Graphene-dendrimer nanostars for targeted macrophage overexpression of metalloproteinase 9 and hepatic fibrosis precision therapy

Pedro Melgar-Lesmes1,2,3,*, Aureli Luquero2, Marina Parra-Robert4, Adriana Mora2, Jordi Ribera4, Elazer R. Edelman1,5, Wladimiro Jiménez2,4.

1. Massachusetts Institute of Technology, Institute for Medical Engineering and Science, Cambridge, MA, US.
2. Department of Biomedicine, School of Medicine, University of Barcelona, Barcelona, Spain.
3. Fundació Clínic per a la Recerca Biomèdica, Hospital Clínic Universitari, IDIBAPS, Barcelona Spain.
4. Biochemistry and Molecular Genetics Service, Hospital Clínic Universitari, IDIBAPS, CIBERehd.
5. Cardiovascular Division, Brigham and Women's Hospital and Harvard Medical School, Boston, MA, USA.

* Corresponding Author

Pedro Melgar-Lesmes, PhD

-Massachusetts Institute of Technology, Cambridge, Massachusetts; 77 Massachusetts Avenue, Building E25-438. Cambridge, MA 02139. USA. Phone: +1 617-715-2026 FAX: +1 617-253-2514.

-Department of Biomedicine, School of Medicine, University of Barcelona, Casanova 143, 08036 Barcelona, Barcelona, Spain. Phone: +34 93-402-4524. FAX: +34 93-227-5697.

e-mails: pedroml@mit.edu; pmelgar@clinic.cat
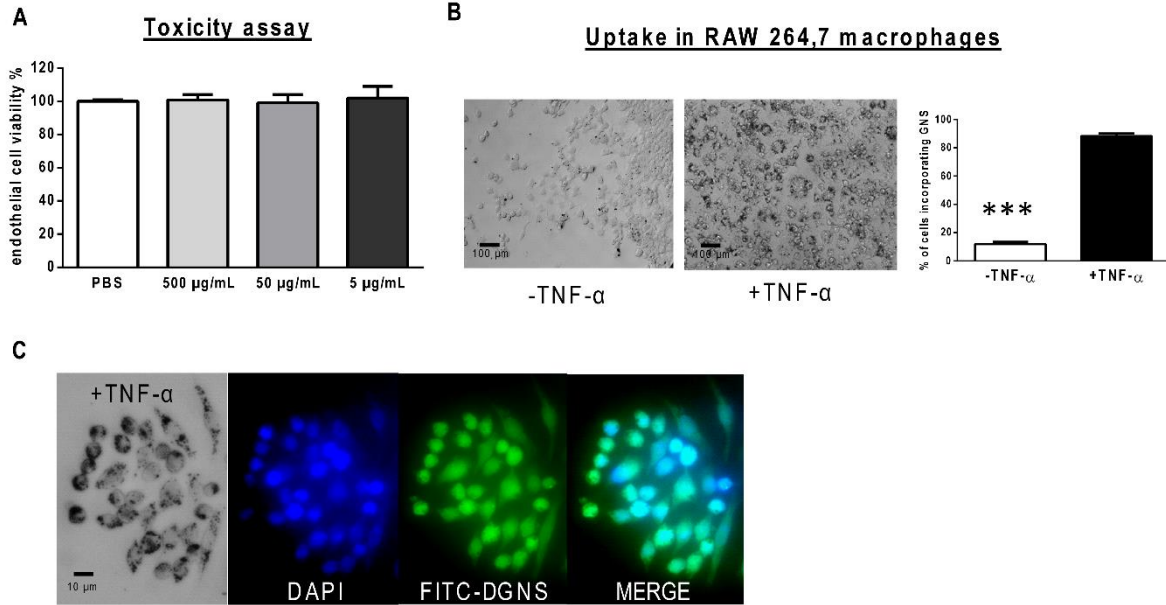
Supplementary Figures

Figure S1. Biological characterization of functionalized graphene nanostars.

