Supplemental Data

Clusterin/Apolipoprotein J Attenuates Renal Fibrosis in Obstructive Nephropathy

Gwon-Soo Jung,[1] Mi-Kyung Kim,[1] Yun-A Jung,[1] Hye-Soon Kim,[1] In-Sun Park,[2] Bon-Hong Min,[3] Ki-Up Lee,[4] Jung-Guk Kim,[5] Keun-Gyu Park,[5*] and In-Kyu Lee[5*]

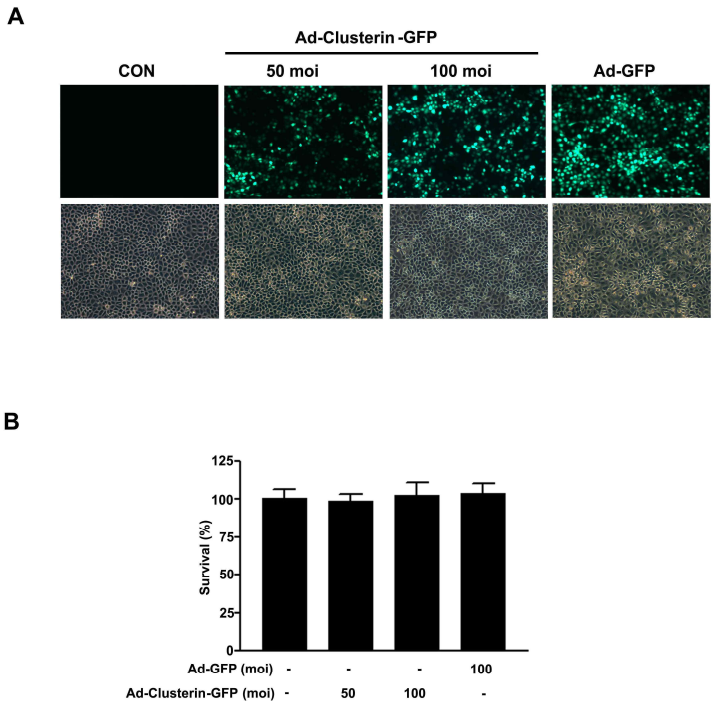


Figure S1. Infection efficiency and viability in NRK-52E cells after Ad-Clusterin-GFP infection.

(A) Representative fluorescence images after infection of adenovirus. Magnification, ×200. NRK-52E cells were infected with the indicated doses of Ad-Clusterin-GFP or

Ad-GFP (100 moi) for 24 h after 24 h serum starvation and then observed with fluorescence microscopy.

(B) MTT assay to demonstrate the effect of Ad-Clusterin-GFP or Ad-GFP on NRK-52E cell viability. Cells were infected with the indicated doses of Ad-Clusterin-GFP or Ad-GFP after 24 h serum starvation. Cell viability was measured using the MTT assay at 24 h after adenovirus infection and expressed as the percentage of viable cells present at the end of culture. Data are the mean ± SEM of three independent measurements.

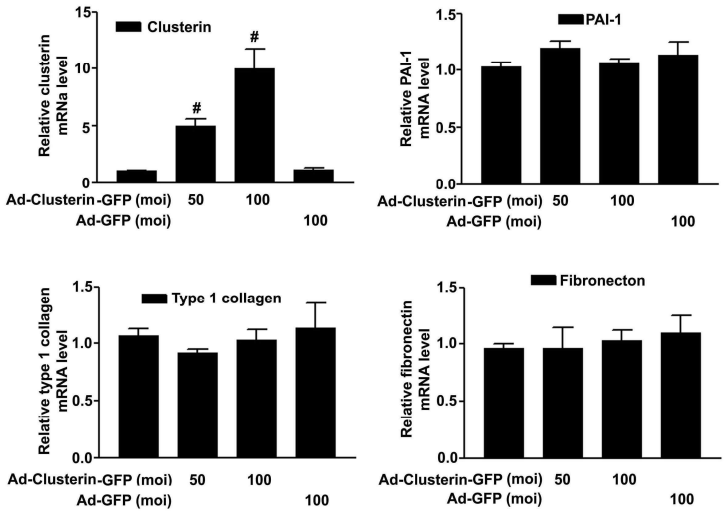


Figure S2. The effects of Ad-clusterin-GFP on the expression of pro-fibrotic genes in the TGF-β-untreated NRK-52E cells.

