Am J Transl Res 2022;14(10):7362-7377
www.ajtr.org /ISSN:1943-8141/AJTR0143661

Original Article

A novel peptide ligand-coated nano-siRNA-lipoplex technology for kidney targeted gene therapy

Xia Liu[1,2], Jiandong Zhang[1], Anna Tang[1], Liang Xu[3], Yufeng Huang[1]

[1]Division of Nephrology & Hypertension, Department of Internal Medicine, University of Utah Health Science, Salt Lake City, Utah, USA; [2]Department of Pathophysiology, University of Nantong College of Medicine, Nantong 226000, Jiangsu, China; [3]Department of Molecular Biosciences, University of Kansas College of Liberal Arts & Sciences, Lawrence, KS, USA

Received April 20, 2022; Accepted July 3, 2022; Epub October 15, 2022; Published October 30, 2022

Abstract: Objectives: Small interfering RNA (siRNA) that silences specific disease-related genes holds the promise for the treatment of renal disease. However, delivery to the intended site of action remains a major obstacle. The goal of this study was to develop glomerulus-specific siRNA particles for targeted gene therapy of kidney diseases. Methods: We used a novel nanoparticle-based system comprised of siRNA in cationic liposomes (Lip) coated with non-inhibitory plasminogen activator inhibitor 1R (PAI-1R) that selectively targets glomerular cells and tested it with transforming growth factor-beta 1 (TGF-β1)-siRNA in nephritic rat model. Results: At the optimized ratio of components, three of PAI-1R, Lip and siRNA formed the compact nanostructured particles with close to neutral surface charge (+5.63 ± 1.45 mV) and relatively uniform size (68.9 ± 4.73 nm). When the fluorescence-conjugated siRNA was used, the labeled siRNA nanoparticles appeared specifically in glomeruli. Targeted delivery of siRNA specific to the TGFβ1 gene reduced elevated TGFβ1 mRNA expression and protein production in glomeruli, but had no effect on TGFβ1 mRNA levels in lung, spleen, artery or renal medulla, and in nephritic rats induced by injection of OX-7, for up to 5 days. PAI-1R-Lip-TGF-β1 siRNA administration significantly reduced increases in glomerular matrix accumulation and expression of PAI-1 and fibronectin. Conclusions: We conclude that a single dose of PAI-1R-Lip-TGF-β1 siRNA inhibited glomerular TGF-β1 gene expression thereby ameliorating glomerulosclerosis specifically and efficiently in nephritic rats without affecting most of other organs. The target silencing of genes critical for glomerular diseases may represent a promising treatment strategy for kidney disease.

Keywords: Nanoparticles, gene therapy, kidney, glomerulosclerosis

Introduction

The discovery of RNA interference (RNAi) that employs small double-stranded RNA molecules to silence gene expression is poised to have a major impact on the treatment of human diseases [1]. However, delivering the RNAi-based therapeutics to specific organs or specific cell types within organs still remains a great challenge. With nanoparticles, a large number of these therapeutic molecules can be loaded into a protected environment and targeted to specific cell type with antibodies or ligands, and then taken up efficiently [2-5]. Of them, the cationic liposome-mediated gene transfer vehicle with compacted structure has been extensively investigated for cancer therapy [6-10]. Cationic liposomes are composed of positively charged lipid bilayers and able to be complexed with negatively charged and naked DNA/RNA by simple mixing of lipids and DNA/RNA. The formed complex (lipoplex) can develop a net positive charge with length scale in the 10 to 100-nanometers range. Those features of lipoplex in particle size and surface charge are important for the preferential delivery of lipoplex to the tendent sites [7, 11, 12]. Furthermore, by bearing a ligand or antibody recognized by a cell surface receptor or protein on the targeted cell [13], the lipoplex-coupled nanoparticles are able to target intended organ or cells specifically. The presence of a ligand on a lipoplex also facilitates the entry of DNA/RNA into cells through initial binding of the ligand to its receptor on the cell surface followed by internalization of the bound lipoplex [7, 8, 13]. Once

internalized, sufficient DNA/RNA escapes the endocytic pathway to be expressed in the cell nucleus. This innovative nanoparticle-based gene delivery or therapy has shown success in vivo to target various tumors [6, 8, 14]. The first-in-human Phase I clinical trial for non-viral p53 gene therapy had completed [15] and is now in Phase II clinical trials for tumor-targeted p53 gene therapy (NCT00470613, NCT-02340117, NCT02340156, ClinicalTrials.gov).

Such advances made us enthusiastic to try this new technology to target glomeruli. The glomerulus is the focus of the inflammatory response in a variety of chronic kidney diseases (CKD). The prevalence of glomerular disease is high and growing because of the increased incidence of hypertension, diabetes and glomerulonephritic diseases [16, 17]. Selective gene delivery to glomerulus is an attractive approach for mechanistic studies of glomerular disease and expected to have profound therapeutic effects on these diseases without adverse systemic effects. The glomerulus is a modified capillary composed of endothelial, mesangial, visceral (known as podocytes) and parietal epithelial cells. Of these cells, endothelial cell (EC) is the first layer of the capillary and can be easily targeted through local circulation. Mesangial cell (MC) plays a pivotal role in the pathogenesis of many glomerular diseases including membranoproliferative glomerulonephritis, lupus nephritis, IgA nephropathy, focal glomerulosclerosis and diabetic nephropathy [18, 19] because MC proliferates and accumulates extracellular matrix that eventually reduces glomerular function. In addition, MC is also a suitable target for nanoparticle-based therapy because MC is located in mesangium where there is no basement membrane between the glomerular capillaries and mesangium. The mesangial space is directly accessible through the glomerular vascular fenestrations, which have a relatively wide width of 50 to 100 nm. Thus, the nanoscale particles with dimensions of less than 100 nm are able to reach the MCs in the kidneys. Podocytes are wrapped around the capillaries. Together with the glomerular basement membrane (GBM) and ECs, podocytes comprise the glomerular filtration barrier of the kidney that possess an effective cutoff size of approximately 10 nm for the entry of molecules/particles into the urinary space from the blood thereby preventing proteins from being filtered. Thus, glomerular filtration barrier likely limits macromolecular vehicles-based drug or gene deliveries to podocytes. However, several studies have shown that podocytes still can be targeted successfully by nanoparticles in animal kidney disease models [20, 21]. It remains unknown whether this action only occurs by limiting the particle size in 5 nm to 30 nm or in diseased kidney where the filtration membrane is injured, and the tiny molecular size cutoff is changed. The goal of the present study was to establish glomerular cell-, particularly MC, targeted delivery of siRNA *in vivo*.

We have developed a new human mutant plasminogen activator inhibitor 1-R (PAI-1R) (50 Kd) that does not bind any protease, but effectively competes with native PAI-1 for vitronectin (Vn) binding sites thereby enhancing plasmin generation locally in glomeruli and resulting in significant plasmin-dependent reductions in glomerular pathological matrix accumulation in anti-Thy1.1 nephritis [22, 23] and diabetic nephropathy in db/db mice [24, 25]. Likely, the human recombinant mutant PAI-1R shows promise as not only a drug that may be useful in combination with others to prevent or slow the progression of glomerulosclerosis but also a ligand to target siRNA nanoparticles to glomerular cells. In the present study, we employed the PAI-1R as the targeting ligand to develop glomerulus-specific, ligand-targeting, nanoparticle-siRNA lipoplex for targeted gene therapy of kidney diseases.

Overexpression of transforming growth factor-beta 1 (TGFβ1) is the most common molecular feature of progressive kidney disease [26]. Targeting of TGFβ1 using several different TGFβ1 inhibitors reduces the progression of chronic kidney disease in a variety of animal models. However, TGFβ1 has profound anti-inflammatory properties and systemic TGFβ1 blockade may have serious inflammatory side effects. Thus, human clinical trials of TGFβ1 blockade are unfortunately hampered by its potential systemic adverse effects [27]. The selective suppression of the TGFβ1 overexpression in diseased kidney or glomerulus is clearly wanted. We then chose TGFβ1 as a glomerular target gene and tested the efficacy of RNAi to glomerular TGFβ1 and the therapeutic potential of TGFβ1siRNA by using the targeted delivery approach in a rat model of human mesan-

gial proliferative glomerulonephritis (anti-Thy-1.1 nephritis model) where high levels of TGFβ1 occur locally in glomerular cells [26, 28, 29]. This new technology showed promising efficiency and specificity in targeted delivery of siRNA against the TGFβ1 gene and glomerulosclerosis to nephritic glomeruli in rats, with limited effect on undesired locations.

Materials and methods

Reagents

The mutant, non-inhibitory PAI-1 (PAI-1R) was produced as described previously [22, 30]. The monoclonal mouse anti-Thy1.1 antibody, OX-7, was obtained from the National Cell Culture Center (NCCC, Biovest International, Inc., Minneapolis, MN, USA). Unless specified, all other reagents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, Missouri, USA).

Animals

The studies *in vivo* were performed on male Sprague Dawley (SD) rats (Strain Code: 400, 180-200 g) obtained from the SAS colony of Charles River Laboratories (Wilmington, MA, USA). Animal housing and care were in accordance with the National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals. The animal studies were approved by the Institutional Animal Care & Use Committee (IACUC) of University of Utah (The IACUC approved protocol number: 10-09001 & 13-09001).

Disease induction

Glomerulonephritis was induced by tail vein injection of the monoclonal anti-Thy1.1 antibody, OX-7, (1.75 mg/kg body weight (BW)) on day 0. OX-7 binds to the Thy1.1 epitope on the surface of mesangial cells and causes complement-dependent cytotoxicity and cell lysis followed by exuberant matrix synthesis and deposition, especially in glomeruli. This model mimics an antigen-triggered immune mesangial proliferative glomerulonephritis in humans. Normal control animals were injected with the same volume of phosphate buffered saline (PBS).

