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Short-Form Paper

Reconstitution of the entire hepatitis C virus life cycle in non-hepatic cells

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Author contribution: MBZ and TFB designed and supervised research. DDC, MT, DJF, EG, SP, GL, RB, MBZ and TFB performed research. DDC, MT, EG, SP, MBZ and TFB analyzed data. RB provided important ideas for the initiation and execution of this study and provided reagents. DDC, MT, MBZ, DJF and TFB wrote the manuscript. DDC, MT, and DJF contributed equally to this work. MBZ and TFB contributed equally.

Abbreviations:

HCV, hepatitis C virus; HCVpp, retroviral HCV pseudo-particle; HCVcc, cell culture-derived HCV; OCLN, occludin; CLDN1, claudin-1; SR-BI, scavenger receptor class B type I; miR-122, microRNA-122; apoE, apolipoprotein E; EGFR, epidermal growth factor receptor; VLDL, very-low-density lipoprotein.

Abstract

Hepatitis C virus (HCV) is a human hepatotropic virus, yet the relevant host factors restricting HCV infection to hepatocytes are only partially understood. We demonstrate that exogenous expression of defined host factors reconstituted the entire HCV life cycle in human non-hepatic 293T cells. This study shows robust HCV entry, RNA replication, and production of infectious virus in human non-hepatic cells, and highlights key host factors required for liver tropism of HCV.

Main Text

Virus-host interactions that determine and restrict specific tissue and host tropisms display complex evolutionary history and have significant consequences on the pathogenesis of viral infection and human disease. Viral hepatitis is a major disease burden. Indeed, infection of hepatocytes by a variety of hepatotropic viruses from different orders and families can lead to tissue inflammation, fibrosis, and hepatocellular carcinoma. Hepatitis C virus (HCV), a member of the family *Flaviviridae,* is a prime example of a virus that causes chronic hepatitis worldwide. While HCV primarily infects hepatocytes of humans and chimpanzees, the virus has been shown to enter neuronal and endothelial cells of the blood-brain barrier. However, infection of these cells occurs at a low level and production of infectious viruses is greatly diminished relative to hepatically derived cells (9, 10). Unlike HCV, other members of the family *Flaviviridae* have a much broader tissue and species tropism. For example Dengue virus infects and replicates both in the midgut epithelia of Aedes aegypti mosquitoes, and in human monocytes and hepatocytes (20, 25, 39). Moreover, a virus closely related to HCV was recently identified from dogs' respiratory samples (18). A large panel of host factors required for HCV has been identified so far (36). However, the key host factors mediating liver tropism of the virus and allowing reconstitution of the viral life cycle in human cells is still only partially understood.

Taking advantage of our current knowledge of host factors involved in HCV infection, we sought to engineer a human kidney cell line (293T) to be capable of sustaining the entire HCV life cycle. The aim was to define those host factors that are

64 necessary and sufficient for allowing the HCV life cycle, in order to understand the liver
65 tissue-specificity of HCV.

66 293T cells were obtained from ATCC and their identity was verified by genomic
67 profile comparison to the LGC Standards database by short tandem repeat profiling as
68 described (1) (Fig. 1A). In order to render them infectable by HCV, we used lentiviral
69 vectors to express the four principal HCV host entry factors: claudin-1 (CLDN1), CD81,
70 occludin (OCLN), and scavenger receptor class B type I (SR-BI) (2, 7, 34, 35) by using
71 previously described expression constructs and methods (3, 24). Four 293T stable cell
72 lines were selected to express either CLDN1 alone, CD81/OCLN with or without CLDN1,
73 or CLDN1/CD81/OCLN together with SR-BI (293T-4R). After verifying stable expression
74 of these proteins using receptor-specific antibodies (Fig. 1B), we infected these cells
75 with HCV pseudoparticles expressing the envelope glycoproteins of HCV genotype 1b
76 (HCVpp; HCV-J strain, described in (31)). While CLDN1 expression alone conferred
77 limited permissiveness for HCV infection as previously described (7), expression of all
78 four factors enhances HCV entry to a level that was around four-fold higher than
79 Huh7.5.1 cells, which is the liver-derived model hepatoma cell line for studying HCV
80 infection (Fig. 1C).

81 Genuine cell culture infection of HCV (HCVcc) was then investigated in 293T-4R
82 cells using a chimeric virus composed of two genotype 2a isolates (designated Jc1 (19,
83 32)) and engineered for Renilla luciferase expression (JcR2a; (38)). However, as shown
84 in Fig. 2A, overcoming the HCV entry block was not sufficient for robust viral RNA
85 replication in 293T cells.

