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Co-regulation of Gremlin and Notch signalling in diabetic nephropathy

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Abstract

Diabetic nephropathy is currently the leading cause of end stage renal disease worldwide, and occurs in approximately one third of all diabetic patients. The molecular pathogenesis of diabetic nephropathy has not been fully characterized and novel mediators and drivers of disease are still being described. Previous data from our laboratory has identified the developmentally regulated gene Gremlin as a novel target implicated in diabetic nephropathy in vitro and in vivo. We used bioinformatic analysis to examine whether Gremlin gene sequence and structure could be used to identify other genes implicated in diabetic nephropathy. The Notch ligand Jagged1 and its downstream effector, hairy enhancer of split-1 (Hes1), were identified as genes with significant similarity to Gremlin in terms of promoter structure and predicted micro RNA binding elements. This led us to discover that transforming growth factor-beta (TGFβ1), a primary driver of cellular changes in the kidney during nephropathy, increased Gremlin, Jagged1 and Hes1 expression in human kidney epithelial cells. Elevated levels of Gremlin, Jagged1 and Hes1 were also detected in extracts from renal biopsies from diabetic nephropathy patients, but not in control living donors. In situ hybridization identified specific upregulation and co-expression of Gremlin, Jagged1 and Hes1 in the same tubuli of kidneys from diabetic nephropathy patients, but not controls. Finally, Notch pathway gene clustering showed that samples from diabetic nephropathy patients grouped together, distinct from both control living donors and patients with minimal change disease. Together, these data suggest that Notch pathway gene expression is elevated in diabetic nephropathy, co-incident with Gremlin, and may contribute to the pathogenesis of this disease.
Introduction

Diabetic Nephropathy (DN) is the primary cause of end-stage renal disease worldwide [1]. Both type 1 and type 2 diabetics develop nephropathy. It occurs in 25-40% of such patients [1]. The initial clinical indicator of DN is microalbuminuria, which reflects functional and potentially reversible abnormalities initiated by glomerular hyperfiltration. Microalbuminuria predicts the development of overt proteinuria in both type 1 and type 2 diabetes patients [2]. Both hyperglycemia and glomerular hypertension are major drivers of DN progression, with current therapies for DN including regulation of blood glucose, angiotensin II receptor antagonists and angiotensin-converting enzyme (ACE) inhibitors which slow, but do not halt the progression to end-stage renal disease once overt nephropathy is established [1]. An underlying genetic susceptibility to DN is suggested by the observation that a cohort of diabetic patients will develop DN despite rigorous hyperglycaemic control.

The pathogenesis of DN is characterized by progressive loss of renal function due to damage to the glomerulus (glomerulosclerosis), and scarring of the kidney tubules due to relentless accumulation and deposition of extracellular matrix (ECM) (tubulointerstitial fibrosis). Epithelial-mesenchymal transition (EMT) is a process in which renal tubular cells lose their epithelial phenotype and acquire characteristic features of mesenchyme, compromising the function of both proximal and distal tubules. There is some disagreement in the literature as to the pathophysiological significance of EMT in diabetic nephropathy in vivo. Those who support a role for EMT in renal fibrosis in vivo suggest that up to 35% of disease-related fibroblasts originate from tubular epithelial cells, with in situ fibroblast proliferation and infiltration of circulating fibroblasts also contributing to this process [3, 4]. EMT is therefore considered by some to be a phenotypic conversion fundamentally linked to
the pathogenesis of renal interstitial fibrosis [5]. Many extrinsic drivers of EMT in kidney have been identified, including transforming growth factor-β (TGFβ1), epidermal growth factor (EGF) and advanced glycation end-products (AGEs) [6], [7], [8]. TGFβ1 signals through complexes of heterotrimeric transmembrane type I and type II receptors, activating Smad-2 or -3 to form complexes with Smad4 and trigger transcriptional changes that drive EMT and fibrosis during diabetic nephropathy. Molecular interventions that act to reduce TGFβ1 activity have been shown to be of benefit in renal fibrosis in vivo [9-11]. Treatment of renal epithelial cells with TGFβ in culture is sufficient to induce EMT, as characterised by loss of E-cadherin expression and increased vimentin expression [12]. In contrast, proteins such as hepatocyte growth factor (HGF) and bone morphogenic factor-7 (BMP-7) have been shown to counteract EMT induction by TGF-beta in vitro and in vivo [13].

The majority of renal tubules in the adult kidney derive from the metanephric mesenchyme via a process called mesenchymal-epithelial transition (MET). Many genes that play a role in embryogenesis and development have been implicated in diabetic nephropathy. Prominent among these is Gremlin, a member of the noggin/chordin like family of bone morphogenic protein (BMP) antagonists [14]. In vitro, Gremlin expression is elevated in kidney mesangial cells treated with high glucose and TGFβ1 [15]. Mice lacking Gremlin die shortly after birth due to renal agenesis [16]. Gremlin expression is low in normal, adult kidney but is highly expressed in areas of tubular fibrosis in kidneys from diabetic nephropathy patients [17]. Increased Gremlin expression during nephropathy fits within the paradigm of aberrant developmental gene expression during disease progression.

