Reduction of Stat3 Activity Attenuates HIV-Induced Kidney Injury

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ABSTRACT

HIV-1 Nef induces podocyte proliferation and dedifferentiation by activating the Stat3 and MAPK1,2 pathways. Activation of Stat3 also occurs in human kidneys affected by HIV-associated nephropathy (HIVAN), but its contribution to the development of HIVAN is unknown. Here, we generated HIV-1 transgenic mice (Tg26) with either 75% Stat3 activity (Tg26-SA/H11001) or 25% Stat3 activity (Tg26-SA/H11002). The kidneys of Tg26-SA/H11001 mice, but not Tg26-SA/H11002 mice, showed increased Stat3 phosphorylation. The Tg26-SA/H11001 phenotype was not different from Tg26 mice, but Tg26-SA/H11002 mice developed significantly less proteinuria, glomerulosclerosis, and tubulointerstitial injury. Tg26-SA/H11001 mice exhibited reduced expression of podocyte differentiation markers and increased expression of VEGF and proliferation markers as compared to Tg26-SA/H11002 mice. Primary podocytes isolated from Tg26-SA/+ mice showed increased Stat3 phosphorylation and reduced expression of podocyte differentiation markers. The tubulointerstitial compartment and isolated tubules of Tg26-SA/+ mice also had increased Stat3 phosphorylation and expression of Stat3 target genes. We confirmed that the expression of the HIV-1 transgene and reduction of Stat3 activity did not affect T and B cell development. In conclusion, Stat3 plays a critical role in the pathogenesis of HIVAN.


Although the natural history of HIV-related kidney disease has changed over the past 10 yr, HIV-associated nephropathy (HIVAN) remains a leading cause of ESRD among African Americans.1–3 Despite growing knowledge on the pathogenesis of this disease, therapeutic options are still lacking. Biopsies from patients with classic HIVAN display collapsing focal segmental glomerulosclerosis, interstitial nephritis, and tubular microcystic dilation.4 Nephropathy seems to be caused primarily by HIV-1 infection of renal epithelium and expression of HIV-1 proteins.5 A distinctive feature of this disease is the finding of podocyte proliferation and dedifferentiation.6 The loss of synaptopodin staining in podocytes is a typical finding in HIVAN.7 Cell cycle regulation is also disrupted in podocytes of HIVAN, characterized by increased staining of Ki67, cyclin A, and cyclin E and downregulation of p27 and p21.8,9

Received August 21, 2008. Accepted May 15, 2009.
Published online ahead of print. Publication date available at www.jasn.org.

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We and others found that Nef, an HIV-1 accessory protein, plays a key role in the pathogenesis of HIVAN.\textsuperscript{10} Furthermore, we have reported that Nef induces podocyte proliferation and dedifferentiation through activation of the Src–Stat3 and Src–mitogen-activated protein kinase (MAPK) 1,2 pathways.\textsuperscript{11} Increased Stat3 and MAPK1,2 phosphorylation is observed in kidneys biopsy specimens from patients with HIVAN.\textsuperscript{12} Inhibition of Stat3 by dominant negative mutants abolishes the effects of Nef on podocytes \textit{in vitro}. Recently, we found that HIV2ox and vascular endothelial growth factor (VEGF) are highly upregulated by Nef through activation of Stat3.\textsuperscript{13} These findings clearly show that Nef induces an abnormal podocyte phenotype by activating distinct cell signaling pathways in which Stat3 is a key mediator. Furthermore, our previous microarray studies suggest that HIV infection activates IL-6, which is a known activator of the Jak–Stat3 pathway, in kidney tubular cells.\textsuperscript{14} Therefore, Stat3 may play an important role in both glomerular and tubular injuries in HIVAN. However, the role of Stat3 \textit{in vivo} has not been determined.

