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Targeted delivery of siRNAs against hepatocellular carcinoma-related genes by a galactosylated polyaspartamide copolymer

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ABSTRACT

Given the lack of effective treatments for Hepatocellular carcinoma (HCC), the development of novel therapeutic approaches is very urgent. Here, siRNAs were delivered to HCC cells by a synthetic polymer containing α,β -poly-(N-2-hydroxyethyl)-D,L-aspartamide (PHEA) derivatized with diethylene triamine (DETA) and bearing in the side chain galactose (GAL) linked via a polyethylene glycol (PEG) to obtain (PHEA-DETA-PEG-GAL, PDPG). The GAL residue allows the targeting to the asialo-glycoprotein receptor (ASGPR), overexpressed in HCC cells compared to normal hepatocytes.

Uptake studies performed using a model siRNA or a siRNA targeted against the enhanced green fluorescence protein, demonstrated the PDPG specific delivery of siRNA to HuH7 cells, a human cellular model of HCC. GAL-free copolymer (PHEA-DETA-PEG-NH₂, PDP) or the chemical block of ASGPR, impaired PDPG targeting effectiveness *in vitro*. The specificity of PDPG delivery was confirmed *in vivo* in a mouse dorsal skinfold window chamber assay.

Functional studies using siRNAs targeting the mRNAs of HCC-related genes (eEF1A1, eEF1A2 and E2F1) delivered by PDPG, significantly decreased HuH7 vitality/number and down regulated the expression of the target genes. Only minor effectiveness was in contrast observed for PDP. In IHH, a human model of normal hepatocytes with reduced ASGPR expression, PDPG barely reduced cell vitality. In a subcutaneous xenograft mouse model of HCC, PDPG-siRNAs reduced HCC tumor growth compared to controls without significant toxic effects.

In conclusion, our study demonstrates the valuable potentials of PDPG for the specific delivery of siRNAs targeting HCC-related genes.

1. Introduction

Hepatocellular carcinoma (HCC), the main form of primary liver cancer, represents the second cause of cancer death and the sixth most commonly diagnosed cancer worldwide [1]. HCC displays high

resistance to chemotherapy and radiation treatments [2,3] and these therapeutic options are abandoned now. Liver transplantation or surgical resection are limited to the very early stages of the disease [4,5]. At present, the first-line treatments widely recommended for un-resectable HCC without both macro-vascular invasion and extrahepatic metastasis

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are loco-regional therapies such as the trans-arterial chemoembolization (TACE) or trans-arterial radio-embolization [6]. Still, also after these procedures, the overall survival is unacceptably short. Even systemic therapies, such as multi tyrosine kinase inhibitor sorafenib (approved for un-resectable HCC for almost a decade) and the new drugs lenvatinib, regorafenib, cabozantinib, and ramucirumab have failed to show a significant survival benefit [6]. It is therefore evident that novel therapeutic options are urgently needed.

Due to the remarkable specificity of action, small interfering RNAs (siRNAs) [7] have the potential to be of therapeutic value in many human diseases, including HCC. However, due to their molecular nature, siRNAs need to be protected in the biological environment from the fast degradation induced by nucleases. In addition, their negative charge and the hydrophilic nature, prevent any efficient cell membrane crossing [7]. To circumvent these and other biological barriers [8,9], siRNAs need to be delivered *via* “smart materials”. In this regard, synthetic/natural polymers are considered particular promising tools due to their general versatility, low cost of production/isolation and biocompatibility [10,11].

In the recent past we have developed different polymer-based delivery systems such as PHEA-DEAEMA (α,β -poly(N-2-hydroxyethyl)-D,L-aspartamide - diethylamino ethyl methacrylate) [12] and Inu-DETA (inulin diethylene triamine) [13] able to protect siRNAs. However, siRNA protection against degradation is not enough for an optimized delivery. Indeed, the release system should be able to specifically targeting pathologic cells leaving the normal counterpart cells as much as possible untouched. This can considerably reduce unspecific side effects and maximize effectiveness.

Recently [14] we have started the development of a HCC targeted delivery systems for siRNAs based on a polymeric backbone containing α,β -poly-(N-2-hydroxyethyl)-D,L-aspartamide (PHEA) derivatized with diethylene triamine (DETA). PHEA-DETA has been conjugated with a galactosylated PEG derivative containing galactose (GAL) molecules at the end of the chain, to obtain PHEA-DETA-PEG-GAL (PDPG). The GAL residues are used as targeting moieties for the asialoglycoprotein receptor (ASGPR), which is overexpressed in HCC cells compared to normal hepatocytes [8]. Because of its restricted expression, ASGPR is considered a valuable surface biomarker to target HCC cells [8]. In our preliminary work [14], we characterized the PDPG polymer, showed the electrostatic interaction with siRNAs, proved the ability to target the HCC cell line JHH6 *in vitro* and its haemo-compatibility. To evaluate the PDPG effectiveness *in vivo*, in this work we have undertaken another HCC cellular model, HuH7, which, in contrast to JHH6, is amenable to generate a xenograft mouse model of HCC [9]. In this work, multiple techniques and functional approaches have been used to prove PDPG specificity in delivering a model siRNA to HuH7, both *in vitro* and *in vivo*. In particular, we developed a novel variant of PDPG linked to a red fluoro-chrome (BODIPY™ TR-X NHS Succinimidyl Ester), which allowed to follow polymer fate in the cell.

In addition to the use of a model siRNA, we have tested the ability of PDPG to deliver functional siRNAs targeted against the eukaryotic elongation factor 1A1 (eEF1A1), eukaryotic elongation factor 1A2 (eEF1A2) and the transcription factor E2F1, all involved in HCC. Besides regulating the elongation step of protein synthesis, eEF1A1/A2 are also implicated in relevant cellular process such as cell cycle and apoptosis [15]. eEF1A1 involvement in HCC is shown by the fact that its overexpression is associated with HCC cell growth and differentiation phenotype [16]. Moreover, eEF1A1 targeting by an aptamer or a siRNA we developed, resulted in a profound impairment of the viability of cultured HCC cell lines [17,18]. eEF1A2 is also overexpressed in HCC tumor specimens [19,20] and its overexpression correlates with cancer cell growth and differentiation phenotype [16]. E2F1 is a transcription factor implicated in the regulation of many cellular processes including cell proliferation and apoptosis. E2F1 has an overall pro-HCC effects [21] and its targeting by siRNAs can significantly impair HCC cell growth [22]. Finally, eEF1A1 depletion resulted in the down regulation

of E2F1 expression [18] thus indicating a correlation E2F1-eEF1A1 in HCC. Here we show both *in vitro* and *in vivo*, that PDPG-mediated delivery of siRNAs against eEF1A1/A2 or E2F1 results in a remarkable and specific HCC growth delay.

2. Material and methods

2.1. Materials

Anhydrous *N,N*-dimethylformamide (a-DMF), anhydrous *N,N'*-dimethylacetamide (a-DMA), deuterium oxide (D₂O), *N,N'*-disuccinimidyl carbonate (DSC), ethyl ether were purchased from Sigma-Aldrich. Diethylene triamine (DETA), triethylamine (TEA), poly(ethylene glycol) bis(amine)2000 (H₂N-PEG-NH₂) and O-(2-aminoethyl)-O'-methyl poly(ethylene glycol) (H₂N-PEG-OCH₃ M_w = 2000 Da), galactose (GAL) were obtained from Fluka (Italy). BODIPY™ TR-X NHS Succinimidyl Ester (Bpy) was purchased from ThermoFisher Scientific.

α,β -poly(N-2-hydroxyethyl)-D,L-aspartamide (PHEA) was prepared and purified according to a procedure elsewhere reported [23].

The O-(2-aminoethyl)-O'-galactosyl polyethylene glycol (H₂N-PEG-GAL) derivative was synthesized and purified according to a procedure elsewhere reported [24,25]. The content of amine-terminated side chains was determined by modified TNBSA assay, while the content of GAL by the anthrone-sulfuric acid colorimetric and fluorometric assays, as reported elsewhere [14].

2.2. General procedures of copolymer synthesis

2.2.1. Synthesis of α,β -poly(N-2-hydroxyethyl)(2-ethylendiamine-aminoethylcarbamate)-D,L-aspartamide (PHEA-g-DETA)

Derivatization of PHEA with diethylenetriamine (DETA) was carried out by using *N,N'*-disuccinimidyl carbonate (DSC) as coupling agent, as previously reported [14]. PHEA-g-DETA copolymer was obtained with a yield of 84.7 wt% based on the starting PHEA.

2.2.2. Synthesis and characterization of α,β -poly(N-2-hydroxyethyl)(2-ethylendiamine-aminoethylcarbamate)-D,L-aspartamide-succinimidyl-BODIPY™ TR-X (PHEA-g-DETA-g-Bpy)

Labeling of PHEA-g-DETA with BODIPY™ TR-X NHS Succinimidyl Ester (Bpy) was carried out following the procedure reported below. To 300 mg PHEA-g-DETA solution in a mixture of a-DMF/ a-DMSO (3,1 v/v) were added 300 μ l of a solution of TEA in a-DMSO and after 5 min, 5 aliquots of a Bpy solution in a-DMSO were added to the PHEA-g-DETA solution and left to react for 4 h at 40 ± 0.1 °C. Reagents were added accordingly to $R_x = (\text{mmol of Bpy} / \text{mmol of functionalizable RU on PHEA} - \text{DETA}) = 0.015$ and $R_y = (\text{mmol of TEA} / \text{mmol of Bpy}) = 0.2$. The product was separated by precipitation in diethyl ether, washing in acetone, dried under vacuum and then purified by dialysis (SpectraPor Dialysis Tubing, at MWCO 12.0–14.0 kDa). After freeze-drying, the product was stored for further characterization. PHEA-g-DETA-g-Bpy copolymer was obtained with a yield of 91.3 wt% based on the starting PHEA-g-DETA.

2.2.3. Synthesis of PHEA-g-DETA-g-PEG-GAL or PHEA-g-DETA-g-Bpy-g-PEG-GAL

Derivatization of either PHEA-g-DETA or PHEA-g-DETA-g-Bpy with H₂N-PEG-GAL was carried out by using *N,N'*-disuccinimidyl carbonate (DSC) as reported elsewhere [14,26]. Briefly, a calculated amount of DSC was added to PHEA-g-DETA solution in a-DMF (33.3 mg/ml) under argon atmosphere; then the catalyst TEA was added drop-wise, according to $R_4 = (\text{mmol of DSC} / \text{mmol of functionalizable RU on PHEA derivative}) = 0.0625$ and $R_6 = (\text{mmol of TEA} / \text{mmol of DSC}) = 1$. The clear solution was stirred at 40 ± 0.1 °C for 4 h under argon atmosphere. At the same time, H₂N-PEG-GAL was dissolved in a-DMF according to $R_5 = (\text{mmol of aminePEGGAL} / \text{mmol of functionalizable RU on PHEA derivatives}) = 0.05$.

After activation time, the polymeric solution was added drop-wise to H₂N-PEG-GAL solution [24,27]. The reaction was carried out under argon atmosphere and continuous stirring at 25 ± 0.1 °C for 18 h. Then, the reaction mixture was purified by dialysis (MWCO 12–14 kDa), then freeze-dried and stored for further characterization. PHEA-g-DETA-g-PEG-GAL and PHEA-g-DETA-g-Bpy-g-PEG-GAL copolymers were obtained with a yield of about 93 wt% based on the starting PHEA-g-DETA and PHEA-g-DETA-g-Bpy, respectively.

2.2.4. Synthesis of PHEA-g-DETA-g-PEG-OCH₃ or PHEA-g-DETA-g-Bpy-g-PEG-OCH₃

Derivatization of PHEA-g-DETA or PHEA-g-DETA-g-Bpy graft copolymers with O-(2-aminoethyl)-O'-methyl poly(ethylene glycol) (H₂N-PEG-OCH₃) was carried out by using DSC as activating agent of terminal amines of DETA linked to PHEA, following the procedure reported above for galactosylated copolymers [14]. PHEA-g-DETA-g-PEG-OCH₃ and PHEA-g-DETA-g-Bpy-g-PEG-OCH₃ graft copolymers were obtained with a yield of 88.0 wt% based on the starting PHEA-g-DETA.

2.3. General procedures of copolymer characterization

2.3.1. ¹H NMR analysis

¹H NMR spectra were obtained by a Bruker Avance II-300 spectrometer, working at 300 MHz (Suppl mat 1A). Spectroscopic data of PHEA, PHEA-g-DETA, PHEA-g-DETA-g-PEG-GAL and PHEA-g-DETA-g-PEG-OCH₃ graft copolymers were in agreement with the attributed structures [14,23].

¹H NMR spectrum of PHEA-g-DETA-g-Bpy graft copolymer (300 MHz, D₂O, 25 °C, TMS): δ 2.50–3.10 (4H_{DETA}-CONHCH₂CH₂NHCH₂CH₂NH₂; 2H_{PHEA}-NHCHCH₂CO-; 2H_{DETA}-CH₂CH₂NH₂), δ 3.1–3.5 (2H_{DETA}-NHCH₂CH₂NHCH₂CH₂NH₂; 2H_{PHEA}-CONHCH₂CH₂OH; δ 3.53 (2H_{PHEA}-NHCH₂CH₂OH); δ 4.00 (2H_{PHEA}-CH₂OCONH-); δ 4.61 (1H_{PHEA}-COCHCH₂NH-); δ 6.55–8.30 (12H_{Bpy}, H-Ar).

¹H NMR spectrum of PHEA-g-DETA-g-Bpy-g-PEG-GAL graft copolymer (300 MHz, D₂O, 25 °C, TMS): δ 2.5–3.1 (4H_{DETA}-CONHCH₂CH₂NHCH₂CH₂NH₂; 2H_{PHEA}-NHCH₂CHCO-; 2H_{DETA}-CH₂CH₂NH₂), δ 3.1–3.5 (2H_{DETA}-NH-CH₂CH₂NHCH₂CH₂NH₂; 2H_{PHEA}-CONHCH₂CH₂OH; 2H_{PHEA}-NHCH₂CH₂OCO-); δ 3.5 (2H_{PHEA}-NHCH₂CH₂OH); 3.5–3.6 (4H_{PEG}-[CH₂CH₂O]₄₄-); δ 4.0 (2H_{PHEA}-CH₂OCONH-); δ 4.6 (1H_{PHEA}-COCHCH₂NH-); δ 6.55–8.30 (12H_{Bpy}, H-Ar).

¹H NMR spectrum of PHEA-g-DETA-g-Bpy-g-PEG-OCH₃ graft copolymer (300 MHz, D₂O, 25 °C, TMS): δ 2.5–3.1 (4H_{DETA}-CONHCH₂CH₂NHCH₂CH₂NH₂; 2H_{PHEA}-NHCH₂CHCO-; 2H_{DETA}-CH₂CH₂NH₂), δ 3.1–3.6 (2H_{DETA}-NHCH₂CH₂NHCH₂CH₂NH₂; 2H_{PHEA}-CONHCH₂CH₂OH; 2H_{PHEA}-NHCH₂CH₂OCO-); δ 3.5 (2H_{PHEA}-NHCH₂CH₂OH); δ 3.5–3.6 (4H_{PEG}-[CH₂CH₂O]₄₄-); δ 4.00 (2H_{PHEA}-CH₂OCONH-); δ 4.6 (1H_{PHEA}-COCHCH₂NH-); δ 6.55–8.30 (12H_{Bpy}, H-Ar).

2.3.2. Evaluation of molar extinction coefficient (ε)

Fluorescence spectra were recorded by a RF-5301PC spectrofluorometer (Shimadzu, Italy). ε of Bpy and PHEA-g-DETA-g-Bpy graft copolymers were evaluated in bidistilled water, in concentration ranging between 10⁻⁷ - 10⁻⁵ M, by recording UV spectra with a RF-5301PC spectrofluorometer (Shimadzu, Italy). The absorption at maxima wavelength was measured and the ε was calculated from the curve obtained by plotting absorbance versus sample solution concentrations. Each test was repeated in triplicate.

2.3.3. Size exclusion Chromatography (SEC) analysis

Weighted-average molecular weight ($\bar{M}_w$) and polydispersity ($\bar{M}_w/\bar{M}_n$) of each copolymer was determined by size exclusion chromatography (SEC) analysis. The SEC system (Waters, Mildford, MA) was

equipped with a pump system, two TSK-GEL columns from Tosoh Bioscience (10³ Å and 10⁴ Å of pores size), and a 410 differential refractometer (DRI) as concentration detector. Analyses were performed with PBS at pH 8 as eluent with a flow of 0.8 ml/min and poly(ethylene oxide) standards (range 145–1.5 kDa) to obtain the calibration curve. The column temperature was set at 37 °C (±0.1 °C).

