original article

Upregulation and function of GADD45γ in unilateral ureteral obstruction

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We performed differential display analysis to determine transcriptional activity in the rat kidney, following unilateral ureteral obstruction and found a 12-fold increase in the expression of Growth Arrest and DNA Damage-45γ (GADD45γ), a stress-responsive molecule that interacts with cell-cycle proteins. GADD45γ was strongly expressed in as little as 6 h following ureteric obstruction in the renal tubules, and was also found in kidney tissue of patients with chronic glomerulonephritis. Adenovirus-mediated expression of GADD45γ in cultured renal tubular cells activated p38 along with a significant upregulation of C–C and C–X3–C chemokine ligands and fibrosis-related factors such as several matrix metalloproteinases, transforming growth factor-β1, decorin, and bone morphogenetic protein 2. Silencing of GADD45γ expression significantly blunted the upregulation of these inflammatory and fibrogenic mediators and monocyte infiltration in the ureteral obstructed rat kidney. Our study shows that GADD45γ is quickly upregulated in the kidney with an obstructed ureter, enhancing the production of factors regulating the pathogenesis of kidney disease.

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Growth Arrest and DNA Damage-45 (GADD45) family of genes has three isoforms, GADD45α, GADD45β, and GADD45γ. GADD45α (= GADD45) was first identified after ultraviolet light-mediated DNA damage to Chinese hamster ovary cells,[1] and two other human isoforms were isolated afterwards and were designated as GADD45β (= MyD118) and GADD45γ (= CR6).[2] The GADD45 proteins are evolutionarily conserved and are about 55–58% identical at the amino-acid level.[2] These proteins have been located mainly in the cell nucleus and less abundantly in the cytoplasm.[2,3] They are induced in response to DNA damage and environmental stresses and interact with various proteins involved in cell-cycle regulation.[4] With regard to kidney diseases, data on the role of GADD45 proteins are scarce. Here, we provide evidence that GADD45γ is upregulated in unilateral ureteral obstruction (UUO) and it regulates various molecules implicated in the pathogenesis of kidney diseases.

RESULTS

Differential display analysis

We cloned and sequenced 37 genes that were differentially expressed in the kidney 1 day post-UUO and found that 18 genes were relevant to rat DNA (data not shown). We performed a gene-specific polymerase chain reaction (PCR) for those rat genes using multiple kidney samples, and results were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression levels. We found that GADD45γ is a molecule that is significantly upregulated in the cortex of the UUO kidneys.

GADD45γ mRNA expression during the course of UUO

We serially examined the changes of GADD45γ expression during the course of UUO from 6 h through 5 days. The PCR analysis showed that GADD45γ was significantly increased as early as 6 h post-UUO ($\sim$12-fold), gradually declined thereafter, and remained elevated up to 3 days post-UUO compared with sham-operated controls (Figure 1). GADD45γ gene expression in the contralateral unobstructed kidneys was similar to that in the sham-operated kidneys at all time points of the experiment. We then performed *in situ* hybridization to localize GADD45γ mRNA in the kidney tissue 1 day post-UUO. We found that GADD45γ was
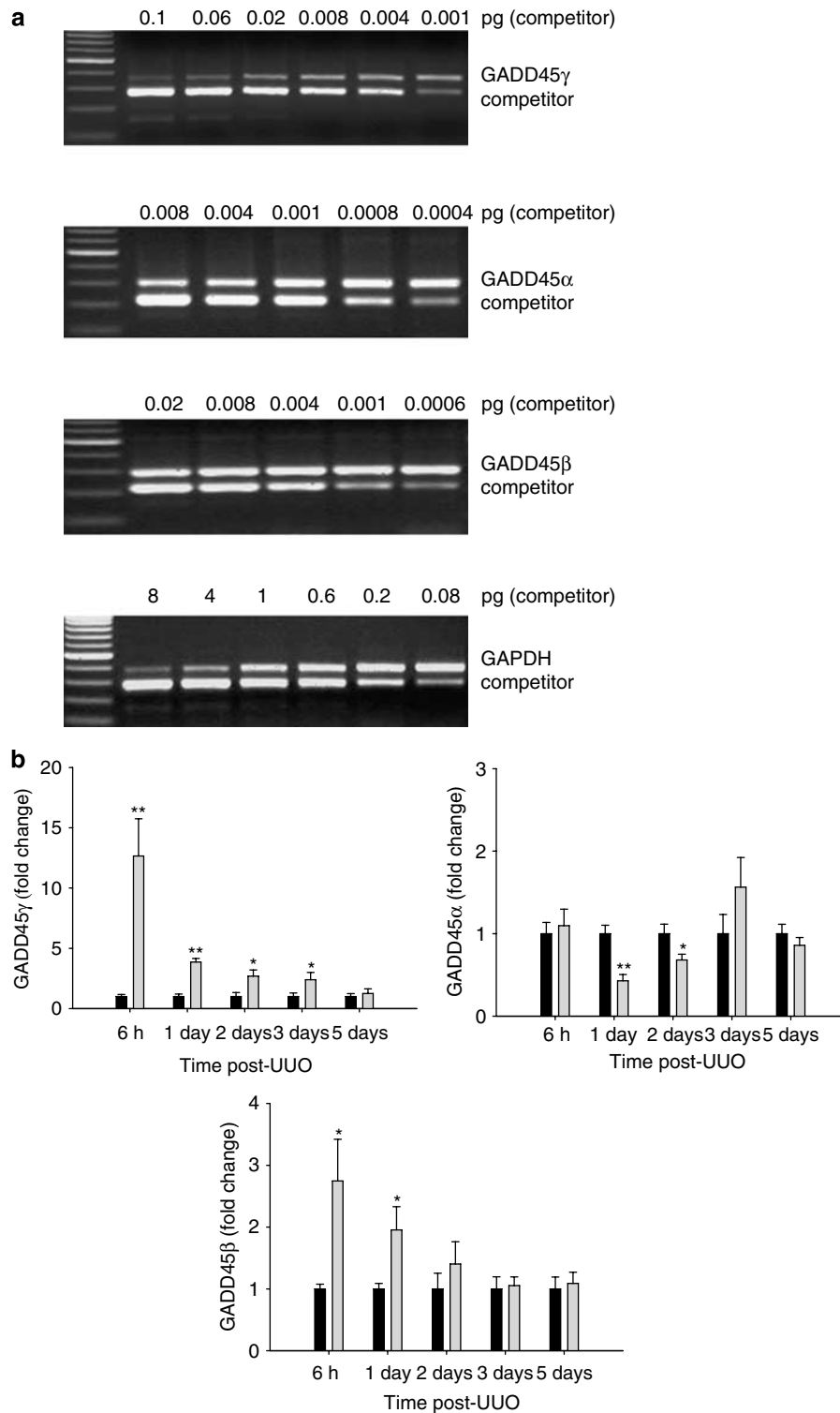
Figure 1 | Quantification of mRNA expression of GADD45-family genes in kidneys after UUO by competitive PCR. (**a**) Competitor constructs were designed to be flanked by sequences recognized by a pair of target gene-specific primers and to contain an intervening sequence that differed in size from the target DNA. (**b**) Rats with UUO (gray bars) were compared with sham-operated controls (black bars) for each corresponding time. Target gene product amounts were normalized to those of GAPDH, and the results were expressed as fold changes compared with the controls, n = 11–14 for each experimental group. Data are mean ± s.e.m.; *P < 0.05, **P < 0.01 compared with controls. UUO, unilateral ureteral obstruction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

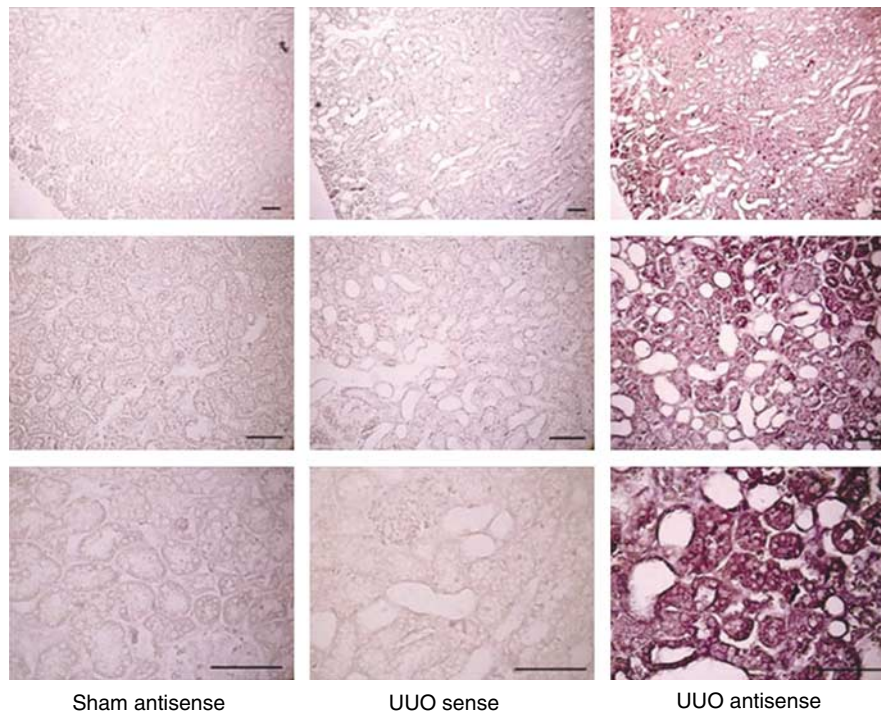Sham antisense   UUO sense   UUO antisense

Figure 2 | Localization of GADD45γ mRNA by *in situ* hybridization. Kidneys were harvested 1 day after UUO or sham operation. Paraffin sections were treated with the sense (control) or the antisense digoxigenin-labeled RNA probe specific for GADD45γ. Strong signals for GADD45γ mRNA were observed in tubular cells of UUO kidneys (UUO antisense) in contrast to weak signals in sham-operated kidneys (Sham antisense). The sense probe for GADD45γ yielded no positive signal (UUO sense). Original magnification × 100 in upper panels, × 200 in middle panels, × 400 in lower panels. Bars = 100 μm.

strongly induced in the kidney tubules even before they were dilated (Figure 2). In addition, we examined the other two members of the GADD45-family genes. Like GADD45γ, GADD45β peaked 6 h post-UUO but with a lesser degree (up to ∼3-fold) and for a shorter duration (up to 1 day post-UUO) compared with GADD45γ (Figure 1). By contrast, in accordance with previous reports,[5] GADD45α (= GADD45) expression was not increased after UUO (Figure 1).

