

Identification of key genes and pathways in diabetic nephropathy by bioinformatics analysis

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Keywords

Bioinformatics analysis, Diabetic nephropathy, Differentially expressed genes

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ABSTRACT

Aims/Introduction: The aim of the present study was to identify candidate differentially expressed genes (DEGs) and pathways using bioinformatics analysis, and to improve our understanding of the cause and potential molecular events of diabetic nephropathy.

Materials and Methods: Two cohort profile datasets (GSE30528 and GSE33744) were integrated and used for deep analysis. We sorted DEGs and analyzed differential pathway enrichment. DEG-associated ingenuity pathway analysis was carried out. The screened gene expression feature was verified in the *db/db* mouse kidney cortex. Then, rat mesangial cells cultured with high-concentration glucose were used for verification. The target genes of transcription factor E26 transformation-specific-1 (ETS1) were predicted with online tools and validated using chromatin immunoprecipitation assay quantitative polymerase chain reaction.

Results: The two GSE datasets identified 89 shared DEGs; 51 were upregulated; and 38 were downregulated. Most of the DEGs were significantly enriched in cell adhesion, the plasma membrane, the extracellular matrix and the extracellular region. Quantitative reverse transcription polymerase chain reaction analysis validated the upregulated expression of *Itgb2*, *Cd44*, *Sell*, *Fn1*, *Tgfb1* and *Il7r*, and the downregulated expression of *Igfbp2* and *Cd55* in the *db/db* mouse kidney cortex. Chromatin immunoprecipitation assay quantitative polymerase chain reaction showed that *Itgb2* was the target gene of transcription factor Ets1. ETS1 knockdown in rat mesangial cells decreased integrin subunit beta 2 expression.

Conclusion: We found that EST1 functioned as an important transcription factor in diabetic nephropathy development through the promotion of integrin subunit beta 2 expression. EST1 might be a drug target for diabetic nephropathy treatment.

INTRODUCTION

Diabetic nephropathy (DN) has emerged as the main cause of end-stage renal disease, but current treatments are still suboptimal. Some genetic and environmental factors can be used to show the pathogenesis of DN. These factors contribute to the progressive and irreversible scarring of the glomeruli and tubulointerstitial parts of the kidneys, leading to the regression of

renal function, eventually leading to renal failure. Genome-wide transcriptome analysis using expression arrays can be used to identify biomarkers for disease progression, and to gain insights into the disease pathogenesis and molecular classification¹. With the wide application of genome transcriptome analysis, a large amount of core slice data has been produced, most of which is already stored in a public database. Re-analyzing these data can provide significant clues for other research. In recent years, some microarray data analysis studies on DN have been carried out, and numerous differentially expressed genes (DEGs) have

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been identified². Because of the heterogeneity of the tissues or samples in independent studies, most of the gene array results are either limited or inconsistent, focused on human DN or generated from a single cohort study. We combine integrated bioinformatics methods and expression profiling techniques to address these disadvantages.

In the present work, we downloaded two original microarray datasets, GSE30528³ and GSE33744⁴, from the National Center for Biotechnology Information–Gene Expression Omnibus (NCBI–GEO) database (available online: <https://www.ncbi.nlm.nih.gov/geo>). Gene expression was profiled in a cohort of type 2 DN patients and in a diabetic BKS *db/db* mouse model of DN. We filtered DEGs using the Morpheus website. Then, we developed a Gene Ontology (GO) analysis and pathway enrichment analysis for the screening of DEGs⁵, and we integrated a DEG ingenuity pathway analysis (IPA) to identify hub genes in DN.

METHODS

Microarray Data Information and DEG Analysis

We obtained two DN renal tissue gene expression profiles, namely GSE30528 and GSE33744, from the NCBI–GEO database. The raw data were analyzed with the Morpheus website. DEGs were identified with a classical *t*-test, and statistically significant DEGs were defined using $P < 0.05$ and $[\log_{2}FC] > 1$ as the cut-off criteria. KEGG PATHWAY, Reactome, BioCyc, Panther⁵, NHGRI and the GO website were used for GO and pathway analysis^{6,7} ($P < 0.05$ was the cut-off criterion). IPA (Ingenuity Systems, Redwood City, CA, USA) was used to analyze biological functions, upstream regulatory molecules/networks, DEG network interactions and pathways.

Experimental Animals and Procedures

The present study was carried out according to the Guide for the Care and Use of Laboratory Animals of the Chinese PLA General Hospital and Military Medical College; the Animal Ethics Committee of the Chinese PLA General Hospital and Military Medical College approved this study.

We used *db/db* mice with the nephropathy-susceptible BKS background⁸ as a model of type 2 diabetes, and *db/m* mice as the control group. We purchased mice from the Model Animal Research Center of Nanjing University (Nanjing, China). The animals were provided with adequate standard rodent chow and water. The 26-week-old male mice were divided into two groups: (i) the control group, namely, *db/m* mice ($n = 8$); and (ii) the DN group, namely, *db/db* mice ($n = 8$). Twenty-six weeks old mice were placed in metabolic cages to collect 24-h urine samples. At the end of 26 weeks, mice were euthanized. The fasting blood glucose level was also measured in all groups. Both kidneys were removed and weighed, and the kidneys were harvested for further processing.

Cell Culture and Transfection

Rat mesangial cells (RMCs) were purchased from American Type Culture Collection. RPMI 1640 medium (Gibco, Grand

Island, NY, USA) containing 10% fetal bovine serum (HyClone Ottawa, Ontario, Canada) at 37°C in a 5% CO₂ atmosphere was used for cell culture. RMCs were cultured for 48 h in normal (5.5 mmol/L) glucose medium or high (30 mmol/L) D-glucose medium; 24.5 mmol/L mannitol was added to the normal glucose medium as an osmotic control. E26 transformation-specific-1 (ETS1) and integrin subunit beta 2 (ITGB2) small interfering ribonucleic acid (siRNA) or control siRNA (siRNA-con: a scrambled siRNA(5'-UCAAGAAGCCAAG-GAUAAU-3')) (GenePharma, Shanghai, China; 100 nmol/L) was transfected into RMCs for 48 h using jetPRIME™ (Polyplus-transfection, Strasbourg, France). Then, ETS1 and ITGB2 protein expression were assessed by western blotting.

Western Blot Analysis

Radioimmunoprecipitation assay lysis buffer containing 50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 0.5% deoxycholate, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate, 1 mmol/L phenylmethylsulfonyl fluoride and 1 µg/mL protease cocktail was used to extract protein from cells and tissues. A bicinchoninic acid assay kit was used to determine the protein concentration. Protein samples (80 µg/lane) were loaded onto gels, separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Then, the membranes were incubated with anti-Ets1, Igtb2 or β-actin antibodies at 4°C overnight. The membrane was washed three times and incubated with a secondary antibody for 1 h. The detection of specific signals was carried out using enhanced chemiluminescence (Amersham Biosciences, Little Chalfont, UK).

Real-Time Polymerase Chain Reaction and Chromatin Immunoprecipitation Assay

To validate the results of the high-throughput microarray analysis, *Itgb2*, *Cd44*, *Sell*, *Fn1*, *Tgfb1*, *Il7r*, *Igfbp2* and *Cd55* were selected and analyzed by quantitative polymerase chain reaction (qPCR). The primers are listed in Table S1. Chromatin immunoprecipitation assay (ChIP) assays were carried out using a kit from Millipore, Inc. (Milford, MA, USA). The PCR primers used for ChIP assays were as follows: *Itgb2* forward, 5'-CCTGCA-GATTGTTCCGGAGT-3' and *Itgb2* reverse, 5'-CTGGGCCACCTTTACTGAG-3'. The $2^{-\Delta\Delta CT}$ method was used to calculate the relative amplification of the promoter sequence of each gene.