Stat3 is a known transducer of signals from growth factors and cytokines such as IL-6. Stat3 is ubiquitously expressed and known to have roles in development, cell growth, differentiation, prevention of apoptosis, and inflammatory response.\textsuperscript{15–17} Phosphorylation of the tyrosine 705 residue is required for Stat3 activation.\textsuperscript{15} However, additional phosphorylation of the serine 727 residue is required for full transcriptional activity.\textsuperscript{18} Stat3 is the only member of the STAT family that leads to embryonic lethality when deleted.\textsuperscript{19} A mouse model with a homozygous mutation in the serine 727 residue (SA/SA) has ∼50% of the Stat3 activity of wild-type mice.\textsuperscript{18} Crossing these mice to heterozygous Stat3 knock-out mice (±) produced mice with either 75% (SA/+) or 25% (SA−/) of Stat3 transcriptional activity. Whereas mice with 50% or more Stat3 transcriptional activity are phenotypically normal, Stat3 SA/− mice have a lower initial body and kidney weight.\textsuperscript{20}

Because our previous \textit{in vitro} studies suggested a critical role of Stat3 in the pathogenesis of HIVAN, here we determined the role of Stat3 \textit{in vivo} using SA/+ and SA−/− mice.

RESULTS

Tg26-SA/− and Tg26-SA/+ and their littermates were generated in the FVB/N background as described in the Concise Methods section. Reduction of total Stat3 expression in SA−/− mice and lack of stimulation of phosphorylation in Tg26 SA−/− mice were confirmed by Western blot of whole kidney lysates (Figure 1A).

By 4 wk of age, Tg26-SA/+ had developed significant proteinuria, which peaked at 10 wk of age. Urine albumin/Cr ratio in Tg26 SA−/− were significantly higher than Tg26 SA−/− and control mice at 4, 7, and 10 wk of age (Figure 1B). Data on body and kidney weights and renal function (blood urea nitrogen [BUN]) are summarized in Table 1. Consistent with previous findings,\textsuperscript{20} Stat3 SA−/− were significantly smaller than Stat3 SA+/+. Tg26 SA−/− had increased kidney weight and kidney to body weight ratio compared with their littermates, whereas Tg26 SA−/+ had kidney weight and kidney to body weight ratio similar to their littermates. Serum BUN levels, as an indicator of renal function, was increased in Tg26 SA−/+ and normal in Tg26 SA−/−. Similar to Tg26 mice, Tg26 SA−/+ developed significant glomerulosclerosis, interstitial nephritis, and tubular microcystic dilation (Table 2; Figure 2). However, Tg26 SA−/− had significantly reduced glomerular and tubular injuries compared with Tg26 SA−/+. These data suggest that inhibition of Stat3 pathway ameliorates HIV-induced kidney disease.

Our previous \textit{in vitro} studies suggest that inhibition of Stat3 activity restores synaptopodin expression in HIV or Nef-infected podocytes.\textsuperscript{11} Therefore, we assessed the expression of synaptopodin in these mice by immunostaining and real-time PCR. As shown in Figure 3, both protein and mRNA levels of synaptopodin were significantly reduced in Tg26 SA−/+ compared with Tg26 SA−/− mice. However, there was no significant difference in synaptopodin expression between Tg26 SA−/− and their littermates.

Podocyte proliferation is considered a hallmark of collapsing FSGS in HIVAN.\textsuperscript{7,21} Our previous studies suggested that Stat3 mediates HIV and Nef-induced podocyte proliferation \textit{in vitro}.\textsuperscript{11} Therefore, we studied the expression of podocyte proliferation markers in kidneys of these mice. We found that Ki67 immunostaining was more pronounced in Tg26 SA−/+ than Stat3 SA−/+ (Figure 4A). Ki67 staining localized predominantly to the podocytes as shown by co-immunostaining using an anti-nestin antibody (Figure 4B). Cyclin E expression as quantified by real-time PCR was also significantly increased in Tg26 SA−/+ mice compared with their littermates (Figure 4C). However, podocyte proliferation markers did not change significantly between Tg26 SA−/− and Stat3 SA−/− mice (Figure 4A–C).