We assessed the Gremlin proximal promoter for conserved regulatory elements and identified members of the Notch signalling pathway sharing common regulatory
elements with Gremlin. We also show that members of the Notch signalling pathway are co-upregulated with Gremlin in an *in vitro* model of TGF-β induced EMT. Coordinated increases in Gremlin and Notch family gene expression were also detected in kidney biopsies from diabetic nephropathy patients, with colocalisation of Gremlin, Jagged1 and Hes1 detected predominantly in the tubular compartments of kidneys from such patients. These data therefore shed new light on the complex alterations in kidney gene expression during diabetic nephropathy.
Material and Methods

Transcription Factor Binding Element Analysis

Experimentally confirmed 5’ capped transcription start sites for human and mouse Gremlin were determined from the Database of Transcription Start Sites (DBTSS) (http://dbtss.hgc.jp/) [18] and the 1kb proximal promoters for Gremlin from both species was retrieved from their database. The proximal promoter for rat Gremlin was retrieved from the UCSC Genome Browser (http://genome.ucsc.edu/) [19] using the Refseq transcription start site. Initial analysis of Gremlin promoter was performed using the sequence alignment programs ClustalW (http://www.ebi.ac.uk/clustalw/) [20] and Pipmaker (http://pipmaker.bx.psu.edu/pipmaker/) [21] to ascertain sequence conservation. In order to determine if the apparent promoter conservation was due to a confounding influence, such as extraneous open reading frames, the locus was analysed using the UCSC Genome Browser. Having established no confounding influence, the human, mouse and rat 1 kb proximal promoters were analysed for the presence of transcription factor binding site consensus sequences using MatInspector (http://www.genomatix.de) [22]. In order to reduce the number of false positives, results were filtered for transcription factor sites appearing in all three species. Clusters of significantly conserved transcription factor binding sites were chosen to search for promoters with comparable sets of binding sites. Matrices corresponding to the conserved transcription factor binding sites were retrieved from MatBase (http://www.genomatix.de). Then a library of approximately 12,000 Unigene accession 1 kb promoters was generated using HOMGL (http://homgl.biologie.hu-berlin.de/homgl/) [23]. In order to determine which promoters in this library contained clusters of these motifs, the promoter library was searched with ClusterBuster (http://zlab.bu.edu/cluster-buster/) [24]. The promoters which were
identified were further filtered by gene ontology, using the tool eGON (http://www.genetools.microarray.ntnu.no/egon/index.php) [25], to increase the probability of finding co-regulated genes. This yielded a list of 10 genes, sharing two gene ontology annotations with Gremlin and whose 1 kb proximal promoters contained clusters of transcription factor binding motifs in common with those conserved in the Gremlin promoter.

3’ UTR Conserved Motif Analysis

We used a set of 106 conserved 3’ UTR 8-mer motifs that were identified in a genome-wide search [26]. Many of these conserved sites match known miRNA binding sites. By parsing a table of the genes harbouring these putative miRNA binding sites (Supplementary Materials – [26]), we created a matrix of Refeq Ids and the conserved motifs. To determine if the Gremlin 3’ UTR contained a conserved motif and if any other genes shared such a motif, the matrix was searched for sites matching the Gremlin 3’ UTR and cross-referenced for other genes in whose 3’ UTR they appear. This process was carried out using R and perl scripts.

CpG Island Motif Analysis

There are two CpG islands in the Gremlin promoter. We decided to examine if any other genes contained similar promoter CpG islands containing similar motifs. Transcription factor binding motifs (1217 motifs) were obtained from TRANSFAC 6.2 Professional [27], Jaspar [28] and from the collection of Xie et al. [26]. Promoter sequences were taken from the 4-way conserved genome sequence (human, mouse, rat and dog) as generated by Xie et al. [26]. The tffind program from the Piptools package [29] was used to generate a frequency matrix of the hits for each of the 1,217
motifs in each of the 11,241 genes. The cpgplot program from the EMBOSS package (http://emboss.sourceforge.net/apps/cvs/cpgplot.html) was then used to detect CpG islands in the human promoter sequences. We scanned the conserved regions of the CpG islands in the Gremlin promoter and identified conserved motifs. We then identified similar conserved motifs in the CpG islands of other genes.

Cell Culture

Human kidney epithelial cells (HK-2) were purchased from the European Collection of Cell Cultures (ECACC). HK-2 cells were cultured at 37 °C in a humidified atmosphere of 95 % air/5 % CO2, and maintained in DMEM-F12 (Sigma) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 10 ng/ml EGF, 36 ng/ml hydrocortisone, 4 pg/ml triiodothyronine and 5 µg/ml insulin-transferrin-selenium (ITS) solution (Sigma). Cells were subcultured using trypsin-EDTA at a ratio of 1:5.

Expression Microarray Analysis

Human kidney proximal tubular epithelial cells (HK-2) were plated in triplicate for stimulation with TGFβ1 for 0, 24 or 48 h. This experiment was carried out three times on different days, with triplicate samples for each time-point being pooled on each occasion, giving a total of 9 samples for Affymetrix expression microarray analysis. Total RNA was isolated using the Trizol method and purified on Qiagen RNeasy columns. Total RNA (5 µg) was used to produce biotinylated cRNA according to the manufacturer’s instructions (Affymetrix, High Wycombe, UK). Affymetrix test3 arrays were used to confirm the quality of the labelled cRNA before hybridization to the Affymetrix HG U133A 2.0 arrays. Raw data were imported into GeneSpring (version 5.0, Agilent Technologies) normalized per chip to the 50th percentile and per
gene to the median. A filtering step was applied to select reliable data and also changing data (determined by two-fold changes in normalized expression intensities between any two time points). The entire data set, which is compliant to the MIAME criteria, is described in Roxburgh et al. (in preparation), and will be deposited at Gene Expression Omnibus upon publication. The data will be available at: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=vlatfewwemych.

