Title: Expression of human ficolin-2 in hepatocytes confers resistance to infection by diverse hepatotropic viruses

Paywast J Jalal[1,2*], Richard A. Urbanowicz[1*], Emma Horncastle[1], Monika Pathak[1], Chun Goddard[1], Amanj Saeed[2], Christopher P. Mason[1], Jonathan K. Ball[1,3], C. Patrick McClure[1,3], Barnabas J. King[1], Alexander W. Tarr[1,3]

[1] School of Life Sciences, Faculty of Medicine and Health Sciences, University of Nottingham, UK. [2] Biology Department, Faculty of Science, University of Sulaimani, Iraq. [3] NIHR Nottingham Biomedical Research Centre, Nottingham University Hospitals NHS Trust and the University of Nottingham, Nottingham, UK.

*These authors contributed equally

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Abstract

The liver-expressed pattern recognition receptors (PRRs) mannose binding lectin (MBL), ficolin-2 and ficolin-3 contribute to the innate immune response by activating complement. Binding of soluble ficolin-2 to viral pathogens can directly neutralize virus entry. We observed that the human hepatoma cell line HuH7.5, which is routinely used for the study of hepatotropic viruses, is deficient in expression of MBL, ficolin-2 and ficolin-3. We generated a cell line that expressed and secreted ficolin-2. This cell line (HuH7.5 [FCN2]) was more resistant to infection with hepatitis C virus (HCV), ebolavirus (EBOV) and vesicular stomatitis virus (VSV), but surprisingly was more susceptible to infection with rabies virus (RABV). Cell-to-cell spread of HCV was also inhibited in ficolin-2 expressing cells. This illustrates that ficolin-2 expression in hepatocytes contributes to innate resistance to virus infection, but some viruses might utilise ficolin-2 to facilitate entry.

Introduction

Many pathogens target the liver for replication. The tolerogenic microenvironment makes this a favourable site of virus infection (Thomson and Knolle, 2010). However, hepatocytes possess innate defence mechanisms against infection. As such, hepatotropic viruses (e.g. hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis A virus (HAV)) must adapt to evade host innate immunity. HCV is a particularly important infection of hepatocytes, transmitted between individuals by direct contact with contaminated blood. Following an initial acute phase of infection, chronic infection is established in approximately 75% of cases. It is currently estimated that 71 million people are chronically infected with HCV (Polaris Observatory, 2017). Infection is associated with progressive fibrosis and loss of liver function, including liver cirrhosis and hepatocellular carcinoma (Fattovich et al., 2004). The mechanisms contributing to resistance to chronic infection remain to be determined. Innate immune resistance to infection is key to limiting HCV infection and contributes to spontaneous resolution of infection and treatment response. The innate immune response to viral infections comprises intracellular sensing, cellular antiviral responses and humoral pattern recognition. Sensing of HCV infections through cytoplasmic pattern recognition receptors (PRRs) (Cao et al., 2015), transmembrane PRRs (Liang et al., 2018), and humoral PRRs (Tarr et al., 2012) can contribute to protection against HCV infection.

The humoral PRRs mannose binding lectin (MBL), ficolin-2 and ficolin-3 are expressed by hepatocytes. They bind to pathogens, recruit complement components and contribute to activation of the cellular response to infection (Fuchs et al., 2011). As such, they are potentially important to inhibition of hepatotropic pathogens. It has previously been demonstrated that the lectins MBL and ficolin-2 possess the capacity to bind to viral pathogens and neutralize entry (Brown et al., 2010; Chang et al., 2011; Fuchs et al., 2010; Hamed et al., 2014; Michelow et al., 2010; Pan et al., 2012; Zhao et al., 2014). However, soluble MBL has also been demonstrated to enhance entry of some viruses, notably Ebolavirus (Brudner et al., 2013). While many studies have investigated the action of soluble lectin PRRs on virus infection, the impact of cellular expression of lectins on permissiveness

to infection has not been studied. The mode of action of secreted ficolin-2 inhibiting HCV infection has been attributed to blockade of interaction of virus particles with virus receptors, including SR-B1 and LDLr (Zhao et al., 2014). However, it is also plausible that intracellular ficolin-2 acts more broadly as a restriction factor for hepatotropic viruses.