It has been shown that overexpression of VEGF in podocytes during development results in collapsing FSGS mimicking HIVAN,\textsuperscript{22} and the loss of the Von Hippel-Lindau gene

![Figure 1](image-url)
causes crescentic glomerulonephritis with significant podocyte proliferation. We recently found that Stat3 mediates HIV-induced HIF2α and VEGF expression in podocytes. Therefore, we determined the level of VEGF expression in these mice. By immunostaining, VEGF expression increased significantly in Tg26-SA/+ but not in Tg26-SA/− mice, compared with their littermates (Figure 5A, A–D). Because it is known that VEGF is highly expressed in the developing kidney, we performed immunostaining of VEGF in kidneys from developing mice (E18) to compare with adult Tg26-SA/+ mice (Figure 5A, E). A low-power image of VEGF staining in Tg26-SA/+ is also shown (Figure 5A, G). In addition, we found that glomerular VEGF mRNA level was increased in Tg26-SA/+ but not in Tg26-SA/−, compared with their Tg26 littermates (Figure 5B). To confirm the activation of the VEGF proangiogenic pathway in kidneys of Tg26 mice, we examined the phosphorylation of vascular endothelial growth factor receptor 2 (VEGFR2) in isolated glomeruli. By Western blot, we found phosphorylation of vascular endothelial growth factor receptor 2 (VEGFR2) in isolated glomeruli. By Western blot, we found that both phospho- and total VEGFR2 were increased in Tg26-SA/+ mice compared to Tg26-SA/− and their littermates (Figure 5C). To determine whether increased VEGFR2 expression is caused by an increase of the endothelial component in Tg26 kidneys, we examined the expression of platelet/endothelial cell adhesion molecule-1 (PECAM-1), an endothelial marker, in glomerular lysates of these mice by Western blot using an anti-PECAM-1 antibody (ABBIOTEC, San Diego, CA). We found that PECAM-1 expression decreased slightly in Tg26-SA/+ mice (Figure 5C), which suggests that increased VEGFR2 expression in Tg26 mice is unlikely because of an increase of the endothelial component. Our data suggest that inhibition of the Stat3 pathway abolishes Nef-induced VEGF expression and activation.

Table 1. Characteristics of mice

<table>
<thead>
<tr>
<th></th>
<th>BW (g)</th>
<th>KW (mg)</th>
<th>KW/BW (mg/g)</th>
<th>BUN (mg/dl)</th>
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<tbody>
<tr>
<td>SA/+</td>
<td>27.5 ± 4.1</td>
<td>223 ± 17</td>
<td>8.1 ± 2.0</td>
<td>21 ± 5</td>
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<td>Tg26-SA/+</td>
<td>26.5 ± 3.1</td>
<td>240 ± 42b</td>
<td>9.2 ± 0.5b</td>
<td>31 ± 9b</td>
</tr>
<tr>
<td>SA/−</td>
<td>24.7 ± 1.4</td>
<td>194 ± 26</td>
<td>7.8 ± 0.92</td>
<td>19 ± 6</td>
</tr>
<tr>
<td>Tg26-SA/−</td>
<td>24.6 ± 1.2</td>
<td>195 ± 57</td>
<td>7.9 ± 1.2c</td>
<td>22 ± 7c</td>
</tr>
</tbody>
</table>

*Mouse body weight (BW), kidney weight (KW), kidney to body weight ratio (KW/BW), and BUN are summarized.

bP < 0.05 compared with SA/+, n = 6.
cP < 0.05 compared with Tg26 SA/+, n = 6.

Table 2. Summary of kidney histology

<table>
<thead>
<tr>
<th>ID</th>
<th>Glomerular Collapse + Sclerosis (%)</th>
<th>Podocyte Hyperplasia (0–3+)</th>
<th>Tubular Degenerative/Regenerative Changes (0–3+)</th>
<th>Percent Cortex with Casts/Cysts (%)</th>
<th>Tubular Atrophy/Interstitial Fibrosis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SA/+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Tg26-SA/+</td>
<td>43.2 ± 14.4</td>
<td>1.7 ± 0.8</td>
<td>1.2 ± 0.4</td>
<td>15 ± 3.5</td>
<td>13 ± 7.4</td>
</tr>
<tr>
<td>SA/−</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tg26-SA/−</td>
<td>13.5 ± 7.2b</td>
<td>0</td>
<td>0</td>
<td>4.3 ± 1.4b</td>
<td>3.5 ± 1.9b</td>
</tr>
</tbody>
</table>

*After PAS staining, the kidney histology from these mice was reviewed and quantified as described in the Concise Methods section.
bP < 0.05 compared with Tg26-SA/+ mice, n = 6.

