Differential Gene Expression in Diabetic Nephropathy in Individuals With Type 1 Diabetes


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Context: Diabetic nephropathy (DN) is the leading cause of end-stage renal disease (ESRD) in the United States.

Objective: The aim of this study was to determine whether there were skin fibroblast gene expression differences between subjects with type 1 diabetes (T1D) with or without DN.

Setting: This was a cross-sectional study conducted in the University of Minnesota.

Patients: Study volunteers were 100 former participants of Genetics of Kidneys in Diabetes: 40 were diabetic nephropathy (DN) Controls, normoalbuminuric after ≥15 years of T1D; and 60 were DN Cases, 25 with proteinuria and 35 with ESRD.

Intervention(s): Skin fibroblasts were grown in high glucose (HG) for five passages (approximately 6 weeks).

Main Outcome Measure(s): SF gene expression was assessed by transcriptome sequencing using the Illumina HiSeq 2000 platform. Pathway analyses tested directionally consistent group differences within the Kyoto Encyclopedia of Genes and Genomes pathways.

Results: Eight pathways, all related to cell cycle and repair, were up-regulated in the DN Controls vs the DN Cases. These pathways markedly overlapped with the pathways up-regulated by HG in T1D monozygotic twins (MZT), but not in their non-T1D MZT. DN Cases showed statistical trends toward up-regulation of these pathways vs non-T1D MZT, but much less so than the DN Controls.

Conclusions: Together, these data suggest that SF from T1D patients undergo epigenetic modifications resulting in increased expression of genes in healing and repair pathways. These responses, much more robust in patients protected from DN, suggest that epigenetic factors are important in DN risk. (J Clin Endocrinol Metab 100: E876–E882, 2015)

Abbreviations: ACR, albumin to creatinine ratio; BP, blood pressure; DN, diabetic nephropathy; ESRD, end-stage renal disease; FPKM, Fragments per Kilobase of transcript Model per Million; GoKinD, Genetics of Kidneys in Diabetes; HbA1c, glycosylated hemoglobin; HG, high glucose; KEGG, Kyoto Encyclopedia of Genes and Genomes; MZT, monozygotic twins; NG, normal glucose; SF, skin fibroblasts; T1D, type 1 diabetes.
have delayed, but have not substantially affected DN-related ESRD. Clearly, new DN pathogenetic concepts are needed for new treatment targets to emerge. Although there is direct association between glycemia and DN risk (3), genetic (4) and epigenetic (5, 6) mechanisms also seem to play important roles in determining DN risk.

A large number of studies from multiple laboratories (7–15), many recently reviewed (16), confirmed that behaviors of cultured skin fibroblasts (SF) differ between individuals with diabetes with and without DN. Given that these SF behavior differences are detectable despite several in vitro passages under identical conditions, they are consistent with either genetic or epigenetic processes related to DN risk. SF sodium-hydrogen (Na+/H+) antiport activity is greater in T1D individuals vs without DN (8). T1D sibling pairs are concordant for DN risk (17), DN lesions (18), and SF Na+/H+ antiport activity (19) and antioxidant enzyme mRNA expression (20), these findings consistent with heritable genetic contributions to disease risk. Consistent with protective mechanisms, SF studies have also found that T1D individuals without DN despite long T1D duration (21) have some SF behaviors that differ from patients with DN and from normal controls (13, 14). These protective mechanisms could be genetic and/or epigenetic in nature. However, the potential role of epigenetic processes in these SF behavioral differences remains relatively unexplored.

In a companion article (22), we reported that SF from T1D members of monozygotic twin (MZT) pairs discordant for T1D had striking SF gene expression changes from normal (NG) to high (HG) glucose in pathways related to cell cycle and DNA repair mechanisms (22). Despite approximately 6 weeks and multiple in vitro passages in these same conditions, none of these pathway changes from NG to HG were seen in their non-T1D MZT (22). Moreover, the non-T1D twins had far fewer genes differentially expressed between the NG and the HG conditions. Given that the twins are genetically identical, these marked differences in response to in vitro HG are necessarily epigenetic phenomena consequent to exposure to longstanding in vivo T1D that persisted for several weeks and passages in the respective in vitro glucose conditions. These findings, along with our earlier MZT studies (23), argue that cultured SF can reflect in vivo epigenetic cellular alterations.

