Enhanced Expression of JAK-STAT Pathway Members in Human Diabetic Nephropathy

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ABSTRACT

Objective. Glomerular mesangial expansion and podocyte loss are important early features of diabetic nephropathy (DN) whereas tubulointerstitial injury and fibrosis are critical for progression of DN to kidney failure. Therefore, we analyzed the expression of genes in glomeruli and tubulointerstitium in kidney biopsies from DN patients to identify pathways that may be activated in humans but are not in murine models of DN that fail to progress to glomerulosclerosis, tubulointerstitial fibrosis and kidney failure.

Research Design and Methods. Kidney biopsies were obtained from 74 patients (controls, early and progressive type 2 DN). Glomerular and tubulointerstitial mRNAs were microarrayed, followed by bioinformatics analyses. Gene expression changes were confirmed by real-time RT-PCR and immunohistological staining. Samples from db/db C57BLKS and streptozotocin-DBA/2J mice, commonly studied murine models of DN, were analyzed.

Results. In human glomeruli and tubulointerstitial samples, the Jak/Stat pathway was highly and significantly regulated. Jak-1, 2 and 3 as well as Stat-1 and Stat-3 were expressed at higher levels in patients with DN than in controls. The estimated glomerular filtration rate significantly correlated with tubulointerstitial Jak1, 2, 3 and Stat1 expression ($R^2=0.30-0.44$). Immunohistochemistry found strong Jak2 staining in glomerular and tubulointerstitial compartments in DN compared to controls. In contrast, there was little or no increase in expression of Jak/Stat genes in the db/db C57BLKS or diabetic DBA/2J mice.

Conclusions. These data suggest a direct relationship between tubulointerstitial Jak/Stat expression and progression of kidney failure in patients with type 2 DN and distinguishes progressive human DN from non-progressive murine DN.
Early clinical diabetic nephropathy (DN) is characterized by progressive increases in albuminuria which are associated with the development of characteristic histopathologic features including thickening of the glomerular basement membrane and mesangial expansion due to accumulation of extracellular matrix proteins (1). As albuminuria progresses and renal insufficiency ensues, glomerulosclerosis, arteriolar hyalinosis, and tubulointerstitial fibrosis develop (2). Mesangial expansion and the degree of tubulointerstitial fibrosis correlate inversely with glomerular filtration rate in humans with diabetic kidney disease and appear to be critical steps in the progression of DN to end stage renal disease (ESRD) (1; 3; 4). Over 20 years ago, Mauer and colleagues established the clear link between mesangial matrix expansion and progression of diabetic kidney disease by demonstrating that measures of mesangial expansion strongly predicted the clinical manifestations of DN [1]. The critical role of tubulointerstitial expansion and fibrosis in the progression of DN has also been recognized for at least 2 decades [1, 5]. Tubulointerstitial changes, including fibrosis, appear to be critical for final progression of DN to kidney failure in Type 1 patients, and may play an even more important, though heterogeneous, role in Type 2 patients [6]. Despite agreement about these pathologic predictors, there is no consensus about the processes that lead to progressive glomerulosclerosis and tubulointerstitial fibrosis.

While hypothesis driven studies have led to many insights about the development and progression of DN, there is recent interest in using more broad-based approaches to understanding the pathogenesis of human renal disease (7-9). Moreover, while current animal models of DN replicate well the early stages of human disease, virtually all fail to develop the severe glomerulosclerosis, progressive tubulointerstitial fibrosis and gradual decline in glomerular filtration rate that characterize human DN (10). Therefore, use of a broad-based approach with tissues from human patients with progressive DN might allow elucidation of pathways that would not be observed in hypothesis based studies of animal or cell models (7-9). In this report, we show that a transcriptomic analysis of glomerular and tubulointerstitial tissues from patients with early and progressive DN reveals regulation of the expression of multiple members of the Jak/Stat pathway.

We focused on these results because the activation of Jak/Stat signaling pathways can be implicated in both tubulointerstitial fibrosis and epithelial to mesenchymal transition in several conditions, including diabetes, in animal models (11-13). Similarly, Jak/Stat activation is reported in rat glomerular cells exposed to high glucose (14;15) and may be important in the glomerular TGF-β activation and fibronectin accumulation critical for extracellular matrix deposition in early DN (15). Moreover, angiotensin converting enzyme inhibitors and angiotensin receptor blockers, which prevent the progression of DN, also prevent Jak/Stat activation in glomerular cells from diabetic rats (14). Therefore, we studied expression and effects of Jak/Stat members in human and mouse DN.

