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Abbreviations: CTGF, connective tissue growth factor; DN, diabetic nephropathy; ECM, extracellular matrix; EMT, epithelial-to-mesenchymal transition; GO, gene ontology; HMC, human mesangial cell; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; PDGF, platelet-derived growth factor; PTC, proximal tubular epithelial cell; TFBS, transcription factor binding site; TGFβ1, transforming growth factor beta1

We have previously identified differentially expressed genes in cell models of diabetic nephropathy and renal biopsies. Here we have performed quantitative DNA methylation profiling in cell models of diabetic nephropathy. Over 3,000 CpG units in the promoter regions of 192 candidate genes were assessed in unstimulated human mesangial cells (HMCs) and proximal tubular epithelial cells (PTCs) compared to HMCs or PTCs exposed to appropriate stimuli. A total of 301 CpG units across 38 genes (~20%) were identified as differentially methylated in unstimulated HMCs versus PTCs. Analysis of amplicon methylation values in unstimulated versus stimulated cell models failed to demonstrate a >20% difference between amplicons. In conclusion, our results demonstrate that specific DNA methylation signatures are present in HMCs and PTCs, and standard protocols for exposure of renal cells to stimuli that alter gene expression may be insufficient to replicate possible alterations in DNA methylation profiles in diabetic nephropathy.

Introduction

Diabetic nephropathy (DN) is a life-threatening microvascular complication affecting approximately one third of persons with diabetes.[1] It is characterized by persistent proteinuria, hypertension, a progressive decline in glomerular filtration rate, retinopathy and accelerated mortality mainly due to cardiovascular disease.[2] Diabetic nephropathy is the leading cause of end-stage renal disease in the USA and Europe.[3,4]

Prolonged hyperglycemia in genetically predisposed individuals leads to chronic metabolic and hemodynamic changes that modify the activity of various intracellular signaling pathways and transcription factors.[5,6] The induction of cytokines, chemokines and growth factors, particularly transforming growth factor beta 1 (TGFβ1) and connective tissue growth factor (CTGF), leads to glomerular basement membrane thickening, podocyte injury and mesangial matrix expansion, with the later development of irreversible glomerular sclerosis and tubulointerstitial fibrosis. The metabolic consequences of prolonged hyperglycemia include inflammatory responses, oxidative stress, dyslipidemia and the formation of advanced glycation end products. Epithelial-to-mesenchymal transition (EMT) plays a key role in the pathogenesis of tubulointerstitial fibrosis in DN.[7,8]

There is increasing evidence from various studies to suggest that epigenetic factors may be implicated in DN.[9-15] We have demonstrated differential expression in several plausible biological candidate genes for DN in various cell models of disease and also renal biopsies from individuals with DN, and have demonstrated upregulation of several genes involved in EMT.[16-20] The objectives of the present study were to perform quantitative DNA methylation profiling in 5' promoter regions of candidate genes (n = 192) previously identified by us to be differentially expressed in cells models of DN or renal biopsies from diabetic individuals with nephropathy to compare DNA methylation signatures in unstimulated human mesangial cells (HMCs) with proximal tubular epithelial cells (PTCs) and assess patterns of DNA methylation in HMCs and PTCs before and after exposure to various stimuli (high extracellular glucose, TGFβ1, CTGF, a combination of TGFβ1 and CTGF, or platelet-derived growth factor (PDGF)). DNA methylation profiling was performed using Sequenom MassCLEAVE technology in combination with matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass

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spectrometry analysis as previously described in detail by us elsewhere.[21,22] The identification of DNA methylation marks in cell models of DN, which if replicated in diabetic individuals with nephropathy, could be useful biomarkers and localize gene targets for novel therapies.