**Real-time PCR analysis of HK-2 cells**

Real-Time TaqMan PCR was used to quantify relative gene expression levels of Gremlin, Jagged1 and Hes1 from total RNA extracted from human kidney proximal tubular epithelial (HK-2) cells. Pooled total RNA samples from the Affymetrix DNA microarray experiment were used as templates for real-time PCR validation. The PCR primers and TaqMan probe for Gremlin were: Forward 5’-GCAAAACCCAGCGCTTAA-3’, reverse 5’-GGTTGATGATGTTGCGACTGTG-3’, Taqman probe 5’-CAGACCATCCACGAGGAAGGCTGC-3’. The primers and probe for E-cadherin, vimentin, Jagged1 and Hes1 were supplied as a pre-optimised single tube primer/probe Gene Expression Assay (Applied Biosystems). The probes for the target genes were labelled with the fluorescent dye, FAM on the 5’ end and a non-fluorescent quencher on the 3’ end. 18s rRNA was used as an endogenous control for normalisation of the target genes. 18s primers and probe were supplied as a pre-developed assay reagent (PDAR, Applied Biosystems) labelled with VIC at the 5’ end and TAMRA on the 3’ end. PCR reactions were set up with Taqman Universal PCR Master Mix from Applied Biosystems. cDNA was amplified on the 7900HT Sequence Detection System (Applied Biosytems) at default thermal cycling conditions: 2 min at 50 °C, 10 min at 95 °C for enzyme activation and then 40 cycles
of 15 sec at 95 °C for denaturation and 1 min at 60 °C for annealing and extension. Results were analysed using the relative standard curve method of analysis/ΔCt method of analysis.

Clinical Biochemistry

Serum creatinine and proteinuria were analyzed at each clinical center by standard, local techniques. Glomerular filtration rate (GFR) was calculated using the Cockcroft-Gault formula.

Real-time PCR and expression microarray analysis of human renal biopsies

Human renal biopsy specimens were collected in an international multi-centre study, the European Renal cDNA Bank-Kroener-Fresenius biopsy bank (ERCB-KFB, see ERCB-KFB, see Lindenmeyer et al., JASN, 2007 for participating centres). Biopsies were obtained from patients after informed consent and with approval of the local human research ethics committees.

Real-time PCR analyses of biopsies from patients with diabetic nephropathy, minimal change disease (MCD) or control living donor (LD) tissue were performed. Following renal biopsy, the tissue was transferred to RNase inhibitor and micro-dissected into glomerular and tubular fragments. Total RNA was isolated from micro-dissected tubulointerstitial tissue (for details see [30]). Reverse transcription and real-time PCR was performed as reported earlier [30]. Pre-developed TaqMan reagents were used for human Gremlin, Jagged1 and Hes1. Expression of target genes were normalized to three different housekeepers (rRNA, GAPDH and cyclophilin) (Applied Biosystems) giving comparable results. The mRNA expression was analyzed by standard curve quantification. Statistical analysis of the correlation of Gremlin, Jagged1 and Hes1
was performed by normalization of the expression of each of the genes to GAPDH, Cyclophilin and rRNA housekeepers separately. We performed Pearson and Spearman analyses to determine if the expression of the three genes correlated significantly. We also performed Mann-Whitney statistical analysis to determine if the normalized expression of Gremlin, Jagged1 and Hes1 differed significantly between the diabetic group, compared to MCD or LD.

A set of samples from this collection was also analyzed by expression microarray analysis (Affymetrix). A total of 24 kidney biopsies from individual patients were included in this study: 3 pre-transplant kidney biopsies from living donors (LD), 4 cadaveric donors (CD), 4 minimal change disease (MCD) and 13 progressive diabetic nephropathy patients (DN). The three groups (LD, CD and MCD) were used as controls in the experiment (see ref. 31). The protocols for Affymetrix microarray analysis and clinical data are given at [http://diabetes.diabetesjournals.org](http://diabetes.diabetesjournals.org) [31]. Subsequently, the expression of genes of interest (Notch pathway gene members) was visualized using the normalized RMAexpress (http://rmaexpress.bmbolstad.com/) data and an Unsupervised Hierarchical Cluster performed by dChip software [31].

**In situ hybridization**

Kidney samples were obtained by percutaneous renal biopsy from patients undergoing diagnostic evaluation at the Division of Nephrology, Austral University, Valdivia, Chile. The samples were studied after obtaining patient consent and the project was approved by the local hospital ethics committee. Renal biopsies from controls or diabetic nephropathy patients diagnosed before specific treatment were studied. Control human kidney specimens (n = 5) were taken from unaffected kidney portions of tumour nephrectomies.
*In situ* hybridization (ISH) was performed as described previously for the TGFβ antisense probe (R&D Systems, Minneapolis, MN, USA; [32]) and with the following modifications for biotin-labeled human Gremlin (Invitrogen, Carlsbad, CA, USA), Jagged1 and Hes1 (Sigma) probes. The Gremlin probe was: anti-sense 5’-CAGGCACTGACTCAGGAAGACA-3´, Jagged1 probe mix was: anti-sense 5’-CCTGACAGTATTATTGAAAAGGCT-3’ and anti-sense 5’-GTACGGCTGGCAAGGCTTGTACTG-3’; Hes1 probe mix was: anti-sense 5’-CACGCCTGCTCTCTGATCCCTGT-3’ and anti-sense 5’-CCTCTCTCCTTGGTCTGGAACAG-3’. For Gremlin, Jagged1 and Hes1 analysis, pre-treatment with endogenous biotin blocking system (Dako Co, Carpinteria, CA, USA) was performed prior to proteinase K digestion. The sections were incubated with a pre-hybridization solution (Dako, mRNA ISH Solution) for 60 min at 37 ºC followed by incubation with the antisense probe overnight at 37 ºC. The slides for Gremlin, Jagged1 and Hes1 were washed twice with 2 X SSC, 1 X SSC and 0.5 X SCC for 5 min at 37 ºC. Detection was performed with streptavidin-alkaline phosphatase conjugate (Dako) for 30 min at room temperature, washed 5 min with 1 X TBS. NBT-BCIP enzyme substrate (R&D Systems) was added for 60 min at 37 ºC. Tissues were then dehydrated in ethanol series and mounted in Canadian balsam (Polysciences Inc., Warrington, PA, USA).
Results