Laboratory investigations of virus entry routinely utilise transformed cell lines as target cells. In particular, the HuH7 cell line has been demonstrated to be permissive for a wide variety of viruses, including for HCV (Bartosch et al., 2003b), ebolavirus (EBOV) (Urbanowicz et al., 2016a), and vesicular stomatitis virus (VSV) (Bartosch et al., 2003a). The innate ability of these cells to resist virus entry requires further definition. Here we assessed the expression of soluble PRRs in HuH7 cells, generated a cell line expressing the PRR ficolin-2, and assessed the impact of ficolin expression on resistance to infection with a range of hepatotropic viruses.

Methods

Cell lines. HEK293T, HuH7, and HuH7.5 (a kind gift from Apath LLC) were cultured as previously described (Urbanowicz et al., 2015). HuH7 and HuH7.5 are liver cell lines derived from a human hepatoma (Blight et al., 2002), and have been thoroughly been described to be permissive for virus infections. HEK293T (ATCC® CRL-3216™) are embryonic kidney-derived neuronal cells.

Assessment of PRR secretion from continuous cell lines. Secretion of MBL, ficolin-2 and ficolin-3 were assessed using ELISAs as previously described (Brown et al., 2010; Hamed et al., 2014; Hein et al., 2010). A standard human serum containing known quantities of each PRR was used as a reference reagent.

Generation of a Huh7.5 cell line expressing the FCN2 gene. A cloned cDNA encoding the ficolin-2 ORF (Gene ID: 2220) has previously been described (Hamed et al, 2014). The ficolin-2 ORF was amplified with specific primers pWPI-F [CAGGCGCGCCGGATCACCAGAAGAGATGGAGCTG], and pWPI-R [CGCCCCGGGGGATCTCAATGGTGATGGTGATGATGA]. PCR products were cloned by In-Fusion® HD Cloning (Takara Bio USA, Inc.), according the manufacturer's instructions, into the pWPI lentiviral

plasmid containing a blasticidin resistance gene (Trono Lab Constitutive Lentiviral Plasmids #12254, Addgene). A positive clone [pWPI-FCN2] was determined by colony-screening PCR and Sanger sequencing. HEK293T cells were co-transfected with pWPI-FCN2, an HIV packaging construct vector and a VSV-g expression plasmid (pHIV, pMD2.G) to generate transducing lentivirus. Lentivirus-containing supernatants were harvested 48 h post-transfection, filtered and inoculated onto naïve HuH7.5 cells. After 48 h, transduced cells were selected with blasticidin (50 $\mu g.mL^{-1}$). Cells were seeded into 96-well tissue culture plates (1000 cells/well) and cultured for three days under selection. Secretion of ficolin-2 was assessed by ELISA. The wells expressing the highest levels of ficolin-2 were further expanded over a period of 54 days with ficolin-2 secretion assessed at each passage. After 54 days cells were designated 'HuH7.5 [FCN2]'.

Assessment of ficolin-2 expression in transduced cells by immunofluorescence. Wild-type HuH7.5 and Huh7.5 [FCN2] cells were seeded onto coverslips and fixed with 4% (v/v) formaldehyde for 15 min. After washing, cells were permeabilised with 0.1% (v/v) Triton X-100 for 15 minutes, followed by blocking [1% (w/v) BSA, PBS] for 1 h. Samples were probed with GN5 anti-FCN2 antibody [1:200 in 0.5% (w/v) BSA, PBS] at RT for 2 h followed by washing in PBS and secondary antibody incubation with goat α-mouse IgG (H+L) AlexaFluor 488 (ThermoFisher) [1:1,000 in 0.5% (w/v) BSA, PBS]. Cells were counter-stained with DAPI (ThermoFisher, 0.04 $\mu g.mL^{-1}$ in PBS) for 5 min, mounted [Dakcytomation fluorescent mounting media, Agilent Dako] and imaged using a Zeiss LSM710 confocal microscope.

Viral entry assays using FCN2-expressing cell lines HuH7.5 [FCN2] and wt HuH7.5 cells were used as targets for infection assays with pseudotyped viruses possessing the glycoproteins of HCV (strain JFH-1), Ebolavirus (EBOV – Makona C15), vesicular stomatitis virus (VSV – Indiana strain), or rabies virus (RABV – challenge virus standard CVS 11) using methods previously described (Urbanowicz et al., 2016a; Urbanowicz et al., 2016b). Secreted ficolin-2 produced by target cells was removed before infection, and infection assays performed for 4 hours. Full-length HCV infection was carried out as

previously described (McClure et al., 2016) using the JFH-1 isolate (Wakita et al., 2005) and a J6/JFH1 chimera (Lindenbach et al., 2005).