Figure 2. Kidney histology. PAS staining was performed in kidneys of Tg26-SA/+ and Tg26-SA/− mice and their littermates. Representative pictures (×200 and ×400) are shown here.

Figure 3. (A) Immunostaining of synaptopodin in kidneys of mice. Immunohistochemistry was performed in paraffin sections of kidneys from Tg26-SA/+ and Tg26-SA/− mice compared with their littermates using anti-synaptopodin antibody. Rabbit IgG was used as a negative control. The top panels were stained without counterstaining, and the bottom panels were stained with H&E counterstaining to visualize tissue structure. The representative pictures (×400) are shown here. (B) Real-time PCR for synaptopodin. Total RNA was isolated from glomeruli of these mice using TRIzol. Real-time PCR was performed as described. The ratios of synaptopodin to tubulin mRNA levels were obtained, and the folds of change to wild-type mice are shown (**P < 0.01 compared with SA/+ or Tg26-SA/− mice, n = 6).
To determine the activation of Stat3 in different renal compartments, we performed immunostaining of phosphorylated Stat3 (pStat3) in kidney sections. By co-immunostaining of kidney sections using both anti-pStat3 and anti-nestin antibodies, we found that Stat3 phosphorylation increased significantly in Tg26 mice and co-localized with the podocyte marker (nestin) (Figure 6A). To further confirm the role of Stat3 in podocytes, we isolated primary cultures of podocytes from these mice and confirmed that pStat3 was increased in podocytes of Tg26-SA+/− mice (Figure 6B). Both phospho- and total-Stat3 were suppressed in podocytes of Tg26-SA/−. We also examined the expression of podocyte differentiation markers in these cells. By both Western blot and immunostaining, we found that the expression of synaptopodin, podocin, and WT-1 were all reduced in Tg26-SA+/− podocytes but normal in Tg26-SA+/− podocytes (Figure 6B and C). To confirm the podocyte lineage, we performed Western blot analysis for nestin in these primary cultures of podocytes. Consistent with our immunostaining data, nestin expression was preserved in podocytes from Tg26 kidney (Figure 6B).

Next, we confirmed that the pStat3 was increased in the tubulo-interstitial compartment of Tg26-SA+/− but not in Tg26-SA/− mice (Figure 7A). Furthermore, we confirmed that infection of RTEC (HK2 cells) with retroviral construct for stable Nef expression induced Stat3 activation (Figure 7B). pStat3 was also increased markedly in tubules isolated from Tg26-SA+/− but not in Tg26-SA/− mice (Figure 7C). To further confirm the activation of Stat3, we measured the expression of ICAM1 and SOCS3, which are known Stat3 target genes. By real-time PCR, we found that both ICAM1 and SOCS3 were stimulated in renal tubules of Tg26-SA+/− but not in Tg26-SA/− (Figure 7D and E). These data suggest that Stat3 is activated in the tubules of Tg26 mice and likely contributes to the tubulo-interstitial lesions observed in Tg26 mice.

Our data suggest that the infiltration of inflammatory cells into the renal interstitium is accompanied by increased Stat3 activation in both podocytes and tubular cells. This activation likely promotes the synthesis of pro-inflammatory molecules, such as ICAM1 and SOCS3, which contribute to the development of the tubulo-interstitial lesions observed in Tg26 mice.
into the kidney was significantly reduced in Tg26-SA/H11002 compared with Tg26-SA/H11001. Because it has been shown that Stat3 plays a role in T and B cell development and HIV genes are expressed ubiquitously in Tg26 mice, we sought to determine whether T and B cell development differs between these mice. As shown in the Supplement Table and Figure, T and B cell development did not differ between these mice.