Results

A total of 5,143 CpG sites were assessed in 192 genes. We previously investigated DNA methylation levels at the promoter regions of these genes in a total of 892 amplicons.[22] From this comprehensive dataset, a subset of these amplicons of most informative content were selected for analysis in the present study. Inclusion criteria for selection included close proximity to transcription start site, evidence of DNA methylation and high CpG content. Exclusion criteria for amplicon selection included presence of PCR amplification bias, low CpG content, or absence of DNA methylation. Here, we assessed one amplicon per gene (192 amplicons). The mean amplicon length was 352 bp (min = 172 bp; max = 583 bp), and an average of 27 CpG sites were assessed per amplicon. DNA methylation at the 5,143 CpG sites was assessed in 3,141 CpG units, in which a CpG unit may correspond to either a single CpG site or multiple adjacent CpG sites.[21,22] Following standard quality checks, CpG units where DNA methylation values were not available, as well as one amplicon derived from the 5' promoter region of the *RBM3* gene were excluded from further analysis. Prior to commencing the present study, potential amplification biases were assessed for each of the amplicons by determining DNA methylation levels in a healthy donor sample, a fully methylated control sample and a 50:50 sample consisting of equal quantities of DNA from the healthy donor and fully methylated samples (data not shown). No significant variation was observed in DNA methylation levels for CpG units between replicate samples, so values were combined to provide a single mean methylation value for each CpG unit. Methylation values for all CpG units in all samples are listed in Supplemental Material Table 1.

DNA Methylation Signatures in HMCs and PTCs

A mean methylation value for each amplicon was obtained from the methylation values of CpG units within an amplicon. Unsurprisingly, the majority of amplicons assessed revealed low levels of DNA methylation (<20%) in both unstimulated HMCs (n = 165; 86%) and PTCs (n = 150; 78%) (Suppl. Fig. 1). High levels of DNA methylation (≥80%) were observed in four 5' promoter regions in HMCs, compared with 16 in PTCs (Suppl. Table 2). Pairwise differences for DNA methylation levels for each CpG unit were then calculated and a total of 335 (~10%) CpG units revealed DNA methylation differences of ≥20% (Suppl. Table 3). Based on overall process variability of bisulfite PCR methods,[23,24] a quantitative value of 20% methylation difference was chosen to represent a greater than two standard deviation difference. In addition, to minimize false positives the data was filtered to include only those genes for which at least two CpG units were differentially methylated, resulting in 301 CpG units across 38 genes (Table 1). We subsequently performed an unsupervised hierarchical clustering analysis based on all CpG units, which revealed a strong association of DNA methylation values in the dendrogram according to HMC and PTC cell type (Fig. 1).

Quantitative real-time PCR was performed to determine whether differences in DNA methylation between unstimulated HMCs and PTCs corresponded with appropriate changes in gene expression. *THBS1* gene expression was assessed in three separate experiments using unstimulated HMCs and PTCs. *THBS1* gene expression was significantly higher in HMCs versus PTCs (~30-fold; $p < 0.005$). Consistent with the effects of DNA methylation on gene expression regulation, elevated methylation in PTCs correlated with decreased expression of *THBS1* (Fig. 2). Similarly, hypomethylation in HMCs correlated with increased *THBS1* expression (Fig. 2).

Gene ontology (GO) analysis was performed on the 38 genes demonstrating differential methylation in HMCs vs. PTCs to determine if specific gene functions were overrepresented. The predominant biological functions were associated with organism development (Suppl. Table 4). Since DNA methylation can affect the binding of transcription factors to their respective transcription factor binding sites (TFBSs), we examined TFBSs in the 38 genes to determine if certain TFBSs were commonly found across genes. For the 11 genes demonstrating higher levels of methylation in HMCs vs. PTCs, 30 TFBSs were significantly overrepresented following Bonferroni correction ($p \leq 0.05$). For the 27 genes demonstrating higher levels of methylation in PTCs vs. HMCs, 24 TFBSs were significantly overrepresented. Comparison of these two groups of enriched TFBSs indicated differing sequence motif profiles in 5' promoters of genes differentially methylated in HMCs and PTCs (Suppl. Table 5).

DNA Methylation Profiles in HMCs and PTCs Exposed to Stimuli

DNA methylation profiling was performed in the following in vitro models of DN: (1) HMCs stimulated with ambient (5 mM) versus elevated (30 mM) extracellular glucose for 24 h, (2) unstimulated HMCs versus HMCs stimulated with TGFβ1, CTGF, TGFβ1 and CTGF, or PDGF for 24 h and (3) unstimulated PTCs versus PTCs stimulated with TGFβ1 for 24 h and 48 h.