Statistical Analysis

Results are presented as the mean ± standard deviation. Statistical analyses were carried out with SPSS 17.0 statistical software (SPSS, Inc., Chicago, IL, USA). Student's *t*-test was used to assess the significance of experimental differences between groups. A *P* value <0.05 was considered statistically significant.

RESULTS

Identification of DEGs in DN

Figure 1 shows the workflow of the present study. Using the free NCBI–GEO database, we obtained the gene expression

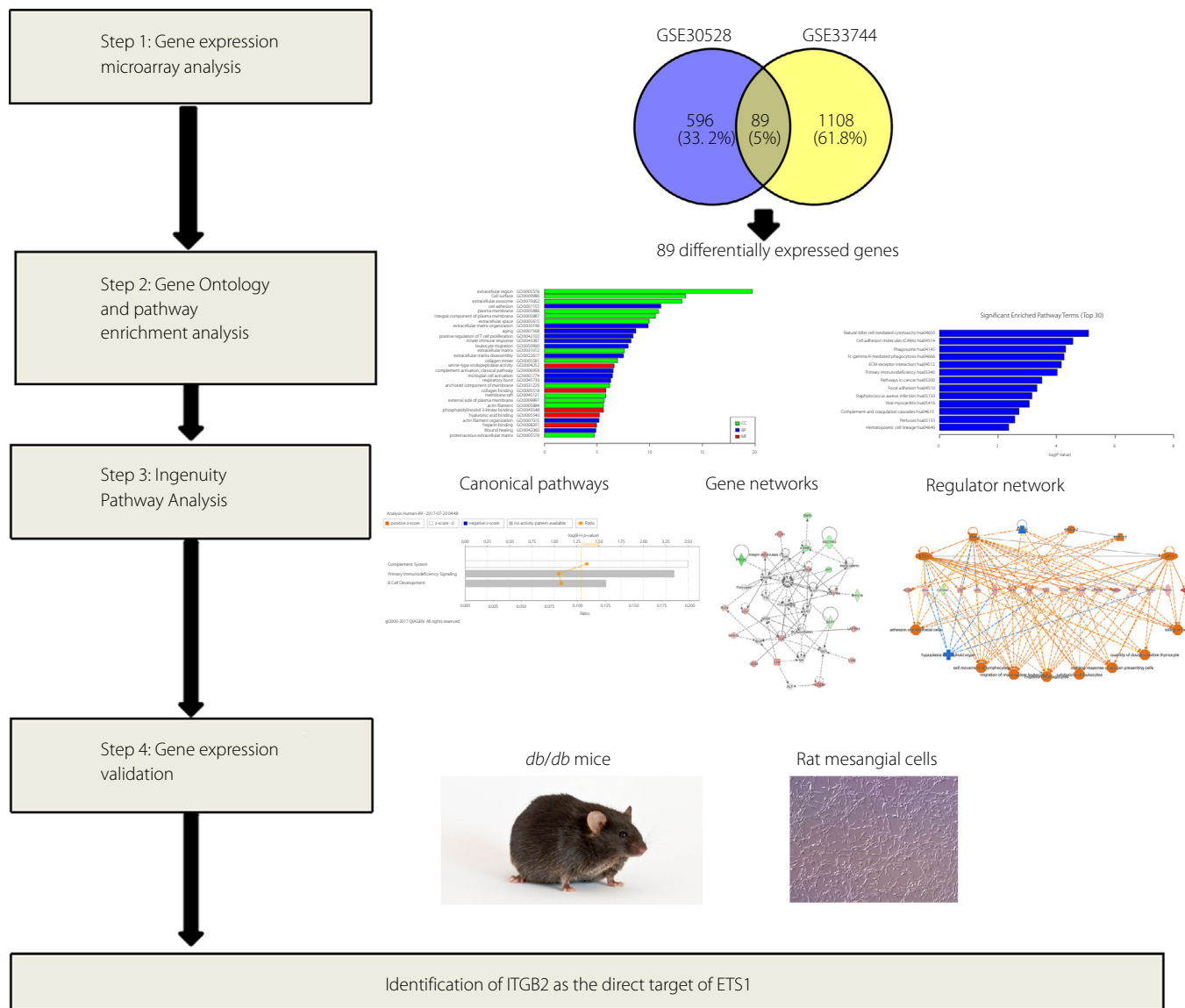


Figure 1 | Workflow of the study. ETS1, E26 transformation-specific-1; ITGB2, integrin subunit beta 2.

profiles of GSE30528 and GSE33744 with the DN renal tissue database. The microarray data of GSE30528 had 22 microdissected human glomerular samples³. The glomerular samples were collected from healthy, living transplant donors; nephrectomies; and diagnostic kidney biopsies. For the glomerular analysis, nine DN and 13 control samples had been chosen. The GSE33744 data included 10 renal glomerular samples from five diabetic BKS *db/db* mice and five non-diabetic BKS *db/m* mice⁴. The diabetic BKS *db/db* mice were an obese type 2 diabetes model with a C57BLKS genetic background combined with a homozygous leptin receptor mutation (*lepr^{db/db}*; BKS *db/db* mice). We obtained 685 and 1,197 DEGs from the expression profile datasets of GSE30528 and GSE33744 using

$P < 0.05$ and $[\logFC] > 1$ as cut-off criteria, respectively. Among the DEGs, 89 were consistently expressed genes; 51 that were upregulated and 38 that were downregulated in the DN glomerular tissues compared with normal glomerular tissues were identified from the two profile datasets (Figure 2a; Table 1). The heat map showed the significantly different distribution of the 89 DEGs using the GSE30528 data profile as the reference with the Morpheus software (Figure 2b).

DEG GO Analysis in DN

Candidate DEG functions and pathway enrichment were analyzed using multiple online databases, including DAVID, KEGG PATHWAY, BioCyc, Panther⁵, NHGRI and the GO