(A) Cell viability in human umbilical vein endothelial cells incubated with Phosphate-Buffered Saline (PBS), or plasmid-dendrimer graphene nanostars (pDNA-DGNS) at concentrations ranging 5 to 500 µg/mL for 24 h using the MTS assay (B) Uptake experiment using RAW 264.7 macrophages incubated with pDNA-DGNS for 3 hours in the presence or absence of TNF-α (5 ng/mL) (C) Intracellular localization experiment in RAW 264.7 macrophages incubated with FITC-DGNS for 24 hours in the presence of TNF-α (5 ng/mL). DAPI was used to stain nuclei in blue. Merge corresponds to DAPI + FITC-GNS merged images. Values are mean ± S.E.M. *** indicates $P \leq 0.001$ using a Student's t-test.
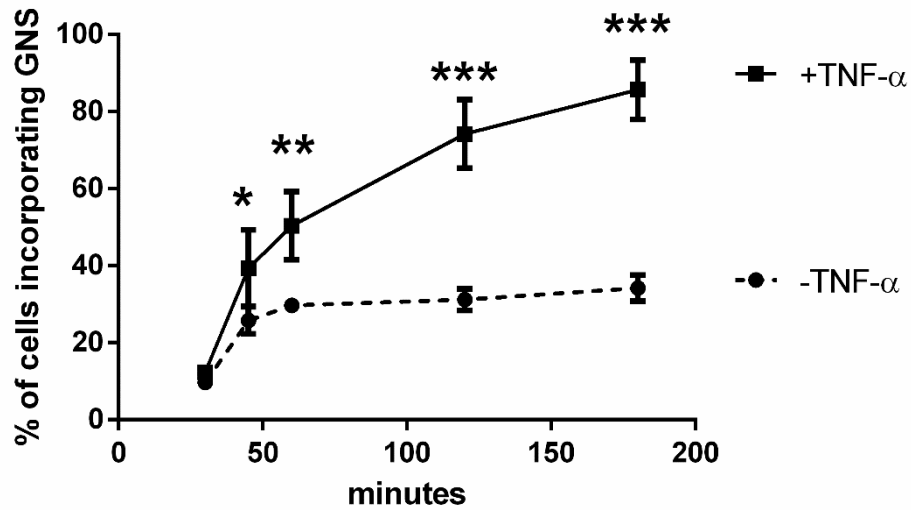
Figure S2. Graphene nanostars (GNS) uptake kinetics in macrophages in cell culture.
Time-course of RAW 264.7 macrophages incorporating GNS (10 µg/mL) in the presence or absence of TNF-α (5 ng/mL) quantified in bright field microscope images obtained after 30, 45, 60, 120 and 180 min of incubation. *** indicates $P \leq 0.0001$, ** indicates $P \leq 0.01$, * indicates $P \leq 0.05$ using a Student's t-test. Values are mean ± S.E.M.
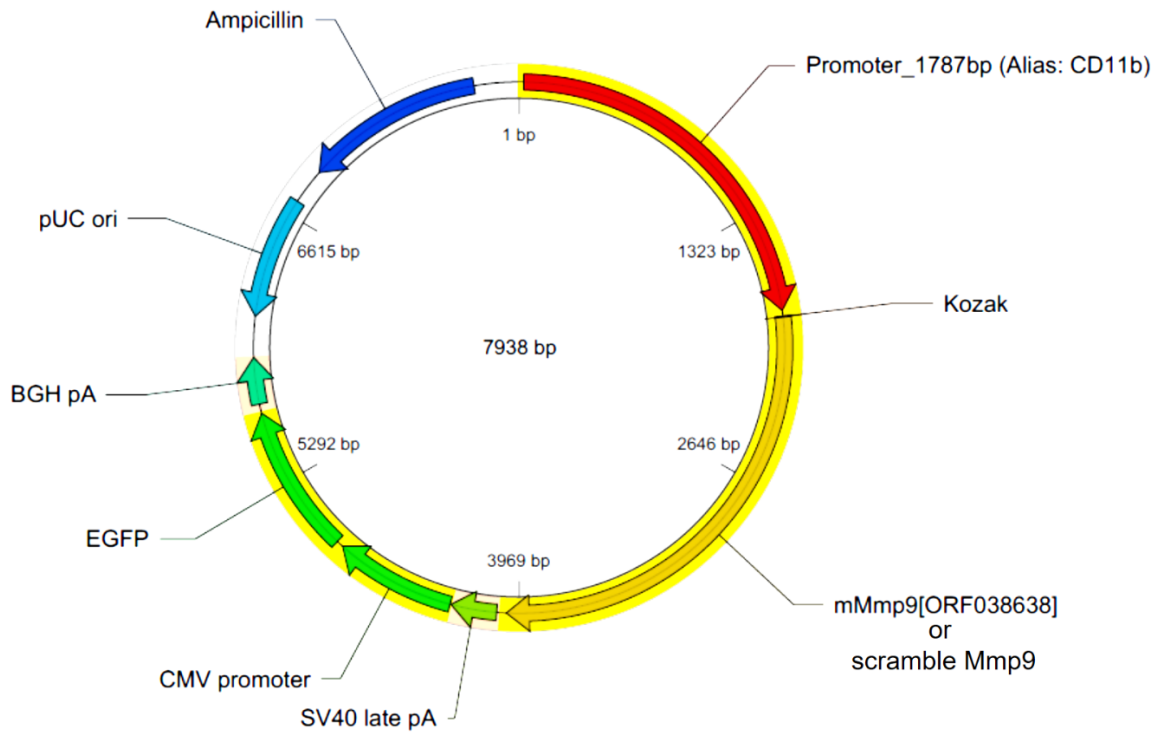
Figure S3. Scramble and MMP-9 plasmid maps. A specific promoter for infiltrated inflammatory macrophages (CD11b) controls the expression of scramble or MMP-9 genes. A strong constitutive promoter promotes the expression of the gene reporter enhanced green fluorescent protein (eGFP) to stain cells incorporating the plasmid and allowing correct transcription. A selection gene for Ampicillin is also present to obtain only the bacterial colonies synthesizing the plasmid.
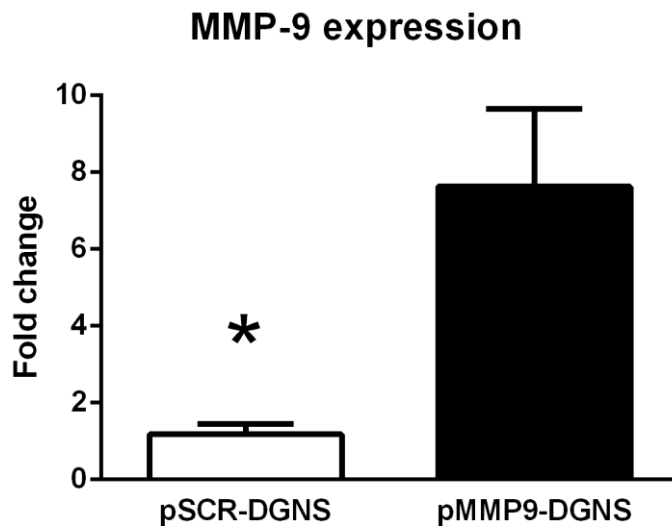
Figure S4. Hepatic expression of MMP-9 in cirrhotic animals treated with pMMP9-dendrimer graphene nanostars (pMMP9-DGNS). Quantification of gene expression of MMP-9 in cirrhotic livers from mice treated with scramble plasmid linked to dendrimer graphene nanostars (pSCR-DGNS) or pMMP9-DGNS by Real-time PCR. Values are mean ± S.E.M. * indicates $P \leq 0.05$ using a Student's t-test.