Analysis of amplicon methylation values in unstimulated versus stimulated cell models failed to demonstrate a >20% difference in methylation levels for any of the amplicons assessed (Suppl. Table 6). Furthermore, the distribution of methylation values for CpG units were similar for HMC and PTC cell models, regardless of stimulus (Suppl. Fig. 2). Hierarchical clustering analysis was performed on all cell models based on methylation values for individual CpG units (Suppl. Fig. 3). All models clustered according to cell type, however no obvious clustering was observed in cell models with respect to stimulus.

Discussion

Epigenetic modification of gene function (mediated in part by environmental factors) may account for a significant

proportion of phenotypic variance in DN. Recently, a study utilizing skin fibroblasts derived from monozygotic twins discordant for type 1 diabetes that were cultured in media containing high levels of glucose, demonstrated differential gene expression between twin pairs in a number of genes involved in epigenetic regulation (Mauer M, University of Minnesota, Minneapolis, MN, USA) personal communication). It has been suggested that cellular memory to hyperglycemia, proposed to result from epigenetic modifications, may account for these findings. Since individuals who develop DN tend to have poor glycemic control, cellular memory to hyperglycemia and associated DNA methylation may, in part, contribute to DN.

In the present study we quantitatively compared DNA methylation levels in the 5' promoter regions of 192 genes in unstimulated HMCs and PTCs, and identified 38 genes (~20%) to be differentially methylated in HMCs versus PTCs. In order to investigate whether these differences in DNA methylation correlate with gene expression we assessed expression levels of thrombospondin (*THBS1*), a constituent of the extracellular matrix involved in cell-to-matrix interactions. Upregulation of *THBS1* is recognized as an important marker of DN[25,26] and was therefore considered an ideal candidate to measure gene expression in unstimulated HMCs and PTCs. *THBS1* differential methylation between HMCs and PTCs (~42% greater methylation in PTCs) correlated with a 30-fold increase in *THBS1* gene expression in HMCs versus PTCs. HMCs are major contributors to the extracellular matrix which contains fibronectin, collagens, laminin and also thrombospondin 1, whereas the primary functions of PTCs include fluid reabsorption and pH regulation. This may explain the relatively large upregulation in expression of *THBS1* in HMCs versus PTCs.

GO analysis of these 38 genes displaying cell-specific methylation profiles revealed that the predominant biological functions were associated with organism development. Analysis of the 5' promoter regions of these genes revealed differing profiles of binding motifs for transcription factors. Hypermethylation at or near these binding sites may prevent the binding of the transcriptional machinery and alter expression of target genes. Of note, the *PDZK1IP1* gene promoter region assessed was hypermethylated in HMCs compared to PTCs (76% vs. 4%) (Fig. 3). The *PDZK1IP1* gene encodes the membrane associated protein PDZK1 interacting protein 1, which is upregulated in carcinoma, and is expressed at high levels in PTCs in the kidney.[27]

Previous studies have demonstrated the utility of using in vitro models of DN in assessing the role of histone acetylation[28] and microRNAs in DN.[29,30] Here, we compared the levels of DNA methylation in HMCs and PTCs before and after exposure with an appropriate stimulus (to reflect the effects of a diabetic milieu) to identify DNA methylation marks; however, no significant differences were observed following stimulation in these in vitro models of DN. It is difficult to explain this outcome, yet a likely explanation is that the length of time that cells were exposed to a given stimulus was inappropriate for inducing alterations in DNA methylation profiles and perhaps should have been considerably longer. This is consistent with the fact that DN is a relatively late-onset disease and develops after prolonged exposure to

Table 1. Genes demonstrating differential methylation in human mesangial cells (HMCs) compared to proximal tubular epithelial cells (PTCs)