Promoter conservation exists between human Gremlin, Jagged1 and Hes1

We initially performed an alignment of human, mouse and rat Gremlin promoter regions. Experimentally confirmed proximal promoters for human and mouse Gremlin were retrieved from the DBTSS (database of transcriptional start sites, http://dbtss.hgc.jp/). The rat Gremlin proximal promoter was retrieved from the UCSC Genome Browser (http://genome.ucsc.edu/). The Gremlin 5kb proximal promoter for human, mouse and rat were then aligned using MultiPipMaker (http://pipmaker.bx.psu.edu/pipmaker/). Conservation was evident up to 2,800bp upstream of the transcription start site and was highest in the 800 bp proximal to the transcription start site (Fig.1a). Due to the high level of conservation, the possibility of a confounding influence such as the presence of another open-reading-frame was investigated using the UCSC Genome Browser. No confounding influence was found, suggesting that the conservation was that of the functional Gremlin promoter. To analyse the potential transcription factors responsible for Gremlin regulation, Matinspector (http://www.genomatix.de/) to search for conserved transcription factor binding sites. Within the 1 kb region of human, rat and mouse Gremlin promoters, two blocks of transcription factor binding sites were detected, from -50 to -600 bp and from -600 to -900 bp (Fig. 1b).

From -50 to -600 bp, transcription factor binding sites were highly conserved in all 3 species. In the -600 to -900 bp region, some conservation was still apparent, but the order and position of the sites was less conserved than in the -50 to -600 bp region. Twenty three conserved transcription factor binding sites were chosen from the -50 to -600 bp region to search the promoter library for the presence of clusters of such binding elements in other promoters (see Table 1, Supplemental Data). The
transcription factor binding sites were chosen due to known TGFβ regulation or strong involvement in embryogenesis (e.g. Smad, Pax, GATA). ClusterBuster analysis (see Materials and Methods) revealed that genes previously shown to be co-regulated with Gremlin in diabetic nephropathy such as Profilin 1 (13.7) and CTGF/Cyr61 (12.5) [33] were amongst the highest scoring promoters detected (Fig. 1b). Gremlin (10.4) itself also scored highly in the analysis. When gene ontology information was added to the resulting list, 10 genes shared two ontological traits (extracellular secreted protein and morphogenesis) with Gremlin. Of those, apart from the Gremlin and CTGF / Cyr61, Inhibin-A and Jagged1 scored most highly in the ClusterBuster analysis. These data suggest that significant homology and similarity exists between the promoters of Gremlin, CTGF/Cyr61 and Jagged1.

To provide further evidence for potential co-regulation of Gremlin and genes such as Jagged1, we scanned the identified gene sequences for predicted microRNA (miRNA) binding sites. miRNA analysis using 3’ UTR conserved sequences in [26] indicated that Gremlin had 5 putative miRNA binding sites in its 3’ UTR. Twenty three genes in the database shared one or more of these sites. Using HOMGL (http://homgl.biologie.hu-berlin.de/homgl/), we then compared the ClusterBuster gene list with the miRNA gene list and found that 4 genes appeared on both lists. These included Gremlin itself, and one other gene which also shared ontological traits with Gremlin- the Notch ligand Jagged1 (Fig. 2a).

Finally, we identified TFBS motifs in CpG islands in Gremlin spanning the transcription start site (tss) and in the region proximal to the coding sequence, that were conserved in human, mouse, rat and dog. A comparison of these genes containing CpG islands revealed that both Gremlin and Hes1 had a conserved NFκB
motif in CpG islands near their promoters (Fig. 2b), suggesting the possibility of co-regulation of Notch pathway expressing genes with Gremlin.

Based on the conservation of promoter sequences between Gremlin and Jagged1, together with common predicted microRNA sites and conserved promoter motifs in Hes1, these data suggest that genes expressing Notch signalling pathway components may be co-regulated with Gremlin. It was therefore of interest to observe if this potential co-regulated expression was associated with renal fibrosis and the pathogenesis of diabetic nephropathy.

**TGFβ1 stimulates Gremlin, Jagged1 and Hes1 gene expression in renal tubular epithelial cells by microarray analysis**

TGFβ1 is a major driver of epithelial-mesenchymal transition (EMT) and renal fibrosis both in vitro and in vivo [34] [35]. To evaluate the effect of TGFβ1 on kidney cell gene expression, HK-2 tubular epithelial cells were treated with TGFβ1 for various times to stimulate EMT-like cellular changes. Induction of EMT was validated by decreased E-cadherin expression (epithelial cell marker) and increased vimentin expression (mesenchymal cell marker, Fig. 3a). RNA was then probed on human HG U133A 2.0 Affymetrix gene arrays, and changes in expression assessed using GeneSpring software. Using a signal-log ratio of 1.5 (representing a 2-fold change in gene expression), expression of 109 genes was upregulated in response to TGFβ1, whereas 97 genes were downregulated in the same experiment (Roxburgh et al., in preparation). Several genes whose altered expression had previously been identified by our group and others in models of EMT were identified, including fibronectin, type 1 collagen alpha 1, type IV collagen alpha 1 and connective tissue
growth factor (CTGF) suggesting that these epithelial cells had indeed undergone EMT-like changes (Fig. 3b).

Interestingly, the most highly upregulated gene in response to TGFβ1 at both 24 h and 48 h time points was Jagged1, with a 17-fold upregulation at 24 h and an 18-fold upregulation at 48 h detected (Fig. 4). Increased expression of hairy enhancer of split (Hes-1) was also detected in the microarray experiment, with 3-fold increases at 24 h and 48 h detected (Fig. 4). As previously demonstrated, TGFβ1 increased Gremlin expression in kidney epithelial cells (3-fold at 24 hr and 4 fold at 48 h, Fig. 4). To verify these data, real-time TaqMan PCR was performed using oligonucleotides specific for Gremlin, Jagged1 and Hes1. Increases in expression in all three genes at both time points were observed using TaqMan PCR, and the fold-change in expression was similar to that observed by microarray expression analysis (Fig. 4 lower panels). These data suggest that TGFβ1, a major pro-fibrotic cytokine, increased the expression of Jagged1 and Hes1, together with Gremlin, in human kidney epithelial cells in vitro.