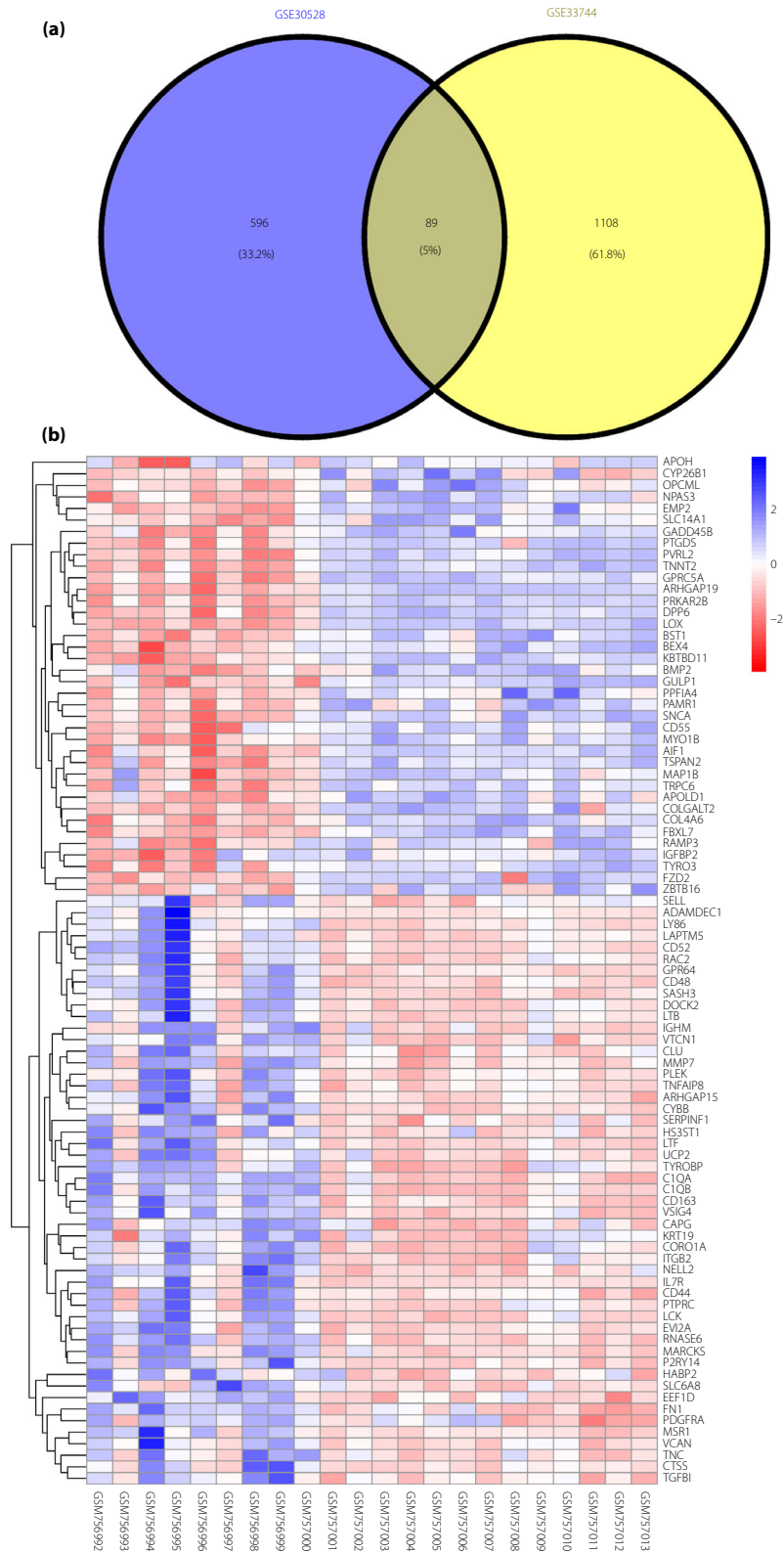


Figure 2 | Identification of 89 commonly changed differentially expressed genes (DEGs) from the two cohort profile datasets (GSE30528 and GSE33744). (a) Different color areas represent different datasets. The cross areas indicate commonly changed DEGs. (b) Heat map of the top 89 DEGs (51 upregulated genes and 38 downregulated genes). Red, upregulation; blue, downregulation.

Table 1 | Differentially expressed genes identified from two profile datasets

DGEs	Gene names
Upregulated	ADAMDEC1, ARHGAP15, C1QA, C1QB, CAPG, CD163, CD44, CD48, CD52, CLU, CORO1A, CTSS, CYBB, DOCK2, EEF1D, EVI2A, FN1, GPR64, HABP2, HS3ST1, IGHM, IL7R, ITGB2, KRT19, LAPTMS, LCK, LTB, LTF, LY86, MARCKS, MMP7, MSR1, NELL2, P2RY14, PDGFRA, PLEK, PTPRC, RAC2, RNASE6, SASH3, SELL, SERPINF1, SLC6A8, TGFB1, TNC, TNFAIP8, TYROBP, UCP2, VCAN, VSIG4, VTCN1
Downregulated	AIF1, APOH, APOLD1, ARHGAP19, BEX4, BMP2, BST1, CD55, COL4A6, COLGALT2, CYP26B1, DPP6, EMP2, FBXL7, FZD2, GADD45B, GPRCSA, GULP1, IGFBP2, KBTBD11, LOX, MAP1B, MYO1B, NPAS3, OPCML, PAMR1, PPFIA4, PRKAR2B, PTGDS, PVRL2, RAMP3, SLC14A1, SNCA, TNNT2, TRPC6, TSPAN2, TYRO3, ZBTB16

The two profile datasets included 51 upregulated genes and 38 downregulated genes in the diabetic nephropathy glomerular tissues, compared with normal glomerular tissues. DGEs, differentially expressed genes; DN, diabetic nephropathy.

website^{6,7,9}. DEG GO analysis was carried out by DAVID and Panther. The DEGs were classified into three groups, namely, cellular component, biological process and molecular function (Figure 3a). As shown in Figure 3b, in the biological process group, DEGs were mainly enriched in cell adhesion, extracellular matrix organization, aging, positive regulation of T-cell proliferation and extracellular matrix disassembly. In the molecular function group, DEGs were mainly enriched in serine-type endopeptidase activity, collagen binding, phosphatidylinositol 3-kinase binding, hyaluronic acid binding and heparin binding. In the cellular component group, DEGs were mainly enriched in the cell surface, extracellular region, plasma membrane, extracellular exosome, integral component of plasma membrane and extracellular space. These results showed that most of the DEGs were significantly enriched in the extracellular region, extracellular matrix, cell adhesion and plasma membrane.

Signaling Pathway Enrichment Analysis

KEGG PATHWAY, Reactome, BioCyc, Panther, NHGRI and GO were carried out for the DEG functional and signaling pathway enrichment. Signaling pathway analysis showed that DEGs were mainly enriched in natural killer cell-mediated cytotoxicity, cell adhesion molecules (CAMs), phagosome, Fc gamma R-mediated phagocytosis, ECM-receptor interaction, primary immunodeficiency, pathways in cancer, focal adhesion, *Staphylococcus aureus* infection, viral myocarditis, complement and coagulation cascades, pertussis, and hematopoietic cell lineage (Figure 4; Table S2).

Key Candidate Gene and Pathway Identification with DEG IPA

We carried out biological functions, pathways and upstream regulatory molecules/networks analysis for 89 DEGs using the QIAGEN IPA (QIAGEN, Redwood City, CA, USA; www.qiagen.com/ingenuity).

Canonical Pathways Identified in IPA

IPA showed that the ingenuity canonical pathways were focused on the following three canonical pathways: the complement system ($P = 0.003$; ratio 0.108), containing ITGB2, CD55, C1QA and C1QB; primary immunodeficiency signaling

($P = 0.004$; ratio 0.083), containing PTPRC, IL7R, LCK and IGHM; and B-cell development ($P = 0.026$; ratio 0.085), containing PTPRC, IL7R and IGHM (Figure 5; Table S3).

Disease and Biological Function Enrichment Analysis

Ingenuity pathway analysis has been used to study potential links with biological findings and diseases. As shown in Table S4, the top biological functions were associated with endocrine system disorders, gastrointestinal disease, metabolic disease, organismal injury and abnormalities. We also compared the DEGs between DN and other disease gene sets to detect any possible correlation between DN and other diseases at the genomic level. As shown, DN was highly associated with diabetes mellitus ($P = 4.4E-20$, 38 molecules), glucose metabolism disorder ($P = 5.75E-20$, 41 molecules), cell movement of blood cells ($P = 3.11E-19$, 34 molecules), activation of blood cells ($P = 4.38E-19$, 31 molecules) and leukocyte migration ($P = 2.19E-18$, 33 molecules).