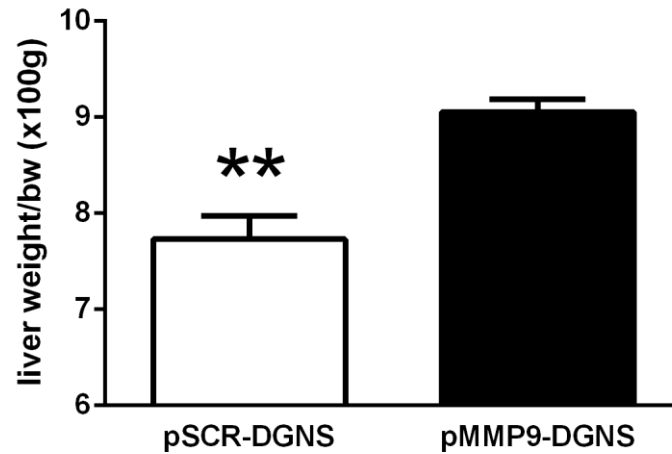**Figure S5.** *In vivo* **effect of pMMP9-dendrimer graphene nanostars (pMMP9-DGNS) on liver size in cirrhotic mice.** Liver size as an indication of hepatic regeneration was calculated as liver weight/body weight×100 in cirrhotic mice treated with scramble plasmid linked to dendrimer graphene nanostars (pSCR-DGNS) or pMMP9-DGNS. Values are mean ± S.E.M. ** indicates $P \leq 0.01$ using a Student's t-test.
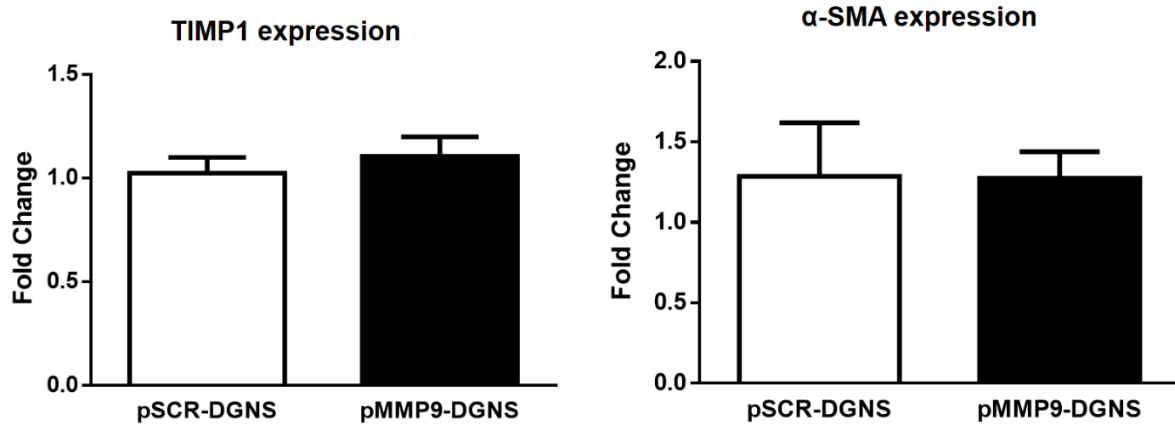
Figure S6. Hepatic expression of hepatic stellate cells activation markers in cirrhotic mice treated with pMMP9-dendrimer graphene nanostars (pMMP9-DGNS). Quantification of gene expression of tissue inhibitor of metalloproteinases 1 (TIMP1) and alpha smooth muscle actin (α-SMA) in cirrhotic livers from mice treated with scramble plasmid linked to dendrimer graphene nanostars (pSCR-DGNS) or pMMP9-DGNS by Real-time PCR. Values are mean ± S.E.M.