Experimental design

Study 1. Organ distribution of PAI-1R in nephritic rats after intravenous injection: Two nephritic rats received intravenous PAI-1R injection (2 mg/kg BW) 24 hours (h) after disease induction. Rats were euthanized at 2 h after PAI-1R administration. The kidneys, liver, spleen, heart, lung and aorta were then removed when blood was completely removed from the body with 60 ml ice-cold PBS. The kidneys were further decapsulated and the cortex was dissected from the medulla. A small piece of organ tissue was snap frozen for immunofluorescent staining of PAI-1R and/or glomerular mesangial cell markers.

Study 2. Prepare and optimize PAI-1R-Lip-siRNA nanoparticles: Cationic liposomes (Lip) consisting of dioleoyl trimethyl-ammonium propane (DOTAP) and dioleoyl phosphatidyl-ethanolamine (DOPE) (Avanti Polar Lipids, Alabaster, AL) at 1:1 molar ratio were prepared by using the ethanol injection method as previously described [6]. PAI-1R at 1.7 mg/ml was stored in 20 mmol 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), 150 mmol NaCl, pH 7.4. In addition, we chose a chemically stabilized siRNA, called Stealth™ siRNA, for this study, synthesized by Invitrogen Life Technologies Inc. (Invitrogen, Carlsbad, CA, USA). The PAI-1R-Lip-siRNA complex was prepared according to the *in vivo* formulation described previously [7, 14] with minor modification. Briefly, for typical preparation with optimized *in vivo* formulation, PAI-1R-Lip-siRNA complex was prepared with various ratios of the components. Finally, 25 μl of PAI-1R (1.7 mg/ml) and 20 μl of Lip (2 mmol total lipids) plus 455 μl of Opti-MEM® I (Invitrogen) were mixed in a polypropylene tube and incubated for 5-15 min at room temperature with frequent rocking. Five μl of siRNA (20 μmol) was first mixed with 480 μl of Opti-MEM for 5 min at room temperature and then it was added to the tube, mixed immediately and thoroughly with PAI-1R-Lip complex, and incubated for 20 min at room temperature with frequent rocking. The final siRNA: Lip: PAI-1R ratio was 1:10:12.5 (μg/nmol/μg). The formed particles were further used for the analyses of surface zeta potential (ZP), size distribution and ultrastructure. The ZP and size distribution of PAI-1R-Lip-siRNA particles were carried out by dynamic laser light scattering (DLS) on a Brookhaven Instruments Corp. system consisting of a BI-200SM goniometer and a BI-9000AT autocorrelator according to the manufacturer's instructions. Each measurement was at least repeat-

ed for three times. The ultrastructure of PAI-1R-Lip-siRNA particles was analyzed by transmission electron microscopy (TEM) and atomic-force microscopy (AFM). The PAI-1R-Lip-siRNA particles prepared freshly were directly loaded on the Formvar/Carbon-coated electron microscope grids (200 Mesh Copper Grids, TED PELLA INC. Redding, CA, USA) and counterstained with uranyl acetate and the image of particles at different magnification were recorded. The AFM image surface of the particles were prepared in a tapping mode by oscillating a cantilever with a sharp tip close to the cantilever resonance frequency. A feedback circuit maintains the oscillation of the cantilever at constant amplitude.

Study 3. In vitro and in vivo validation of the cell-specific targeting of the PAI-1R-Lip-siRNA particles: The BLOCK-iT™ Alexa Fluor® Red Fluorescent Control Stealth™ siRNA (Invitrogen) was employed as an indicator of the lipoplex-mediated transfection efficiency both in vitro and in vivo. The sequence of the control siRNA is not homologous to any known gene. It does not produce any cellular effect. Also, the red fluorescence labeling is chosen to avoid the false-positive results in kidneys that have endogenous green fluorescence.

In vitro, primary MCs derived from intact rat glomeruli of four-to-six-week-old male SD rats were used between passages 5 and 8. Cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 20% fetal bovine serum (FBS) (Hyclone Laboratory, Logan, UT, USA), 100 U/ml penicillin, 100 μg/mL streptomycin, 0.1 U/mL insulin, 25 mmol Hepes buffer at 37°C in a 5% CO_2 incubator. One day before transfection of Stealth™ siRNA, rat MCs were plated in six-well-plates and maintained in antibiotic-free RPMI 1640 medium supplemented with 10% FBS, 0.66 U/ml insulin, 25 mmol Hepes buffer for 24 h. At the time of transfection, 2 ml of antibiotic-free Dulbecco's Modified Eagle Medium (DMEM) supplemented with 2.5% FBS, 2 mmol glutamine, 0.11 U/ml insulin, 25 mmol Hepes buffer with PAI-1R-Lip carrying 50 nmol red fluorescent-labeled control siRNA were added to cells of ~50% confluence. Transfection was performed for 2 h, 4 h, 6 h, 24 h, 48 h, and 72 h at 37°C in a CO_2 incubator. Medium was replaced with 2.5% FBS, antibiotic-free DMEM every 24 h. After transfection, the culture medium was discharged, and cells were washed with phosphate buffered saline (PBS) twice and fixed with cold ethyl alcohol for 5 min. The efficiency of siRNA transfection on MCs was monitored using the confocal microscopy directly.

In vivo, 6 nephritic rats received intravenous injection of the PAI-1R-Lip carrying the red fluorescent-labeled siRNA (at a dose of 8 μg of siRNA per animal) 24 h after disease induction. Nephritic rats received the same amount of naked red fluorescent-labeled siRNA alone or unliganded Lip-siRNA, or unspecific liganded transferrin (Tf)-Lip-siRNA (targets the transferrin receptor for the delivery of therapeutic agents into cancer cells) [6] severed as controls. The animals were euthanized under isoflurane anesthesia at 2 h, 4 h and 6 h after injection (n=2/per time point). Kidneys were perfused in situ with cold PBS (pH 7.4), and then excised. Glomeruli isolated from individual rats using graded sieving [22] were suspended in PBS and subsequently examined and photographed with a light microscopy and a fluorescent stereoscope.

Study 4. Effect of RNA interference (RNAi) to glomerular TGFβ1 on experimental glomerulonephritis by targeted delivery using PAI-1R-Lip-siRNA particles in vivo: The efficiency of the rat TGFβ1siRNA transfected by the established PAI-1R-Lip-siRNA nanoparticle approach was firstly assessed, *in vitro*, in cultured rat MCs using a similar protocol as described in the Study 3. We chose Stealth™ siRNA, which is stable in serum and in tissue homogenates for at least 10 days [31]. The effective sequences of the Stealth™ siRNA oligonucleotides targeting 1017 base pair (bp) -1041 bp of the rat TGFβ1 gene (GenBank accession number, NM_021578) were designed using the BLOCK-iT™ RNAi Designer (Invitrogen) and synthesized by Invitrogen Life Technologies Inc. The sense strand included 5'-AGA ACU GCU GUG UAC GGC AGC UGU A-3' and the antisense strand included 5'-UAC AGC UGC CGU ACA CAG CAG UUC U-3'. MCs were harvested at 72 h after transfection for total RNA isolation followed by TGFβ1 mRNA measurement. The latter was determined by real time reverse transcription-polymerase chain reaction (RT-PCR).

In vivo, eighteen rats were randomly assigned to the following three groups: normal control, nephritic control rats treated with PAI-1R-Lip

without siRNA, and nephritic rats treated with PAI-1R-Lip-TGFβ1siRNA (n=6/per group). The treated rats received a single tail vein injection of 48 μg/kg BW, Stealth™ siRNA against TGFβ1 carried by the PAI-1R-Lip nanoparticles. Animals were placed in metabolic cages for 24 h urine collection from day 4 to day 5 and were euthanized at day 5 after OX-7 injection.

Euthanasia

Rats were euthanized under isoflurane anesthesia. After blood was drawn from the lower abdominal aorta, the kidneys were perfused with 60 ml ice-cold PBS and harvested. For histological examination, cortical tissue was snap-frozen and fixed in 10% neutral-buffered formalin. Glomeruli from individual rats were isolated by graded sieving as described previously [22]. The silencing efficacy of Stealth™ siRNA in injected rats was determined by measuring glomerular TGFβ1 mRNA expression by real time RT-PCR and glomerular TGFβ1 protein production by the enzyme linked immunosorbent assay (ELISA) as described [32]. In addition, the downstream targets of TGFβ1 including plasminogen activator inhibitor-1 (PAI-1) and matrix protein fibronectin (FN) mRNA and protein production were determined by real time RT-PCR and ELISA. In order to determine the specific targeting of PAI-1R-Lip-siRNA nanoparticles, pieces of liver, lung, artery, heart and spleen tissues were taken from each rat for measurements of TGFβ1 mRNA expression by real time RT-PCR.

Urinary protein excretion

Twenty-four-hour urinary protein excretion was determined using the Bradford method (Bio-Rad Protein Assay, Bio-Rad Laboratories Inc., Hercules, CA, USA).

Light microscopy

All microscopic examinations were performed in a blinded fashion on 3-μm sections of paraffin-embedded tissues stained with periodic acid-Schiff (PAS). Glomerular matrix expansion was evaluated as previously described [22]. Briefly, in 30 glomeruli from each rat, the amount of mesangial extracellular matrix (ECM) occupying each glomerulus was scored as 0 (0%), 1 (25%), 2 (50%), 3 (75%) or 4 (100%).

Immunofluorescent staining for PAI-1R and glomerular cells

The indirect immunofluorescence was performed on 3 μm cryostat sections as described previously [22]. The goat anti-human PAI-1 IgG (American Diagnostic Inc. Greenwich CT) (which does not cross react with rat or mouse endogenous PAI-1), was used as the primary antibody. Fluorescein isothiocyanate (FITC)-conjugated rabbit anti-goat IgG or tetramethyl rhodamine (TRITC)-conjugated donkey anti-goat IgG and FITC-conjugated donkey anti-mouse IgG (at 1:100 dilution, Jackson ImmunoReseach Laboratories Inc.) were used as the secondary antibodies. Dual-immunostained sections were analyzed using a fluorescence microscope with double filters.

