan models, and elucidate
16 host-pathogen interactions in authentic host cells.

17

18 ***3.3.3. Production of hepatocyte-like cells from cell reprogramming***

19 The development of induced pluripotent stem cells (iPSCs) [151] has shown a great
20 potential in disease modeling, drug screening, organ reconstruction and cancer therapy.
21 Human iPSCs offer the ability to produce host-specific differentiated cells and thus have
22 the potential to transform the study of infectious disease. In 2011 Yoshida *et al* reported
23 that iPSC-derived hepatocytes expressed HCV receptors, including CD81, SR-BI,

1 CLDN1, and OCLN; in contrast, the iPSC showed no expression of SR-BI or CLDN1.
2 HCV RNA genome replication occurred in the iPSC-derived hepatocytes. Anti-CD81
3 antibody, an inhibitor of HCV entry, and interferon, an inhibitor of HCV genomic
4 replication, dose-dependently attenuated pseudotyped HCV entry and HCV subgenomic
5 replication in iPSC-derived hepatocytes, respectively [152].

6 Accordingly, in 2012 Schwartz *et al* [153] and Roelandt *et al* [154] reported that
7 iPSC-derived hepatocytes, but not iPSCs, support the entire life cycle of HCV, including
8 inflammatory responses to infection, enabling studies of how host genetics impact viral
9 pathogenesis. Wu *et al* also reported that permissiveness to infection was correlated with
10 induction of the liver-specific microRNA-122 and modulation of cellular factors that
11 affect HCV replication [155].

12 A humanized mouse model consisting of triple knockout, combined
13 immunodeficient (IL2R γ ^{-/-}, Rag2^{-/-}) native hepatocyte-poor (fumarylacetoacetate
14 hydrolase^{-/-}) mice reconstituted with human primary hepatocytes in the liver provides a
15 novel experimental opportunity which mimics the *in vivo* growth of the human
16 hepatocytes [156, 157]. Zhou *et al* introduced the personalized mouse model using
17 human iPSCs (Figure 3) [158]. Accordingly, Carpentier *et al* reported that hepatocytes
18 differentiated from hiPSCs could be engrafted in the liver parenchyma of immune-
19 deficient transgenic mice carrying the urokinase-type plasminogen activator gene driven
20 by the major urinary protein promoter. The hepatocytes were maintained for more than 3
21 months in the liver of chimeric mice, in which they underwent further maturation and
22 proliferation. These engrafted and expanded human hepatic-like cells were permissive to

1 *in vivo* infection with HCV-positive sera and supported long-term infection of multiple
2 HCV genotypes [159].

3 Recently, the field of HCV research has been revolutionized by the finding that
4 pigtail macaque (*Macaca nemestrina*) hepatic cells derived from iPSCs support the entire
5 HCV life cycle, and pigtail macaques may serve as a suitable, clinically relevant model
6 for the study of HCV infection [160].

7 Moreover, the technologies generating vascularized and functional human liver
8 from human iPSCs by transplantation of liver buds created *in vitro* have also been
9 established. These findings will play a very important role in understanding the
10 mechanism of HCV infection, spread in non-hepatic cells and tissues, and therapy for
11 HCV related hepatocellular carcinoma [161].

12

13 **3.3.4. *In vitro* models of extrahepatic HCV infection**

14 Beside hepatocytes and as described in previous chapters, HCV RNA and proteins
15 have been detected at low levels in lymphatic system, brain, gut, thyroid, etc. [49, 162-
16 166]. Whereas several non-hepatic disease manifestations point to a direct role of HCV, it
17 is not yet clear whether these tissues are truly infected (and potential extrahepatic
18 reservoirs [167, 168]) or score positive due to infiltrating HCV-infected mononuclear
19 cells. In this respect, an ever-growing body of literature suggests that blood cells are
20 indeed an important site of HCV replication and persistence [39, 51, 52, 169]. It is
21 unknown and object of intense research whether the antiviral compounds, tested in
22 hepatic cells, possess same efficacy at blocking virus replication and curing infection.

