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EGFR and EphA2 are host factors for hepatitis C virus entry and targets for antiviral therapy

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ABSTRACT

Hepatitis C virus (HCV) is a major cause of liver disease. Therapeutic options are limited and preventive strategies are absent. Entry is the first step of infection and requires the cooperative interaction of several host cell factors. Using a functional RNAi kinase screen we identified epidermal growth factor receptor and ephrin receptor A2 as host co-factors for HCV entry. Blocking of kinase function by approved inhibitors broadly inhibited HCV infection of all major HCV genotypes and viral escape variants in cell culture and an animal model *in vivo*. Receptor tyrosine kinases (RTKs) mediate HCV entry by regulating CD81-claudin-1 co-receptor associations and membrane fusion. These results identify RTKs as novel HCV entry co-factors and uncover that kinase inhibitors have significant antiviral activity. Inhibition of RTK function may constitute a novel approach for prevention and treatment of HCV infection.

INTRODUCTION

Hepatitis C virus (HCV) is a major cause of liver cirrhosis and hepatocellular carcinoma. Current antiviral treatment is limited by resistance, toxicity and high costs¹. Although the clinical development of novel antiviral substances targeting HCV protein processing has been shown to improve virological response, toxicity and resistance remain major challenges². Thus, novel antiviral preventive and therapeutic strategies are urgently needed. Since HCV entry is required for initiation, dissemination and maintenance of viral infection, it is a promising target for antiviral therapy^{3,4}.

HCV entry is a multistep process involving several attachment and entry factors⁵. Attachment of the virus to the target cell is mediated through binding of HCV envelope glycoproteins to glycosaminoglycans⁶. HCV is internalized in a clathrin-dependent endocytic process requiring CD81⁷, scavenger receptor type B class I (SR-BI)⁸, claudin-1 (CLDN1)⁹ and occludin (OCLN)¹⁰. The impact of other host factors for HCV entry is poorly understood.

RESULTS

EGFR and EphA2 are host co-factors for HCV entry. Using a siRNA screen we identified a network of kinases with functional impact on HCV entry (**Supplementary results, Supplementary Figs. 1 and 2**). To study the relevance of the identified kinases on the HCV life cycle, we further validated and characterized the functional impact of epidermal growth factor receptor (EGFR) and ephrin receptor A2 (EphA2) on HCV entry. We focused on these kinases because they (i) are key components in the identified networks (**Supplementary Fig. 2c**), (ii) are highly expressed in human liver (**Supplementary Table 2**) and (iii) their kinase function is inhibited by clinically approved protein kinase inhibitors (PKIs)¹¹⁻¹³ allowing us to explore the potential of these molecules as therapeutic targets.

Using individual siRNAs we first confirmed that silencing of mRNAs reduced EGFR and EphA2 mRNA and protein expression (**Fig. 1a,b** and **Supplementary Fig. 3a,b**). Inhibition of infection with cell culture-derived HCV (HCVcc) in silenced cells demonstrated that both EGFR and EphA2 are important for initiation of a productive infection (**Fig. 1c** and **Supplementary Fig. 3c**). Silencing of kinase expression inhibited the entry of HCV pseudoparticles (HCVpp) derived from major genotypes including highly diverse HCV strains¹⁴ (**Fig. 1d** and **Supplementary Fig. 3d**). Effects of silencing of endogenous EGFR or EphA2 on HCV infection were rescued by RNAi-resistant ectopic expression of wild-type EGFR or EphA2 (**Fig. 1e,f** and **Supplementary Fig. 3e,f**). These results largely excluded the possibility of off-target effects causing the observed phenotype. Furthermore, silencing and rescue experiments using well characterized lentiviral vectors expressing EGFR-specific shRNA unambiguously and specifically demonstrated a key role for EGFR in HCV entry into primary human hepatocytes (PHH) (**Fig. 1f**). The functional impact of EGFR as a co-factor for HCV entry was further

confirmed by expressing human EGFR in mouse hepatoma cell lines engineered to express the four human entry factors CD81, SR-BI, CLDN1 and OCLN (AML12 4R, **Supplementary Fig. 4**). Cell surface expression of human EGFR in AML12 4R cells markedly increased HCVpp entry into mouse cells (**Supplementary Fig. 4**).

RTK kinase function is important for HCV entry. The functional relevance of the identified kinases and their kinase function for HCV entry and infection was further studied using PKIs. *Erlotinib* (an EGFR inhibitor) and *Dasatinib* (an EphA2 inhibitor) inhibited HCV infection in a dose-dependent manner whilst having no detectable effect on replication of the corresponding subgenomic replicon (**Fig. 2a–c** and **Supplementary Fig. 5a–c**). The formally calculated IC₅₀ values for *Erlotinib* and *Dasatinib* to inhibit HCVpp entry (*Erlotinib* $0.45 \pm 0.09 \mu\text{M}$, *Dasatinib* $0.53 \pm 0.02 \mu\text{M}$) and HCVcc infection (*Erlotinib* $0.53 \pm 0.08 \mu\text{M}$, *Dasatinib* $0.50 \pm 0.30 \mu\text{M}$) of Huh7.5.1 cells were comparable (**Fig. 2a** and **Supplementary Fig. 5a,b**). These data suggest that RTKs inhibited by *Erlotinib* and *Dasatinib* predominantly play a role during the HCV entry process.