Representative real time RT-PCR analysis of the expression of PAI-1, type I collagen and fibronectin in TGF-β untreated NRK-52E cells. Cells were infected with the

indicated doses of Ad-Clusterin-GFP or Ad-GFP after 24 h serum starvation. The bar graphs show pro-fibrotic gene expression relative to the internal control GAPDH mRNA expression (=1). Data are the mean ± SEM of three independent measurements. $^{\#}P < 0.001$ compared with control.

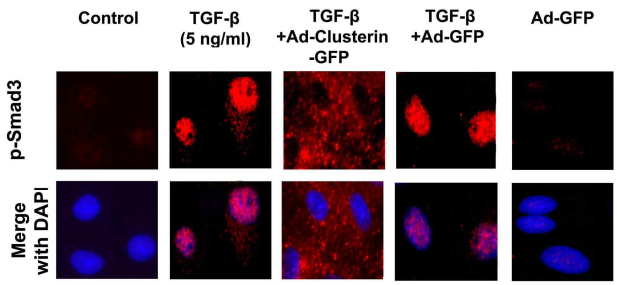


Figure S3. Effect of clusterin on p-Smad3 nuclear translocalization in TGF-β-treated NRK-52E cells.

Cells were incubated with TGF-β (5 ng/ml) after 24 h serum starvation and then infected with Ad-Clusterin-GFP (100 moi) or Ad-GFP (100 moi). Control cells were untreated. The cellular localization of p-Smad3 (red) and DAPI nuclear staining (blue) was examined using fluorescence microscopy. Magnification, ×400.

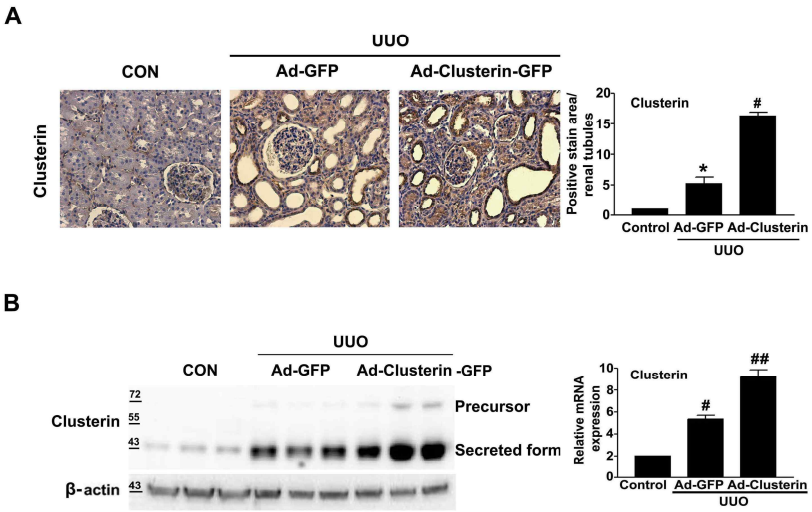
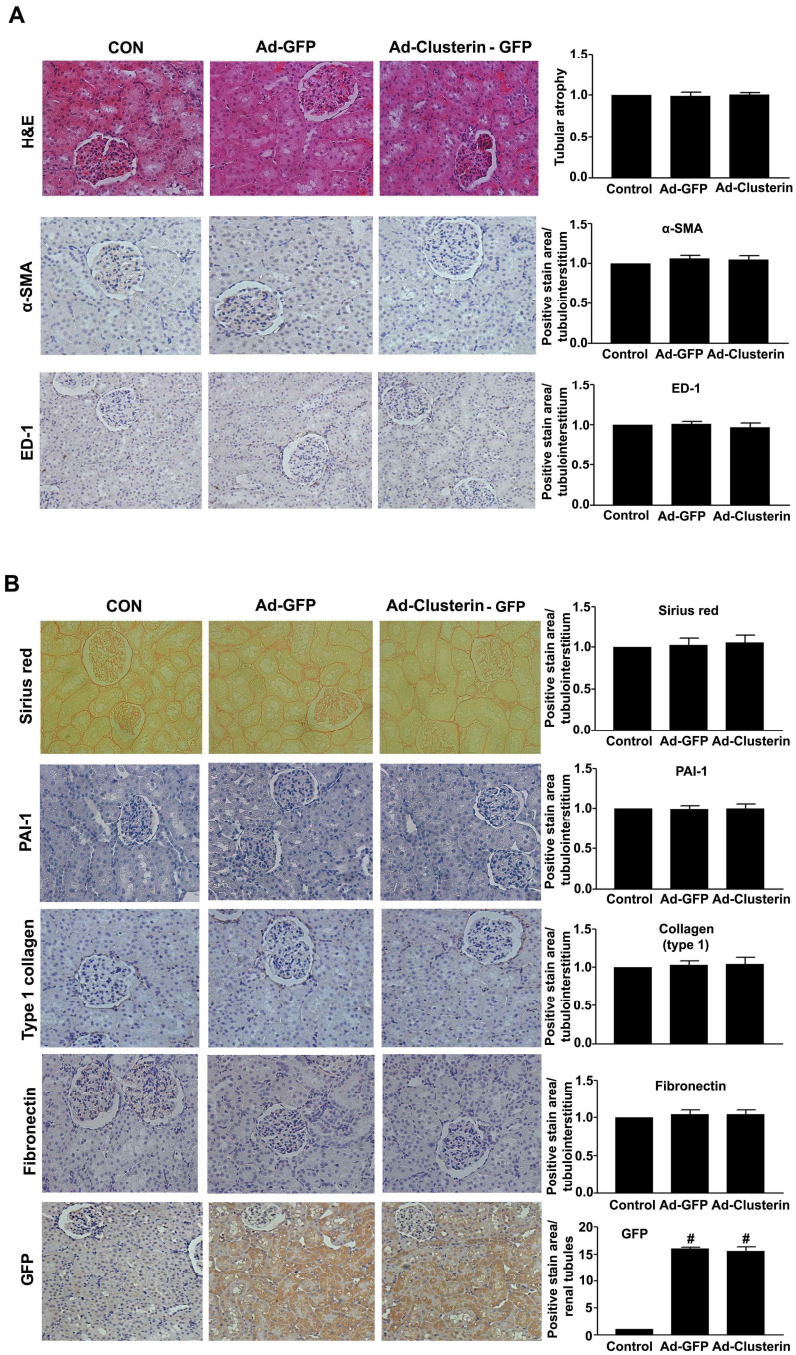