1 Many attempts to establish an *in vitro* model for replication in non-hepatic cells
2 have been attempted with controversial results. Several reports claimed successful
3 replication of HCV_{cc} or HCV_{TCP} in B- and T-lymphocytes, monocytes/macrophages, and
4 dendritic cells of healthy donors, in macrophages of HIV-infected patients, and cells of
5 different histotype [33, 41, 170-176]). Unfortunately, HCV replication was detectable at
6 low levels at best and all these models lacked consistency and reproducibility. The
7 availability of an *in vitro* model for HCV replication using non-hepatic cells is therefore
8 still an open issue. As further additional problem, there are no laboratory methods
9 capable to firmly distinguish mere HCV detection from productive infection. Standard
10 virological assays used for hepatoma cells are often inapplicable. Methods based on the
11 detection of viral antigens are poorly sensitive to ascertain HCV infection [177]. In
12 contrast, quantitative molecular assays that need to be highly sensitive for clinical
13 diagnosis (see below) suffer from high background due to contamination by viral inocula
14 or input RNA used for transfection. Various approaches, ranging from use of molecular
15 clones with gene reporters, monitor expression of non-structural proteins, detection of
16 replicative intermediate, etc., have been pursued to overcome this hurdle but there are no
17 suitable *in vitro* method based on non-hepatic cells supporting robust HCV replication
18 and exploitable for antiviral testing.

19

20 **4. FINE-TUNING OF IN VITRO CULTURE METHODS AND MOLECULAR**
21 **DIAGNOSIS TO MEET NEW PARADIGMS OF HCV THERAPY: AN EVER-**
22 **CHANGING PROCESS**

23

1 Availability of culture methods to replicate HCV *in vitro* prompted the study of
2 virus and host factors as candidate targets for antiviral therapy. Structure and function of
3 HCV protease and polymerase were predicted by molecular modeling before the advent
4 of culture systems, but then again it was necessary an *in vitro* model of replication to
5 assess efficacy of the approach and which lead compounds work best. The first proof of
6 concept in the path of HCV DDAs came in 2003 with a small molecule from Bayer that
7 inhibited the HCV protease *in vitro* and significantly reduced viremia *in vivo* [178]. In the
8 same years the development of the replicon system represented a major breakthrough as
9 it allowed direct testing of potential candidates to block HCV replication in a laboratory
10 setting. The payback was substantial; there has been an explosion of new agents,
11 targeting almost every phase of HCV replication and ranging from entry into the
12 hepatocyte to assembly and virion release. Also thanks to the lessons learned with HIV,
13 key targets of HCV therapy were found the HCV protease (NS3/4) and the polymerase
14 (NS5 A/B).

15 As described in chapter 6, most clinically approved DDAs superseded the mainstay
16 of IFN-RBV combined treatments to the point that IFN-free treatments are now available
17 and the main goal of treatment has become cure, rather than halting disease progression.
18 Such high stake has changed criteria and parameters defining response to therapy and
19 residual viremia (see in the next paragraphs) and led to refocusing on use of *in vitro*
20 systems. Up to few years ago, these methods were essentially used to test potency of
21 HCV protease and polymerase inhibitors in hepatocytes. In the last years these methods
22 where essentially used to investigate: 1. *Variability and mechanisms of resistance*. First
23 *in vivo* trials clearly demonstrated that a single agent is not sufficient to produce sustained

1 viral suppression. Due to infidelity of viral RNA polymerases and high production
2 (around 10^{12} virions per day [179], HCV rapidly accumulates an ensemble of viral
3 variants, called quasispecies, each of those containing one or more mutations compared
4 to parental virus. Using a single drug targeting a single block point will allow the rapid
5 development of resistance. This led to the development of combinations of drugs
6 targeting a different step of HCV replication steps or attacking the same viral molecule
7 but having different sensitivities to mutations conferring resistance. Here, *in vitro*
8 methods are essential to identify drug combinations that have synergistic effects and,
9 usually following prolonged *in vitro* cultivation in the presence of suboptimal amounts of
10 drug(s), identify viral pathways and mutations blunting the drug(s); 2. *Antiviral efficacy*
11 *across HCV genotypes*. Accumulation of mutations caused progressive divergence of
12 viral variants intra- and inter-host that have been organized in genotypes and
13 subgenotypes. Hierarchical classification is important for epidemiological properties and
14 responsiveness to therapy. As discussed below, identification of infecting genotype
15 (genotyping) is essential to define optimal IFN-RBV treatment and likelihood of clinical
16 and virological responses. Structural differences among genotypes also impact current
17 DDAs that have different levels of effectiveness against different block points, different
18 regimes and combinations according to genotype. *In vitro* models are crucial to define the
19 breadth of antiviral activity against genotypes (and subgenotypes) and to assist in the
20 design of broad-spectrum therapeutic options; 3. *Virus-receptor(s) interplay and*
21 *development of entry inhibitors*. As described above, pathway and cellular actors
22 involved in the entry process are only partly known. For most putative receptors,
23 identified *in vitro*, it is not proved that they interact with the virus also *in vivo*, and work

