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Identification and Validation of Disulfidptosis-Related Genes in DKD via Integrative Machine Learning and Single-Cell Transcriptomics

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ABSTRACT

Background: The global occurrence of diabetes is steadily increasing, with DKD representing a major and severe complication arising from this condition. Disulfidptosis is a recently identified form of programmed cell death strongly associated with various diseases. The objective of this investigation was to identify hub genes related to disulfide in DKD and to reveal their potential functions via integrative bioinformatic analyses.

Method: To screen for DEGs that are related to DKD, we obtained transcriptomic profile data from GEO. GO, KEGG, and GSEA functional enrichment analyses revealed that the DEGs were associated with cellular immunity, cytoskeletal organization, and ferroptosis. Through the joint application of WGCNA, differential expression, PPI network, and machine learning algorithms, we pinpointed hub genes that exhibit strong diagnostic capability. The diagnostic performance and predictive accuracy of these genes were evaluated using ROC curves, a nomogram model, and the Nephroseq database. Immune infiltration analysis and single-cell RNA sequencing were employed to investigate the effects of hub genes on immune and renal cells involved in DKD progression. The protein and mRNA expression of the hub genes were subsequently corroborated through Western blotting and quantitative real-time

PCR assays. The functional role of PDLIM1 in disease pathogenesis was assessed through CCK-8 viability assays, siRNA-mediated knockdown, and DTT-based rescue experiments.

Results: Four hub genes were ultimately identified via the combined use of differential expression analysis, WGCNA, PPI network analysis, and machine learning: DSTN, RAC1, ACTB, and PDLIM1. AUC for each of the four genes exceeded 0.60, and calibration curves showed a high degree of agreement between predicted and observed risks, indicating robust diagnostic performance. Inverse correlations with GFR were found for all four genes through correlation analysis, PDLIM1 was positively correlated with serum creatinine. Significant correlations between these genes and diverse immune cell subsets were observed via immune infiltration analysis. Cell-type-specific expression profiles in proximal tubular cells and macrophages were uncovered through single-cell analysis, with enhanced signaling between macrophages and neutrophils. Experimental validation confirmed the association of the four hub genes with DKD severity.

Conclusion: These findings identify four hub genes as promising DKD biomarkers. Notably, PDLIM1 may contribute to DKD pathogenesis by modulating SLC7A11-mediated disulfide stress, and its upregulation appears to represent a pathogenic driver rather than a compensatory protective response.

Introduction

Globally, diabetes burden is escalating rapidly. In 2019, the International Diabetes Federation (IDF) reported global diabetes prevalence at roughly 463 million, with projections suggesting that this figure will climb beyond 700 million by 2045.¹ For patients with diabetes, the risk of developing diabetic kidney disease (DKD) is 30% for type 1 and can be as high as 50% for type 2.²

As a leading cause of end-stage kidney disease (ESKD), DKD also represents a major microvascular complication of diabetes.³⁻⁶ Hypertension, proteinuria, and progressive deterioration in renal function are its principal manifestations. The prognosis for DKD patients remains poor. In 2021, DKD accounted for 477,300 deaths globally,⁷ representing 30%–40% of all ESKD etiologies.⁸ Beyond the high mortality, DKD progression imposes a substantial burden on healthcare systems, as ESKD patients typically require long-term and costly renal replacement therapy.⁹ Despite extensive research, early clinical manifestations of DKD are often insidious, presenting only as subtle proteinuria¹⁰. Consequently, reliable biomarkers for early diagnosis or prognostic prediction are still lacking.

Liu et al. proposed that, under glucose-deprived conditions, In SLC7A11-high cells, aberrant disulfide buildup triggers a distinct cell

death process, termed disulfidptosis, which is clearly separable from both ferroptosis and apoptosis.¹¹ Elevated SLC7A11 accelerates NADPH consumption, leading to NADPH/NADP⁺ imbalance, disulfide stress, and ultimately cytoskeletal collapse and cell death.^{12,13} Given its relationship with disulfidptosis and glucose metabolism, we hypothesized that disulfidptosis-related genes may be involved in diabetes and its complications. DKD pathogenesis has been linked to oxidative stress, hypoxia, apoptosis, inflammation, ferroptosis, and pyroptosis in previous studies.¹⁴⁻¹⁶ As a novel cell death modality, disulfidptosis shares mechanistic links with ferroptosis,¹⁷ suggesting a potential connection with DKD.