Herein we report the results of SF gene expression studies in HG in 100 former Genetics of Kidneys in Diabetes (GoKinD) participants (24) rerecruited at the University of Minnesota and at the Joslin Diabetes Center.

Materials and Methods

Subjects

Subjects were 100 T1D participants in the original GoKinD study (24). GoKinD entry criteria were previously published (24). Briefly, subjects were at least 18 years old when entering the study, 30 or less years old at T1D onset and were on insulin within 6 months. Criteria for DN in the Cases were T1D duration of at least 10 years and presence of proteinuria (urinary albumin to creatinine ratio [ACR] ≥ 300 μg/mg) in at least two of the three consecutive urine samples, or ESRD. DN Controls had at least 15 years’ T1D duration and at least two of the last two ACR measurements were < 20 μg/mg, with no ACR value exceeding 39 μg/mg. DN Controls could not be receiving renin angiotensin system blocking agents. Using these GoKinD baseline criteria and classifications, we recruited 40 DN Controls and 60 DN Cases; 25 of the DN Cases met the proteinuria criterion and 35 had ESRD, all having undergone renal transplantation.

Also included in these studies were nine non-T1D members of T1D-discordant MZT pairs who served as nondiabetic controls. These studies were approved by the Committee for the Use of Human Subjects in Research at the University of Minnesota and The Committee of Human Studies at the Joslin Diabetes Center.

Clinical studies

Urinary albumin was measured using an immunoturbidimetric method (Cobas 6000 instrument; Roche Diagnostics) and expressed as ACR. Creatinine was measured by an enzymatic method. Glycated hemoglobin was measured at time of skin biopsy by HPLC (Bio-Rad; normal range, 4.3–6.0%).

Skin biopsy and cell culture

The methods for skin biopsy, SF culture, and RNA isolation are detailed elsewhere (13–15, 20, 23). SF were grown from acquisition in HG (25 mmol/L). Stored SFs were thawed and passaged before a final seeding (fifth passage) into T150 tissue culture flasks at 10 000 cells/cm² in DMEM with 10% fetal bovine serum. SFs were then grown for 60–72 hours until approximately 90% confluence, collected with 0.125% Trypsin-EDTA in Hanks’ Balanced Salt Solution without Ca²⁺ or Mg²⁺, washed in DMEM with 10% fetal bovine serum, pelleted by centrifugation, and stored at −80°C until RNA isolation. Cells were coded by the single technician performing the original explants while the technician performing the gene expression studies was masked as to the subjects’ DN status. SFs were grown in batches of 10 containing a random mix of DN Cases and DN Controls.

RNA isolation

Total RNA was isolated and its integrity confirmed using the RNA 6000 LabChip kit and Agilent 2100 bioanalyzer (Hewlett Packard).

mRNA-Seq

SF sample transcriptome sequencing was carried out on the Illumina HiSeq 2000 instrument in paired-end 2 × 50-bp cycle mode using library creation kits from Illumina (Tru-Seq RNA sample prep kit) (25). Sets of eight sample libraries were indexed together in sequencing flow cells lanes using Illumina-supplied indexing adaptors and an adequate number of flow-cell lanes run...
to generate 10 million filter-passing high-quality reads per sample.

**Sequence data analysis**

Illumina read files were sent to the Minnesota Supercomputing Institute server, converted to Fastaq data files, checked for resynchronization as paired end reads (two files/sample), sent to the Galaxy/University of Minnesota server at Minnesota Supercomputing Institute for data quality assessment, further file conversion and alignment with University of California–Santa Cruz, Homo sapiens reference genome (build hg19), using a TopHat algorithm (26, 27). The mapping results were then used to identify transcript and exon expression.