Gene Networks

Ingenuity pathway analysis identified nine gene networks, with scores ranging from 2 to 33, of which the top six (score >20) were associated with: (i) network 1: cell-to-cell signaling and interaction, hematological system development and function, and cellular development (score = 33; Figure 6a); (ii) network 2: cellular development, cellular growth and proliferation, and hematological system development and function (score = 31; Figure 6b); (iii) network 3: cardiovascular system development and function, organismal development, and cellular movement (score = 28; Figure 6c); (iv) network 4: cell death and survival, skeletal and muscular disorders, and cell signaling (score = 28; Figure 6d); (v) network 5: hematological system development and function, cellular function and maintenance, and cell-to-cell signaling and interaction (score = 24; Figure 6e); and (vi) network 6: neurological disease, organismal injury and abnormalities, and developmental disorder (score = 24; Figure 6f).

Regulator Network Analysis

Figure 7 shows the number of focus genes in the network relative to the IPA "Global Molecular Network." IPA identified five

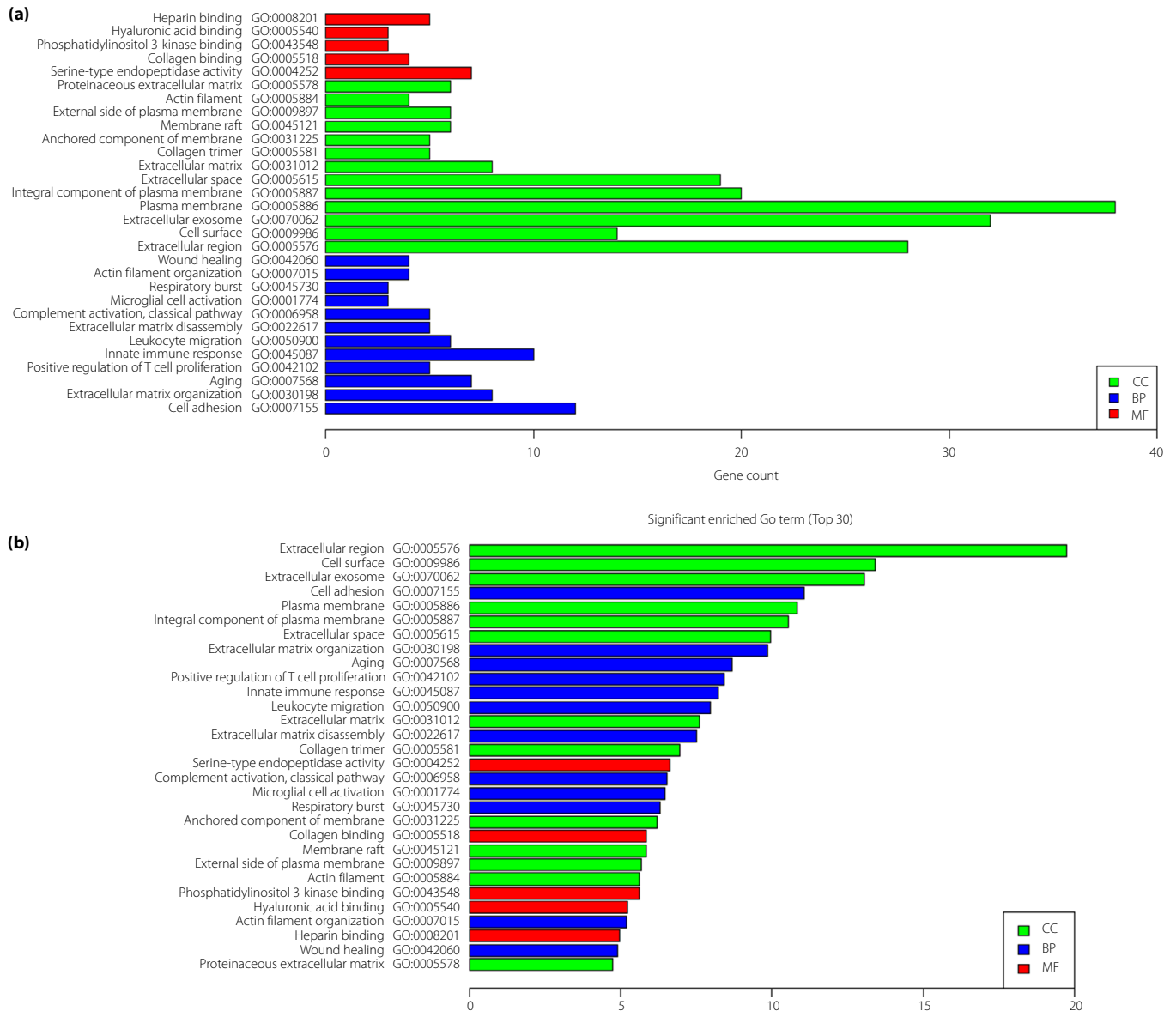


Figure 3 | Gene Ontology (GO) analysis and significantly enriched GO terms of differentially expressed genes in diabetic nephropathy. (a) GO analysis classification of differentially expressed genes in three groups. (b) Significantly enriched GO terms of differentially expressed genes in diabetic nephropathy based on function. BP, biological process; CC, cellular component; MF, molecular function.

regulator networks. Networks 4 and 5 identified the key upstream regulator ETS1, and showed a small number of genes involved in the quantity of lymphatic system cells, which included *CD44*, *EEF1D*, *IL7R*, *ITGB2*, *LCK*, *LTB* and *SELL* (Figure 7d), and tyrosine phosphorylation of protein, which includes *CD44*, *FN1*, *ITGB2*, *LCK* and *SELL* (Figure 7e). ETS1, an upstream regulator, is also evident in network 1 in Figure 7a and network 2 in Figure 7b.

Upstream Regulator Analysis

Potential upstream regulators of proteins could be identified by upstream regulator analysis. Statistically significant enrichment

of upstream regulators that were included in the regulator network analysis was carried out for DN using IPA (Table S5). For DN, positive upstream regulators included TNF, ETS1, Hbb-b2, Hbb-b1, CD2, EGF, SPI1, TCF4, PDGF, BB, PRL and TGM2. Interestingly, the only negative upstream regulator was APOE. ETS1, SPI1 and TCF4 were three transcription regulators among the 13 upstream regulators. ETS1, which had the highest activation z-score, was selected for future analysis.