To confirm that the inhibitors reduced HCV entry into cells more closely resembling HCV target cells *in vivo*, we investigated their effect(s) on HCVpp entry into polarized HepG2-CD81 cells¹⁵ and PHH. PKIs markedly and significantly ($P < 0.005$) inhibited HCVpp entry into polarized HepG2-CD81 cells (**Fig. 2c** and **Supplementary Fig. 5d**) and PHH (**Fig. 2d** and **Supplementary Fig. 5e**). Similar results were obtained for infection of PHH with HCVcc and serum-derived HCV (**Fig. 3h,i** and **Supplementary Fig. 5f**) confirming the relevance of the kinases as auxiliary host cell co-factors in models which most closely mimic *in vivo* infection.

A specific effect of *Erlotinib* on EGFR-mediated HCV entry was further confirmed by the investigation of additional EGFR inhibitors: EGFR-inhibitors *Gefitinib* and *Lapatinib* markedly inhibited HCVpp entry and HCVcc infection in PHH and Huh7.5.1 similar to *Erlotinib* (**Fig. 2e,f**). The specific action of PKIs on RTKs as HCV entry factors was further corroborated by absent effects on MLV and measles virus (**Fig. 2c** and **Supplementary Fig. 10**) and silencing/rescue experiments: PKIs specifically reversed rescue of HCV entry when added to silenced Huh7.5.1 cells expressing EGFR and EphA2 in trans (data not shown). Taken together, these results suggest that the RTK kinase function is important for HCV entry.

RTK-specific ligands and antibodies modulate HCV entry. To investigate the functional role of RTK ligand binding domains for viral entry, we assessed virus entry in the presence of RTK-specific ligands and antibodies. Epidermal growth factor (EGF) and transforming growth factor alpha (TGF- α) are well characterized EGFR ligands, where ligand binding promotes receptor homodimerization and subsequent phosphorylation of the intracytoplasmic kinase domain¹⁶. To confirm the biological activity of EGFR-specific reagents in the target cells of our HCV model systems, we first studied their effect on EGFR phosphorylation. Pre-incubation of PHH with EGF markedly increased basal levels of EGFR phosphorylation (**Fig. 3a**, upper and middle panel). In contrast, EGF had no effect on the phosphorylation of an unrelated kinase. EGF-induced enhancement of basal EGFR phosphorylation was markedly inhibited by *Erlotinib* and an EGFR-specific antibody (**Fig. 3a**, lower panel) demonstrating their specific effect(s) on EGFR phosphorylation and activation.

To elucidate the role of the EGFR ligand domain, we assessed the effect of EGFR ligands on HCV entry. Binding of EGF and TGF- α markedly enhanced entry of HCVpp into serum-

starved Huh7.5.1, HepG2-CD81 and PHH (**Fig. 3b,c**) whereas TGF- β had no effect (data not shown). These data suggest that direct interaction of EGF or TGF- α with the EGFR ligand-binding domain modulates HCV entry. The higher affinity of EGF for EGFR on hepatocytes¹⁷ may explain the differences between EGF and TGF- α in enhancing HCVpp entry. *Erlotinib* at doses used in HCV entry inhibition experiments reversed the enhancing effect(s) of EGF (**Fig. 3d**) and TGF- α (data not shown) on HCV entry. These data confirm that *Erlotinib* inhibits HCV entry by modulating EGFR activity.

We screened a large panel of EGFR-specific antibodies and identified a monoclonal human EGFR-specific antibody that bound to PHH (**Fig. 3e**) and dose-dependently inhibited HCV entry (**Fig. 3f**) with an IC₅₀ value of $1.82 \pm 0.3 \mu\text{g mL}^{-1}$. The antibody inhibited EGFR phosphorylation (**Fig. 3a**) and reversed the EGF-induced enhancement of HCV entry (**Fig. 3g**). Ligand-induced enhancement and EGFR-specific antibody-mediated inhibition of HCV entry were also observed for infection of PHH with HCVcc (**Fig. 3h**) and serum-derived HCV (**Fig. 3i**). Taken together, these results suggest that the EGFR ligand domain plays a crucial role in HCV entry. Similarly, EphA2 ligands and EphA2-specific antibodies modulated HCV entry confirming a functional relevance of the EphA2 ligand-binding domain for HCV entry (**Supplementary results and Supplementary Fig. 6**).

