Materials and Methods

Plasmids and viruses:

Development of the reporter virus genomes Luc-Jc1, Luc-JFH1, Luc-JFH1/ NS3-5B, Luc-JFH1 /NS2-5B have been described recently [1]. The replication-defective mutant Luc-Jc1 GDD has an in-frame deletion of 10 amino acids encompassing the GDD motif of NS5B [2]. Short hairpin RNA`s (shRNA) Raptor_1 shRNA (Addgene plasmid 1857), rictor_1 shRNA (Addgene plasmid 1853), mTOR1 shRNA (Addgene plasmid 1855) scramble shRNA (Addgene plasmid 1864) and pLJM1 Flag raptor (Addgene plasmid 26633) were all purchased from Addgene, Cambridge, USA [3, 4]. Plasmid pFK-Jc1 [5] and monocistronic *Renilla* luciferase reporter virus genomes designated JcR2a and SA13/5a/R2a were described recently [6]. Luc Ubi Neo JFH1 NS3-3`has been described before [7].

Cell culture:

Huh-7.5 cells were maintained in Dulbecco Modified Eagle Medium supplemented with 10% fetal calf serum, non-essential amino acids, L-glutamine and Penicillin/Streptomycin. Cells harboring small hairpin RNA (shRNA) constructs were kept in the presence of neomycin or puromycin. For differentiation, Huh-7.5 cells were seeded in 12 well plates and treated with DMEM + 1% DMSO for at least two weeks as described before [8].

Primary human hepatocytes (PHH) were isolated from patients undergoing partial hepatectomy, plated at a density of $1.3x10^6$ on Collagen in P6 dishes, and kept in William's E medium (Invitrogen, Karlsruhe, Germany).

Preparation of lentiviral pseudo-particles and HCVpp infection:

Lentiviral-based pseudotypes bearing vesicular stomatitis virus glycoproteins (VSV-G) or HCV E1-E2 proteins of the Con1 or JFH isolate were generated as described recently [2]. Huh-7.5 target cells were seeded on a 12 well plate and infected with pseudoparticles and

mTOR inhibitors in different concentrations for 5h. Luciferase was measured 72h post transduction.

HCV infection assay on primary human hepatocytes

In general, 1000 µl containing virus supernatant with a viral titer of 2×10^5 $TCID_{50}$ (MOI 0.5-1) was used to inoculate target cells. After 6 hours, cell culture medium supplemented with different concentrations of rapamycin or everolimus was added. After 24 and 48 hours, supernatant was harvested, filtered and then used to infect naïve Huh-7.5 cells. HCV titers were determined as published recently [9].

Luciferase reporter cell line

To test for anti-proliferative and/or cytotoxic effects, lentiviral VSV-G pseudoparticles were assembled as described above and used to transduce Huh-7.5 cells to stably express firefly luciferase driven by a CMV promoter (Huh-7.5/Fluc). Drugs were added to Huh-7.5/Fluc cells at different concentrations for 48 hours. After 48h luciferase signal was measured.

EdU Proliferation Assay

Huh-7.5 cells +/- rapamycin were stained with EdU (5-ethynyl-2`-deoxyuridine) Flow Cytometry Kit (Base Click GmbH, Tutzing, Germany) following manufacturer´s instruction. As a positive control we used mitomycin. A BD FACS Canto flow cytometer (Becton Dickinson, Heidelberg, Germany) was used for acquisition and FlowJo software (Tree Star; Ashland, OR) for data analysis.

Core ELISA

Core protein was measured using HCV core antigen kit received from Wako Chemicals (Neuss, Germany).

Western Blot Analysis

Proteins, resolved by electrophoresis and blotted onto a polyvinylidene difluoride membrane, were detected using anti-E2 (AP33 from Arvind Patel), anti-NS2 (6H6 provided by Charles Rice), anti-NS5A (9E10 provided by Charles Rice) and anti-Core (C7-50, provided by Darius Moradpour). Anti-ß-actin (Sigma-Aldrich, Germany) was used as described recently.

Anti-raptor antibody (EP539Y, anti-rabbit monoclonal) and Anti-rictor antibody (7B3, anti-mouse monoclonal) were bought from Abcam, Cambridge, USA. Anti-mTOR antibody (anti-rabbit) was purchased from Cell Signaling Technology, USA. For raptor, rictor and mTOR western blots, sample buffer was used without 2% mercaptoethanol. The ECL Plus Western Blotting Detection System (GE Healthcare Europe, Freiburg, Germany) was used for final protein detection.

Freeze and thaw lysates of HCV transfected cells

Huh7.5 cells were transfected with Jc1 RNA, 48 h post transfection cell culture supernatants were harvested and virus titers determined by $TCID_{50}$. Cell-associated infectivity was prepared essentially as described [10]. Briefly, cells were extensively washed with PBS, scraped and centrifuged for 5 minutes at 400 x g. Cell pellets were resuspended in 1 ml of DMEM containing 5% FCS and subjected to three cycles of freeze thaw using liquid nitrogen and a thermoblock set to 37°C. Samples were then centrifuged at 10,000 x g for 10 minutes at 4°C to remove cell debris, and cell-associated infectivity was determined by $TCID_{50}$ assay.

Iodixanol density-gradient

Density Gradient centrifugation was performed as described recently [11]. Briefly, viruses were separated by an overnight centrifugation through a 0% - 40% iodixanol step gradient at 154,000 x g in a TH-641 swing-out rotor at 4°C using a Sorvall Ultra WX80 centrifuge. Ten fractions of 1 ml were collected from the bottom and analysed for virus infectivity, core protein levels and viral RNA copies, respectively.

RNA quantification by reverse-transcription PCR (RT-PCR)

RNA was isolated from cells by RNAeasy Kit (Qiagen) as recommended by the manufacturer. HCV-specific RNA and GAPDH (glyceraldehyde-3-phosphate dehydrogenase)-specific mRNA were determined as described previously [6]. HCV RNA and GAPDH were quantified using a Light Cycler 480 II (Roche,Mannheim, Germany) .

MTT viability assay

Cell viability was measured using an MTT assay according to the manufacturer instructions. 3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide was purchased from Life Technologies (Life Technologies GmbH, Darmstadt, Germany).

Propidium Iodide (PI) staining

Cytotoxicity was measured using propidium iodide (PI) staining. Briefly, cells were incubated with the desired drug concentrations for 48h. Medium was collected and adherent cells were trypsinized for 5 minutes at 37ºC and centrifuged, together with the culture medium, at 4ºC 300g for 5 minutes. The pellet was washed with cold PBS. Cells were resuspended in 350uL of fresh media and conserved on ice. 600ng of PI (Life Technologies GmbH, Darmstadt, Germany) were added to the cell suspension before measuring and the mixture was incubated on ice for 2 minutes and transferred to 7.5mL tubes for immediate FACS analysis. 488nm laser was used for excitation.

References

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