Activation of STAT3/Smad1 Is a Key Signaling Pathway for Progression to Glomerulosclerosis in Experimental Glomerulonephritis*

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Mesangial cell proliferation is a significant event in the development of progressive glomerular injuries. However, the issue of how cell proliferation is involved in the development of glomerulosclerosis is unclear. Recently, we have demonstrated that the overexpression of type IV collagen (Col IV), a major component of mesangial extracellular matrix, is transcriptionally regulated by Smad1 in diabetic glomerulosclerosis. In this study, we have demonstrated the effect of the administration of an anti-platelet-derived growth factor (PDGF) β-receptor antibody (APB5) blocking activation by the PDGF-B chain on rat glomerulonephritis and have examined the signaling pathways that regulate both mesangial cell proliferation and glomerulosclerosis in vivo and in vitro. Experimental mesangial proliferative glomerulonephritis (Thy1 GN) was induced by a single intravenous injection of anti-rat Thy-1.1 monoclonal antibody. In Thy1 GN, mesangial cell proliferation and the expression of Col IV peaked at day 6. Immunohistochemical staining for the expression of Smad1, phospho-Smad1 (pSmad1), and phospho-STAT3 (pSTAT3) revealed that the peak for glomerular Smad1 expression occurred at day 6, consistent with the peak for mesangial cell proliferation. The expression of pSmad1 was up-regulated at day 1, and the peak for glomerular pSmad1 expression occurred at day 4 of the disease. When treated with APB5, both mesangial proliferation and sclerosis were reduced significantly. The expression of Smad1, pSmad1, and pSTAT3 was also significantly reduced by the administration of APB5. PDGF induced both mesangial cell replication and Col IV synthesis in association with an increased expression of pSTAT3 and pSmad1 on cultured mesangial cells. In addition, APB5 reduced mesangial cell proliferation in association with decreased pSmad1, pSTAT3, and Col IV protein expressions in vitro. The introduction of dominant negative STAT3 significantly decreased the expression of Col IV in cultured mesangial cells. These data suggest that the activation of STAT3 and Smad1 participates in the developing process of glomerulosclerosis in experimental glomerulonephritis.

Both mesangial cell proliferation and glomerulosclerosis are major important pathological features in progressive glomerular damage. In many glomerular sclerosing diseases, mesangial cell proliferation is a critical process in progressive glomerular injuries (1, 2). Both events are observed simultaneously in most glomerular diseases; however, the issue of how cell proliferation contributes to the development of glomerulosclerosis remains unclear.

Platelet-derived growth factor (PDGF)1 is known to be a critical mitogen for mesangial cells in vivo and in vitro (3, 4). Several lines of evidence indicate that PDGF plays a key role in the development of glomerulosclerosis not only in experimental models but also in human glomerular diseases (3). PDGF-BB has also been reported to be essential for mesangial cell proliferation (5), which is followed by development of glomerulosclerosis in the remnant kidney model (6). The introduction of a neutralizing anti-PDGF antibody has shown that both mesangial proliferation and glomerulosclerosis can be markedly ameliorated in a rat glomerulonephritis model (7), but little is known concerning the mechanisms of how the suppression of cell proliferation reduces glomerular sclerotic lesions.

Glomerulosclerosis is characterized by an increase in the levels of ECM. Col IV is a major component of expanded ECM in glomerular diseases, but the molecular mechanism of regulating Col IV gene transcription had not been cleared until our recent report in which we showed that Smad1 transcriptionally regulates the overexpression of Col IV in diabetic nephropathy (8). Smad1 directly transduces signals to downstream target genes that are related to renal damage such as osteopontin (9), inhibition of differentiation (10), and type I collagen (11) and is critically important for the development of kidney disease (12). These findings suggest that Smad1 is a critical transcriptional factor in the progression of glomerulosclerosis.

