Comprehensive genomic profiling in diabetic nephropathy reveals the predominance of proinflammatory pathways

K. J. Kelly,1 Yunlong Liu,2 Jizhong Zhang,1 Chirayu Goswami,2 Hai Lin,2 and Jesus H. Dominguez1,3

1Department of Medicine, Indiana University School of Medicine, Indianapolis, Indiana; and 2Department of Medical and Molecular Genetics, Indiana University School of Medicine and the 3Roudebush Veterans’ Affairs Medical Center, Indianapolis, Indiana

Submitted 19 February 2013; accepted in final form 6 June 2013

DIABETIC NEPHROPATHY (DN) is the leading cause of progressive chronic kidney disease (CKD) and end-stage renal disease (ESRD) (41), reflecting a failure of available therapies to protect diabetic patients (41). This alarming situation has reached global proportions, illustrated by a worldwide study showing prevalence for microalbuminuria and macroalbuminuria in 49% and impaired renal function in 22% of Type 2 diabetics who thought were free of renal disease (70). The prevalence of end-stage renal disease, demands more effective approaches to prevent and treat progressive CKD. We undertook next-generation sequencing in a rat model of diabetic nephropathy to study in depth the pathogenic alterations involved in DN with progressive CKD. We employed the obese, diabetic ZS rat, a model that develops diabetic nephropathy, characterized by progressive CKD, inflammation, and fibrosis, the hallmarks of human disease. We then used RNA-seq to examine the combined effects of renal cells and infiltrating inflammatory cells acting as a pathophysiological unit. The comprehensive systems biology analysis of progressive CKD revealed multiple interactions of altered genes that were integrated into morbid networks. These pathological gene assemblies lead to renal inflammation and promote apoptosis and cell cycle arrest in progressive CKD. Moreover, in what is clearly a major therapeutic challenge, multiple and redundant pathways were found to be linked to renal fibrosis, a major cause of kidney loss. We conclude that systems biology applied to progressive CKD in DN can be used to develop novel therapeutic strategies directed to restore critical anomalies in affected gene networks.

diabetic nephropathies; inflammation; renal failure

 Lean and obese diabetic male ZS rats (Charles River, Wilmington, MA) were acquired at 8 wk of age and fed Purina diet #5008 with 27% protein, 17% animal fat, and 56% carbohydrate. Their body
weights were recorded, and sera plus urine samples were collected for analyses at biweekly intervals until termination. The rats underwent intervention at 10 wk of age as follows: They were anesthetized with pentobarbital (50 mg/kg ip) and placed on a homeothermic table to maintain core body temperature at ∼37°C. After insuring adequate anesthesia, we induced renal ischemia by occluding both renal pedicles for 25 min with microaneurysm clamps in one obese/diabetic group [diabetic/ischemia (DI)] (40, 42). The lean (LS) control group and a second obese/diabetic group [diabetic sham surgery (DS)] were subjected to sham surgery: the kidneys were surgically exposed in an identical manner but not clamped. Changes in renal and metabolic parameters were monitored by sequential measurements of serum creatinine, blood urea nitrogen, urinary protein/creatinine ratios, serum glucose, triglycerides, and cholesterol with the autoanalyzer of the clinical laboratory at the Indianapolis VA Hospital. The rats were terminated at 28 wk of age. Kidneys were removed from anesthetized animals and immediately frozen with liquid nitrogen, and RNA extraction was then performed as below. To examine all renal and inflammatory cells as a unit, we performed no perfusion to avoid removing cells potentially critical in the pathophysiology of diabetic nephropathy.

Histology and Immunohistochemistry

Kidney sections were fixed in 3.8% paraformaldehyde, paraffin embedded, and 4 μM sections obtained for periodic acid-Schiff (PAS) and Leder stains to visualize intrinsic renal cell morphology and neutrophils, respectively. Masson’s trichrome was used to stain collagen (20, 44).