Figure S4. Expression of clusterin in the kidneys after UUO and Ad-Clusterin delivery.

(A) Representative images of immunohistochemical staining for clusterin expression in UUO kidneys infected with Ad-GFP or Ad-Clusterin-GFP. Magnification, ×200. Areas of positive immunostaining with clusterin were quantified by computer-based morphometric analysis. All data were normalized to the control (=1) and, in all bar graphs, expressed as fold increase in clusterin expression relative to the control. Data are the mean ± SEM of five random fields per kidney (n=5 in each group). $^{*}P < 0.01$ compared with control and $^{\#}P < 0.001$ compared with Ad-GFP.

(B) Representative Western blot analysis of clusterin protein expression in kidneys 7 days after UUO surgery and Ad-GFP or Ad-Clusterin-GFP infection. Data are the mean ± SEM of three independent measurements. $^{\#}P < 0.001$ compared with control and $^{\#\#}P$

< 0.001 compared with Ad-GFP.



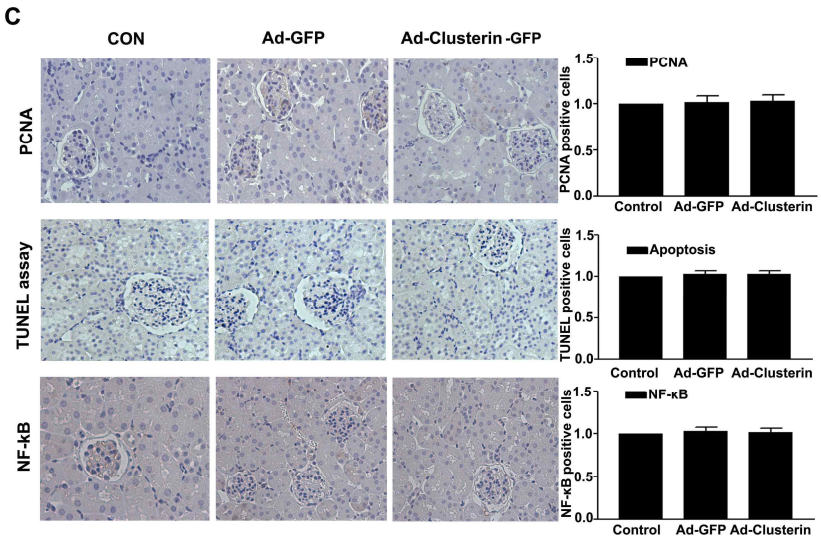


Figure S5. The effect of Ad-GFP and Ad-Clusterin-GFP on unobstructed kidneys.

(A) Representative images of H&E and immunohistochemical staining for α-SMA and ED-1 expression from the unobstructed rat kidneys infected with Ad-GFP or Ad-Clusterin-GFP. Magnification, ×200. The number of atrophic tubules in the kidneys was determined by measurement of abnormal irregular and dilated tubular basement membrane identified by H&E staining. Five random fields per kidney (n=5 in each group) were counted under high-power magnification. Areas of positive immunostaining with α-SMA and ED-1 were quantified by computer-based morphometric analysis. All data were normalized to the control (=1) and, in all bar graphs, were expressed as fold increase relative to the control. Data are the mean ± SEM of five random fields per kidney (n=5 in each group).

(B) Representative images of Sirius red and immunohistochemical staining for PAI-1,

type I collagen fibronectin and GFP expression from the unobstructed kidneys infected with Ad-GFP or Ad-Clusterin-GFP. Magnification, ×200. All data obtained by computer-based morphometric analysis of the positive area in the kidneys were normalized to the control (=1) and, in all bar graphs, were expressed as fold increase relative to the control. Data are the mean ± SEM of five random fields per kidney (n=5 in each group). $^{\#}P < 0.001$ compared with control.

(C) Representative images of immunohistochemical staining for PCNA, NF-kB and TUNEL from the unobstructed kidneys infected with Ad-GFP or Ad-Clusterin-GFP. Magnification, ×200. The number of PCNA-positive, NF-kB-positive and apoptotic cells was determined by counting positively stained tubular epithelial cells of renal tubules in five random fields per kidney (n=5 in each group) under high-power magnification. All data were normalized to the control (=1) and, in all bar graphs, were expressed as fold increase relative to the control.