In the study, gene expression differences between normal and DKD samples were examined using the GEO database, multiple machine learning algorithms were employed to identify key genes and establish a predictive model, and immune infiltration along with single-cell analyses were carried out to investigate the potential roles of disulfidptosis-related genes in DKD from both cellular and intercellular communication perspectives. Together, these findings point to shared regulatory mechanisms and potential therapeutic targets for DKD.

2. Materials and methods

2.1 Data collection and proccession

Three raw datasets (GSE96804, GSE30122, and GSE30528) were retrieved from the Gene Expression Omnibus (GEO) database via the “GEOquery” R program. They include gene expression profiles from DKD and control samples: GSE96804 includes 41 DKD samples and 20 control samples, GSE30122 contains 19 DKD samples and 50 control samples, and GSE30528 consists of 9 DKD and 13 control samples. Additionally, single-cell RNA sequencing data encompassing 6 immune cell types and 10 kidney cell types were obtained from the dataset GSE209781.

2.2 Screening and Functional Enrichment Analysis of Differentially Expressed Genes

Three datasets were merged into Merge-GSE, comprising 60 DKD samples and 70 control samples. Differential gene analysis was conducted using the R package “limma”. Volcano plots were constructed using the ggplot2 package, and genes with $P\text{-value} < 0.05$ along with $|\log_2 \text{FC}| > 0.75$ were regarded as statistically significant¹⁸. Gene set enrichment analysis (GSEA), Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis, and Gene Ontology (GO) enrichment analysis were conducted using “clusterProfiler”, with Benjamini–Hochberg adjusted P values < 0.05 considered significant. The resulting DEGs were then intersected with a previously reported set of 24 disulfidptosis-related genes¹¹.

2.3 Weighted correlation network analysis (WGCNA)

Based on the constructed WGCNA, co-expressed gene modules associated with DKD were identified. First, based on the pickSoftThreshold function and scale-free topology criterion, we set the soft threshold at 14 and built a weighted adjacency matrix. The resulting data were converted into a topological overlap matrix (TOM), and module determination was subsequently performed using the dynamic tree-cut algorithm with a module size cutoff of 50. Modules with similarity ≥ 0.7 were merged, and unassigned genes were grouped into the gray module. Pearson correlation was calculated between module eigengenes and DKD phenotypes to identify trait-associated modules. The correlation between gene modules and patients with DKD was subsequently evaluated using Gene significance (GS) and module membership (MM) values.

2.4 Screening of Hub Genes and Functional Enrichment Analyses

The obtained genes were intersected with the 24 disulfidptosis-related genes and the DEGs, yielding 11 key genes, as shown in a Venn diagram. Subsequently, a protein–protein interaction (PPI) network was generated via the STRING database, and hub genes were predicted and visualized with Cytoscape (v3.9.0). GO and KEGG enrichment analyses further highlighted the functional roles of these

genes in DKD.

2.5 Integrating machine learning models to screen Hub Genes

The LASSO regression approach, namely the least absolute shrinkage and selection operator, is commonly applied in fitting generalized linear models. It enables variable selection and complexity adjustment, thereby improving prediction accuracy and identifying important feature variables. Using this method, eight genes were screened. Random Forest (RF), a randomization algorithm that generates multiple decision trees to enhance predictive accuracy and prevent overfitting, was used to select the top five most important feature genes. The genes identified as important by both methods were retained as core diagnostic genes for DKD.

2.6 Validation of Hub Genes

The expression profiles of the four hub genes were evaluated via an unpaired two-sample t-test applied between the Merge-GSE training cohort and the GSE30529 validation cohort. To verify the accuracy of the hub genes, receiver operating characteristic (ROC) curves were plotted using the 'pROC' package and visualized using the 'ggplot2' package. A larger area under the curve (AUC) indicates greater diagnostic accuracy. A nomogram model was then constructed using version 6.5.0 of the rms R package, and calibration and decision

curves were generated to further assess the nomogram's predictive capability. Using the Nephroseq v5 database, we analyzed target gene expression in DKD datasets and calculated correlations with GFR using the built-in "Gene Summary View" module, outputting Pearson coefficients and P values. Collectively, these analyses corroborated the diagnostic potential of the hub genes in DKD.