1 the same way with all *in vitro* systems[180]. Identification of *bona fide* receptors and
2 regions binding to E1 and/or E2 glycoproteins is essential to design molecules inhibiting
3 viral entry. Since binding regions between viral and cellular receptors are usually highly
4 conserved, the resulting entry inhibitor should be broad-range and possess a high genetic
5 barrier, i.e. drug resistance occurs following acquisition of several critical mutations that
6 usually heavily affects protein structure and function [181]; 4. *Host target agents (HTAs)*.
7 Like any virus, HCV utilizes multiple host-encoded factors for cell entry, genome
8 replication and virus assembly. In principle, all these factors are potential drug targets for
9 HCV therapeutics, provided that blocking their function arrests HCV propagation without
10 compromising their physiological activity or harming the cellular pathway to which they
11 belong. *In vitro* studies have shown that this can be achieved with a number of HTAs that
12 however need extensive testing *in vitro* and in suitable *in vivo* models before clinical
13 approval comes into reach[138]. Maraviroc and similar drugs antagonizing chemokine
14 receptors of HIV-1 provide proof of concept that safe and well-tolerated HTAs can be
15 developed; 5. *Antivirals and HTAs in extrahepatic cell target of HCV replication*. Even
16 though DDAs in infected liver cells are extremely potent, there are no data for their
17 efficacy in extrahepatic cells and tissues considered important site of HCV replication
18 and persistence [33]. In this respect, the development of non-hepatic cell culture systems
19 supporting HCV replication with which to test current DDAs and assist in the design of
20 entry inhibitors or HTAs also effective in these cells will represent a great leap for HCV
21 therapy; 6. *Intracellular mechanisms of viral persistence and pathogenesis*. Since HCV
22 does not integrate its viral genome during replication it is generally believed that upon
23 successful and sufficiently long treatment the viral genome is degraded and the infected

1 tissue is eventually cured from infection. This fate has been postulated to occur and
2 corroborated by the fact that most patients cleared the infection. It is not know whether
3 this holds true in the long run, i.e. years after suspension of treatment. Ad hoc *in vitro* and
4 *in vivo* experiments aimed to understand whether and how the virus persists in a non-
5 replicative form intracellularly and if current therapy is effective are warranted.
6 Similarly, the direct and indirect pathogenetic mechanisms leading to cell transformation
7 and other ailments need to be elucidated and, albeit suggesting by numerous clinical trials
8 as described in this thematic issue, no firm conclusions with *in vitro* studies have been
9 reached. 7. *Innate and genetic mechanisms of resistance*. Similarly to what observed with
10 HIV, population studies of individuals at high risk for HCV infection suggest that
11 protective mechanisms, whether immune and/or genetic, allowing clearance of infection
12 without seroconversion do exist [182-184]. This evidence has been corroborated by *in*
13 *vitro* studies showing that genetic polymorphisms influence susceptibility to infection and
14 response to therapy [136]. *In vitro* culture systems to examine innate immunity and HCV
15 interactome, and to manipulate genes associated with resistance to infection could
16 identify potential targets for vaccine design and inform novel therapies.

17 As mentioned, efficacy of current HCV therapy has changed the requirements of
18 clinically-approved molecular assays, and paradigms and clinical interpretation of the
19 results achieved with molecular assays.