RNA preparation and real-time RT-PCR

Total RNA was extracted immediately from MCs or renal glomeruli using Trizol™ reagent (GIBCO BRL) according to the manufacturer's instructions. Total RNA (2 μg) was reverse-transcribed using the Superscript III first-strand synthesis system for RT-PCR kit (Invitrogen). Real-time RT-PCR was then performed using SYBR Green I gene assay kit (Applied Biosystems, Foster City, CA, USA) with ABI 7900 HT system as described previously [33]. Samples were run as triplicates in separate wells to permit quantification of the target gene normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) used for equal loading. Primers for TGFβ1, PAI-1, FN and GAPDH were constructed as described previously [34]. In addition, total RNA from liver, lung, artery, heart and spleen and renal medulla from each group was pooled and analyzed for TGFβ1 mRNA expression as described above.

Statistical analysis

Values are expressed as mean ± standard deviation (SD). Groups were analyzed by one-way ANOVA, and the Student-Neuman-Keuls comparison was employed to compare differences among groups. A value of $P<0.05$ was considered statistically significant. In study 4, duplicate wells of cell culture were analyzed for each experiment, and data were generated from a pool of at least three independent experiments.

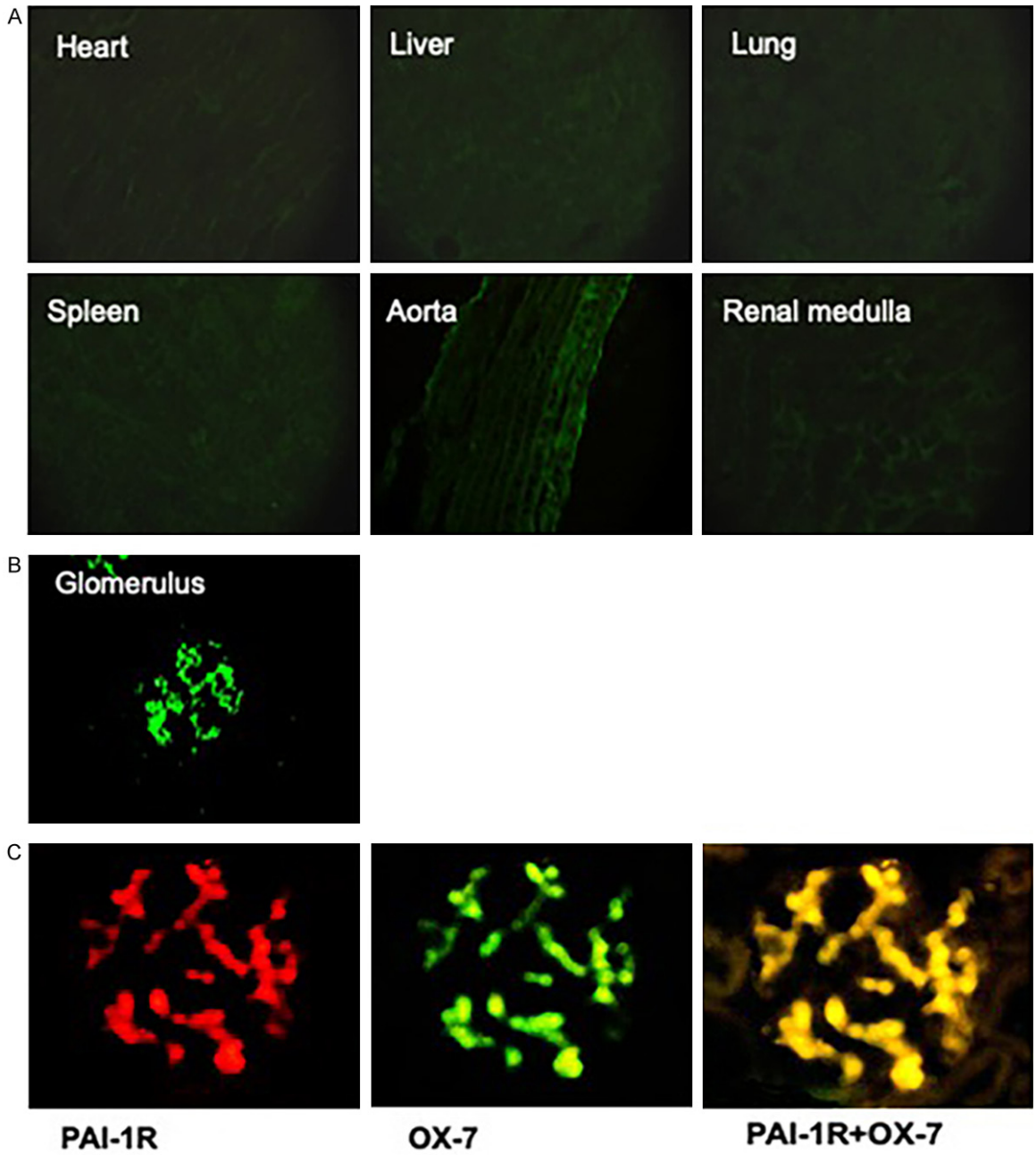


Figure 1. Injected PAI-1R mainly targeted glomerular cells in nephritic rats. (A) Representative photomicrographs of major extra-glomeruli organs and (B) glomeruli at 2 h after PAI-1R injection, stained by goat-anti-human plasminogen activator inhibitor 1 (hPAI-1) IgG and FITC-conjugated rabbit anti-goat IgG. Magnification: 250×. (C) Colocalization of PAI-1R with glomerular mesangial cells. Staining for PAI-1R (red) and glomerular mesangial cells (green) and double staining for PAI-1R and glomerular cells shown in yellow. Magnification: 400×. No PAI-1R staining was seen without PAI-1R injection in each tissue.

Results

Organ target of the injected PAI-1R

To identify the distribution of injected PAI-1R by immunofluorescent staining at a dose of 2 mg/kg BW, which was 5.8-fold higher than the dose required when forming siRNA nanoparticles, nephritic rats were sacrificed at 2 h after injection. As shown in **Figure 1**, PAI-1R was extensively stained in renal glomeruli (**Figure 1B**). A few signals of PAI-1R were observed in the

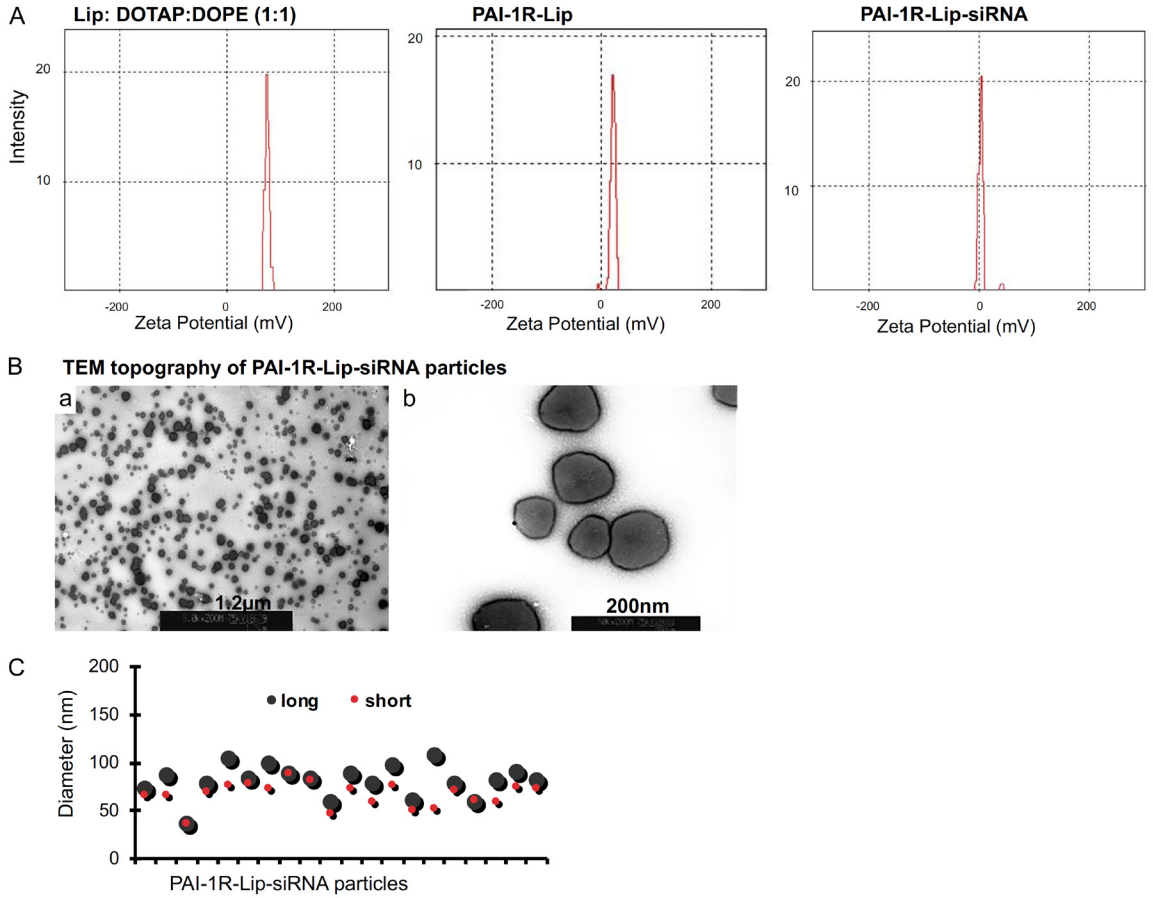


Figure 2. The surface change and particle size of PAI-1R-Lip-siRNA. (A) Surface zeta potential and size distribution of the optimized PAI-1R-Lip-siRNA particles, compared with cationic liposome alone, and PAI-1R coated liposome, PAI-1R-Lip. (B) TEM (Ba, Bb) images of PAI-1R-Lip-siRNA particles. Images of the complexes were prepared according to the optimized in vivo formulation. (Bb) Higher magnification images of (Ba) with strong defocus to improve the contrast of the surface structure of the particles. Magnification bar was present in the bottom of each photo. (C) Representative graphic size distribution of the optimized PAI-1R-Lip-siRNA particles determined by TEM. The short diameter size of particles (short): 65 ± 13.0 nm and the long diameter size of particles (long): 80.9 ± 15.6 nm.