DISCUSSION

In this study, we identified the critical role of Stat3 in the pathogenesis of HIVAN in vivo using a mutant Stat3 knockout model. In our study, Tg26-SA/+ (75%) and Tg26-SA− (25%) littermates were used for comparison because it has been shown that 25% reduction in Stat3 activity is required for phenotypic changes, and the severity of the renal phenotype did not differ between Tg26-SA/+ and Tg26 mice (data not shown). Furthermore, using Tg26-SA/+ as the control for Tg26-SA− mice allowed us to exclude potential confounding effects introduced by the SA mutation in analysis of study results.

A limitation of our study is that Stat3 is ubiquitously expressed, and downregulation of Stat3 in our animal model occurs in all tissues expressing Stat3. Therefore, our study does not address the relative contribution of Stat3 inactivation in podocytes, tubules, and other cell types including lymphocytes. We previously reported that Stat3 is activated mostly in podocytes, which contributes to podocyte proliferation and dedifferentiation in HIVAN. This is further confirmed here by co-immunostaining studies. We also confirmed that primary podocytes from Tg26-SA/, but not from Tg26-SA−, had increased Stat3 phosphorylation and reduced expression of podocyte differentiation markers.

It has been shown that overexpression of VEGF by 10- to 20-fold during development causes collapsing FSGS, mimicking HIVAN. Our data confirm that Stat3 mediates upregulation of VEGF in adult Tg26 mice. Our immunostaining data suggest that expression levels of VEGF in Tg26 kidney are relatively low compared with those in the developing kidney. However, semaphorin 3A, a negative regulator of VEGF activation, is significantly downregulated in Tg26 kidney as shown

Figure 6. (A) Co-immunostaining was performed in kidney sections of Tg26 mice and their littermates using rabbit polyclonal anti-pStat3 and mouse monoclonal anti-nestin antibodies. The representative pictures are shown (x400). The third panel shows overlapping pictures and arrows indicate overlapping cells. (B) Western blot. Primary cultures of podocytes from both Tg26 and control Stat3 SA−/− and Stat3 SA−/− mice were lysed for Western blot analysis with anti-pStat3, anti-Stat3, anti-synaptopodin, anti-podocin, anti-WT-1, anti-nestin, anti-β-actin, and anti-Nef antibodies. (C) Immunostaining. Primary cultures of podocytes isolated from Tg26-SA+/+ and Tg26-SA−/− mice were placed on fibronectin-coated coverslips for immunostaining with anti-synaptopodin, anti-podocin, anti-WT1, or IgG control. The representative pictures (x400) are shown; scale bars = 10 mm. The matched exposures were used for taking these pictures. A nuclear staining for synaptopodin was noticed and is likely nonspecific.

Figure 7. (A) Immunostaining of pStat3 in kidneys of mice. Immunohistochemistry was performed in kidney sections of Tg26-SA+, Tg26-SA−, and their littermates using rabbit polyclonal anti-pStat3 antibody (Cell Signaling). The representative pictures of tubulo-interstitial areas are shown (x400). (B) Western blot. HK2 cells were infected with a retroviral construct expression Nef as described. Infected cells were lysed for Western blot analysis for pStat3, total Stat3, and Nef. (C) Renal tubuli from both Tg26 and Stat3 SA+/+ and Stat3 SA−/− mice were isolated and lysed for Western blot analysis with anti-pStat3, anti-total Stat3, anti-β-actin, and anti-Nef antibodies. (D and E) Real-time PCR for ICAM1 and SOCS3. Total RNA was isolated from renal tubules of these mice using TRizol. Real-time PCR was performed as described. The ratios of ICAM1 and SOCS3 to tubulin mRNA levels were obtained and the folds of change to WT mice are shown (**P < 0.01 compared with Tg26-SA+/+ or SA−/− mice, n = 4).
previously. Therefore, we believe that the combination of VEGF upregulation and semaphoring 3A downregulation in Tg26 leads to a highly activated VEGF signaling pathway and contributes to the pathogenesis of HIVAN in these mice.