Signal transducer and activator of transcription (STAT) proteins have been shown to be involved in signaling by numerous cytokines and growth factors. It is well established that STAT3 activation is a key step in PDGF-induced mitogenesis (13). Nakashima et al. (14) report that transcriptional coactivator p300 physically interacts with STAT3 and Smad1, followed by the subsequent activation of the target gene transcription in

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1 The abbreviations used are: PDGF, platelet-derived growth factor; PDGFβ-R, PDGFβ receptor; ECM, extracellular matrix; Col IV, type IV collagen; STAT, signal transducer and activator of transcription; pSmad1, phospho-Smad1; PAM, periodic acid-methenamine; PCNA, proliferating nuclear antigen; FCS, fetal calf serum; BrdUrd, bromodeoxyuridine; ELISA, enzyme-linked immunosorbent assay; GN, glomerulonephritis.
astrocyte differentiation. We have postulated from these findings that PDGF is able to activate the STAT3-Smad1 cross-talk pathway in mesangial proliferative glomerulonephritis and that the process is essential for the progression of mesangial cell proliferation to glomerulosclerosis. The goal of this study was to determine how the STAT3 and Smad1 signaling pathways are involved in the development of glomerulosclerosis, using anti-PDGFβ-receptor antibody in a rat glomerulonephritis model.

**EXPERIMENTAL PROCEDURES**

**Animals**

Male Wistar rats (CLEA Japan, Inc.) weighing 180–200 g were used in this study. Rats were housed under specific pathogen-free conditions. All animal experiments were performed in accordance with institutional guidelines, and the Review Board of Tokushima University granted ethical permission for this study.

**Induction of Thy1 Glomerulonephritis**

Experimental mesangial proliferative glomerulonephritis (Thy1 GN) was induced by a single intravenous injection of anti-rat Thy-1 monoclonal antibody (1 mg/kg) (Cedarlane Laboratories, Ontario, Canada) as described elsewhere (15). These rats were sacrificed at days 1, 2, 4, 6, and 12 (n = 6/group) after the administration of anti-Thy-1 antibody. Six age-matched rats were injected with vehicle only and were sacrificed as controls.

**Protocol of Treatment with Anti-PDGFR-R Antibody in Thy1 GN**

A rat monoclonal anti-PDGFRβ-receptor antibody (APB5) and its antagonistic effects on the PDGFβ-R signal transduction pathway in vivo and in vitro have been described previously (16, 17). The rats were injected intraperitoneally at daily intervals with 400 μg of APB5 or irrelevant isotype-matched control rat IgG after the administration of anti-Thy-1.1 antibody from day 0. They were sacrificed at days 1, 2, 4, 6, and 12 (n = 6/group).

**Histological Examination**

**Light Microscopy**—After removal of the kidney, tissue blocks for light microscopy examination were fixed in methyl Carnoy’s solution and embedded in paraffin. Sections (2 μm) were stained with hematoxylin and eosin, periodic acid-Schiff’s reagent, and periodic acid-methenamine (PAM) silver.

**Immunohistochemistry**—Kidney sections were processed for immunohistochemistry following standard procedures. To study proliferating nuclear antigen (PCNA), Col IV, and Smad1, methyl Carnoy’s solution-fixed and paraffin-embedded tissue blocks were used. Kidney sections were dehydrated and treated with 0.3% hydrogen peroxide in methanol for 30 min. To eliminate nonspecific staining, sections were incubated with the appropriate preimmune serum for 20 min at room temperature and then incubated with avidin-D- and biotin-blocking solutions (Vector, Burlingham, CA) for 15 min each. Sections were incubated with the anti-PCNA antibody (1:200 dilution), anti-Col IV antibody (1:200 dilution), and anti-Smad1 antibody (1:100 dilution) (Santa Cruz Biotechnology) for 60 min at room temperature and then incubated with the appropriate biotinylated secondary antibodies followed by incubation with the avidin-biotin peroxidase complex (Vectastain Elite ABC kit, Vector). Peroxidase conjugates were subsequently localized using diaminobenzidine tetrahydrochloride. To study pSmad1 and pSTAT3, the tissues were snap-frozen in cold acetate in OCT compound (Miles Inc., Elkhart, IN), cut in 4-μm-thick sections, fixed in acetone for 5 min, and treated with 0.3% hydrogen peroxide in methanol for 30 min. Sections were treated in the same manner as PCNA with the following primary antibodies: anti-pSmad1 antibody (1:100 dilution) (Calbiochem) and anti-pSTAT3 antibody (1:100 dilution) (Santa Cruz Biotechnology). The treated sections were then analyzed.