GADD45γ expression in human kidney tissue

To determine whether GADD45γ is a relevant molecule in human kidney disease, we examined GADD45γ protein expression in the kidney biopsy specimens. Immunohisto-chemistry was performed in 12 cases of chronic glomerulo-nephritis, including IgA nephropathy and membranous glomerulonephritis, and in five cases of normal findings. Positive GADD45γ staining was observed in five patients with chronic glomerulonephritis. The staining was most evident in tubular cells and was also present in glomerular cells, interstitial cells, and infiltrating lymphocytes (Figure 3). All of the patients with normal biopsy findings showed negative GADD45γ immunostaining. These patients were those who requested kidney biopsy although they had only minor urine abnormalities. The staining was localized to the nucleus as expected from immunostaining of cultured cells by other investigators (Figure 3).[4] The specificity of staining was verified by immunohistochemistry in which the primary antibody was omitted (data not shown).
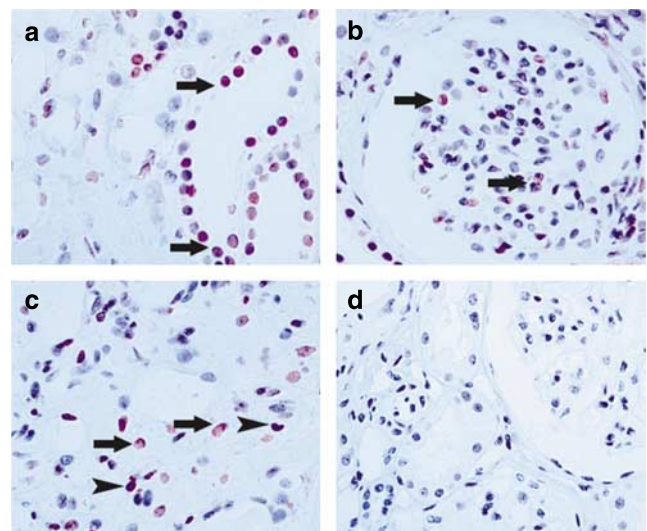

Figure 3 | GADD45γ expression in human kidneys. Representative kidney biopsy sections demonstrating positive GADD45γ staining (red-purple) in tubular cells (**a**), glomerular cells (**b**), interstitial cells (arrows), and infiltrating lymphocytes (arrowheads) (**c**). A normal kidney section with negative GADD45γ staining (**d**). Only a few exemplary cells are marked with arrows or arrowheads in each figure. Original magnification × 400.

Activation of MAPK pathways in HRE cells

To determine whether GADD45γ induces activation of mitogen-activated protein kinase (MAPK) pathways in renal tubular cells, we infected human renal epithelial (HRE) cells

Figure 4 | Expression of GADD45γ protein in human renal epithelial cells. Cells were infected with a serial amount of adenoviral vectors harboring the open reading frame of GADD45γ. After a 24-h incubation, cells were harvested. GADD45γ proteins could be detected at an MOI of 25 and above. V represents vehicle only and G represents GADD45γ. Each number following G represents an MOI.

with recombinant adenoviral vectors containing the open reading frame, which encodes GADD45γ protein (Ad-GADD45γ). First, we infected HRE cells at multiplicities of infection (MOIs) of 1, 10, 25, 50, 75, and 100 for 24 h; cells were then harvested and western blotting was carried out to verify the expression of GADD45γ protein. The GADD45γ protein could be detected at an MOI of 25 and above (Figure 4). Next, we infected HRE cells at MOIs of 1, 10, 100, and 250 for 24 h and examined the expression of MAPK proteins. Recombinant adenoviral vectors containing LacZ (Ad-LacZ) served as control. The result showed that p-p38 MAPK was significantly increased compared with LacZ controls even at an MOI of 10 where the GADD45γ protein level was below our detection limits. By contrast, phosphorylated-c-Jun-N-terminal kinase (P-JNK) and phosphorylated-extracellular signal-regulated kinase (P-ERK) did not show any significant changes (Figure 5). These findings suggest that GADD45γ activates the p38 MAPK pathway, but not the extracellular signal-regulated kinase (ERK) or the c-Jun-N-terminal kinase (JNK) pathways in renal tubular cells.

Microarray analysis
To further define the function of GADD45γ in renal tubular cells, we infected HRE cells with Ad-GADD45γ and Ad-LacZ and determined differentially expressed genes using micro-array slides containing 24 000 human genes. The results showed that the expression level of 164 genes differed by more that twofold (Table 1). Among those 164 genes, we selected 12 genes that have been investigated with regard to pathogenesis of kidney disease, and verified the microarray results using semiquantitative PCR: these genes include chemokine (C–C motif) ligand 20 (CCL20), chemokine (C–X3–C motif) ligand 1 (CX3CL1, fractalkine), interleukin 8 (IL-8), IL-11, matrix metalloproteinase 1 (MMP1), MMP10, bone morphogenetic protein 2 (BMP2), decorin, integrin-α5, thrombospondin1, integrin-β1, and collagen XI. Apart from microarray results, we additionally selected genes that have been proposed to be implicated in kidney disease progression; these include collagen I, collagen III, collagen IV, fibronectin, MMP2, MMP9, monocyte chemotactic protein-1, vascular endothelial growth factor A, vascular endothelial

growth factor B, connective tissue growth factor, plasminogen activator inhibitor-1, and transforming growth factor-β1 (TGF-β1). After normalization to GAPDH expression, we were able to identify nine genes whose expression was clearly altered by GADD45γ; these genes were CCL20, CX3CL1, IL-8, MMP1, MMP9, MMP10, TGF-β1, decorin, and BMP2, all of which showed significant upregulation (Figure 6). Next, we examined the production of proinflammatory chemokines, including CCL20, CX3CL1, and IL-8, in cell culture media using enzyme-linked immunosorbent assay. After 24 h of incubation of HRE cells with adenoviral vectors, cell culture media were collected for analysis. In accordance with the PCR results, production of all these chemokines was significantly enhanced by GADD45γ in renal tubular cells (Figure 6).

Apoptosis and proliferation of HRE cells
To investigate the role of GADD45γ in apoptosis in renal tubules, HRE cells were infected with Ad-GADD45γ at an MOI of 500 for 24 h and we evaluated the extent of apoptosis by detecting poly(ADP-ribose) polymerase (PARP) fragmentation and cleaved DNA with immunoblotting and terminal deoxynucleotidyl transferase (TdT)-mediated dUTP–biotin nick-end labeling (TUNEL) staining, respectively. The result showed absence of TUNEL-positive apoptotic cells and of cleaved PARP despite strong expression of GADD45γ protein (Figure 7), which indicates that GADD45γ may not induce apoptosis in HRE cells. The role of GADD45γ in HRE cell proliferation was determined using 5-bromo-2-deoxyuridine (BrdU) labeling and we found that HRE cell proliferation was significantly reduced in a dose-dependent manner by GADD45γ (Figure 7).

***In vivo* silencing of the GADD45γ gene**
To delineate the relationship between GADD45γ and inflammatory and fibrogenic mediators *in vivo*, we knocked down the GADD45γ gene in the kidney using a 'hydronamic' intravenous injection of small interfering RNA (siRNA) into rats.[6–8] Kidneys were harvested 2 days after UUO when GADD45γ and several molecules of interest, such as CCL20, CX3CL1, cytokine-induced neutrophil chemoattractant-1 (CINC-1, rat homolog for human IL-8),[9] TGF-β1, and p-p38 MAPK, were found to be significantly upregulated (Figures 1 and 8). In rats injected with GADD45γ siRNA, mRNA expression of GADD45γ was inhibited by 90% in the UUO kidneys compared with that in the controls groups treated with phosphate-buffered saline (PBS) or negative control siRNA (Figure 8). The inhibition of GADD45γ resulted in a significant reduction of mRNA expression of CCL20, CX3CL1, CINC-1, TGF-β1, and p-p38 MAPK in the UUO kidneys (Figure 8). To evaluate monocyte/macrophage infiltration into the tubulointerstitium, 20 high-power fields (× 400) per section for each rat (six rats per group) were obtained from the cortical region and ED1-positive cells were counted. We found that ED1-positive cells were significantly reduced in UUO kidneys treated with GADD45siRNA-γ
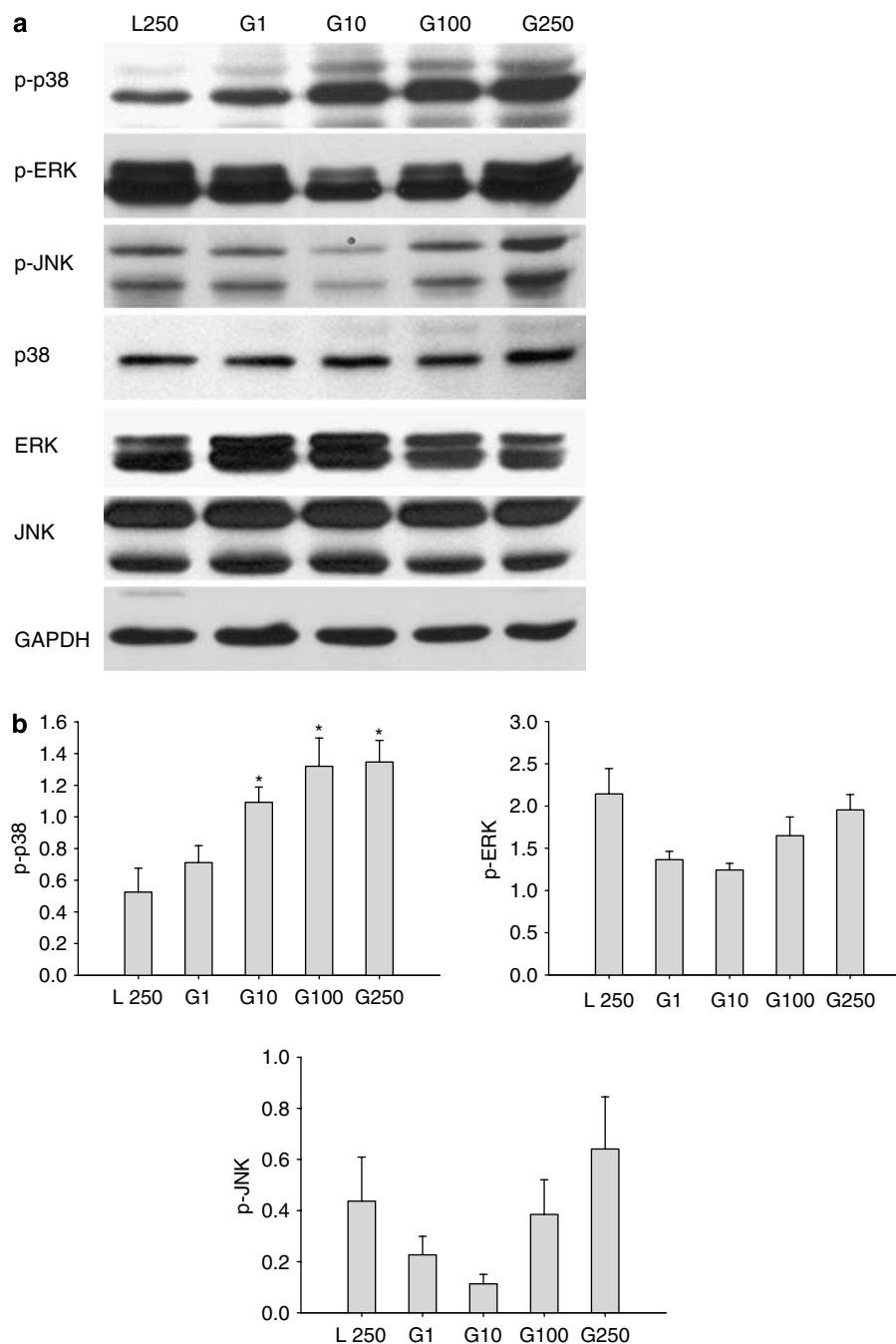
Figure 5 | Activation of p38 MAPK by GADD45γ in human renal epithelial cells. After a 24-h incubation with adenoviral vectors, cells were harvested. GADD45γ protein activated p38 but not ERK or JNK. Total MAPK proteins did not show any significant changes. The intensity of the MAPK protein band was normalized to that of re-probed GAPDH band. Results are the mean of three independent sets of experiment, $n = 4$ for each experimental group. Data are mean ± s.e.m.; *$P < 0.05$ compared with LacZ controls. L represents LacZ; G, GADD45γ. Each number following L or G represents an MOI. MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun-N-terminal kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