Several studies have shown that microRNA (miR)-122 is a liver-specific host factor critical for HCV replication (5, 16, 17, 28). Since Northern blot analyses demonstrated non-detectable miR-122 expression in 293T-4R cells (Fig. 2C), we investigated whether exogenous miR-122 expression reconstituted viral RNA replication. Indeed, stable expression of this factor, by using miR-122 encoding lentiviruses in the 293T-4R line, conferred the cells permissive for bona fide HCVcc infection, with replication to comparable levels as Huh7.5.1 cells as assessed by luciferase reporter activity (Fig. 2B). Further confirmation of genuine infection was garnered by observing similar infectivity ($TCID_{50}$) with HCVcc (Jc1) without a reporter gene, by detecting expression of viral protein NS5A (Fig. 2B). We verified expression of miR-122 in transduced 293T-4R/miR122 cells, and the level was comparable to that of Huh7.5.1 cells as assessed by Northern blot (Fig. 2C), and the cell proliferation rate of the different cell lines was similar (data not shown). Kinetics of HCV replication in 293T-4R/miR122 cells matched those of Huh7.5.1 cells, suggesting that aside from miR-122, cell factors present in human liver- and kidney-derived cells are equally efficient for replication as assayed by luciferase reporter gene expression (Fig. 2D). Expression of viral proteins in infected cells was further confirmed using HCV core-specific immunofluorescence (Fig. 2E) and flow cytometry (data not shown).

To further confirm whether viral entry and replication in stably transduced 293T cells is mediated by the same host and virus factors as in human Huh7.5.1 hepatoma cells, we used well-characterized entry and replication inhibitors. Antibodies directed against the HCV entry factors CD81, CLDN1, and SR-BI (JS-81, BD Biosciences, (11), Zahid et al., unpublished, respectively) were effective in inhibiting infection (Fig. 2F).

Moreover, both a polyclonal serum recognizing apolipoprotein E (apoE) (29), and a monoclonal antibody recognizing the LDL receptor binding domain of apoE (37) effectively neutralized HCV infection of 293T-4R/miR122 cells (Fig. 2F). The same was true for the recently identified HCV entry inhibitor, erlotinib, which targets the kinase activity of the host entry regulatory protein, epidermal growth factor receptor (EGFR) (Fig. 2F) (24). Likewise, well characterized inhibitors of HCV NS3 protease or polymerase, telaprevir (VX950) and mericitabine (R7128), impaired HCV replication in 293T-4R/miR122 cells (Fig. 2F). These data demonstrate that HCVcc RNA replication in kidney-derived 293T-4R/miR122 cells is efficient, and dependent on similar mechanisms as in liver-derived Huh7.5.1 cells.

Despite efficient entry and RNA replication of 293T-4R/miR122 cells infected with recombinant HCVcc, these cells did not release infectious virions, suggesting that kidney-derived cells lack factors required for viral assembly and release. Therefore, we aimed to reconstitute virus production by expression of HCV assembly factors. HCV production shares factors involved in very-low-density lipoprotein (VLDL) assembly, a process that occurs exclusively in hepatocytes (13, 14, 27). While the necessity of apolipoprotein B (apoB) in HCV production is controversial (15), apoE is known to be critical, and is incorporated into the virion (26). We therefore expressed the most common isoform of apoE (apoE3) in 293T-4R/miR122 cells by using a lentiviral vector encoding human apoE3 as described previously (23), and confirmed its expression by flow cytometry using an apoE-specific antibody (Fig. 3A). We then infected 293T-4R/miR122/apoE cells. Subsequently, the production and release of viral particles was assessed by incubating naïve Huh7.5.1 cells with the supernatants from these cells.

Indeed, 293T-4R/miR122/apoE released infectious HCV particles as shown by a marked and highly significant increase in infectivity (as assessed by luciferase activity of JcR2a virus and $TCID_{50}$ of Jc1 virus without a reporter gene) of the supernatant compared to the supernatant of 293T-4R/miR122 cells without apoE expression (Fig. 3B). Although the production of infectious particles was lower than in Huh7.5.1 cells studied in side-by-side experiments, these data indicate that apoE is a key factor for virus production in reconstituting the viral life cycle in non-hepatic cells. This diminished HCV production was not due to diminished replication levels as apoE transduced cells had similar HCV replication levels to 293T-4R/miR122 cells prior to apoE expression (data not shown). To test if HCV produced by these cells is reliant only on human apoE3 isoform or could use other forms of apoE, we similarly transduced human apoE2 and apoE4 isoforms, as well as murine apoE (Fig. 3C). Viruses produced from 293T cells expressing these apoE isoforms and the mouse ortholog had similar infectivity compared to human apoE3 isoform (Fig. 3D).