20

21 **4.1. Molecular assays to diagnose HCV infection and response to therapy**

22 Screening for HCV detection is carried out by examining blood samples with a
23 combination of immunological and molecular assays. These latter have rapidly acquired a

1 pivotal role in the diagnosis of HCV infection and now are essential for the correct
2 clinical management of infected patients, because they are used to quantitate the copies of
3 viral RNA at baseline, during the course of treatment, and at the end of therapy [185,
4 186]. At least four “*in vitro* diagnostics” molecular platforms are currently available
5 (Table 1). Although the intrinsic performances of these tests may differ, all use an
6 automated RNA extraction followed by automated amplification and detection of HCV
7 genome, and all are characterized by the same molecular principle based on real-time
8 PCR technology. Currently, the assays demonstrate lower limits of detection range of 12-
9 21 IU/ml across all HCV genotype and they are linear from 10 to 10⁸ IU/ml with high
10 accuracy and precision. Genetic variability of HCV is very high, thus correct selection of
11 the viral genome segment targeted is critical for satisfactory performance of gene
12 amplification assays. The 5'-untranslated region (UTR) is the most conserved portion of
13 the genome and the more extensively used for HCV amplification even if it presents
14 sufficient diversity between genotypes to affect assay sensitivity. The more recent
15 versions of the assays have been redesigned to reduce this shortcoming, also with the use
16 of a dual probe approach providing a broader detection and quantification of rare HCV
17 sequences. It is important to note that, except for rare samples, currently the results of
18 different assays are quite well comparable; however it is valuable that each patient is
19 constantly monitored by using the same type of assay.

20 In the monitoring of HCV patients other molecular assays are used. Those for
21 determining the infecting HCV genotype are essential because they help to decide type
22 and duration of therapy as well as to predict treatment outcomes. Sequence analysis of
23 the viral genome is considered the gold standard for genotyping HCV, and it also has

1 been shown that sequencing selected segments of the viral genome (i. e. E1, C, or NS5B
2 regions) suffices for correct HCV genotype and subtype recognition [187, 188]. For
3 practical purposes sequencing the 5' UTR is considered acceptably accurate albeit not
4 completely efficient at distinguishing some subtypes. Commercial kits for semiautomated
5 sequencing are available, but alternative, easier-to-perform methods exist. A widely used
6 commercial test exploits the ability of selected HCV amplicons to hybridize to type-
7 specific probes immobilized on nitrocellulose strips (line probe assay). A recently
8 updated version of this assay uses primers and probes that simultaneously target 5' UTR
9 and C regions improving identification of genotype 1 subtypes and accurately
10 distinguishing genotype 1 from 6 [189].

11

12 **4.2. Key considerations, interpretation of results and screening recommendation in** 13 **the post-DAA era.**

14 The goal of HCV therapy is a sustained virological response (SVR), defined as
15 HCV undetectable in blood 24 weeks after the end of treatment. Early predictors of a
16 future SVR have been investigated and a rapid virological response (RVR) characterized
17 by “undetectable HCV RNA” (e.g < 50 IU/ml) after 4 weeks of standard PEG INF/RBV
18 has been considered a good marker to shorten at 24 weeks (*vs* 48 weeks) therapy in
19 genotype 1 infected patients. The recent development of DAAs and the approval of triple-
20 therapy regimens have given new hopes for higher virus eradication rate in infected
21 patients. That implies more accurate and sensitive quantitative HCV RNA tests for
22 monitoring faster virus kinetics and promptly detecting treatment failures. These new
23 drugs have revolutionized HCV treatment and they have led to the need of interpreting

1 more carefully the results of HCV RNA load (Tables 1 and 2). The concept of
2 “undetectable HCV RNA” has been revised [186, 190]. Only a result defined as “Target
3 not detected” at both 4 and 12 weeks of treatment is now believed discriminating for
4 choosing patients eligible for shorten therapy being considered not more equivalent to an
5 “detectable but below the lower limit of quantification (LLOQ)” HCV RNA result.
6 Again, values above 25 IU/ml at 4, 12 or 24 weeks post-drug administration entail the
7 stop of therapy while a “<25 IU/ml, HCV RNA detected” result is still considered
8 acceptable for defining a patient with SVR. Not all commercial assays work similarly, in
9 particular when very low amounts of HCV RNA have to be quantified. A study
10 demonstrated that a large number of samples at 4 weeks of treatment resulted “Target not
11 detected” when tested by a molecular assay but they have “detectable but below the
12 LLOQ “HCV RNA levels with another commercial assay[191]. Then, depending to the
13 HCV RNA assay used, the medical decisions on which patients have had to receive a
14 shortened treatment regimen can be affected.