2.3.4. siRNA binding efficiency to the copolymer

This parameter was estimated from gel electrophoresis test (Suppl mat 1B) where a major condensation of siRNA to PDPG was observed starting from R (weight ratio polymer/siRNA, corresponding to a N/P ratio of 3.5) equal to 5. From this data, it was possible to estimate the binding efficiency, expressed as the amount of siRNA on the total weight of polyplex at R = 5, which turned out to be 20 wt%.

2.3.5. TEM analysis

For TEM analysis, siRNA+PDPG complex was prepared as describe in section 2.5 except that distilled water was used to allow siRNA binding to PDPG. Afterwards, the sample was dry for 30 min at room temperature on a carbon support film 200 mesh copper (Electron Microscopy Sciences) and subsequently visualized by a transmission electronic microscopy (Philips EM 208) equipped with an acquisition system Camera OLYMPUS QUEMESA, Software RADIUS (EMSIS electron microscopy imaging company, Muenster, Germany) (Suppl mat 1C).

2.4. Cell cultures

The human HCC cell line HuH7 and the 293 T (ATCC® CRL-3216™) cell line, used for lentivirus production, were cultured in Dulbecco's modified Eagle's medium (DMEM) (Euroclone) containing 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine (Euroclone). The non-tumorigenic immortalized human hepatocytes (IHH) were cultured in Ham's Nutrient Mixture-F12 (Euroclone) containing 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 1 µM Dexamethasone (Sigma-Aldrich) and 10 µM of recombinant human insulin (Humulin). All cell lines were mycoplasma-free as determined by standard detection methods.

2.5. siRNA in vitro transfection

For uptake studies, siGL2 delivered by PDPG or PDP conjugated with BODIPY™ TR-X (Ex/Em = 588/616 nm) was used. Copolymer powders were weighted each time before the use and re-suspended in RNase free water in order to obtain the final concentration of 1 mg/ml. Optimal transfection conditions were set at the weight ratio siRNAs-copolymers of 1:1 and administrating the mix siGL2 + PDPG/PDP (220 nM in 0,8 ml of transfection solution) to the cells for 4 h at 37 °C in the presence of serum-free medium Optimem (Invitrogen). Afterwards, transfection medium was removed, HuH7 were washed with PBS and 3 ml of complete medium were added to the cells. HuH7 were previously seeded on slides placed in 6-wells plates at the density of 7.4 × 10³ cells/cm² and transfected the following day. Cells were then fixed 4 h after transfection in 4% paraformaldehyde (Sigma-Aldrich) for 20 min at RT, washed twice with PBS, stained by DAPI (1:2500 in 1× PBS; n-9564; Sigma-Aldrich) for 5 min at RT and mounted using Mowiol Mounted solution (Mowiol® 4-88, Fluka). The % of transfected cells was obtained by counting BODIPY™ TR-X positive cells in ten fields of two slides from three independent experiments (40× magnification). In the studies using siEGFP (Dharmacon™, P-002048-01-20 GFP Duplex I), HuH7-EGFP were seeded on slides placed in 6-well plates at the density of 7.4 × 10³ cells/cm². The day after, cells were transfected by PDPG/PDP or Lipofectamine 2000 delivering siGL2 or siEGFP using the same protocol adopted for siGL2-polymer transfection, with or without pretreatment (10 min before transfection) with 5 nM of EGTA. Forty-eight hours after transfection, slides were fixed as above described and analyzed for fluorescence intensity. The % of fluorescence intensity was

evaluated by FiJi (ImageJ) software (<https://imagej.net/Citing>) measuring the fluorescence intensity of each cell in ten fields of three slides from three independent experiments and normalized to the respective siGL2 treatment. Images were acquired by Leica DM-2000 microscope.

The anti eEF1A1 siRNA (siA1), anti eEF1A2 siRNA (siA2), anti E2F1 siRNA (siE2F1) and the control siRNA (siGL2) were previously developed by us [18,22] and here used for the functional studies. The day before transfection, HuH7 cells were seeded at the density of 3.8×10^3 cells/cm² in 6 well plate in 3 ml of DMEM complete medium. Transfections were performed using the functional siRNAs (siA1, siA2, siE2F1) and the control siGL2 delivered by PDPG/PDP/PD under the same condition reported for the uptake studies except that for the dose dependent studies different siA1 concentrations were used (55–110–220 nM). To test the effect of serum on transfection effectiveness, the procedure above reported was followed except that 10% FBS was added to the transfection medium. In the competition experiments of siA1 + PDPG with free galactose (Fig. 4C), the transfection was conducted as above reported except that HuH7 have been pre-treated for 1 h with an excess (400×) of free galactose (5.6×10^{-3} nM) compared to the amount of the galactose bound to the polymer.

2.6. Co-localization test

For the ASGPR-PDPG co-localization analysis, HuH7 were seeded on slides placed in 6-wells plates at density of 7.4×10^3 cells/cm² and then transfected by siGL2 + PDPG-Bpy (220 nM in 0.8 ml of transfection solution, weight ratio siRNA-copolymers of 1:1) for 5 min or 1 h. Thereafter, cells were fixed in 4% paraformaldehyde, incubated with 0.1 M glycine, washed with PBS and incubated overnight in a humid chamber in the dark with Mouse anti-ASGPR antibody (Santa Cruz Biotechnology, dilution 1:100) in 1% BSA/PBS. Cells were then incubated with anti-mouse Alexa Fluor® 488 secondary antibody (Jackson ImmunoResearch, 1:500 dilution) for 50 min at RT in a humid chamber in the dark, stained by DAPI and mounted with Moviol Mounted solution. All images were acquired using Carl Zeiss Laser Scanning Confocal Microscopy LSM800 Zeiss Airyscan equipped with a 63×, NA = 1.3 objective. Images were processed using Fiji (ImageJ) software; the co-localization coefficient (Manders' Coefficient) was calculated by Coloc2 (Fiji's plugin for co-localization analysis) setting a threshold manually and considering a regions-of-interest (ROI) delimitating each single transfected cell. Experiments were performed in triplicates and at least 6 cells for fields were analyzed in each experiment.

2.7. Immunofluorescence

HuH7, IHH and H441 cells were seeded on slides placed in each well of 6-wells plates at density of 7.4×10^3 cells/cm². The day after, cells were fixed in 4% paraformaldehyde for 20 min at RT, washed twice with PBS, incubated with glycine 0.1 M and washed twice with PBS. Before primary antibody incubation, cells on slides were saturated in 1% BSA/PBS and then incubated overnight in a humid chamber in dark with a specific primary antibody targeting the ASGPR at 1:100 dilution (Mouse anti-ASGPR antibody, Santa Cruz Biotechnology). After incubation, cells were washed twice with PBS and then incubated with secondary antibody (anti-mouse Alexa Fluor 488, 1:500 dilution) for 50 min at RT in a humid chamber in dark. Finally, cells were stained with DAPI for 5 min at RT and mounted using Moviol Mounted solution. Images were acquired using Carl Zeiss Laser Scanning Confocal Microscopy LSM800 Zeiss Airyscan equipped with a 63×, NA = 1.3 objective.

2.8. HuH7-EGFP stable cell line generation

The HuH7-EGFP stable cell line was obtained transfecting the 293 T cells by pLenti PGK GFP Puro (w509-5) (a gift from Eric Campeau & Paul Kaufman (Addgene plasmid #19070; <http://n2t.net/addgene:19070>;

RRID:Addgene_19,070))³⁹⁸, pMDLg/pRRE (a gift from Didier Trono (Addgene plasmid #12251))³⁹⁹, pRSV-Rev (a gift from Didier Trono (Addgene plasmid #12253))³⁹⁹ and pMD2.G (a gift from Didier Trono (Addgene plasmid #12259)) plasmids. 293 T cells were plated at the density of 1.3×10^5 cells/cm² in 10 ml of medium in 100 mm plate and after 12–16 h the medium was replaced with 8.0 ml of fresh DMEM complete medium (without antibiotic). For each plate, a mix containing all plasmids and Lipofectamine 2000 in Optimem without serum and antibiotics was added dropwise onto the medium in each dish and incubated for 6 h. After 14–16 h the medium was replaced with 5 ml of complete DMEM for the lentivirus collection. Viral particles were harvested after 24 h from 293 T cells by supernatant centrifugation at 3000 rpm for 10 min at RT and then filtered by a 0.45 µm filter (Millipore, Bedford, MA) to remove cell debris.

For the HuH7 infection, cells were seeded at the density of 1.8×10^4 cells/cm² in 10 ml of medium in 100 mm plate. The day after, the medium was removed, cells were washed with PBS and 5 ml of supernatant containing viral particles were added. After 5–6 h, 5 ml of fresh medium was added. Two days after infection, the medium was replaced with fresh medium and 5 µg/ml of Puromycin (Puromycin dihydrochloride from *Streptomyces alboniger*, Catalog Number P8833, Sigma-Aldrich®) were added for the transduced cells selection. In order to select cells with similar fluorescence intensity, HuH7-EGFP cells were sorted using the FACS Calibur cytofluorimeter (BD Biosciences).

2.9. Cell viability, necrosis, autophagy and apoptosis

Cell viability, evaluated by MTT test, was performed as described [28] seeding HuH7 or IHH at the density of 5×10^3 cells/cm² in 24-wells plates. Cell necrosis was evaluated by lactate dehydrogenase (LDH) assay kit according to manufacturer instructions (BioVision Prod., Mountain View CA) and as described [28]. Apoptosis and autophagy were performed evaluating PARP (Poly (ADP- ribose) polymerase) cleavage and LC3B forms as previously described [28–30].

2.10. Western blotting

Protein extraction was performed as described [22]. Briefly, 30/40 µg of protein extract were resolved onto 15% (LC3B), 12% (eEF1A1, eEF1A2 and E2F1) or 8% (PARP) SDS- PAGE and blotted onto a 0.22 mm nitrocellulose membrane (Schleicher & Schuell, Keene, NH). The rabbit monoclonal antibody anti-eEF1A1 (AbCam, dilution 1:1000), the rabbit polyclonal antibody anti-eEF1A2 (Santa Cruz, dilution 1:1000), the rabbit polyclonal antibody anti E2F1 (Santa Cruz, dilution 1:100), the rabbit polyclonal antibody anti-LC3B (Genetex, dilution 1:1000) and the mouse monoclonal anti-PARP (BD Pharmingen, dilution 1:1000) were used. On the same membrane, the loading control protein GAPDH (Santa Cruz, dilution 1:1000) was probed. Blots were developed using the corresponding secondary horseradish peroxidase antibodies (Santa Cruz,) by enhanced chemi-luminescence detection system (Pierce) and exposed to Kodak film (Sigma-Aldrich). Particular care was taken to appropriately developing Kodak film in order to generate non-saturated bands. Band intensities were measured by the densitometer (Model GS-700 Imaging Densitometer, Bio-Rad) equipped with the Molecular analyst software (Bio-Rad).

2.11. QRT-PCR and digital droplet PCR

Total RNA was extracted, quantified and the quality evaluated as reported [22]. Reverse transcription was performed using 1 µg of total RNA in the presence of random hexamers and MuLV reverse transcriptase (Applera Corporation). The primers (Eurofins Genomics, 300 nM) and the Real-Time amplification conditions for eEF1A1 [31], for E2F1 [28] and for 28S RNA [32] have been already highlighted. EGFP mRNA reverse transcription was performed as reported for the other mRNA targets using the EGFP reverse primer: 5'- TGC CGT CGT CCT TGA AGA-

3' (Eurofins Genomics). Real time amplification consisted of 40 cycles (denaturation 95 °C for 15 s, annealing 60 °C for 60 s and extension 72 °C for 30 s), using the primers EGFP fw: 5'-TAC GGC AAG CTG ACC CTG-3' and EGFP rev: 5'- TGC CGT CGT CCT TGA AGA-3' (Eurofins Genomics, 300 nM). Amplification cycles were preceded by a pre-denaturation step at 95 °C for 10 min and followed by a final extension step at 72 °C for 10 min. The relative amounts of the target mRNA were normalized by 28S rRNA content.

Absolute quantification of mRNA levels of eEF1A2 were determined using the QX200 Droplet Digital PCR System (ddPCR) due to the higher precision and sensitivity of the ddPCR. This was very useful for the very low abundance of this transcript in the cells. The reverse transcription was performed using the iScript™ gDNA Clear cDNA Synthesis Kit (Bio-Rad) to avoid genomic DNA contamination and the cDNA amplification was performed using expression primers probe assay for eEF1A2 FAM (200 nM) (Bio-Rad) and GAPDH HEX (200 nM) (Bio-Rad). Then, the fluorescence of the plate containing the samples was read in the QX200™ Droplet Reader machine; data were normalized to GAPDH mRNA content and analyzed using the QuantaSoft™ (Bio-Rad) software.

2.12. In vivo studies

Mice were kept in quarantine for 2 weeks before the experiment. Specific-pathogen-free housing conditions were provided throughout the period of quarantine and experiment. All *in vivo* experiments were performed following the guidelines of the EU directive (2010/63/EU) and the permission from the Veterinary Administration of the Ministry of Agriculture, Forestry and Food of the Republic of Slovenia (permission no. U34401-16/2017/9).

The dorsal skinfold window chamber model was performed for *in vivo* uptake studies as described [33]. The most superficial layer of a small area of the mouse skin was removed. Subsequently, tumors were induced in female Severe Combined Immunodeficiency (*Prkdc^{scid}*) mice (Envigo, Udine, Italy) by injecting a solution of 5 µl of 1×10^6 HuH7-EGFP cells re-suspended in saline mixed with 5 µl of Matrigel (Corning® Matrigel® Basement Membrane Matrix Phenol Red Free) into the dermis of intact skin in the dorsal window chamber. The area of implant was then covered by a fixed glass cover slip. Six days after surgery, mice were randomly divided into experimental group and treated by intra-tumor injection of PDPG/PDP-siEGFP (13,5 µg siRNA, weight ratio 1:1 in 10 µl final). Bright-field and fluorescent images of tumors growing in window chambers were taken on day 0, day 1, day 4 and day 7 using a Carl Zeiss SteREOLumar V12 (Carl Zeiss, Jena, Germany) fluorescence stereomicroscope equipped with a Neo-Lumar S 0.8× objective (Carl Zeiss) and a AxioCam MRc5 digital camera (Carl Zeiss). During image acquisition, animals were subjected to isoflurane inhalation anesthesia and placed on a plexiglas custom-designed holder, so that the mice rested in a lateral position during imaging. Images from fluorescence stereomicroscope were analyzed using the FiJi software. On the bright-field images a ROI (Region Of Interest) was drawn around the tumors and overlaid over the fluorescent image of the same tumor. Fluorescent area was separated from the background by adjusting the lower threshold value, and mean fluorescence intensity over the threshold and inside the ROI was measured. The limits of the tumors were drawn at day 0 in two dimensions. Since these tumors were growing inside the window chamber, the third dimension (height) of the tumor is basically the same for all the tumors. Therefore, 2D ROI was chosen as sufficient for this type of experiments. Finally, the mean fluorescence intensity measured at each day was normalized to the mean fluorescence intensity at day 0. The window chamber model above described, was undertaken for the possibility to follow over time in a single animal the effect of the siRNA on the expression of EGFP in HuH7-EGFP. We could therefore dramatically reduce the number of animals needed down to 1/3 (one animal for time points 1, 4 and 7 days per repetition, instead of three animals for each time point per repetition).

Two types of *in vivo* functional experiments were performed, *i.e.* *long-*

term and *short-term experiments*. In both experiments, tumors were induced in the right flanks of female SCID mice by a subcutaneous injection of 10×10^6 of HuH7 cells, re-suspended in 0.1 mL of saline solution. The size of the tumor was measured in the three perpendicular tumor diameters every second day using the digital Vernier caliper. Tumor volume was calculated using the formula for ellipsoid $V = a \times b \times c \times \pi/6$, where a, b and c represent the three tumor diameters. When tumors reached the volume size around 30–40 mm³, mice were randomly divided into different groups and subjected to specific experimental protocols (6 animals in siA1 + PDPG, siA2 + PDPG, siE2F1 + PDPG and siGL2 + PDPG groups, 4 animals in PDPG and NaCl 0,9% groups). Fifty µl of solution containing 150 µg of specific siRNAs or siGL2 were incubated with 150 µg of PDPG for 20 min at room temperature and then injected intratumorally. This dosage was chosen based on our previous work [30]. In this last case we used 300 µg of siA1 delivered by lipofectamine to subcutaneous chronic lymphatic leukemia tumors with a starting volume of about 250 mm³. Considering that in the present work, the tumor starting volume is of about 40 mm³ and that in a preliminary experiment (data not included in the manuscript) the use of 90 µg of siRNAs did not result in an evident effect on tumor mass, we decided to increase the amount to 150 µg. As a control, 50 µl of saline (NaCl 0,9%) was injected. Tumors were measured every second day to draw a tumor growth curve. Mice were humanely sacrificed by CO₂ when tumor volume reached approximately 300 mm³ (*long-term experiment*) or three days after treatments injections (*short-term experiment*, 5 animals in siA1 + PDPG, siA2 + PDPG, siE2F1 + PDPG groups and 4 in the siGL2 + PDPG group). Tumor growth delay was calculated using the formula: $GD = \overline{DT}_{tr} - \overline{DT}_{untr}$; where DT_{tr} and DT_{untr} correspond to the average doubling times of the treated or untreated tumors, respectively (*long-term experiment*). RNA was extract from tumors of the short-term experiment using the AllPrep DNA/RNA/Protein Mini Kit (QIAGEN®). In all experiments mice were weighed every second day.