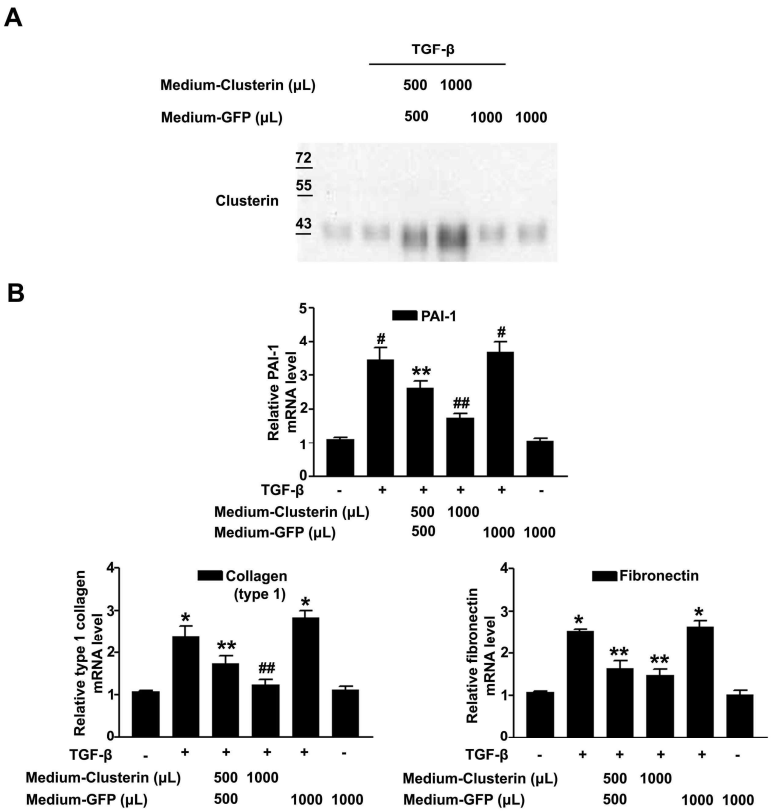
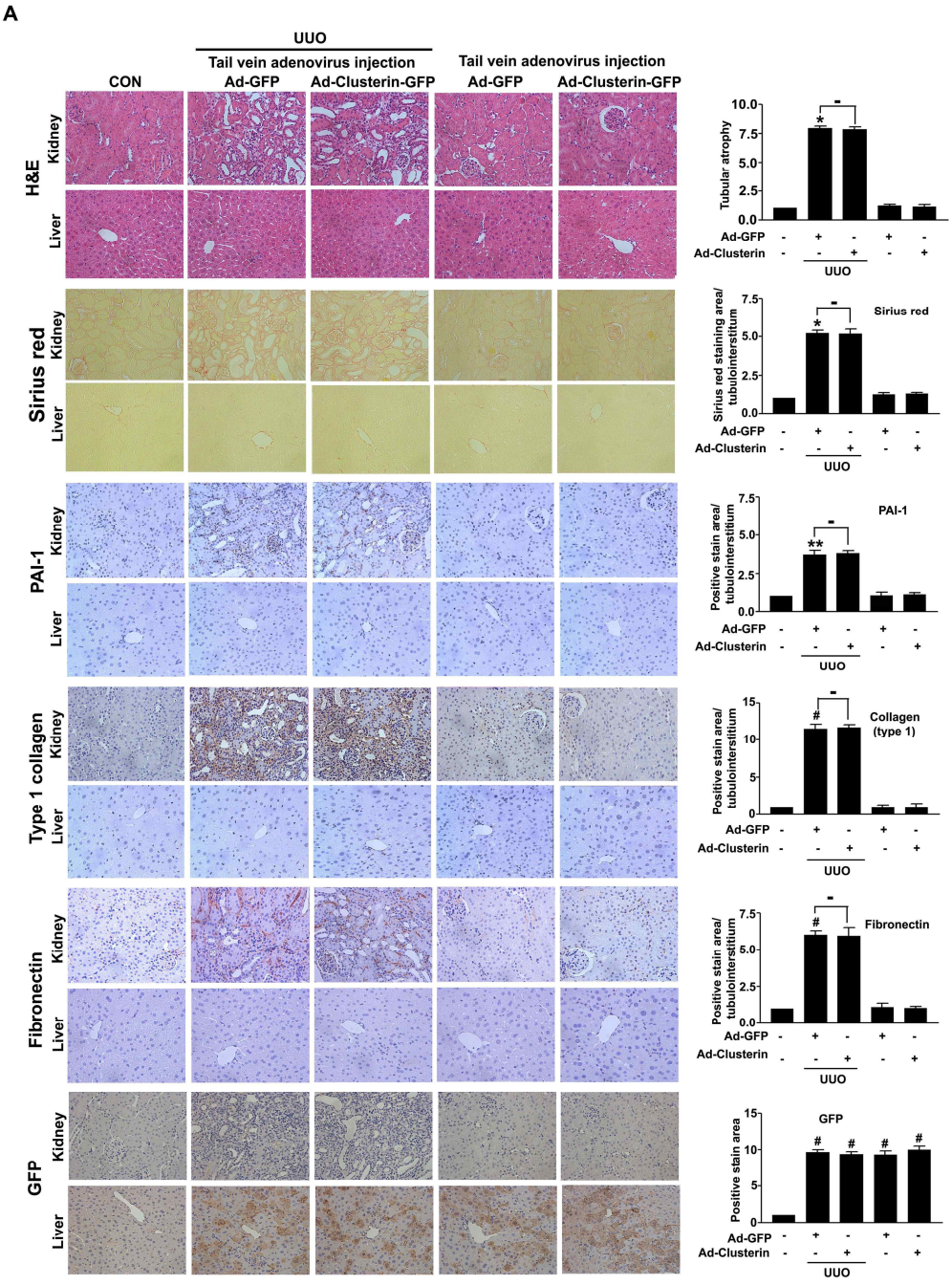


Figure S6. Effect of secreted clusterin on TGF-β induced PAI-1, type I collagen, and fibronectin expression in cultured NRK-52E cells.