RTKs promote CD81-CLDN1 associations and membrane fusion. To understand the mechanistic role of EGFR and EphA2 in HCV entry, we first investigated whether the RTKs regulate HCV entry factor expression. Silencing RTK expression with specific siRNAs or inhibiting RTK function with PKIs had no significant effect on HCV entry factor expression

(**Fig. 4a,b**) suggesting that RTKs do not mediate entry by modulating expression levels of the known human HCV entry factors.

Next, we aimed to fine-map the entry step(s) affected by the RTKs. Viral attachment is the first step of viral entry. To ascertain whether PKI-inhibition of RTK function modulates HCV binding we used a surrogate model that measures association of the recombinant soluble form of HCV envelope glycoprotein E2 with cells¹⁸. RTK-specific antibodies or silencing RTK expression by siRNAs had no significant effect on E2 binding to target cells, whereas pre-incubation with SR-BI-specific antibodies or silencing SR-BI expression markedly reduced E2 binding (**Fig. 4c** and **Supplementary Fig. 7a**). Furthermore, in contrast to CD81¹⁹ and SR-BI¹⁹, RTKs did not confer cellular E2 binding when expressed on the cell surface of CHO cells (data not shown). These data suggest that (i) RTKs are not required for HCV binding and that (ii) direct E2-RTK binding is not required for RTK-mediated HCV entry.

Following viral envelope binding, HCV enters its target cell in a multistep temporal process. To identify the time in which the PKIs exert their effect(s), we used a well characterized HCV binding/postbinding assay¹⁹⁻²¹. In contrast to heparin (an inhibitor of HCV binding) but similarly to CD81- and SR-BI-specific antibodies and *Concanamycin A* (ConA — an inhibitor of endocytosis) PKIs inhibited HCVcc infection when added after virus binding to target cells (**Fig. 4d**). Similar results were obtained for HCVpp entry into PHH after treatment with an EGFR-specific antibody (**Fig. 4e**). These data suggest that the RTKs act at postbinding steps of viral entry.

To further elucidate the entry steps targeted by the RTKs, we performed a kinetic entry assay^{19,21} (**Supplementary Fig. 7b**). Interestingly, the half-maximal times ($t_{1/2}$) for *Erlotinib* ($t_{1/2}=+20$ min) and *Dasatinib* ($t_{1/2}=+26$ min) to inhibit HCV entry were similar to the half-

maximal time of a CD81-specific antibody ($t_{1/2}=+26$ min) (**Fig. 4f** and **Supplementary Fig. 7d**). Moreover, similar to ConA, PKIs also had an inhibitory effect when added at late times post-infection. The role of EGFR as a postbinding factor was further confirmed by kinetic assays under serum-free conditions. In line with previous reports²², HCV entry kinetics are delayed under serum-free conditions (**Fig. 4g**). EGF significantly ($P<0.05$) reduced the time for HCVcc to escape the inhibiting effects of an CD81-specific antibody in serum-starved cells from 44 ± 8 min to 27 ± 6 min (mean \pm SD of three independent experiments), suggesting that EGF markedly and significantly ($P<0.05$) accelerates the rate of HCV entry (**Fig. 4g**). In summary, these data suggest that EGFR is required for efficient viral entry by modulating early and late steps of postbinding events.

Postbinding steps of HCV entry are mediated by HCV co-entry factors SR-BI, CD81, CLDN1 and OCLN. Since PKIs inhibited HCV entry at similar time-points as an CD81-specific antibody we investigated whether PKIs interfere with CD81-CLDN1 co-receptor interactions using a FRET-based assay^{15,23,24}. PKIs significantly ($P<0.0005$) reduced CD81-CLDN1 FRET in polarized HepG2 cells (**Fig. 4h** and **Supplementary Fig. 7e**). Similar results were obtained using RTK-specific siRNAs (**Fig. 4h** and **Supplementary Fig. 7e**) confirming that the observed inhibition is RTK-specific and not mediated by off-target effects of the PKIs. These results suggest that EGFR and EphA2 regulate the formation of the CD81-CLDN1 co-receptor complexes that are essential for HCV entry²³ and that *Erlotinib* and *Dasatinib* inhibit HCV entry by interfering with the CD81-CLDN1 co-receptor association(s).

Since kinetic assays demonstrated that PKIs inhibited late steps of viral entry (**Fig. 4f** and **Supplementary Fig. 7d**), we investigated the impact of these kinases in a viral glycoprotein-dependent cell-cell fusion assay²⁵. Both PKIs significantly ($P<0.05$) inhibited membrane fusion

of cells expressing glycoproteins derived from genotypes 1a (H77), 1b (Con1) and 2a (J6) (**Fig. 4i** and **Supplementary Fig. 7f**), whereas the EGFR ligand EGF enhanced membrane fusion of cells expressing HCV envelope glycoproteins (**Fig. 4i**). In contrast, neither *Erlotinib* nor EGF had a marked effect on the membrane fusion of cells expressing measles virus envelope glycoproteins. Comparable results were obtained in EGFR and EphA2 silenced cells (**Fig. 4i**, data not shown) confirming a functional role of the RTKs during viral fusion.