Use of *db/db* Mice and *db/m* Mice for Verification

The *db/db* mouse with the BKS background comprises a type 2 diabetes model and has been shown to develop diabetic renal

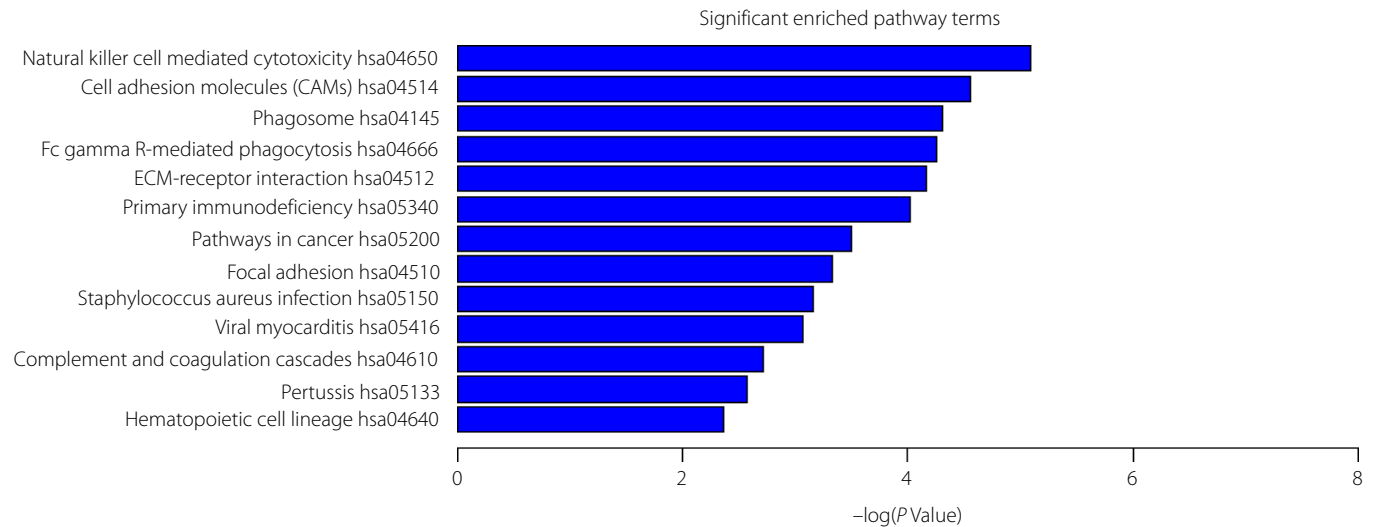


Figure 4 | Significantly enriched pathway terms of differentially expressed genes in diabetic nephropathy. Differentially expressed gene functional and signaling pathway enrichment was carried out using the following online websites: KEGG PATHWAY, Reactome, BioCyc, Panther, NHGRI and Gene Ontology.

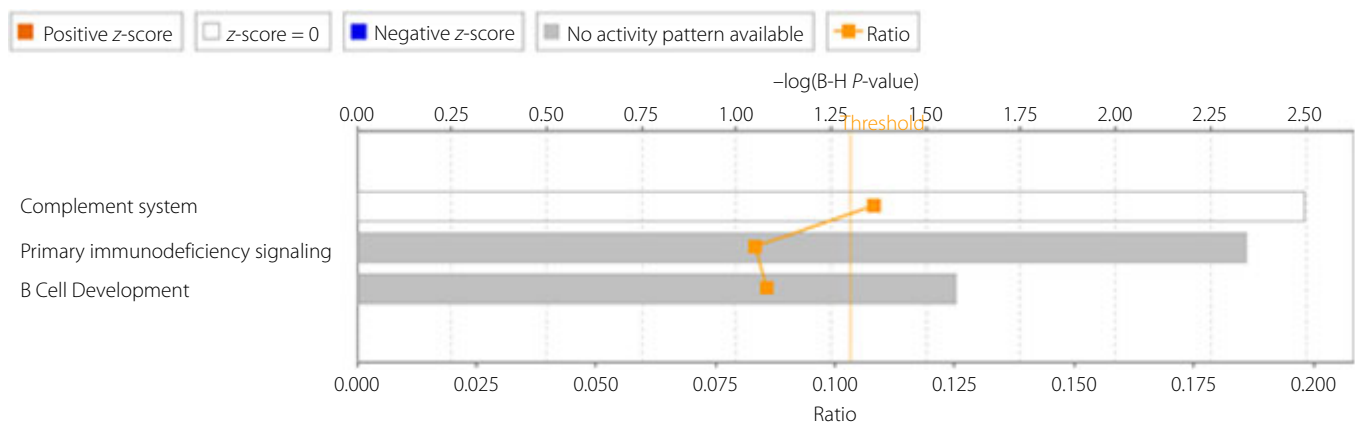


Figure 5 | Ingenuity pathway analysis shows three canonical pathways of the differentially expressed genes.

injury¹⁰. At 26 weeks-of-age, the bodyweight and blood glucose level were increased remarkably in *db/db* mice compared with *db/m* mice (Figure 8a,b). The 24-h urinary albumin and kidney weight of the *db/db* mice were also significantly higher than those of the *db/m* mice (Figure 8c,d). We used *db/db* mice to verify the DEGs (*Itgb2*, *Cd44*, *Sell*, *Fn1*, *Tgfbi*, *Il7r*, *Igfbp2* and *Cd55*) and *db/m* mice as a control. The results showed that compared with the *db/m* group, the messenger RNA expression levels of *Itgb2*, *Cd44*, *Sell*, *Fn1*, *Tgfbi* and *Il7r* were significantly upregulated. Additionally, the significantly reduced expression levels of *Igfbp2* and *Cd55* were also validated in the *db/db* mice versus the *db/m* mice (Figure 8e).

Identification of ITGB2 as the Direct Target Gene of Transcription Factor ETS1

Multiple transcription factors are activated under diabetic conditions in DN. Regulator network analysis showed that networks 4 and 5 identified the key upstream regulator ETS1. At the same time, ETS1 had the highest activation z-score in the upstream regulator analysis. We also used the TRRUST (transcriptional regulatory relationships unraveled by sentence-based text-mining, <http://www.grnpedia.org/trrust>) database to analyze the target genes of *ETS1* in humans and mice. *ITGB2*, *IL7R* and *SELL* were the common genes between the IPA upstream analysis and TRRUST database. *ITGB2*, an upregulated gene of