Focusing on the most common apoE isoform (apoE3), we further characterized the kinetics and attributes of these viruses. First, we confirmed that HCV particles from engineered 293T cells could establish infection by monitoring the increase in HCV genomes over time in Huh7.5.1 target cells after exposure to the supernatant of HCVcc-infected 293T-4R/miR122/apoE cells (Fig. 4A). Next, we characterized the kinetics of HCV RNA production from infected 293T-4R/miR122/apoE cells by measuring HCV RNA in the media at serial time points following infection (Fig. 4B). Interestingly, the levels of HCV RNA released into the culture media of 293T-4R/miR122/apoE cells was similar to levels of HCV RNA in the media of Huh7.5.1 cells after 72h, whereas cells that

were not transduced with apoE released minimal amounts of HCV RNA, likely due to previously reported non-specific release of HCV RNA during replication (Fig. 4B)(33). These data suggest that the specific infectivity differs between virus produced from Huh7.5.1 cells and 293T cells engineered to express essential host factors. An estimation of the specific infectivity of the released viruses ($TCID_{50}$/HCV RNA genomes) revealed approximately a 30-fold difference between the differently derived viruses (1/900 for Huh7.5.1-derived virus and 1/26,000 for 293T-4R/miR122/apoE-derived virus). It should be noted that HCV particles produced from 293T-4R/miR122/apoE cells proved to have a similar route of infection to hepatically-derived HCVcc, in that entry into Huh7.5.1 cells was neutralized by well-characterized HCV entry inhibitors including CD81-, SR-BI-, CLDN1-, apoE-specific antibodies, and erlotinib (Fig. 4C). Fractionating the virus by iodixanol density gradients revealed that the infectious virions produced from 293T-4R/miR122/apoE cells have similar buoyant density as those from Huh7.5.1 cells (Fig. 4D).

The data presented here demonstrate that trans-expression of OCLN, CD81, CLDN1, SR-BI, miR-122, and apoE endow 293T human kidney-derived cells with the capacity to support the complete HCV life cycle. Expression of four principal entry factors and miR-122 generated cells with higher entry and similar replication kinetics as the extensively optimized Huh7.5.1 cells (4, 41). It should be noted in this context, that the recently identified entry factor EGFR is also expressed in 293T cells (data not shown, 24, 40). We confirmed that expression of CLDN1 alone appears to be sufficient for infection of 293T cells (7), and expand these findings in that high-level expression of the four canonical HCV entry factors make previously impenetrable cells four-fold more

permissive than Huh7.5.1 cells. These observations were confirmed by HCVcc infection of 293T cells engineered to express miR-122 in addition to variable sets of entry factors (data not shown). While the present study focused on engineering a human cell line for infection, it has been demonstrated that concomitant high level expression of the four human entry factors is required for robust entry of mouse hepatocytes *in vivo* (6). Since none of the identified entry factors are exclusively expressed in the liver, it is likely that the combined expression of these host factors at substantial levels allows the virus to productively infect the human liver, rather than a single liver-specific entry factor restricting HCV infection.

Investigators have shown that miR-122 expression increases HCV replication in mouse embryonic fibroblasts and other hepatoma cell lines such as HepG2 cells (21, 17, 28). Furthermore, HEK-293 cells modified to express miR-122 are capable of sustaining selectable HCV subgenomic replicons, although expression of mutated miR-122, at sites required for HCV RNA binding, can also sustain these replicons (5). We demonstrate here *de novo* replication following an infection event of a non-hepatic cell line engineered to express HCV host factors. Our data also demonstrate that there is no restrictive factor of HCV entry and viral RNA replication that is present in 293T cells. HCV entry and replication in human blood brain barrier endothelial and neuronal cells have been described (9, 10). In contrast to the kidney-derived cells described here, HCV replication in blood brain barrier endothelial cells occurred via a miR-122 independent mechanism, yet at a diminished level (9). Thus, the cell lines developed in this study may be useful as a tool to further understand the molecular mechanisms of extra-hepatic infection.

The production of HCV from 293T-4R/miR122/apoE cells was diminished relative to Huh7.5.1 cells, but markedly and significantly higher than in cells without apoE expression. This demonstrates that apart from apoE, all the other factors necessary for the production of infectious particles are present in 293T cells, yet additional host factors may increase efficient production levels. The cell line generated in this study is likely to allow further discovery of the minimal set of host factors required for robust viral production. Additional relevant factors enhancing viral production may be apoB (27), DGAT1 (13), or microsomal triglyceride transfer protein (MTP) (12, 14). Notably, apoE has recently been demonstrated to be essential for virus production; apoE-deficient mouse hepatocytes with trans-expression of HCV RNA and proteins along with apoE are able to produce high levels of infectious virions (23).

In summary, this study demonstrates that a small set of defined host factors is sufficient to reconstitute the complete viral life cycle in non-hepatic cells. These results advance our knowledge on tissue-specific factors for HCV infection and provide novel tools to elucidate host and restriction factors for the HCV life cycle.