(1.83 ± 0.31 per ×400 magnification field) compared with control UUO kidneys (13.17 ± 1.35 per ×400 magnification field) ($P < 0.001$) (Figure 9).

DISCUSSION

Regardless of the underlying causes, tubulointerstitial fibrosis is closely associated with disease progression in chronic kidney disease.[10] During this process, tubular cells play a dominant role by secreting inflammatory and fibrogenic molecules that are activated by insults.[11,12] UUO is a commonly used animal model of tubulointerstitial injury and fibrosis.[13] Our recent research has been focused on examining genes that are differentially expressed in the kidney following UUO, and we found that GADD45γ is

Table 1 | Genes whose expression is altered by overexpression of GADD45γ in human renal epithelial cells

Genes	GenBank accession	Fold ± s.d.
B-cell CLL/lymphoma 9 (BCL9), mRNA	NM_004326.2	7.66 ± 0.10
Resistin-like-β (RETNLB), mRNA	NM_032579.1	7.60 ± 0.18
Interleukin 11 (IL-11), mRNA	NM_000641.2	6.69 ± 0.16
Colony-stimulating factor 2 receptor-β, low affinity (granulocyte–macrophage) (CSF2RB), mRNA	NM_000395.1	5.29 ± 0.10
Dopamine-β-hydroxylase (dopamine-β-monooxygenase) (DBH), mRNA	NM_000787.2	5.05 ± 0.11
Jagged 1 (Alagille syndrome) (JAG1), mRNA	NM_000214.1	5.05 ± 0.07
Heat-shock 70-kDa protein 6 (HSP70B′) (HSPA6), mRNA	NM_002155.3	5.01 ± 0.16
Heat-shock 70-kDa protein 1B (HSPA1B), mRNA	NM_005346.3	4.87 ± 0.11
Heat-shock 70-kDa protein 1A (HSPA1A), mRNA	NM_005345.4	4.80 ± 0.10
Zinc finger, AN1-type domain 2A (ZFAND2A), mRNA	NM_182491.1	3.99 ± 0.26
Chemokine (C–C motif) ligand 20 (CCL20), mRNA	NM_004591.1	3.48 ± 0.12
Growth differentiation factor 15 (GDF15), mRNA	NM_004864.1	3.47 ± 0.31
Interleukin 8 (IL-8), mRNA	NM_000584.2	3.44 ± 0.38
DnaJ (Hsp40) homolog, subfamily A, member 4 (DNAJA4), mRNA	NM_018602.2	3.34 ± 0.03
Inhibin, β-B (activin AB β-polypeptide) (INHBB), mRNA	NM_002193.1	3.24 ± 0.08
Somatostatin (SST), mRNA	NM_001048.3	3.21 ± 0.14
Histone 1, H2bk (HIST1H2BK), mRNA	NM_080593.1	3.12 ± 0.11
Coagulation factor III (thromboplastin, tissue factor) (F3), mRNA	NM_001993.2	3.10 ± 0.13
Chemokine (C–X3–C motif) ligand 1 (CX3CL1), mRNA	NM_002996.3	3.06 ± 0.22
Regulator of G-protein signalling 4 (RGS4), mRNA	NM_005613.3	3.05 ± 0.07
Histone 1, H2bd (HIST1H2BD), transcript variant 2, mRNA	NM_138720.1	3.02 ± 0.07
Histone 2, H2ac (HIST2H2AC), mRNA	NM_003517.2	2.86 ± 0.26
FK506-binding protein 4, 59 kDa (FKBP4), mRNA	NM_002014.2	2.76 ± 0.29
Matrix metallopeptidase 1 (interstitial collagenase) (MMP1), mRNA	NM_002421.2	2.70 ± 0.10
Chorionic somatomammotropin hormone 1 (placental lactogen) (CSH1), transcript variant 3, mRNA	NM_022641.2	2.69 ± 0.14
Pregnancy-associated plasma protein A, pappalysin 1 (PAPPA), mRNA	NM_002581.3	2.66 ± 0.11
Potassium voltage-gated channel, subfamily G, member 1 (KCNG1), transcript variant 1, mRNA	NM_002237.2	2.62 ± 0.22
Histone 1, H1c (HIST1H1C), mRNA	NM_005319.3	2.62 ± 0.17
Inhibin, β-E (INHBE), mRNA	NM_031479.3	2.57 ± 0.12
Glucosaminyl (*N*-acetyl) transferase 3, mucin type (GCNT3), mRNA	NM_004751.1	2.57 ± 0.16
Dual-specificity phosphatase 5 (DUSP5), mRNA	NM_004419.3	2.54 ± 0.05
Histone 2, H2aa (HIST2H2AA), mRNA	NM_003516.2	2.54 ± 0.15
Crystallin, α-B (CRYAB), mRNA	NM_001885.1	2.54 ± 0.07
UDP glycosyltransferase 8 (UDP-galactose ceramide galactosyltransferase) (UGT8), mRNA	NM_003360.2	2.53 ± 0.11
ClpB caseinolytic peptidase B homolog (*E. coli*) (CLPB), mRNA	NM_030813.3	2.49 ± 0.12
Serine/threonine kinase 25 (STE20 homolog, yeast) (STK25), mRNA	NM_006374.3	2.48 ± 0.27
Chloride intracellular channel 1 (CLIC1), mRNA	NM_001288.4	2.47 ± 0.53
Protein phosphatase 1G (formerly 2C), magnesium-dependent, γ-isoform (PPM1G), transcript variant 1, mRNA	NM_177983.1	2.45 ± 0.19
Hypothetical protein DKFZp564N2472 (DKFZp564N2472), mRNA	NM_182595.2	2.45 ± 0.05
Secretogranin II (chromogranin C) (SCG2), mRNA	NM_003469.3	2.45 ± 0.07
H2B histone family, member S (H2BFS), mRNA	NM_017445.1	2.43 ± 0.16
Transcobalamin I (vitamin B12-binding protein, R binder family) (TCN1), mRNA	NM_001062.2	2.42 ± 0.06
Serpin peptidase inhibitor, clade A (α-1 anti-proteinase, antitrypsin), member 3 (SERPINA3), mRNA	NM_001085.4	2.39 ± 0.21
Potassium voltage-gated channel, subfamily F, member 1 (KCNF1), mRNA	NM_002236.4	2.36 ± 0.28
Tubulin tyrosine ligase-like family, member 11 (TTLL11), mRNA	NM_194252.1	2.36 ± 0.25
Dual-specificity phosphatase 1 (DUSP1), mRNA	NM_004417.2	2.35 ± 0.18
Heat-shock 105/110-kDa protein 1 (HSPH1), mRNA	NM_006644.2	2.32 ± 0.20
BRF2, subunit of RNA polymerase III transcription initiation factor, BRF1-like (BRF2), mRNA	NM_018310.2	2.31 ± 0.15
Actin-related protein 2/3 complex, subunit 2, 34 kDa (ARPC2), transcript variant 2, mRNA	NM_005731.2	2.31 ± 0.09
Matrix metallopeptidase 10 (stromelysin 2) (MMP10), mRNA	NM_002425.1	2.31 ± 0.09
Solute-carrier family 26, member 6 (SLC26A6), transcript variant 3, mRNA	NM_134426.1	2.30 ± 0.49
Bone morphogenetic protein 2 (BMP2), mRNA	NM_001200.1	2.30 ± 0.06
Mannose-*P*-dolichol utilization defect 1 (MPDU1), mRNA	NM_004870.1	2.28 ± 0.57
Hypothetical protein HSPC111 (HSPC111), mRNA	NM_016391.2	2.27 ± 0.29
Hypothetical protein FLJ13639 (FLJ13639), transcript variant 1, mRNA	NM_001031719.1	2.26 ± 0.13
N-myc downstream regulated gene 1 (NDRG1), mRNA	NM_006096.2	2.26 ± 0.16
Interferon-stimulated exonuclease gene 20 kDa (ISG20), mRNA	NM_002201.4	2.25 ± 0.19
MHC class I polypeptide-related sequence B (MICB), mRNA	NM_005931.2	2.25 ± 0.37
Interleukin-1 receptor-associated kinase 2 (IRAK2), mRNA	NM_001570.3	2.24 ± 0.14
Tubulin, α1 (testis specific) (TUBA1), mRNA	NM_006000.1	2.24 ± 0.34
Keratin, hair, acidic, 4 (KRTHA4), mRNA	NM_021013.3	2.21 ± 0.09
Decorin (DCN), transcript variant B, mRNA	NM_133504.2	2.19 ± 0.05
Histone 1, H3d (HIST1H3D), mRNA	NM_003530.3	2.19 ± 0.10
Golgi autoantigen, golgin subfamily a, 2 (GOLGA2), mRNA	NM_004486.4	2.18 ± 0.40
Colony-stimulating factor 2 (granulocyte–macrophage) (CSF2), mRNA	NM_000758.2	2.17 ± 0.24
Elastin microfibril interfacer 2 (EMILIN2), mRNA	NM_032048.2	2.17 ± 0.28