Tumors collected in the short-term experiment were fixed in zinc fixative (BD Biosciences, Franklin Lakes, NJ, USA) for 1 day and then stored in 70% ethanol until embedding in paraffin. In the direction perpendicular to the skin layers, 5-µm thick sections were cut and stained with hematoxylin and eosin. Images of the whole tumor sections were taken with BX51 optical microscope equipped with a DP70 CCD digital camera (Olympus) under 32× magnification. The percentage of cell death was estimated by 3 independent observers.

2.13. Mathematical modelling of siRNA effects on tumor growth in vivo

To finely describe the effects of the delivered siRNAs, we developed a mathematical model based on the classical idea [34] that, in the condition of unperturbed tumor expansion (*i.e.* in the absence of antitumor drugs and/or any problem linked to tumor cells feeding), tumor volume (and, thus, the number of tumor cells) increases according to an exponential evolution. To account for the presence of external perturbing agents such as anti-tumor drugs, it is supposed that the fraction (*f*) of cells that can undergo division depends on the time (*t*) according to the following exponential law:

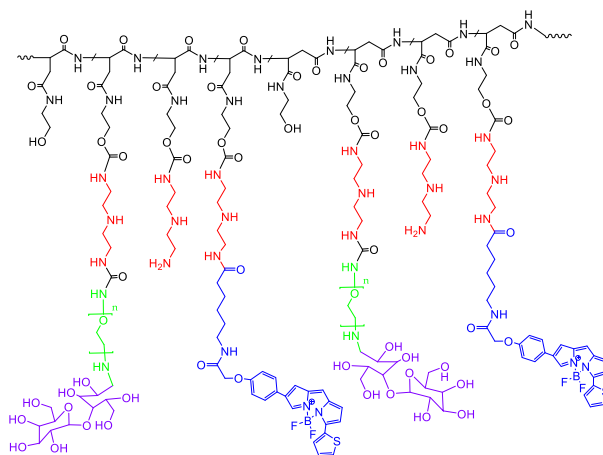
$$f = (f_0 - f_\infty)e^{-k_t t} + f_\infty \quad (1)$$

where f_0 and f_∞ are, respectively, the initial ($t = 0$) and the final ($t \rightarrow \infty$) fraction of cells that undergo division and k_t is a kinetic constant connected to the kinetics of *f* decrease. Eq. (1) simply represents the physiological condition of a time independent *f* (when $f_0 = f_\infty$) or an exponentially decreasing *f* from f_0 to f_∞ . Thus, it can be viewed as a correcting factor for the classical kinetics equation describing the time increase of the tumor cells number *N*:

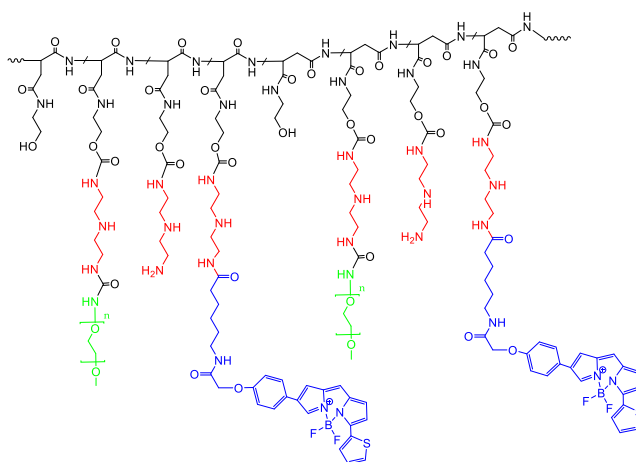
$$\frac{dN}{dt} = k_D N ((f_0 - f_\infty)e^{-k_t t} + f_\infty) = V_D \quad (2)$$

where k_D is the kinetic constant connected to cells division speed V_D

A PHEA-DETA-Bpy-PEG-GAL



B PHEA-DETA-Bpy-PEG



C PHEA-DETA-Bpy

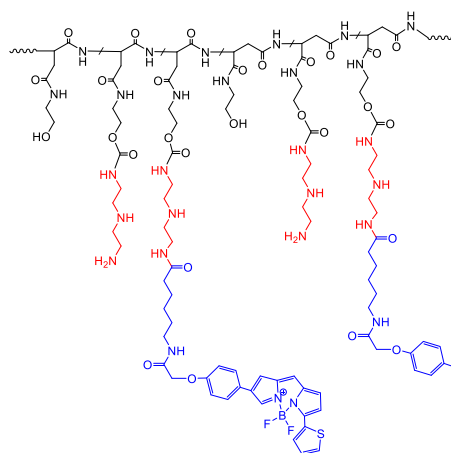


Fig. 1. Chemical structures of the copolymers BODIPY TR-X conjugated.

In black, red, green, violet and blue are indicated PHEA, DETA, PEG, GAL and BODIPY TR-X, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1

Weight-average molecular weight ($\overline{M}_w$), polydispersity index ($\overline{M}_w/\overline{M}_n$), and chemical composition of obtained copolymers.

Copolymers	Molecular weight		Degree of derivatization (DD)			
	$\overline{M}_w$ (g/mol)	$\overline{M}_w/\overline{M}_n$	DETA ^a	Bpy ^b	PEG ^a	GAL ^c
PHEA	38,700	1.6	–	–	–	–
PHEA-g-DETA	30,800	1.5	19.3	–	–	–
PHEA-g-DETA-g-PEG-GAL	38,000	1.4	19.3	–	3.6	3.6
PHEA-g-DETA-g-PEG-OCH ₃	34,200	1.4	19.3	–	2.2	–
PHEA-g-DETA-g-Bpy	31,400	1.3	19.3	1.6	–	–
PHEA-g-DETA-g-Bpy-g-PEG-GAL	39,200	1.4	19.3	1.6	3.8	3.7
PHEA-g-DETA-g-Bpy-g-PEG-OCH ₃	34,800	1.4	19.3	1.6	2.2	–

^a Quantification by ¹H NMR analysis (SD = 0.5).

^b Quantification by UV analysis (SD = 0.3).

^c Quantification by colorimetric assays (SD = 0.4).

(cells/time). Eq. (2) analytical solution is:

$$N = N_0 \exp \left(k_D \left((f_0 - f_\infty) \left(\frac{1 - e^{-k_f t}}{k_f} \right) + f_\infty t \right) \right) \quad (3)$$

Eq. (3) is mathematically very powerful as it can describe the entire *in vivo* tumor evolution composed by an initial exponential growth, followed by a linear zone reaching, eventually, a plateau [35]. Interestingly, when f_∞ assumes negative values, Eq. (2) leads to negative V_D values so that Eq. (3) predicts that the initial N increase is followed by a subsequent decrease. This model lies inside the Gompertzian kinetics [36].

2.14. Blood chemistry

The peripheral blood was collected in EDTA-treated tubes (BD Microtainer® MAP) from orbital sinus of mice (*short-term experiment*) and stored at room temperature for approximately 30 min. Serum was isolated by centrifuging blood samples at 3000 rpm for 5 min and stored at -20°C until analysis. Samples analysis was performed at the Veterinary faculty University of Ljubljana using ADVIA 120 Hematology System (Siemens Healthineers, Erlangen, Germany). C reactive protein (PCR) levels were analyzed using the ELISA kit (Abcam® ab157712-C Reactive Protein (PTX-1) Mouse ELISA kit). The measured values were compared to reference normal ranges.

2.15. Statistical analysis

All data are expressed as mean $\pm$ SEM and they were tested for normality of distribution with the Kolmogorov–Smirnov test. P values were calculated by the GraphPad InStat tools (Graph-Pad Software, Inc., La Jolla, CA, USA) using unpaired t -test with or without Welch correction and the Mann-Whitney Test, Wilcoxon matched-pairs signed-ranks test, as appropriate. P values <0.05 were considered statistically significant.

3. Results

3.1. Synthesis and characterization of PHEA-g-DETA-g-PEG-GAL and PHEA-g-DETA-g-Bpy-g-PEG-GAL graft copolymer

The novel graft copolymer developed in this work is composed of α,β -poly(N-2-hydroxyethyl)-D,L-aspartamide (PHEA), bearing diethylene triamine (DETA), the fluorescent NIR probe BODIPY™ TR-X (Bpy) and galactosylated polyethylene glycol (PEG) chains (PHEA-g-DETA-g-

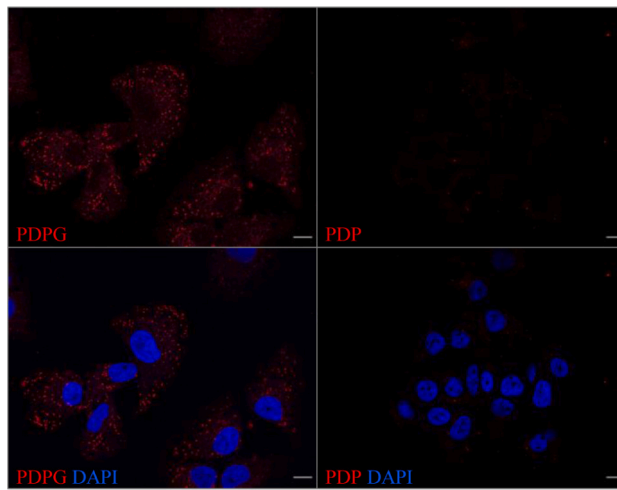
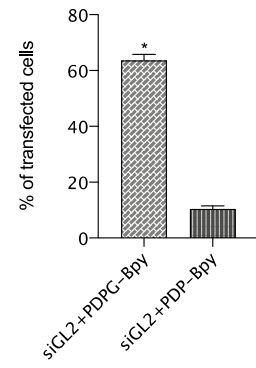
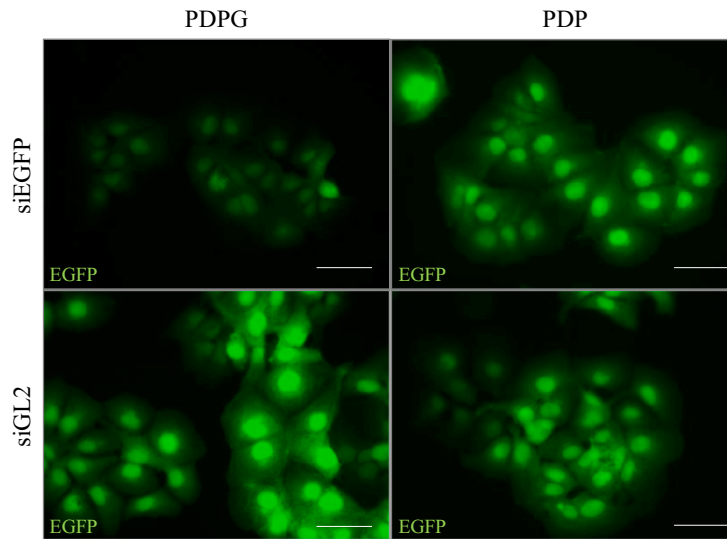
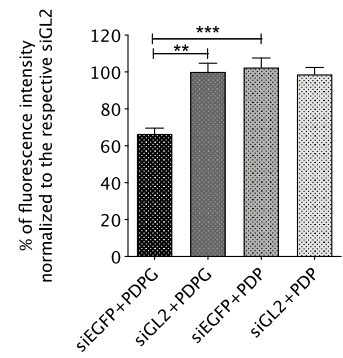
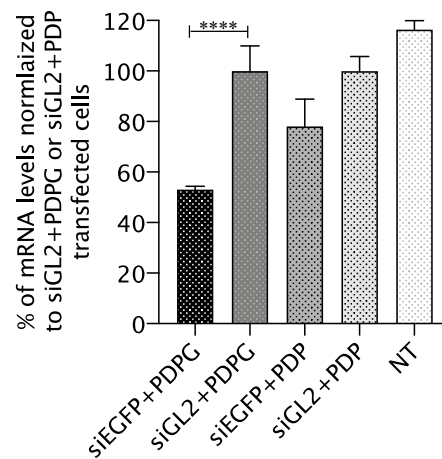
Bpy-g-PEG-GAL, Fig. 1A) [14,23]. The functionalization of the repeating units of PHEA with DETA allowed to introduce a permanently conjugated positive charge to the polymeric backbone, conferring to the resulting material the potential to form stable polyplexes with the negative-charged siRNAs, being DETA protonated at physiological pH [14]. Bpy was added to have the possibility to follow the *in vitro* and *in vivo* the copolymer fate. To verify whether the binding of the Bpy probe to the polymer modifies the probe absorbance, *i.e.* causes a reduction in the molar extinction coefficient or the disappearance of the fluorescence, the molar extinction coefficient (ϵ) of the PHEA-g-DETA-g-Bpy copolymer was compared to Bpy alone. While for the free Bpy ϵ is equal to $68,000\text{ M}^{-1}\text{ cm}^{-1}$ (at a maxima λ of 588 nm), for PHEA-g-DETA-g-Bpy it is of $53,600\text{ M}^{-1}\text{ cm}^{-1}$ (at a maxima λ of 592 nm). This demonstrates that the probe bound to the copolymer maintains a high fluorescence capacity, equal to about 78% of the free one. Moreover, from UV analysis and from the ϵ value of the molar extinction coefficient of the probe, it was possible to determine that the degree of derivatization in Bpy (DD_{Bpy}) was equal to $1.58 \pm 0.3\text{ wt\%}$ (Table 1). In particular, the absorbance value of a known concentration of copolymer solution was compared to the straight line equation determined graphically by plotting the absorbance values of the free Bpy as a function of Bpy aqueous concentrations. Galactose (GAL) residue was introduced to allow the targeting to ASGPR, overexpressed in HCC cells [37]. Both PHEA-g-DETA-g-Bpy-g-PEG-GAL (PDPG-Bpy) (Fig. 1A) and PHEA-g-DETA-g-Bpy-g-PEG (PDP-Bpy) (Fig. 1B) copolymers were prepared starting from PHEA-g-DETA-g-Bpy (Fig. 1C). In parallel to the Bpy-conjugated copolymers (used in uptake studies), Bpy non-conjugated copolymers (PDPG and PDP) were prepared for functional studies. The difference between the pegylation degree (DD_{PEG}) among the galactosylated copolymers (PDPG-Bpy and PDPG) and the not-galactosylated ones (PDP-Bpy and PDP) resulted to be statistically significant although the reaction parameters and experimental conditions were the same. This result could be explained with a better capability of the H₂N-PEG-GAL than the H₂N-PEG-OCH₃ to react with the pending amines on PHEA-g-DETA-g-Bpy or PHEA-g-DETA copolymers, probably due to a conformational difference due to the GAL moiety.

For all the copolymers, $\overline{M}_w$ and the $\overline{M}_w/\overline{M}_n$ values were determined by SEC analysis (Table 1). As expected, the reaction of PHEA with amine causes a significant reduction in $\overline{M}_w$, while the copolymers obtained with the subsequent functionalizations show $\overline{M}_w$ values in accordance with the amount of actually bounded PEG [25]. However, the use of materials with low $\overline{M}_w$ is often preferred as polymeric carriers for complexing genetic material [38].

The siRNA binding efficiency to the copolymer was estimated to be of 20% at a siRNA to polymer weight ratio of 5 (Suppl mat 1B). Despite this, the R used in this work is of $R = 1$. This decision is based on our previous investigation [14] where we observed that at R higher than 1, the effects on cell viability were progressively lost. We think that at R higher than 1, a too tight binding between the siRNA and the polyplexes occurs thus disfavoring siRNA release from the carrier when in the cell. Finally, size, zeta potential, and polydispersity index for the generated copolymers, already determined in [14], are reported in Suppl mat 1D together with TEM images (Suppl mat 1C) that show particles with a grossly spherical shape.

3.2. In vitro uptake of a model siRNA

PDPG-Bpy was employed to study the uptake in HuH7, a HCC cell line suitable for *in vivo* tests. PDP-Bpy, lacking the GAL residue, was used as control. Both copolymers were complexed with the model siRNA siGL2 (targeting the luciferase mRNA, not expressed in HuH7) to generate the complexes siGL2 + PDPG-Bpy and siGL2 + PDP-Bpy. Optimal time point to detect the red fluorescence into the cells by confocal microscopy was of 4 h. Whereas siGL2 + PDPG-Bpy was clearly visible in HuH7 cytoplasm, siGL2 + PDP-Bpy was barely detectable

A**B****C****D****E**

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Fig. 2. siRNA uptake *in vitro*.