15

16 **CONCLUSION**

17

18 The development of methods that faithfully recapitulate in vitro the HCV life cycle
19 has massively increased since the construction of the first replication-competent
20 molecular clone from an isolate of an infected patient. Novel technologies such as three-
21 dimensional cultures and cell reprogramming also promise to dramatically expand the
22 possibility to study individual aspects of the HCV life cycle in primary hepatocytes, in
23 vitro reconstructed liver tissue, or extrahepatic cells and tissues know to be target of HCV

1 infection in vivo. These novel substrates, together with HCV_{cc} and HCV_{TCP} or similar
2 systems permitting generation of inter-genotypic chimeras should allow studying some
3 still obscure aspects of virus release and spread in a model more complicated but
4 certainly closer to what takes place in vivo. Given the different in vivo relationships and
5 outcomes of infection and disease exhibited by HCV, there is urgent need for in vitro
6 systems permissive to patient-derived HCV isolates. The development of protocols to
7 differentiate human and animal iPSC to fully functional hepatocytes and creation of
8 mouse models engrafted with human iPSC-derived liver cells supporting HCV replication
9 is certainly a big step toward this goal. Gene manipulation techniques with which to
10 tweak hepatic cell genes permitting or restraining HCV replication combined with cell
11 reprogramming and novel culture systems will offer the opportunity to finely dissect
12 virus–host interactions as well as to understand the contribution of the host to resistance
13 to infection or disease progression. Novel systems of HCV replication in vitro may help
14 studying innate and immune mechanisms of cell resistance and assisting in the design of
15 vaccine candidates. In more immediate terms, HCV culture systems already had
16 important payoffs mainly consisting in the definition of targets for antiviral therapy, and
17 development and testing of novel drugs so effective that in mere two years swiped away
18 standard IFN-RBV therapy and revolutionized objectives and concepts of HCV therapy
19 and patient management.

20 New concepts and goals of therapy also significantly impacted *in vitro* research and
21 diagnostics. *In vitro* culture systems will be necessarily refocused toward the
22 development of personalized medicine approaches using patient-derived HCV isolate to
23 optimize the therapeutic regimen, analyze and characterize the emergence of resistance

1 mutations, and define the role of chemokines in the pathogenesis of HCV chronic
2 infection and extrahepatic manifestations as well as markers of response to therapy [192-
3 194].

4 More potent regimens with higher cure rates created complexity for laboratory
5 diagnosis and clinicians. This has led to definition of new guidelines and interpretations
6 of HCV RNA viral load defining SVR and response to therapy in general. Here, accurate
7 quantitation, high sensitivity, and reproducibility and interchangeability of results among
8 clinically-approved molecular platforms are even more important compared to what
9 required with IFN treatment. Standardization of methods and equivalence of results is
10 badly needed as well as definition of protocols to monitor adherence on-therapy, and
11 early prediction of disease worsening and development of hepatocellular carcinoma.

12 Given the high cost of the new regimens and global burden of HCV chronic
13 infection and disease in vitro culture systems and in vitro diagnosis will progressively
14 increase their importance.

15

16

17

18 **Conflict of interest**

19 The authors declare that this article content has no conflicts of interest.

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Table 1. HCV RNA quantitative assays commercially available.

| Assay | Manufacturer | Method (target region) | Lower limit of detection (IU/ml) | Lower limit of quantification (IU/ml) | Linear range of quantification (IU/ml) | In vitro diagnostic registration |
|--|------------------------|------------------------------------|----------------------------------|---------------------------------------|--|----------------------------------|
| Abbott RealTime HCV test | Abbott Molecular | Real-time PCR (5'-UTR) | 12 | 12 | 12 to 1×10^8 | CE, FDA |
| Artus HCV QS RGQ kit | Qiagen | Real-time PCR (target proprietary) | 21 | 35 | 35 to 1.77×10^7 | CE |
| COBAS AmpliPrep / COBAS TaqMan v2.0 test | Roche Molecular System | Real-time PCR (5'-UTR) | 15 | 15 | 15 to 1×10^8 | CE, FDA |
| VERSANT HCV RNA 1.0 assay | Siemens | Real-time PCR (<i>pol</i> gene) | 15 | 15 | 15 to 1×10^8 | CE |

CE, European Community.