**Quantitation of Light Microscopy**—Glomerular morphometry was evaluated in PAM-stained tissues. The glomerular surface area and the PAM-positive area/glomerular area (%) were measured using an image analyzer with a microscope (IPAP, Image Processor for Analytical Pathology; Sumitomo Chemical Co., Osaka, Japan) as described (18–20). For each animal, 50 glomeruli were analyzed.

**Quantitation of Immunohistochemistry**—For the quantification of proliferating cells (PCNA-positive cells), a blind test evaluated 50 glomeruli in each specimen, and the mean values/glomerulus were calculated. To quantitate the expression of pSmad1, pSmad1-positive cells/glomerular cell were counted, and the mean percentages of pSmad1-positive cells were calculated. For Col IV, Smad1, and pSTAT3, the brown area on an immunoperoxidase-stained section was selected for its color range, and the percentages of this area to the total mesangial area were quantitated using IPAP. In each animal, 50 glomeruli were evaluated.

**Cell Culture Experiment**

A glomerular mesangial cell line was established from glomeruli isolated from normal, 4-week-old mice (C57BL/6xSJLJ) and identified according to a previously described method (21). Mesangial cells were maintained in B medium (a 3:1 mixture of minimal essential medium/F12 modified with trace elements) supplemented with 1 mM glutamine, penicillin at 100 units/ml, streptomycin at 100 μg/ml, and 20% fetal calf serum (FCS). The cultured cells fulfilled the generally accepted criteria for glomerular mesangial cells (22). Mesangial cells were plated in B medium/20% FCS onto 100-mm dishes. After 24 h of incubation, the cells were starved for 2 days in B medium/0.1% bovine serum albumin, cultured in B medium/2% FCS with 5 ng/ml of PDGF-B (Calbiochem), and then incubated with 100 ng/ml of APB5 or control rat IgG for 24 h.

**Cell Proliferation Test by BrdUrd ELISA**

The proliferation of mesangial cells was also determined using a colorimetric immunoassay, based on the measurement of BrdUrd incorporation during DNA synthesis (Amersham Biosciences). The BrdUrd ELISA was performed according to the manufacturer’s instructions. Briefly, mesangial cells were plated out at a low density in 96-well flat-bottomed microtiter plates in B medium/10% FCS and allowed to adhere overnight. The subconfluent cells were then starved for 2 days in B medium/0.1% bovine serum albumin. 100 ng/ml of APB5 was added to cells in B medium/2% FCS with 5 ng/ml PDGF-B and 10 μg/ml BrdUrd. After 6 h of culture, the plates were centrifuged and the cells denatured with a fixative solution and then incubated for 30 min with 1:100 diluted anti-BrdUrd monoclonal antibodies conjugated to peroxidase. After removing the antibody conjugate, substrate solutions were added for 15 min, and the reaction was terminated by adding 1 M sulfuric acid. The absorbance was measured within 5 min at 450 nm with a reference wavelength at 690 nm using an ELISA plate reader (Model550; Bio-Rad Laboratories). The blank corresponded to 100 μl of culture medium with or without BrdUrd.

**Western Blot Analysis**

Cultured mesangial cells were starved for 24 h in B medium/0.1% bovine serum albumin. The cells were stimulated with 5 ng/ml PDGF-BB with 100 ng/ml APB5 or control IgG for 120 min. Cells were suspended in lysis buffer, resolved by SDS-polyacrylamide gel electrophoresis, and transferred to a nitrocellulose membrane. They were subjected to Western blot analysis using a 1:1000 dilution of antibodies for pSTAT3, 1:1000 dilution of antibodies for pSmad1, and 1:2000 dilution of antibodies for Col IV and detected using an enhanced chemiluminescence detection system (Amersham Biosciences).

**Treatment of AG490 in Cultured Mesangial Cells**

Cultured mesangial cells were starved for 24 h in B medium/0.1% bovine serum albumin. The cells were stimulated with 5 ng/ml PDGF-BB with 50 μM AG490 (Calbiochem), a well characterized inhibitor of STAT3 phosphorylation (23, 24), or control vehicle for 8 h. Cell lysis and Western blot analysis were performed as previously described. The proliferation of mesangial cells was also determined using a BrdUrd ELISA system.