aorta intimae. Heart, liver, lung, spleen and collecting ducts of the kidney remained negative (**Figure 1A**). Within glomeruli, injected PAI-1R stained in red and Thy1.1 stained in green were merged and produced clear yellow staining (PAI-1R/Thy1.1), indicating that PAI-1R binds to renal MCs. Furthermore, we have tested PAI-1R distribution in diabetic db/db mice at 20 weeks of age after intraperitoneal injection. Similar to the distribution of PAI-1R in nephritic rats observed here, the majority of injected PAI-1R stained red was colocalized with glomerular MCs and ECs stained green when both staining were merged and yielded clear yellow staining (PAI-1R/Thy1.1 or PAI-1R/rat endothelial cell antigen (RECA)-1), while PAI-1R (in red) and Glepp1 (in green) only yielded a few yellow color (PAI-1R/glomerular epithelial protein 1 (Glepp1)) (Supplementary Figure 1), indicating that a small portion of PAI-1R might be colocalized with glomerular podocytes. These results indicate that intravenously injected PAI-1R mainly targets glomerular MCs and ECs. Thus, PAI-1R was chosen as the targeting ligand for the following experiments by targeting glomerular cells, especially MCs.

Characteristics of PAI-1R-Lip-siRNA particles

To characterize the optimized PAI-1R-Lip-siRNA formulation, we measured the particle distribution and zeta potential of the PAI-1R-lipoplex particles using dynamic laser light scattering (DLS). The DOTAP: DOPE liposomes (Lip) themselves had a net positive charge of ~+66 mV (+66.46 ± 3.15 mV). PAI-1R coated liposome,

PAI-1R-Lip, had a less net positive charge of ~+29 mV (+29.36 ± 0.50 mV). When mixed with siRNA, the PAI-1R-Lip-siRNA particles attained a close to neutral surface charge of ~+5.6 mV (+5.63 ± 1.45 mV). As shown in the **Figure 2A**, one narrow peak of particle distribution of each mixture indicates that the vast majority of PAI-1R-Lip or PAI-1R-Lip-siRNA mixture formed a major particle of similar size, which was consistent with the changes in the surface charge of these formed particles.

The size of the PAI-1R-Lip-siRNA particle was then examined by transmission electron microscopy (TEM) and atomic-force microscopy (AFM). The DOTAP: DOPE liposomes used here are small unilamellar vesicles with a diameter of 28.7 ± 5.1 nm measured by TEM [7]. When using PAI-1R as the targeting ligand, we observed a highly compact structure of the PAI-1R-Lip-siRNA complex with a relatively uniform size of 65.8 ± 13.0 nm (short diameter of particles) to 80.9 ± 15.6 nm (long diameter of particles) determined by TEM, as shown in **Figure 2Ba**, **2Bb**, **2C**. Approximately 90% of the particles are smaller than 100 nm. The features of these nanoparticles are consistent with previously reported nanoparticles targeted by transferrin (Tf) or anti-Tf receptor antibody (TfR-scFv) [6-8, 35], which are currently in Phase II clinical trials for tumor-targeted p53 gene therapy. Measurement of particles by AFM also revealed fairly similar uniform size of 80.98 ± 4.73 nm. This size range of particles is consistent with the size that is allowed to cross the fenestrated endothelium of glomeruli but too large to pass through the extraglomerular tight endothelium and the glomerular basement membrane. Thus, the PAI-1R-Lip-siRNA nanoparticles gain direct access to the endothelial cells and the mesangium but are not filtered by the glomerular filtration barrier and therefore not excreted or available for internalization by tubular cells. These results indicate that the structure of the PAI-1R-Lip-siRNA particles is most likely responsible for the high efficiency and efficacy of glomerular mesangial cells and/or endothelial cells-targeted gene delivery.

In vitro and in vivo validation of cellular and organ targeting of PAI-1R-Lip-siRNA particles

Fluorescently labeled control siRNAs were first directly tracked and imaged intracellularly by a confocal laser scanning microscopy or a fluorescent stereoscope. The red fluorescence indicates the presence of siRNA. The labeled siRNA nanoparticles appeared in MCs at the 4 h time point after transfection. After 6 h virtually all transfected cells exhibited intense and diffuse red fluorescence staining that lasted at least 72 h (**Figure 3A**). When photographs of different layers of transfected fluorescence were merged and a higher magnification was used, the transfected siRNA nanoparticles were clearly observed in cellular cytoplasm (**Figure 3Ab**, **3Ac**). These results confirm the high transfection efficiency of these particles in rat MCs.

For the *in vivo* study, only intravenous (iv) injection of PAI-1R-Lip-siRNA to nephritic rats showed a specific targeting of glomeruli in both kidneys from 2 h to at least 6 h after injection (**Figure 3B**). No fluorescent signal was observed in the glomeruli from control rats at 2 h, 4 h, or 6 h after injection (**Figure 3C-E**). Those untargeted siRNAs were mostly seen in renal tubular cells, indicating nonspecific uptake by these cells. Minimal signal was observed in the liver, spleen, heart and artery after systemic PAI-1R-Lip-siRNA injection. These results indicate that siRNA-nanoparticle conjugated with a targeting ligand increases the specificity as well as the potency of siRNA delivery compared to free siRNA or untargeted-lipoplex carried siRNA. Glomerulus-specific, ligand-targeting, siRNA-nanoparticles are achievable, and they are able to selectively target glomeruli and stay in glomeruli long enough to be internalized by glomerular cells after systemic injection.

Functional assessment of PAI-1R-Lip-TGFβ1siRNA for the treatment of nephritic rats

It has been well established that TGFβ1 perpetuates the disease process including kidney disease through continued induction of its own production in cells at the site of injury [28]. In the anti-Thy1.1 nephritic rat model, TGFβ1 is significantly raised by glomerular cells in glomerulonephritis and involved in the development of glomerulosclerosis [36]. SiRNA to TGF-β1 was used to test the efficiency of the new PAI-1R-Lip-siRNA nanoparticle technology both in cultured MCs *in vitro* and in anti-Thy1.1 nephritic rat model *in vivo* using the same protocols described above.