2.7 Immune infiltration analysis

Gene expression data were deconvoluted using CIBERSORT to evaluate immune microenvironment variations between DKD and control groups. Using ggplot2 violin plots, we visualized the disparities in immune infiltration between the DKD and control groups, based on the relative abundances of 22 immune cell types derived from the infiltration matrix. P-value < 0.05 was considered statistically significant. Immune cell correlation patterns were presented in a heatmap constructed with the corrplot package, while violin plots generated via ggplot2 illustrated the relationships between hub genes and immune cells. A P-value threshold of <0.05 was adopted to denote statistical significance.

2.8 Single-cell RNA-seq data acquisition and processing

Single-cell RNA-seq transcriptomic profiles (GSE209781) were obtained from GEO and subsequently processed using the Seurat

package (v4.4.1). After filtering cells with 200–7,000 UMIs and mitochondrial UMI proportions below 20%, as well as removing genes detected in fewer than three cells, we selected 3,000 highly variable genes (HVGs) for subsequent downstream analyses. Cells were clustered using the “Findneighbors” and “FindClusters” functions, and the results were visualized using t-SNE. Cell types were annotated according to the classification criteria provided in the original study, in order to facilitate interpretation of the results. Cell–cell interaction analysis was performed using CellChat (version 2.1.2). The “FindMarkers” function was used to assess differential expression between groups, with the default parameters selected to identify group-specific changes.

2.9 Verify the expression of the target gene using real-time PCR

The human renal tubular epithelial cell line HK-2 was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and subsequently maintained in low-glucose DMEM containing 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, at 37 °C in a humidified incubator with 5% CO₂ and 95% air. Medium was refreshed or cells were passaged at 2- to 3-day intervals. Once cells had reached 70–80% confluence, the medium by incubating them in a medium containing 1% FBS for 24 hours prior to the experiment. High glucose (HG) concentration was selected based on our previous experiments

and the literature to mimic hyperglycemic effects under diabetic pathological conditions¹⁹. Total RNA was isolated with TRIzol reagent (Invitrogen, 15596018). Following quantification and quality assessment, the RNA was converted to cDNA using HI Script III RT SuperMix (Vazyme, R323-01). Quantitative PCR was then carried out with SYBR Green master mix (Vazyme, Q711-02), and the expression of target genes was standardized against Tubulin as the reference. The thermal cycling profile comprised an initial 95 °C for 30 s, then 40 cycles at 95 °C for 10 s and 60 °C for 30 s. The primers used for DSTN were as follows: forward primer, GGCACCAGAACTAGCACCTC , reverse primer, AGCCCGATTGAGATCTTCTGG; the primers used for RAC1 were as follows: forward primer , TGTCCCAACACTCCCATCATCC, reverse primer, CAGCACCAATCTCCTTAGCCATG; the primers used for PDLIM1 were as follows: forward primer, CCACATCCTTCCTGGTTCTG, reverse primer, TGGTGATCCCTCAGCTTCAC; the primers used for ACTB were as follows: forward primer, TGAGCTGCGTTTTACACCCT, reverse primer, CGCCTTCACCGTTCCAGTTT), with amplification efficiencies ranging from 90% to 110% for all primer pairs.

2.10 Cell Viability Assay

At a density of 2×10^4 cells/mL, HK-2 cells were plated into 96-well plates, with 100 μ L of medium added to each well. Following 48 h of

cultivation, 10 μ L of CCK-8 solution was introduced per well as specified by the Cell Proliferation and Cytotoxicity Assay Kit protocol, and the plates were then maintained at 37 °C for 2 h. Absorbance at 450 nm was subsequently recorded with a microplate reader, and cell viability was derived from these measurements.