Expression of Gremlin, Jagged1 and Hes1 is elevated in biopsies from diabetic nephropathy patients by real-time PCR

To examine whether increased expression of Notch signalling pathway genes was a feature of human diabetic nephropathy, samples from human patients were analysed. The clinical profile of our patient cohort of living donor (LD) cadaveric donor (CD), minimal changes disease (MCD) and diabetes showed significantly reduced glomerular filtration rates (GFR) in diabetic patients versus controls (Diabetes, 36.6 ml/min +/- 14.7, minimal change disease (MCD) 102.25 ml/min +/-24.99, p < 0.05), suggesting stage III to IV chronic kidney disease (CKD). Renal biopsy cores from
these patient cohorts were micro-dissected, and RNA was extracted from the isolated tubulointerstitial segments. Tubulointerstitial RNA from diabetic patients, transplant donor controls and minimal change disease controls was analysed for the expression of Jagged1, Hes1 and Gremlin using qRT-PCR. Figure 5 shows that, using the Mann-Whitney-Test to analyze differences, the cohort of diabetic nephropathy patients exhibited a significantly higher expression of Gremlin, Hes1 and Jagged1 compared to control patients (LD) and minimal changes disease (MCD, Fig. 5). Furthermore, the expression of all three genes in the renal tubulointerstitium correlated with each other when normalized to any of 3 housekeeping genes separately (see legend of Fig. 5). These data suggest that Jagged1 and Hes1 gene expression is upregulated in a coordinated fashion in the tubulointerstitial of kidneys from diabetic nephropathy patients with that of Gremlin.

*Increased expression of Jagged1 and Hes1 and Gremlin is detected in the tubular compartment of renal biopsies from diabetic nephropathy patients by in situ hybridisation*

To identify the specific region of the nephron where Gremlin, Jagged1 and Hes1 levels were upregulated in diabetic nephropathy, their expression was studied by *in situ* hybridization (Fig. 6). No expression of Gremlin, Jagged1 or Hes1 was observed in normal human kidney (Fig. 6, upper panels). In contrast, abundant Gremlin, Jagged1 and Hes1 mRNA expression was co-expressed in tubular epithelial cells in serial sections of kidney biopsies from patients with diabetic nephropathy (Fig. 6, lower panels). Expression of all three genes appears to localize to the same tubular structures, based on staining of serial sections. These data suggest that upregulation of
Gremlin, Jagged1 and Hes1 occurs in the tubuloepithelium during the progression of diabetic nephropathy.

*Notch-signalling pathway genes are upregulated in biopsies from diabetic nephropathy patients*

To broaden our analysis, expression microarray data of tubulointerstitium from patients with established diabetic nephropathy (DN) *versus* either living donor pre-transplant biopsies (LD) alone or including minimal change disease (MCD) or cadaveric donor (CD) as controls were analysed for the expression levels of all detectable members of the Notch signalling pathway. The clinical profile of these patients was previously published by Schmid and colleagues [31]. Using a cutoff expression value of 5.83 and a p-value < 0.05, ten Notch-pathway related genes were found to be significantly differentially expressed in DN *versus* LD and MCD. Included among these were the Notch ligand Jagged1-1, two Notch receptors Notch-2 and Notch-3 and SKI-Interacting Protein (SKIP, Fig. 7). Of these genes, MECT1 and NOTCH3 were found to be significantly downregulated in the DN group, while genes such as Jagged1, Notch2 and ADAM10 are all upregulated (Fig. 7). Thus, the expression levels of multiple genes in the Notch signal transduction pathway are altered in diabetic nephropathy.
Discussion

The data presented in this report explore the parallel potentially coordinated upregulation of Gremlin and Notch family genes in the kidneys of diabetic nephropathy patients. Using a bioinformatics approach, common clusters of transcription factor binding and regulatory elements in the promoters of Gremlin and Jagged1 (the Notch ligand) were identified. TGFβ1, the primary pro-fibrotic cytokine involved in renal fibrosis, increased the expression of Gremlin, Jagged1 and Hes1 (a downstream target of the Notch signalling pathway) within the same time-frame in tubular epithelial cells. Elevations in all three genes were observed in cDNA samples isolated from kidney biopsies from diabetic nephropathy patients, but not controls. In situ hybridization identified the kidney tubules as the main site of Gremlin, Jagged1 and Hes1 upregulation during DN. Finally, changes in expression of other members of the Notch signalling pathway were also detected in renal biopsies from DN patients, suggesting that upregulation of Notch pathway signalling is a feature of diabetic nephropathy.

Bioinformatic analysis of Gremlin genomic sequences from human, mouse and rat revealed an unusually high degree of conservation up to -2800 bp upstream of the predicted transcription start site, with particularly strong alignment in the first 800 bp (Fig. 1a). Recent studies suggest that the average sequence identity between orthologous human and mouse promoter regions peaks at approximately 70% at the TSS [36, 37] and falls rapidly with distance from the TSS. The comparatively high degree of conservation evident in orthologous Gremlin human, mouse and rat promoters suggested that common sequence elements exist and are functionally relevant in the promoters of Gremlin in all three species.
Consistent with the high degree of conservation in the Gremlin promoter, clusters of transcription factor binding sites common to human, rat and mouse were identified, many of which had previously been implicated in TGFβ signalling and embryogenesis (Fig. 1b). These included Smad3 [38], Fast1 [39] and Mitf [40].