**Cell Transfection**

Plasmid constructs of expression vectors of wild type STAT3 and dominant negative STAT3 were kindly provided by Jackie Bromberg (Rockefeller University) (25). Mesangial cells (60-mm dish) were transfected with an expression vector encoding wild type STAT3 (8 mg) or dominant negative STAT3 (8 mg) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. After 6 h of transfection, the medium was changed to growth medium (60% Dulbecco’s modified Eagle’s medium, 20% F12, 20% fetal calf serum). After 48 h, cells were suspended in lysis buffer, and Western blot analysis was performed as previously described. The proliferation of transfected mesangial cells was also determined using a BrdUrd ELISA system.
**Statistical Analysis**

All values were expressed as the mean ± S.E. and analyzed by Mann-Whitney nonparametric analysis or one-way analysis of variance with a modified t test. Statistical analysis of the cell proliferation test and expression of Smad1 mRNA in cultured mesangial cells were done by t test. Quantitation of immunohistochemistry and expression of Smad1 mRNA in glomeruli were analyzed by one-way analysis of variance followed by the post hoc test. p values <0.05 were considered significant. Data are expressed as mean ± S.D.

**RESULTS**

**Morphological Changes in Thy1 GN—**We examined the in vivo role of Smad1 phosphorylation in glomerulonephritis. We utilized an acute model of mesangial proliferative glomerulonephritis known as Thy1 glomerulonephritis. In Thy1 GN, the proliferation of mesangial cells begins at day 2, peaks at day 6, and subsides in 12 days after the injection. Fig. 1 shows a representative light microscopic picture at day 6 for each group. The Thy1 GN group showed the increase of mesangial matrix, which peaked at day 6 (Fig. 1B). Increased replication of glomerular cells was assessed by immunostaining of PCNA. PCNA-positive cells were markedly increased in the Thy1 GN group and peaked at day 6 (data not shown). Col IV is a main component of the ECM in the glomerulosclerosis. Col IV was weakly visible along the glomerular basement membrane and insignificant in the mesangial area in the normal control group (Fig. 1D). On the other hand, the Thy1 GN group showed a strong positive reaction for Col IV in the expanded mesangial area (Fig. 1F).


Anti-PDGFβ-receptor Antibody Inhibits Both Glomerular Cell Proliferation and Glomerulosclerosis in Vivo—APB5 inhibited the objective PDGFβ-R-mediated signaling pathway as described previously. Treatment with APB5 resulted in significant reductions of both glomerular cell numbers and glomerular PCNA-positive cells in Thy1 GN at each point studied (Figs. 1C and 2, A and B). Overexpression of the PDGF-B chain and PDGFβ-R were significantly reduced after the administration of APB5 (Fig. 1, I and L). APB5 treatment also reduced the increase in mesangial matrix in Thy1 GN, as assessed by the PAM-positive area/glomerular area using an image analyzer with a microscope (Fig. 2C). The mesangial expression of Col IV in Thy1 GN was suppressed by treatment with APB5 (Fig. 2D). These data indicate that APB5 is able to inhibit mesangial cell proliferation and the mesangial matrix expansion of Thy1 GN.

**Time Course for the Expression of Smad1, Phospho-Smad1 (pSmad1), and Phospho-STAT3 (pSTAT3) in Thy1 GN—**We examined the expression of Smad1 in a Thy1 GN rat kidney by
immunostaining. Smad1 was barely detectable in control glomeruli (Fig. 3A). However, in the glomerulus of Thy1 GN at day 6, Smad1 was extensively expressed with a typically expanded mesangial pattern (Fig. 3B). An IFAP system was used to quantitate the expression of Smad1. Glomerular Smad1 expression peaked at day 6 (Fig. 4A), consistent with the peak for mesangial proliferation. We next examined whether the phosphorylation and translocation of Smad1 are affected in Thy1 GN. By immunohistochemistry, pSmad1 was barely observed in control glomeruli. pSmad1 was markedly observed in the Thy1 rat glomeruli. APB5 treatment led to a significant decrease. A, C, Smad1. D–F, pSmad1. G–I, pSTAT3. A, D, G, normal control rat. B, E, H, untreated Thy1 rat glomeruli at day 6. C, F, I, treated with APB5 at day 6.

The APB5-treated groups had significantly reduced expression of Smad1 and pSmad1 proteins in glomeruli in Thy1 GN (Figs. 3, C and F, and 5, A and B). In addition, the overexpression of pSTAT3 was significantly inhibited by the administration of APB5 at each point studied (Figs. 3I and 5C).