2.11 Western blot analysis

Protein was isolated with RIPA buffer (Beyotime, #P0013B) containing phosphatase/protease inhibitors. Following 30 min on ice with vortexing every 10 min, lysates were centrifuged at 12,000 $\times$ g for 15 min at 4 °C, and supernatants were quantified via BCA assay (Solarbio, #PC0020). Equal protein amounts (30 μ g/lane) were resolved by 10 % SDS-PAGE and transferred onto PVDF membranes (wet transfer, 300 mA, 90 min, ice bath). Membranes were blocked with 5 % non-fat milk in TBST for 2 h at room temperature, then probed with primary antibodies overnight at 4 °C. After three 10-min TBST washes, HRP-conjugated secondary antibodies (1:5000) were applied for 1 h at room temperature, followed by additional washes, ECL detection, and imaging. Densitometry was performed with ImageJ, using Tubulin as loading control. For non-reducing Western blotting, samples were mixed with LDS sample buffer (Beyotime, #P0731) without reducing agents, divided into two aliquots—one for non-reducing analysis and the other supplemented with

2-mercaptoethanol (2-ME, Beyotime, #P0733) to 2 % as reducing control—and heated at 70 °C for 10 min. All subsequent electrophoresis, transfer, and immunoblotting steps followed the same protocol as above. Primary antibodies used for WB included DSTN (Cat No. 10962-2-AP), RAC1 (Cat No. 24072-1-AP), PDLIM1 (Cat No. 11674-1-AP), ACTB (Cat No. 66009-1-Ig), Tubulin (Cat No. 11224-1-AP), and SLC7A11 (Cat No. 26864-1-AP).

2.12 PDLIM1 Knockdown

For transfection, HK-2 cells were plated in 6-well plates and grown overnight until reaching 60%–70% confluence. The PDLIM1-targeting siRNA (si-PDLIM1) and its negative control (si-NC) were synthesized according to previously validated sequences (reference20). Transfections were carried out with Lipofectamine™ 3000 (Invitrogen, Carlsbad, CA, USA) per the manufacturer's instructions, with the final siRNA concentration adjusted to 50–100 nM. Cells were collected 48–72 h post-transfection for downstream assays.

2. 13 Statistical analysis

All statistical analyses were conducted using R software (version 4.1.1) and SPSS (version 26.0). Comparisons between groups were performed using Student's t-test or one-way ANOVA (with Dunnett ' s multiple comparison test). For non-normally

distributed data, Wilcoxon rank-sum test was applied. P-value<0.05 was considered statistically significant. No-template controls (NTC) were included in each run to exclude reagent contamination; all samples were run in triplicate wells.

3. Results

The complete analytical procedure is depicted in Figure 1. Three datasets, GSE96804, GSE30122, and GSE30528, were combined into a merged cohort (Merge-GSE), with batch effects removed via the "sva" package (Figures 2A-B). Differential expression analysis using the "limma" package yielded 2,283 DEGs (995 down-regulated and 1,288 up-regulated), as visualized in the volcano plot of Figure 2C. To investigate the potential functions of these DEGs, we conducted GO, KEGG, and GSEA enrichment analyses within the R. GO enrichment analysis revealed that the biological processes (BP) of DEGs were associated with humoral immune response, response to metal ion, cell-substrate adhesion, cell surface receptor signaling pathway via JAK-STAT, positive regulation of MAPK cascade, and focal adhesion. For cellular components (CC), they were linked to the cell-substrate junction, secretory granule lumen, phagocytic vesicle, actin-based cell, and ubiquitin protein ligase binding projection. For molecular functions (MF), they were related to the structural constituent of cytoskeleton, cytokine receptor binding, TAP binding, and cadherin

binding involved in cell-cell adhesion structural (Figure 2D). The GSEA enrichment analysis indicated that the DEGs were associated with Mitophagy – animal, NOD-like receptor signaling pathway, IgSF CAM signaling, Ferroptosis, Adherens junction, Th17 cell differentiation, Leukocyte transendothelial migration, Chemokine signaling pathway, cAMP signaling pathway, and Calcium signaling pathway (Figure 2E). KEGG enrichment analysis indicated that the DEGs were involved in Focal adhesion, cAMP signaling pathway, Regulation of actin cytoskeleton, JAK-STAT signaling pathway, AGE-RAGE signaling pathway in diabetic complications, Cell adhesion molecules, Th17 cell differentiation, Renin-angiotensin system, Regulation of actin cytoskeleton, Ferroptosis, ECM-receptor interaction (Figure 2F).