These conserved transcription factor binding elements were then used to identify other genes with common promoter regulatory elements. This approach identified the previously implicated TGFβ1 co-regulated gene CTGF, as well as Gremlin itself (Fig. 1b). Also present in this group was Jagged1, the ligand for the Notch receptor which regulates multiple developmental processes [41]. Mutations in Jagged1 can lead to the genetic disorder Alagille syndrome [42], which results in multi-organ defects, including, but not limited to cardiac, ocular and facial [43]. A similar approach was taken by Kielbasa et al. [44], who described a method for predicting muscle-specific gene targets for a set of transcription factors also using a strategy centred on analysis within Cluster-Buster.

The presence of common motifs in CpG islands in Gremlin and Hes1 (a downstream target of Jagged1/Notch signalling) genes, as well as micro RNA binding sites in the 3’ UTR of Gremlin and Jagged1 genes further support our suggestions that common regulatory strategies exist for expression of Gremlin and genes of the Notch signalling pathway. Both Gremlin and Jagged1 control developmental processes in many species [16, 45-47]. Gremlin is a BMP antagonist whose temporal and spatial expression is crucial to normal development [16]. Mice lacking Gremlin exhibit limb deformities in both fore and hind-limb [16]. Of relevance to our study, mice lacking Gremlin die shortly after birth due to renal agenesis [16], suggesting an important role for Gremlin in kidney development. Jagged1 mutations cause Alagille syndrome in humans, an autosomal dominant condition affecting the liver, kidneys, heart and craniofacial
structures [48]. To date, common mechanisms of regulation for Gremlin and Jagged1 have not been described, although coordinated expression of both genes may be required for correct sonic hedgehog signalling during epithelial-mesenchymal signalling in mouse limb bud development [49].

TGFβ1 is a key pathogenic driver of renal fibrosis in diabetic nephropathy and other forms of kidney disease [50]. TGFβ1 stimulates increases in expression of genes such as fibronectin, CTGF and Gremlin in models of glucose-induced damage in kidney cells [15]. Cells treated with TGFβ1 underwent EMT-like changes, as confirmed by decreased E-cadherin levels and increased vimentin expression (Fig. 3a). Treatment of renal epithelial cells with TGFβ1 is known to induce EMT in epithelial cells [13]. Several genes that are also characteristic of TGFβ1-induced fibrosis were also increased, including fibronectin, collagen and CTGF (Fig. 3b). TGFβ1 increased the expression of Gremlin, as well as Jagged1 and Hes1 in human proximal epithelial cells (HK-2) in a cell culture model of epithelial-mesenchymal transition (EMT, Fig. 4). In agreement with our identification of common regulatory elements within the Gremlin and Jagged1 promoters, our data suggested that signal transduction cascades triggered by TGFβ1 activated the expression of Gremlin and Jagged1, as well as Hes1 in tubular epithelial cells. Morrissey and colleagues have also observed TGFβ1-mediated Jagged1 upregulation in human kidney cells [51]. These authors also identified the kidney tubules as the primary site of Jagged1 upregulation in a mouse model of acute renal fibrosis [51]. Other data suggests that TGFβ-induced EMT in human keratinocytes is associated with the expression of the hairy enhancer of split-like genes in the Notch signalling pathway [12]. Functional inactivation of the Notch ligand Jagged1 or Notch has also been shown to inhibit TGFβ1 induced EMT [52]. Notch signalling induced by TGFβ has been shown to occur in a biphasic manner
At the onset of EMT a subset of Notch target genes, together with its ligand Jagged1 are induced by TGFβ1, Smad3-dependent, and Notch independent. After prolonged TGFβ1 exposure, a second, sustained wave of Notch signalling occurs through TGFβ-induced synthesis of Jagged1, Notch dependent [52]. Together, these data suggest that TGFβ1 mediated increases in Notch signalling may act to sustain or amplify its pro-fibrotic effects in kidney cells.

Based on bioinformatic and in vitro data, we hypothesised that coordinated upregulation of Gremlin with members of the Notch signalling pathway such as Jagged1 and Hes1 is a feature of renal fibrosis. Our diabetic patient cohort displayed a significantly decreased GFR resulting from DN. The scatter in GFR observed in the diabetic patient group is indicative of a heterogeneity in renal function, with values in these patients ranging from 60 ml/min down to 6 ml/min. Thus, the majority of these patients can be classified as either moderate or severe CKD as a result of DN. Levels of all three gene transcripts of interest were elevated in biopsies from diabetic nephropathy patients compared to normal living donor or minimal change disease controls (Fig. 5). Further analysis revealed that the upregulation of all three genes correlated with each other in a statistically significant manner, when compared to any of three housekeeping genes. Our correlation analysis highlights that Gremlin, Jagged1 and Hes1 follow a shared pattern of upregulation in the kidney tubules during diabetic nephropathy. Supporting this conclusion, we observed significant staining of Gremlin, Jagged1 and Hes1 in the same kidney tubules in diabetic nephropathy, but not in control sections (Fig. 6). Weaker staining for all three genes was observed in glomeruli. Previous reports have identified Gremlin upregulation in the tubular compartment of diabetic nephropathy kidneys [17]. Gremlin mRNA levels correlated well with renal dysfunction in these patients [17]. Similar to Dolan et al. (2005),
Gremlin upregulation was predominantly detected in the tubulointerstitium, with lower levels evident in the glomerulus. These data suggest that Gremlin, and indeed Notch family signalling may play a more significant role in tubulointerstitial fibrosis than in glomerulosclerosis during DN. To our knowledge, our data is the first to report increased expression of Notch family genes such as Jagged1 and Hes1 in the kidneys of diabetic nephropathy patients. Furthermore, the strong correlation between Gremlin and Jagged1/Hes1 expression suggests that drivers of renal fibrosis such as TGFβ1 may be triggering coordinated waves of such gene expression that contribute to the pathogenesis of renal disease. Based on the tight co-regulation of Gremlin with two genes of the Notch signalling pathway (Jagged1 and Hes1), we then identified other genes in the Notch pathway whose expression was also elevated in kidney biopsies from DN patients compared to controls (Fig. 7). Genes encoding the Notch receptors, ADAM-10 (a metalloproteinase essential for Notch signalling [53]) and Ski-interacting protein (SKIP, a co-activator of transcription that binds to the activated intracellular portion of the Notch receptor [54]) were all highly expressed in biopsies from human DN patients compared to controls (Fig. 7). These data fit within the paradigm of the re-emergence of developmental gene expression in the context of diabetic nephropathy, where developmental gene expression may be part of an inappropriate repair mechanism to reverse the progression of renal fibrosis.