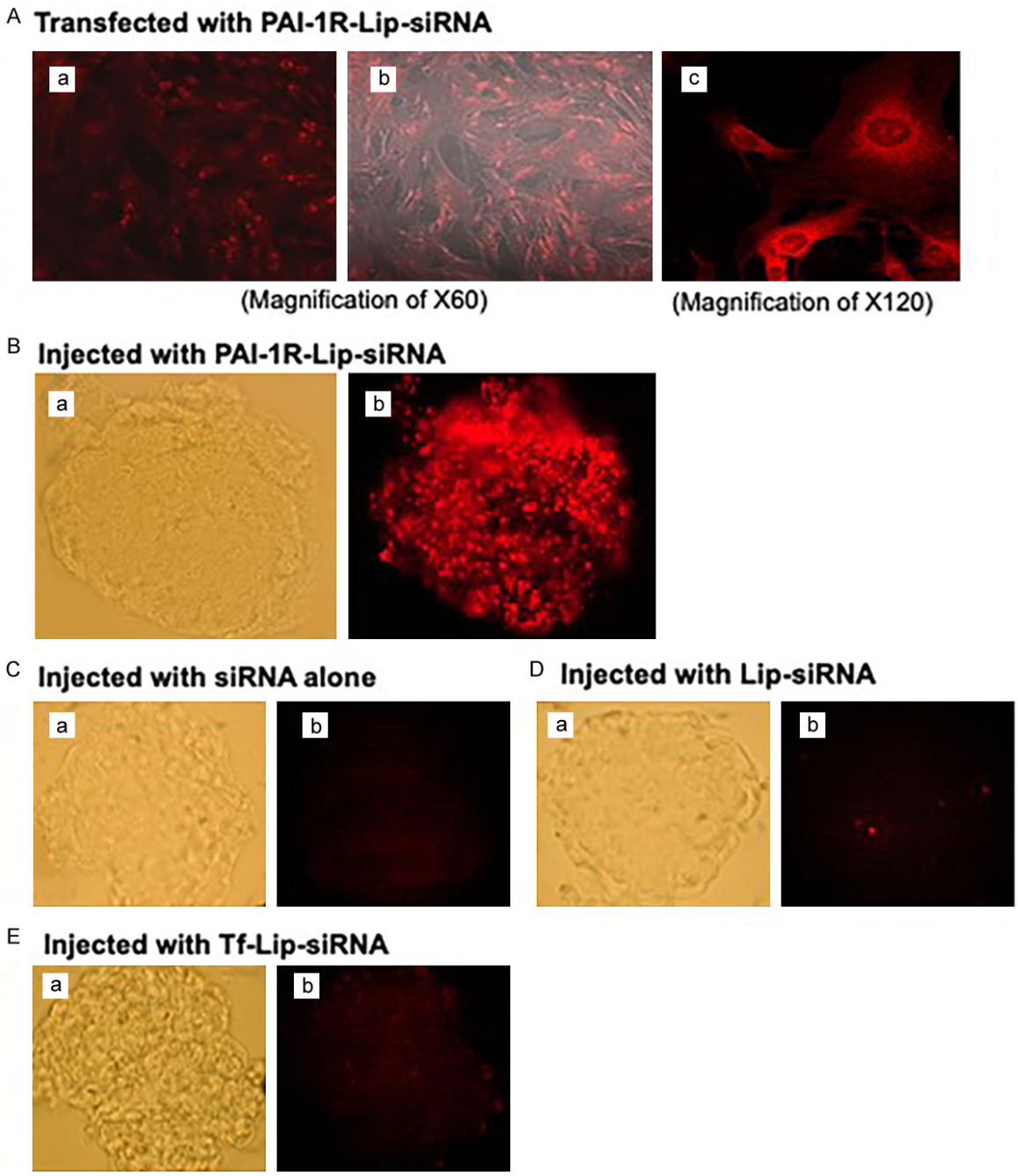


Figure 3. Intracellular and glomerular target of PAI-1R-Lip-sRNA. (A) Location of fluorescently labeled siRNA in renal mesangial cells transfected with PAI-1R-targted nano-liposomal siRNA complex in vitro. Transfected cells were imaged by a confocal microscopy at an original magnification of 60× or 120×. (B) In vivo specific glomerulus targeting of the PAI-1R targeted siRNA nanoparticles injected via the tail vein. A glomerulus from a rat sacrificed 6 h after PAI-1R-Lip-siRNA injection. The isolated glomerulus verified by light microscopy (light, a) showed strong red fluorescence staining of delivered siRNA (Fluorescence, b). (C-E) A glomerulus from rats sacrificed 2 h after injection with naked siRNA (C), Lip-siRNA (D), or Tf-Lip-siRNA (E), respectively. All isolated glomeruli verified by light microscopy (a, left panel of each paired photos,) did not show any significant red fluorescent staining of delivered siRNA when observed by fluorescent microscopy and shown on the right panels (b). Magnification: 400×.

First, the efficiency of PAI-1R-Lip-TGFβ1siRNA on MCs was monitored by assessing cellular TGFβ1 mRNA expression by real time RT-PCR. In cultured rat MCs, administration of the PAI-1R-Lip-siRNA that targets the TGFβ1 gene was shown to induce a dose-dependent decrease

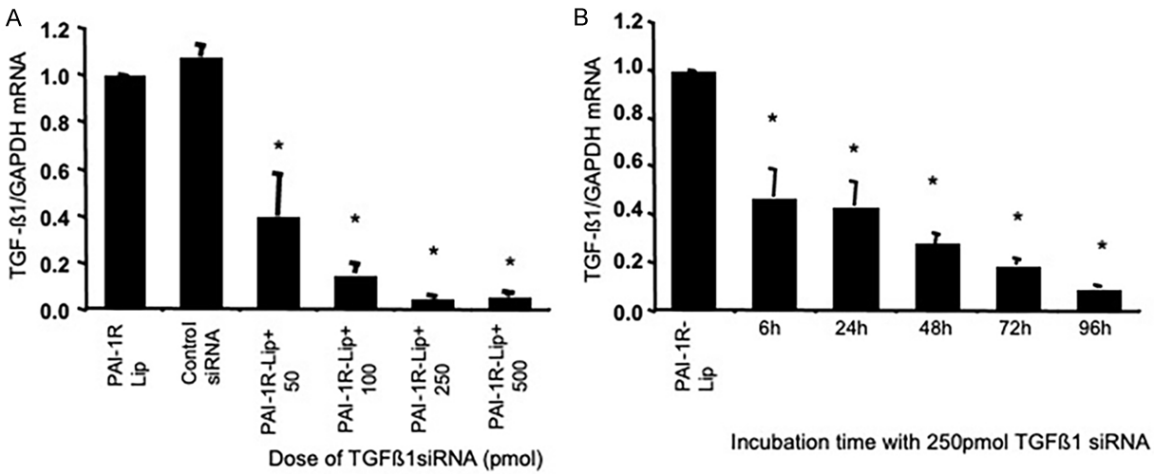


Figure 4. Effect of TGFβ1-siRNA *in vitro* on TGFβ1 mRNA expression by mesangial cells transfected with PAI-1R-targeted nanoparticles. The TGFβ1 mRNA levels were determined by real time RT-PCR and normalized to GAPDH mRNA levels for equal loading. Relative mRNA levels are expressed relative to PAI-1R-Lip transfected, no siRNA added control (PAI-IR-Lip), which is set at 1. Transfection of a Stealth™ siRNA molecule that targets the TGFβ1 was shown to induce dose-dependent (A) and time-dependent decreases (B) in TGFβ1 mRNA expression. *P<0.05 vs. cells transfected with PAI-1R-Lip control.

in TGFβ1 mRNA levels after 48 h (**Figure 4A**) and a time-dependent, 90% reduction in TGFβ1 mRNA levels at 96 h (**Figure 4B**), as compared with controls transfected with PAI-1R-Lip without siRNA added. These results demonstrate the efficient siRNA sequences against TGFβ1 gene.

Second, using the PAI-1R coated Lip-TGFβ1-siRNA particles for targeted gene delivery in vivo we investigated the therapeutic potential of TGFβ1siRNA in experimental glomerulonephritis in rats where high levels of TGFβ1 occur locally in glomeruli. Based on a pilot experiment (data not shown), a single injection of 48 μg siRNA/kg BW was given at 24 h (day 1) after disease induction and all rats were sacrificed at day 5, when the maximal reduction in glomerular TGFβ1 mRNA levels was achieved. As shown in **Figure 5**, TGFβ1siRNA administration significantly reduced elevated glomerular TGFβ1 mRNA expression and protein production seen in disease group by 89% and 65% respectively, determined by real time RT-PCR and ELISA (**Figure 5A**, **5B**). Of note, there was no changes on TGFβ1 mRNA expression in renal medulla where no glomeruli exist, as well as in other extra-renal organs such as lung, spleen, and artery (**Figure 5C**). However, liver TGFβ1 mRNA levels were unexpectedly reduced by 61.2% in disease group after treatment (**Figure 5D**). Treatment had no effect on body weight and food intake (data not shown).

To assess the biological effect of reduced TGFβ1 levels we measured the urine protein and disease severity for glomerulosclerosis in this nephritic model. As shown in **Figure 6A**, this treatment showed a trend of reduction on disease-induced urinary protein excretion but did not reach the significant levels (P>0.05). However, a significant reduction in glomerular matrix accumulation was observed after one single injection of PAI-1R-Lip-TGFβ1siRNA particles (**Figure 6B**). Furthermore, increased glomerular PAI-1 (**Figure 6C**) and FN (**Figure 6D**) mRNA expression and FN protein production (**Figure 6E**) seen in disease group were decreased by 79.1%, 48.8% and 53.4% after treatment. These results indicate that a single dose of PAI-1R-Lip-TGFβ1siRNA inhibits glomerular TGFβ1 specifically and efficiently *in vivo* thereby ameliorating glomerulosclerosis without affecting most of other organs.

Discussion

It has been well evidenced that the glomerulus is appropriate for nanotechnology-based targeted drug/gene delivery on account of the enhanced permeability and retention effect of its unique architecture. Therefore, nanoparticles designed to have diameters between 50 and 130 nm with selective charge are able to extravasate through the glomerular vasculature and further deposited and retained in the mesangial space. The present study has dem-

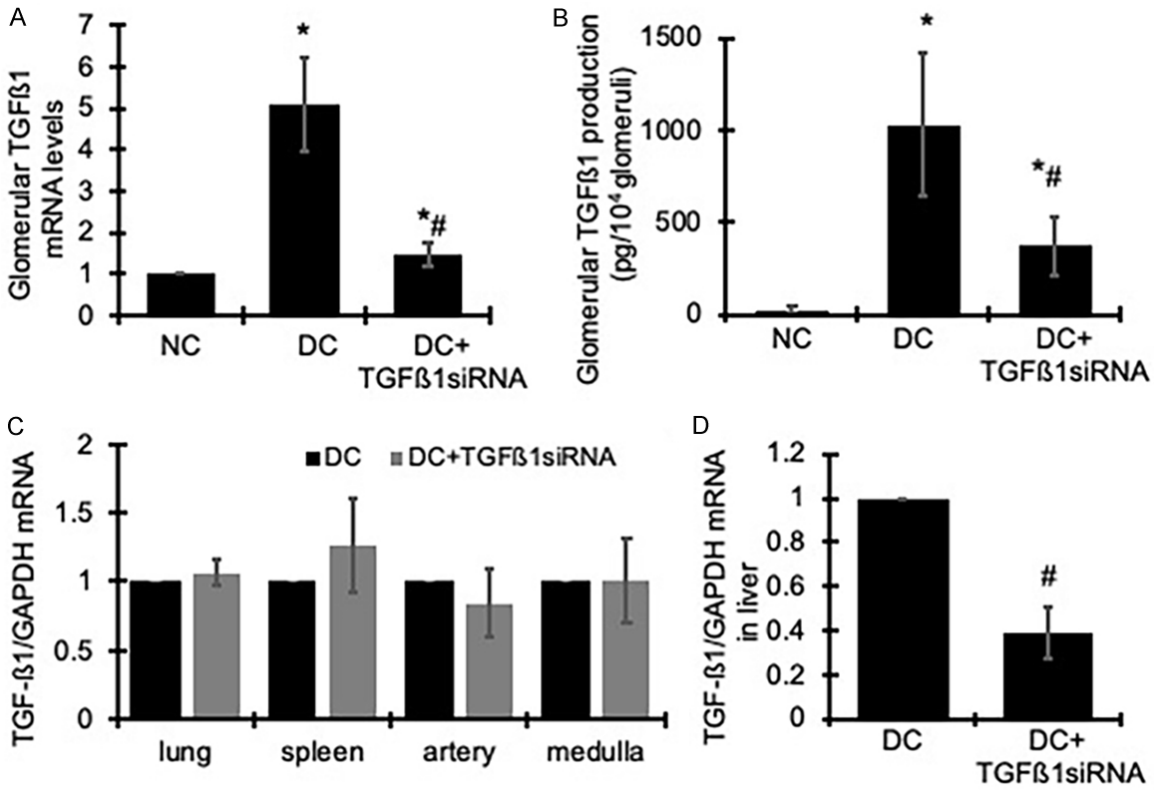


Figure 5. Effect of TGFβ1-siRNA *in vivo* on glomerular and extra-glomerular TGFβ1 mRNA expression in the nephritic rat model at day 5, delivered by PAI-1R targeted nanoparticles. (A) Glomerular TGFβ1 mRNA levels were determined by real time RT-PCR and normalized to GAPDH mRNA levels for equal loading. (B) Glomerular TGFβ1 protein levels were determined by ELISA. Relative mRNA or protein levels are expressed relative to their levels in normal control rats, which were set at unity. (C, D) TGFβ1 mRNA levels in lung, spleen, artery and renal medulla (C) and liver (D) were determined by real time RT-PCR and normalized to GAPDH mRNA levels for equal loading. Relative mRNA levels are expressed relative to their levels in disease control rats (DC), which are set at unity. *P<0.05 vs. normal control rats (NC); #P<0.05 vs. disease control rats (DC). DC+TGFβ1siRNA, diseased rats treated with PAI-1R-Lip-TGFβ1siRNA. N=6/per group.