Table 1 | Continued

Genes	GenBank accession	Fold ± s.d.
Runt-related transcription factor 3 (RUNX3), transcript variant 2, mRNA	NM_004350.1	2.16 ± 0.14
Hairy and enhancer of split 4 (*Drosophila*) (HES4), mRNA	NM_021170.2	2.16 ± 0.19
Chromosome 22 open reading frame 19 (C22orf19), transcript variant 1, mRNA	NM_001002878.1	2.15 ± 0.37
Immediate early response 3 (IER3), transcript variant long, mRNA	NM_052815.1	2.15 ± 0.39
AHA1, activator of heat-shock 90-kDa protein ATPase homolog 1 (yeast) (AHSA1), mRNA	NM_012111.1	2.14 ± 0.25
Rho/rac guanine nucleotide-exchange factor (GEF) 2 (ARHGEF2), mRNA	NM_004723.2	2.14 ± 0.13
Serpin peptidase inhibitor, clade B (ovalbumin), member 1 (SERPINB1), mRNA	NM_030666.2	2.14 ± 0.10
Cyclin A1 (CCNA1), mRNA	NM_003914.2	2.13 ± 0.09
Major facilitator superfamily domain containing 7 (MFSD7), mRNA	NM_032219.2	2.13 ± 0.14
Sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6A (SEMA6A), mRNA	NM_020796.2	2.12 ± 0.08
S100 calcium-binding protein A2 (S100A2), mRNA	NM_005978.3	2.11 ± 0.15
MCM7 minichromosome maintenance deficient 7 (*S. cerevisiae*) (MCM7), transcript variant 2, mRNA	NM_182776.1	2.10 ± 0.25
Insulin-like growth factor-binding protein 5 (IGFBP5), mRNA	NM_000599.2	2.10 ± 0.20
Pleckstrin homology domain containing, family O member 1 (PLEKHO1), mRNA	NM_016274.3	2.09 ± 0.32
KIAA0690 (KIAA0690), mRNA	NM_015179.2	2.09 ± 0.16
Enolase 2 (γ, neuronal) (ENO2), mRNA	NM_001975.2	2.09 ± 0.12
Sialic acid-binding Ig-like lectin 7 (SIGLEC7), transcript variant 1, mRNA	NM_014385.1	2.09 ± 0.12
Hypothetical protein FLJ90166 (FLJ90166), mRNA	NM_153360.1	2.08 ± 0.17
Limb region 1 homolog (mouse)-like (LMBR1L), mRNA	NM_018113.1	2.08 ± 0.25
Short stature homeobox (SHOX), transcript variant SHOXb, mRNA	NM_006883.1	2.08 ± 0.05
DnaJ (Hsp40) homolog, subfamily B, member 6 (DNAJB6), transcript variant 1, mRNA	NM_058246.3	2.08 ± 0.14
SAP30-binding protein (SAP30BP), mRNA	NM_013260.5	2.07 ± 0.16
Breast cancer membrane protein 11 (BCMP11), mRNA	NM_176813.3	2.07 ± 0.06
Hairy and enhancer of split 1, (*Drosophila*) (HES1), mRNA	NM_005524.2	2.07 ± 0.18
Integrin, α5 (fibronectin receptor, α-polypeptide) (ITGA5), mRNA	NM_002205.2	2.07 ± 0.37
Family with sequence similarity 32, member A (FAM32A), mRNA	NM_014077.1	2.06 ± 0.44
Transmembrane protein 44 (TMEM44), transcript variant 1, mRNA	NM_138399.3	2.05 ± 0.48
ATPase, H+ transporting, lysosomal 31-kDa, V1 subunit E2 (ATP6V1E2), mRNA	NM_080653.3	2.05 ± 0.12
Histone 2, H2be (HIST2H2BE), mRNA	NM_003528.2	2.05 ± 0.08
Keratin, hair, acidic, 6 (KRTHA6), mRNA	NM_003771.3	2.04 ± 0.07
Hypothetical protein FLJ20551 (FLJ20551), mRNA	NM_017875.1	2.04 ± 0.23
Dual-specificity phosphatase 4 (DUSP4), transcript variant 2, mRNA	NM_057158.2	2.04 ± 0.16
Integrin-β4-binding protein (ITGB4BP), transcript variant 4, mRNA	NM_181466.1	2.03 ± 0.25
Signal-induced proliferation-associated 1-like 2 (SIPA1L2), mRNA	NM_020808.1	2.02 ± 0.07
RNA-binding motif protein 28 (RBM28), mRNA	NM_018077.1	2.01 ± 0.09
Interleukin 23, α-subunit p19 (IL-23A), mRNA	NM_016584.2	2.01 ± 0.31
Hypothetical protein LOC283932 (LOC283932), mRNA	NM_175901.3	2.01 ± 0.04
VGF nerve growth factor-inducible (VGF), mRNA	NM_003378.2	2.01 ± 0.08
N-myristoyltransferase 1 (NMT1), mRNA	NM_021079.3	2.00 ± 0.45
Growth arrest and DNA damage-inducible, α (GADD45A), mRNA	NM_001924.2	−5.82 ± 0.26
Transforming growth factor, β-receptor-associated protein 1 (TGFBRAP1), mRNA	NM_004257.3	−3.96 ± 0.18
AHNAK nucleoprotein (desmoyokin) (AHNAK), transcript variant 1, mRNA	NM_001620.1	−3.57 ± 0.72
Phosphoribosyl pyrophosphate synthetase 1 (PRPS1), mRNA	NM_002764.2	−3.28 ± 0.60
Tubulin, β6 (TUBB6), mRNA	NM_032525.1	−3.26 ± 0.84
Chromosome 10 open reading frame 65 (C10orf65), mRNA	NM_138413.2	−3.13 ± 0.12
Protease, serine, 23 (PRSS23), mRNA	NM_007173.3	−3.01 ± 0.16
CXYorf1-related protein (LOC349338), mRNA	NM_182905.1	−2.97 ± 0.16
Transmembrane, prostate androgen induced RNA (TMEPAI), transcript variant 1, mRNA	NM_020182.3	−2.95 ± 0.82
Cytokine-like 1 (CYTL1), mRNA	NM_018659.2	−2.79 ± 0.13
Thrombospondin 1 (THBS1), mRNA	NM_003246.2	−2.78 ± 0.62
RAP1, GTPase-activating protein 1 (RAP1GA1), mRNA	NM_002885.1	−2.77 ± 0.19
Profilin 1 (PFN1), mRNA	NM_005022.2	−2.75 ± 1.09
Wingless-type MMTV integration site family, member 10A (WNT10A), mRNA	NM_025216.2	−2.73 ± 0.18
Myosin, light polypeptide kinase (MYLK), transcript variant 6, mRNA	NM_005965.3	−2.71 ± 0.60
Olfactomedin-like 2A (OLFML2A), mRNA	NM_182487.1	−2.66 ± 0.15
Lymphocyte antigen 6 complex, locus E (LY6E), mRNA	NM_002346.1	−2.62 ± 0.48
CD151 antigen (CD151), transcript variant 4, mRNA	NM_004357.3	−2.59 ± 0.94
Small nuclear ribonucleoprotein polypeptide N (SNRPN), transcript variant 1, mRNA	NM_003097.3	−2.56 ± 0.85
Ras homolog gene family, member B (RHOB), mRNA	NM_004040.2	−2.55 ± 0.28
Kv channel interacting protein 1 (KCNIP1), transcript variant 2, mRNA	NM_014592.2	−2.52 ± 0.26
Cyclin D2 (CCND2), mRNA	NM_001759.2	−2.52 ± 0.40
Ribosomal protein, large, P0 (RPLP0), transcript variant 2, mRNA	NM_053275.3	−2.45 ± 0.85
Zinc finger, BED-type containing 2 (ZBED2), mRNA	NM_024508.3	−2.42 ± 0.14
Small EDRK-rich factor 2 (SERF2), mRNA	NM_001018108.1	−2.40 ± 0.35
Chromobox homolog 3 (HP1 γ homolog, *Drosophila*) (CBX3), transcript variant 2, mRNA	NM_016587.2	−2.38 ± 0.87
Actin-like protein (FKSG30), mRNA	NM_001017421.1	−2.36 ± 0.89