FDA, Food and Drug Administration.

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Table 2. Specimen results and interpretations.

| Reported result | Interpretation of the result |
|--|---|
| Target not Detected | No PCR amplification or detection, Ct value for HCV is above the limit for the assay or no Ct value for HCV is obtained |
| HCV RNA detected, less than the LLOQ IU/ml HCV RNA | Calculated IU/ml is below the lower limit of quantification (LLOQ) of the assay |
| "number" IU/ml, HCV RNA detected | Calculated result is quantifiable, greater than or equal to the LLOQ and less than or equal to upper limit of quantification (ULOQ) |
| "greater than the ULOQ IU/ml HCV RNA | Calculated result is above the linear range of the assay |

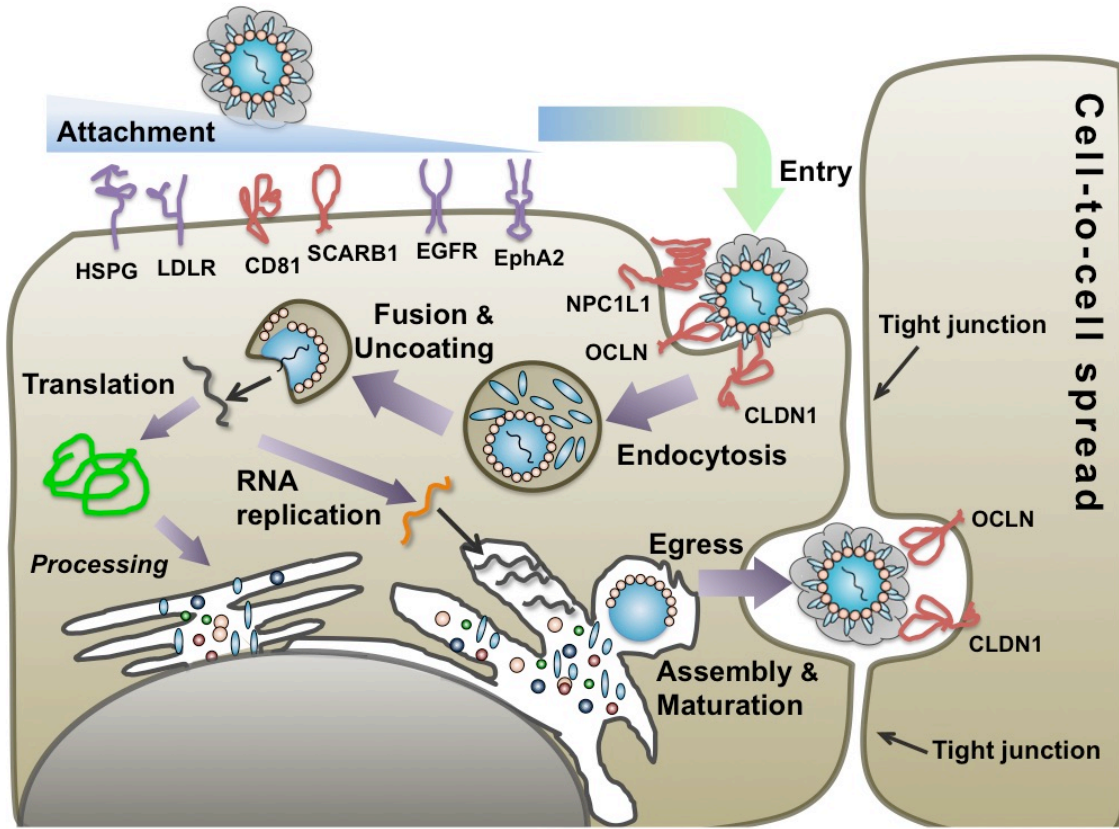
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1 **Figures**

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5 **Fig. (1). Schematic of HCV life cycle and the five major steps of replication.** The

6 virus interacts with target cells by binding to receptors that, based on in vitro and in vivo