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Figure Legends

Figure 1. Expression of four HCV entry factors renders 293T cells highly permissive to HCVpp entry. (A) Short tandem repeat (STR) profile of the 293T cells used in this study (Cell line authentication, LGC Standards) was performed as described previously (1). The names of tested loci are indicated in bold and peak positions from STR profile of 293T cells were compared to LGC Standards database. (B) 293T cells (cultured in DMEM high glucose, Life Tech) were transduced with lentiviruses (as described in (3)) to express given HCV entry factors. After transduction, cells were selected with blasticidin (12 µg/ml) for 2 weeks. Blasticidin-resistant cells were assessed by flow cytometry using monoclonal antibodies (CLDN1 (11), OCLN (Cat.# 33-1500 Invitrogen), SR-BI (Zahid et al., submitted manuscript)) recognizing indicated entry factors. Entry factor transduced cells (dark grey histograms) were compared to naïve 293T cells (light grey histograms) and isotype control antibody (Cat.# 10400C, Life Technologies, white dashed histograms). X axis: fluorescence intensity, Y axis: number of events. (C) Transduced 293T cells were assessed for HCVpp (genotype 1b; HCV-J strain; produced as described in (31)) entry by determining luciferase activity 72h post-infection as previously described (35). Results were first normalized to vesicular stomatitis virus pseudoparticle entry (VSV-Gpp; produced as described in (8)), and then compared to Huh7.5.1 cells (cultured as described in (41)). Results are expressed as means +/- SD of percentage HCVpp entry relative to entry into Huh7.5.1 cells from three independent experiments performed in triplicate, and 100% relative infectivity is represented by a solid line. Statistical analysis for entry factor expressing cells relative to naïve 293T cells was performed using the Student's *t* test, *P<0.05.

Figure 2. 293T-4R cells support robust HCV infection upon miR-122 expression. (A) Stable 293T-4R cells described in Fig. 1 were challenged with HCVcc (JcR2a; produced as described in (38)) or were mock infected and luciferase activity was assessed 72h post-infection as described previously (38). Results are expressed as means +/- SD of relative light units (RLU) from three independent experiments performed in triplicate. (B) 293T-4R cells were stably transduced using miR-122 encoding lentiviruses (Cat.# mh15049, ABM Good) and puromycin (2,5 µg/ml) resistant cells were selected over 2 weeks. 293T-4R/miR122 cells and Huh7.5.1 cells were then infected with HCVcc or mock infected for 6h. Infection was assayed by monitoring luciferase activity 72h post-infection. Results are expressed as means +/- SD of relative light units (RLU) from three independent experiments performed in triplicate. Jc1, an HCVcc without a luciferase reporter (32) was likewise used to infect Huh7.5.1 and 293T-4R/miR122 cells and its infectivity was assessed by limiting-dilution assay ($TCID_{50}$) by detecting viral protein NS5A using immunohistochemistry, represented as grey bars (22). Results are expressed as means +/- SD of $TCID_{50}$/ml from three independent experiments. (C) Northern blots of miR-122 and miR-16, and U6 RNA as a loading control, extracted from 293T-4R, 293T-4R stably expressing miR-122, and Huh7.5.1 cells as positive control. Northern blots using a miR-122-specific probe were performed as described previously (30). Oligonucleotide lengths (nt) are indicated on the left of each blot. (D) 293T-4R, 293T-4R/miR122 and Huh7.5.1 cells were incubated side-by-side with HCVcc (JcR2a) and luciferase activity was monitored every 24h over a 72 h period. Results are expressed as means +/- SD of relative light units (RLU) of three independent experiments performed in triplicate. (E) Huh7.5.1, 293T-4R, and 293T-

4R/miR122 cells were infected for 72 h and HCV core protein (core antibody C7-50, Thermo Scientific,), or non-specific IgG, as a control (Cat.# 10400C, Life Technologies) were observed by immunofluorescence; nuclei were stained using DAPI. (F) 293T-4R/miR122 cells were pre-incubated for 1h at 37°C with the indicated entry inhibitors, antivirals or controls (monoclonal antibodies (mAb), anti-CD81 (JS81, BD Biosciences), anti-CLDN1 (11), anti-SR-BI (Zahid et al. submitted manuscript), polyclonal (pAb) anti-apoE (Cat #178479, Calbiochem), anti-apoE mAb was described in (37), 20 µg/ml, erlotinib: 10 µM (Cat.# E-4997, LC Laboratories), protease inhibitor telaprevir VX950: 1 µM; polymerase inhibitor mericitabine R7128: 1 µM; both synthesized by Acme Bioscience Inc. , DMSO: 0.7%, and then infected with HCVcc (JcR2a) in the presence of given entry inhibitors or antivirals. Cell lysates were assessed for luciferase activity 72h post-infection. Results are expressed as means +/- SEM of percentage HCVcc infection compared to controls, from three independent experiments performed in triplicate, and 100% relative infectivity is represented by a solid line. In panels A, B, and D, detection limits are represented by dashed lines. Statistical analysis relative to control was performed using the Student's *t* test, *P<0.05.