Results

Low levels of ficolin-2 are secreted from hepatoma cells. It has previously been demonstrated that secreted ficolin-2 binds to viral glycoproteins, including the E2 protein of HCV (Liu et al., 2009). To investigate the impact of cell-expressed ficolin 2 on HCV entry and spread in cell culture, we assessed the presence of ficolin-2 in the media of HuH7, HuH7.5, and HEK 293T cell lines by ELISA. HEK 293T cells were used as a control cell line of a non-hepatocyte origin, and baseline signal in the assay was determined using cell culture media only. Media from cultured cells (80-90% confluent) was incubated with either acetylated BSA or mannan to measure ficolin, or MBL, respectively. Surprisingly, very low levels of ficolin-2 secretion (<0.4 $\mu g.mL^{-1}$) were observed in the media from all three cell lines with no significant difference between the hepatocyte and non-hepatocyte cell lines (figure 1A).

Expression of ficolin-2 in lentivirus-transduced cells. It has been reported that the serum concentrations of ficolin-2 in healthy individuals ranges from 0.7 – 6 $\mu g.mL^{-1}$ (Hummelshoj et al., 2005). Having found that the two HuH7 cell lines expressed very low levels of ficolin-2, we generated a lentiviral transduction cassette encoding the human FCN2 coding region of the splice variant 0 form. Actively replicating HuH7.5 cells were transduced with these recombinant lentiviruses. Secreted levels of ficolin-2 were assessed 3 days post-transduction of the mixed culture and compared to control non-transduced cell lines (figure 1A). The supernatant of HuH7.5-FCN2 cells possessed greater than 2 $\mu g.mL^{-1}$ of ficolin-2. We also analysed the levels of two other liver-expressed PRRs, MBL and ficolin-3, and observed that they were low and comparable across all cell lines, confirming the transduction had not altered expression of these PRRs. To obtain a high-expressing sub-culture, HuH7.5-FCN2 cells were seeded into a 96 well dish (1×10^3 cells/well). Ficolin-2 secretion was assessed after 3 days and the highest-expressing sub-culture was expanded under blastacidin selection, passaging at between 80-90% confluence. Ficolin-2 in the culture

supernatant was assessed at regular intervals. It was observed that secreted ficolin-2 concentrations increased with time, to a maximum of ~7 $\mu g.mL^{-1}$ (figure 1B). A control non-transduced HuH7.5 cell line passaged similarly demonstrated no increase in ficolin-2 over this period. At day 54, the resultant cell line 'HuH7.5 [FCN2]' was assessed for intracellular expression of ficolin-2 by immunofluorescence (figure 1C). Intracellular expression of ficolin-2 was clearly elevated, with a pattern of expression consistent with expression into vesicles.

As transduction of actively replicating cells may alter cell growth kinetics, we tested growth kinetics of the two cell lines over the period of our standard virus infection assays. Seeding 3 $x10^5$ cells into a 6 well dish, cells were harvested and counted 72 h after seeding. Cell numbers during this period were highly comparable between the cell lines (figure 1D).

Virus infection of HuH7.5 cells overexpressing ficolin-2. To determine the impact of ficolin-2 expression on the infection of HuH7.5 cells with HCV, we performed infection assays using a well-characterised retrovirus pseudotype model of HCV entry (HCVpp) using the envelope glycoproteins of strain JFH-1. Infections were performed using equal numbers of either the wild-type Huh7.5 cells or Huh7.5 [FCN2] cells. HCV entry was inhibited by 50 % ($p = 0.0074$) in the ficolin-2- transduced cells (figure 2A). We next tested whether adding soluble ficolin-2 at the time of infection resulted in increased inhibition. However, performing infections in the presence of media collected from the ficolin-2-expresisng HuH7.5 [FCN2] cell line did not have a significant additive effect on inhibition of HCV entry (data not shown). To exclude the possibility that the observed inhibition was an artefact of the retrovirus pseudotype infection model, we infected the two cell lines with two infectious HCV clones (HCVcc); full-length JFH-1 and a chimeric J6/JFH virus. We observed similar significant levels of inhibition ($p = 0.0005$ and 0.0034, respectively) in the ficolin-2-transduced cells (figure 2B). The J6/JFH chimera is known to efficiently spread between cells in culture. We hypothesised that if ficolin-2 was impairing virus entry, as determined by the HCVpp model, this might also prevent cell-to-cell spread, reducing the size of the foci resulting from successful infections. The number of cells