A) Representative confocal images of HuH7 transfected by siGL2 + PDPG-Bpy or siGL2 + PDP-Bpy taken 4 h after transfection. Scale bar: 10 μ m. Nuclei have been labelled by DAPI (blue). **B)** Cumulative data reporting the % of transfected cells by siGL2 + PDPG-Bpy or siGL2 + PDP-Bpy in HuH7 cells. Data are shown as mean $\pm$ SEM. $^{*}p = 0.0001$ compared to siGL2 + PDP-Bpy; $n = 4$. **C)** Representative images of HuH7-EGFP transfected by siEGFP/siGL2 + PDPG or siEGFP/siGL2 + PDP taken 48 h after transfection, scale bar: 50 μ m **D)** Cumulative data reporting the % of fluorescence intensity normalized to the respective siGL2 transfections. Data are shown as mean $\pm$ SEM. $^{**}p = 0.0001$ compared to siGL2 + PDPG transfected cells; $^{*}p = 0.0001$ compared to siEGFP+PDP transfected cells; $n = 3$. **E)** EGFP mRNA levels following siEGFP+PDPG or siEGFP+PDP transfection compared to the respective siGL2 + PDPG or siEGFP/PDP transfected cells. EGFP mRNA data, normalized to 28S rRNA levels, are expressed as the % of the respective siGL2 + PDPG/PDP-transfected cells and are shown as mean $\pm$ SEM; $^{****}p < 0.002$ compared to control siGL2 + PDPG transfected cells; $n = 3$. NT = non treated cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Fig. 2A). A quantitative analysis of polymer entry into the cells revealed that siGL2 + PDPG-Bpy was visible in about 65% of the HuH7; in contrast, only 10% of HuH7 were stained by siGL2 + PDP-Bpy (Fig. 2B).

3.3. *In vitro uptake of a functional siRNA*

To prove that PDPG can effectively deliver a functional siRNA, we delivered a siRNA (siEGFP) directed against the enhanced green fluorescence protein (EGFP) to HuH7 stably expressing EGFP (HuH7-EGFP). A decrease in the fluorescence intensity 48 h after siEGFP+PDPG transfection was observed compared to the control siGL2 + PDPG-transfected HuH7-EGFP (Fig. 2C and 2D). In contrast, no significant difference in the fluorescence intensity was observed in HuH7-EGFP following the transfection by either siEGFP+PDP or siGL2 + PDP. As a further prove of siEGFP+PDPG effectiveness, EGFP mRNA was quantitated resulting in about 50% expression down regulation of EGFP compared to the control siGL2 + PDPG (Fig. 2E).

3.4. *Functional role of ASGPR in PDPG uptake*

To prove the functional involvement of ASGPR in the uptake process of PDPG, the interaction ASGPR/PDPG was explored. To this end, HuH7-EGFP were treated by EGTA (5 mM), 10 min before the transfection. EGTA impairs ASGPR activity by chelating calcium ions, which are essential for receptor functionality [39]. EGTA pretreatment prevented siEGFP-PDPG effect (Fig. 3A and B upper panels). Notably, the effect of siEGFP+PDP was invariably modest and not modified by EGTA (Fig. 3A and B middle panels). Additionally, EGTA pretreatment did not affect siEGFP released by lipofectamine, a delivery agent whose uptake mechanism is independent from ASGPR (Fig. 3A and B lower panels). In line with these findings, EGTA pretreatment prevented siGL2 + PDPG-Bpy entry into HuH7 (Suppl mat 2A).

To further proving ASGPR involvement in PDPG uptake, co-localization experiments were conducted. As ASGPR is rapidly internalized after ligand binding and as the turnover of the ASGPR on hepatic cell membranes is around 20 min [39], we have chosen short time points for the analysis, i.e. 5 min (Fig. 3D) and 1 h (Fig. 3C and D). The calculation of Manders' coefficient, which reflects the overlapping between the red (siGL2 + PDPG-Bpy) and green (ASGPR) fluorescence, revealed a remarkable degree of co-localization. Notably, significantly less co-localization was detected in HuH7 cells transfected by siGL2 + PDP-Bpy (Fig. 3D). These results further support the ASGPR-dependent uptake of PDPG.

3.5. *In vitro functional studies: Effect on cell viability*

Following the demonstration of the effective and specific (ASGPR dependent) uptake of PDPG in cultured HuH7, we tested the effects of the delivery of three siRNAs targeted against the mRNAs of eEF1A1 (siA1), eEF1A2 (siA2) and E2F1 (siE2F1). These genes are potentially valuable targets for HCC treatments, since they are overexpressed in HCC and their expression down-regulation can impair HCC tumor cells growth [16–22]. An evident decrease in HuH7 cell viability was observed three days from the transfection by siA1/siA2/siE2F1 + PDPG, compared to the control siGL2 + PDPG transfected cells (Fig. 4A left).

The effect was dose (Suppl mat 2B) and time dependent being reduced at day 6 and substantially not detectable at day 10 from transfection. Notably, the addition of serum in the transfection medium did not substantially affect transfection efficacy (Fig. 4A right), as evaluated using siA1 + PDPG, randomly selected within the three siRNAs considered to test serum effect on transfection. The same siA1 was chosen to test PDP (lacking the GAL moiety) and PD (polymer lacking both PEG and GAL residues) transfection ability. Our data indicate only a minor effect at day 6 for siA1-PDP and no effects of siA1-PD (Fig. 4B left and right, respectively). Notably, the addition of free galactose to HuH7 before transfection by siA1 + PDPG, abrogated the down regulation of cell vitality (Fig. 4C); this suggests that the free galactose saturated ASGPR preventing siA1 + PDPG entry into the cells. Moreover, in IHH, a model of normal hepatocytes [40], the effect of siA1/siA2/siE2F1 + PDPG was very modest (Fig. 4D). This most likely depends on the reduced expression level of ASGPR (Suppl mat 2C/D and [14]) compared to HuH7. Finally, in HuH7, the viability data of siA1/siA2/siE2F1 + PDPG were confirmed by cell counting (Fig. 4E).

We have previous evidences that the siRNAs here considered can down regulate HuH7 proliferation [18]. Thus, here we explored the possible contribution of cell death evaluating necrosis, apoptosis and autophagy. Our data show that neither relevant necrosis (Suppl. mat 3A) nor apoptosis (data not shown) were elicited by our siRNAs-PDPG. In contrast, significant autophagy induction was observed for siE2F1 + PDPG and siA2 + PDPG (Suppl. mat 3B), according to the autophagy marker LC3B; for siA1 + PDPG a tendency towards autophagy induction was detected (Suppl. mat 3B).

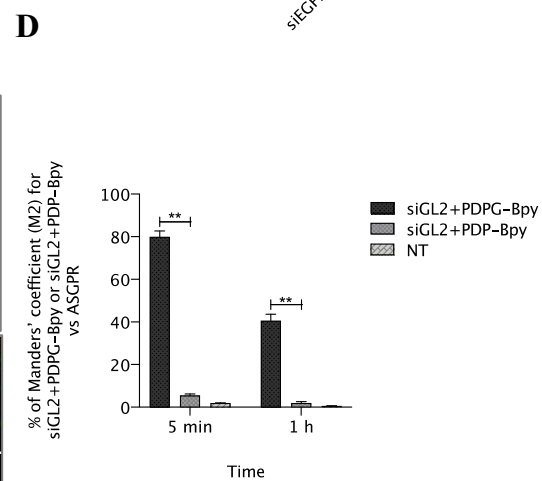
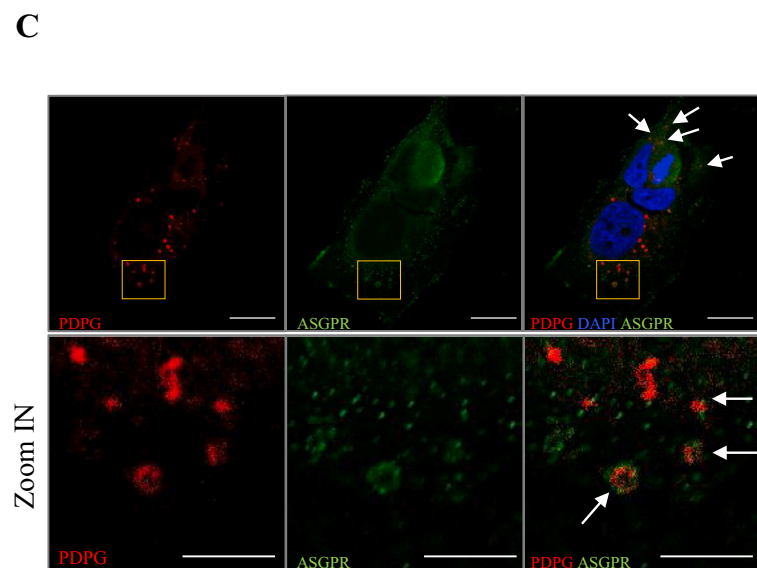
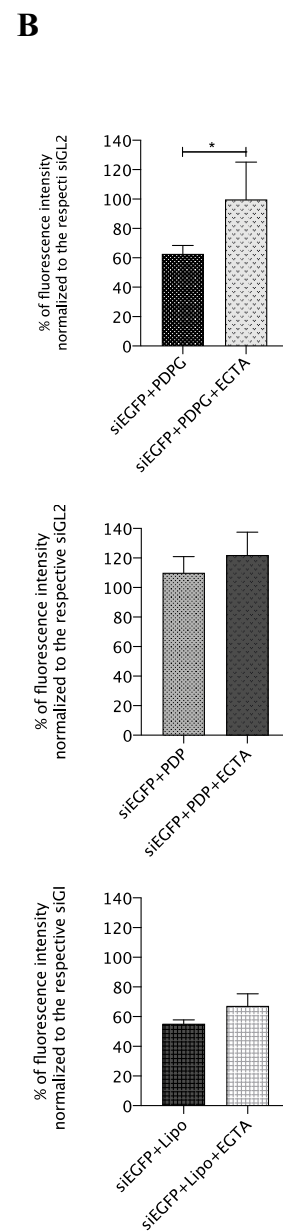
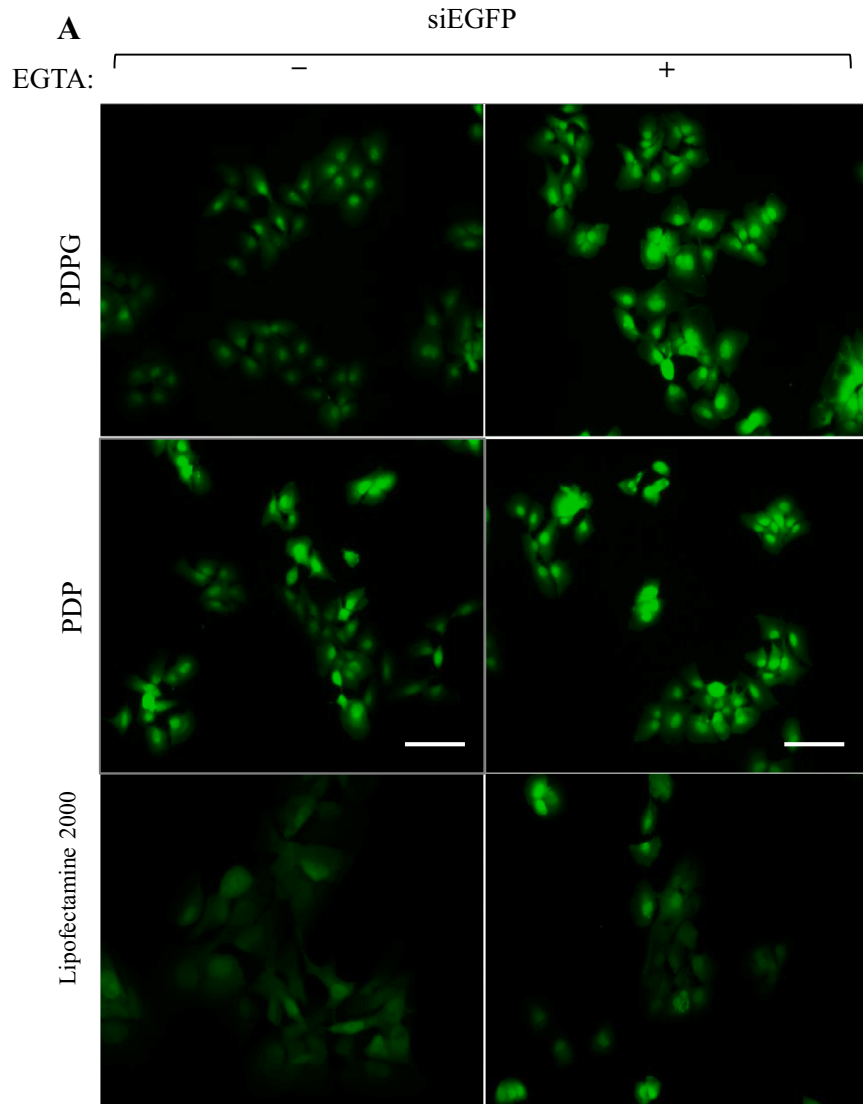
3.6. *In vitro functional studies: Effect on target gene expression*

To prove the specific action of siA1/siA2/siE2F1 + PDPG, the mRNA levels of the respective targets were evaluated three days post transfection in HuH7, based on the observed effects on cell viability. A decrease of around 80%, 40% and 60% was observed for eEF1A1, eEF1A2 and E2F1 mRNA levels, respectively (Fig. 5A-C). Conversely, siA1 + PDP had only a marginal effect on the target mRNA level (Fig. 5D). The effects on the protein levels (Fig. 5E-H) where in line with the mRNA data, even if protein decrease for siA1-siA2-siE2F1 + PDPG treatments was more contained compared to the decrease of the respective mRNA levels.

3.7. *In vivo uptake*

The promising results obtained in *in vitro* experiments, prompted us to investigate the effectiveness of PDPG also *in vivo*. For this purpose, the dorsal skinfold window chamber experiment was undertaken [33]. The test allows to follow the growth and the fluorescence intensity of HuH7-EGFP xenografts growing inside a window chamber over time by bright-field and fluorescence microscopy.

To minimize animal discomfort due to the window chamber, six days after HuH7-EGFP inoculation was chosen as the time point to start the intra-tumor injection of siEGFP+PDPG or siEGFP+PDP. Fluorescence images were acquired on the day of the treatment (day 0) and on day 1, day 4 and day 7. Although the fluorescence intensity was somewhat decreased also in tumor treated by siEGFP+PDP, our data demonstrate



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Fig. 3. Functional role of ASGPR in PDPG uptake.

A) Representative images of HuH7-EGFP transfected by siEGFP/siGL2 + PDPG, siEGFP/siGL2 + PDP or siEGFP/siGL2 + Lipofectamine 2000 48 h after transfection with or without EGTA pre-treatment, scale bar: 50 μ m. **B)** Cumulative data reporting the % of fluorescence intensity normalized to the respective siGL2 treatment. Data are expressed as mean $\pm$ SEM. $*p = 0.0006$ compared to siEGFP+PDPG; $n = 3$. **C)** Confocal images showing the co-localization of PDPG-Bpy (red) and ASGPR (green) following siGL2 + PDPG-Bpy transfection. Scale bar = 10 μ m; arrows indicate representative co-localization regions. **D)** Manders' coefficient for siGL2 + PDPG-Bpy or siGL2 + PDP-Bpy vs ASGPR co-localization $**p < 0.0001$ compared to siGL2 + PDP, $n = 12$.

that siEGFP+PDPG was always superior to siEGFP+PDP in reducing green fluorescence (Fig. 6A, 6C and Suppl. mat 3C). The green fluorescence decrease was not due to a reduction of the tumor mass, as the tumor size remained rather constant throughout the experimental time, as evaluated by bright field microscopy (Fig. 6B). These data demonstrate the superiority of PDPG vs PDP to effectively target siEGFP to HuH7-EGFP cells *in vivo*.

3.8. *In vivo* functional studies: Effects on tumor growth

The very promising uptake data obtained *in vivo*, prompted us to test *in vivo* the effectiveness of our HCC-related siRNAs, *i.e.* siA1, siA2 and siE2F1, in analogy to the *in vitro* tests. To this end, a xenograft subcutaneous mouse model of HCC using HuH7 was employed. Due to the targeting ability proved for PDPG vs PDP *in vitro/vivo* (Figs. 2–6) and to fulfill the 3Rs rule, in the functional *in vivo* test the control PDP treated group was omitted. The results (Fig. 7A) showed an evident slowdown of the tumor growth in mice treated by siA1/siA2/siE2F1 + PDPG compared to the control siGL2 + PDPG, PDPG and NaCl treated animals (*long term experiment*). Notably, the difference between specific siRNAs and controls was evident already at day 1 and was maintained up to day 6–7. The tumor growth delay was approximately three days for siA1 + PDPG, siA2 + PDPG and siE2F1 + PDPG treated mice compared to the control mice (Fig. 7B).