Figure 3. Infectious HCV particles are released from 293T-4R/miR122 cells upon apoE expression. (A) 293T-4R/miR122 cells were transduced with an apoE3 encoding lentiviral vector described in (23). 72h post-transduction, cells that were or were not transduced were stained for flow cytometry analysis. ApoE expression was analyzed using a specific apoE antibody (clone D6E10, Cat.# ab1906, Abcam, untransduced cells are represented as light grey histogram and transduced cells are shown as dark grey histogram) and an isotype antibody (Cat.# 10400C, Life Technologies) was used as

445 control (white dashed histograms). Huh7.5.1 cells were used for control of apoE
446 expression and PBS is presented as control of the isotype antibody (thick black
447 histogram). (B) Transduced 293T-4R/miR122/apoE cells were infected with HCVcc
448 (JcR2a, or Jc1). 6h post-infection, cells were washed three times with PBS, and fresh
449 culture medium was added. 72h post-infection, media from infected cells was passaged
450 onto naïve Huh7.5.1 cells. Cell lysates of JcR2a infected cells were assessed for
451 luciferase activity 72h post-infection. Results are expressed as means +/- SD of relative
452 light units (RLU) of three independent experiments performed in triplicate. The detection
453 limit is represented by a dashed line. The infectivities of Jc1 derived from Huh7.5.1 or
454 293T-4R/miR122/apoE infected cells were assessed by limiting-dilution assay ($TCID_{50}$)
455 by detecting NS5A by immunohistochemistry, represented as grey bars. Results are
456 expressed as means +/- SD of $TCID_{50}$/ml from three independent experiments. #
457 represents below detectable levels. Statistical analysis relative to the control was
458 performed using the Student's *t* test, *P<0.05. (C) 293T-4R/miR122 cells were
459 transduced with indicated apoE isoform-encoding lentiviral vectors (24), or mock
460 transduced (Control). 72h post-transduction, cells were either lysed or seeded for
461 HCVcc infection. Cell lysates were assessed for apoE expression by Western blot either
462 by using apoE antibody (clone D6E10, Cat.# ab1906, Abcam) for human apoE (h-apoE)
463 expression or using a mouse apoE specific antibody for mouse apoE (m-apoE)
464 expression (Cat# ab20874, Abcam). Huh7.5.1 and primary mouse hepatocytes (PMH)
465 were used as controls for human and mouse apoE expression, respectively. (D) The
466 different apoE isoform-expressing 293T-derived cells were assessed for their capacity to
467 produce infectious virus by infecting them with HCVcc (JcR2a) and 72h post-infection,
468 supernatants of infected 293T-derived cells were passaged onto naïve Huh7.5.1 cells.

72h after initiating this infection, Huh7.5.1 cells were lysed and luciferase activity assessed. Results are expressed as means +/- SD of relative light units (RLU) from a representative experiment performed in triplicate. The dashed line represents the detection limit.

Figure 4. Characterization of HCVcc derived from 293T-4R/miR122/apoE cells (A) Culture media from Jc1-infected 293T-4R/miR122, 293T-4R/miR122/apoE, and Huh7.5.1 cells were passaged onto naïve Huh7.5.1 target cells. Total RNA from these Huh7.5.1 target cells was extracted at indicated time points and HCV RNA was quantitated by RT-qPCR as described (11). Values were normalized to the internal control gene GAPDH and are represented as HCV RNA to GAPDH RNA ratio. Results are expressed as means +/- SD from an experiment performed in quadruplicate. (B) HCV RNA production was measured by infecting 293T-4R/miR122, 293T-4R/miR122/apoE and Huh7.5.1 cells side-by-side with HCVcc (Jc1). RNA from supernatants of infected cells was extracted at indicated time points and HCV RNA quantitated by RT-qPCR. Results are expressed as means +/- SD of copies/ml from an experiment performed in triplicate. (C) Culture media of infected 293T-4R/miR122/apoE cells were harvested 72h post-infection and passaged onto naïve Huh7.5.1 cells that were pre-incubated with either control IgG, DMSO, or with indicated entry inhibitors. Results represent mean percentages of HCV infection (as assessed by luciferase activity) relative to control +/- SD from a representative of two independent experiments performed in triplicate, and 100% relative infectivity is represented by a solid line. Virus used was JcR2a with a $TCID_{50}$ of 10^5 to 10^6/ml. (D) Density distributions of infectious 293T-4R/miR122/apoE- and Huh7.5.1-derived HCVcc (Jc1) were determined by

493 overlaying 0.5 ml of culture media on a 5 ml, 4-40% iodixanol step gradient, and
494 ultracentrifuging samples for 16h at 40,000 rpm on a SW-55 rotor (Beckman Coulter).
495 Fractions were carefully harvested from the top of each tube, and density was
496 determined by weighing 0.5 ml of each fraction. Each fraction was assayed for infectivity
497 by $TCID_{50}$ by detecting NS5A as described (22).
498
499