onstrated that the lipoplex-coupled nanoparticles with an appropriate size and selective charge, and decorated with specific cell targeting agents such as PAI-1R (PAI-1R-Lip-siRNA) can be used as potent vehicles for targeted siRNA/or gene delivery to the glomerulus, specifically to the glomerular mesangial cells, with little extraneous binding to other tissues following systemic injection into the tail vein in rats. Internalization of PAI-1R-Lip-siRNA by glomerular cells, followed by cytoplasmic accumulation, was demonstrated by confocal microscopy both *in vitro* and *in vivo,* as shown by the increase in fluorescence. The mechanisms of internalization have been extensively described previously [7, 8, 13]. The ability of PAI-1R to bind to vitronectin and complex with uPAR and $a_v\beta_3$ integrin may drive the particles to bind to glomerular cells and be internalized [37]. After cellular internalization, the PAI-1R-Lip-siRNA nanoparticles, similar to other lipoplex-coupled nanoparticles, may deliver siRNA into the cytoplasm by cell membrane fusion or lysosome membrane fusion after endocytosis or both phenomena. We have shown that PAI-1R reaches and/or binds to glomerular cells as early as 10 min after iv injection and stays there for at least 12 h [22]. In the present study, we also observed PAI-1R-Lip-siRNA accumulation in glomeruli at 2 h and lasting at least 6 h after intravenous injection. Although we did not check whether glomerular PAI-1R-Lip-siRNA uptake occurred at early time point (<2 h) or later than 6 h after injection, such time period of 6 h is sufficient for PAI-1R-Lip-siRNA to bind to the surface of glomerular cells and to be internalized to intracellular cytoplasm. Our in vitro observation that all transfected cells

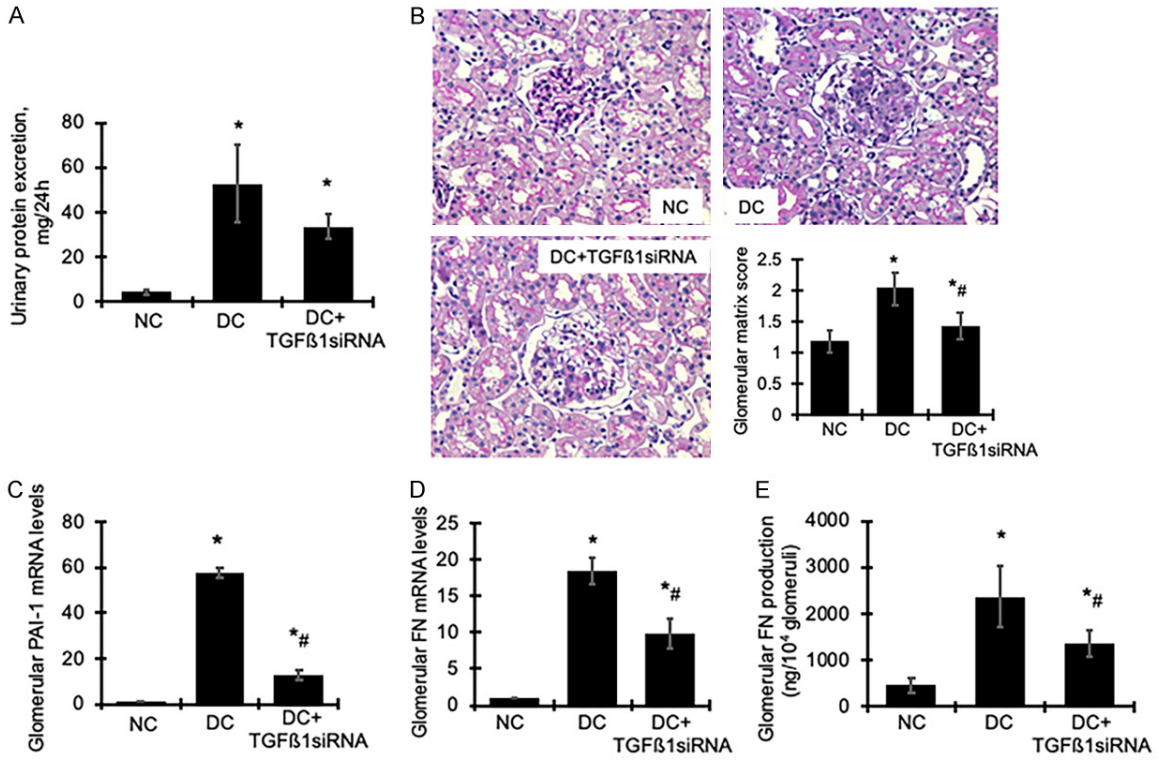


Figure 6. Effect of TGFβ1-siRNA *in vivo* on disease severity measurement in the nephritic rats at day 5, delivered by PAI-1R targeted nanoparticles. (A) 24-h urinary protein excretion from day 4 to day 5. (B) Representative photomicrographs of glomeruli from normal control (NC), disease control (DC) and PAI-1R-Lip-TGFβ1siRNA treated disease rats (DC+TGFβ1siRNA) stained with PAS at day 5. Graphic representation of glomerular matrix score was shown on the right corner. (C-E) The relative levels of glomerular mRNA expression of PAI-1 (C) and FN (D) were standardized to GAPDH mRNA levels. Relative mRNA levels are expressed relative to their levels in normal control rats (NC), which are set at unity. (E) Glomerular FN protein levels were determined by ELISA. *P<0.05 vs. normal control rats (NC); #P<0.05 vs. disease control rats (DC). N=6/per group.

exhibited intense, diffuse red fluorescence in cellular cytoplasm at 6 h after delivery also supports this expectation in cellular internalization of PAI-1R-Lip-siRNA in vivo. In addition, the surface charge of nanoparticles is a key factor affecting tissue uptake of nanoparticles. It has been shown that nanoparticles with a surface charge of <15 mV have minimal macrophage uptake and long circulation time [21]. Apparently, the PAI-1R-Lip-siRNA nanoparticles with a close to neutral surface charge of ~+5.6 mV may be ideal for the kidney uptake but avoiding circulating macrophage uptake or degradation after intravenous injection. In contrast, Lip-siRNA particles without targeting or specific targeting were almost not observable in glomeruli at either 2 h or 6 h after injection. Together with no PAI-1R binding in most of important organs such as heart, lung and spleen, these findings demonstrate that PAI-1R-Lip-siRNA particles delivering siRNA into glomerular cells are specific and effective and mediated by the specific peptide targeting. As expected, specific TGFβ1siRNA delivered by the targeted nanoparticles resulted in significant inhibition of TGFβ1 gene expression and action in glomeruli thereby specifically ameliorating glomerulosclerosis in the nephritic rat model. The success of this nanoparticle delivery system suggests that the targeted siRNA delivery to glomeruli is achievable. The selective silencing of the TGF-β1 overexpression in diseased glomeruli may represent a promising strategy for the treatment of fibrotic glomerular disease without remarkable systemic side-effects. This targeted delivery method has been repeatedly producing a similar targeted therapeutic effect in glomeruli in this nephritic rat model when a different profibrotic molecule gene such as PAI-1 or prorenin receptor (PRR) was targeted by RNAi in our laboratory (unpublished data). Furthermore, besides the therapeutic potential of

PAI-1R itself that has been demonstrated previously for glomerulosclerosis induced by nephritis or diabetes [22, 24], the human peptide, PAI-1R, is capable to directing the nanoparticle-siRNA lipoplex therapeutics to diseased kidneys in humans. Therefore, the present study may serve as a translational stage for future development and application of a novel targeted therapeutic tool for the treatment of kidney disease.

However, in agreement with the fact that a few of injected PAI-1Rs bind to glomerular podocytes (**Figure 1C**), PAI-1R targeted-Lip-siRNA particles that become much larger than PAI-1R alone in molecular size may become even hard to reach at glomerular podocytes in the present studies. It has been revealed that the marked proteinuria in anti-Thy1.1 glomerulonephritis also results from the podocyte dysfunction such as podocyte foot enfacement and even podocyte loose [34]. We did not examine podocyte number and structural changes after treatment here. Nonetheless, the limitation of this delivery targeting may predict the limited inhibitory effect of PAI-1R-Lip-TGFβ1siRNA particles on podocytes, which may explain no significant changes or less effect found in disease-induced urinary protein excretion after treatment (**Figure 6A**). In addition, a study with increased animal number may be needed in order to further determine the effect of this treatment on proteinuria.