included in each foci was therefore calculated. With cells at the same density at the time of infection, Huh7.5 [FCN2] cells clearly had reduced numbers of multicellular foci (>10 cells/foci, p = 0.0019) (figure 2C and representative images figure 2D), indicating that ficolin-2 expression results in reduced spread of virus through these cultures.

To determine if the ficolin-2-mediated restriction of virus entry was limited to HCV, we employed the retrovirus pseudotype model to investigate entry of Ebola virus (EBOV), vesicular stomatitis virus (VSV) and rabies virus (RABV) in parallel (figure 2E). Entry of EBOV and VSV were significantly reduced in the HuH7.5 [FCN2] cells (p = 0.0098 and 0.0163, respectively), however the effect on entry was not as marked as that seen for HCV (inhibition of ~15 % and 30%, respectively). In contrast, entry of the rabies virus (RABV) was significantly enhanced (~40%, p = 0.0007) in the HuH7.5 [FCN2] cells. This provides evidence that the functional consequences of ficolin-2 interactions with viral envelope glycoproteins is dependent on the virus species.

Discussion

The cellular innate immune recognition of virus infections has been extensively studied, but the contribution of secreted PRRs such as MBL, ficolin-2 and ficolin-3 in limiting virus infections is still poorly understood. While it is well described that these proteins recruit complement and contribute to inflammation, direct antiviral properties of these PRRs require further investigation. We and others have previously demonstrated that recombinant-expressed soluble ficolin-2 has antiviral activity by preventing virus entry into susceptible cells (Hamed et al., 2014; Zhao et al., 2014). However, it was unclear if cellular expression of ficolin-2 contributes to innate resistance to virus infection. Ficolin-2 is predominantly expressed in the liver, with immunohistochemistry revealing expression in hepatocytes (Human Protein Atlas, (Uhlen et al., 2015)). Our initial investigations revealed that human hepatoma cells used routinely for infection studies with HCV are deficient in expression of ficolin-2. HuH7 and HuH7.5 cells have been extensively used to interrogate the entry pathway of HCV using both cell cultured HCV and retroviruses pseudotyped with the HCV E1E2

proteins. Low expression of MBL, ficolin-2 and ficolin-3 in these cells may contribute to their permissive phenotype. The modified HuH7.5 [FCN2] hepatocyte cell line had increased expression of ficolin-2, as determined by intracellular staining and measurement of secreted protein. Intracellular expression patterns were consistent with expression into secretory vesicles, although no evidence of localisation to the Golgi was observed. This is consistent with the intracellular localisation of ficolin-1 in monocytes (Liu et al., 2005). Concentrations of secreted ficolin-2 were similar to those observed in the serum of healthy individuals (Chen et al., 2015; Luo et al., 2013; Mishra et al., 2015). While localised concentrations of ficolin-2 in the liver are likely to be elevated compared to that in the peripheral plasma, the concentrations produced by this cell line may model physiological conditions.

Having validated ficolin-2 expression in these cells, we assessed their permissiveness to infection with different hepatotropic viruses. HCV was selected as an example of a strictly hepatocyte-tropic virus, while EBOV and VSV have much broader cell tropism. RABV has tropism mainly for neuronal cells, the ability to infect a broader range of cells has been demonstrated (Nie et al., 2017; Thoulouze et al., 1997). The transduced cells were most resistant to HCV infection, but entry of both EBOV and VSV entry was also inhibited, suggesting a common mechanism for ficolin-2 inhibiting entry of genetically diverse virus species. This was consistent with our previous observations using exogenous addition of soluble ficolin-2 (Hamed et al., 2014). The inhibitory effect of ficolin-2 expression on HCV was confirmed using authentic virus particles possessing the structural genes of either the JFH1 strain or J6 strain, as these two viruses have different susceptibility to antibody-mediated neutralization (Urbanowicz et al., 2015). Replication of both viruses was inhibited to similar levels in the ficolin-2 expressing cells. Cell-to-cell spread of HCV was also reduced in cells expressing ficolin-2, highlighting a potential functional role for this PRR in preventing spread of HCV infection between hepatocytes. However, it is possible that as the assay used for assessing cell-to-cell spread involves entry of the virus, which was inhibited, the reduced foci size could be an artefact of the reduced infection.