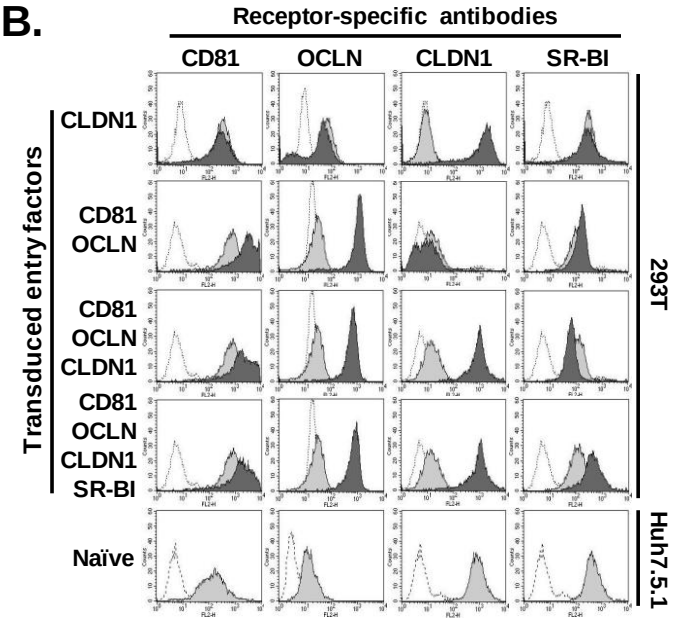
500 **Figure 1**

A.

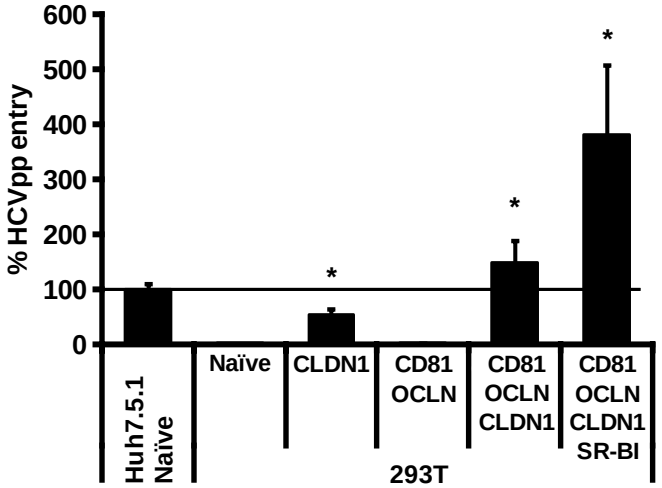
Loci Tested	AMELO	THO1	D5	D13	D7
ATCC Reference: CRL-1573 (HEK293)	*X, X*	*7, 9.3*	*8, 9*	*12, 14*	*11, 12*
293T cells	X, X	9.3, 9.3	8, 9	12, 13, 14	11, 12

Loci Tested	D16	CSF	VWA	TPOX
ATCC Reference: CRL-1573 (HEK293)	*9, 13*	*11, 12*	*16, 19*	*11, 11*
293T cells	9, 13	11, 12	16, 19	11, 11

B.