Our data describe a novel link between Gremlin upregulation and increased Notch pathway signalling downstream of TGFβ1 in diabetic nephropathy. Further experiments are required to determine whether increased Notch signalling acts to amplify or prolong TGFβ1 signalling in the diabetic kidney and what is the precise role of Gremlin in this paradigm. Elucidation of these cellular mechanisms will allow
potentially novel interventions that aim to delay or prevent the onset of renal fibrosis in diabetic nephropathy.
Acknowledgments

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Figure Legends

Figure 1. Conservation of clusters of transcription factor binding sites in the Gremlin proximal promoter and their occurrence in other promoters. (A) Combined dot-plots (upper panel) and pip-plots (lower panel) for the 5 kb proximal promoters of human Gremlin versus mouse Gremlin and human Gremlin versus rat Gremlin respectively. The human proximal promoter sequence is represented by the horizontal axis in all cases. The dot-plots represent each of the gap-free segments of alignment as a diagonal line indicating position in both the human and mouse sequences and the human and rat sequences. The pip-plots depict each one of these segments of alignment as a horizontal line, the left-to-right position of which is determined by the human coordinates and the vertical placement of which indicates the percent identity of the mouse or rat sequence. In both comparisons regions of sequence identity extend up to 2.8 kb upstream of the transcription start site. With the exception of an apparent deletion in the mouse genome corresponding to -700 to -1200 bp in human, the sections of gap-free alignments are almost uninterrupted. (B) Matinspector analysis of human, mouse and rat Gremlin 1 kb proximal promoters (-1 to -400 bp shown). Transcription factor matrices common to all 3 species are shown. Each coloured tab represents an individual binding site identified by Matinspector, [sites are identified in the legend to its right]. The lower panel shows the ClusterBuster score output for genes identified as having clusters of the transcription factors found conserved in the Gremlin promoter. Shown here are those genes whose score was higher than the 7.0 cut-off and also shared gene ontology with Gremlin. Highlighted is the Jagged1 1 result, indicating its score of 8.27, the position of the cluster within the promoter region, and to the right a graphical representation of the
cluster. Once again the coloured tabs represent individual transcription factor matrix sites, and the colours correspond to the matrices above.

**Figure 2. Gremlin shares regulatory elements with Jagged1 and Hes1** (A) Using conserved 3’ untranslated (UTR) motif data [26], we identified 5 potential miRNA binding sites in the Gremlin 3’ UTR. Further analysis revealed 23 genes (Table 2, Supplemental data) that shared one or more of these miRNA target sites. Of these the Notch ligand Jagged1 shared a TGTGAATA motif in its 3’ UTR with Gremlin. (B) The Gremlin proximal promoter contains two CpG islands. Within those we found 6 conserved motifs. Of the 6, 5 of them are NFKappa B related, including MA0105 (Jaspar accession) p50 subunit of NFKappaB. We searched for Notch pathway members sharing a similar motif in its CpG island with Gremlin. We found that Hes1 also contain a CpG island in its proximal promoter, and similarly contains a MA0105 motif.

**Figure 3. TGFβ1 induces gene expression-like changes in human kidney epithelial cells consistent with EMT.** Human proximal tubule kidney epithelial cells (HK-2) were treated for 0, 24 or 48 h with 10 ng/ml TGFβ1. Total RNA was extracted and processed for quantitative TaqMan PCR and Affymetrix microarray analysis as described. (A) Specific TaqMan probes for E-cadherin and vimentin were used to amplify individual RNA transcripts. Fluorescence intensities were normalised to 18s levels for each gene. Mean fold-change in expression for each experiment (in triplicate) were calculated and plotted versus TGFβ1 exposure time (h). Error bars represent standard error of the mean (SEM). Statistical significance was calculated using two-tailed t-tests. * p<0.02, ** p<0.05, *** p<0.0001, **** p<0.00005. (B) Total RNA was analysed on HG U133A 2.0 Affymetrix chips as described in
Materials and Methods. Fold change in expression of fibronectin, collagen, Gremlin and CTGF at 24 and 48 h compared to control are indicated.

Figure 4. TGFβ1 induces Gremlin, Jagged1 and Hes1 expression in human kidney epithelial cells in culture. Human proximal tubule kidney epithelial cells (HK-2) were treated for 0, 24 or 48 h with 10 ng/ml TGFβ1. Total RNA was extracted and processed for Affymetrix microarray analysis as described in Materials and Methods. (A) Changes in gene expression were determined using Affymetrix GeneSpring® analysis software. Each time-point is the average of three independent experiments carried out on separate days. Fold change in expression of Gremlin, Jagged1 and Hes1 are plotted versus duration of TGFβ1 treatment (h). (B) Quantitative TaqMan PCR was carried out on the total RNA samples used in the Affymetrix experiment above. Specific TaqMan probes for Gremlin, Jagged1 and Hes1 were used to amplify individual RNA transcripts. Fluorescence intensities were normalised to 18s RNA levels for each gene. Mean fold change in expression for each experiment (in triplicate) were calculated and plotted versus duration of TGFβ1 treatment (h). Error bars represent standard error of the mean (SEM). Statistical significance was calculated using two-tailed t-tests. * p<0.01, ** p<0.05, *** p<0.005, **** p<0.00005, ***** p<0.000001.