Previous studies suggest that IL-6 expression is increased in tubular cells of HIVAN kidneys. We confirmed that the phosphorylation of Stat3 and expression of Stat3 target genes (ICAM1 and SOCS3) were stimulated in the tubules isolated from Tg26-SA/+ but not Tg26-SA/-. Interestingly, recent studies suggested that activation of the JAK–Stat3 pathway occurs in the tubulo-interstitial area of kidneys from human with diabetic nephropathy but not murine models of diabetic nephropathy. This observation might explain why there is a lack of tubulo-interstitial changes in murine models of diabetic nephropathy. Stat3 activation may contribute to tubulo-interstitial injury observed in human DN. Consistent with these findings, Tg26 mice had increased Stat3 phosphorylation and developed significant tubulo-interstitial disease.

We also noted a significant reduction in the inflammatory infiltrate in kidneys of Tg26-SA/− compared with Tg26-SA/+ . It has been shown that Stat3 plays a role in T and B cell development. We confirmed that knock-down of Stat3 activity and expression of HIV transgene did not affect T and B cell development in these mice. In a prior study, we found that homozygous Tg26 mice had a small thymus, large spleen, and lymphadenopathy. In these homozygous Tg26 mice, the percentage of CD4-positive cells in the lymph node was significantly reduced compared with wild-type littermates. The reasons for the differences between homozygous and heterozygous Tg26 mice are likely because of the level of transgene expression. Studies from Jolicoeur’s laboratory showed that transgenic mice expressing Nef under the control of the human CD4 gene promoter develop a severe AIDS-like disease, with manifestations including premature death, failure to thrive, thymic atrophy, and an especially low number of peripheral CD8+ T cells as well as a low number of peripheral CD4+ T cells. These phenotypes are quite similar to those observed in homozygous Tg26 mice but not in heterozygous mice. The observed difference in phenotype could also be caused by the level of transgene expression in CD4+ cells, although it has never been examined. In renal cells, Nef produces a program of host response with activation of downstream signaling pathways that are different than the response elicited by Nef in CD4+ cells. Therefore, the level of Nef expression required to cause renal phenotype could be different from that required to downregulate CD4 expression in immune cells.

Stat3 also plays an important role in other kidney diseases. The JAK–Stat3 pathway plays an important role in the pathogenesis of diabetic nephropathy. Selective pharmacologic inhibition of JAK2 (AG490) ameliorates nephrotic syndrome in adriamycin-induced nephropathy. Stat3 is also a key mediator in Gas6 nephropathy. In addition, Stat3 plays an important role in acute kidney injury.

In conclusion, our previous in vitro studies and current in vivo studies suggest that Stat3 is a key mediator in the development of HIVAN. Inhibition of the Stat3 pathway should be considered as a potential drug target for the treatment of HIVAN.

CONCISE METHODS

Generation of Tg-26-SA/− Mice

Stat3 SA/SA and Stat3 +/+ mice were provided by the Dr. J. E. Darnell and Dr. D. E. Levy groups at Rockefeller University as described. Derivation of a transgenic mouse line that bears a defective HIV-1 provirus lacking gag-pol (Tg26) has been described. Stat3 SA/SA and Stat3 +/+ mice in the C57/B6 background were first backcrossed to the FVB/N background for six generations. Then, a female Stat3 +/+ mouse was crossed with a male HIV-1 transgenic mouse (heterozygote Tg26) to generate Tg26-SA +/− mice. We crossed male Tg26-SA +/− mice with female Stat3 SA/SA mice to generate Tg26 SA/+ and Tg26 SA/− mice. SA/+ and SA− mice without the Tg26 gene from the same litter were used as controls. Mice were generated from the same original litter of Tg26 mice. Genotyping by tail prep and PCR were performed at 3 wk of age as described.

Experimental Design

Mice (n = 6 per group including 3 male and 3 female mice in each group) were provided with unrestricted food and water throughout the duration of the experiment. The mice were killed at 10 wk of age for tissue collection by exposure to carbon monoxide. Urine samples were collected at ages of 4, 7, and 10 wk. Blood samples were also collected at the end of study (10 wk). Body and kidney weight were recorded. All animal studies were performed according to the protocols approved by Institutional Animal Care and Use Committee at the Mount Sinai School of Medicine.