All of the viruses tested have an entry pathway requiring internalisation via clathrin-coated vesicles, albeit by interactions with different host membrane proteins, and entry is likely to result in the virus encountering the ficolin-2 in endosomes. As all the pseudotypes were produced in an identical manner, yet we observed different effects on entry, we conclude that the specificity of the inhibition is due to interactions with different patterns of glycosylation of these proteins. The enhancement of infection with pseudotypes bearing the RABV glycoprotein was unexpected raises important questions about the mechanism of action of ficolin-2 mediated modulation of virus infectivity. To our knowledge this is the first evidence to suggest that RABV entry is enhanced by lectins, but it has recently been reported that ficolin-1 enhances entry EBOV (Favier et al., 2016). The mechanism of neutralisation, and enhancement, clearly requires definition, but it is likely that the patterns of glycosylation on these viruses influence interactions with ficolin-2, as well as other lectins. It has previously been demonstrated that ficolin-2 interacts with the glycoproteins of HCV and VSV (Hamed et al., 2014; Liu et al., 2009). Ficolin-2 has binding specificity for N-acetylglucosamine and mannose, and while HCV particles are strictly modified with sugars bearing GlcNAc cores with terminal mannose structures, RABV glycoprotein may have more complex-type glycan modifications (Wojczyk et al., 2005). The glycan specificity of lectins combined with virus receptor usage might determine impact of lectin interactions with different viruses. Together, we have demonstrated that commonly used hepatoma cell lines used for infection assays with HCV are deficient in expression of ficolin-2, and when transduced to express physiologically relevant levels of this PRR these cells are more refractive to infection by hepatotropic viruses. However, at least in a model of virus entry, rabies virus infection is enhanced in these cells. It is intriguing that viruses normally targeting the liver have not adapted to utilise ficolin-2 to augment entry. The cell line we describe will be a useful tool with which to interrogate further the mechanism of ficolin-2 mediated inhibition/enhancement of virus entry. It will be important to directly compare ficolin-2 expression in primary human hepatocytes and determine the impact of genetic polymorphisms in the FCN2 gene on the antiviral properties of ficolin-2.

Conflict of Interests

The authors declare no conflicts of interest.