Impact of RTKs in cell-cell transmission and viral spread. To investigate the relevance of RTK-mediated virus-host interactions for cell-cell transmission and viral spread, we used a cell-cell transmission assay²⁶(**Fig. 5a–c**). *Erlotinib* and *Dasatinib* significantly ($P < 0.0005$) blocked HCV cell-cell transmission during short-term co-culture experiments (24 h) (**Fig. 5d–f** and **Supplementary Fig. 8a–c**). A marked inhibition of cell-cell transmission was also observed when EGFR and EphA2 were silenced using specific siRNAs: infection of GFP-positive target cells directly correlated with levels of RTK cell surface-expression (**Fig. 5g,h** and **Supplementary Fig. 8d,e**). Since PKIs markedly inhibited cell-cell transmission, we investigated whether *Erlotinib* and *Dasatinib* inhibit viral spread in the HCVcc system when added post-infection during long-term experiments. Both PKIs dose-dependently inhibited viral spread when added 48 h post-infection to HCV-infected cells for up to 14 days (**Fig. 5f** and **Supplementary Fig. 8c**). Cell viability was not affected by long term PKI treatment. A specific decrease in viral spread was also observed in cells with silenced RTK expression (**Fig. 5i** and **Supplementary Fig. 8f**). Taken together, these data demonstrate that PKIs reduce viral spread and suggest an important functional role of these RTKs in cell-cell transmission and dissemination.

***Erlotinib* inhibits HCV infection *in vivo*.** To address the *in vivo* relevance of the identified virus-host interactions, we assessed the effect of *Erlotinib* on HCV infection *in vivo* in the chimeric uPA-SCID mouse model²⁷⁻²⁹. *Erlotinib* dosing and administration was performed as described previously for cancer xenograft models³⁰. *Erlotinib* treatment caused a marked and significant ($P<0.05$) delay in the kinetics of HCV infection (**Fig. 6**). The median time to reach steady-state levels of infection increased from 15 days (placebo group) to 30 days (*Erlotinib* group) (median of pooled data from 6 placebo- and 8 *Erlotinib*-treated animals). Furthermore, *Erlotinib* treatment decreased steady-state HCV RNA levels 11.4 fold (mean of pooled data from 6 placebo- and 8 *Erlotinib*-treated animals; $P<0.05$). Following discontinuation of treatment, viral load reached similar levels as in control animals (**Fig. 6**). The treatment was well tolerated and did not induce any marked changes in safety parameters such as alanine transaminase (ALT), albumin or body weight (data not shown). *Erlotinib* plasma concentrations were similar as described previously in preclinical studies of cancer mouse models³⁰ (data not shown). Taken together, these data support a functional role of EGFR as a co-factor for HCV entry and dissemination *in vivo* and demonstrate that *Erlotinib* has antiviral activity *in vivo*.

DISCUSSION

Using RNAi screening we uncovered a network of kinases with functional impact for HCV entry and identified EGFR and EphA2 as novel co-factors for HCV entry. The implications of our results are two-fold: (i) the identification of kinases as novel HCV entry factors advances our knowledge on the molecular mechanisms and cellular requirements of HCV entry and (ii) the discovery of PKIs as antivirals defines a novel strategy for prevention and treatment of HCV infection.

EGFR is a RTK that regulates a number of key processes, including cell proliferation, survival, differentiation during development, tissue homeostasis, and tumorigenesis³¹. EphA2 mediates cell positioning, cell morphology, polarity and motility³². Since PKIs had no effect on HepG2 polarization (**Supplementary Fig. 9**), it is unlikely that changes in polarity explain their mode of action. Our results rather highlight a role of these RTKs in the formation of HCV entry factor complexes and membrane fusion. EGF accelerated HCV entry suggesting that EGFR plays a key role in the HCV entry process allowing HCV to efficiently enter its target cell (**Fig. 4g**). Applying FRET proximity analysis we found that inhibition of EGFR or EphA2 activity reduced CD81-CLDN1 association(s) (**Fig. 4h** and **Supplementary Fig. 7e**). Since EGFR activation has been reported to promote CLDN1 redistribution^{33,34} and CD81 or CLDN1 cell surface expression levels were not modulated by EGFR silencing (**Fig. 4a,b**), we hypothesize that EGFR activation modulates CLDN1 and/or CD81 trafficking that are necessary to form receptor complexes.

The observations that *Erlotinib* inhibited late steps in the kinetic infection assay (**Fig. 4f** and **Supplementary Fig. 7d**) and HCV cell fusion assay²⁵ suggest a functional role for EGFR in pH-dependent fusion of viral and host cell membranes^{25,35}.