RNA-seq and RT-PCR

Total kidney RNA was isolated with a Trizol purification kit as recommended by vendor (Cat. #12183-555; Invitrogen, Grand Island, NY) and cleaned with an RNeasy Mini kit as recommended by vendor (Cat. #74104; Qiagen, Valencia, CA). For RNA-seq, 3 μg were fragmented with RNase III, cDNA libraries constructed with SOLiD adaptors by reverse transcription (RT), and then subjected to RNA sequencing (strand-specific RNA-seq) of short 50 bp reads using the SOLiD 4 platform (Center for Medical Genomics at Indiana University School of Medicine) (16). RNA-seq yielded 12 complete transcriptomes, corresponding to four kidneys randomly chosen from each of the three groups of rats: LS, DS, and DI.

The RNA-seq data analysis includes three major steps: quality control (QC) filtering, sequence alignment, and differential expression analysis.

QC filtering. We first used SOLiD Instrument Control Software and SOLID Experiment Tracking System software for the read quality recalibration. Sequences containing more than two “n” or wildcards were discarded. Each sequence was scanned for low-quality regions, and if a 5-base sliding window had an average quality score <20, the read was further truncated at that position. Any read with a length of <35 bases was discarded. Our experience suggests that this strategy effectively eliminates low-quality reads while retaining high-quality regions (8, 37, 84).

Sequence alignment. We used the RNA-seq module in the NGSpipe for RNA-seq analysis. In brief, BFAST (33) was used as our primary alignment algorithm because it has high sensitivity on color-space data for aligning the reads on the loci containing small insertions and deletions comparing with the rat reference genome (m4). We used a TopHat-like strategy (85) to align the sequencing reads that cross splicing junctions. After aligning the sequence reads to a filtering index including repeats, ribosome RNA, and other sequences that are not of interest, we conducted sequence alignment at three levels: genomic, known junctions (UCSC), and novel junctions (based on the enriched regions identified in the genomic alignment). For the subsequent analysis, we used only the uniquely aligned sequences with no more than two mismatches.

Differential expression analysis. Gene expression was calculated in the form of reads per kilobase exon model per million mapped reads (RPKM), which is a standard method for quantifying gene expression levels from RNA-seq data (10, 63, 82). When calculating RPKM values, we did not consider sequencing reads falling into the genes with highest 25% expression levels; this is done to avoid the biases due to the potential expression changes of highly expressed genes. Among the total of 16,536 annotated rat genes (based on UCSC annotation), 13,453 genes had credible expression level in at least half of samples in at least one of the conditions. To identify the differentially expressed genes, we further conducted Student’s t-test on the logarithmically transformed RPKM values, comparing DI vs. LS and DS vs. LS. Pathway analysis was performed with Metacore Software (GeneGo, Carlsbad, CA) using the RPKM expression data for each sample as the input. Motif analysis was performed with the MotifModeler informatics program as described (54).

For RT-PCR, 3 μg of total kidney RNA were used to synthesize cDNA with an AffinityScript QPCR cDNA Synthesis Kit as recommended by vendor (Cat. #500559; Agilent Technologies, Santa Clara, CA). Real-time quantitative PCR was run on a MX3005P Multiples Quantitative PCR System (Agilent) with Brilliant SYBR Green PCR Master Mix (Agilent), and an RT-PCR primer set for each target gene from Qiagen. Standard curves for each target gene were obtained with the same system before the running of corresponding samples. The samples were always run in quadruplicate. The data were normalized to the mRNA content of β-actin in each sample and are reported as relative amount of mRNA compared with control (LS) samples.