Table 1 | Continued

Genes	GenBank accession	Fold ± s.d.
Phosphoglycerate mutase 1 (brain) (PGAM1), mRNA	NM_002629.2	−2.33 ± 0.83
Guanine nucleotide-binding protein (G protein), γ-10 (GNG10), mRNA	NM_001017998.1	−2.33 ± 0.71
Actin, γ-1 (ACTG1), mRNA	NM_001614.2	−2.32 ± 0.94
p53 target zinc-finger protein (WIG1), transcript variant 2, mRNA	NM_152240.1	−2.29 ± 0.48
Filamin A, α (actin-binding protein 280) (FLNA), mRNA	NM_001456.1	−2.28 ± 0.43
LanC lantibiotic synthetase component C-like 1 (bacterial) (LANCL1), mRNA	NM_006055.1	−2.26 ± 0.22
Ribosomal protein, large, P0 (RPLP0), transcript variant 1, mRNA	NM_001002.3	−2.25 ± 0.57
Nucleosome assembly protein 1-like 1 (NAP1L1), transcript variant 1, mRNA	NM_139207.1	−2.23 ± 0.44
Matrilin 2 (MATN2), transcript variant 2, mRNA	NM_030583.2	−2.19 ± 0.14
Cingulin-like 1 (CGNL1), mRNA	NM_032866.3	−2.19 ± 0.16
EF-hand domain family, member D1 (EFHD1), mRNA	NM_025202.2	−2.19 ± 0.14
Integrin, β1 (fibronectin receptor, β-polypeptide, antigen CD29 includes MDF2, MSK12) (ITGB1), transcript variant 1E, mRNA	NM_133376.1	−2.18 ± 0.77
Tensin 3 (TNS3), mRNA	NM_022748.9	−2.18 ± 0.16
Immunoglobulin superfamily, member 4 (IGSF4), mRNA	NM_014333.2	−2.16 ± 0.32
Chromosome 9 open reading frame 58 (C9orf58), transcript variant 1, mRNA	NM_031426.2	−2.16 ± 0.08
Transmembrane protein 14A (TMEM14A), mRNA	NM_014051.2	−2.15 ± 0.21
Collagen, type XI, α1 (COL11A1), transcript variant B, mRNA	NM_080629.1	−2.15 ± 0.31
Integrin, αV (vitronectin receptor, α-polypeptide, antigen CD51) (ITGAV), mRNA	NM_002210.2	−2.14 ± 0.53
Nuclear factor I/X (CCAAT-binding transcription factor) (NFIX), mRNA	NM_002501.2	−2.14 ± 0.20
Transmembrane protein 123 (TMEM123), mRNA	NM_052932.1	−2.13 ± 0.60
ADP-ribosylation factor-like 6 interacting protein (ARL6IP), mRNA	NM_015161.1	−2.12 ± 0.29
Zinc-finger protein 503 (ZNF503), mRNA	NM_032772.3	−2.11 ± 0.20
Chromosome 2 open reading frame 28 (C2orf28), transcript variant 2, mRNA	NM_080592.2	−2.11 ± 0.10
Adaptor-related protein complex 1, σ1 subunit (AP1S1), transcript variant 1, mRNA	NM_001283.2	−2.10 ± 0.71
Transgelin 2 (TAGLN2), mRNA	NM_003564.1	−2.09 ± 0.85
Hypoxia-inducible factor 1, α-subunit (basic helix–loop–helix transcription factor) (HIF1A), transcript variant 1, mRNA	NM_001530.2	−2.07 ± 0.78
Thyroid-hormone receptor interactor 6 (TRIP6), mRNA	NM_003302.1	−2.07 ± 0.12
Calsyntenin 2 (CLSTN2), mRNA	NM_022131.1	−2.03 ± 0.09
Family with sequence similarity 84, member B (FAM84B), mRNA	NM_174911.3	−2.02 ± 0.50
COMM domain containing 6 (COMMD6), transcript variant 2, mRNA	NM_203495.1	−2.02 ± 0.38
Arachidonate 5-lipoxygenase-activating protein (ALOX5AP), mRNA	NM_001629.2	−2.01 ± 0.15
Latent transforming growth factor-β-binding protein 3 (LTBP3), mRNA	NM_021070.2	−2.01 ± 0.45

GADD45γ, Growth Arrest and DNA Damage-45γ.
Gene expression of human renal epithelial cells overexpressing GADD45γ (*n*=4) was compared with that of control human renal epithelial cells infected with LacZ-containing adenoviruses (*n*=4). The values shown are the means of the fold ratios of (GADD45γ/LacZ) ± s.d. Genes, which displayed a greater than twofold difference, are shown.

substantially upregulated in UUO kidneys. Our findings that GADD45γ peaked as early as 6 h post-UUO suggest that GADD45γ activation may be an early, upstream event in the pathogenesis of tubular injury. Given that majority of the tubules remain undilated at this early time of UUO, such an early peak is consistent with our *in situ* hybridization findings where GADD45γ was strongly induced even in the undilated tubules.

Recent studies have presented evidence linking GADD45 proteins to activation of JNK and p38 MAPK. It has been demonstrated that GADD45α, -β, and -γ mediate activation of the p38/JNK pathways, via MTK1/MEKK4, in response to environmental stresses in COS cells.[2] Accordingly, GADD45α has been shown to be required for p38 MAPK activation in mouse embryonic fibroblasts.[14] Other studies have shown that GADD45β and GADD45γ are implicated in the activation of p38 and/or JNK in subsets of T cells.[15,16] By contrast, several other studies have not confirmed these results and have reported conflicting findings. The observations that the induction of the GADD45 proteins was delayed relative to JNK/p38 activation in mouse fibroblasts during acute stress suggests that induction of JNK/p38 does not depend on these