EXPERIMENTAL PROCEDURES:

Materials

Carbon graphene oxide nanohorns were supplied by Sigma (St. Louis, MO). Both scramble and MMP-9 expression plasmids under a CD11b promoter, with an enhanced green fluorescence protein (EGFP) sequence under a cytomegalovirus promoter were obtained from Cyagen Biosciences (Guangzhou, China). One Shot® Top 10 Chemically Competent E.coli and Qiagen® Endofree Plasmid Maxi Kit, used for transformation, amplification, and purification of ultrapure, transfection-grade plasmid DNA, were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA) and Qiagen Inc. (Chatsworth, CA, USA), respectively. Super-optimal broth with catabolite repression (SOC) medium, Luria broth (LB broth), LB agar ampicillin-100 plates, gelatin and FITC were obtained from Sigma (St. Louis, MO). Deionized water was obtained from a Milli-Q water purification system (Millipore, Molsheim, France). Primary human umbilical vein endothelial cells (HUVECs) and mouse macrophages (RAW 264.7) were supplied by Thermo Fisher Scientific Inc. (Waltham, MA, USA) and ATCC (Manassas, VA, USA), respectively. Endothelial growth medium (EGM) supplemented with EGM-2 growth supplements (Lonza, Walkersville, MD). Dulbecco's phosphate buffered saline (DPBS), Dulbecco's Modified Eagle Medium (DMEM and penicillin/streptomycin were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). donkey polyclonal anti-mannose receptor antibodies was obtained from Abcam (Cambridge, UK). Cy3-conjugated was supplied by Jackson ImmunoResearch Laboratories (West Grove, PA). The mounting medium containing 4′,6-diamidino-2- phenylindole (DAPI) was supplied from Vectashield (Vector laboratories, Burlingame, CA). Generation 5 PAMAM dendrimer was purchased from Dendritech Inc (Midland, MI).