**RESEARCH DESIGN AND METHODS**

*Human renal biopsy samples for genome-wide expression profiling and real-time RT-PCR.* Gene expression
profiling of 74 kidney biopsies was performed essentially as reported by Cohen CD et al. (16) and Lindenmeyer et al. (17). For a detailed description, see the Supplementary Material. Clinical data for these patients are provided in the Supplementary Tables 1 and 2.

**Microarray target preparation, data processing, analysis and pathway mapping.** Microarrays from extracted microdissected renal compartment RNAs were processed following a previously published protocol (18). The CEL files were processed by the ChipInspector software (Genomatix Software GmbH; http://www.genomatix.de). Briefly and as described in the user manual, ChipInspector extracted the single probes significantly differentially expressed in Affymetrix GeneChip microarrays (false discovery rate (fdr) = 0). In the designed analysis treatment/control experiment, LD and MCD patients were the “control” group and the early DN (Pima Indians) or progressive DN (European cohort) were the “experiment” group. The ChipInspector analysis steps were: normalization, log2 transformation, statistical analysis and mapping to transcripts. The resulting lists were uploaded into the Ingenuity Pathway Analysis software (IPA, www.ingenuity.com). Differential regulation was displayed in hierarchical manner with color-coded expression levels. All the genes of a family were combined and represented by the generic family name.

**Histology and immunohistochemistry.** Following the protocol previously described (19), immunohistochemistry studies were performed from an independent cohort of controls (histologically verified unaffected regions from tumor nephrectomies), type 2 progressive DN cases and other kidney disease biopsies (Supplementary Table 3). Jak2 staining was performed using a primary monoclonal antibody at 1:100 (AHO1352, Biosource Camarillo, CA). A biotinylated goat anti-mouse antibody (Histoline, Zymed, Milan, Italy) was used as secondary antibody (1:200).

Morphometric analysis of the progressive DN patients included analysis of the numbers of glomeruli that had segmental or global glomerulosclerosis or were sclerotic. The presence of tubulointerstitial damage was determined by the presence of tubular atrophy, presence of myofibroblasts and/or accumulation of extracellular matrix proteins (Supplementary Table 4).

**Animals.** Mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Db/db and db/m mice on a C57BKLS background (BKS.Cg-m +/+ Lepr<sup>db</sup>/J) become obese around 3-4 weeks and develop hyperglycemia between 4-8 weeks of age. Male 10 week-old DBA/2J mice were allowed to acclimate to their environment for at least 3 days before injection. Mice were fasted for 4 hours and then given intraperitoneal injections of 40 mg/kg streptozotocin (STZ) or the vehicle (control mice) daily for five consecutive days as previously reported (10,20). Mice were subcutaneously implanted with pellets impregnated with bovine insulin (LinBit tablets, LinShin Canada, Inc., Toronto, Ontario, Canada) at 10 weeks, 15 weeks and 20 weeks post-STZ. The administered dose of insulin was approximately 0.1 U/day. These animals were euthanized at 24 weeks after completion of STZ injections.

Phenotypic evaluation of the DBA/2J diabetic and control mice was reported previously (21). Albuminuria was increased 3-fold, mesangial matrix was increased ~65% and podocyte number was decreased ~30% in the diabetic
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DBA/2J mice compared to controls (21). The db/db C57BLKS mice were not phenotyped in this study. However, this model has been extensively phenotyped by others in previous studies and has very similar changes of early diabetic nephropathy (22).

The procedures used in this study were in accordance with the guidelines of the University of Michigan Committee on the Use and Care of Animals and conformed to “The Guide for the Care and Use of Laboratory Animals,” Department of Health, Education, and Welfare Publication No. (NIH) 86-23. Veterinary care was provided by the University of Michigan Unit for Laboratory Animal Medicine. The University of Michigan is accredited by the American Association of Laboratory Animal Care.