In order to get a deeper insight about the effect of siRNAs+PDPG complexes on tumor growth, we developed a simple, but powerful mathematical model (see material and methods). Eq.(3) fits the experimental data normalized with respect to the initial tumor volume/mass/cells number (Suppl. mat.4), assuming $f_0 = 1$ and $f_\infty = 0$. This equation allows evaluating the ratio V_D/N_0 (number of cells generated per unit time for each one of the original N_0 cells) for the six different systems considered (Fig. 7C). The mathematical model indicates that while controls share similar growth speed constant k_D (average value $\approx (0.41 \pm 0.02 \text{ day}^{-1})$) and similar fraction of growing cells represented by k_f (average value $\approx (0.15 \pm 0.02) \text{ day}^{-1}$), siRNAs+PDPG treated tumors are characterized by a very different average value of k_D and k_f ($k_{D\text{av}} \approx (0.17 \pm 0.02 \text{ day}^{-1})$; $k_{f\text{av}} \approx 0 \text{ day}^{-1}$). In the case of controls the determined constants (k_D and k_f), provide the quantitative indication that the increase of cell number is high in the beginning and tends to decrease after 4–5 days, probably because of a suboptimal cells feeding occurring when tumor volume exceeds a critical threshold. On the contrary, in the case of siRNAs+PDPG treatments, the increase in cell number is much lower because k_D is about one half of that of controls. Consequently, being the cells number (or volume or mass) lower, a fraction of cells maintains the capacity to divide ($k_{f\text{av}} \approx 0 \text{ day}^{-1}$). Notably, the reduced k_D observed in siRNA+PDPG treated tumors may depend on the increase of tumor cell death revealed *in vitro* (Suppl. mat 3B) and to the reduction in cell growth we previously observed [18].

3.9. *In vivo* functional studies: Effect on target gene expression

To prove *in vivo* that siRNA mechanisms of action was dependent on the down regulation of the specific targets, we performed a *short-term experiment* limited to day three post intra tumor injection of siRNAs. This time point was chosen based on the *in vitro* and *in vivo* experiments. A longer time point was not considered as the molecular effects of the siRNAs could have been lost both due to siRNA processing in the cells and to the over-growth of un-transfected cells. As shown in Fig. 8A, the

trend of the tumor growth curve is comparable to that of Fig. 7A. Importantly, siA1-siA2 + PDPG significantly reduced the mRNA levels of the respective target; siE2F1 + PDPG resulted in the clear tendency to E2F1 mRNA reduction (Fig. 8B), in line with the *in vitro* data. Notably, the levels of the target proteins were reduced as well (Fig. 8C). Finally, compared to the control siGL2 + PDPG, siA1-siA2-siE2F1 + PDPG induced increased cells death (Fig. 8D) in tumors in agreement with the *in vitro* data (Suppl. mat 3B).

3.10. *In vivo* functional studies: Evaluation of toxic effects

Finally, we evaluated the possible toxicity of our siRNAs+PDPG complexes. To this end, the markers of liver (albumin, Aspartate transaminases-AST- and Alanine aminotransferases-ALT) and kidney (creatinine) functions were evaluated in mice sacrificed at day three post siRNA injection in the tumor as at this time point the siRNA phenotypic (Fig. 8A) and the molecular effects (Fig. 8B and C) were well evident. Additionally, glucose (marker of glycemic homeostasis) and C-reactive protein (marker of systemic inflammation) levels were measured. For all the markers tested, no significant variations were detected compared to the normal reference values (Fig. 9A-F). These data were also confirmed by the evaluation of the animal body weight, which did not change over the three days of the experiments (Fig. 9G). Notably, comparable results were obtained by monitoring animal weight in the *long-term experiment* (Fig. 9H).

4. Discussion

4.1. Uptake studies

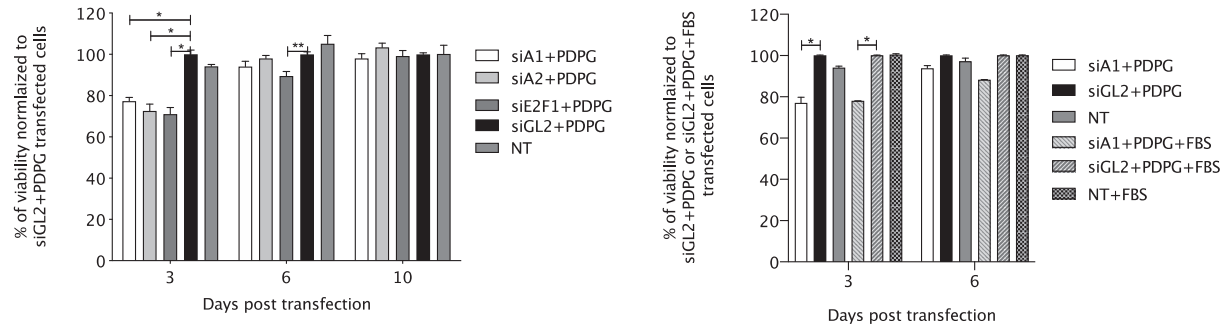
The necessity to have effective and specific therapeutic options for HCC prompted us to explore the effectiveness of HCC specific siRNAs delivered by a novel polymeric carrier (PDPG) we developed. To improve the targeting to HCC cells, PDPG bearing in the side chains GAL residues linked to the distal end of the PEG chain was prepared. PEG molecules are inserted as spacer for GAL group and to improve stealth properties of polyplexes (Fig. 1) [41].

Our data demonstrate an efficient and specific uptake of PDPG in HuH7 cells (Fig. 2A and 2B), a cell model of HCC overexpressing the ASGPR (Suppl mat 2C/D and [14]). Moreover, we show that PDPG can deliver a functional siRNA (siEGFP) to HuH7 as demonstrated by the significant decrease in EGFP fluorescence (Fig. 2C and 2D) and EGFP mRNA levels (Fig. 2E).

The PDPG delivery specificity is related to the presence of the GAL residues for different reasons. First, PDP lacking GAL residues has far less transfection ability compared to PDPG (Fig. 2). Second, EGTA, a Ca^{++} chelating agent that impairs ASGPR activity, reduces the effects of siEGFP+PDPG delivered to HuH7-EGFP (Fig. 3A and B upper panels). Notably this is not the case for siEGFP delivered by lipofectamine (Fig. 3A and B lower panels) whose transfection mechanism does not depend on ASGPR/ Ca^{++} . Third, the treatment of HuH7 by free galactose before siRNA+PDPG transfection abrogates the effect on cell vitality (Fig. 4C), most likely due to ASGPR saturation by free galactose that eventually impairs siRNA+PDPG binding. Fourth, in IHH, undertaken as a model of normal hepatocytes [40], the low ASGPR expression (Suppl. mat 2C/D and [14]), is paralleled by negligible effects on cell vitality following siRNA+PDPG transfection (Fig. 4D). Finally, we have evidences of a co-localization between PDPG and ASGPR (Fig. 3C and D).

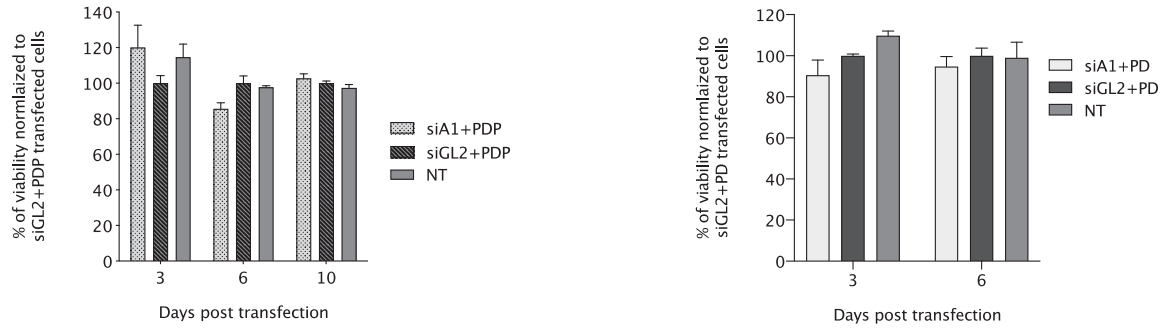
A

Cell viability in HuH7-siRNA/PDPG



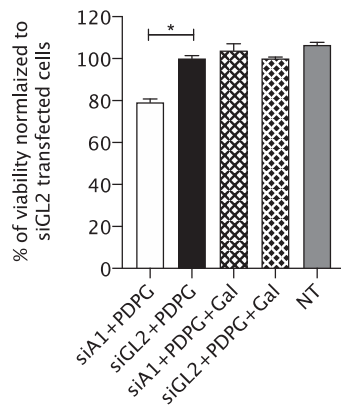
B

Cell viability in HuH7-siRNA/PDP or PD



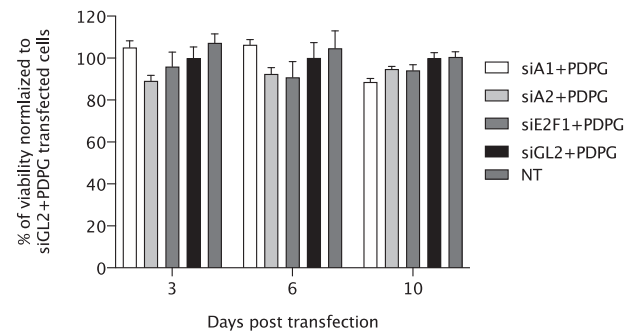
C

Cell viability in HuH7 pretreated by galactose -siRNA/PDPG



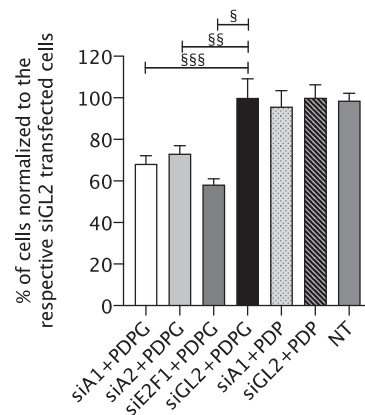
D

Cell viability in IHH-siRNA/PDPG



E

Cell number in HuH7-siRNA/PDPG



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Fig. 4. siRNA effects on cell viability in vitro.

siRNAs targeting either eEF1A1, eEF1A2 or E2F1 mRNAs were delivered by PDPG or PDP in HuH7 cells. Effects on cell viability were evaluated by MTT test at different time points following siRNAs+PDPG (A) transfection performed in the absence (left) or presence of serum (for siA1 + PDPG only, right) compared to the control siGL2 + PDPG transfected cells. B) Similar experiment to (A) but evaluating siA1 + PDP or siA1 + PD effectiveness compared to the respective control siGL2 + PDP or siGL2 + PD transfected cells. Data are expressed as % of viable cells transfected by siGL2 + PDPG/PDP/PD and are shown as mean $\pm$ SEM. * p < 0.0001 and ** p = 0.0008 compared to control siGL2 + PDPG transfected cells; n = 6. C) Effects on cell viability of free galactose administration before siA1-PDPG/PDP transfection. Data are expressed as % of viable cells transfected by siGL2 + PDPG/PDP and are shown as mean $\pm$ SEM. * p < 0.0001 compared to control siGL2 + PDPG transfected cells; n = 6. D) Effects on cell viability in IHH were evaluated by MTT test at different time points following siRNAs+PDPG transfection. Data, expressed as the % of siGL2 + PDPG transfected cells, are shown as mean $\pm$ SEM; n = 6. E) Three days after transfection by siRNAs+PDPG or siA1 + PDP, the variation in the HuH7 cell number was measured. Data, expressed as the % of siGL2 + PDPG/PDP transfected cells, are shown as mean $\pm$ SEM; § p = 0.0049, §§ p = 0.031, §§§ p = 0.016 and compared to the control siGL2 + PDPG transfected cells; n = 6. NT = non-treated cells.

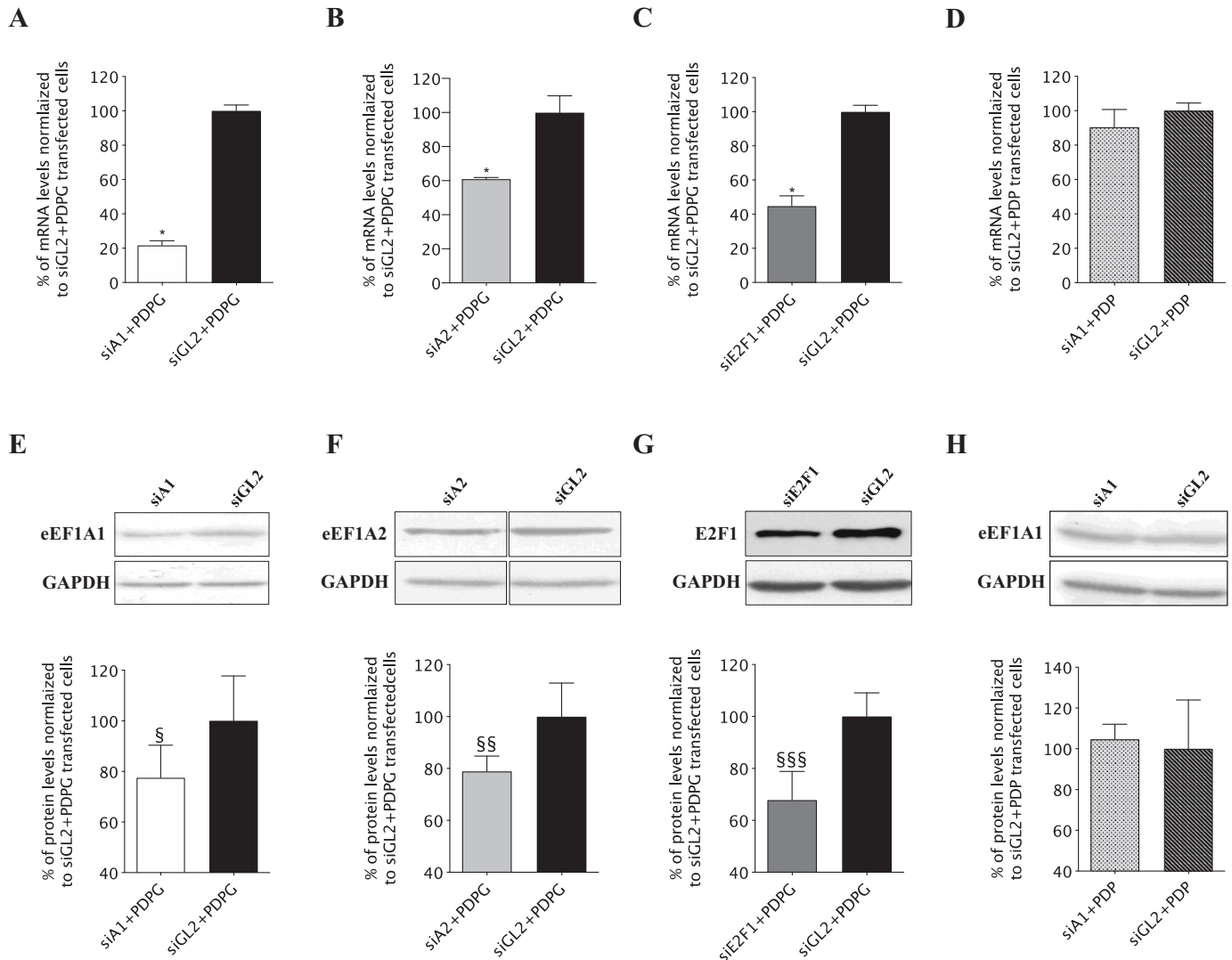


Fig. 5. siRNA effects on target gene expression in vitro.

A-C) Evaluation of the target mRNA levels in HuH7 three days after siA1-siA2-siE2F1 + PDPG transfection, compared to the control transfected cells siGL2 + PDPG. D) eEF1A1 mRNA levels following siA1 + PDP transfection compared to siGL2 + PDP-transfected cells. eEF1A1 and E2F1 mRNA data were normalized to 28S rRNA levels, while eEF1A2 mRNA data were normalized to GAPDH mRNA levels. In A-D data are expressed as the % of the respective siGL2 + PDPG/PDP-transfected cells and are shown as mean $\pm$ SEM; * p < 0.0001 compared to control siGL2 + PDPG transfected cells; n = 6. E-G) Evaluation of the protein levels three days after siA1-siA2-siE2F1 + PDPG transfection in HuH7, compared to the control transfected cells siGL2 + PDPG. H) Evaluation of the protein levels three days after siA1 + PDP transfection compared to siGL2 + PDP-transfected HuH7 cells. In E-H data, normalized to GAPDH levels and expressed as % of the respective siGL2 + PDPG/PDP-transfected cells, are shown as mean $\pm$ SEM; § p = 0.0431, §§ p = 0.0313 and §§§ p = 0.0462 compared to control siGL2 + PDPG transfected cells; n = 6.