3.2 Co-Expression Network of DKD-Related Genes

To screen for crucial genes relevant to the DKD phenotype, WGCNA was applied to both normal and DKD samples. Hierarchical clustering of the Merge-GSE was then used to remove outlier samples (Figure 3A). The soft-thresholding power was selected as 14, according to the scale-free topology fit and average connectivity (Figure 3B). Dynamic module identification was then performed, requiring each module to contain at least 50 genes (Figure 3C). The grey module, which had a low correlation, was excluded. A total of 4 modules were identified (Figure 3D). The turquoise module was positively associated with DKD

(cor = 0.22, P = 0.01). Applying MM > 0.4 and GS > 0.1 as cutoffs, 4,414 genes within this module were deemed candidate DKD-associated genes (Figure 3E).

3.3 Identification and functional enrichment of key genes

Intersection of DKD-related genes, DEGs, and disulfidptosis-related genes yielded 11 core genes (Figure 4A): DSTN, RAC1, RPN1, NCKAP1, ACTB, PRDX1, CD2AP, MYL6, PDLIM1, ACTN4, and TM9SF2. CytoHubba plug-in software in Cytoscape predicted 10 key genes from the PPI network (Figure 4B), displayed as a heatmap (Figure 4C). The ssGSEA was carried out based on these 10 key genes, and the subsequent GO and KEGG enrichment analyses employed the top 200 genes most closely associated with them (Figure 4D), highlighting pathways related to actin cytoskeleton regulation, cAMP signaling, leukocyte transendothelial migration, antigen processing and presentation, and cytoskeletal functions. KEGG enrichment analysis revealed multiple signaling pathways, including regulation of actin cytoskeleton, cAMP signaling pathway, leukocyte transendothelial migration, neuroactive ligand-receptor interaction, antigen processing and presentation, and Shigellosis (Figure 4E). Significant GO terms among BP, CC, and MF included structural constituent of cytoskeleton, cortical cytoskeleton, cytokinetic process, regulation of actin cytoskeleton organization, cytoskeleton-dependent cytokinesis,

and regulation of DNA damage response (Figure 4F).

3.4 Identification and Validation of DKD Hub Genes

Overlapping genes were screened using LASSO and RF machine-learning algorithms. LASSO identified 8 genes (Figure 5A-5B). RF selected the optimal fitting model from 154 models (Figure 5C-5D), and genes with scores >5 were selected based on importance scores (Figure 5E), identifying 5 genes. The two methods intersected to yield 4 hub genes: DSTN, RAC1, ACTB, and PDLIM1 (Figure 5F). Consistently upregulated transcript levels of the four hub genes were observed in DKD samples from both the Merge-GSE training cohort and the GSE30529 validation cohort (Figure S3). AUC was calculated for each of the four hub genes to evaluate their clinical diagnostic performance. For the Merge-GSE training set, the corresponding values of DSTN, RAC1, ACTB, and PDLIM1 were 0.78, 0.78, 0.81, and 0.86, in that order (Figure 5G). In the external validation cohort GSE30529, the values of the four hub genes were all greater than 0.6 (Figure S3E). A nomogram model was developed to predict DKD diagnosis (Figure 5I). The calibration curve demonstrated negligible discrepancy between observed and predicted risks, reflecting the nomogram's excellent predictive accuracy (Figure 5H). The DCA curve indicated a high net benefit of the model (Figure 5J). Expression of all four genes was negatively correlated with GFR (Figures S1A-D), and PDLIM1

expression was positively correlated with serum creatinine (Figure S1E), supporting their association with renal function decline.

3.5 Immune infiltration analysis and correlation analysis of hub genes with infiltrating immune cells.

Using the CIBERSORT algorithm, we found marked disparities in immune infiltration between DKD and controls (Figure 6A). DKD patients had increased Monocytes ($p < 0.05$) and Macrophages M2 ($p < 0.001$), but decreased T cells follicular helper ($p < 0.05$) compared with the control group (Figure 6B). For further characterization of these distinct immune subsets, pairwise correlation analysis revealed that monocytes were inversely correlated with follicular helper T cells and positively associated with macrophages (Figure 6C). These findings suggest that myeloid cells, represented by Macrophages and Monocytes, play an important role in DKD pathogenesis. Correlation analysis further revealed interactions between different cell types.