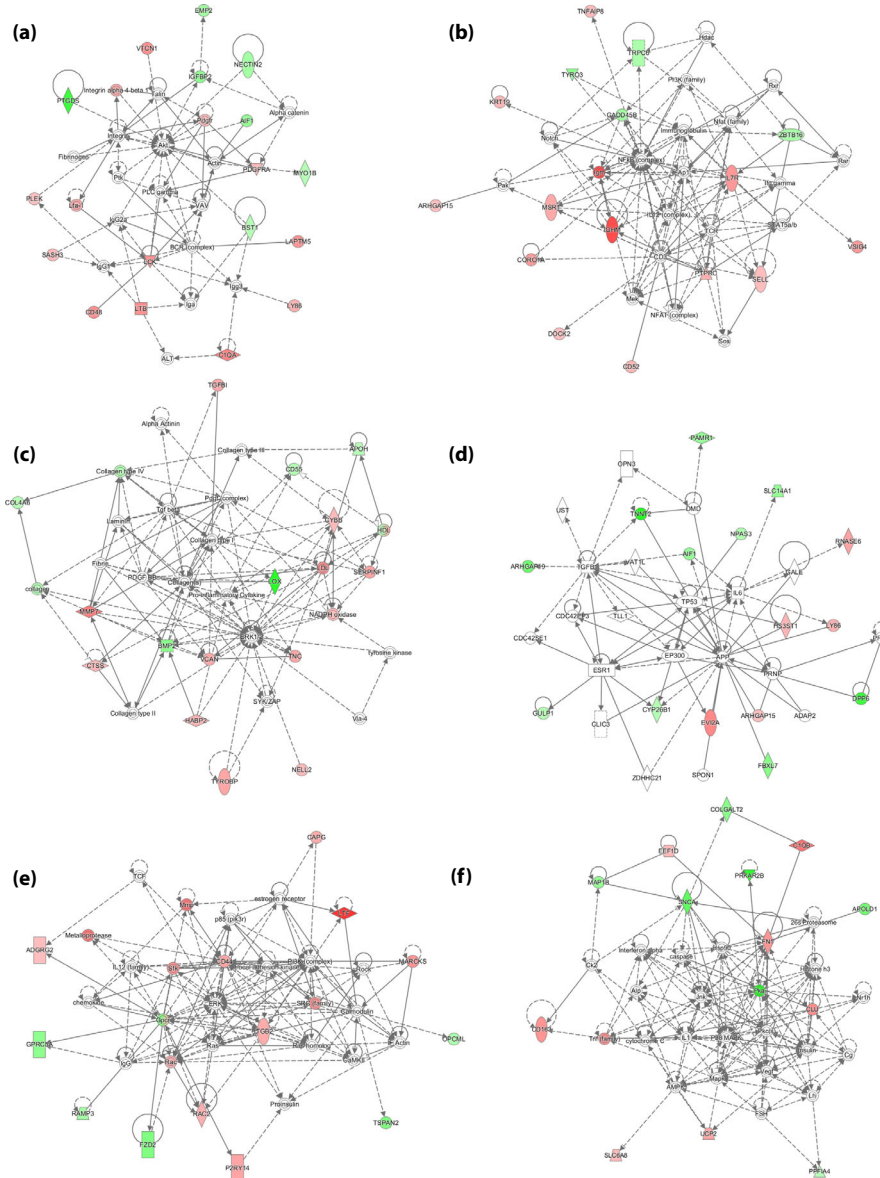


Figure 6 | Genes forming the top six gene networks, as identified by ingenuity pathway analysis (score >20).

DEGs, was included in the CAMs pathway and had functions in cell adhesion, and in cell surface and extracellular matrix organization. Therefore, we selected ETS1 and ITGB2 for future analysis.

To examine the roles of ETS1 and ITGB2 in DN, we used *db/db* mice for validation *in vivo*. Western blot analysis showed increased expression levels of ETS1 and ITGB2 in the *db/db* mice (Figure 9a). We evaluated ETS1 expression in high glucose (HG)-treated RMCs *in vitro*. After 48 h of incubation in HG medium, the protein levels of ETS1 and ITGB2 were markedly increased compared with the normal glucose medium and mannitol medium groups. We next examined whether the glucose-induced modulation of ETS1 activity was accompanied

by changes in ITGB2 expression in HG medium. We used ETS1 siRNA to knock down its protein level. The expression of ETS1 in the siRNA group was significantly lower than that of the HG and siRNA-con groups ($P < 0.05$). Additionally, the siRNA inhibition of ETS1 significantly reduced the activation of ITGB2 protein ($P < 0.05$; Figure 9b) compared with the HG alone and siRNA-con groups. We also used ITGB2 siRNA to knock down the protein expression of ITGB2 in RMCs. The expression of ITGB2 was significantly lower in the siRNA-ITGB2 group than in the HG alone and siRNA-con groups ($P < 0.05$). However, the siRNA inhibition of ITGB2 did not reduce the expression of ETS1 protein ($P < 0.05$; Figure 9c) in the siRNA-ITGB2 group.

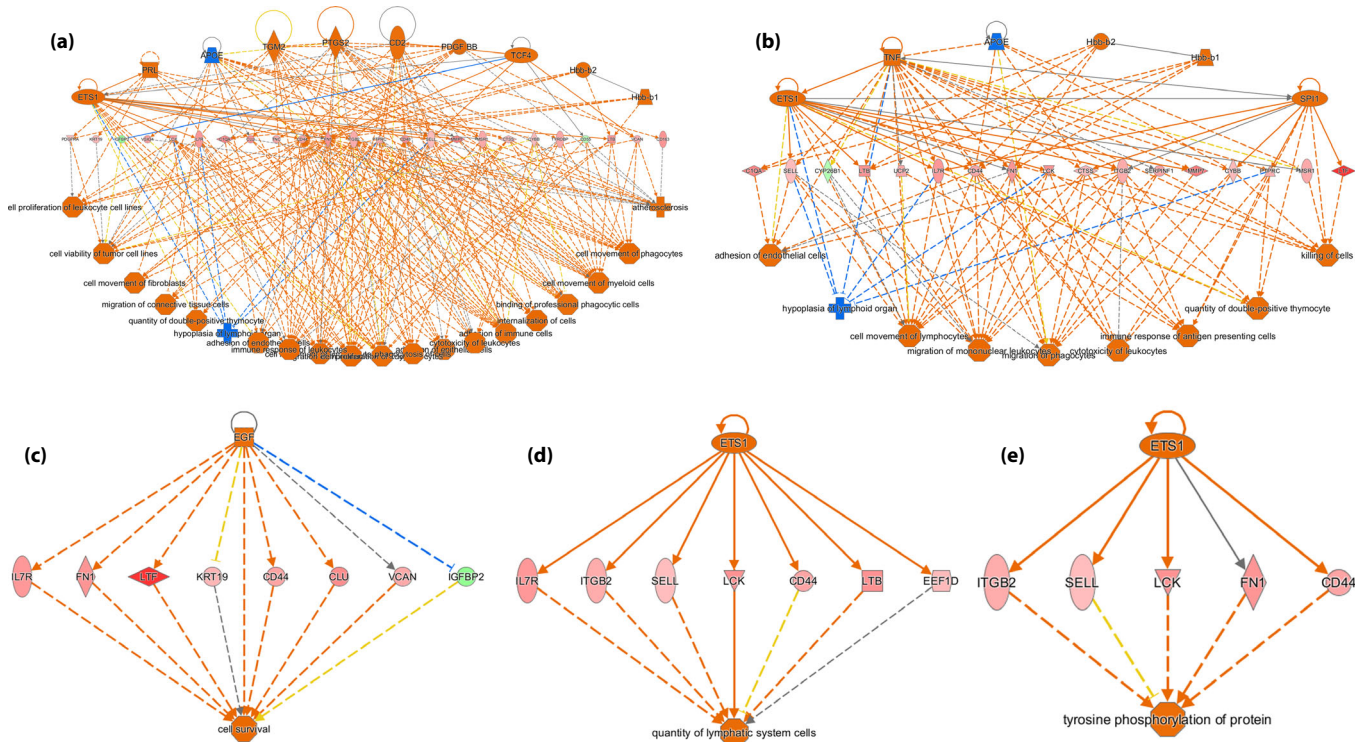


Figure 7 | Regulatory network analysis using analysis. (a–e) Regulatory networks generated in IPA for genes that were differentially expressed. Orange represents an activated upstream regulator or biological process; blue represents an inhibited upstream regulator or biological process; red indicates that gene expression is upregulated; green indicates that gene expression is downregulated.