Whereas co-localization *per se* does not necessarily demonstrate a physical binding, it is highly suggestive of an interaction PDPG-ASGPR.

The specificity of the uptake is also corroborated by the *in vivo* studies (Fig. 6) where siEGFP+PDPG resulted to be superior to siEGFP+PDP in reducing EGFP expression. Notably, fluorescence

slightly decreased over time also for siEGFP+PDP. This may be because a modest amount of siEGFP+PDP can enter HuH7 cells as observed by the *in vitro* uptake study (Fig. 2A and B). Moreover, functional studies in cultured HuH7 (Fig. 5D) indicate a somewhat decrease of eEF1A1 mRNA following siA1 + PDP treatment. Despite this, our data clearly indicate

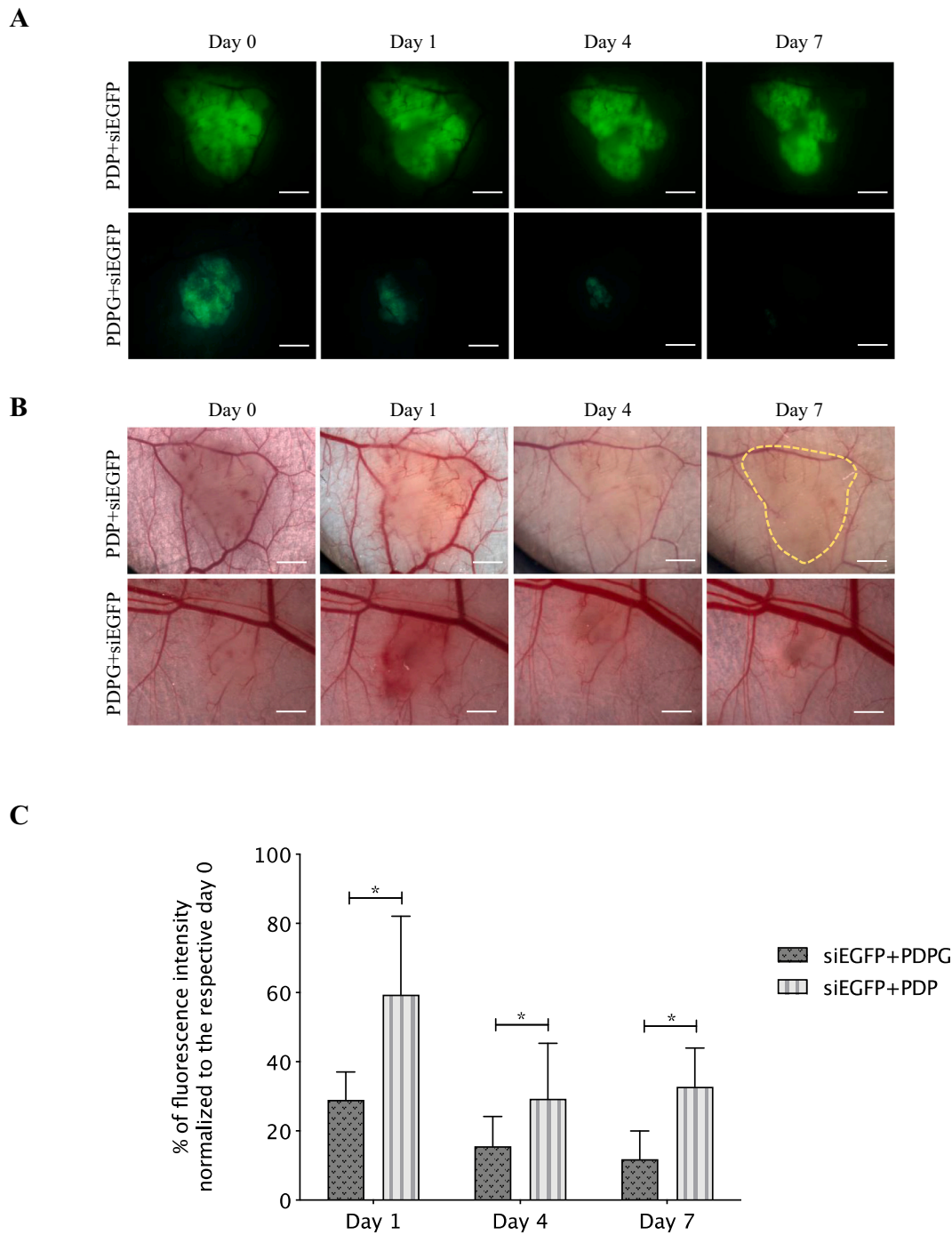


Fig. 6. siRNA uptake in vivo.

Dorsal skinfold window chamber model: representative images from tumors (HuH7-EGFP) treated by siEGFP+PDPG or siEGFP+PDP. Fluorescence images (A, green) and bright-field images (B) taken at different time points after intra-tumor injection of siEGFP+PDPG or siEGFP+PDP. Scale bar = 50 μ m. C) Cumulative data reporting the fluorescence intensities normalized to the fluorescence intensity observed in siEGFP+PDPG/PDP treated tumors on day 0. Data, shown as mean $\pm$ SEM, are expressed as %; $n = 5$, $*p < 0.0463$ compared to siEGFP+PDP.

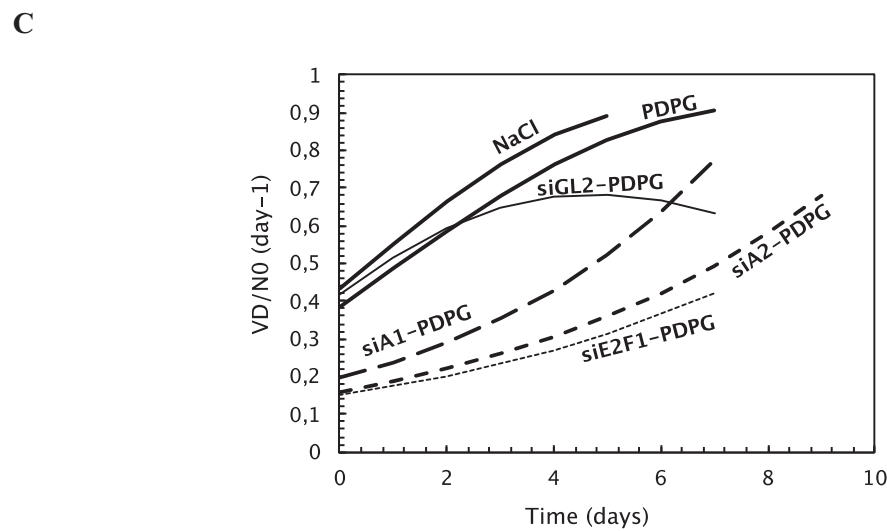
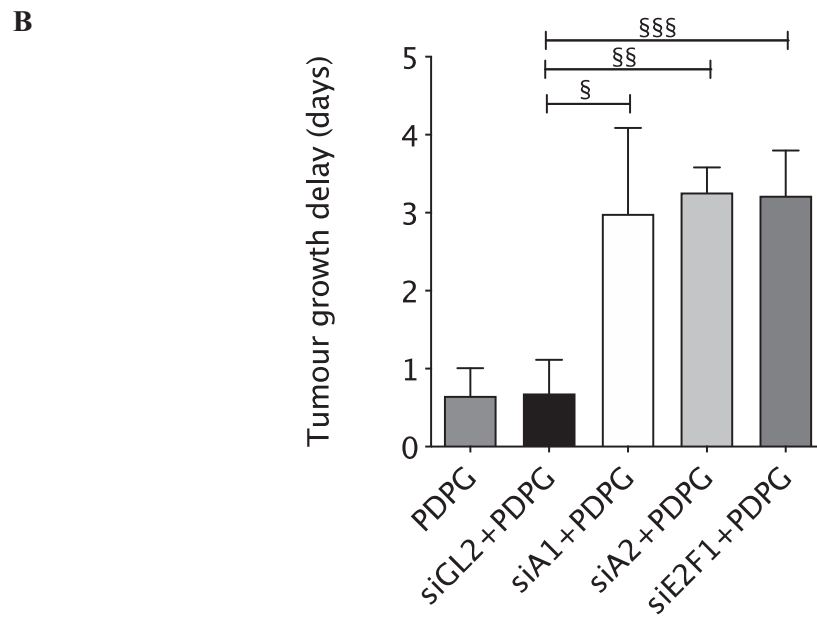
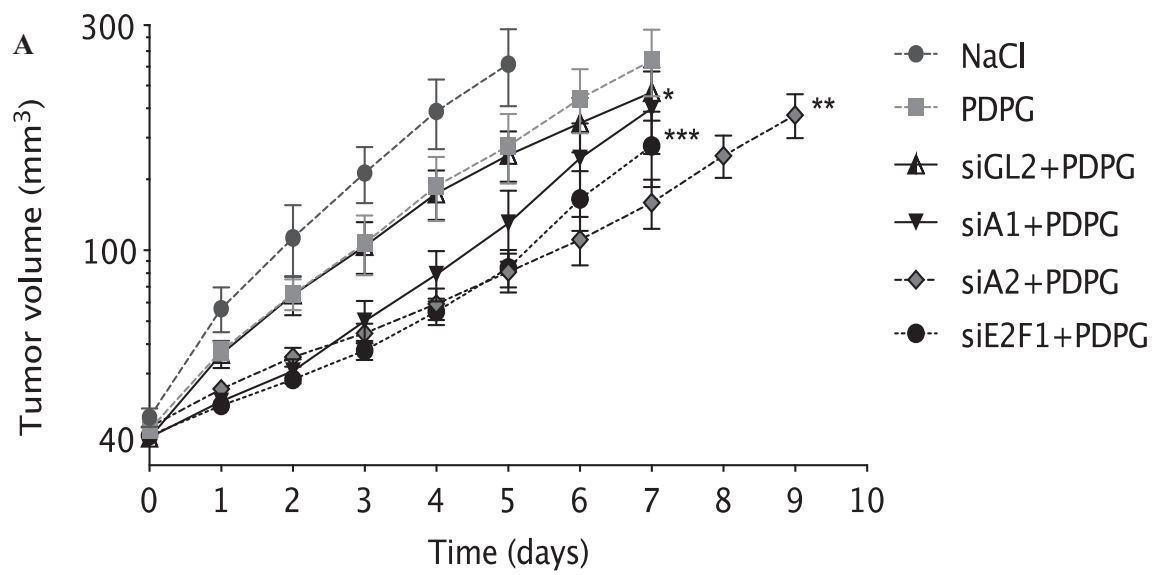
that PDP is not efficient in siRNA transfection to HCC cells.

Together, the above considerations support the ASGPR-dependent uptake of siRNAs+PDPG.

4.2. In vitro functional studies

The first level of specificity of our strategy derives from the ASGPR-dependent mechanism of uptake. The second level of specificity descends from the fact that our siRNAs target HCC related genes. Besides

being overexpressed in HCC, eEF1A2 is not expressed in the normal hepatocytes [15]. E2F1 is overexpressed in HCC [21] and it is not crucial for the growth of normal hepatocytes [42]. Thus, siA2/siE2F1 are expected to predominantly exert their effect in HCC cells and not in normal hepatocytes, thus preserving their functions. With regard to eEF1A1, we have preliminary evidences (unpublished results) that its depletion by lipofectamine-mediated delivery of siA1 to IHH does not greatly affect cell viability. Whereas further investigations are necessary, this opens the possibility that also eEF1A1 may not be crucial for the survival of



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Fig. 7. siRNA effects on tumor growth: long term experiment.

A) Reported are the tumor growth curves following siA1 + PDPG, siA2 + PDPG or siE2F1 + PDPG intra-tumor injections in comparison to control treated tumors (NaCl 0.9%, PDPG and siGL2 + PDPG). Data are shown as mean $\pm$ SEM, $n = 6$. $*p = 0.0008$ $**p = 0.003$; $***p = 0.0004$ compared to siGL2 + PDPG treated mice. **B)** Tumor growth delay following siRNAs+PDPG treatments compared to siGL2 + PDPG or PDPG treated mice. Data are shown as mean $\pm$ SEM. $***p = 0.009$ and $^{**}p = 0.0063$ $^{*}p = 0.045$ compared to siGL2 + PDPG treated mice. **C)** Temporal trend of the cells generated per day for each one of the original N_0 cells (V_D/N_0). It is clear the different trend of V_D/N_0 for the three controls (NaCl, PDPG, siGL2-PDPG) and the three siRNAs (siA1-PDPG, siA2-PDPG, siE2F1-PDPG). Eq.(3) fitting parameters are: NaCl ($k_D = (0.43 \pm 0.02) \text{ day}^{-1}$; $k_f = (0.15 \pm 0.026) \text{ day}^{-1}$; $F(1, 4, 0.95) < 1916$); PDPG ($k_D = (0.38 \pm 0.012) \text{ day}^{-1}$; $k_f = (0.13 \pm 0.014) \text{ day}^{-1}$; $F(1, 6, 0.95) < 2284$); siGL2-PDPG ($k_D = (0.42 \pm 0.011) \text{ day}^{-1}$; $k_f = (0.18 \pm 0.012) \text{ day}^{-1}$; $F(1, 6, 0.95) < 4894$); siA1-PDPG ($k_D = (0.19 \pm 0.028) \text{ day}^{-1}$; $k_f \approx 0$; $F(1, 6, 0.95) < 109$); siA2-PDPG ($k_D = (0.16 \pm 0.01) \text{ day}^{-1}$; $k_f \approx 0$; $F(1, 8, 0.95) < 911$); siE2F1-PDPG ($k_D = (0.15 \pm 0.017) \text{ day}^{-1}$; $k_f \approx 0$; $F(1, 6, 0.95) < 143$). $p < 0.05$ for k_D and k_f of treatments vs control.

normal hepatocytes. Thus, our approach combines the ASGPR-dependent targeting specificity to the biological specificity related to the siRNAs delivered, a fact that can contribute to further increase the overall specificity.

The *in vitro* data indicate a clear dose (Suppl mat 2B) and time-dependent reduction of cell viability for the siRNAs tested (Fig. 4A). This last behavior most likely descends from the processing of siRNA into the cells and/or to the expansion of non-transduced cells that “dilute” the viability down-regulation of transduced cells. Despite this, target mRNA down regulation was evident for the siA1/siA2 (Fig. 5A-B) and it was fully comparable to that we previously observed following Lipofectamine 2000-mediated delivery (same siRNAs, concentration and time point of analysis) [18]. For siE2F1, the target mRNA down regulation of 60% we measure here (Fig. 5C) is only slightly inferior from that (80%) observed following Lipofectamine 2000-mediated delivery [22]. We believe that the modest reduction in target mRNA down-regulation can be paid in favour of a remarkable increased delivery specificity. Together the above data indicate that our novel PDPG has comparable effectiveness as Lipofectamine 2000 *in vitro*, but with an extraordinary increased delivery specificity. Moreover, while Lipofectamine 2000 has a remarkable unspecific effect on HuH7 viability/number (about 40% reduction observed in our previous studies [18,22]) PDPG has negligible unspecific effects, showing an additional advantage of PDPG (Fig. 4).

The considerable decrease in the eEF1A1 mRNA (80%, Fig. 5A) induced by siA1 + PDPG, seems to be in disagreement with the more contained reduction (25%, Fig. 5E) in the protein level. We believe this may be explained by the huge abundance of the eEF1A1 protein (among the most abundant proteins in human cells) together with its long half-life [15]. Thus, despite eEF1A1 mRNA is down-regulated effectively, protein decrease is attenuated by the huge amount of protein in the cell. Notably, we observed a similar phenomenon [18] when siA1 (same concentration and time point of analysis) was delivered by Lipofectamine 2000 to HuH7. Thus, the divergence between mRNA and protein down regulation seems to be independent from the delivery system used. For siA2 + PDPG and siE2F1 + PDPG we observe a more contained divergence between the mRNA (40 or 55%, Fig. 5B and 5C, respectively) and protein (20 or 30%, Fig. 5F and G, respectively) reduction, most likely related to the higher turnover of the two proteins compared to eEF1A1. Notably, also for siA2 the divergence mRNA/protein reduction is comparable to that we observed delivering siA2 by Lipofectamine 2000 (same concentration and time point of analysis) to HuH7 [18].