Quantitative Histopathology

Mice were perfused with PBS, and kidneys were fixed in 4% paraformaldehyde for 2 h. Kidney tissue was embedded into paraffin by American Histolabs (Gaithersberg, MD). Kidney histology was examined after periodic acid-Schiff (PAS) staining. Glomerulosclerosis was scored as described previously in collaboration with Dr. D’Agati. Briefly, each specimen received a score for three parameters: percentage of collapsing glomerular sclerosis, percentage of tubular cysts or casts, and podocyte hypertrophy. The percentage of collapsing glomerulosclerosis was obtained by identifying the total number of glomeruli with any sclerosis and dividing this number by the total number of glomeruli seen. The percentage of tubular cysts or casts score was obtained by the number of tubules with either microcystic dilation or filled with casts divided by the total number of tubular cross sections in a representative area. Finally, the degree of podocyte hypertrophy was scored as 1+ (podocyte hypertrophy observed in <25% of all glomeruli), 2+ (podocyte hypertrophy observed in between 25 and 50% of all glomeruli), and 3+ (podocyte hypertrophy in >50% of all glomeruli).
Measurement of BUN, Urine Protein, and Creatinine

Mouse serum was collected for measurement of BUN in the laboratory of Mount Sinai Hospital using urease-based assay (Instrument Vitros S4). Urine albumin was quantified by ELISA using a kit from Bethyl Laboratory (Houston, TX). Urine creatinine levels were measured in the same samples using QuantiChrom Creatinine Assay Kit (DICT-500; BioAssay Systems) according to the manufacturer’s instructions. The urine albumin excretion rate was expressed as the ratio of albumin to creatinine.

Western Blot

Tissue or cells were lysed with a buffer containing 1% Triton, a protease inhibitor cocktail, and tyrosine and serine-threonine phosphorlylation inhibitors. Lysates were subjected to immunoblot analysis using anti-phospho-Stat3 and anti-total Stat3 antibodies (Cell Signaling Technology), anti-β-actin antibody (Sigma), anti-synaptopodin and anti podocin (gifts from Dr. Peter Mundel, University of Miami), anti-WT-1 (Santa Cruz), anti-phospho-VEGFR2 (Cell Signaling Technology), anti-VEGFR2 (Santa Cruz), anti-nestin (Santa-Cruz), and anti-PECAM-1 (ABBIOTEC).

Immunohistochemistry

Kidney sections from these mice and from an E18 mouse were prepared in identical fashion. Immunostaining was performed using anti-total Stat3 (Cell Signaling Technology), anti-synaptopodin (gifts of Dr. Peter Mundel), anti-Ki-67 (Dako), and biotinylated secondary antibodies (Vector Laboratories) as described. For VEGF staining, antigen retrieval was performed after deparaffinizing and rehydrating the sections. The slides were immersed in citrate buffer (10 mM citric acid, 0.05% Tween 20, pH 6.0), heated to boiling point for 5 min, cooled to room temperature for 20 min, washed in PBS two times, and followed by standard staining protocol. An anti-VEGF antibody from R&D Systems was used. After staining, slides were mounted in Aqua Poly/Mount (Polysciences) and photographed under an Olympus BX60 microscope (Olympus Optical) with a digital camera. For colocalization studies, sections were incubated with either anti-pStat3 or anti-Ki67 antibodies together with anti-nestin antibody (Santa Cruz) at room temperature for 1 h. After washing, sections were incubated with a fluorophore-linked secondary antibody (Alexa Fluor 488 anti-rabbit IgG and Alexa Fluor 568 anti-mouse IgG from Invitrogen). After mounting, slides were examined by fluorescence microscopy.