(A) Representative Western blot analysis of secreted clusterin protein. NRK-52E cells infected with 100 moi of Ad-GFP or Ad-Clusterin-GFP for 2 h were incubated with serum free medium for 48 h, and culture (conditioned) medium was collected.

(B) Representative real time RT-PCR analysis of the effects of secreted clusterin on the expression of PAI-1, type I collagen, and fibronectin in TGF-β stimulated NRK-52E cells. Cells were incubated with conditioned medium (Medium-Clusterin or Medium-GFP) with TGF-β (2 ng/ml, 3 h) for 24 h. GAPDH mRNA was used as an internal

control. Data are the mean ± SEM of three independent measurements (three separate experiments). $^{*}P < 0.01$, $^{\#}P < 0.001$ compared with control, $^{**}P < 0.05$, $^{\#\#}P < 0.001$ compared with TGF-β alone.


A
UUO
Tail vein adenovirus injection
Tail vein adenovirus injection
CON
Ad-GFP
Ad-Clusterin-GFP
Ad-GFP
Ad-Clusterin-GFP
H&E
Kidney
Liver
Sirius red
Kidney
Liver
PAI-1
Kidney
Liver
Type 1 collagen
Kidney
Liver
Fibronectin
Kidney
Liver
GFP
Kidney
Liver
Tubular atrophy
Sirius red
Sirius red staining area/ tubulointerstitium
PAI-1
Positive stain area/ tubulointerstitium
Collagen (type 1)
Positive stain area/ tubulointerstitium
Fibronectin
Positive stain area/ tubulointerstitium
GFP
Positive stain area
Ad-GFP
Ad-Clusterin
UUO

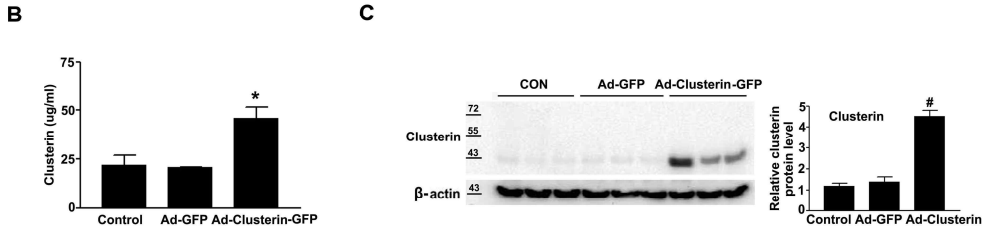


Figure S7. The effect of hepatic overexpression of clusterin on UUO-induced renal fibrosis.

(A) Representative images of H&E, Sirius red and immunohistochemical staining for PAI-1, type I collagen, fibronectin and GFP in mice kidneys and livers after hepatic overexpression of clusterin by tail vein injection of Ad-GFP or Ad-Clusterin-GFP. Magnification, ×200. The number of atrophic tubules was determined by counting the numbers of abnormal irregular and dilated tubular basement membranes identified by H&E staining in five random fields per kidney tissue sample. Sirius red stained areas (renal fibrotic areas) and areas of positive immunostaining for PAI-1, type I collagen, fibronectin (renal fibrotic regions) and GFP (liver) in five random fields per kidney and liver under high-power magnification were quantified by computer-based morphometric analysis. All data were normalized to the control (=1) and in all bar graphs, the data were expressed as fold increase relative to the control. Data are the mean ± SEM of five random fields per kidney (n=5 in each group). $^{*}P < 0.01$, $^{**}P < 0.05$, $^{\#}P < 0.001$ compared with control; -: no significant effect compared with Ad-GFP with UUO.

(B) Measurement of plasma clusterin after hepatic overexpression of clusterin following tail vein injection of Ad-GFP or Ad-Clusterin-GFP. The plasma was collected and assayed in triplicate for levels of clusterin by using commercial ELISA kits. Data are the mean ± SEM of three independent measurements. $^{*}P < 0.01$ compared with control.