Gene	Chromosome	No. of differentially methylated CpG units	Average difference in methylation*
Greater methylation in HMCs vs. PTCs			
AGT	12	3	40%
CAPZA1	1	2	30%
FXYD2	11	4	66%
KIAA0182	16	10	28%
KLK1	19	3	30%
LY6E	8	2	36%
PAX6	11	2	25%
PDZK1IP1	1	5	72%
PRODH2	19	4	36%
RAB11FIP3	16	2	39%
SERPINA1	7	6	36%
Greater methylation in PTCs vs. HMCs			
BDKRB1	14	4	36%
C1S	12	3	40%
CASKIN1	16	9	56%
CD9	12	20	48%
CYP1B1	2	24	71%
DAO	12	5	37%
DPYSL2	8	7	41%
EGFR	7	13	62%
FGFR2	10	15	41%
FOLH1	11	8	30%
GPR56	16	4	44%
GSTM5	1	11	51%
IFI16	1	2	32%
MACF1	1	4	42%
NEUROG1	5	17	56%
NID1	1	7	46%
NPHS2	1	13	34%
PPP1R16B	20	9	40%
PROM1	4	9	85%
SCUBE3	6	19	53%
TAGLN	11	8	69%
TCEA2	20	5	34%
TETRAN	4	7	66%
THBS1	15	5	42%
THY1	11	13	94%
TNFRSF11B	13	11	92%
XPNPEP2	X	6	61%

*An average value was calculated for differentially methylated (i.e., >20% difference) CpG units in the same gene.

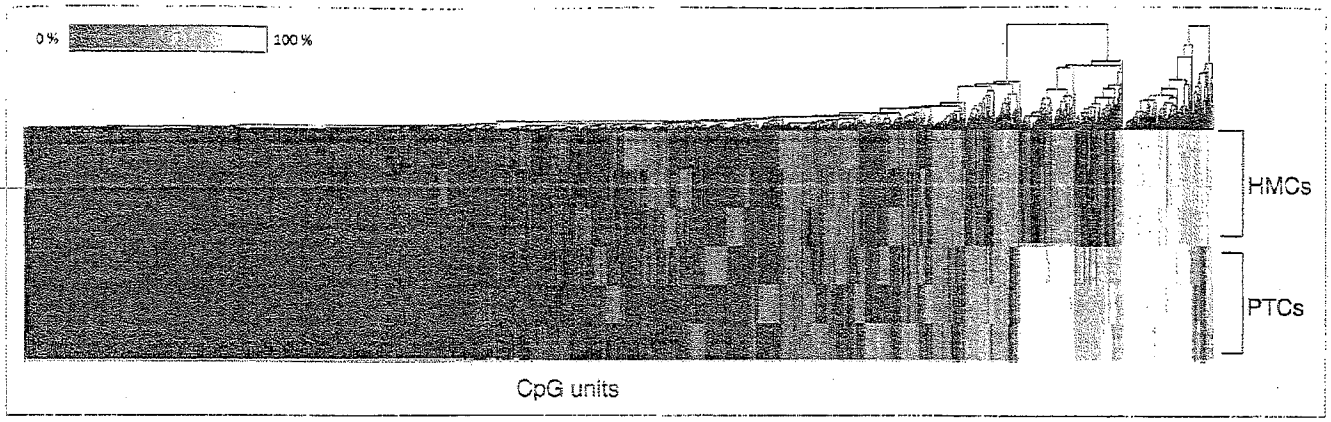

Figure 1. Hierarchical cluster analysis of human mesangial cells (HMCs) and proximal tubular epithelial cells (PTCs) (rows) and DNA methylation values of CpG units in candidate genes. DNA methylation values are represented on a continuous scale from non-methylated (red) to fully methylated (yellow).

hyperglycemia over several years. The exposure times employed here were standard, and we validated each cell model prior to use in this study by confirming the presence of protein markers for EMT and renal fibrosis. Interestingly, others have found similar inconsistencies between short term stimulus exposure and DNA methylation changes.[31]