Functional experiments using specific ligands, antibodies and kinase inhibitors demonstrate that both ligand-binding and kinase domains are required for EGFR to promote HCV entry. EGFR ligands enhance HCV infection and an EGFR-specific antibody inhibits HCV infection (**Fig. 3**). This antibody binds between ligand binding domain III and the autoinhibition (tether) domain IV of the extracellular part of EGFR³⁶ and prevents EGF and TGF- α -induced receptor dimerization³⁷. Thus, it is likely that the domain and/or receptor dimerization targeted by the antibody are required for HCV entry. Taken together, these findings support a model in which EGFR-ligand binding activates EGFR kinase function that is required for HCV entry.

Similar results were obtained for EphA2 where antibodies specific for the extracellular domain of EphA2 inhibited HCV entry into PHH and EphA2 surrogate ligands decreased viral entry (**Supplementary Fig. 6**). Since addition of surrogate ligands only reduced HCV entry to a small extent it is conceivable that the effect of EphA2 on HCV entry could be mediated as well in a ligand-independent and ligand-dependent manner. This is consistent with other well characterized EphA2 functions such as cell invasion and migration³⁸.

Since functional and mechanistic studies demonstrate that the expression and activity of EGFR and EphA2 appear to be involved in similar entry steps, it is likely that both RTKs are part of the same entry regulatory pathway. Since *Erlotinib* and EGF modulated entry of HCVpp but showed minimal effects on other viruses studied (**Supplementary Fig. 10**), it is likely that the uncovered molecular mechanisms on co-receptor interactions are most relevant for HCV entry.

Finally, our results have important clinical implications for the prevention and treatment of HCV infection as they demonstrate for the first time that clinically licensed PKIs have significant antiviral activity *in vitro* and *in vivo*. Furthermore, the identification of a RTK-specific antibody inhibiting HCV infection (**Fig. 3**) provides a new strategy to target RTKs to prevent and

treat HCV infection. Taken together, these results demonstrate that small molecules or antibodies targeting RTKs as HCV entry factors hold promise as a novel class of antivirals and may offer a perspective for urgently needed antiviral strategies for prevention and treatment of resistant HCV infection.

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AUTHOR CONTRIBUTIONS

J. L., M. B. Z. and T. F. B. wrote the manuscript. J. L., M. B. Z., F. X., D. L., F.-L. C, J. A. McK, and T. F. B. designed experiments and analyzed data. J. L., M. B. Z, F. X., C. T., I. F., L. Z., C. D., C. J. M., M. T., S. G., C. R., M. N. Z., D. L. and J. F. performed experiments. S. M. R., T. P., A. H. P., P. P. and M. D. contributed essential reagents, W. R. and O. P. performed bioinformatic analyses, J.L., B. F. and L. B. implemented the siRNA screen, T. F. B. designed and supervised the project.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests. Inserm and the University of Strasbourg have filed a patent application on “Host cell kinases as targets for antiviral therapy against HCV infection”.

FIGURE LEGENDS

Fig. 1. EGFR is a co-factor for HCV entry. (a,b) Silencing EGFR expression in HCV-permissive cells. (a) EGFR mRNA (qRT-PCR analysis) and (b) protein expression (Western blot) in Huh7.5.1 cells transfected with EGFR-specific individual siRNAs (si1–4). Silencing of CD81 mRNA expression by CD81-specific siRNA served as control. EGFR mRNA (relative to GAPDH mRNA) and protein expression compared to cells transfected with control siRNA (siCTRL) is shown. (c,d) Inhibition of HCV infection and entry in cells with silenced EGFR expression. (c) HCVcc infection in Huh7.5.1 cells transfected with individual siRNAs shown in panels a,b. siCTRL and CD81-specific siRNA served as internal controls. Data are expressed as percentage HCVcc infection relative to siCTRL-transfected cells. (d) Entry of HCVpp containing envelope glycoproteins of various isolates^{14,39} in Huh7.5.1 cells transfected with si4. VSV and measles virus pp entry or cells transfected with CD81 siRNA served as controls. Data are expressed as percentage pp entry relative to siCTRL-transfected cells. (e,f) Rescue of HCV entry in cells with silenced EGFR expression by exogenous EGFR. (e) HCVpp entry and EGFR protein expression in Huh7.5.1 cells co-transfected with EGFR-specific individual si3 and a cDNA encoding for RNAi-resistant EGFR (pEGFR-WT)⁴⁰. (f) HCVpp entry and EGFR protein expression in PHH co-transduced with lentiviruses expressing shEGFR and wild-type EGFR cDNA (EGFR-WT)⁴⁰. Protein expression was quantified using Image Quant analysis of Western blots. Data are expressed as percentage HCVpp entry relative to CTRL cells or as percentage EGFR expression normalized for β -actin expression. *** $P < 0.0005$.

Fig. 2. Inhibition of EGFR activation by kinase inhibitors reduces HCV entry and infection.