A variety of targeting peptides and antibodies have been developed and utilized with nanoparticles to target the kidney and kidney cells [38, 39]. Compared with those practicing methods for glomerular targeting drug/gene delivery, our study characterizes a unique targeted-nanoparticle siRNA/gene carrier using a kidney-specific and safe peptide, PAI-1R, to achieve very high renal specificity, and the optimized lipoplex to form a size-selective nanoparticle with an optimum surface charge to facilitate the efficacy of the delivery carrier to kidney cells. The intrarenal distribution of PAI-1R that was mostly in glomerular cells, not tubular, is consistent with the effect of PAI-1R-Lip-carried TGFβ1siRNA after intravenous systemic administration in vivo that only inhibits TGFβ1 mRNA expression and protein production in glomeruli, not renal tubular. This demonstrates the efficient and specific kidney cell targeting. In addition, our study revealed that sustained TGFβ1 inhibition in glomeruli with a single intravenous systemic injection of PAI-1R-Lip-TGFβ1siRNA provided significant therapeutic benefits to glomerular fibrosis in this acute nephritic rat model, indicating a long-lasting effect of this delivery method. However, increasing dosage or administration frequency of this treatment may be needed for chronic glomerular disease such as diabetes. Since PAI-1R itself is also therapeutic for glomerular diseases [22, 24], it is unlikely that increased amount of peptide targeting, PAI-1R, due to increased administration dosage or frequency of PAI-1R-coated nanoparticles, will cause any cytotoxicity. In addition, multiple doses of the lipoplex-coupled nanoparticles using the same lipoplex have been shown to be well-tolerated in cancer treatment in both animal models and humans, which are currently in Phase II clinical trials for tumor-targeted gene therapy (NCT00470613, NCT02340117, NCT02340156, ClinicalTrials.gov). Thus, our delivery system with these two main components (PAI-1R and lipoplex) should be safe and effective if multiple administration is needed for a long-term treatment. One question is whether the therapeutic effect of PAI-1R-Lip-TGFβ1siRNA is due to PAI-1R itself in the anti-Thy1.1 nephritic rat model. First, the dose of PAI-1R used for coating Lip-siRNA molecules is 0.34 mg/kg BW for a single iv injection, which is much less than the therapeutic dosage (1 mg/kg BW, iv, twice a day for 5 days) [22]. Second, treatment with PAI-1R alone reduced glomerular TGFβ1 secretion but did not affect TGFβ1 mRNA expression, possibly through increasing TGFβ1 clearance [22], which is different with the renal outcome achieved in the present treatment. Therefore, it is less possible that the reduction of glomerular TGFβ1 expression and generation and the amelioration of glomerular ECM accumulation after treatment with PAI-1R-Lip-TGFβ1siRNA in nephritic rats are due to a direct effect of PAI-1R on diseased glomeruli.

Based on the liver's microanatomical features, liver Kupffer cells that comprise 80% of the entire macrophage population within the body will first interact with Lip-siRNA nanoparticles and have high phagocytic ability for these particles following intravenous injection and entering into liver sinusoid. It has been shown that three major factors such as negative charges

that can be easily recognized by class-A scavenger receptor on Kupffer cells, or mannose- and fucose-type receptors coated Lip-nanoparticles that can be leveraged to selectively target those particles to Kupffer cells, or blood complement factors and serum proteins coated lip-nanoparticles that usually cause mononuclear phagocyte sequestration, may result in Kupffer cell clearance of Lip-siRNA nanoparticles [40, 41]. Although the design of PAI-1R-coated Lip-siRNA maximally avoids the influence associated with these three factors, it is still possible that a small portion of PAI-1R-Lip-TGFβ1siRNA particles is entrapped by liver Kupffer cells following intravenous injection, thereby resulting in reduction of TGFβ1 mRNA expression in liver tissue. On the other hand, Kupffer cells may be the main resource to express TGFβ1 in liver tissue. It is needed to further modify the Lip or PAI-1R to prevent the loss of PAI-1R-coated Lip-siRNA from the liver Kupffer cells. Nonetheless, it is apparent that the majority of intravenously administrated PAI-1R-Lip-TGFβ1siRNA particles still reach the kidneys since glomerular delivery targeting and action of those particles have been well evident in the present study.

In conclusion, our study demonstrates that the nanoparticles with an appropriate size and decorated with specific and safe cell targeting agents can target glomerular mesangial cells with little extraneous binding to other tissues. Specific siRNA therapeutics delivered by the targeted nanoparticles resulted in significant inhibition of target gene expression in glomeruli *in vivo*. The success of this nanoparticle delivery system suggests that the targeted siRNA delivery to glomeruli is achievable. Undoubtedly, the efficacy of this targeted delivery of siRNA or genes to glomeruli in a chronic kidney disease model by repeated intravenous injections needs to be further determined. Nonetheless, the selective silencing of the TGF-β1 overexpression in diseased kidney may represent a promising strategy with translational potential for the treatment of fibrotic glomerular disease in humans in future once the long-term fate and toxicity of those nanoparticles in the body are further defined.

Acknowledgements

We thank Linda Hoge for her excellent technical assistance with animal studies, and Dr. You Bae for providing the dynamic laser light scattering (DLS) measurement. The work was supported by National Institute of Health Grants K01DK077955 (Y.H.), R21DK081815 (Y.H.), the American Diabetes Association (No. 1-17-IBS-312) (Y.H.) and the National Natural Science Foundation of China (No. 81670665 to Xia Liu).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Yufeng Huang, Division of Nephrology & Hypertension, Department of Internal Medicine, University of Utah Health Science, Wintrobe Rm 403, Salt Lake City, Utah 84132, USA. Tel: 801-585-0581; Fax: 801-213-2563; E-mail: yufeng.huang@hsc.utah.edu

References

[1] Hede K. Blocking cancer with RNA interference moves toward the clinic. J Natl Cancer Inst 2005; 97: 626-628.

[2] Liu H, Marquez RT, Wu X, Li K, Vadlamani S, Li S, Wang Y, Xu L and Wu D. A non-intrusive evaluation method for tumor-targeting characteristics of nanomedicines based on in vivo near-infrared fluorescence imaging. J Mater Chem B 2019; 7: 4751-4757.

[3] Li K, Liu H, Gao W, Chen M, Zeng Y, Liu J, Xu L and Wu D. Mulberry-like dual-drug complicated nanocarriers assembled with apogossypolone amphiphilic starch micelles and doxorubicin hyaluronic acid nanoparticles for tumor combination and targeted therapy. Biomaterials 2015; 39: 131-144.

[4] Lu J, Zhao W, Liu H, Marquez R, Huang Y, Zhang Y, Li J, Xie W, Venkataramanan R, Xu L and Li S. An improved D-alpha-tocopherol-based nanocarrier for targeted delivery of doxorubicin with reversal of multidrug resistance. J Control Release 2014; 196: 272-286.

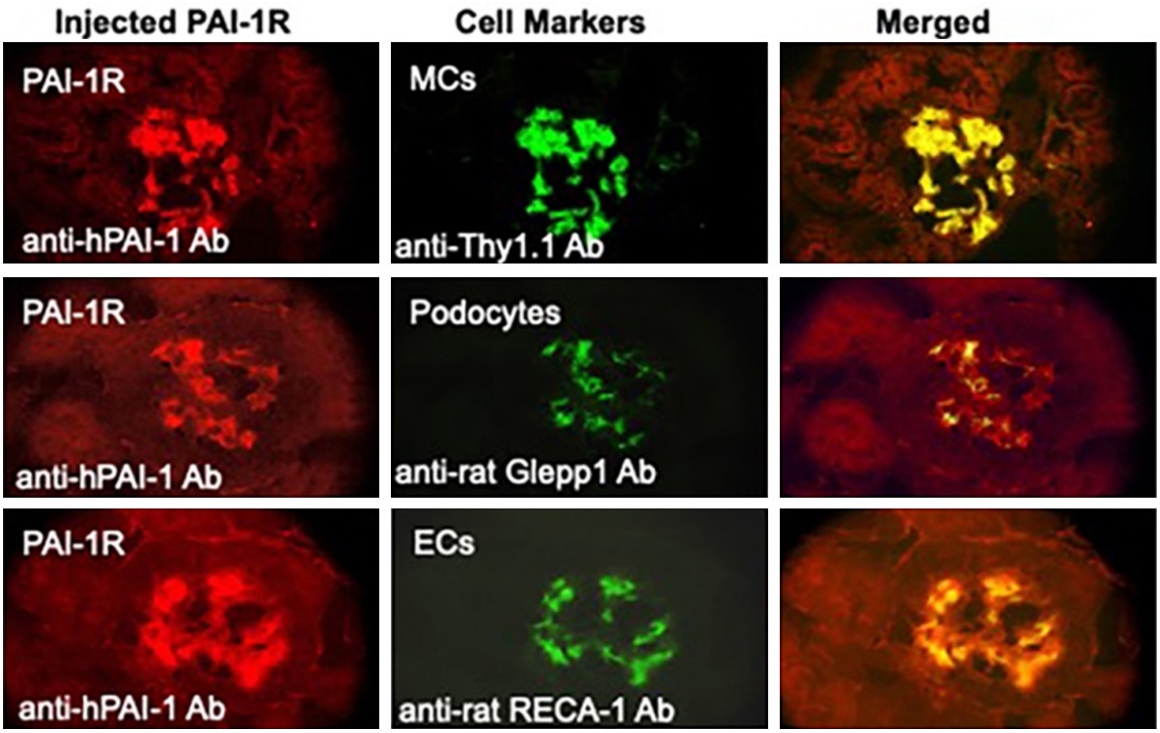
[5] Lu J, Zhao W, Huang Y, Liu H, Marquez R, Gibbs RB, Li J, Venkataramanan R, Xu L and Li S. Targeted delivery of doxorubicin by folic acid-decorated dual functional nanocarrier. Mol Pharm 2014; 11: 4164-4178.