Tophat BAM files were exported to GeneData Expressionist Genome Refiner 7.6 (GeneData AG) (http://www.genedata.com) for transcript Fragments per Kilobase of transcript Model per Million reads (FPKM) quantification transcript and splice junction assembly. FPKM values were imported into the GeneData Analyst module for quality control/quality assurance analyses and statistical significant differences in gene expression were tested by t test (GeneData Expressionist v7.6, Refiner Genome Reference Manual).

**Statistical analyses**

After the codes for patient’s grouping were broken, the gene-data expressionist Analyst module was used for statistical testing by t test (P ≤ .05) for differential gene expression and data plotting (25). The selected gene expression level data were then used for pathway analysis.

**Pathway analysis**

Previously described methodologies were applied to determine whether differentially expressed genes were overrepresented in a given pathway as defined by the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (28) (http://www.genome.jp/kegg/pathway.html). KEGG’s gene set enrichment analysis annotations were used in pathway analyses. For each pathway, a 2 × 2 contingency table was constructed with columns labeled “significant genes” (K) and “nonsignificant genes” (N-K) and rows labeled “present in pathway” (M) and “not present in pathway” (N-M) for a total of N genes. We then determined whether the significance of gene expression differences were independent of being present in a given pathway by using a Fisher’s exact test based on hypergeometric distribution. Also, as we previously described (15, 23) and discussed in some detail (23), we performed a 1000-fold permutation test that randomly redistributes genes across pathways to generate a null distribution of significant gene counts within each pathway. Both approaches to determining statistical significance gave nearly identical results (23). Enrichment analyses were conducted using the Exploratory Visual Analysis software (http://discovery.dartmouth.edu/~pandrews/EVA.html) (15, 23, 29, 30). Given their more informational nature, results presented are those emanating from directional pathway analysis. Directional pathway analysis, performed as we reported (23), tested directionally consistent group differences on expression levels within the KEGG biological pathways. This system also used the 1000-fold permutation test on each pathway to evaluate whether the proportion of statistically significantly increased/decreased genes in a pathway is higher than expected by chance. Thus, the P value for directional pathway analyses represents the likelihood of obtaining the observed number of genes with increased (or decreased) expression levels in a given biological pathway other than by chance.

A pathway was considered to have a statistically significant enrichment of differentially expressed genes at a type I error rate of α = 0.001. This level of significance was selected to maximize our power to identify pathways that are associated with in vivo DN risk. To help guard against type I error, we determined, as part of our interpretation, whether prior evidence exists from human, in vitro, or animal studies for the pathways’ relationship to diabetes and/or its complications. Moreover, we also considered whether these pathways were functionally interrelated. All but one of the up-regulated pathways in the comparison of DN Controls vs the proteinuric subjects overlapped with the up-regulated pathways in the combined analyses of the proteinuric and ESRD DN subgroups; all but one of the up-regulated pathways in the DN Controls vs the ESRD subjects overlapped with pathways up-regulated in the combined analysis; and only one of these pathways differed in direct comparisons of the proteinuric and ESRD DN groups. Also, six of the eight down-regulated pathways in the DN Controls vs the ESRD group and six of the six down-regulated pathways in DN Controls vs the proteinuric group overlapped with the down-regulated pathways in the DN Control vs the combined DN Cases cohort. For these reasons, only the analyses of the combined proteinuric and ESRD DN Cases are presented. Two pathways, TGF-ß signaling and adherens junction pathways, both down-regulated in DN Controls vs the combined DN Cases group, were also different between the two DN subgroups, and these are presented in Results.

Finally, pathways that achieved this level of significance (α = 0.001 because of differential expression of genes that overlapped with genes in a major pathway are not presented. These included Alzheimer’s, Parkinson’s, and Huntington’s disease pathways, which achieved statistical significance because of genes shared with the oxidative phosphorylation pathway; mismatch repair and homologous recombination pathways, which overlapped with the DNA replication pathway; and oocyte meiosis and progesterone-mediated oocyte maturation pathways, which overlapped with the cell cycle pathway.