**Mouse kidney samples.** Kidney cortex and glomeruli were harvested from 32 week-old diabetic db/db C57BLKS mice and their control db/m littermates and from STZ-diabetic and control DBA/2J mice 24 weeks after STZ or vehicle injection. Mice were anesthetized by IP pentobarbital (0.06 mg/g of body weight). Briefly, blood was flushed from the mice with 0.1M Phosphate Buffered Saline (PBS sterile, pH 7.4) via a catheter inserted into the abdominal aorta. A 20 ml iron oxide bolus (5 mg/ml in 0.9 % NaCl) followed. The right kidney was then removed, minced and pushed through a 90 μm nylon mesh (Catalog # 03-90/49, Sefar America, Kansas City, MO) into a beaker over a magnet (grade 8), used to retain the iron-perfused glomeruli, then washed 2 times with sterile PBS (pH 7.4). One fifth of the glomeruli harvested were then stored in RNA later (Ambion, Austin-TX) for RNA analysis; the remaining glomeruli were lysed and stored at -80°C for protein analysis.

**Mouse RNA and protein extraction.** Total RNA was isolated from the microdissected tissues using the RNeasy mini kit; Qiagen GmbH, Hilden, Germany, following the manufacturer’s instructions. Harvested mouse kidney samples were lysed in a buffer containing 10 mM Tris-HCl pH7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM Na3VO4, 50 mM NaF, 1 mM PMSF and complete protease inhibitor cocktail (Roche, Indianapolis, IN), sonicated and centrifuged 20 min at 15,000 g and 4 °C. The supernatant was obtained and stored at -80°C until analyzed.

**Human and mouse real-time RT-PCR.** Reverse transcription of RNA and amplification was performed as described previously (7) using commercially available pre-developed TaqMan reagents for Jak2, Stat3 and 18S ribosomal RNA for result normalization (Assay-on-demand, Applied Biosystems, Darmstadt, Germany). Quantification of the given templates was performed according to the standard curve method for the human samples and the delta Ct value for the mouse samples. Serial dilutions of kidney cDNA were included in all PCR runs and served as standard curve. All measurements were performed in duplicate. Controls consisting of double distilled H2O were negative in all runs.

**Mesangial cell culture and transfection.** Murine mesangial cells obtained from the American Type Cell Collection (ATCC: MES-13, cloned from mice transgenic for the early region of the SV-40 virus) were grown in low glucose DMEM (Gibco, BRL) containing 5% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified air/5% CO2 atmosphere at
37°C. Nucleofection of mesangial cells was performed according to the optimized protocols provided by the manufacturer (Amaza, Gaithersburg, MD). Briefly, 1.5 x 10^6 cells were gently resuspended in 100 µl of Amaza® Nucleofector Solution (Cat No.VCA 1003), mixed with 1.5 µg Jak2 prk-5 or vector plasmid (23). Following transfection using the L-29 program on the Amaza Nucleofector, cells were cultured for 12 h and incubated in 5.5 mM D-glucose + 24.5 mM mannitol, or 30 mM D-glucose serum free DMEM. Cells were assayed 24 h later for Western blotting or quantification of intracellular reactive oxygen species (ROS). Transfection efficiency as quantified by nucleofection of the pMAX-GFP construct (included with the Amaza® kit) was ~60% (not shown).

**Western blot analysis.** Equal amounts of microdissected cortex, glomerular, or cultured mesangial cell protein samples were loaded onto 7.5% SDS-PAGE gels, electrophoresed, and transferred onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH). The blots were then incubated overnight at 4°C with a monoclonal mouse anti-Jak2 antibody (AHO1352, Biosource, Camarillo, CA; dilutions: 1:1000 [glomeruli], 1:2000 [cortex]), STAT3 (Cell Signaling Technology, Beverly, MA; dilutions: 1:1000 [glomeruli], 1:2000 [cortex]), phospho-STAT3 (Tyr705) (Cell Signaling Technology, Beverly, MA; dilutions: 1:1000 [glomeruli], 1:2000 [cortex]); or one hour at room temperature with an anti-β-tubulin antibody (Millipore, Lake Placid, NY; dilution: 1:6000). Subsequently, the blots were washed and incubated for 1 h at room temperature with an HRP-conjugated goat anti-mouse antibody (dilution: 1:1000 [glomeruli] - SuperSignal West Dura Extended Duration Substrate kit, Pierce, Rockford, IL; or 1:10000 [cortex] - SC-2031, Santa Cruz Biotechnology, Inc, CA). The peroxidase luminescence intensity was measured using NIH ImageJ Software.