proteins.[17] Accordingly, JNK/p38 activation was not impaired in the absence of GADD45 expression in GADD45$^{−/−}$ mouse embryonic fibroblasts.[18] Furthermore, GADD45β has been shown to inhibit JNK signaling via blockade of the catalytic activity of MKK7.[19] To explain this discrepancy, it has been proposed that depending on the cell types, GADD45 proteins may preferentially interact with particular pathways, leading to differential regulation of p38/JNK activities.[20] To explore the link between the GADD45γ and MAPK proteins in renal tubular cells, we overexpressed GADD45γ in HRE cells that are of kidney tubular origin; we found that only p38 was significantly activated by the GADD45γ protein. Indeed, an increasing number of studies have suggested that p38 MAPK activation contributes to pathogenesis of kidney disease. In cultured renal tubular cells, p38 has been shown to mediate TGF-β1-induced generation of reactive oxygen species,[21] tumor necrosis factor-α production in ischemic stress,[22] and TGF-β1-induced generation of procollagen-Iα1.[23] In rodent models, p38 activation has been associated with renal inflammation and fibrosis.[24–26] Moreover, it has been demonstrated that p38 activation contributed to the pathogenesis of various progressive kidney diseases in humans.[27,28]
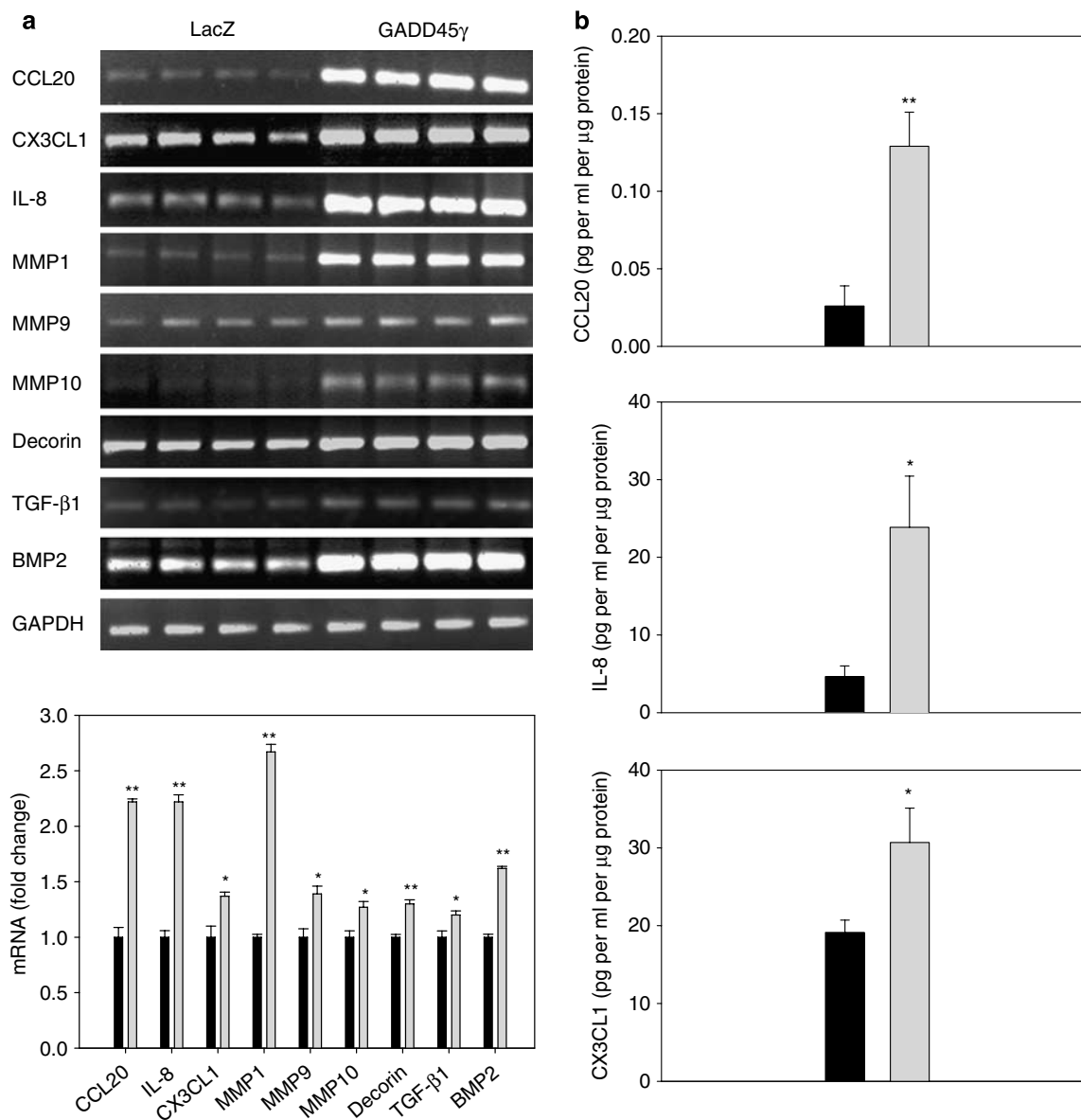
Figure 6 | Enhanced production of chemokines and fibrosis related molecules by GADD45γ in human renal epithelial cells. (a) After a 24-h incubation with adenoviral vectors, cells were harvested for reverse transcription-PCRs. Target gene product amounts were normalized to those of GAPDH, and the results were expressed as fold changes compared with LacZ controls, $n = 4$ for each experimental group. (b) After a 24-h incubation of adenoviral vectors, cell culture media were harvested for enzyme-linked immunosorbent assay. The protein levels were corrected for total protein amount of the cell monolayer, $n = 8$ for each experimental group. Data are mean ± s.e.m.; *$P < 0.05$, **$P < 0.01$ compared with LacZ controls (black bar). CCL20, chemokine (C–C motif) ligand 20; CX3CL1, chemokine (C–X3–C motif) ligand 1; IL-8, interleukin 8; MMP, matrix metalloproteinase; TGF-β1, transforming growth factor-β1; BMP2, bone morphogenetic protein 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Additionally, we found that GADD45γ stimulates the expression of chemokines including CCL20, CX3CL1, and IL-8. CCL20 is a molecule mainly expressed by epithelial cells. CCL20 plays an important role under inflammatory conditions by chemoattracting immature dendritic cells, T cells, and B cells.[29] CCL20 expression was markedly increased in renal tubular epithelium during allograft rejection episodes, which suggests CCL20 attracts inflammatory cells to the kidney.[30] CX3CL1 is a transmembrane molecule with an extracellular chemokine domain and mucin stalk. Soluble CX3CL1 promotes chemotaxis of target leucocytes, whereas the membrane-anchored form function as an adhesion molecule for monocytes, natural killer cells, and subsets of CD8+ T cells. It has been shown that CX3CL1 enhanced leukocyte adhesion to the luminal surface of renal tubular cells.[31] Accordingly, expression of CX3CL1 was increased by tubular epithelial cells in acute renal allograft rejection, and this correlated with infiltrating leukocyte subsets.[32] In addition, CX3CL1 was upregulated by albumin overload in cultured renal tubular cells.[33] IL-8 is a member of the CXC
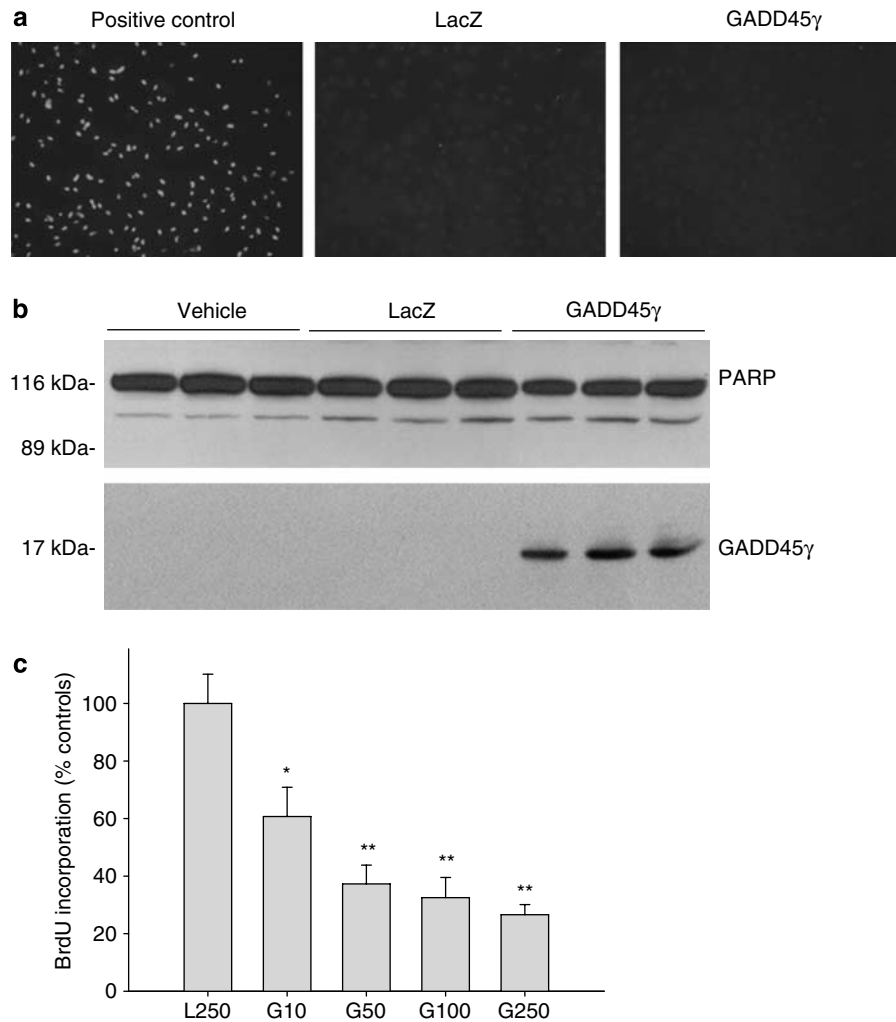
Figure 7 | The effect of GADD45γ on HRE cell apoptosis and proliferation. (a, b) GADD45γ did not induce apoptosis in HRE cells. The cells were incubated with adenoviral vectors containing the GADD45γ gene or the control LacZ gene at an MOI of 500 for 24 h. (a) TUNEL staining. TUNEL-positive apoptotic cells were not visualized in HRE cells overexpressed with LacZ or GADD45γ. For positive controls, DNA strand breaks were induced by DNaseI. (b) Western blot analysis for PARP cleavage. PARP cleavage was not observed; only the intact PARP (116 kDa), and not the cleaved form (89 kDa), was observed in HRE cells overexpressed with LacZ or GADD45γ. (c) GADD45γ significantly reduced HRE cell proliferation. After a 24-h incubation with adenoviral vectors, cells were labeled with BrdU. LacZ 250 served as controls. Values are expressed as percentage of controls and are means ± s.e. of three independent experiments, each of which used five replicate wells per each group. *$P < 0.05$, **$P < 0.01$ compared with LacZ controls. L represents LacZ and G represents GADD45γ. Each number following L or G represents an MOI. PARP, poly(ADP-ribose) polymerase; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick-end labeling; BrdU, 5-bromo-2'-deoxyuridine.

chemokine family and causes chemotaxis of neutrophils and T-lymphocytes.[34] In human glomerular diseases, IL-8 was increased in patients' urine[35] and in renal tissue.[36] In addition, a pathogenetic role of IL-8 in antineutrophil cytoplasmic antibody-associated glomerulonephritis has been addressed.[37]

We also found that GADD45γ induces the upregulation of fibrosis-related factors, including MMP1; MMP9 and MMP10; decorin; TGF-β1; and BMP2. It has historically been considered that MMPs degrade matrix, thereby reducing renal fibrosis. However, recent studies challenge such a concept. A decrease in MMP9 by knockout of tissue-type plasminogen activator preserved the integrity of tubular

basement membrane in the obstructed kidneys.[38] Similarly, it has been shown that MMP9 disrupted cell barrier function in the kidney cells.[39] Furthermore, transgenic renal tubular expression of MMP2 produced tubular atrophy, fibrosis, and renal failure in the kidney.[40] TGF-β is a multifunctional cytokine that has been studied extensively as a mediator of kidney fibrosis.[41] Decorin is an extracellular proteoglycan, which binds TGF-β1, hence it has been suggested that decorin neutralizes the profibrotic effect of TGF-β1.[42] However, accumulating studies showed that decorin actually contributes to development of glomerular and tubulointerstitial fibrosis.[43,44] Lastly, increased BMP2 production in response to high glucose[45] and induction of reactive oxygen
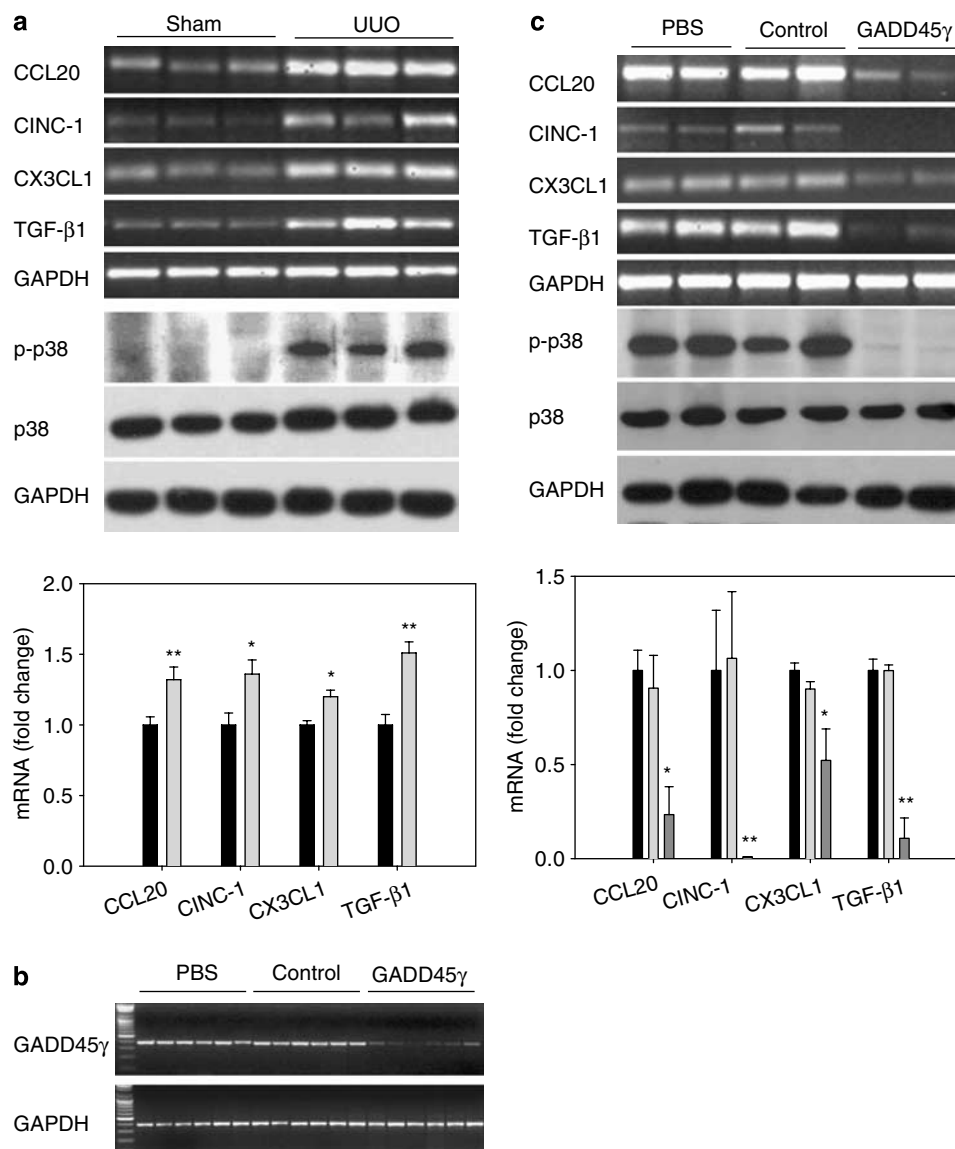
Figure 8 | GADD45γ siRNA reduced the expression of chemokines, TGF-β1, and p-p38 MAPK. (**a**) The mRNA expression of CCL20, CX3CL1, CINC-1, and TGF-β1 was significantly increased in the kidneys 2 days after UUO (gray bars) compared with sham-operated controls (black bars). The western blot shows that p-p38 MAPK was increased significantly in the kidneys 2 days after UUO compared with sham-operated controls, $n = 10–12$ for each experimental group. (**b**) The expression of GADD45γ mRNA was significantly decreased in UUO kidneys treated with GADD45γ siRNA. Subsequent competitive PCRs confirmed a 90% reduction of GADD45γ mRNA in GADD45γ siRNA-treated kidneys, $n = 6$ for each experimental group. (**c**) The expression of CCL20, CX3CL1, CINC-1, and TGF-β1 was significantly decreased in GADD45γ siRNA-treated UUO kidneys (dark gray bars) compared with the control UUO kidneys treated with PBS (black bars) or negative control siRNA (light gray bars). The western blot shows that p-p38 MAPK was significantly reduced by GADD45γ siRNA infusion, $n = 6$ for each experimental group. Data are mean ± s.e.m.; *$P < 0.05$, **$P < 0.01$ compared with controls. UUO, unilateral ureteral obstruction; PBS, phosphate-buffered saline; CCL20, chemokine (C–C motif) ligand 20; CX3CL1, chemokine (C–X3–C motif) ligand 1; CINC-1, cytokine-induced neutrophil chemoattractant-1 (rat homolog for human IL-8); TGF-β1, transforming growth factor-β1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