Effect of Anti-PDGFβ-R Antibody in Vitro—To determine whether APB5 inhibits the proliferation of mesangial cells, we examined the proliferation of mesangial cells in the absence and the presence of APB5 by using the BrdUrd ELISA system. As shown in Fig. 6A, the addition of APB5 suppressed PDGF-induced DNA synthesis in cultured mesangial cells. Next we examined whether the presence of phosphorylation inhibitors can prevent the actions of STAT3 in mesangial cells. AG490, an inhibitor of STAT3 phosphorylation, significantly inhibited STAT3 phosphorylation in cultured mesangial cells and mesangial proliferation (Fig. 6B). We also examined by Western blot analysis whether APB5 reduces the expression of pSTAT3, pSmad1, and Col IV in mesangial cells after stimulation with PDGF-B. We found that APB5 decreased phosphorylation of STAT3 and Smad1 and the expression of Col IV (Fig. 6C). These data suggest that STAT3 and Smad1 are involved to a significant extent in the expression of Col IV induced by stimulation of the PDGF-B signaling pathway.

Interaction between STAT3 and Smad1—To elucidate the role of the interaction between STAT3 and Smad1 for the increased expression of Col IV, transfection with a vector encoding dominant negative STAT3 was performed in cultured mesangial cells. Transfection with the dominant negative STAT3 clearly decreased the expression of pSmad1 and Col IV compared with wild type STAT3 (Fig. 6D). Using the BrdUrd ELISA system, we further examined whether transfection with dominant negative STAT3 reduces mesangial proliferation. The dominant negative STAT3 suppressed PDGF-induced DNA synthesis in cultured mesangial cells.
DISCUSSION

Many glomerular diseases are characterized by both mesangial cell proliferation and progressive glomerulosclerosis, but the common mechanisms related to both of these important pathological findings remain unresolved. The findings herein have shown that the activation of STAT3 and Smad1 plays a key role in regulating both of these critical events of progressive glomerular damage. Based on these findings, we have proposed a new direction of research concerning the pathogenesis and a therapeutic approach for chronic glomerulonephritis and diabetic nephropathy, which are major problems in the 21st century.

Glomerulosclerosis is characterized by an increased amount of ECM mainly in the mesangium. Col IV is one of the major components of ECM and is overproduced in glomerulosclerosis (27). We recently reported that Smad1 is a key transcriptional factor in the regulation of Col IV expression in diabetic nephropathy in vitro and in vivo (8). In Thy1 GN, Col IV is strongly expressed in the sclerotic lesions of glomeruli as previously described (19). We showed here that phosphorylated Smad1 is strongly expressed in parallel with the up-regulated expression of Col IV and the expanded ECM in this glomerulonephritis. Moreover, the area in which Smad1 is strongly expressed is consistent with the Col IV-positive area. These findings suggest that Smad1 is a critical factor in the development of glomerulosclerosis not only in diabetic nephropathy but also in glomerulonephritis as well.

Glomerulosclerosis has the pathological features of progressive glomerular injuries, including chronic glomerulonephritis, IgA nephropathy, and diabetic nephropathy. Glomerular cell proliferation at an early stage in a number of glomerular diseases progresses to the subsequent development of glomerulosclerosis, which eventually progresses to end stage glomerular damages (1, 2). This process is seen in IgA nephropathy, membranoproliferative glomerulonephritis, diabetic nephropathy, and light chain systemic diseases in humans as well as in animals such as the anti-Thy1 glomerulonephritis and the rat renal ablation models (2, 28). Blocking glomerular cell proliferation with an anti-PDGF antibody (7), anti-coagulant heparin (30), or vitamin D analogue (19) has been demonstrated to abolish the subsequent development of progressive glomerulosclerosis, but responsibility for this remains unclear. In the

![Fig. 4. Time course of expression of Smad1, pSmad1, and pSTAT3. Kidney sections from Thy1 GN rats on days 0, 1, 2, 4, 6, and 12 were subjected to immunohistochemical staining with antibodies against Smad1, pSmad1, and pSTAT3. A, expression of Smad1 in Thy1 GN. Expression of Smad1 peaked at day 6 and subsided on day 12. B, the time course for the ratio of pSmad1-positive cells to total glomerular cell number. Expression of pSmad1 peaked at day 4. C, time course of expression of pSTAT3. The ratio of pSTAT3-positive area to mesangial area increased by day 6 and subsided on day 12. *, p < 0.001 versus Control. **, p < 0.001 versus each day.](http://www.jbc.org/)