Western Blot

Proteins were separated from renal homogenates on a 12% acrylamide SDS-PAGE gel, electrophoretically transferred to a BIO-RAD Immuno-Blot polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA) at 15 mA, and labeled with anti-α-2-macroglobulin (A2M) goat antibody (Cat. #M-0140; Sigma, St. Louis, MO), anti-collagen I rabbit antibody (Cat. #600-401-103s; Rockland, Gilbertsville, PA), anti-acyl-CoA oxidase 2, branched chain (ACOX) goat antibody (Cat. #PAS-18671; Thermo Scientific, Waltham, MA) and anti-actin mouse antibody (Cat. #A6267; Abcam, Cambridge, MA). The secondary antibodies were donkey anti-rabbit IRDye 800CW (Cat. #926-32213; donkey anti-goat IRDye 800CW, Cat. #926-32214; and donkey anti-mouse IRDye 680CW, Cat. #926-32222, all of them from LI-COR Biosciences, Lincoln, NE). The membranes were analyzed with a fluorescence scanner (Odyssey Classic, model 9120, LI-Cor Biosciences).

Animal Use Statement

The experiments were conducted in conformity with the American Physiological Society’s “Guiding Principles for Research Involving Animals and Human Beings.” The investigations were approved by the Institutional Animal Care and Use Committee of Indiana University School of Medicine.

Statistics

Results are expressed as means ± 1 SE. Differences in renal and metabolic parameters were determined by one-way ANOVA with subsequent t-tests (when ANOVA indicated statistical significance; GraphPad Prism, La Jolla, CA). Bonferroni correction was used for multiple comparisons. The null hypothesis was rejected at P < 0.05.

RESULTS AND DISCUSSION

Renal/metabolic Phenotype

ZS rats were assigned to three study groups: lean sham controls (LS, n = 6) only had their kidneys surgically exposed. The obese-diabetic rats were randomly divided into a sham...
surgery group (DS, \( n = 7 \)), which only had their kidneys exposed, and an ischemia/reperfusion surgery group (DI, \( n = 11 \)), which had both kidney pedicles clamped for 25 min. The rats were acclimatized for 2 wk, and then surgeries were performed at age 10 wk. Serum creatinine levels at 8 wk of age were similar in LS control rats and presurgery DS and DI: 0.30 ± 0.00, and 0.34 ± 0.02, and 0.33 ± 0.03 mg/dl, respectively. Subsequently, 24 h after surgery, mean serum creatinine peaked to 0.71 ± 0.08, returning to presurgery levels at 48 h (0.47 ± 0.06) in DI. Serum creatinine was not altered postoperatively in the LS or DS groups: 0.43 ± 0.04 and 0.37 ± 0.03 for the 24 h time points. However, later on, when DI rats were 20 wk of age, there was a marked and progressive increase in serum creatinine, while in DS the increase in serum creatinine was more gradual and less pronounced (Fig. 1).

Mean serum urea nitrogen levels, kidney weights, urine volumes, and urine protein excretion were all higher in DS and DI than in LS (Fig. 1). Initial serum glucose levels were higher in DS, 151 ± 7 mg/dl, and in DI, 142 ± 3, than in their lean litter mates (LS), 125 ± 2, \( P < 0.01 \). This difference was magnified over time until the conclusion of the study at 28 wk of age (Fig. 1). Obese-diabetic rats had dyslipidemia: mean initial serum cholesterol levels were 91 ± 21 mg/dl in DS rats, 109 ± 10 in DI, and 61 ± 10 in LS, and increased further in DS and DI (Fig. 1). Mean initial serum triglycerides were 306 ± 18 mg/dl in DS rats, 305 ± 18 in DI, and 129 ± 7.5 in LS, and also increased further in DS and DI. The liver enzyme aspartate aminotransferase was also slightly increased in DS and DI (Fig. 1).