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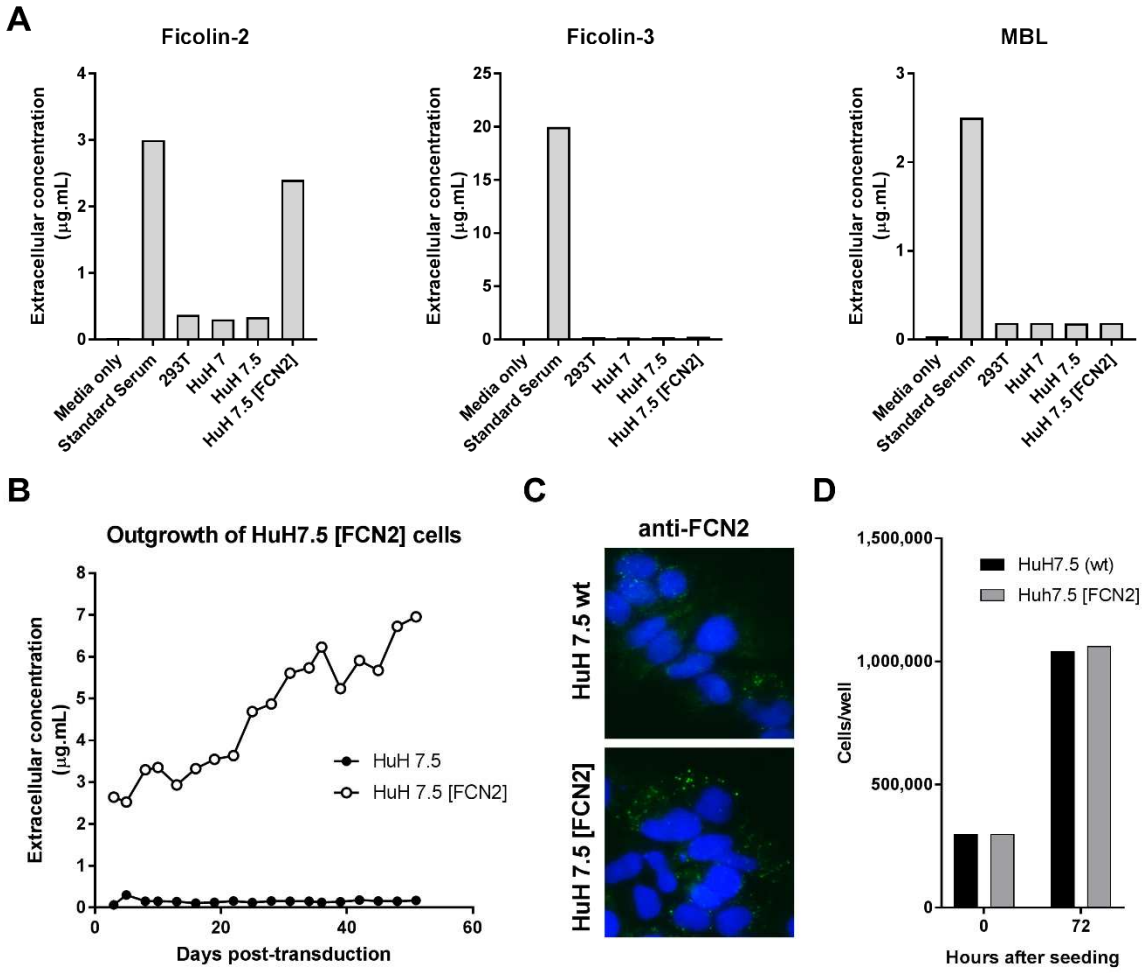
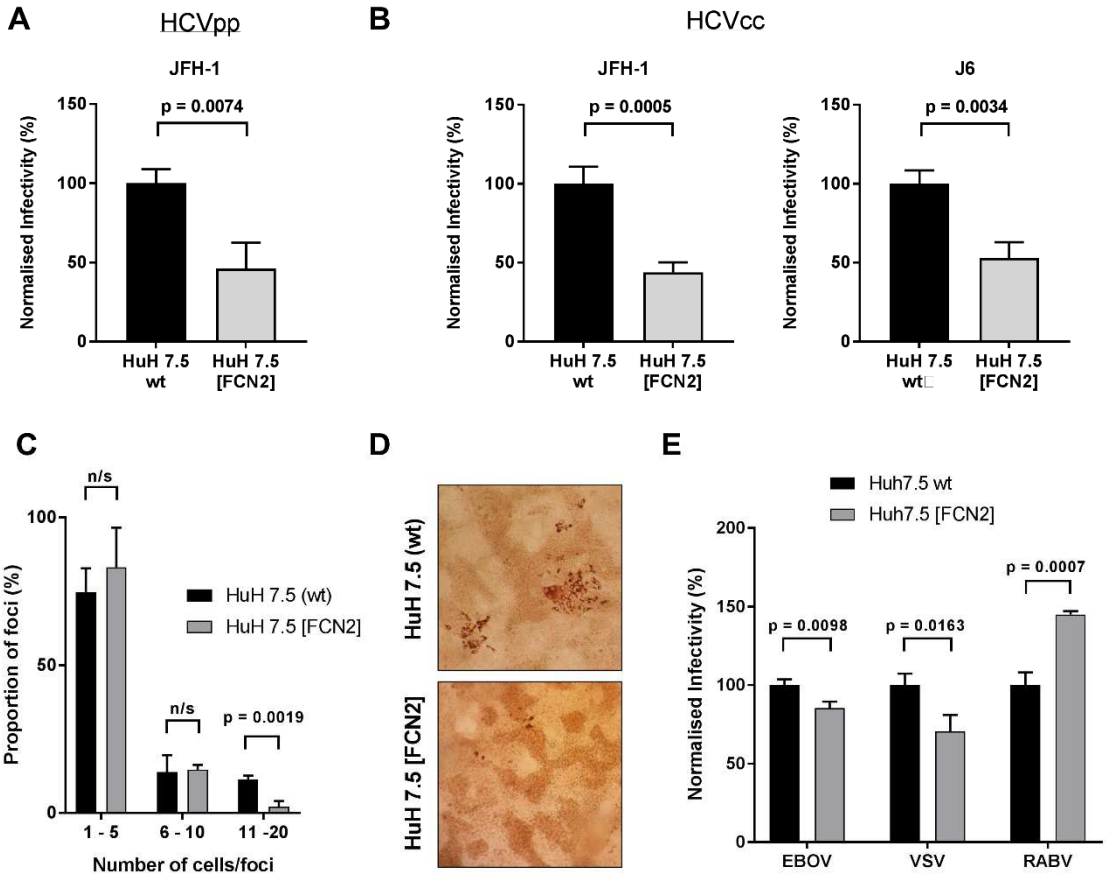
Figure legends