Synthesis and functionalization of graphene-dendrimer nanostars

GNS oxidized were dispersed in DMSO (500 µg/mL) and carbon nanohorns separated by incubating the dispersion in an ultrasound bath (Selecta, Barcelona, Spain), at a frequency of 50 kHz and potency 360 W. for 15 min. Afterwards 100 µL of carbon nanohorns were mixed with 900 µL of 1 mg/mL EDC/NHS 1:1 containing 30 µL of PAMAM dendrimer 25% v/v, and incubated for 2 hours in the ultrasound bath to have the nanohorns separated to react, and keeping the temperature constant at 25±2 ºC with ice. Then dispersions were centrifuged at 21000 Gs for 10 min, washed three times with DMSO, and then three times with PBS for subsequent *in vitro* and *in vivo* experiments. Plasmids were incubated with dispersions of GNS in a ratio 1:10 for 2 h in a shaker, centrifuged and washed with PBS before use for transfection and functional assays. Ratio of plasmid/nanoparticles was established using the variations in Zeta potential from positive (DGNS) to negative when coating dendrimers-nanostars with plasmid.

Physicochemical characterization of nanoparticles.

Nanoparticle size properties were determined by dynamic light scattering (DLS), using a Zetasizer nano ZS (Malvern Instruments Ltd., UK). Measurements were carried out at 25 °C and at fixed angle of 173°, by analyzing the intensity of the scattered light supplied by a helium-neon laser (4 mW, λ = 633 nm). DLS data were calculated from the autocorrelation function of scattered light by means of two mathematical approaches; the cumulants method and Dispersion Technology Software nano v. 5.10 (Malvern Instruments Ltd.). Through the cumulants analysis, two important parameters were obtained; the mean hydrodynamic diameter (Z-Average) and the width of the particle size distribution

(polydispersity index-PDI). To prepare samples for the measurements, 20 μL of nanostars suspension were dispersed in 1,480 μL of PBS, in an ordinary cuvette. Reported values of Z-Average and PDI corresponded to the average of approximately 40 measurement runs. The size and morphology of different nanoparticles were characterized by TEM, using a JEOL JEM 1010 microscope (JEOL, Akishima, Japan) equipped with an AMT XR40 digital imaging camera, at a magnification of 75,000× and a maximum accelerating voltage of 100 kV. Particle diameter was determined in approximately 300 randomly selected nanoparticles from different TEM images using the morphometry software ImageJ v. 1.44 (U.S. National Institutes of Health, Bethesda, Maryland, USA). Osmolality was determined from osmometric depression of the freezing point (Advanced Instruments Osmometer 3300, Needham, HTs MA, USA).

Cell culture

HUVECs were cultured in pre-gelatinized plates with endothelial growth medium supplemented with EGM-2 growth supplements medium and 10% fetal bovine serum (FBS), and 50 U/mL penicillin/streptomycin. Cells were grown at 37 °C and 5% CO_2, in a water jacketed incubator. HUVECs were passaged when they reached 80% confluence and passages 2–5 were used for all experiments. RAW 264.7 mouse macrophages were cultured with DMEM supplemented with 10% FCS and 50 U/mL penicillin/ streptomycin. Cells were grown at 37 °C and 5% CO2, in a water jacketed incubator.

Biological characterization of nanoparticles

GNS cytotoxicity was analyzed on HUVECs using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (MTS assay, Promega, Madison, WI, USA). Briefly, cells

were seeded in pre-gelatinized 96-well plates at a cell density of 5×10^3 cells per well, serum starved for 6 h and then incubated with GNS at different concentrations (500, 50 and 5 µg/mL). Just before determination of cell viability, cells were washed with PBS and transferred into starvation medium. Cytotoxicity was determined by adding 20 µL of MTS solution to each well. After 2 h, the absorbance was measured at 490 nm using a microplate spectrophotometer (Varioskan Flash spectrophotometer (Thermofisher Scientific). Cell viability was expressed as the absorbance of cells treated with GNS relative to cells treated with PBS (control). Each condition was performed in sextuplicate and reported as mean ± SEM.