species by BMP2 in glomerular cells suggest that BMP2 may contribute to glomerular cell injury.[46] To further elucidate the link between GADD45γ and inflammatory and fibrogenic mediators, we knocked down the GADD45γ gene in the kidney using *in vivo* infusion of siRNA and were able to demonstrate that silencing of GADD45γ gene significantly blocks upregulation of CCL20, CX3CL1, CINC-1, and TGF-

β1 in UUO kidneys. We also demonstrated that silencing of GADD45γ gene significantly reduces inflammatory cell infiltration in UUO kidneys.

In this study, we show that GADD45γ is upregulated in UUO and is expressed in kidney biopsy tissue obtained from patients with chronic glomerulonephritis. We further provide *in vitro* and *in vivo* evidence that GADD45γ regulates various
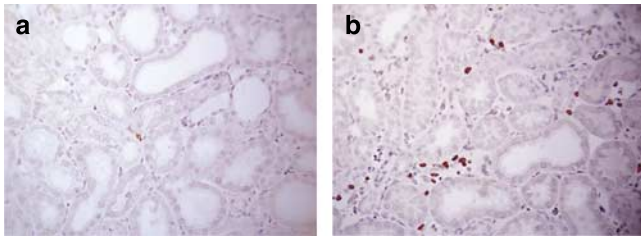
Figure 9 | ED1 immunostaining to assess monocyte/ macrophage infiltration in the interstitium. ED1-positive cells (brown-red cytoplasmic staining) were significantly reduced in UUO kidneys treated with GADD45γ siRNA (**a**) compared with the control UUO kidneys treated with negative control siRNA (**b**). Original magnification ×400.

molecules that have been implicated in the pathogenesis of kidney disease. Further studies are warranted to determine the role of GADD45γ in the pathogenesis of kidney disease.

MATERIALS AND METHODS
Animal surgery
Male Wistar rats (Japan SLC, Hamamatsu, Japan) weighing 250–300 g were used for UUO or a sham operation. After anesthesia, the left kidney and the ureter were exposed by an abdominal incision and the left ureter was ligated with 4-0 silk. The sham operation for control kidneys consisted of a similar abdominal incision and visualization of the left ureter without further manipulation. The harvested kidneys were bisected and cortical tissues were immediately snap frozen in liquid nitrogen and kept at −70 °C until required. For *in situ* hybridization, the halved kidneys were immediately fixed in 4% paraformaldehyde. All animal experiments were undertaken in accordance with the guidelines of the animal committee of our institution.

Differential display analysis
Differential display was performed using a Gene Fishing DEG kit (Seegene, Seoul, South Korea) according to the manufacturer's instructions. Total RNA was extracted from the rat kidney harvested 1 day following UUO. The unobstructed kidney on the other side served as a control. First-strand complementary DNA was synthesized by reverse transcription using the annealing control primer, dT-ACP1 (5′-CTGTGAATGCTGCGACTACGATXXXXX(T)$_{18}$-3′), and then PCRs were performed with combinations of an arbitrary ACP (ACP01-ACP20) and dT-ACP2 (5′-CTGTGAATGCTGCGACTACG ATXXXXX(T)$_{15}$-3′). The PCR products were separated by 2% agarose gel electrophoresis. Differentially expressed genes were cloned into a TOPO TA cloning vector (Invitrogen, Carlsbad, CA, USA); the inserts were then sequenced.

In situ hybridization
To synthesize DIG-labeled cRNA probes, a DNA fragment containing bases +341 to +782 (442 bp) of rat GADD45γ (GenBank accession no. AB020978) were ligated into the *Xba*I–*Xma*I restriction sites of a vector pSPT18 (Roche, Mannheim, Germany), which contains SP6 and T7 polymerase sites flanking the cloned insert. After linearization with *Xba*I or *Xma*I, the DNA insert was transcribed into antisense and sense cRNA probes in the reaction mixture containing DIG-11-UTP by T7 or SP6 RNA polymerase, respectively. Sections (7 µm) were cut from paraffin-embedded

blocks, dewaxed, and then permeabilized using 12.5 µg ml^{-1} RNase-free proteinase K for 30 min at 37 °C. The sections were refixed with 4% paraformaldehyde for 5 min at 4 °C, treated with 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8), and then incubated in pre-hybridization buffer (4 × saline-sodium citrate, 50% deionized formamide). We then applied 150 ng DIG-labeled sense or antisense probes in 75 µl hybridization buffer (40% deionized formamide, 10% dextran sulfate, 1 × Denhardt's solution, 4 × SSC, 10 mM DTT, 1 mg ml^{-1} yeast t-RNA, 1 mg ml^{-1} salmon sperm DNA) to the sections, coverslipped, and then incubated them at 42 °C for overnight in a humid chamber. The sense probe served as control. After hybridization, the sections were treated with 20 µg ml^{-1} RNase A in NTE buffer (500 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 8) for 30 min at 37 °C. After incubation with blocking solution (0.1% Triton X-100, 2% normal sheep serum), the sections were incubated with 1:500 anti-DIG alkaline phosphatase Fab fragments in blocking solution for 2 h at room temperature. We then developed color with nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in buffer (100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 50 mM MgCl$_2$) containing 5 mM levamisole.

RNA isolation
Kidney specimens were homogenized in Trizol reagent (Invitrogen) using a glass–Teflon homogenizer. RNA was extracted with chloroform, precipitated with isopropanol, washed with 75% ethanol, and then redissolved in TE buffer. The isolated RNA was quantified spectrophotometrically.

Reverse transcription
After removing contaminated DNA from the isolated RNA using DNaseI (Invitrogen), 1 µg of total RNA was reverse transcribed into cDNA in 20 µl reaction mixtures containing 200 U of Moloney murine leukemia virus reverse transcriptase; 100 ng per reaction of random hexanucleotide primers; and 0.5 mM each of dNTPs, dATP, dCTP, dGTP, and dTTP. The reaction mixture was then incubated at 37 °C for 1 hour and at 65 °C for 10 min to deactivate the reverse transcriptase.

Polymerase chain reactions
Competitive PCRs were performed for GADD45γ and GAPDH of rat kidneys using the competitor constructs as described previously.[47,48] Semiquantitative PCRs were performed for multiple gene analysis of HRE cells. Individual PCRs was performed over 28–33 cycles using a Perkin Elmer 9600 thermocycler. The amplification profile typically consisted of denaturation at 94 °C for 20 s, primer annealing at 57 °C for 30 s, and primer extension at 72 °C for 20 s. Target gene-specific primers are shown in Table 2. The target gene product amounts were normalized to those of GAPDH.

Immunohistochemistry
For antigen retrieval, the 4-µm paraffin sections obtained from human biopsy samples were heated in citrate buffer (pH 6) in a microwave oven , and then incubated with the GADD45γ primary antibody for 1 h at room temperature in a humidified chamber. For immunostaining of ED1 (= CD68, a specific monocyte/macrophage marker), the paraffin sections obtained from rat kidneys were treated with 0.1% proteinase K (Sigma, St Louis, MO, USA) for 30 min and incubated with ED1 primary antibody (Serotec, Oxford, UK) for 1 h at 37 °C in a humidified chamber. The sections were