To identify whether *ITGB2* was the direct target gene of *ETS1*, we treated RMCs with 30 mmol/L glucose (HG) or 24.5 mmol/L mannitol+5.5 mmol/L glucose as the control for 48 h. We also used *ETS1* siRNA to knock down the expression of *ETS1* in RMCs. Using a ChIP assay followed by qPCR, we tested binding of *ETS1* to the putative *ETS1*-binding sites in the *ITGB2* promoter in response to HG treatment. Compared with the control cells, *ETS1* binding to the putative *ETS1*-binding sites in the *ITGB2* promoter was significantly increased in HG-treated RMCs (Figure 9d). In contrast, this finding was not observed in the HG-treated RMCs isolated from *ETS1* knock-down cells (Figure 9e). These findings suggest that *ETS1* likely mediates the HG-induced expression of *ITGB2*-specific genes.

DISCUSSION

DN is emerging as the primary cause of end-stage renal disease worldwide¹¹. In the present research, we identify shared pathways and networks of transcriptional dysregulation in the kidney glomeruli of DN by carrying out an unbiased transcriptome comparison of glomerular gene expression in human and mouse models of DN. Signaling pathway enrichment analysis showed that CAMs and the ECM–receptor interaction are the two major pathways in the extracellular matrix category in DN. CAMs are expressed on the cell surface, and

mediate cell–cell binding and cell–extracellular matrix adhesion, recognition, activation and migration¹². There is evidence that adhesion molecules are important in the development of DN¹³. *ITGB2*, an upregulated gene among the DEGs, is involved in the CAM pathway and has functions in cell adhesion, and in cell surface and extracellular matrix organization. *ITGB2* is a protein-coding gene. *ITGB2* is the beta subunit of four different structures: LFA-1 (CD11a), macrophage-1 antigen (CD11b), integrin alphaXbeta2 (CD11c) and integrin alphaDbeta2 (CD11d). CD11b, a component of the beta2-integrin adhesion molecule Mac-1 that is expressed on neutrophils, closely relates to neutrophil adhesion. This could result in more neutrophil adherence in DN at the activated sites and provide a focus for platelet aggregation. Mac-1 blockade in a model of lung injury can reduce neutrophil accumulation and inflammatory damage, even after the injury begins¹⁴. These results indicate that an increase in cellular adhesion and the production of CAMs might lead to accelerated small vessel lesions, a process typical of DN.

Canonical pathway analysis showed that the top differentially regulated pathway in DN was the complement system. The transcript levels of the C3 complement component were increased in human DN glomeruli. The deposition of C3 in glomeruli and glomerular capillaries has been observed in KK

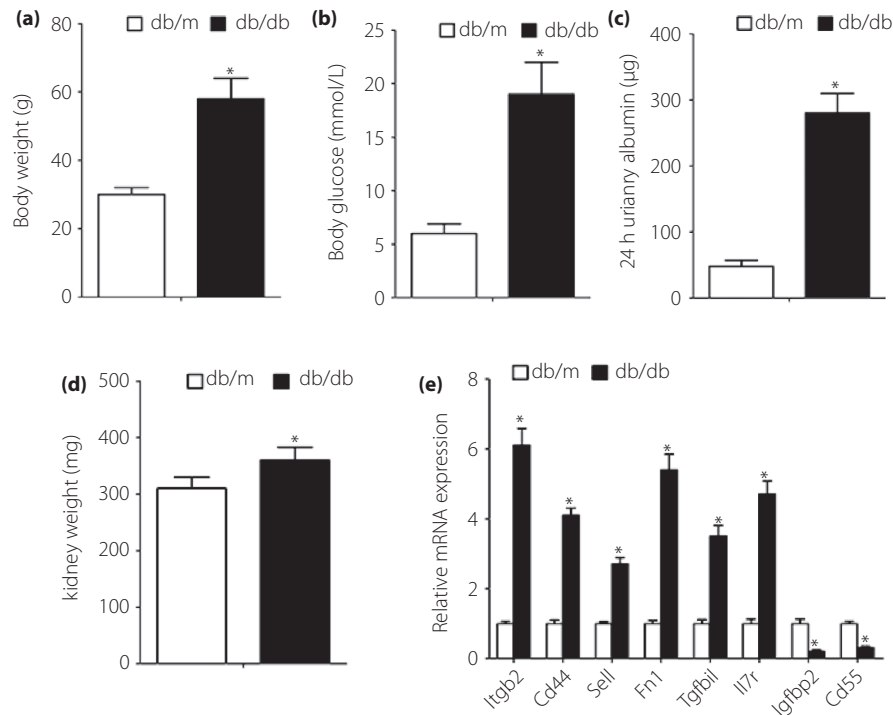


Figure 8 | *db/db* and *db/m* mouse-based verification of microarray data analysis. (a) Bodyweight, (b) blood glucose level, (c) 24-h urinary albumin and (d) kidney weight were measured in 26-week-old mice. (e) Quantitative polymerase chain reaction results showed that compared with *db/m* mice, the messenger ribonucleic acid (mRNA) expression levels of *Igf2* and *Cd55* were significantly decreased, and the mRNA expression levels of *Itgb2*, *Cd44*, *Sell*, *Fn1*, *Tgfb1* and *Il7r* were significantly increased in *db/db* mice. * $P < 0.05$ versus *db/m*; $n = 8$ per group.

mice, a model of type 2 diabetes mellitus¹⁵. Clinical studies have shown a sixfold increase in the glomerular levels of C3 in kidney biopsy samples from patients with overt diabetic kidney disease³. These results suggested that the complement system might play a functional role in DN and glomerulosclerosis.

To validate the accuracy of the DEGs identified by microarray data analysis, we used *db/db* mice and *db/m* mice for verification. We selected several genes closely related to the pathogenesis of DN for further verification by qPCR, including *ITGB2*, *SELL*, *CD44*, *FN1*, *TGFBI*, *IL7R*, *IGFBP2* and *CD55*. *CD44* and *FN1* are involved in ECM–receptor interactions. *SELL* and *ITGB2* are involved in the CAM pathway. *TGFBI* encodes a protein that is induced by *TGFβ*, plays a role in cell–collagen interactions and can be found in the gene network. *IL7R* is a molecule that is involved in both primary immune deficiency signaling and the B-cell development pathway. *IGFBP2* and *CD55* were significantly downregulated genes among the DEGs. Furthermore, *CD44*, *ITGB2*, *SELL*, *IL7R* and *FN1* had the same upstream components as transcription factor ETS1. The regulation trends of those genes were similar between the microarray and qPCR data.

Gene regulatory networks can be regarded as essential to the understanding of some diseases and might also lead to new therapeutic approaches. The present investigation showed that

ETS1 could be detected in regulatory networks 1, 2, 4 and 5. Additionally, ETS1 had the highest activation z-score in the upstream analysis. ETS1 is a member of the ETS family of transcription factors, and has functions in tumor invasion and metastasis^{16,17}. ETS1 regulates various types of genes, and plays roles in stem cell development, tumorigenesis and cell senescence or death. The multifunctional cytokine, *TGFβ*, has been implicated as a principal mediator of diabetic kidney disease^{18,19} and induces ETS1 expression²⁰. ETS1 is crucial for kidney development, glomeruli integrity and matrix metalloproteinase expression²¹. Liu *et al.*²² showed that ETS1 expression can be associated with DN progression. Decreased ETS1 expression and increased TIMP-2 expression might play an important role in the progression of glomerulosclerosis and renal interstitial fibrosis in DN.²² Expression of ETS1 in the kidney is closely related to matrix remodeling in DN. Therefore, we suspect that ETS1 plays a role in matrix remodeling by regulating matrix-degrading enzymes.