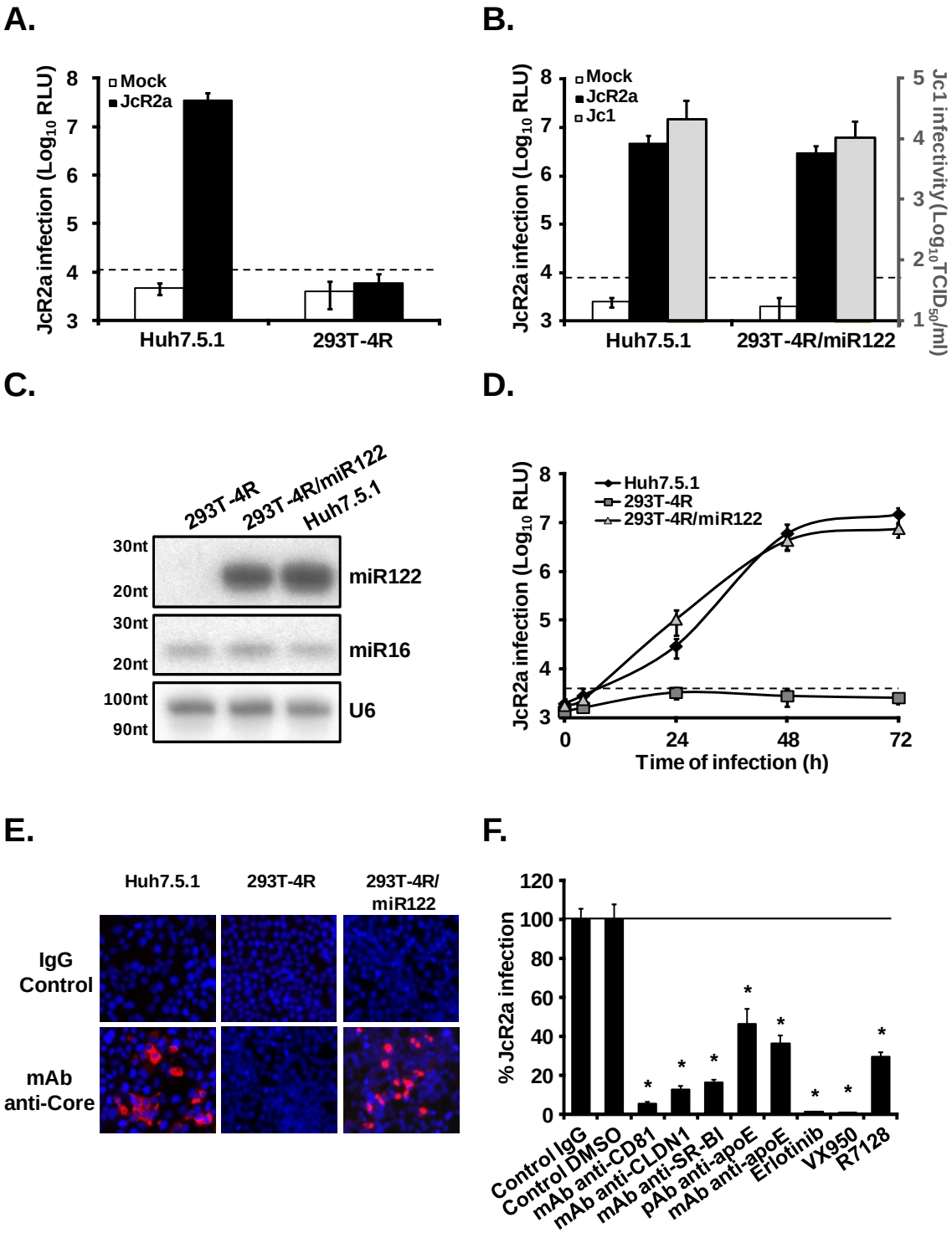


C.



501

502 **Figure 2**



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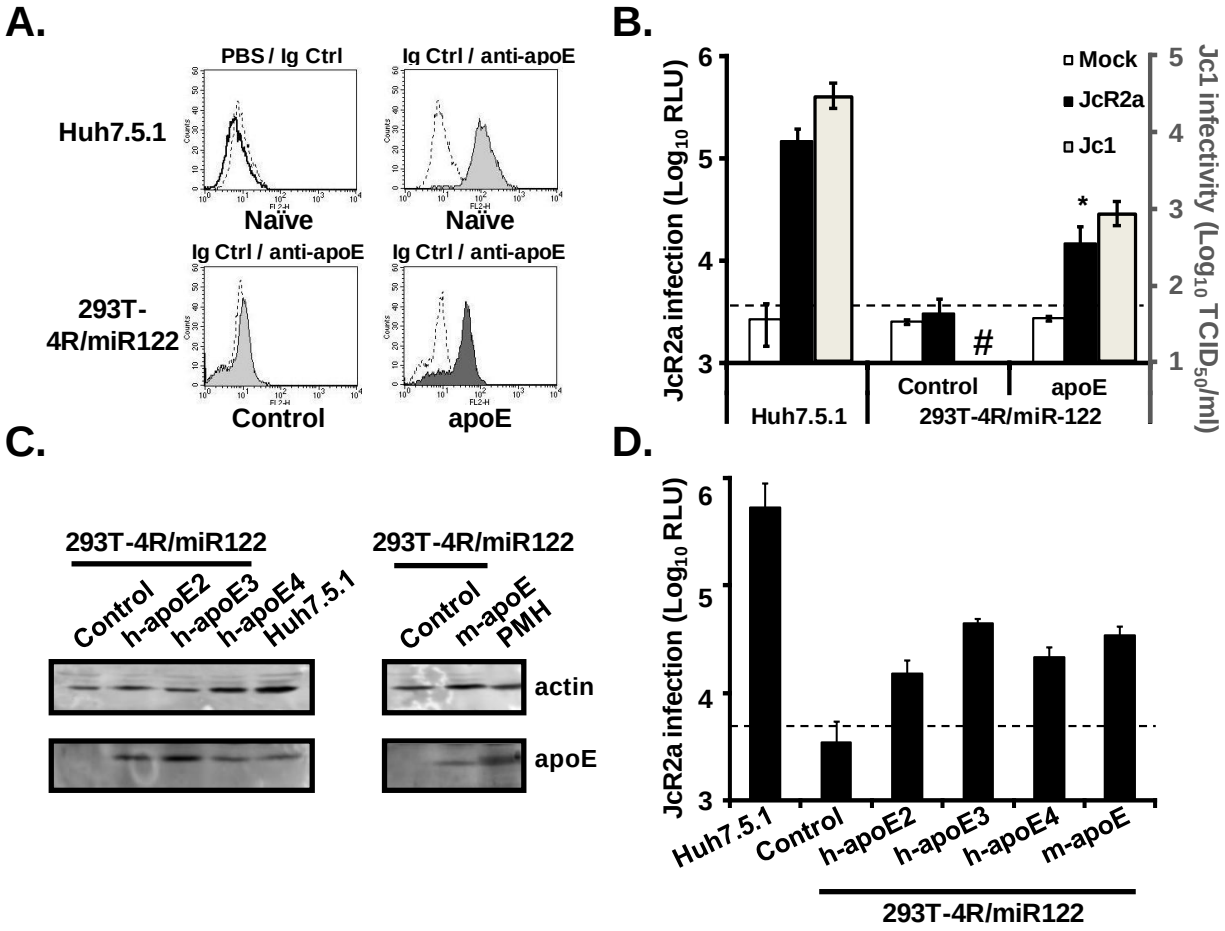
504 **Figure 3**