**Results**

**DN Cases and DN Controls**

DN Cases were, on average, approximately 8 years older than DN Controls (55.5 ± 6.8 vs 47.9 ± 10.5 y, respectively), and had 9 years’ longer T1D duration (43.4 ± 7.6 vs 34.5 ± 6.7 y, respectively; Table 1). However, duration of T1D from diagnosis to time of recruitment for the current study in the DN Controls was not different (P = .67) from duration of T1D from diabetes onset to kidney transplant in the DN Cases (35.2 ± 10.1 y), indicating that the time after ESRD largely accounted for the group difference in T1D duration. Glycemic control at skin biopsy was similar between these groups (Table 1). Systolic, but not diastolic, BP was higher in DN Cases vs DN Controls. All GoKinD Controls were confirmed to
have remained normoalbuminuric and on no renin angiotensin system blockers.

### Nondiabetic MZT

Nondiabetic MZT were 40.5 ± 12.2 years old and had normal glycosylated hemoglobin (HbA1c) (5.2 ± 0.4%).

### DN Cases vs DN Controls

Eight pathways had a statistically significant greater number of genes with increased expression in DN Controls vs DN Cases (Table 2). These included spliceosome, cell cycle, DNA replication, proteasome, RNA degradation, base excision repair, ribosome, and terpenoid backbone biosynthesis pathways. All but one of these pathways has primary functions directly related to cell replication and repair. However, within the only apparent exception, the terpenoid backbone biosynthesis pathway, the up-regulated genes in DN Controls vs DN Cases were predominantly in the mevalonate pathway, which is involved in cell membrane maintenance (data not shown) (31). Table 2 also denotes that five of these eight pathways (spliceosome, cell cycle, DNA replication, proteasome, and base-excision repair) overlapped with pathways up-regulated by HG in T1D MZT but not in their non-T1D MZT (22). Three pathways, related to vascular tone regulation and extracellular matrix dynamics (vascular smooth muscle contraction, extra cellular matrix receptor interaction, and TGF-ß signaling) were upregulated in DN Cases vs DN Controls (Table 3).

### DN Controls vs nondiabetic MZT

The six pathways that were up-regulated in HG in DN Controls compared with nondiabetic members of MZT discordant for T1D (cell cycle, DNA replication, homologous recombination, ubiquitin-mediated proteolysis, mismatch repair, and RNA degradation) are shown in Table 4. Three of these six pathways (cell cycle, DNA replication, and RNA degradation) overlapped with pathways up-regulated in DN Controls vs DN Cases (Table 2).

### DN Cases vs nondiabetic MZT

There was a complete overlap between pathways up-regulated in DN Cases vs non-T1D MZT (ubiquitin-mediated proteolysis, mismatch repair, homologous recombination, cell cycle, DNA replication, and RNA degradation; Table 5) with pathways up-regulated in DN Controls vs non-T1D twins (Tables 4). However, these differences were statistically much more robust in DN Controls vs non-T1D twins (Table 4) than in DN Cases vs non-T1D twins (Table 5). Furthermore, the pathways up-regulated in the DN Controls compared with the DN Cases (proteasome, DNA replication, cell cycle, base-excision repair, spliceosome, and nucleotide-excision repair) overlapped extensively with the pathways up-regulated by...