[6] Xu L, Huang CC, Huang W, Tang WH, Rait A, Yin YZ, Cruz I, Xiang LM, Pirollo KF and Chang EH. Systemic tumor-targeted gene delivery by anti-transferrin receptor scFv-immunoliposomes. Mol Cancer Ther 2002; 1: 337-346.

[7] Xu L, Frederik P, Pirollo KF, Tang WH, Rait A, Xiang LM, Huang W, Cruz I, Yin Y and Chang EH. Self-assembly of a virus-mimicking nanostructure system for efficient tumor-targeted

gene delivery. Hum Gene Ther 2002; 13: 469-481.
[8] Xu L, Tang WH, Huang CC, Alexander W, Xiang LM, Pirollo KF, Rait A and Chang EH. Systemic p53 gene therapy of cancer with immunolipoplexes targeted by anti-transferrin receptor scFv. Mol Med 2001; 7: 723-734.
[9] Xu L, Pirollo KF, Tang WH, Rait A and Chang EH. Transferrin-liposome-mediated systemic p53 gene therapy in combination with radiation results in regression of human head and neck cancer xenografts. Hum Gene Ther 1999; 10: 2941-2952.
[10] Kostarelos K and Miller AD. Synthetic, self-assembly ABCD nanoparticles; a structural paradigm for viable synthetic non-viral vectors. Chem Soc Rev 2005; 34: 970-994.
[11] Torchilin VP. Recent advances with liposomes as pharmaceutical carriers. Nat Rev Drug Discov 2005; 4: 145-160.
[12] Kaneda Y and Tabata Y. Non-viral vectors for cancer therapy. Cancer Sci 2006; 97: 348-354.
[13] Varga CM, Wickham TJ and Lauffenburger DA. Receptor-mediated targeting of gene delivery vectors: insights from molecular mechanisms for improved vehicle design. Biotechnol Bioeng 2000; 70: 593-605.
[14] Pirollo KF, Zon G, Rait A, Zhou Q, Yu W, Hogrefe R and Chang EH. Tumor-targeting nanoimmunoliposome complex for short interfering RNA delivery. Hum Gene Ther 2006; 17: 117-124.
[15] Senzer N, Nemunaitis J, Nemunaitis D, Bedell C, Edelman G, Barve M, Nunan R, Pirollo KF, Rait A and Chang EH. Phase I study of a systemically delivered p53 nanoparticle in advanced solid tumors. Mol Ther 2013; 21: 1096-1103.
[16] Perico N, Benigni A and Remuzzi G. Present and future drug treatments for chronic kidney diseases: evolving targets in renoprotection. Nat Rev Drug Discov 2008; 7: 936-953.
[17] McGrogan A, Franssen CF and de Vries CS. The incidence of primary glomerulonephritis worldwide: a systematic review of the literature. Nephrol Dial Transplant 2011; 26: 414-430.
[18] Striker L, Peten E, Elliot S, Doi T and Striker G. Mesangial cell turnover: effect of heparin and peptide growth factors. Lab Invest 1991; 64: 446-456.
[19] Kashgarian M and Sterzel RB. The pathobiology of the mesangium. Kidney Int 1992; 40: 524-529.
[20] Visweswaran GR, Gholizadeh S, Ruiters MH, Molema G, Kok RJ and Kamps JA. Targeting rapamycin to podocytes using a vascular cell adhesion molecule-1 (VCAM-1)-harnessed SAINT-based lipid carrier system. PLoS One 2015; 10: e0138870.
[21] Bruni R, Possenti P, Bordignon C, Li M, Ordanini S, Messa P, Rastaldi MP and Cellesi F. Ultrasmall polymeric nanocarriers for drug delivery to podocytes in kidney glomerulus. J Control Release 2017; 255: 94-107.
[22] Huang Y, Haraguchi M, Lawrence DA, Border WA, Yu L and Noble NA. A mutant, noninhibitory plasminogen activator inhibitor type 1 decreases matrix accumulation in experimental glomerulonephritis. J Clin Invest 2003; 112: 379-388.
[23] Huang Y, Border WA, Lawrence DA and Noble NA. Noninhibitory PAI-1 enhances plasmin-mediated matrix degradation both in vitro and in experimental nephritis. Kidney Int 2006; 70: 515-522.
[24] Huang Y, Border WA, Yu L, Zhang J, Lawrence DA and Noble NA. A PAI-1 mutant, PAI-1R, slows progression of diabetic nephropathy. J Am Soc Nephrol 2008; 19: 329-338.
[25] Zhang J, Gu C, Lawrence DA, Cheung AK and Huang Y. A PAI-1 mutant retards diabetic nephropathy in db/db mice through protecting podocytes. Exp Physiol 2014; 99: 802-815.
[26] Border WA and Noble NA. Transforming growth factor beta in glomerular injury. Exp Nephrol 1994; 2: 13-17.
[27] Ruiz-Ortega M, Rayego-Mateos S, Lamas S, Ortiz A and Rodrigues-Diez RR. Targeting the progression of chronic kidney disease. Nat Rev Nephrol 2020; 16: 269-288.
[28] Border WA and Noble NA. Transforming growth factor beta in tissue fibrosis. N Engl J Med 1994; 331: 1286-1292.
[29] Tracey WR, Xue C, Klinghofer V, Barlow J, Pollock JS, Förstermann U and Johns RA. Immunochemical detection of inducible NO synthase in human lung. Am J Physiol 1994; 266: L722-L727.
[30] Huang Y, Border WA, Lawrence DA and Noble NA. Mechanisms underlying the antifibrotic properties of noninhibitory PAI-1 (PAI-1R) in experimental nephritis. Am J Physiol Renal Physiol 2009; 297: F1045-F1054.
[31] Stealth RNAi: from in vitro to in vivo. Quest an Invitrogen Publication for Discovery 2006; 3: 55-59.
[32] Yu L, Border WA, Anderson I, McCourt M, Huang Y and Noble NA. Combining TGF-β inhibition and angiotensin II blockade results in enhanced anti-fibrotic effect. Kidney Int 2004; 66: 1774-1784.
[33] Zhang J, Noble N, Border PW, Owens RT and Huang Y. Receptor-dependent prorenin activation and induction of PAI-1 expression in vascular smooth muscle cells. Am J Physiol Endocrinol Metab 2008; 295: E810-E819.
[34] Gu C, Zhou G, Noble NA, Border WA, Cheung AK and Huang Y. Targeting reduction of pro-

teinuria in glomerulonephritis: maximizing the antifibrotic effect of valsartan by protecting podocytes. J Renin Angiotensin Aldosterone Syst 2014; 15: 177-189.
[35] Xu L, Pirollo KF and Chang EH. Tumor-targeted p53-gene therapy enhances the efficacy of conventional chemo/radiotherapy. J Control Release 2001; 74: 115-128.
[36] Border WA, Okuda S, Languino LR, Sporn MB and Ruoslahti E. Suppression of experimental glomerulonephritis by antiserum against transforming growth factor beta 1. Nature 1990; 346: 371-374.
[37] Wei C, Moller CC, Altintas MM, Li J, Schwarz K, Zacchigna S, Xie L, Henger A, Schmid H, Rastaldi MP, Cowan P, Kretzler M, Parrilla R, Bendayan M, Gupta V, Nikolic B, Kalluri R, Carmeliet P, Mundel P and Reiser J. Modification of kidney barrier function by the urokinase receptor. Nat Med 2008; 14: 55-63.
[38] Zuckerman JE and Davis ME. Targeting therapeutics to the glomerulus with nanoparticles. Adv Chronic Kidney Dis 2013; 20: 500-507.
[39] Liu CP, Hu Y, Lin JC, Fu HL, Lim LY and Yuan ZX. Targeting strategies for drug delivery to the kidney: from renal glomeruli to tubules. Med Res Rev 2019; 39: 561-578.
[40] Poon W, Zhang YN, Ouyang B, Kingston BR, Wu JLY, Wilhelm S and Chan WCW. Elimination pathways of nanoparticles. ACS Nano 2019; 13: 5785-5798.
[41] Witzigmann D, Kulkarni JA, Leung J, Chen S, Cullis PR and van der Meel R. Lipid nanoparticle technology for therapeutic gene regulation in the liver. Adv Drug Deliv Rev 2020; 159: 344-363.



Supplementary Figure 1. Injected PAI-1R mainly targeted glomerular cells in male diabetic db/db mice at 20 weeks of age after intraperitoneal (ip) injection. The indirect glomerular immunofluorescent staining for PAI-1R and glomerular cells was performed on 3 μm cryostat sections at 3 h after PAI-1R injection at 2 mg/kg body weight (n=3). The goat anti-human PAI-1 (hPAI-1) IgG (American Diagnostic Inc. Greenwich CT) (which does not cross react with mouse endogenous PAI-1), mouse anti-rat-glomerular epithelial protein 1 (Glepp1) IgG (to stain glomerular podocytes) (kindly provided by Dr. Roger Wiggins, Division of nephrology, University of Michigan, Ann Arbor, MI, USA), mouse anti-rat endothelial cell antigen (RECA-1) IgG (to stain glomerular endothelial cells (ECs)) (Serotec Ltd, Oxford, UK) and mouse anti-Thy1.1 IgG (to stain glomerular mesangial cells (MCs)) were used as the primary antibodies. TRITC-conjugated donkey anti-goat IgG and FITC-conjugated donkey anti-mouse IgG (1:100 dilution, Jackson ImmunoResearch Laboratories Inc.) were used as the secondary antibodies. Dual-immunostained sections were analyzed using a fluorescence microscope with double filters. Staining for PAI-1R (red) and glomerular cells (green) and double staining for PAI-1R and glomerular cells including glomerular mesangial cells, podocytes and endothelial cells shown in yellow. No PAI-1R staining was seen without PAI-1R injection in glomeruli. Magnification: 400×.