Real-Time PCR

Total RNA was isolated from kidney glomeruli or tubuli of mice using TRIzol (Invitrogen). Real-time PCR was performed with a Roche Lightcycler and Qiagen QuantiTect One Step RTPCR SYBR green kit (Qiagen) according to the manufacturer’s instructions. Predesigned primer sets were obtained from Qiagen (GeneGlobe) for synaptopodin, VEGF, cyclin E, ICAM1, and SOCS3. The following sequences were used for the tubulin primers: 5’TCGAGGCAGTTTGGAGACAC3’ and 5’TGAGGCAGTTTGGAGACAC3’. Light cycler analysis software was used to determine crossing points using the second derivative method. Data were normalized to housekeeping genes (tubulin) and presented as fold increase compared with RNA isolated from WT animals using the 2−ΔΔCT method.42

Isolation of Glomeruli and Renal Tubules from Mice for Western Blot and Real-Time PCR

Mouse glomeruli and tubuli were isolated as described.43 Briefly, animals were perfused with Hank’s buffered salt solution (HBSS) containing 2.5 mg/ml iron oxide and 1% BSA. At the end of perfusion, kidneys were removed, decapsulated, minced into 1-mm3 pieces, and digested in HBSS containing 1 mg/ml collagenase A and 100 U/ml deoxyribonuclease I. Digested tissue was passed through a 100-μm cell strainer and collected by centrifugation. The pellet was resuspended in 2 ml of HBSS, and glomeruli were collected using a magnet. Those not collected by the magnetic purification process were collected as the tubular fraction. The purity of glomerular and tubular preparation was verified under microscopy.

Isolation of Primary Podocytes

Primary podocyte cultures were developed from Tg26-Stat3 SA/+ and Tg26-Stat3 SA/− mice as described.44 Glomeruli were isolated by the sieving method in the absence of collagenase. The glomerular fraction was placed into the fibronectin-coated dish and cultured in RPMI medium with 10% FBS. After 5 d of culture, podocytes were attached and started to grow around glomeruli. Both glomeruli and single cells were detached by trypsinization. Single cells were separated from glomeruli by passing through a cell strainer. These single cells were mostly podocytes as identified by morphology under the microscope and by immunostaining for podocyte specific markers.

Immunostaining for Podocyte-Specific Markers

Cells were plated onto a 12-well plate with fibronectin-coated coverslips and fixed with 4% paraformaldehyde/0.1% Triton X-100 in 1× PBS. The fixed cells were incubated with the primary antibodies and secondary antibody conjugated to fluorescein. The coverslips were mounted onto glass slides and were visualized using a fluorescence microscope.

Infection of HK2 Cells

The HK2 line of human proximal tubule cells was obtained from the American Type Culture Collection (ATCC, Manassas, VA). A retroviral expression vector (pBabe-puro) was used to clone nef into the BamHI–SalI site. Moloney murine leukemia virus gag/pol genes and VSV.G envelope were provided in trans to generate pseudotyped virus particles. HK2 cells were infected with control vector or Nef-containing vector as described previously.11 The expression of Nef in HK2 cells was confirmed by Western blot analysis.11

Analysis of Immune Cells of Mice

Cells from the thymus, spleen, and lymph nodes were harvested, and single-cell suspensions were generated by disruption with ground glass slides. Before enumeration, red blood cells were removed from spleen preparations by lysis using ammonium chloride. Cells were preincubated with 2.4G2mAb to block FcγR for 15 min and followed by incubation with specific antibodies for 30 min on ice. Labeled cells were collected and analyzed using a BD FACSCalibur flow cytometer.
system. All antibodies were obtained from BD Biosciences Pharmingen.

Statistical Analysis
Data were expressed as mean ± SD. The unpaired t test was used to analyze data between two groups. Statistical significance was considered when \( P < 0.05 \).

ACKNOWLEDGMENTS
J.C.H. is supported by NIH R01 DK078897. This work was also supported by NIH Grants P01 DK056492 to P.K. and R01 GM54508 to R.I.

DISCLOSURES
None.

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See related editorial, “Insight versus Quagmire with Compound HIV Transgens,” on pages 2085–2086.
Supplemental information for this article is available online at http://www.jasn.org/.