Uptake kinetics of GNS

RAW 264.7 mouse macrophages were cultured with DMEM with 10% FCS in 24 wells (10^5 cells/well), for 24 hours and then serum-starved for 6 hours. Afterwards cells were incubated with GNS 100 ng/mL in the presence or absence of TNF-α (5 ng/mL) and images were taken at different time points (30, 60, 120 and 180 minutes) with a light microscope. Black aggregates of GNS were visualized at high magnification to establish the number of cells incorporating GNS. Percentage of cells incorporating GNS is calculated with the formula: number of cells with black aggregates/total number of cells per field x 100. At least 30 different fields were used to calculate the uptake percentage per time point.

Intracellular localization of DGNS in macrophages

DGNS (10 µg/mL) were incubated with FITC (2 mg/mL, Sigma) for 1 h at room temperature in the dark. Afterwards GNS were centrifuged at 21000 Gs for 10 min, washed three times with DMSO, and then three times with PBS for subsequent *in vitro* experiments.

FITC-DGNS were incubated with inflamed RAW 264.7 mouse macrophages for 24 h, washed with PBS, and visualized with an epifluorescence microscope (Fluo Zeiss Axio Observer Z1, Zeiss, Oberkochen, Germany) and a digital imaging system (Ret Exi, Explora Nova, La Rochelle, France). DAPI was used as mounting medium to counterstain cell nuclei.

Functional assay of plasmid transfection efficiency and M2-subset formation

The transfection efficiency of pDNA-DGNS complexes was studied in inflamed RAW 264.7 macrophages. Cells were seeded at a concentration of 5×10^4 cells in 2-well Labtek II chamber slides, grown to 80% confluence, and inflamed with TNF-α (5 ng/mL) for 16 h. After that, cells were serum-starved for 6 h and incubated for 3 h with GNS 10 μg/mL containing 1 μg/mL of plasmid DNA expressing MMP-9 and EGFP reporter or plasmid alone. Cells were then washed and incubated for 3 days. Afterwards, cells were washed with PBS and mounted with a coverslip using a DAPI mounting medium to counterstain cell nuclei. Intracellular presence of synthesized EGFP was visualized with an epifluorescence microscope. To analyze the possible switch from M1 to M2 macrophages, cells were stained with rabbit polyclonal anti-mannose receptor (1:100, Abcam, Cambridge, MA) and revealed with Cy3-conjugated donkey-anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) incubated for 1h at room temperature. The presence of synthesized mannose receptor was visualized with an epifluorescence microscope.

Collagen degradation assay

The preparation FITC-conjugated gelatin and the quantitative analysis of collagen degradation assay was performed as previously described.[40] Briefly, 1 mg/ml of gelatin were dissolved in a buffer containing 61 mM NaCl and 50mM Na2B4O7 (pH 9.3) and then

incubated at 37°C for 1 hour. After this incubation time, 2 mg/ml of FITC were added and mixed for 2 hours in complete darkness. This mixture was then dialyzed at RT in PBS in complete darkness for 4 days with 2-3 buffer changes per day. After a quick spin to remove insoluble material, small aliquots were stored in the dark at 4°C. FITC-conjugated gelatin coated plates were prepared covering the surface of each well FITC-gelatin and fixed with 1 drop of 0.5% ice-cold formaldehyde in PBS at 4°C for 15 minutes. Wells were then gently washed three times with PBS and finally quenched in complete medium for 1 hour at 37°C. Cells were cultured for variable lengths of time up to 7 days and then supernatants were collected. Cells were fixed, washed, stained with mounting medium containing DAPI and visualized with an epifluorescence microscope. Supernatants were centrifuged and fluorescence quantified with a Hitachi F-2500 Fluorescence Spectrophotometer (Hitachi High Technologies Corp., Tokyo, Japan).