Figure 5. Increased expression of Gremlin, Jagged1 and Hes1 mRNA was detected in biopsies from diabetic nephropathy patients, by RT-PCR. Real-time Taqman PCR was performed on RNA extracted from renal biopsies from control living donor (LD, n=10), minimal change disease (MCD, n=5) or diabetic nephropathy (diabetes, n=18) as described in Materials and Methods. Shown is the
expression level in arbitrary units for Gremlin, Jagged1 and Hes1 normalized to the housekeeper GAPDH. Using Mann-Whitney non-parametric tests we found that the diabetic group shows significantly higher expression of Gremlin (p = 0.006), Jagged1 (p = 0.001) and Hes1 (p = 0.011) compared to LD and MCD. This was true for all housekeepers used. Both latter groups do not show a significant difference in expression levels. Using Pearson (numeric) and Spearman (rank) tests we analyzed the expression levels of the 3 genes for statistical correlation, normalized to each housekeeper separately. The expression levels of the 3 genes were found to correlate significantly. This was true for each housekeeper and for both statistical tests. In the Pearson/Parametric tests Gremlin and Jagged1 had correlation coefficients of 0.627 (rRNA), 1.000 (GAPDH) and 0.756 (Cyclo), in the non-parametric/Spearman-Rho tests the coefficients were 0.530 (rRNA), 0.876 (GAPDH) and 0.674 (Cyclo). For Gremlin and Hes1 the Pearson coefficients were found to be 0.519 (rRNA), 0.999 (GAPDH) and 0.544 (Cyclo), the Spearman-Rho coefficients were 0.346 (rRNA) (0.05 level significance), 0.809 (GAPDH) and 0.527 (Cyclo). In all cases, unless specified, p = 0.01.

**Figure 6.** Gremlin, Jagged1 and Hes1 expression co-localizes to tubular epithelial cells in serial kidney sections of DN patients. *In situ* hybridization of renal biopsies from control (upper panel) and diabetic nephropathy (lower panel) patients were performed as described in Materials and Methods. Serial sections of 4 µm were stained for Gremlin (left panel), Jagged1 (middle panel) or Hes1 mRNA (right panel). Glomeruli (*) and tubules (arrows) are indicated.
Figure 7. Key genes of the Notch signalling pathway are upregulated in Diabetic Nephropathy. Hierarchical cluster analysis of gene expression profiles from DN renal biopsies and different ‘controls’. Tubulointerstitial compartments of renal biopsies from patients with DN and MCD, as well as pre-transplant biopsies from related LD and CD kidneys, were analyzed. Gene expression profiles were determined using Affymetrix oligonucleotide arrays HG-U133A. Transcript abundance is displayed on a red-green color scale, with red expression above and green below the median. The cluster dendrogram sorts the patients with the most similar gene expression profiles together with the shortest branches. Hierarchical clustering showed a clear distinction between the DN patients and the LD/MCD/CD patients, with CD4 being the one exception, clustering within the DN group. The above cluster shows non-redundant genes expressed higher than the cutoff (expression value = 5.83) that are significantly differentially regulated (p < 0.05). Included in this cluster are genes that function at key points in the Notch pathway: ligand (Jagged1), receptor (Notch2 & Notch3), receptor cleavage (ADAM10), transcriptional activators (RBPSUH, SKIP), notch ligand activators (MIB1), notch repressor (NLE1) as well as other genes shown to interfere with Notch signaling (APP, MECT1).
References


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Figure 1

A.

B.
**Figure 2a**

![Diagram of gene structures](image)

**Gremlin (NM_013372)**

- CDS: 3079487 to 30812027
- 3' UTR: 30812027 to 30814558

**Jagged (NM_000214)**

- CDS: 10502590 to 10581460
- 3' UTR: 10581460 to 10560334
Figure 2b

Gremlin (NM_013372)

30796080

30794057

30794080

30790091

CDS

CpG Island

HES1 (NM_005524)

153331342

153331013

NFκB/MA105

153330028

CDS
Figure 3a

![Graph showing the fold change in expression of E-cadherin and Vimentin over time (0hr, 24hr, 48hr). The graph indicates significant changes in expression levels, with E-cadherin showing a decrease over time and Vimentin showing an increase.]
**Figure 3b**

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Accession number</th>
<th>Fold change 24 hr</th>
<th>Fold change 48hr</th>
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<tr>
<td>Fibronectin 1</td>
<td>NM_212482</td>
<td>2.64</td>
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<td>Type I collagen, alpha 1</td>
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<td>Type IV collagen, alpha 1</td>
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<td>Gremlin</td>
<td>NM_013372</td>
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<td>CTGF</td>
<td>NM_001901</td>
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</tr>
</tbody>
</table>
Figure 4

**Graphs**

**Gremlin**

- Time (h): 0, 24, 48
- Fold change in intensity

**Jagged1**

- Time (h): 0, 24, 48
- Fold change in intensity

**Hes1**

- Time (h): 0, 24, 48
- Fold change in intensity

**Bar Graphs**

**Gremlin**

- Fold change in expression: 0hr, 24hr, 48hr

**Jagged1**

- Fold change in expression: 0, 24hr, 48hr

**Hes1**

- Fold change in expression: 0, 24hr, 48hr
Figure 5

**Gremlin**

**Jagged1**

**Hes1**

Diabetes LD MCD

mRNA copy number (arbitrary units)

Diabetes LD MCD

mRNA copy number (arbitrary units)

Diabetes LD MCD

mRNA copy number (arbitrary units)
Figure 6

**Control**

**Diabetes**

*Gremlin*  *Jagged1*  *Hes1*
Figure 7