![Fig. 5. Effects of APB5 treatment on Smad1, pSmad1, and pSTAT3 expression. Immunohistochemical staining and quantitation of Smad1, pSmad1, and pSTAT3 expression revealed treatment with APB5 reduced the expression of these proteins similar to mesangial matrix expansion and Col IV expression in glomeruli. A, expression of Smad1. B, expression of pSmad1. C, expression of pSTAT3. *, p < 0.001 versus non-treated disease control.](http://www.jbc.org/)
mesangial cells and is constitutively synthesized as an autocrine growth factor by these cells in vivo (32, 33). PDGF plays an important role in the development of pathological conditions, including glomerulonephritis, diabetic nephropathy, and progressive glomerulosclerosis in vivo and in vivo (3, 4). It has been previously reported that the activation of PDGF receptor tyrosine kinase induces the tyrosine phosphorylation of STAT3 proteins (13, 34). This activation is associated with growth regulation and differentiation (28, 29). The findings herein have demonstrated that the overexpression of phosphorylated STAT3 occurs simultaneously with the increased expressions of both PDGF and its β-receptor in this experimental glomerulonephritis and that APB5 ameliorates glomerulonephritis in association with reduced expression of PDGF, its β-receptor, and STAT3 in vivo. We have also shown that treatment with APB5 reduces the expression of Smad1 in Thy1 GN, indicating that the PDGF pathway can affect Smad1 production in vivo.

We confirmed the interaction of STAT3 and Smad1 in regulating the critical gene of glomerulosclerosis. The introduction of dominant negative STAT3 significantly decreased the expression of Col IV in cultured mesangial cells. The activation of STAT3 and Smad1 appears to be independent, although both factors are activated by PDGF. Furthermore, the activation of Smad1 appears to be involved in the activation of STAT3, based on the findings of a partial reduction of phosphorylated Smad1 by the introduction of dominant negative STAT3. These findings suggest that STAT3 activation by PDGF interacts with the overexpression of Smad1, followed by the subsequent activation of Col IV in experimental glomerulonephritis. Nakashima et al. (14) report that the transcriptional coactivator p300 physically interacts with STAT3 and Smad1 and that the formation of a complex between STAT3 and Smad1, bridged by p300, is involved in the cooperative signaling of the pathway. Thus, we concluded that the blocking of PDGF could affect the signaling of Smad1 and reduce the overproduction of Col IV in vitro and in vivo. The clear elucidation of both signaling pathways is essential for developing a complete understanding of the pathological process for development of progressive glomerular injury.

Therapeutic approaches for sclerosis in diverse organs are currently limited to supportive therapy to slow the loss of function of these organs. Our findings offer insights into the nature of the proliferative diseases that lead to sclerosis. Because both Smad1 and STAT3 are nearly absent in normal glomeruli, blocking Smad1 and/or STAT3 signaling may be beneficial for inhibiting the progression of various renal diseases leading to sclerosis by suppressing the pathologically activated proliferation and production of ECM.

FIG. 6. Effect of APB5 in vivo and Western blot analysis of transfected mesangial cells. A, inhibitory effects of APB5 on mesangial cell proliferation. Addition of PDGF-B increased the proliferation of mesangial cell, and APB5 significantly inhibited this proliferation. *, p < 0.05 versus control. **, p < 0.05 versus PDGF-B-stimulated control. B, AG490 significantly inhibited STAT3 phosphorylation in PDGF-B-stimulated mesangial cells and mesangial proliferation. *, p < 0.05 versus Control. **, p < 0.05 versus PDGF-B-stimulated Control. C, Western blot analysis revealed that expression of pSTAT3, pSmad1, and Col IV protein was reduced by addition of APB5. One of three independent experiments is shown. D, expression of pSmad1 and Col IV protein was reduced by dominant negative STAT3. One of three independent experiments is shown. Mesangial proliferation was reduced by dominant negative STAT3. *, p < 0.05 versus wild type.

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