Postmortem renal sections were stained with PAS for a detailed examination of renal histology (20, 21, 40, 83); Leder’s stain was used to evaluate renal inflammation represented by infiltrating leukocytes (20, 21, 40, 83), and Masson’s trichrome was utilized to determine the extent of renal fibrosis (20, 21, 40, 83) (Fig. 2). The renal pathology in LS, DS, and DI ZS male rats has been described extensively (19 –21, 40, 58, (20, 21, 40, 83) (Fig. 2). The renal pathology in LS, DS, and DI trichrome was utilized to determine the extent of renal fibrosis by infiltrating leukocytes (20, 21, 40, 83), and Masson’s trichrome was used for RNA-seq mapping statistics of the RNA-seq experiment are provided in Supplementary Table S1. After we removed the genes that are not expressed in any condition, the log transformed RPKM were normally and equally distributed for all groups, which indicated that sequencing data were consistent for all 12 samples contained in the three groups, demonstrating that differences were not secondary to read distribution among the groups (Fig. 3). In addition, principal component analysis (PCA) was conducted to examine the main source of variance in the data (23, 59). Widely used in microarray data analysis, PCA is a dimensionality reduction strategy for visualizing the overall variability among different samples (Fig. 3). PCA verified that the source of variability was mostly biological, i.e., there was greater variability among LS, DS, and DI groups than within group variability of the individual samples in each group (Fig. 3). There were 1,442 upregulated genes in DI compared with LS and 184 upregulated genes in DS compared with LS. In DI, 1,297 genes were uniquely upregulated. In DS 39 genes were uniquely upregulated, and 145 upregulated genes overlapped the two diabetic rat groups (Fig. 3). Moreover, there were 1,239 downregulated genes in DI compared with LS. In DS, 396 genes were downregulated compared with LS. There were also 864 uniquely downregulated genes in DI, 21 uniquely downregulated genes in DS, and 375 downregulated genes overlapped in the two diabetic groups. Hence, about half of the 3,261 differentially expressed genes in DI and DS were upregulated and half downregulated, and 9% of the upregulated genes overlapped in the two diabetic groups, while 30% of the downregulated genes overlapped in the two diabetic groups. (A detailed list of up- and downregulated genes is in Supplementary Table S2.) A random set of eight differentially expressed genes in DI and DS was selected for comparison, and their mRNA levels measured by RT-PCR in the same RNA used for RNA-seq. The changes in the levels of these eight mRNAs were highly correlated with the mRNA levels obtained.

The mechanisms responsible for these and other manifestations of diabetic and ischemic nephropathy are complex, and their understanding is still inadequate to develop effective therapies. Moreover, hypertension, part of the metabolic syndrome phenotype in these obese rats (21) and in humans with Type 2 diabetes (41), was a likely contributor to progressive CKD, although blood pressure was not recorded in this study. In our prior studies (40) systolic blood pressure was significantly elevated (166 ± 9.7) in postischemia diabetic rats compared with 113 ± 9.5 in lean rats and 143 ± 5.6 in the diabetes/sham group. One novel and potentially fruitful approach to this challenging problem is to study the relationships of participating renal genetic elements. Accordingly, we conducted RNA sequencing [RNA-seq, (1, 16)] to capture the interacting genetic elements, renal and nonrenal. Diabetic nephropathy is extremely complex, and renal injury results from the interplay between migrating inflammatory cells and multiple types of stressed renal cells, all included in the analysis. The overall mapping statistics of the RNA-seq experiment are provided in Supplementary Table S1.

Fig. 1. Phenotype in experimental diabetic nephropathy. Renal injury in ZS rats is characterized by progressive renal failure (inset), the sine qua non of human disease. The rats also have nephromegaly, polyuria, proteinuria, as well as dyslipidemia. For each parameter, mean values ± SE are presented. The units are mg/dl for creatinine, blood urea nitrogen (BUN), glucose, cholesterol, and triglycerides; mg for kidney weight (kid wt); ml24 h for urine volume (ur vol); and units/l for aspartate aminotransferase (AST). *\( P < 0.05 \) vs. lean; ‡\( P < 0.05 \) vs. diabetes/sham.
by RNA-seq (Fig. 4). In addition, Western blots of proteins encoded by selected transcripts showed changes consistent with the RNA-seq and RT-PCR data (Fig. 4).