7 entry inhibition tests with antibodies against the cellular molecules, are considered

8 essential (shown in red) or accessory (purple) entry factors. Following attachment the

9 virus is internalized through clathrin-mediated endocytosis, and the cellular and viral

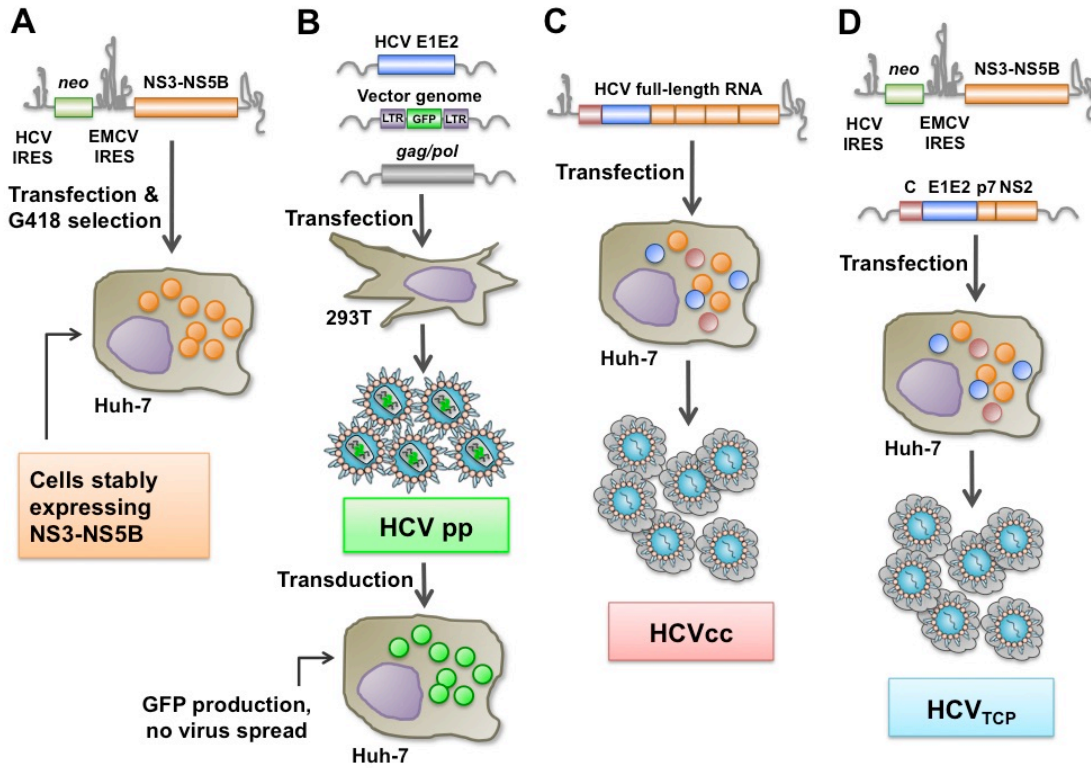
10 membranes are fused and capsid disorganized with a process triggered by the low pH of

11 the endosome. After uncoating the positive-strand RNA genome is released into the

12 cytoplasm and directly translated in a polyprotein precursor that is then cleaved into