Figure 1. Transduction of HuH7.5 cells with human FCN2. A) Secreted levels of ficolin-2, ficolin-3 and mannose-binding lectin (MBL) were assessed from continuous cell lines and HuH7.5 cells transduced with the human *FCN2* gene (HuH7.5 [FCN2]). Levels were determined using a binding ELISA and compared to a standard serum possessing known PRR concentrations**. B)** Following transduction of HuH7.5 cells with the FCN2 a single high-expressing sub-culture was expanded over 54 days in increasing culture volume. Supernatants were regularly monitored for FCN2 expression by binding ELISA and compared with the progenitor HuH7.5 cell line. **C)** Immunofluorescence staining of HuH7.5 [FCN2] and wild-type HuH7.5 cells. Cells were fixed, permeabilised and probed with anti-FCN2 labelled with AlexaFluor 488 and counter-stained with DAPI. Representative images are shown. **D**) $3x10^5$ of wild-type and transduced HuH 7.5 cells were seeded in parallel into a 6 well dish and incubated for 72 h. Cells were harvested by trypinisation and counted.

Figure 2. Over-expression of human FCN2 alters entry efficiency of enveloped viruses. Wild type and FCN2-transduced Huh7.5 cells were infected with (**A**) HCV pseudoparticles (HCVpp), or (**B**) full length cultured HCV (HCVcc). (**C**) HCVcc foci (J6 isolate) were counted and grouped by the number of cells per foci. (**D**) Microscope images of HCVcc infected cells (dark red) in HuH7.5 and HuH7.5 [FCN2] cells (20x magnification, representative images are shown). (**E**) Wild type and FCN2-transduced Huh7.5 cells were infected with pseudoparticles bearing the glycoproteins of EBOV, VSV and RABV. Entry levels were normalised to wt cell line values. Differences were analysed using an unpaired t-test with p-values noted. EBOV, Ebola virus; VSV, vesicular stomatitis virus; RABV, rabies lyssavirus.

Figures


A
Ficolin-2
Ficolin-3
MBL
Extracellular concentration (µg.mL)
Media only
Standard Serum
293T
HuH 7
HuH 7.5
HuH 7.5 [FCN2]
B
Outgrowth of HuH7.5 [FCN2] cells
Extracellular concentration (µg.mL)
HuH 7.5
HuH 7.5 [FCN2]
Days post-transduction
C
anti-FCN2
HuH 7.5 wt
HuH 7.5 [FCN2]
D
HuH7.5 (wt)
Huh7.5 [FCN2]
Cells/well
Hours after seeding


A
HCVpp
JFH-1
Normalised Infectivity (%)
150
100
50
0
p = 0.0074
HuH 7.5 wt
HuH 7.5 [FCN2]
B
HCVcc
JFH-1
p = 0.0005
J6
p = 0.0034
C
Proportion of foci (%)
n/s
HuH 7.5 (wt)
HuH 7.5 [FCN2]
p = 0.0019
1 - 5
6 - 10
11 -20
Number of cells/foci
D
HuH 7.5 (wt)
HuH 7.5 [FCN2]
E
Huh7.5 wt
Huh7.5 [FCN2]
200
p = 0.0098
p = 0.0163
p = 0.0007
EBOV
VSV
RABV

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