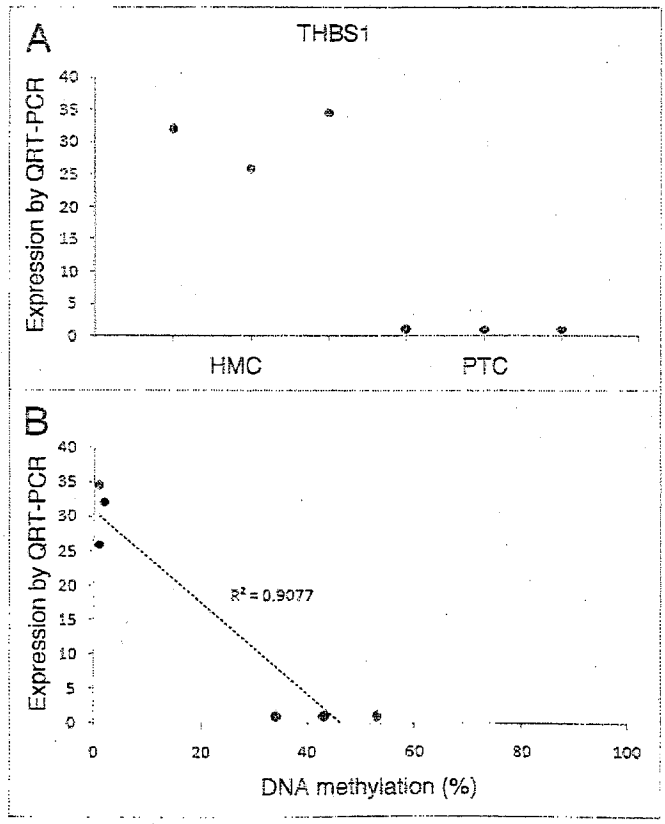

Figure 2. Differential DNA methylation between human mesangial cells (HMCs) and proximal tubular epithelial cells (PTCs) correlates with differential gene expression. (A) Gene expression for *THBS1* assessed by quantitative PCR in three unstimulated HMC and PTC samples. (B) Correlation between gene expression and DNA methylation in differentially methylated CpG units of *THBS1* using Spearman's rank correlation coefficient. HMC and PTC samples are color-coded red and blue, respectively.

An alternative approach would be to assess DNA methylation in mouse models of DN monitored over several months. Several studies using mouse models have shown that long-term stimulus exposure leads to global changes in DNA methylation and that these changes vary with duration of exposure.[32,33] It is highly unlikely that DNA methylation differences in cell models were not observed due to technical difficulties associated with quantitative DNA methylation profiling, as the Sequenom MassCLEAVE assay in combination with mass spectrometry used here is extremely accurate and robust,[21] and appropriate quality control checks were employed in this study. Finally, it is possible that DNA methylation in the gene promoters assessed may not be crucial in inducing or maintaining changes in gene expression; alternatively, other untested sites of DNA methylation (such as in intronic CpG islands) in certain genes might explain some of the heritability in DN.

In conclusion, the results of our study demonstrate that specific DNA methylation profiles are present in HMCs and PTCs that likely reflect the role of DNA methylation in renal cell differentiation, and relatively short-term in vitro exposure of renal cells to stimuli such as TGFβ1 that are sufficient to lead to alterations in gene expression may be insufficient to replicate possible alterations in DNA methylation profiles that may contribute to development or progression of DN. Future studies involving cell models of DN should consider the use of extended exposure times to stimuli and consideration should be given to extending screening of CpG sites for differential methylation beyond promoter regions in candidate genes.

Methods

Cell culture and DNA extraction. Primary HMCs and PTCs were obtained from Clonetics™ and the American Tissue Type Culture Collection, respectively. Following overnight growth in serum-free medium, HMCs and PTCs were treated with

various stimuli and validated by Western blotting as detailed in Supplementary Methods. DNA extraction from cells was performed using a Qiagen DNeasy kit according to the manufacturer's instructions (Qiagen, Crawley, UK).