1 single proteins by both host and viral proteases. The non-structural (NS) proteins and
2 some host factors form a replication complex that synthesized multiple copies of the
3 HCV RNA genome via a minus-strand replicative intermediate. Assembly of the
4 nucleocapsid occurs into the endoplasmic reticulum from which the virion acquires the
5 envelope with E1 and E2 glycoproteins. Maturation and association with endogenous
6 lipoproteins to form lipoviral particles immediately follow. Virions are eventually
7 released from the cells and transmitted to other cells via a cell-free mechanism or directly
8 through tight junctions to neighboring cells by cell-to-cell spread. This process is likely
9 mediated by only two receptors.
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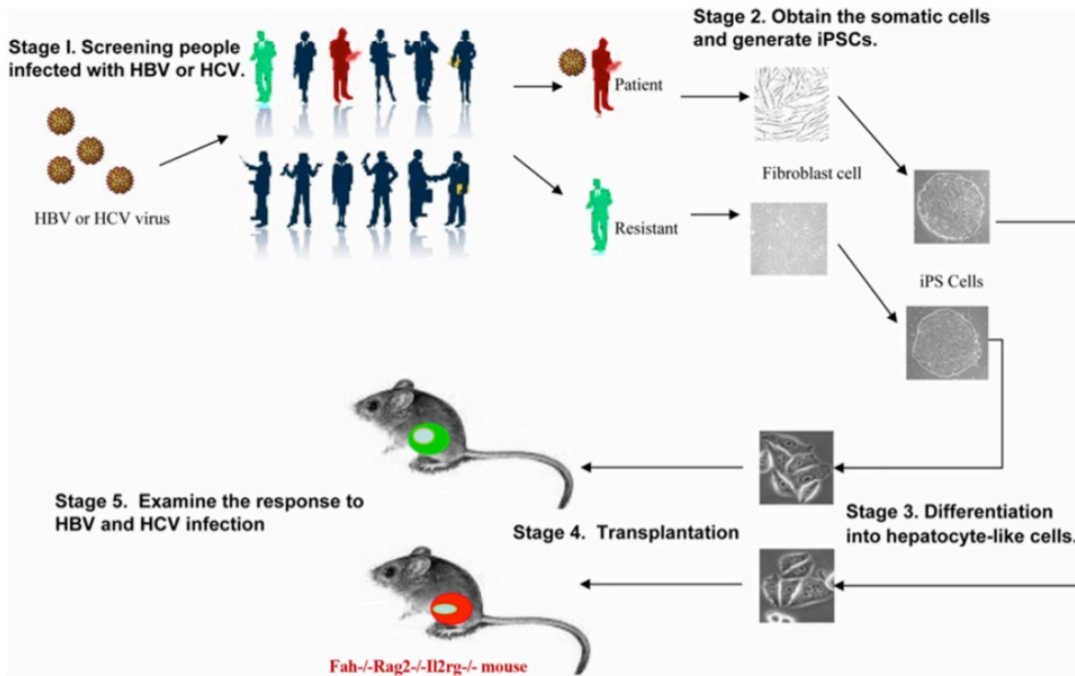
3 **Fig. (2). Standard HCV cell culture systems.** (A) The HCV replicon encodes the
4 selection marker neomycin (*neo*) and the HCV replicase proteins (NS3-NS5B). The
5 coding sequences are placed under control of the internal ribosome entry site (IRES) of
6 HCV and encephalomyocarditis picornavirus (EMCV), respectively. Huh-7 cells are
7 transfected with the HCV replicon and treated with G418 (an antibiotic) treatment, to
8 select for cells harboring the HCV replicon and thereby expressing the antibiotic
9 resistance gene (*neo*). (B) HCV pseudoparticles (HCVpp) are produced by co-
10 transfection of human epithelial 293T cells with three expression vectors encoding HCV
11 E1 and E2, HIV or murine leukemia virus (MLV) Gag-Pol, and HIV or MLV genome
12 encoding a reporter gene (typically luciferase or green fluorescent protein [GFP]).
13 HCVpp produced and released by 293T cells are collected and used to evaluate/quantitate

1 HCV entry into Huh-7 or other target cells. (C) Cell-culture derived HCV (HCV_{cc}) is
2 generated by transfecting Huh-7 with a full-length HCV RNA genome. The released
3 virions are replication competent and associated with lipoproteins as for the virus
4 produced in vivo. (D) Trans-complemented HCV particles (HCV_{TCP}), are obtained by co-
5 transfection of Huh-7 with the HCV replicon shown in Fig. 1A and an expression vector
6 encoding the structural and NS genes missing in the HCV replicon and necessary to
7 produce HCV virions. These particles are associated with lipoproteins but incapable to
8 replicate. This system is used to overcome the hurdle of isolate specificity and provide
9 isolate-specific information for HCV entry, replication and assembly. Typically uses the
10 subgenomic replicon from the replication-competent molecular clone of JFH1 and
11 missing genes derived from a specific isolate.

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4 **Fig. (3).** Strategies for developing a personalized mouse model for hepatitis B virus and
5 HCV using personalized human induced pluripotent stem (iPS) cells (adapted from ref
6 [158], with permission from the Author).