Table 2 | Primers used in the PCRs

Primers (expected size)	Sequences	GenBank accession
GAPDH, rat (390 bp)	F: 5′-TGTTCCAGTATGACTCTACCC-3′ R: 5′-TCATGAGCCCTTCCACGATG-3′	AF106860
GADD45γ, rat (378 bp)	F: 5′-ACTCTGGAAGAAGTCCGTGG-3′ R: 5′-TTCATTAGGGTTCGAAATGAGG-3′	AB020978
GADD45α, rat (314 bp)	F: 5′-AGGAAGTGCTCAGCAAGGCT-3′ R: 5′-CGTCACCAGCACACAGTGTA-3′	NM_024127
GADD45β, rat (302 bp)	F: 5′-TCAGAAGATGCAGGCGGTGA-3′ R: 5′-CCTCGTTTGTGCCTAGAGTC-3′	NM_001008321
CINC-1, rat (669 bp)	F: 5′-CTCCAGCCACACTCCAACAGA-3′ R: 5′-CACCCTAACACAAAACACGAT-3′	NM_030845
CX3CL1, rat (160 bp)	F: 5′-TTTCATCTGTGTACTCTGCTGGC-3′ R: 5′-GTCTCGTCTCCAGGATGATGG-3′	NM_030845
CCL20, rat (301 bp)	F: 5′-GATGGAATTGGACAAGCCC-3′ R: 5′-AAGGGCCATATAAACTTACTGC-3′	NM_019233
GAPDH (299 bp)	F: 5′-GTCGGAGTCAACGGATTTGG-3′ R: 5′-ATGGTGGTGAAGACGCCAGT-3′	NM_002046
CCL20 (270 bp)	F: 5′-GTGCTGTACCAAGAGTTTGCT-3′ R: 5′-TTACTGAGGAGACGCACAATAT-3′	NM_004591
CX3CL1 (347 bp)	F: 5′-TTGGCCACCTTCTGCCATCT-3′ R: 5′-AGGACCACAGACTCGTCCAT-3′	NM_002996
IL-8 (258 bp)	F: 5′-CTGATTTCTGCAGCTCTGTGT-3′ R: 5′-TGAATTCTCAGCCCTCTTCAAA-3′	NM_000584
IL-11 (355 bp)	F: 5′-ACCACAACCTGGATTCCCTG-3′ R: 5′-CAGTCAAGTGTCAGGTGCAG-3′	NM_000641
MMP1 (432 bp)	F: 5′-GTATTGGAGGGGATGCTCATT-3′ R: 5′-ATTCGTAAGCAGCTTCAAGCC-3′	NM_002421
MMP10 (240 bp)	F: 5′-ACTCATTCACAGAGCTCGCC-3′ R: 5′-TCCAGTGGGATCTTCGCCAA-3′	NM_002425
BMP2 (351 bp)	F: 5′-CCTGCAACAGCCAACTCGAA-3′ R: 5′-GCTGGACTTAAGGCGTTTCC-3′	NM_001200
Decorin (246 bp)	F: 5′-GTGGTCCAGTGTTCTGATTTG-3′ R: 5′-GAAACTCAATCCCAACTTAGCC-3′	NM_133504
Integrin-α5 (351 bp)	F: 5′-TGCACCACCAGCAAAAACGG-3′ R: 5′-CTAGGATGATGATCCACAGTG-3′	NM_002205
Thrombospondin 1 (320 bp)	F: 5′-GGAGAATGCTGAGTTGGACG-3′ R: 5′-CCATGCTGGACAGCTCATCA-3′	NM_003246
Integrin-β1 (382 bp)	F: 5′-ACCACAGCAGTTGGTTTTGCG-3′ R: 5′-AGATATGCGCTGTTTTCCAACA-3′	NM_133376
Collagen XI (360 bp)	F: 5′-ATCACAGGTGATCCCAAGGC-3′ R: 5′-ATTTGGCTCATTTGTCCCAGAA-3′	NM_080629
Collagen I (348 bp)	F: 5′-TGCCGTGACCTCAAGATGTG -3′ R: 5′-CACGCTGTTCTTGCAGTGGTA-3′	NM_000088
Collagen III (440 bp)	F: 5′-TGGAGGTGAAAAAGCTGGCG-3′ R: 5′-CCAGCTGCACATCAAGGACA-3′	NM_000090
Fibronectin (501 bp)	F: 5′-AGGTAGCAGCACAGAACTCAA-3′ R: 5′-CATCTCACCAGGACAGTAGAA-3′	NM_212482
MMP1 (392 bp)	F: 5′-ACAACTTACATCGTGTTGCGG-3′ R: 5′-GACTTCATCTCTGTCGGCAAA-3′	NM_002421
Collagen IV (391 bp)	F: 5′-GTATTGGTGGCTCTCCAGGA-3′ R: 5′-CCTGGAAATCCTCTTGGACC-3′	NM_001845
MMP9 (380 bp)	F: 5′-TGCCTGCAACGTGAACATCTT-3′ R: 5′-TCTGCGCCTTCACGTCGAAC-3′	NM_004994
MCP-1 (292 bp)	F: 5′-AAAGTCTCTGCCGCCCTTCT-3′ R: 5′-TCTTCGGAGTTTGGGTTTGCT-3′	X14768
CTGF (320 bp)	F: 5′-CCCGGGTTACCAATGACAAC-3′ R: 5′-GCAGGCACAGGTCTTGATGA-3′	NM_001901
VEGFB (320 bp)	F: 5′-CACCAGAGGAAAGTGGTGTC-3′ R: 5′-CTGTCTGGCTTCACAGCACT-3′	NM_003377
PAI-1 (360 bp)	F: 5′-AAAGAGGTGCCTCTCTCTGC-3′ R: 5′-GACCACAAAGAGGAAGGGTC-3′	M16006
VEGFA (370 bp)	F: 5′-CTGAGGAGTCCAACATCACC-3′ R: 5′-AACTCAAGCTGCCTCGCCTT-3′	NM_001025366
MMP2 (347 bp)	F: 5′-TTGATGCGGTATACGAGGCC-3′ R: 5′-GGCACCCTTGAAGAAGTAGC -3′	NM_004530

BMP2, bone morphogenetic protein 2; CCL20, chemokine (C–C motif) ligand 20; CINC-1, cytokine-induced neutrophil chemoattractant-1; CTGF, connective tissue growth factor; CX3CL1, chemokine (C–X3–C motif) ligand 1; F, forward primers; GADD45γ, Growth Arrest and DNA Damage-45γ; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; MCP-1, monocyte chemotactic protein-1; MMP, matrix metalloproteinase; PAI-1, plasminogen activator inhibitor-1; R, reverse primers; TGF-β1, transforming growth factor-β1; VEGFA, vascular endothelial growth factor A; VEGFB, vascular endothelial growth factor A.

then incubated with biotinylated secondary antibodies (Dako, Carpinteria, CA, USA) followed by peroxidase-labeled streptavidin. Sections were developed with diaminobenzidine and counterstained with hematoxylin. All protocols for obtaining human samples were approved by the institutional review board of our institution.

Cell culture

Primary HRE cells (Cambrex, Walkersville, MD, USA) were prepared and maintained in renal epithelial basal medium with recommended supplements included in the REGM Singlequot Bulletkit (Cambrex). Renal epithelial basal medium supplemented only with 0.5% fetal bovine serum was used for serum starvation.

Western blot analysis

Two wells of a six-well plate were pooled for each analytical sample in all cases. Equal amounts of total protein (20–30 μg) were subjected to 6–15% acrylamide gel sodium dodecyl sulfate-polyacrylamide gel electrophoresis, blotted with primary antibodies for GADD45γ (sc-8778; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or MAPK proteins (Cell Signaling, Danvers, MA, USA), and then incubated with peroxidase-conjugated secondary antibodies. We then detected the proteins by enhanced chemiluminescence (Amersham, Piscataway, NJ, USA). Band intensity was analyzed using the Scion Image software alpha 4.0.3.2 (Scion Corp., Frederick, MD, USA).

Microarray analysis

After 24 h of serum starvation, HRE cells were infected with Ad-GADD45γ at an MOI of 50 ($n = 4$, sample group) and with Ad-LacZ at an MOI of 100 ($n = 4$, control group). After 24 h of incubation, cells were harvested and total RNA was isolated as described above. Two wells of the six-well plate were pooled for each analytical sample. Biotinylated cRNA was prepared using the Illumina amplification kit (Ambion, San Diego, CA, USA) and was purified using RNAeasy columns (Qiagen, Valencia, CA, USA). The product was hybridized to Sentrix Human Ref-8 Expression Beadchips (Illumina, San Diego, CA, USA) and was detected with streptavidin-Cy3. The arrays were scanned using the Illumina Bead Station 500x array reader (Illumina). The scanned images were analyzed using the BeadStudio software (Illumina).

Enzyme-linked immunosorbent assay

After 24 h of serum starvation, HRE cells were infected with Ad-GADD45γ at an MOI of 50 ($n = 8$, sample group) and with Ad-LacZ at an MOI of 100 ($n = 8$, control group). After 24 h of incubation, cell culture media were collected and analyzed for CCL-20, CX3CL1, and IL-8 using commercially available enzyme-linked immunosorbent assay kits according to the manufacturer's specifications (R&D Systems, Minneapolis, MN, USA).

Apoptosis assay

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP–biotin nick-end labeling staining was performed to label DNA strand breaks generated during apoptosis with fluorescein isothiocyanate-conjugated dUTP (Roche). Cells grown on coverslips were incubated in permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) and then with mixture of TdT and labeled dNTP. For positive controls, DNA strand breaks were induced by incubating fixed and permeabilized cells with 10 U of DNaseI. The apoptotic cells were visualized by fluorescence microscopy (Axiostar plus; Carl

Zeiss, Jena, Germany). We also observed apoptosis by western blotting for PARP cleavage using the monoclonal anti-PARP antibody (Cell Signaling).

Cell proliferation assay

The effect of GADD45γ on HRE cell proliferation was determined using the BrdU Labeling and Detection kit (Roche) according to the manufacturer's instructions. Briefly, cells were incubated with various concentrations of Ad-GADD45γ for 24 h, the medium was then replaced, and cells were incubated with 10 μM BrdU for 18 h. BrdU-labeled DNA was detected using an anti-BrdU antibody conjugated with peroxidase and visualized with a soluble chromogenic substrate. Optical density was measured using a microplate reader at 405 nm.

In vivo silencing of the GADD45γ gene

We performed *in vivo* gene silencing based on hydrodynamic injection of siRNAs, which has been used successfully to silence target gene expression in the kidney.[6–8] The sense and antisense strands of siRNA targeted to GADD45γ were annealed and dissolved in RNase-free PBS. Rats were rapidly injected into the tail vein with 500 μg GADD45γ siRNA or negative control siRNA (Bioneer, Daejeon, Korea) in 6 ml PBS or PBS only. The sequences of siRNA were as follows: GADD45γ siRNA: CUGUUCGUGGAUCGCAC AAdTdT (sense), UUGUGCGAUCCACGAACAGdTdT (antisense); negative control siRNA: 5′-CCUACGCCACCAAUUUCGUdTdT-3′ (sense), 5′-ACGAAAUUGGUGGCGUAGGdTdT (antisense). Ureters were ligated on the following day and the kidneys were harvested 2 days after UUO.

Statistical analysis

Data are expressed as means ± s.e.m. (s.d. for microarray results). Unpaired *t*-test was used to compare continuous variables between two groups. A *P*-value of < 0.05 was considered significant.

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