(a) Effect of *Erlotinib* on HCV entry and infection in Huh7.5.1 cells. HCVcc (Luc-Jc1; J6-JFH1) infection and HCVpp (J6) entry in Huh7.5.1 cells pre-incubated with indicated concentrations of *Erlotinib* are shown. Data are expressed as percentage HCVcc infection or HCVpp entry relative to solvent-treated control cells (means \pm SEM). (b) Effect of *Erlotinib* on HCV replication. Northern blot analysis of HCV RNA and GAPDH mRNA in Huh7.5 cells electroporated with RNA from subgenomic HCV JFH1 replicon and incubated with solvent CTRL, HCV protease inhibitor BILN-2061 or *Erlotinib* (ERL) is shown. Analysis of HCV RNA in cells transfected with replication incompetent HCV RNA (GND, Δ) served as negative control. (c) Effect of *Erlotinib* on HCVpp and MLVpp entry in HepG2-CD81 cells. Pseudovirus entry into non-polarized and polarized HepG2-CD81 cells (generated as described¹⁵) pre-incubated with *Erlotinib* (10 μ M) is shown. (d) Effect of *Erlotinib* on HCVpp entry into PHH. HCVpp entry in PHH pre-incubated with *Erlotinib* is shown relative to entry into solvent-treated control cells. IC₅₀ value is expressed as median of three independent experiments \pm standard error of the median. (e,f) Effect of PKIs on HCV entry and infection in PHH and Huh7.5.1 cells. (e) HCVpp entry into PHH and (f) HCVcc infection in Huh7.5.1 pre-incubated with 1 μ M *Erlotinib* (ERL), *Gefitinib* (GEF), *Lapatinib* (LAP), *Blebbistatin* (BLEB) or *Wortmannin* (WORT) is shown. Cell viability was assessed using MTT assay.

Fig. 3. Modulation of HCV entry by EGFR ligands and an EGFR-specific antibody. (a)

Modulation of EGFR phosphorylation by EGF, *Erlotinib* and EGFR-specific antibody. EGFR activation was assessed in PHH incubated with the indicated compounds using the Human Phospho-RTK Array Kit. Phospho-tyrosine (P-Tyr) and phosphorylation of an unrelated kinase

(MERTK) served as internal positive and negative controls. **(b,c)** Effect of EGFR ligands on HCVpp entry. HCVpp entry (HCV-J) into serum-starved Huh7.5.1, polarized HepG2-CD81 and PHH in the presence of EGF **(b)** and TGF- α **(c)** is shown. **(d)** Reversion of EGF mediated-enhancement of HCVpp entry by *Erlotinib*. HCVpp entry into Huh7.5.1, polarized HepG2-CD81 and PHH incubated with EGF or EGF and *Erlotinib* is shown. **(e)** Flow cytometric analysis of non-permeabilized PHH binding EGFR-specific or control monoclonal antibody (mAb). **(f)** Inhibition of HCV entry by EGFR-specific mAb. HCVpp entry into PHH pre-incubated with EGFR-specific or control mAb is shown. Viability of cells was assessed using MTT assay. IC₅₀ value is expressed as median of three independent experiments \pm standard error of the median. **(g)** Reversion of EGF-induced enhancement of HCV entry by an EGFR-specific antibody. HCVpp entry into PHH pre-incubated with EGF and EGFR-specific mAb. **(h,i)** Effect of EGF, EGFR-specific mAb and *Erlotinib* on HCV infection in PHH. Intracellular HCV RNA in PHH infected with **(h)** HCVcc or **(i)** serum-derived HCV (one representative experiment) was measured by qRT-PCR. **, $P < 0.005$; ***, $P < 0.0005$. Unless otherwise indicated: EGFR-specific and control mAbs: $10 \mu\text{g mL}^{-1}$, EGF: $1 \mu\text{g mL}^{-1}$, ERL= *Erlotinib*: $10 \mu\text{M}$.

Fig. 4. EGFR mediates HCV entry at postbinding steps by promoting CD81-CLDN1 co-receptor interactions and membrane fusion. **(a,b)** HCV entry factor expression after RTK silencing or PKI treatment. **(a)** Cell surface expression of entry factors in EGFR or EphA2-silenced Huh7.5.1 cells assessed by flow cytometry. SR-BI silencing served as positive control. **(b)** Western blot analysis of HCV entry factor expression in PKI- or siRNA-treated Huh7.5.1 cells. **(c–e)** Effect of *Erlotinib* and EGFR-specific mAb on HCV binding and postbinding steps. **(c)** Flow cytometric analysis of HCV glycoprotein sE2-binding to Huh7.5.1 cells incubated with