Functional pathways or gene networks (Metacore) were assembled using the differentially expressed gene data sets. The statistical relevance ($P < 1 \times 10^{-8}$ for the pathways presented) of this mapping is calculated as the probability of a chance match between specific genes in our data sets and those existing in the defined pathways (Metacore, http://www.genego.com). This probability is expressed as the $P$ value of the hypergeometric distribution (17, 23). The genes included in the networks (Fig. 5) were those differentially expressed in both conditions, DS and DI, compared with controls (LS). The inflammation pathway is shown in Fig. 5A, and genes in this set are primarily involved in the interaction of renal and inflammatory cells. The illustration includes 35 designated genes plus 10 additional genes encoding G protein-coupled receptors (GPCRs). These 45 genes are upregulated in both DS and DI with respect to LS. The network also includes five genes that were downregulated. In the diabetic kidney, PARP-1 is upregulated and it becomes one of several activators of upregulated NF-$\kappa$B (30). The NF-$\kappa$B complex is then seen in positive interaction with upregulated CXCR3, its ligand CXCL10, plus the inflammatory ligands CCL19 and CXCL1. NF-$\kappa$B is positioned upstream of upregulated atherogenic CD40L (68) and mitogenic myc (25). On the other hand, NF-$\kappa$B is shown suppressing downregulated proangiogenic CXCL12 (29, 57), also suppressed by activated MMP2 and MMP3 (STMY1) (60). Moreover, the antioxidation genes SOD1 and VDAC1 are attenuated in DS and DI, despite stimulation from activated NF-$\kappa$B and SP1, an effect consistent with the dependence of VDAC1 on SOD1 activity (11). TLR4 is activated by TLR6 and IRF4, while positively
interacting with MyD88 (in Fig. 5C, MyD88 is also seen receiving stimulatory signals from NF-κB) (80) and is potentially involved in cell proliferation via the NF-κB/Myc signaling axis (25). Motif analysis (Table 1) demonstrates a significant level of regulation by NF-κB that involves a total of 82 regulated genes. Other transcription factors with highly significant transcription contribution scores include myc and stat5.

The network also reports that Myc has negative interaction with TLR4, as reported elsewhere (94), although this conclusion is not shared by others (62). Antioxidant CAT is suppressed, receiving inhibitory signals via casp1 and upregulated KLF11 (24, 75). The inflammation pathway includes interactions of activated CXCL1, 6, 10, 17, and CCL19 with their respective GPCRs and subsequent activation of HCK, VAV1, FGR, STAT3, ERB2, and GpB (G protein β2), followed by positive interaction with PREX1, RAC1, NCF2 (p67phox), which lead to assembly and activation of NADPHox (22). Thus, renal inflammation in DS and DI is associated with combined attenuation of antioxidant systems and activation of pro-oxidant systems.

The apoptosis pathway shown in Fig. 5B includes 39 genes; 27 genes were upregulated in DS and DI with respect to LS. These 27 upregulated genes are interacting with 12 genes not differentially expressed in DS and DI. Figure 5B, top left corner, addresses the intrinsic apoptotic pathway, represented by casp3 acting on p53 and from which released fragments can induce mitochondrial membrane permeabilization (74). p53 also stimulates upregulated Apaf1, which forms the apoptosome with the initiator caspase 9 (7). Figure 5B, top right corner, focuses on the extrinsic apoptotic pathway, illustrated...
by FADD and its activating relationship with upregulated casp8 and subsequent activation of the execution caspases casp3 and 4 (3). The pathway also portrays positive interaction between upregulated casp3, p53, and Apaf1 (77). Thus, the data point to activation of intrinsic and extrinsic apoptotic pathways in the nephropathy of DS and DI (18). TNFR1 is shown activating IRAK1/2 and then diverging: one branch signals a proapoptotic stimulus via TRADD/FADD/Casp8 axis and the other inflammation via TRAF2/TRAF6/MAPK pathway (89), illustrating one intersection of inflammatory and apoptotic pathways. TNFR1 also interacts positively with STAT3 and JAK2 (92). Activated SP1-dependent transcription
Table 1. Motif analysis