**Detection of intracellular ROS.** Intracellular ROS generation was monitored by confocal microscopy using the fluoroprobe carboxymethyl-H₂-dichlorofluorescein diacetate (CM-H₂DCFDA; Molecular Probes, Inc., Eugene, OR). Transfected cells were grown on glass Lab-Tek Chamber Slides (Nunc Inc., Naperville, IL) in serum-free DMEM for 24 h with either 5.5 mM D-glucose + 24.5 mM mannitol, or 30 mM D-glucose. They were washed with PBS and incubated in the dark for 30 min in phenol red-free DMEM containing 5 µM CM-H₂DCFDA. CM-H₂DCFDA passively diffuses into cells, where its acetate groups are cleaved by intracellular esterases and its thiol-reactive chloromethyl group reacts with intracellular glutathione and other thiols. Subsequent oxidation yields a fluorescent adduct that is trapped inside the cell. After incubation, images were captured with an Olympus FluoView 500 Laser Scanning Confocal Microscope. Three fields from each of 5 separate slide wells were assessed for each condition. Fluorescence was quantified from 50 randomly chosen cells from each slide well using NIH ImageJ software.

**Statistics.** A t-test was carried out between the groups of patients. A Bonferroni correction was performed when multiple samples were being compared. For protein analyses, a one-way ANOVA followed by Tukey-Kramer post hoc analysis was performed. p values < 0.05 were considered statistically significant. All data are presented as mean ± SD or SEM, as noted.
RESULTS

Jak/Stat pathway in human DN.
Pathway mapping from Affymetrix microarray analysis identified Jak/Stat signaling as one of the highly regulated pathways in the glomerular and tubulointerstitial compartments of patients with early and progressive DN compared to controls (Figure 1). In the tubulointerstitial compartment, several Jak/Stat family members were down-regulated in early DN patients, whereas most were expressed at higher levels in progressive DN patients compared to controls (Figure 1, upper panel). These included Jak1, Jak2, Jak3, Stat1, Stat3, Stat4, Stat5B. Conversely, in the glomerular compartment, Jak/Stat pathway members were mainly increased in early DN compared to controls then down-regulated in progressive DN (Figure 1, lower panel).

Real time RT-PCR analysis of RNA from control, early and progressive DN microdissected biopsies was performed to confirm the mRNA changes of Jak/Stat pathway members. As suggested by the microarray data, a significant regulation of Jak1, Jak2, Jak3, Stat1 and Stat3 mRNAs was demonstrated in glomeruli from patients with early and progressive DN, and in the tubulointerstitial of patients with progressive DN. In contrast to the microarray data, real time RT-PCR found that mRNA expression of most Jak/Stat family members remained elevated in glomeruli from progressive DN patients compared to control samples (Supplementary Table 5A). In the remainder of our study, we focused on Jak2 since it appears to play a key role in proinflammatory responses in both glomerular and tubular cells (24). Jak2 mRNA levels were significantly increased in the glomeruli of early DN patients and in the tubulointerstitium of patients with progressive DN compared to controls (Figure 2A). Jak2 mRNA levels were not increased in glomeruli or tubulointerstitium from patients with other progressive kidney diseases (Supplementary Table 5B).

The estimated glomerular filtration rate (eGFR) of patients with DN (early and progressive) as calculated by the Modification of Diet in Renal Disease study formula (25) was strongly and inversely correlated with Jak2 tubulointerstitial (Figure 2B) but not glomerular mRNA levels as determined by qRT-PCR; this is also true with 1/serum creatinine (Supplementary Table 5C). Tubulointerstitial, but not glomerular mRNA expression of Jak1, Jak3, and Stat1 were also inversely and significantly correlated with eGFR and 1/serum creatinine (Supplementary Table 5C). Mean blood pressure also inversely correlated with tubulointerstitial Jak3 mRNA expression (Supplementary Table 5C).

Jak2 immunohistochemistry showed a robust increase in Jak2 protein expression in proximal tubular epithelia and glomerular cells in DN compared to controls and other progressive kidney diseases (Figure 2C), indicating that protein expression corresponded with mRNA levels. In the tubulointerstitial regions, contribution from cellular elements other than tubular cells was minimal (data not shown).