EGFR-specific mAb or transfected with siEGFR. SR-BI-specific reagents served as positive controls. **(d)** HCVcc infection of Huh7.5.1 cells and **(e)** HCVpp entry into PHH after inhibition of binding and postbinding steps by the indicated compounds (EGFR-specific mAbs: 10 and 50 $\mu\text{g mL}^{-1}$) **(f,g)** Effect of *Erlotinib* and EGF on HCV entry kinetics. Time-course of HCVcc infection of Huh7.5.1 cells following incubation with **(f)** *Erlotinib* or indicated compounds or **(g)** EGF at different time-points during infection (**Supplementary Methods**). **(h)** Effect of *Erlotinib* and EGFR silencing on CD81-CLDN1 association(s). FRET of CD81-CLDN1 co-receptor associations in HepG2-CD81 cells incubated with *Erlotinib* or EGFR-specific siRNA (means \pm SEM). **(i)** Role of EGFR in viral membrane fusion. Viral glycoprotein-dependent fusion of 293T with Huh7 cells incubated with EGF, *Erlotinib* or EGFR-specific siRNA was assessed as described²⁵. *, $P < 0.05$; ***, $P < 0.0005$. Unless otherwise indicated: EGFR-specific and control mAb: 10 $\mu\text{g mL}^{-1}$, EGF: 1 $\mu\text{g mL}^{-1}$, *Erlotinib*: 10 μM .

Fig. 5. Functional role of EGFR in viral cell-cell transmission and spread. **(a)** Experimental set-up. HCV producer cells (P_i = HCV RNA-electroporated Huh7.5.1) co-cultivated with non-infected target cells (T = GFP-expressing Huh7.5)²⁶ were incubated with siEGFR or PKIs. Cell-free HCV transmission was blocked by an E2-neutralizing antibody (25 $\mu\text{g mL}^{-1}$)²⁶. HCV-infected target cells (T_i = GFP+, HCV NS5A+) were quantified by flow cytometry²⁶. **(b)** Immunofluorescence analysis of P_i , T and T_i cells stained with an NS5A-specific antibody. **(c)** Infectivity of P_i - T cell co-cultivation supernatants (cell-free HCV transmission). **(d,e)** Quantification of infected T_i cells during *Erlotinib* (ERL, 10 μM) treatment in the absence (total transmission) and presence (cell-cell transmission) of E2-specific antibody by flow cytometry. **(f)** Effect of PKIs on viral spread. Long-term HCVcc infection of Huh7.5.1 cells incubated with

Erlotinib 48 h post-infection at the indicated concentrations. Medium with solvent (CTRL) or PKI was replenished every 2nd day. Cell viability was assessed using MTT test. **(g)** EGFR expression in target cells with silenced EGFR expression. Cell surface EGFR expression was analyzed by flow cytometry and target cells were divided in three groups displaying high, medium and low EGFR expression. **(h)** HCV infection in GFP-positive target cells expressing EGFR at high, medium and low levels (see panel **g**) assessed as described above. **(i)** Effect of EGFR silencing on viral spread. Long-term analysis of HCVcc infection in Huh7.5.1 cells transfected with EGFR-specific or control siRNA 24 h post-infection. Cell viability was assessed using MTT test. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$.

Fig. 6. Erlotinib modulates HCV kinetics and inhibits infection *in vivo*. Chimeric uPA/SCID mice repopulated with PHH^{27,28} were treated with *Erlotinib* or placebo during infection with patient-derived HCV as indicated by the bar and dashed lines. *Erlotinib* administration and dosage were performed as previously described for xenograft tumor mouse models³⁰. Serum HCV load was analyzed at the time points indicated. Results are shown as median viral load of *Erlotinib*- ($n=4$) or placebo-treated control mice ($n=3$). **(a,b)** Two independent studies (with a total of 2 x 7 mice, respectively) are shown. **(c)** Pooled data of results shown in **a,b** ($n=14$); *, $P < 0.05$.

METHODS

For more detailed Methods see **Supplementary Methods**. Unless otherwise stated, data are presented as means \pm SD of at least three independent experiments performed in duplicate or triplicate.

Infection of cell lines and primary human hepatocytes with HCVpp, HCVcc and serum-derived HCV. Pseudotyped particles (pp) expressing envelope glycoproteins from different HCV strains (**Supplementary Methods**), VSV, MLV, influenza, measles and endogenous feline leukemia virus (RD114) and HCVcc were generated as described^{14,15,21,41-45}. Infection of Huh7, Huh7.5.1 cells and PHH with HCVpp, HCVcc (TCID₅₀ 10³-10⁴ mL⁻¹ for Huh7.5.1 experiments, TCID₅₀ 10⁵-10⁶ mL⁻¹ for PHH experiments) and serum-derived HCV (genotype 1b)⁴⁶ was performed as described^{14,19,21,47}. Polarization of HepG2-CD81, determination of TJ integrity and cell polarity index were performed, measured and calculated as previously described¹⁵. Gene silencing was performed 3 d prior to infection as described for the RNAi screen (**Supplementary Methods**). Inhibitors, antibodies or ligands were added 1 h prior to HCVpp or HCVcc infection and during infection unless otherwise stated. Experiments with RTK ligands were conducted with serum-starved cells. Unless otherwise stated, HCV entry and infection was assessed by luciferase reporter gene expression.