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### Table 1. Clinical Characteristics of T1D Subjects with (DN Cases) and without (DN Controls) Diabetic Nephropathy

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>DN Cases</th>
<th>DN Controls</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>60</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Sex, male/female</td>
<td>32/28</td>
<td>14/26</td>
<td>NS</td>
</tr>
<tr>
<td>Age, y</td>
<td>55.5 ± 6.8</td>
<td>47.9 ± 10.5</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Age at T1D onset, y</td>
<td>12.1 ± 6.6</td>
<td>13.5 ± 8.1</td>
<td>NS</td>
</tr>
<tr>
<td>T1D duration, y</td>
<td>43.4 ± 7.6</td>
<td>34.5 ± 6.7</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>HbA1c, %</td>
<td>7.5 ± 1.1</td>
<td>7.3 ± 0.9</td>
<td>NS</td>
</tr>
<tr>
<td>SBP, mm Hg</td>
<td>130.9 ± 19.1</td>
<td>120.7 ± 13.9</td>
<td>.003</td>
</tr>
<tr>
<td>DBP, mm Hg</td>
<td>70.4 ± 9.0</td>
<td>68.6 ± 6.9</td>
<td>NS</td>
</tr>
</tbody>
</table>

Abbreviations: DBP: diastolic blood pressure; NS, not significant; SBP: systolic blood pressure.

Data are the means ± sd or No. of subjects.

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### Table 2. KEGG Pathways Up-Regulated in High Glucose in DN Controls Versus DN Cases

<table>
<thead>
<tr>
<th>KEGG Pathway</th>
<th>Up-regulated/Expressed Genes</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spliceosome</td>
<td>59/110</td>
<td>8.485 e−17</td>
</tr>
<tr>
<td>Cell cycle</td>
<td>57/111</td>
<td>3.734 e−15</td>
</tr>
<tr>
<td>DNA replication</td>
<td>27/34</td>
<td>1.851 e−14</td>
</tr>
<tr>
<td>Proteasome</td>
<td>23/40</td>
<td>8.943 e−10</td>
</tr>
<tr>
<td>RNA degradation</td>
<td>24/56</td>
<td>2.063 e−5</td>
</tr>
<tr>
<td>Base-excision repair</td>
<td>16/32</td>
<td>5.139 e−5</td>
</tr>
<tr>
<td>Ribosome</td>
<td>31/86</td>
<td>8.360 e−5</td>
</tr>
<tr>
<td>Terpenoid backbone biosynthesis</td>
<td>9/13</td>
<td>8.567 e−5</td>
</tr>
</tbody>
</table>

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### Table 3. KEGG Pathways Up-Regulated in HG in DN Cases Versus DN Controls

<table>
<thead>
<tr>
<th>KEGG Pathway</th>
<th>Up-regulated/Expressed Genes</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vascular smooth muscle contraction</td>
<td>20/59</td>
<td>1.53 e−4</td>
</tr>
<tr>
<td>ECM receptor interaction</td>
<td>18/69</td>
<td>6.26 e−4</td>
</tr>
<tr>
<td>TGF-ß signaling</td>
<td>18/73</td>
<td>.001</td>
</tr>
</tbody>
</table>

Abbreviation: ECM, extra cellular matrix.

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### Table 4. KEGG Pathways Up-Regulated in HG in T1D DN Controls Versus Non-diabetic MZTs

<table>
<thead>
<tr>
<th>KEGG Pathway</th>
<th>Up-regulated/Expressed Genes</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell cycle</td>
<td>56/111</td>
<td>1.785 e−9</td>
</tr>
<tr>
<td>DNA replication</td>
<td>21/34</td>
<td>3.537 e−6</td>
</tr>
<tr>
<td>Homologous recombination</td>
<td>17/25</td>
<td>4.582 e−6</td>
</tr>
<tr>
<td>Ubiquitin-mediated proteolysis</td>
<td>54/128</td>
<td>5.292 e−6</td>
</tr>
<tr>
<td>Mismatch repair</td>
<td>15/22</td>
<td>1.692 e−5</td>
</tr>
<tr>
<td>RNA degradation</td>
<td>25/55</td>
<td>4.619 e−4</td>
</tr>
</tbody>
</table>
null
The greatest proportions of up-regulated genes in the DN Controls are all functionally related.