Here, like in our previous study [18], we notice that a submaximal target protein (eEF1A1, eEF1A2, E2F1) reduction is sufficient to induce a significant slow-down of HuH7 viability (Fig. 4). The question is how a submaximal reduction in the level of protein target level can result in a clear impairment in cell growth? We do not have an unequivocal answer to this question. However, our previous observation [18] about eEF1A1 silencing may provide a reasonable hypothesis. We noticed that below a certain eEF1A1 protein decrease (around 40% decrease) cell growth (measured by cell counting) was no further reduced. For example, despite a decrease of eEF1A1 protein level of about 80%, the reduction in cell number was comparable to that of a protein decrease around 40%. It is thus possible that for gene products related to cell proliferation (like eEF1A1/A2 and E2F1) a submaximal decrease in their protein level

is sufficient to perturb the delicate equilibrium of the proliferative pathway resulting in cell growth slow down and/or cell death induction.

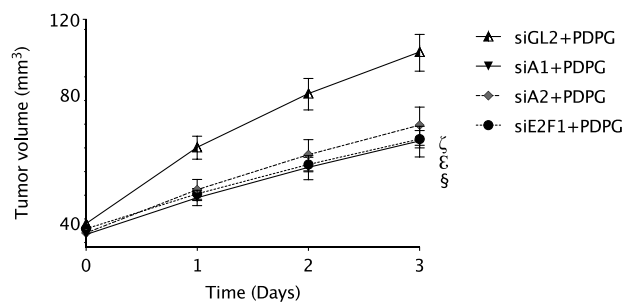
While no evident signs of cell necrosis/apoptosis were detected following siRNAs+PDPG delivery, we observed a propensity towards autophagy induction (Suppl mat 3B). Autophagy [43] is a process by which cytoplasmic components are delivered into the lysosome for degradation leading to cell death. Does autophagy induction mean that our siRNAs+PDPG elicit non-specifically a certain type of cell death? We do not believe this is the case. Indeed, we have evidences that in Mec-1 cell line (chronic lymphocytic leukemia cells [30]) and PC-3 (prostatic adenocarcinoma cell line, unpublished results) siA1 elicits autophagy. The phenomenon seems to be totally unrelated to the delivery agent as in Mec-1 and PC-3 we used electroporation and lipofection, respectively, as delivery strategies. Which is the connection eEF1A1/A2/ E2F1 depletion with autophagy induction? So far it is not clear, however, for example, eEF1A1 is known to form complexes with actin [44] which in turn is related to autophagy since its filamentous form is requested for autophagy to progress [45]. Thus, it is possible that perturbation of eEF1A1 (and perhaps eEF1A2, which shares with eEF1A1 many functions) levels affects actin leading to autophagy. The connection of E2F1 with autophagy may be related to the functional crosstalk between eEF1A1 and E2F1 we described in HuH7 [18]. Notably, the connection of eEF1A1/A2 and E2F1 with cell death are also substantiated by the observation that tumors injected by siA1/siA2/siE2F1 + PDPG display an increased cell death rate compared to controls (Fig. 8D). Obviously, further investigation are necessary to define this aspect.

4.3. *In vivo* functional studies

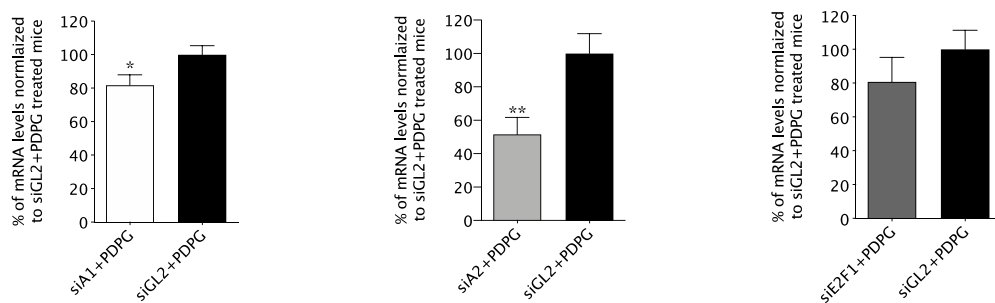
The effectiveness of siA1/siA2/siE2F1 + PDPG observed *in vitro* is fully confirmed *in vivo* following a single injection into the tumor (Fig. 7A and 7B). The mathematical analysis (eq.(1)) of the *in vivo* data leads to the conclusion that controls (NaCl, PDPG, siGL2 + PDPG) and active compounds (siA1 + PDPG, siA2 + PDPG, siE2F1 + PDPG) show very different behaviors. This is shown by the cells duplication speed (V_D/N_0 ; Fig. 7C) depending on the proliferation speed constant k_D and on the fraction of dividing cell k_f . Without this analysis, the effects of siRNAs-PDPG could have been only grossly inferred from the growth curve. Thus, our mathematical model represents a powerful tool to compare precisely and objectively the effectiveness of diverse anti-tumor approaches (different anti-tumor drug, dosage, delivery systems *etc.*) just considering the tumor growth curve. Notably, the different values of the constants between control and active siRNAs may depend on the increased cell death we observed (Fig. 8D and Suppl. mat 3B). However, we believe that a reduction of cell proliferation may also contribute. Indeed, we have previous evidences that siA1, siA2 and siE2F1 effectively down regulate HuH7 proliferation [18,22].

Compared to the *in vitro* data, the *in vivo* effect of the siRNAs+PDPG seems to have a longer duration, as *in vitro* the effect peaks at day 3. Considering the differences between the two experimental set up, many variables can account for this observation. For example, it is possible that *in vivo* siRNAs+PDPG can stay in contact with tumor cells longer than in the *in vitro* situation, where the optimized contact time was of 4 h. Thus, the longer interaction of tumor cells with siRNAs+PDPG could have enhanced transfection. This improved effectiveness of siRNAs *in*

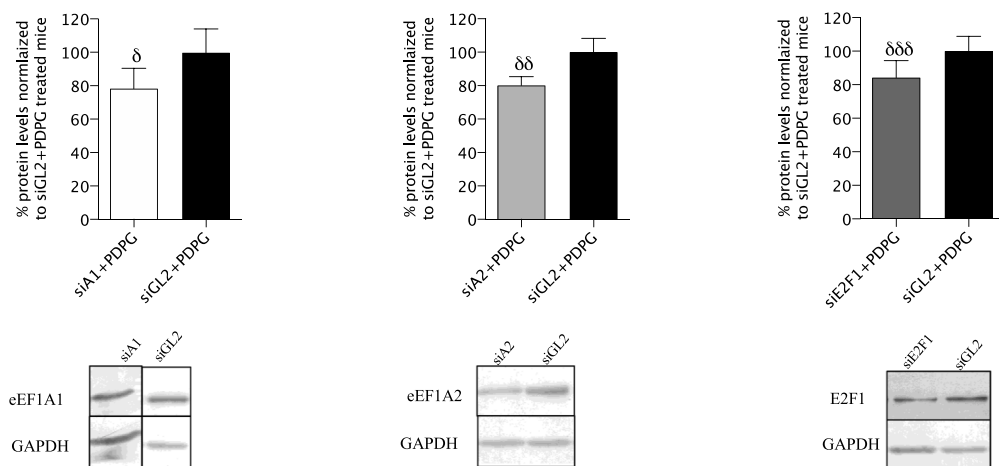
A)



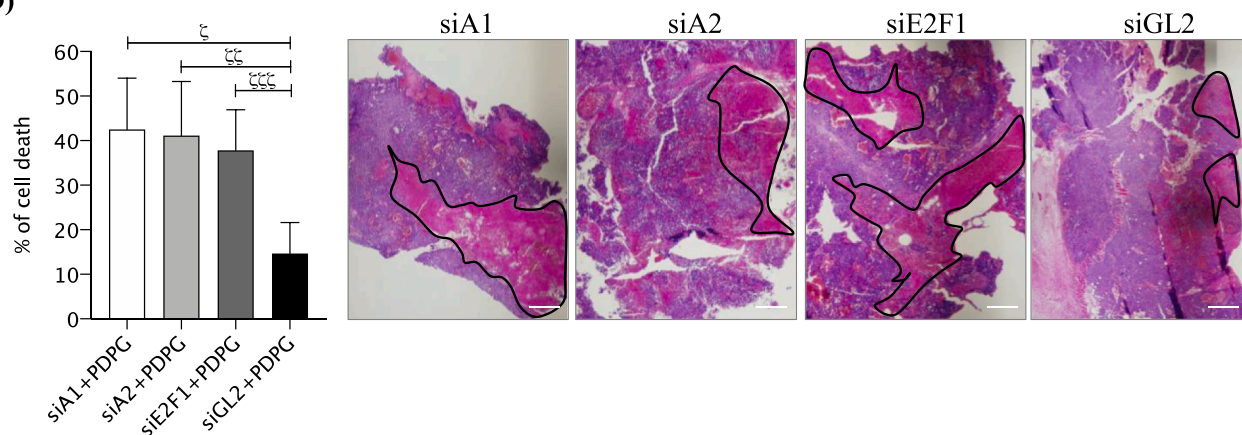
B)



C)



D)



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Fig. 8. siRNA effects on tumor growth: short term experiment.

A) Reported are the tumor growth curves following siA1 + PDPG, siA2 + PDPG or siE2F1 + PDPG intra-tumor injections in comparison to control treated tumors (siGL2 + PDPG). Data are shown as mean $\pm$ SEM, $n = 5$, $^{\epsilon}p = 0,038$, $^{\delta}p = 0,031$ and $^{\zeta}p = 0,041$ compared to siGL2 + PDPG treated mice. **B)** mRNA levels of eEF1A1, eEF1A2 and E2F1 following siRNAs+PDPG intra-tumor injections, compared to siGL2 + PDPG treated mice (*short-term* experiment, day 3). Data, normalized to 28S rRNA levels (eEF1A1 and E2F1) or GAPDH (eEF1A2) and expressed as the % of the respective siGL2 + PDPG treatment, are shown as mean $\pm$ SEM; $^*p = 0.0439$ and $^{**}p = 0.0159$ compared to control siGL2 + PDPG treatment. **C)** Evaluation of the protein levels three days after siA1-siA2-siE2F1 + PDPG injection in tumors, compared to the control transfected tumors siGL2 + PDPG (*short-term* experiment, day 3). Data are shown as mean $\pm$ SEM, $n = 5$. $^{\delta}p < 0.0348$, $^{85}p = 0.0232$ and $^{86}p = 0.0349$ compared to control siGL2 + PDPG. **D)** Representative images of hematoxylin-eosin stained tumor tissues obtained from the *short-term* experiment (day 3). The black lines indicate the cell death areas. The histogram shows the % of cell death (in relation to the total tissue area) in tumors three days following siRNAs+PDPG injections. Scale bar = 100 μ m. Data are shown as mean $\pm$ SEM, $n = 5$. $^{\zeta}p < 0,049$, $^{\zeta\zeta}p = 0,048$ and $^{\zeta\zeta\zeta}p = 0,0045$ compared to control siGL2 + PDPG.

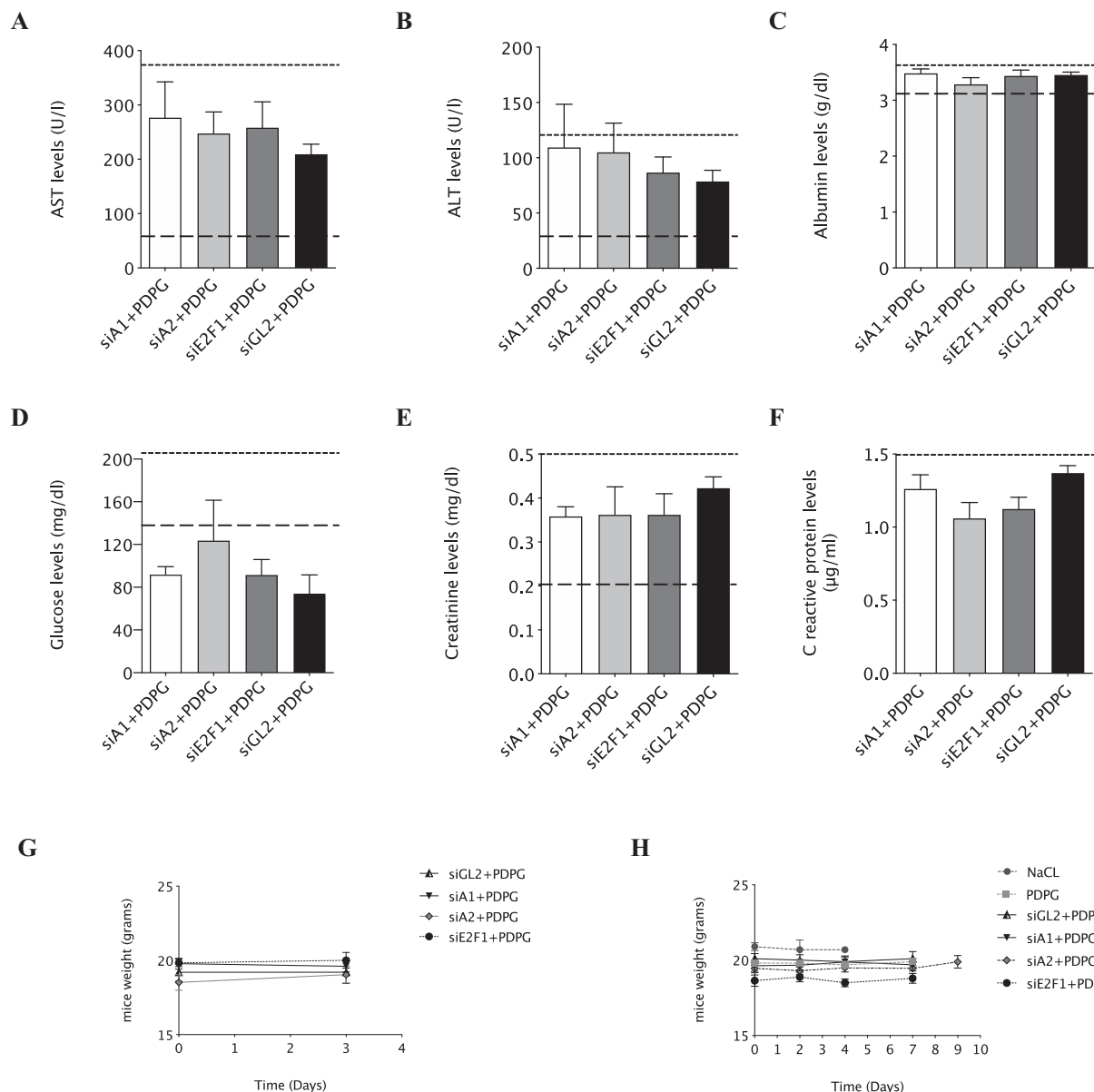


Fig. 9. Evaluation of siRNA toxicity *in vivo*.

Reported are the levels of AST (**A**), ALT (**B**), albumin (**C**), Glucose (**D**), Creatinine (**E**) and C reactive protein (**F**) measured in mice following siRNAs+PDPG or siGL2 + PDPG intra-tumor injection. The dashed line indicates the normal range (upper and lower) in mice. Data are shown as mean $\pm$ SEM, $n = 5$. **G** and **H**) Values for mice body weight from the *short-term* experiment ($n = 5$) and *long-term* experiment ($n = 6$), respectively. Data are shown as mean $\pm$ SEM.

in vivo compared to *in vitro* is a phenomenon we have already observed. In particular, the transfection (by lipofectamine) of the same siA1 into the tumor formed by Mec-1 cells *in vivo*, resulted in a longer down regulation in tumor growth compared to siA1 delivered to cultured Mec-1 cells [30]. Thus, the phenomenon seems to be independent from the

transfection method used.

In addition of being effective and specific, the siRNAs+PDPG we developed have negligible toxic effect in the treated animals both in the short (**Fig. 9A-G**) and long range (**Fig. 9H**) after intra-tumor injection. This feature together with the proved hemo-compatibility of

siRNAs+PDPG [14] indicate the safety of the delivery system developed.

4.4. Conclusions

The data presented support the concept that our ASGPR-targeted PDPG, loaded by siA1, siA2 or siE2F1 displays high specificity and a remarkable anti HCC effect following a single intra-tumor injection. Based on these very promising results, we are in the process to evaluate the effects of a systemic delivery of siRNAs+PDPG focusing on the optimization of the dosage and administration modality. In this regard, however, it should be considered that an intravenous delivery of an anti HCC drug might not be strictly necessary. Indeed, an anti HCC drug can be locally administered *via loco-regional approaches* [6]. In this regard, sorafenib, the first line chemotherapy approved by FDA for HCC, is often administered *via TACE* [46]. A part from the possibility to confine the effect of the drug and maximize the local concentration, this delivery approach guarantees an arterial delivery. This is a relevant aspect as the arterial supply in HCC increases with the progression of the disease becoming preponderant in advanced HCC [47]. Thus, to target HCC and preserve the normal liver tissue, it may be desirable to consider a local arterial delivery rather than a systemic intravenous delivery.