Analysis of HCV replication. Electroporation of RNA derived from plasmid pSGR-JFH1 or replication-deficient mutant pSGR-JFH1/GND (Δ) was performed as described⁴². Four hours after electroporation, cells were incubated with inhibitors. Total RNA was isolated and HCV RNA was analyzed by Northern blot as described⁴⁸.

Rescue of gene silencing. To assess whether silencing of endogenous RTKs could be rescued by expression of RNAi-resistant RTK expression, 4×10^6 Huh7.5.1 cells were co-electroporated with 10 μg siRNA targeting the 3'UTR of the endogenous cellular mRNA (siEGFR si3, siEphA2 si4, HS-CDC2_14) and a RTK encoding plasmid expressing siRNA-resistant mRNA containing a deletion of the 3'UTR (pEGFR, pEphA2, pCDC2)^{40,49,50}. 2.5×10^4 cells cm^{-2} were seeded 72 h prior to infection with HCVcc (Luc-Jc1; genotype 2a/2a) or HCVpp (H77; genotype 1a). EGFR rescue in PHH was performed by co-transduction with lentiviruses expressing shEGFR⁴⁰ and/or EGFR⁴⁰ 72 h prior to infection with HCVpp (HCV-J; genotype 1b).

Analysis of EGFR phosphorylation in PHH and Huh7.5.1 cells. EGFR phosphorylation was assessed in cell lysates using the Human Phospho-RTK Array Kit (R&D Systems Inc.), where RTKs are captured by antibodies spotted on a nitrocellulose membrane. Levels of phospho-RTK were assessed using an HRP-conjugated pan phospho-tyrosine-specific antibody followed by chemiluminescence detection as described by the manufacturer. Phospho-tyrosine (P-Tyr) and phosphorylation of the unrelated c-mer proto-oncogene tyrosine kinase (MERTK) served as internal positive and negative controls. PHH were incubated in EGF-free William's E medium. Huh7.5.1 cells were serum-starved overnight prior to addition of ligands, inhibitors and antibodies.

Analysis of HCV binding, postbinding and entry kinetics. HCV glycoprotein E2 binding to cells was performed as described¹⁸ using polyclonal SR-BI²¹- or monoclonal EGFR-specific antibodies ($100 \mu\text{g mL}^{-1}$) or SR-BI- or EphA2-specific serum (1:100) and corresponding

controls. HCV postbinding and entry kinetics were performed as described^{19,21} (**Supplementary Methods and Supplementary Fig. 7**).

Receptor association using fluorescence resonance energy transfer (FRET). Homotypic and heterotypic interactions of CD81 and CLDN1 were analyzed as described^{15,23,24}. The data from 10 cells were normalized and the localized expression calculated.

Membrane fusion. HCV membrane fusion during viral entry was investigated using a cell-cell fusion assay as described²⁵.

Cell-cell transmission of HCV. Cell-cell transmission of HCV was assessed as described²⁶. Briefly, producer Huh7.5.1 cells were electroporated with HCV Jc1 RNA and co-cultured with gene-silenced or naïve target Huh7.5-GFP cells in the presence or absence of PKIs (10 μ M). An HCV E2-neutralizing antibody (25 μ g mL⁻¹) was added to block cell-free transmission²⁶. After 24 h of co-culture cells were fixed with PFA, stained with a NS5A-specific antibody (0.1 μ g mL⁻¹), and analyzed by flow cytometry²⁶. Total and cell-cell transmission were defined as percentage HCV infection of Huh7.5-GFP+ target cells (Ti) in the absence (total transmission) or presence (cell-cell transmission) of an HCV E2-specific antibody.

HCV infection and treatment of chimeric uPA/SCID mice. Chimeric mice repopulated with PHH^{27,28} were infected with serum-derived HCV (genotype 2a, 1 x 10⁴ HCV IU per mouse) via the orbital vein during isofluoran anesthetization (PhoenixBio Inc., Japan). *Erlotinib* administration and dosage (50 mg kg⁻¹ day⁻¹) were performed as previously described in

xenograft tumor mouse models³⁰. Four mice received 50 mg kg⁻¹ day⁻¹ *Erlotinib* and three mice placebo (p. o.) from day -10 until day 20 of infection in two independent experiments (total 2 x 7 animals). Plasma HCV RNA, ALT, albumin and *Erlotinib* were monitored as described^{28,51}. All experimental procedures used to treat live animals in this study had been approved by the Animal Ethics Committee of PhoenixBio in accordance with the Japanese legislation.

Toxicity assays. Cytotoxic effects on cells were assessed in triplicates by analyzing the ability to metabolize 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)⁵². Formazan crystals were solubilized 5 h after adding MTT (0.6 mg mL⁻¹) as described⁵².

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