Candidate genes and MassCLEAVE assay. We have previously performed DNA methylation profiling studies in 318 genes in peripheral blood leukocytes and lymphoblastoid cell lines using the Sequenom MassCLEAVE assay.[22] Of the 318 genes, a total of 210 genes had previously been demonstrated by us to be differentially expressed in various cell models of DN. Of these, 192 genes were included in the present study and 18 genes were excluded on the basis of PCR failure, low CpG content or absence of CpG islands. In our previous study,[22] a total of 892 amplicons were designed and assessed for these 192 genes. Amplicons were designed to cover CpG islands in the 5' promoter region, or where no CpG island was defined according to the UCSC genome browser criteria (http://genome.ucsc.edu/), we typically selected genomic sequence in the 5' gene region. One amplicon per gene (192 amplicons) was selected for assessment in this present study. Primer sequences for all amplicons are listed in Supplemental Material Table 7. Genomic DNA (1 μg) derived from the various cell models was bisulfite-treated followed by in vitro transcription and quantitative detection of DNA methylation levels using MALDI-TOF mass spectroscopy as previously described.[21,22] Control samples included a fully methylated DNA sample (CpGenome™, Millipore, CA, USA), and a DNA sample from a healthy donor.

RNA extraction, cDNA synthesis and quantitative PCR. RNA extraction was performed using an RNeasy RNA extraction kit according to the manufacturer's protocol (Qiagen, Crawley, UK). cDNA was synthesised from 1 μg of total RNA, using the Superscript-II RNase H-Reverse transcriptase cDNA synthesis kit (Invitrogen, Paisley, UK). Quantitative real-time PCR for *THBS1* and the house keeping gene *18S* was performed in a 10 μl reaction using 1:20 diluted cDNA and Taqman Gene Expression Assays for *THBS1* and *18S* according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA).

Data analysis. Quantitative real-time PCR analysis was performed using the Student's t-test and two-tailed p-values were used to determine statistical significance ($p < 0.05$). Analysis for the association between DNA methylation and gene expression was performed using Spearman's rank order correlation coefficient. Gene ontology analyses for differentially methylated genes were performed using GOstat (http://gostat.wehi.edu.au/), which uses a Fisher's exact test (p-value cut-off: 0.01) to identify overrepresented GO terms in the differentially methylated subset versus all genes in the GO gene-associations database. Transcription factor binding sites were identified using Pscan software (http://159.149.109.9/pscan/). Motifs within 1,000 bp upstream of the transcription start site for each promoter were analyzed. The TRANSFAC motif profile database was selected for analysis. A Bonferroni corrected p-value was used to determine significantly overrepresented motifs (z-test p-value multiplied by the number of motif profiles used in the analysis). Hierarchical clustering was performed using Hierarchical Clustering Explorer 2.0 (http://www.cs.umd.edu/hcil/hce/).

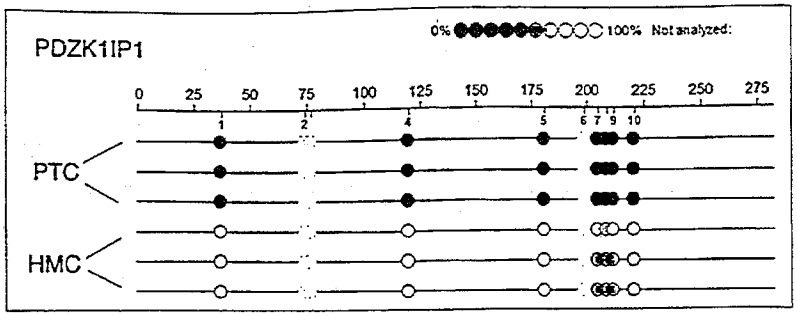

Figure 3. Representative epigram of DNA methylation in the *PDZK1IP1* CpG island in proximal tubular epithelial cells (PTCs) and human mesangial cells (HMCs). Each circle represents a single CpG site. Methylation levels are graded on a continuous scale ranging from 0% (red) to 100% (yellow).

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Conflict of interest

M. Ehrich and D. van den Boom are shareholders and full-time employees of Sequenom, Inc.

Note

Supplementary materials can be found at: www.landesbioscience.com/supplement/BrennanEPI5-5-Sup.pdf

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