<table>
<thead>
<tr>
<th>Transcription Factor</th>
<th>Diabetes Ischemia</th>
<th>Diabetes Sham</th>
</tr>
</thead>
<tbody>
<tr>
<td>AML1a</td>
<td>220</td>
<td>88</td>
</tr>
<tr>
<td>AML1</td>
<td>220</td>
<td>88</td>
</tr>
<tr>
<td>E2F</td>
<td>111</td>
<td></td>
</tr>
<tr>
<td>HNF1</td>
<td>55</td>
<td>26</td>
</tr>
<tr>
<td>E2F-1</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>Xvent-1</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td>Rb:EF2-1:DP-1</td>
<td>77</td>
<td></td>
</tr>
<tr>
<td>AML</td>
<td>64</td>
<td>25</td>
</tr>
<tr>
<td>PU.1</td>
<td>67</td>
<td>23</td>
</tr>
<tr>
<td>Osf2</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>Ets</td>
<td>82</td>
<td></td>
</tr>
<tr>
<td>PEBP</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td>Major T-antigen</td>
<td>71</td>
<td></td>
</tr>
<tr>
<td>E2F</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>ETF</td>
<td>78</td>
<td>28</td>
</tr>
<tr>
<td>MZF1</td>
<td>68</td>
<td></td>
</tr>
<tr>
<td>myc</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>c-Ets-2</td>
<td>69</td>
<td></td>
</tr>
<tr>
<td>E2F</td>
<td>81</td>
<td></td>
</tr>
<tr>
<td>Ncx</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>STAT5</td>
<td>93</td>
<td></td>
</tr>
<tr>
<td>HSF</td>
<td>73</td>
<td></td>
</tr>
<tr>
<td>PEA3</td>
<td>163</td>
<td></td>
</tr>
<tr>
<td>TEF-1</td>
<td>198</td>
<td></td>
</tr>
<tr>
<td>MyoD</td>
<td>66</td>
<td>31</td>
</tr>
<tr>
<td>c-myc:max</td>
<td>66</td>
<td></td>
</tr>
<tr>
<td>AP-2rep</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>core-binding factor</td>
<td>57</td>
<td>27</td>
</tr>
<tr>
<td>IRF1</td>
<td>66</td>
<td></td>
</tr>
<tr>
<td>NF-κB</td>
<td>67</td>
<td>32</td>
</tr>
<tr>
<td>FOX3</td>
<td>52</td>
<td></td>
</tr>
</tbody>
</table>

Transcription factors with the most significant effects on gene expression (diabetes vs. control) as well as the number of regulated genes are presented.

is seen acting on transforming growth factor receptor 1 (TGF-βR1), which also stimulates TRAF6, followed by activation of the upregulated MAPK system (92). In addition, SP1 has positive interaction with genes encoding fibrogenic proteins; collagens I, III, IV, and fibronectin (FN-1) (87). SP1 is activated by STAT3, and in turn, SP1 interacts with interleukin (IL)-24, which signals upregulated JAK1, as well as the chemokine CXCL1, which signals JAK3 (31). SP1 is also shown activating upregulated ICAM1 (93), a critical adherence molecule in ischemia and diabetic nephropathy (40, 44). Upregulated LIF (32), OSM (79), and unchanged IL-9 stimulate shown activating upregulated ICAM1 (93), a critical adherence

and IL4R, although the latter receptor was suppressed in diabetic rats (64, 65, 81). On the other hand, cell cycle inhibitory signals emanating from p21 can inhibit STAT3 signaling (14). Other upregulated targets of STAT6 include Col I (6), NF-κB (52), and IL-4, which in turn activates STAT6 (64) in an activating loop previously reported in inflammatory (50) and epithelial cells (67). IL4 interacts with unchanged IRS2 (88) and upregulated JAK3 (34), which is upstream of activated JAK1, also stimulated by the upregulated receptor IL6ST (gp130) (56). It is noteworthy that upregulation of the JAK-STAT pathway is also seen in human DN but not in mouse models without renal failure or tubulointerstitial fibrosis (90).