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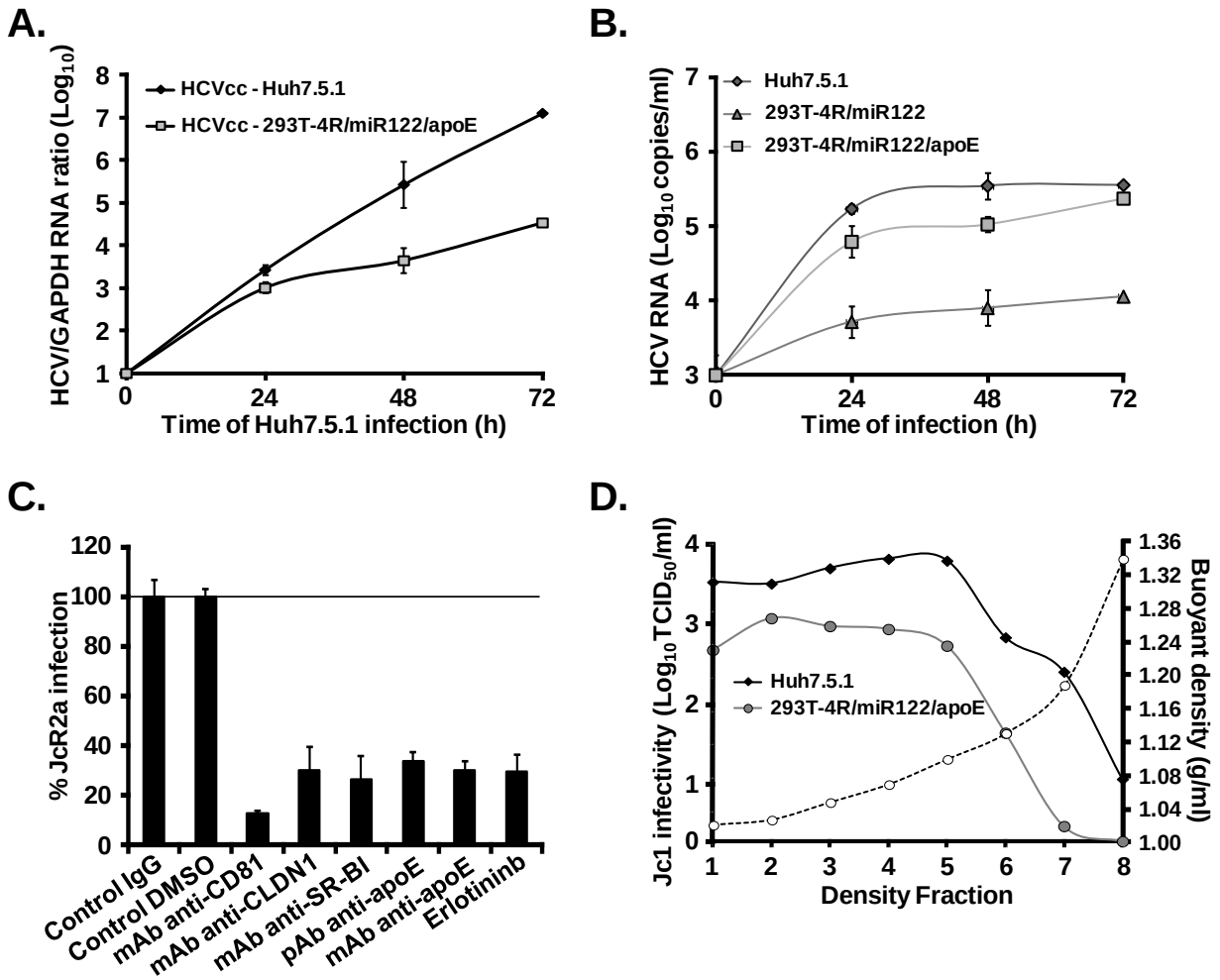
507 **Figure 4**



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