Jak2 expression in db/db C57BLKS and STZ-DBA/2J diabetic mice. Since common murine DN models fail to develop severe glomerulosclerosis or tubulointerstitial fibrosis and kidney failure, we hypothesized that these models would also fail to manifest critical gene expression changes that occur in humans with progressive DN. Db/db C57BLKS mice and STZ-diabetic DBA/2J
mice develop robust changes of early DN (albuminuria, mesangial expansion, podocyte loss) but do not develop the significant tubulointerstitial fibrosis and decline in kidney function that characterize progressive DN in humans (10) (23;24). Therefore, we specifically hypothesized that Jak2 expression would be unchanged in the glomeruli and renal cortex (largely tubulointerstitial tissue) of these two mouse models. Indeed, Jak2 mRNA and protein levels were similar in diabetic mice and their non-diabetic controls (Figure 3). Jak2 protein expression was also sought in glomerular lysates of STZ-DBA/2J mice and their controls only 12 weeks after STZ (or vehicle) injections. There was no difference in Jak2 protein levels on immunoblots of glomerular lysates from these animals (n = 4 in each group, data not shown).

**Effects of increased Jak2 expression in murine mesangial cells.** To determine whether increases in Jak2 expression alone could induce downstream signaling, we analyzed the overexpression of Jak2 in cultured mouse mesangial cells for 36 h. Jak2 overexpression in cells incubated in normal (5.5 mM) glucose resulted in a 3-fold increase in phosphorylation of Stat3 on Tyr705, consistent with Stat3 activation (Figure 4). There was no significant change in the level of total Stat3 protein. Incubation in high (30 mM) glucose medium alone did not significantly enhance Stat3 phosphorylation compared to normal (5.5 mM glucose), but did lead to an additive increase in Stat3 phosphorylation in the Jak2 transfected cells. We also determined whether increased Jak2 expression could enhance ROS species, since enhanced ROS generation appears to be a hallmark of progressive DN in humans (28). As determined by CM-H$_2$DCF fluorescence, a significant increase in ROS was observed in Jak2 overexpressing cells compared to control vector cells when both cell types were grown in 5.5 mM glucose (Figure 5). A similar increase in ROS generation was demonstrated in control vector cells incubated in 30 mM glucose media compared to cells grown in 5.5 mM glucose medium. There was a further, but non-significant, increase in ROS in Jak2 transfected cells incubated in high glucose medium compared to Jak2 transfected cells grown in normal glucose medium.

**DISCUSSION**

DN in humans requires activation of multiple molecular programs to become manifest. The current pathophysiological understanding of DN is derived largely from analysis of animal and cellular models. Several pathways were identified by these approaches and were confirmed to play critical roles in the evolution of DN in humans (29; 30). However, despite pathogenic changes in a variety of different signaling pathways, current murine models of DN fail to completely replicate progressive human DN (glomerulosclerosis, tubulointerstitial fibrosis and decline in glomerular filtration rate) (10). This discrepancy could be due to partial, incomplete or temporary activation of critical pathogenic responses and/or to distinctive protective responses that counteract or prevent nephropathy in mice that are not manifest in humans. To identify those human-specific pathogenic or murine protective pathways, we used a comparative transcriptomic approach to DN in both species.

In human diabetes, as assessed by microarray analysis, and confirmed by RT-PCR and immunohistochemistry, the
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Jak/Stat signaling pathway appeared to be one of the top regulated pathways and was consistently altered in both glomeruli and tubulointerstitium from patients with DN. Marrero's group previously has identified Jak/Stat activation as potentially pathogenic in mediating angiotensin II signaling, in inducing TGF-β expression, and in stimulating extracellular matrix protein production in cultured mesangial cells and animal models of DN (14; 15; 24; 31; 32). However, to our knowledge the current report is the first study to demonstrate enhanced Jak/Stat expression in human DN. While multiple members of the Jak/Stat family showed increased mRNA expression in microarrays and RT-PCR expression studies, we focused on the expression patterns of Jak2, since it is a critical upstream regulator of many Jak/Stat signaling events, and was already implicated in processes that enhance fibrosis and epithelial mesenchymal transition in DN (11; 13-15; 24; 31; 32).

Our findings suggest an interesting compartmental and temporal association of enhanced Jak2 expression. Glomerular Jak2 mRNA levels increase several-fold in diabetic patients with early DN and then decline in later stages as tubulointerstitial Jak2 increases along with progressive tubulointerstitial fibrosis and reduction in kidney function. Thus, enhanced Jak2 expression temporally corresponds to the evolution of human DN, with glomerulopathy followed by tubulointerstitial fibrosis. The impressive inverse correlation between tubulointerstitial Jak2 expression and that of other Jak/Stat members with eGFR suggests a potential causal relationship between enhanced Jak/Stat expression and progressive tubulointerstitial fibrosis and renal failure. While some of this apparent correlation could be due to population differences between the early DN group, who are all Pima Indians, and the mostly European progressive DN group, a strong correlation between tubulointerstitial Jak2 expression and eGFR remained in the progressive DN group alone, suggesting that this association was not due to population differences.