Renal expression of the ETS1 proto oncogene is closely related to matrix remodeling in DN. We further investigated the potential mechanism of ETS1 in HG-treated RMCs. ETS1 is a transcription factor²³. Previous studies have shown that HG treatment can increase the nuclear accumulation of ETS1 protein in podocytes. Additionally, blocking the ERK pathway

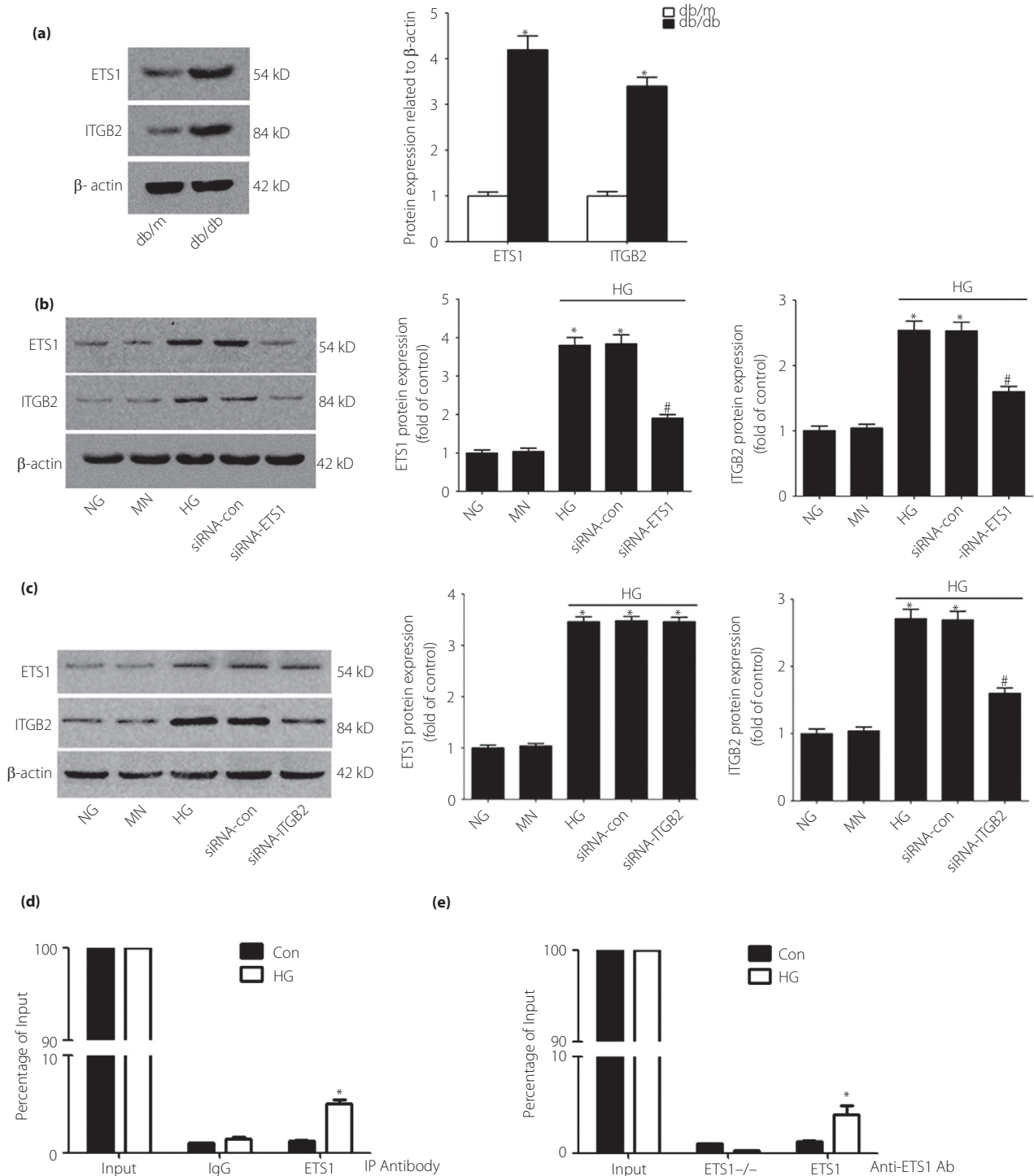


Figure 9 | Western blot analysis of the protein levels of E26 transformation-specific-1 (ETS1) and integrin subunit beta 2 (ITGB2) in *db/db* or *db/m* mice and cultured rat mesangial cells after 48 h of incubation. (a) Western blot analysis shows increased protein expression of ETS1 and ITGB2 in *db/db* mice. (b,c) Incubation with high-glucose (HG) medium increased the protein levels of ETS1 and ITGB2 compared with incubation with normal-glucose (NG) medium or mannitol control (MN) medium. The expression levels of ETS1 and ITGB2 were also detected by western blotting after inhibition of ETS1 and ITGB2 by small interfering ribonucleic acid (siRNA). Data are the mean \pm standard deviation of every experiment. * $P < 0.05$ compared with NG or MN; # $P < 0.05$ compared with HG or siRNA-con (control siRNA). (d,e) Exposure to high glucose increased the binding of ETS1 to the promoter region of *Itgb2*. Rat mesangial cells were cultured with HG or 24.5 mmol/L mannitol + normal glucose (control [Con]) (d) without ETS1-siRNA or (e) with ETS1-siRNA to knock down ETS1, and proteins were extracted for chromatin immunoprecipitation assay analysis ($n = 3$, * $P < 0.05$ vs control).

can suppress the nuclear accumulation of ETS1 in podocytes²⁴. The upstream regulator analysis showed that ETS1 is the upstream regulator of ITGB2. We further examined the relationship between ETS1 and ITGB2. *In vivo*, the ETS1 and ITGB2 levels are significantly increased in diabetic mice. *In vitro*, we showed that HG treatment upregulates the expression levels of ETS1 and ITGB2. Furthermore, inhibiting ETS1 significantly reduces the HG-induced expression of ITGB2, but inhibiting ITGB2 does not reduce the protein expression of ETS1 in HG-induced mesangial cells. This suggests that ETS1 plays a crucial role in the process. Our upstream regulator analysis shows that the ETS1 transcription regulator is the upstream regulator of ITGB2. According to the ChIP-qPCR analysis, ETS1 binding to the promoter regions of ITGB2 was increased in HG-treated renal mesangial cells. We conclude that HG stimulates the expression of ETS1 and ITGB2 through the binding of ETS1 directly to the promoter of ITGB2. These findings might indicate that ETS1 is a novel transcriptional regulator in DN.

In summary, the present data provide a comprehensive bioinformatics analysis of DEGs that might be related to the progression of DN. We have identified 89 candidate DEGs with multiple cohort profile datasets and integrated bioinformatics analyses. A variety of new genes and pathways might be involved in the pathogenesis of DN. We also conclude that ETS1 binding to the promoter regions of ITGB2 is increased in HG-treated RMCs. These findings could lead to a significant increase in our understanding of the etiology and underlying molecular events of DN.

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DISCLOSURE

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1 | Primer sequences used in real-time polymerase chain reaction.

Table S2 | Signaling pathway enrichment analysis of differentially expressed gene function in diabetic nephropathy.

Table S3 | Three canonical pathways identified by ingenuity pathway analysis.

Table S4 | Top 10 biological functions and diseases identified through ingenuity pathway analysis.

Table S5 | Upstream regulators identified through ingenuity pathway analysis.