Nondiabetic members of the MZT pairs discordant for T1D were considered the most appropriate nondiabetic controls given that they share the genetic propensity to T1D. Interestingly, compared with these nondiabetic MZT, the T1D DN Controls had up-regulation of pathways (cell cycle, DNA replication, DNA degradation and base excision repair) that were similar to those up-regulated in these DN Controls vs the DN Cases. Similarly, DN Cases had statistical trends toward up-regulation of the same pathways compared with the nondiabetic MZT. These observations are consistent with the hypothesis that, exposed to HG, SF of individuals with longstanding T1D have in vitro up-regulation of similar gene expression pathways associated with healing responses to cell injury, and that more robust up-regulation of these pathways is associated with protection from DN. Oxidative stress activates DNA damage and repair mechanisms, and it is conceivable that the damage caused by oxidative stress and the consequent activation of pathways of injury (TGF-β, etc.), could be reversed (repaired) in these protected individuals, what could, in turn, affect the rate of development of kidney lesions. Given that the SF were studied after being in culture from acquisition through approximately 6 weeks and multiple passages, it is reasonable to postulate that these findings reflect prolonged differential epigenetic influences of the in vivo diabetic state and/or genetic differences in SF behaviors.

The directional pathway gene expression changes in SF between NG and HG glucose conditions differed markedly between T1D members of MZT pairs discordant for T1D and their nondiabetic MZT (22). Thus, many more genes changed their expression levels in HG vs NG media in the T1D twins than in their genetically identical nondiabetic twins. Moreover, most of the changes from NG to HG in the T1D twins represented increases in gene expression vs their nondiabetic MZT, whereas the genes altered from NG to HG in the non-T1D MZT were mostly down-regulated. These systematic differences between genetically identical T1D and non-T1D twins are necessarily the result of epigenetic cellular alterations that persisted despite prolonged and identical in vitro conditions. There was, in fact, as shown here, remarkable overlap in the pathways up-regulated by HG in the T1D twins and the pathways with increased gene expression in HG in the DN Controls vs the DN Cases. This leads us to posit that prolonged in vivo exposure to hyperglycemia induces epigenetic cellular changes representing efforts to repair the injurious influences of the diabetic state. We also suggest that failure to mount a strong epigenetically mediated healing response may increase the risk of DN.

In summary, the up-regulated pathways in DN Controls vs DN Cases markedly overlap with the pathways up-regulated by HG in T1D MZT, but not in non-T1D twins. These systematic differences between MZT discordant for T1D are necessarily epigenetic. SF of DN Controls in HG also have up-regulation of many of these pathways compared with SF of nondiabetic MZT in HG. DN Cases also showed up-regulation of these pathways, but this was much less so than observed in DN Controls. Together, these data are consistent with the hypothesis that SF from patients with T1D have undergone epigenetic modifications resulting in increased expression of healing and repair pathways, perhaps in response to oxidative stress and other injury processes. When compared with nondiabetic individuals with genetic propensity to T1D, these presumed epigenetic responses seem to be much more robust in the T1D patients who are protected from DN. These studies are consistent with the possibility that epigenetic factors may be important determinants of DN risk. Although it is certainly important for validation studies to be conducted in the kidney, culturing kidney-derived cells in different conditions for prolonged periods of time has been a major challenge. It is possible, however, to study kidney biopsy tissues obtained from research volunteers with T1D and similar clinical characteristics, and this will likely be informative.

Acknowledgments

We thank Cathy Bagne for clinical coordinator efforts, Paul Walker and Jhuma Saha for technical work, and Patricia Erickson and Tanya Double for assistance in manuscript preparation. We also thank Dr Rama Natarajan for reviewing this manuscript and for her valuable comments. This work was carried out, in part, using computing resources at the University of Minnesota Supercomputing Institute.

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This work was supported by a research grant from Juvenile Diabetes Research Foundation (JDRF) (JDRF-42–2009-755; Dr code 101, Minneapolis, MN 55455. E-mail: caram001@umn.edu
Caramori) and by funds from the Pennock Professorship (M.L.C.). M.L.C. was a recipient of a JDRF Career Development Award.

Disclosure Summary: The authors have nothing to disclose.

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