Jak2 mRNA induction in DN was confirmed by immunochemistry and appears to be DN specific, as there was no induction of Jak2 mRNA in glomeruli or tubulointerstitium in lupus nephritis, IgA Nephropathy, or hypertensive nephrosclerosis. Jak2 protein expression was increased in both glomeruli and proximal tubular cells in humans with DN, but variable expression in other nephropathies with tubular damage and proteinuria, consistent with the notion that Jak2 activation is not generic to all progressive renal diseases. This observation confirmed the results obtained by Affymetrix GeneChip compared to control patients (living donors, n=21), where Jak2 mRNA showed no consistent regulation in patients with hypertensive nephropathy (n=20), IgA nephropathy (n=27) and lupus nephritis (n=32) with fold changes between 0.93 and 1.10 in the glomerular and tubulointerstitial compartments.

Similarly, there was no correlation between gene expression of Jak2 or other Jak/Stat members in DN samples with blood pressure, duration of diabetes, glycosylated hemoglobin or body mass index (with the sole exception of Jak3 and blood pressure), suggesting that the correlation with eGFR was specific and not due to some confounding factor. Nonetheless, a significant correlation between Jak2 and eGFR does not necessarily imply a causal connection between the two parameters, nor a
directionality if a causal relationship exists. It will be imperative to follow up these studies with prospective analysis of patients with DN to determine whether either glomerular or tubulointerstitial Jak2 expression truly predicts progression of DN in humans, as well as tissue-specific Jak2 overexpression studies in animal models of DN.

The absence of enhanced Jak2 expression in the db/db C57BLKS and STZ-diabetic DBA/2J mouse DN models is interesting since these models, frequently used for studies of DN (10; 22; 26; 27), develop high levels of albuminuria and undergo extensive early glomerular changes of DN (22; 26; 27), but rarely progress to severe glomerulosclerosis and do not develop significant tubulointerstitial fibrosis and progressive kidney failure (10; 26; 27). No increases in Jak2 mRNA or protein expression were found in either glomerular or tubulointerstitial compartments suggesting one possible reason for the lack of progressive DN induction in these mouse models. It is certainly likely that additional responses critical for progression of human DN are missing in conventional murine models of DN and that protective responses in mouse models may be absent from humans with progressive DN. Thus, the strategy of uncovering divergent responses between human progressive DN and murine models of early DN may reveal other pathways that are essential for the pathogenesis of DN and that are potential targets for therapeutic or prevention strategies.

Since Jak/Stat pathways are activated by growth factors, cytokines or other upstream signals, and because Jak2 protein activation is via autophosphorylation, enhanced Jak2 expression should result in Stat tyrosine phosphorylation and activation only with Jak2 activation. Thus, it was important to test whether Jak2 overexpression alone could induce Stat phosphorylation and other downstream responses. While in vivo confirmation of such a response necessarily awaits the generation and testing of tissue specific Jak2 transgenic mice, we have confirmed that Jak2 overexpression alone, without additional growth factors or cytokines, substantially increases Stat3 phosphorylation on tyrosine 705. This observation confirms activation of Jak2/Stat3 signaling simply by overexpressing Jak2 in mouse mesangial cells. Interestingly, high glucose alone had only a modest and statistically insignificant effect on Stat3 phosphorylation implying that high glucose alone is not sufficient to trigger Jak2 signaling in our system. However, high glucose had an additive effect on Jak2 overexpression on Stat3 phosphorylation. The molecular mechanism by which Jak2 overexpression induces Stat3 phosphorylation remains to be determined.

Enhanced production of ROS has been described as a potential major activator of Jak/Stat signaling (33) and can occur independently of the addition of exogenous cytokines (34). This phenomenon appears to be relatively general since ROS mediate induction of Jak2 activation in tissues such as cardiac myocytes and vascular smooth muscle cells (34-36) as well as kidney cells (12; 14; 15; 32). We did not test whether ROS could further augment the signaling to Stat3 in our Jak2 overexpressing cells but we did show that Jak2 overexpression induced ROS production in mesangial cells, especially those cultured in high glucose medium. This observation suggests the presence of an amplification
loop in diabetic glomerular and proximal tubular cells in which there is both enhanced Jak2 expression, as shown in this study, as well as enhanced ROS production, due to metabolic alterations resulting from enhanced glucose flux (26; 37). These 2 independent processes can each enhance the other, leading to progressive downstream signaling from both pathways and inexorable fibrosis and kidney failure.