(C) Measurement of the renal clusterin level after hepatic clusterin overexpression. Renal clusterin levels were measured by Western blot analysis after tail vein injection of Ad-GFP or Ad-Clusterin-GFP. Data are expressed as the mean ± SEM of three independent experiments (n=5 in each group). β-actin levels were analyzed as an internal control. $^{\#}P < 0.001$ compared with control

Materials & Methods

Cell viability assay

NRK-52E cells were plated at a density of 1×10^5 cells per well in triplicate in 96-well plates. Plates were then incubated at 37°C in a 5% CO_2 atmosphere overnight. Cells were rendered quiescent by incubation for 24 h in medium containing 0.5% FBS. Cells were infected with Ad-Clusterin in serum-free medium for 2 h, after which it was changed to the medium containing 0.5% FBS for 24 h. Cells were incubated at 37°C for 24 h. The 3-(4,5-Dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (MTT) viability

assays were carried out after 72 h, according to the manufacturer's protocol (Roche Diagnostics, Mannheim, Germany). This assay measures cell viability based on mitochondrial conversion of MTT from a soluble tetrazolium salt into an insoluble formazan precipitate. After dissolving in N,N-dimethylformamide, it can be quantified using spectrophotometry. All experiments were repeated three times.

Histological Analysis

Immunohistochemical staining was performed using anti-clusterin (1:100, Santa Cruz, CA), NF-kB (1:100, Santa Cruz) and PCNA (1:100, BD Bioscience, San Jose, CA) primary antibodies and horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG secondary antibodies (Dako, Glostrup, Denmark), according to the manufacturer's instructions. *In situ* detection of DNA fragmentation was performed by terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate (dUTP) nick-end labeling (TUNEL) using the ApopTag Peroxidase *In Situ* Apoptosis Detection Kit (Chemicon International, Temecula, CA) according to the manufacturer's instructions. The number of PCNA-positive, NF-kB-positive and apoptotic cells was determined by counting positively stained tubular epithelial cells of renal tubules in five random fields under high-power magnification. All data were normalized to the control (=1) and, in all

bar graphs, were expressed as fold increase relative to the control.

Preparation of conditioned medium (secreted clusterin) and treatment of conditioned medium (secreted clusterin) to NRK-52E cells.

To prepare conditioned medium containing secreted clusterin, NRK-52E cells infected with 100 moi of Ad-GFP or Ad-Clusterin-GFP for 2 h were incubated with serum free medium for 48 h, and culture (conditioned) medium was collected. NRK-52E cells were seeded on 6-well plates at 90% confluency. After 24 h serum starvation, NRK-52E cells were incubated with conditioned medium (Medium-Clusterin or Medium-GFP) with TGF-β (2 ng/ml, 3 h) for 24 h. To confirm the expression of secreted clusterin, the medium was harvested and Western blotting for clusterin was performed. To exclude the possibility that excessive secretion of clusterin by Ad-Clusterin may influence renal fibrosis, cells were subsequently processed for the isolation of RNA and real time RT-PCR was performed.

Animals

Male 8-week-old C57BL/6 mice and male 8-week-old Sprague–Dawley (SD) rats weighing 250 g were purchased from Samtako (Osan, Korea). Adenovirus encoding rat

clusterin or GFP (3×10^9 p.f.u./kidney, n=5/group) was infused into the liver via the tail vein and unilateral urinary obstruction (UUO) surgery was performed. Seven days after UUO and adenovirus infection, rats were euthanized. The left UUO kidneys and the liver were removed and fixed for 20 h in 4% paraformaldehyde, and embedded in paraffin for histological examination. All procedures were performed in accordance with the institutional guidelines for animal research.

ELISA for clusterin measurements in plasma

The mouse clusterin ELISA kit was purchased from Alpha Diagnostic International (San Antonio, TX). Mouse clustein ELISA was performed according to the manufacturer's protocol (plasma diluted 1:50). Clusterin values were read at 450nm and expressed as concentration units. Samples were run in triplicate and averaged.

Statistical Analyses

Data were evaluated using analysis of variance (ANOVA) followed by a *post hoc* least significant difference test and expressed as means ± SEM. Values of $P < 0.05$ were considered statistically significant. All experiments were performed at least three times.