Animal Studies

Male Balb/c mice were purchased from Charles River Laboratories (Charles River, Saint Aubin les Elseuf, France). All animals were maintained in a temperature-controlled room (22 °C) on a 12-h light-dark cycle. The study was performed according to the criteria of the Investigation and Ethics Committees of the Hospital Clínic Universitari of Barcelona. After arrival, mice were continuously fed ad libitum until euthanasia. To induce liver cirrhosis, mice were injected i.p. twice a week with CCl_4 diluted 1:8 v/v in corn oil for 8 weeks. Dispersions of pDNA-DGNS were then intravenously injected (50 µg/Kg in a ratio plasmid/DGNS 1:10) every 3 days. Animals were euthanized after 10 days of treatment. Liver samples and serum were collected and frozen for further analysis. Serum parameters

were measured using a BS-200E Chemistry Analyzer (Mindray Medical international Ltd, Shenzhen, China).

Immunofluorescent staining in liver tissue

Liver was excised and tissue was washed with PBS and fixed with 10% buffered formaldehyde solution for 24h. Afterwards the tissue was cryo-protected with 30% sucrose solution (in PBS) and then embedded using Tissue-Tek OCT compound (Sakura Fineteck USA, Torrance, CA) and frozen. Liver sections underwent 1% SDS solution antigen retrieval for 5 minutes at room temperature and then were blocked with 5% normal goat serum, and incubated with rabbit polyclonal anti-mannose receptor (1:100, Abcam, Cambridge, MA) and revealed with Cy3-conjugated donkey-anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) incubated for 1h at room temperature. The presence of mannose receptor was visualized with an epifluorescence microscope. DAPI was used to counterstain cell nuclei.

Fibrosis quantification

Liver sections (4 µm) were stained in 0.1% Sirius Red F3B (Sigma) with saturated picric acid (Sigma). Relative fibrosis area (expressed as a percentage of total liver area) was analyzed in 20 fields of Sirius red-stained liver sections per animal using the morphometry software ImageJ v 1.37. To evaluate the relative fibrosis area, the measured collagen area was divided by the net field area and then multiplied by 100. From each animal analyzed, the amount of fibrosis as percentage was measured and the average value presented.

Gene expression assay with Real-Time PCR

Total RNA from liver was extracted using commercially available kits: RNeasy (Gibco-Invitrogen, Paisley, UK). A 1 µg aliquot of total RNA was reverse transcribed using a complemen-tary DNA synthesis kit (High-Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Foster City, California, USA). Primers and probes for gene expression assays (Applied Biosystems) were selected as follows: MMP-9 (Taqman assay reference from Applied Biosystems: Mm00442991_m1), NOS2 (Mm00440502_m1), COX-2 (Mm00478374_m1), IL1-β (Mm00434228_m1), ARG-1 (Mm00475988_m1), MRC1 (Mm00485148_m1), RETN1A (Mm00445109_m1), TIMP-1 (Mm01341360_g1), α-SMA (Mm01204962_gH), and hypoxanthine phosphoribosyltransferase (HPRT), used as an endogenous standard (Mm03024075_m1). Expression assays were designed using the Taqman Gene Expression assay software (Applied Biosystems). Real-time quantitative PCR was analyzed in duplicate and performed with a Lightcycler-480 II (Roche Diagnostics). A 10 µl aliquot of the total volume reaction of diluted 1:8 cDNA, Taqman probe and primers and FastStart TaqMan Master (Applied Biosystems) were used in each PCR. The fluorescence signal was captured during each of the 45 cycles (denaturing 10s at 95 $^{\circ}$C, annealing 15s at 60 $^{\circ}$C and extension 20s at 72 $^{\circ}$C). Water was used as a negative control. Relative quantification was calculated using the comparative threshold cycle (CT), which is inversely related to the abundance of mRNA transcripts in the initial sample. The mean CT of duplicate measurements was used to calculate ΔCT as the difference in CT for target and reference. The relative quantity of the product was expressed as fold induction of the target gene compared with the control primers according to the formula $2^{-\Delta\Delta CT}$, where ΔΔCT represents ΔCT values normalized with the mean ΔCT of control samples.

Statistical analysis

All data were expressed as mean ± standard error (SEM). Statistical analysis of the results was performed by one-way analysis of variance (ANOVA) with the posthoc Newman-Keuls test or by Student's t-tests, where appropriate (GraphPad Prism v6.0a). Differences were considered to be statistically significant at a p-value ≤ 0.05.