Enhanced Jak2 expression in both glomerular and proximal tubule cells may stimulate a host of downstream processes that lead to the glomerulosclerosis, tubulointerstitial fibrosis and kidney failure that affects up to 40% of diabetic patients. The absence of this response may protect mice from these late, but deadly, aspects of progressive DN. Further exploration of Jak/Stat pathways in DN as well as additional systems analysis of the differences between mice and humans may elucidate targets for therapies that could help humans with DN be more like mice.

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Figure 1. Jak/Stat canonical pathway in human DN as assessed by Ingenuity Pathway Analysis (IPA) software. The Jak/Stat family members represented include Jak1, Jak2, Jak3, Stat1, Stat2, Stat3, Stat4, Stat5A, Stat5B and Stat6. The mRNA expression changes for Jak/Stat family members in early (PIMA Indians, n=22-24) (left panel) and progressive (European, n=7-11) (right panel) human DN (DN) are indicated in red for increased expression and green for decreased expression, compared to the corresponding control group (LD+MCD, n=7-12). 2000-2008 Ingenuity Systems, Inc. All rights reserved.
Figure 2. Jak2 mRNA expression levels in human DN: correlation with renal function. A. Increased expression of Jak2 mRNA in the glomeruli of early DN patients and in the tubulointerstitium of progressive DN patients compared to controls (n = 6-9 in the control group, n = 11-12 in the early DN group, n = 10-12 in the progressive DN group). B. Correlation of tubulointerstitial Jak2 mRNA expression (as measured by real-time RT-PCR) with eGFR in patients with early (black cross, n=11) and progressive (black circles, n=12) DN. C. Representative Jak2 immunohistographs of kidney biopsies from patients with no kidney disease (Control), progressive DN (Prog. DN), hypertensive nephropathy (HTN), IgA nephropathy (IgAN) or lupus nephritis (LN). Jak2 expression was substantially and significantly increased in the proximal tubular cells and glomeruli of the patients with DN compared to the control and not in other progressive kidney diseases.
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Figure 3. Jak2 in the C57BLKS type2-DN and STZ-DBA/2J type1-DN mouse models. 
A. Jak2 real-time RT-PCR mRNA expression in cortex (n=6 control, n=7 diabetic) and glomeruli (n=5 control, n=7 diabetic) was not altered in db/db mice compared to db/m control animals. 
B. Jak2 immunoblot (upper panel) and densitometry (lower panel). Jak2 protein levels were unchanged in the cortex (n=3 control, n=5 diabetic) and glomeruli (n=4 in both groups) of db/db mice vs. db/m mice. 
C. Jak2 qRT-PCR mRNA expression in cortex and glomeruli was not altered in DBA/2J diabetic mice compared to control animals (n=5 in each group). 
D. Jak2 immunoblot (upper panel) and densitometry (lower panel) in DBA/2J mice. Jak2 protein levels were not significantly different in the cortex or glomeruli of DBA/2J diabetic mice. n.s. = statistically non-significant.
Figure 4. Effects of Jak2 overexpression and high glucose on Stat3 phosphorylation in Jak2 mesangial cells.

**Left panel:** Jak2 (130 kDa), total Stat3 (79 kDa), phosphorylated Stat3 (79kDa) and β-tubulin (50 kDa) immunoblots of murine mesangial cells transfected with a Jak2 prk-5 plasmid or a control vector \( (n=3\) per group). **Right panel:** Phosphorylated Stat3 levels normalized to total Stat3 levels.
Figure 5. Effects of Jak2 overexpression and high glucose on ROS generation in mesangial cells.  

Left panel: DCF fluorescence in murine mesangial cells transfected with either Jak2 prk-5 or control vectors exposed to 5.5 mM D-glucose + 24.5 mM mannitol, or 30 mM D-glucose for 24h. Image exposure times were identical and are representative of those from 3 separate fields on 5 separate slides for each condition. Right panel: ROS quantitation from the DCF fluorescence experiments. Fluorescence intensity was determined using NIH image J. (n=5 per group; 50 cells were assessed for each slide).