The cell cycle arrest network shown in Fig. 5D contains 35 activated genes, four suppressed genes, and three unchanged genes. In Fig. 5D, right center, is the upregulated cyclin-dependent kinase inhibitor p21, the target of upregulated p53 (86), also stimulated by TGF-β signaling, including SMAD5, SMAD1, SMAD3 (69) by upregulated IRF5 (4), SPI (91), RUNX3 (45), and RUNX1 (71). In turn, p21 is shown suppressing the downregulated cyclins CCNB, D, and E, as well as upregulated CDK4 and PLK1 (13). In Fig. 5D, top left corner, upregulated TGF-β is seen interacting positively, via a non-SMAD pathway, with the upregulated SHS and SOS axis, although expression of effector signaling molecule HRAS is suppressed in diabetic kidneys (51). In addition, TGF-β via TGF-βR1 activates SMAD3, shown interacting positively with upregulated PAI1 (72), TIMP1 (61), and FN1 (55). Furthermore, TGF-β receptors (49) and IRAK4 (39) stimulate upregulated TRAF6, which is shown signaling Src (53), upregulated IL1R1 and upregulated IL17R, the latter a component of a positive feedback loop that includes TRAF6 and unchanged CIKS upstream of the NF-κB complex (not shown) (78). SMAD1 activates collagen genes that link positively to upregulated ITG-α5 and upregulated ITG-β1 (38), which stimulate upregulated RUNX2 via upregulated transcriptional cofactor Fhl2 (28). Figure 5D also illustrates upregulated RUNX3 activating p21, a process mediated by TGF-β signaling (15), as well as RUNX1 (2). RUNX2 is seen interacting in a complex way with genes regulating fibrogenesis: RUNX2 activates profibrotic genes and also has positive interactions with TIMP1 (5) and with MMP14 (35). Activated TIMP1 is shown inhibiting MMP2 and MMP12, and upregulated TIMP2 is shown inhibiting MMP2. Furthermore, the gene encoding the hyaluronic acid (HA) receptor, CD44, is cooperating with MMP2 and SRC (46). The end-result is renal fibrosis, as these interactions seem to overwhelm the role of metalloproteinases.

Figure 5D also includes the known association of cell cycle arrest and fibrogenesis (93), exemplified herein by the positive interaction of upregulated TGF-β with p15, which inhibits CDK4 (73). It is noteworthy that many of these mediators (including CXCR4, CCL19, CD44, collagens, TIMP1 and FN1) are also increased in human biopsy specimens evaluated by microarray technology (90).

RNA-seq and bioinformatic protocols were used to derive interacting renal gene networks in progressive CKD of DN. This extensive systems biology analysis revealed exceedingly complex interactions among inflammatory and renal cells, a reality made evident even by a single cross-sectional snapshot of the events. For example, it is clear from our data that multiple paths, TGF-β1, IL6ST, BMP1, lead to destructive
fibrosis, and we surmise that this redundancy is a major challenge to be considered in anti-fibrosis drug design. The networks identified other interlinked areas of morbidity and potential opportunity. Thus, the pro-inflammatory NF-κB/CXCL1 axis, which drives neutrophil migration, was increased 10-fold in both DS and DI kidneys and is a potentially rewarding target. Accordingly, we previously showed that immune suppression with mycophenolate mofetil attenuates upregulated renal CXCL1, improving renal function, inflammation, and fibrosis (19). The networks also revealed critical renal apoptosis mechanisms that can be targeted in DN. Another important finding is the pervasive role of p21 in cell cycle arrest, where multiple inputs activate p21, a state that must be considered when trying to restart the arrested cell cycle in DN.

GRANTS

This work was supported by the National Institute of Diabetes and Digestive and Kidney Diseases Grant R01 DK-082739 and a research grant from DCI Paul Teschan Research and Development Fund to K. J. Kelly and VA Merit Review funds to J. H. Dominguez.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES

TRANSCRIPTOME IN DIABETIC NEPHROPATHY


56. Shen X, Tian Z, Holtzman MJ, Gao B. Cross-talk between interleukin 1beta (IL-1beta) and IL-6 signalling pathways: IL-1beta selectively inhibits its IL-6-activated signal transducer and activator of transcription factor 1...