In addition to the effectiveness, the developed PDPG has several other attracting features such as the negligible toxicity, the low production cost and the simplicity of siRNA loading requiring just siRNA mixing with PDPG at room temperature form 20 min. The data we present here are in line with the effects observed for other ASGPR-targeted delivery systems containing chemically synthesized siRNAs [48–51]. The methodologies undertaken in the published works differ significantly from our (chemical nature of the delivery material, siRNA targets, HCC animal model, delivery route, siRNA amount administered *etc.*). However, in all cases, the use of delivery materials equipped with ASGPR targeting moieties, lead to an effective HCC targeting and down regulation of tumor growth. This indicates that delivery materials, like our PDPG, able to target ASGPR are particularly promising for the development of future siRNA-based anti HCC therapeutic approaches especially when carrying HCC specific siRNAs.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jconrel.2020.11.020>.

References

- [1] J. Ferlay, I. Soerjomataram, R. Dikshit, S. Eser, C. Mathers, M. Rebelo, D.M. Parkin, D. Forman, F. Bray, Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012, *Int. J. Cancer* 136 (2015) E359–E386.
- [2] R. Lencioni, J. Marrero, A. Venook, S.L. Ye, M. Kudo, Design and rationale for the non-interventional global investigation of therapeutic DEcisions in hepatocellular carcinoma and of its treatment with Sorafenib (GIDEON) study, *Int. J. Clin. Pract.* 64 (2010) 1034–1041.
- [3] A. Gabrielson, A.A. Tesfaye, J.L. Marshall, M.J. Pishvaian, B. Smaglo, R. Jha, K. Dorsch-Vogel, H. Wang, A.R. He, Phase II study of temozolomide and veliparib combination therapy for sorafenib-refractory advanced hepatocellular carcinoma, *Cancer Chemother. Pharmacol.* 76 (2015) 1073–1079.
- [4] J. Bruix, M. Sherman, Management of hepatocellular carcinoma: an update, *Hepatology* 53 (2011) 1020–1022.
- [5] S.D. Ryder, Guidelines for the diagnosis and treatment of hepatocellular carcinoma (HCC) in adults, *Gut* 52 (Suppl. 3) (2003) iii1–iii8.
- [6] D.H. Palmer, K. Malagari, L.M. Kulik, Role of locoregional therapies in the wake of systemic therapy, *J. Hepatol.* 72 (2020) 277–287.
- [7] M. Grassi, G. Cavallaro, S. Scirè, B. Scaggiante, B. Daps, R. Farra, D. Baiz, C. Giansante, G. Guarnieri, D. Perin, G. Grassi, Current strategies to improve the efficacy and the delivery of nucleic acid based drugs, *Current Signal Transduction Therapy* 5 (2010) 92–120.
- [8] R. Farra, F. Musiani, F. Perrone, M. Cemazar, U. Kamensek, F. Tonon, M. Abrami, A. Rucigaj, M. Grassi, G. Pozzato, D. Bonazza, F. Zanconati, G. Forte, B.M. El, L. Scarabel, M. Garziera, S.C. Russo, S.L. De, B. Salis, G. Toffoli, F. Rizzolio, G. Grassi, B. Daps, Polymer-Mediated Delivery of siRNAs to Hepatocellular Carcinoma: Variables Affecting Specificity and Effectiveness, *Molecules* 23 (2018).
- [9] L. Scarabel, F. Perrone, M. Garziera, R. Farra, M. Grassi, F. Musiani, S.C. Russo, B. Salis, S.L. De, G. Toffoli, F. Rizzolio, F. Tonon, M. Abrami, G. Chiarappa, G. Pozzato, G. Forte, G. Grassi, B. Daps, Strategies to optimize siRNA delivery to hepatocellular carcinoma cells, *Expert. Opin. Drug Deliv.* 14 (2017) 797–810.
- [10] R. Farra, M. Maruna, F. Perrone, M. Grassi, F. Benedetti, M. Maddaloni, B.M. El, S. Parisi, F. Rizzolio, G. Forte, F. Zanconati, M. Cemazar, U. Kamensek, B. Daps, G. Grassi, Strategies for Delivery of siRNAs to Ovarian Cancer Cells, *Pharmaceutics* 11 (2019).
- [11] A.A. Barba, G. Lamberti, C. Sardo, B. Daps, M. Abrami, M. Grassi, R. Farra, F. Tonon, G. Forte, F. Musiani, M. Licciardi, G. Pozzato, F. Zanconati, B. Scaggiante, G. Grassi, G. Cavallaro, Novel lipid and polymeric materials as delivery Systems for Nucleic Acid Based Drugs, *Curr. Drug Metab.* 16 (2015) 427–452.
- [12] G. Cavallaro, M. Licciardi, G. Amato, C. Sardo, G. Giammona, R. Farra, B. Daps, M. Grassi, G. Grassi, Synthesis and characterization of polyaspartamide copolymers obtained by ATRP for nucleic acid delivery, *Int. J. Pharm.* 466 (2014) 246–257.
- [13] C. Sardo, R. Farra, M. Licciardi, B. Daps, C. Scialabba, G. Giammona, M. Grassi, G. Grassi, G. Cavallaro, Development of a simple, biocompatible and cost-effective inulin-Diethylenetriamine based siRNA delivery system, *Eur. J. Pharm. Sci.* 75 (2015) 60–71.
- [14] G. Cavallaro, R. Farra, E.F. Craparo, C. Sardo, B. Porsio, G. Giammona, F. Perrone, M. Grassi, G. Pozzato, G. Grassi, B. Daps, Galactosylated polyaspartamide copolymers for siRNA targeted delivery to hepatocellular carcinoma cells, *Int. J. Pharm.* 525 (2017) 397–406.
- [15] B. Scaggiante, B. Daps, R. Farra, F. Tonon, M. Abrami, M. Grassi, F. Musiani, F. Zanconati, G. Pozzato, G. Grassi, Translational elongation, in: A. Parysyan (Ed.), *In translation and its regulation in cancer biology and medicine*, Springer, Heidelberg New York London, 2014, pp. 241–266.
- [16] G. Grassi, B. Scaggiante, R. Farra, B. Daps, F. Agostini, D. Baiz, N. Rosso, C. Tiribelli, The expression levels of the translational factors eEF1A 1/2 correlate with cell growth but not apoptosis in hepatocellular carcinoma cell lines with different differentiation grade, *Biochimie* 89 (2007) 1544–1552.
- [17] B. Scaggiante, R. Farra, B. Daps, G. Baj, G. Pozzato, M. Grassi, F. Zanconati, G. Grassi, Aptamer targeting of the elongation factor 1A impairs hepatocarcinoma cells viability and potentiates bortezomib and idarubicin effects, *Int. J. Pharm.* 506 (2016) 268–279.
- [18] R. Farra, B. Scaggiante, C. Guerra, G. Pozzato, M. Grassi, F. Zanconati, F. Perrone, C. Ferrari, F. Trotta, G. Grassi, B. Daps, Dissecting the role of the elongation factor 1A isoforms in hepatocellular carcinoma cells by liposome-mediated delivery of siRNAs, *Int. J. Pharm.* 525 (2017) 367–376.
- [19] F.N. Qiu, Y. Huang, D.Y. Chen, F. Li, Y.A. Wu, W.B. Wu, X.L. Huang, Eukaryotic elongation factor-1alpha 2 knockdown inhibits hepatocarcinogenesis by suppressing PI3K/Akt/NF-kappaB signaling, *World J. Gastroenterol.* 22 (2016) 4226–4237.
- [20] C. Schlaeger, T. Longerich, C. Schiller, P. Bewerunge, A. Mehrabi, G. Toedt, J. Kleeff, V. Ehemann, R. Eils, P. Lichter, P. Schirmacher, B. Radlwimmer, Etiology-dependent molecular mechanisms in human hepatocarcinogenesis, *Hepatology* 47 (2008) 511–520.
- [21] R. Farra, G. Grassi, F. Tonon, M. Abrami, M. Grassi, G. Pozzato, N. Fiotti, G. Forte, B. Daps, The role of the transcription Factor E2F1 in hepatocellular carcinoma, *Curr. Drug Deliv.* 14 (2017) 272–281.
- [22] R. Farra, B. Daps, G. Pozzato, B. Scaggiante, F. Agostini, C. Zennaro, M. Grassi, N. Rosso, C. Giansante, N. Fiotti, G. Grassi, Effects of E2F1-cyclin E1-E2 circuit down regulation in hepatocellular carcinoma cells, *Dig. Liver Dis.* 43 (2011) 1006–1014.
- [23] G. Giammona, B. Carlisi, S. Palazzo, Reaction of poly(N-2-hydroxyethyl)-D,L-aspartamide with derivatives of carboxylic acid, 1987, pp. 2813–2818.
- [24] E. Craparo, M. Licciardi, G. Cavallaro, Hepatocyte-targeted fluorescent nanoparticles based on a polyaspartamide for potential theranostic applications, 2015, pp. 257–270.
- [25] E.F. Craparo, D. Triolo, G. Pitarresi, G. Giammona, G. Cavallaro, Galactosylated micelles for a ribavirin prodrug targeting to hepatocytes, *Biomacromolecules* 14 (2013) 1838–1849.
- [26] E.F. Craparo, G. Cavallaro, M.L. Bondi, D. Mandracchia, G. Giammona, PEGylated Nanoparticles based on a polyaspartamide. preparation, physico-chemical characterization, and intracellular uptake, *Biomacromolecules* 7 (2006) 3083–3092.

- [27] E.F. Craparo, B. Porsio, N. Mauro, G. Giammona, G. Cavallaro, Polyaspartamide-Polylactide graft copolymers with tunable properties for the realization of fluorescent nanoparticles for imaging, *Macromol. Rapid Commun.* 36 (2015) 1409–1415.
- [28] D. Baiz, G. Pozzato, B. Dapas, R. Farra, B. Scaggiante, M. Grassi, L. Uxa, C. Giansante, C. Zennaro, G. Guarnieri, G. Grassi, Bortezomib arrests the proliferation of hepatocellular carcinoma cells HepG2 and JHH6 by differentially affecting E2F1, p21 and p27 levels, *Biochimie* 91 (2009) 373–382.
- [29] M. Zanetti, A. Stocca, B. Dapas, R. Farra, L. Uxa, A. Bosutti, R. Barazzoni, F. Bossi, C. Giansante, F. Tedesco, L. Cattin, G. Guarnieri, G. Grassi, Inhibitory effects of fenofibrate on apoptosis and cell proliferation in human endothelial cells in high glucose, *J. Mol. Med.* 86 (2008) 185–195.
- [30] B. Dapas, G. Pozzato, S. Zorzet, S. Capolla, M. Paolo, B. Scaggiante, M. Coan, C. Guerra, C. Gnan, V. Gattei, F. Zanconati, G. Grassi, Effects of eEF1A1 targeting by aptamer/siRNA in chronic lymphocytic leukaemia cells, *Int. J. Pharm.* 574 (2020) 118895.
- [31] B. Scaggiante, B. Dapas, S. Bonin, M. Grassi, C. Zennaro, R. Farra, L. Cristiano, S. Siracusano, F. Zanconati, C. Giansante, G. Grassi, Dissecting the expression of EEF1A1/2 genes in human prostate cancer cells: the potential of EEF1A2 as a hallmark for prostate transformation and progression, *Br. J. Cancer* 106 (2012) 166–173.
- [32] B. Dapas, R. Farra, M. Grassi, C. Giansante, N. Fiotti, L. Uxa, G. Rainaldi, A. Mercatanti, A. Colombatti, P. Spessotto, V. Lacovich, G. Guarnieri, G. Grassi, Role of E2F1-cyclin E1-cyclin E2 circuit in human coronary smooth muscle cell proliferation and therapeutic potential of its downregulation by siRNAs, *Mol. Med.* 15 (2009) 297–306.
- [33] B. Staresinic, T. Jesenko, U. Kamensek, F.S. Krog, G. Sersa, J. Gehl, M. Cemazar, Effect of calcium electroporation on tumour vasculature, *Sci. Rep.* 8 (2018) 9412.
- [34] T. La, W. Sun, X. Zhao, J. Zhang, W. Guo, A mathematical model to study the effects of calcium electroporation on tumor vasculature, 2019, pp. 1–9.
- [35] M. Simeoni, P. Magni, C. Cammia, N.G. De, V. Croci, E. Pesenti, M. Germani, I. Poggesi, M. Rocchetti, Predictive pharmacokinetic-pharmacodynamic modeling of tumor growth kinetics in xenograft models after administration of anticancer agents, *Cancer Res.* 64 (2004) 1094–1101.
- [36] L. Norton, R. Simon, Growth curve of an experimental solid tumor following radiotherapy, *J. Natl. Cancer Inst.* 58 (1977) 1735–1741.
- [37] E.F. Craparo, C. Sardo, R. Serio, M.G. Zizzo, M.L. Bondi, G. Giammona, G. Cavallaro, Galactosylated polymeric carriers for liver targeting of sorafenib, *Int. J. Pharm.* 466 (2014) 172–180.
- [38] G. Cavallaro, C. Sardo, E.F. Craparo, B. Porsio, G. Giammona, Polymeric nanoparticles for siRNA delivery: production and applications, *Int. J. Pharm.* 525 (2017) 313–333.
- [39] A.A. D'Souza, P.V. Devarajan, Asialoglycoprotein receptor mediated hepatocyte targeting - strategies and applications, *J. Control. Release* 203 (2015) 126–139.
- [40] T.H. Nguyen, G. Mai, P. Villiger, J. Oberholzer, P. Salmon, P. Morel, L. Buhler, D. Trono, Treatment of acetaminophen-induced acute liver failure in the mouse with conditionally immortalized human hepatocytes, *J. Hepatol.* 43 (2005) 1031–1037.
- [41] E.F. Craparo, B. Porsio, M. Bondi, G. Giammona, G. Cavallaro, Evaluation of biodegradability on polyaspartamide-poly(lactic acid) based nanoparticles by chemical hydrolysis, 2015, pp. 56–67.
- [42] E.A. Conner, E.R. Lemmer, M. Omori, P.J. Wirth, V.M. Factor, S.S. Thorgeirsson, Dual functions of E2F-1 in a transgenic mouse model of liver carcinogenesis, *Oncogene* 19 (2000) 5054–5062.
- [43] J.M.M. Levy, C.G. Towers, A. Thorburn, Targeting autophagy in cancer, *Nat. Rev. Cancer* 17 (2017) 528–542.
- [44] O. Novosylina, A. Doyle, D. Vlasenko, M. Murphy, B. Negrutskii, A. El'skaya, Comparison of the ability of mammalian eEF1A1 and its oncogenic variant eEF1A2 to interact with actin and calmodulin, *Biol. Chem.* 398 (2017) 113–124.
- [45] J. Wojnacki, T. Galli, A new actin-binding domain glues autophagy together, *J. Biol. Chem.* 293 (2018) 4575–4576.
- [46] F.X. Wu, J. Chen, T. Bai, S.L. Zhu, T.B. Yang, L.N. Qi, L. Zou, Z.H. Li, J.Z. Ye, L. Q. Li, The safety and efficacy of transarterial chemoembolization combined with sorafenib and sorafenib mono-therapy in patients with BCLC stage B/C hepatocellular carcinoma, *BMC Cancer* 17 (2017) 645.
- [47] Z.F. Yang, R.T. Poon, Vascular changes in hepatocellular carcinoma, *Anat. Rec. (Hoboken.)* 291 (2008) 721–734.
- [48] A.A. Khan, A.M. Alanazi, M. Jabeen, A. Chauhan, M.A. Ansari, Therapeutic potential of functionalized siRNA nanoparticles on regression of liver cancer in experimental mice, *Sci. Rep.* 9 (2019) 15825.
- [49] K.W. Huang, Y.T. Lai, G.J. Chern, S.F. Huang, C.L. Tsai, Y.C. Sung, C.C. Chiang, P. B. Hwang, T.L. Ho, R.L. Huang, T.Y. Shiue, Y. Chen, S.K. Wang, Galactose derivative-modified nanoparticles for efficient siRNA delivery to hepatocellular carcinoma, *Biomacromolecules.* 19 (2018) 2330–2339.
- [50] H.X. Wang, M.H. Xiong, Y.C. Wang, J. Zhu, J. Wang, N-acetylgalactosamine functionalized mixed micellar nanoparticles for targeted delivery of siRNA to liver, *J. Control. Release* 166 (2013) 106–114.
- [51] L. Han, C. Tang, C. Yin, Enhanced antitumor efficacies of multifunctional nanocomplexes through knocking down the barriers for siRNA delivery, *Biomaterials* 44 (2015) 111–121.