Immune infiltration analysis of hub genes revealed that DSTN, RAC1, ACTB, and PDLIM1 are associated with specific immune cells (Figure 6D). For the four hub genes, ACTB, DSTN, and RAC1 consistently showed positive correlations with the infiltration of Macrophages M2 and Monocytes, and inverse correlations with follicular helper T cells. In contrast, PDLIM1 was positively linked only to Macrophages M2,

while also exhibiting a negative correlation with follicular helper T cells (Figure 7A-7O).

3.6 Single-cell sequencing analysis of hub gene expression

To examine the relationship between hub genes and immune cells, we mapped four hub genes to a refined single-cell RNA sequencing dataset from the public GSE209781 cohort, which includes three control samples and three DKD samples. Based on marker gene expression, the cells were classified into 16 clusters (Figure 8A), comprising ten renal cell types (distal convoluted tubules-loop of Henle [DCT-LOH], proximal tubules [PT], distal convoluted tubules [DCT], podocytes, fibroblasts [FIB], endothelial cells [EC], loop of Henle [LOH], collecting duct [CD]-principal cells [PC], CD-intercalated cells [IC], and mesangial cells [MC]) and six immune cell types (B cells, macrophages, monocytes, mast cells, neutrophils, and T natural killer cells). The marker genes are shown in Figure 8B. We then quantified cell–cell communication across all subtypes (Figure 8C). Compared with the control group, the disease group exhibited markedly greater communication counts and interaction intensities (Figure 8D), with the largest increases between neutrophils and monocytes (Figure 8E–8F). These findings highlight neutrophils and monocytes as key interacting immune cells in DKD-associated aberrant glucose metabolism. The SPP1-CD44 pathway was identified as a major signaling axis

mediating interaction between proximal tubule cells and monocytes. Relative to the control group, PT cells and monocytes from the disease group exhibited markedly elevated transcript levels of the four hub genes (Figure 8G-8H), which aligned with the bulk RNA-seq results. Collectively, these data point to critical cell populations and common pathways that may drive DKD initiation and progression.

3.7 Expression validation of hub genes and preliminary functional exploration of PDLIM1

The mRNA levels of DSTN, RAC1, PDLIM1, and ACTB were found to be upregulated in the DKD cell model relative to the control group (Figure 9A), which was established to verify the reliability of core gene expression via RT-qPCR and Western blotting. However, at the protein level, DSTN, RAC1, and PDLIM1 were upregulated, while ACTB showed no significant change (Figure 9B-9F). These findings indicate that DSTN, RAC1, and PDLIM1 may be key regulators in DKD. Given the superior clinical diagnostic performance of PDLIM1 and its dual role in inflammation regulation, we further knocked down PDLIM1 to determine whether its upregulation in DKD reflects a compensatory protective response or directly contributes to pathological injury. CCK-8 cell viability assay revealed that high-glucose stimulation significantly suppressed cell viability

compared with the normal-glucose control group, whereas PDLIM1 knockdown reversed the high-glucose-induced reduction in cell viability (Figure 10C). Concurrently, Western blot results demonstrated that SLC7A11 and disulfide-crosslinked proteins were upregulated following high-glucose stimulation (Figure 10A). At the same time, PDLIM1 knockdown downregulated their expression (Figure 10B), suggesting that PDLIM1 may regulate the disulfidptosis-related pathway. In addition, experiments using the disulfide-reducing agent DTT showed that DTT treatment reversed the high-glucose-induced reduction in cell viability (Figure 10C), indicating that the cell injury observed under high-glucose stimulation may be partially attributed to aberrant intracellular disulfide bond accumulation.

4. DISCUSSION

Diabetes mellitus results from defects in insulin secretion or action and is classified into four main types, with type 2 diabetes being the most prevalent.²⁰ The disease is characterized by a chronic course, high prevalence, and substantial healthcare costs. As of 2021, the global prevalence of diabetes stood at 10.5% (536.6 million), and it is forecasted to climb to 12.2% (783.2 million) by 2045.²¹ As the most frequent microvascular complication of diabetes and a major

contributor to ESKD, diabetic nephropathy has been widely investigated. Early studies have implicated oxidative stress, hypoxia, apoptosis, inflammation, ferroptosis, and pyroptosis in DKD pathogenesis.¹⁴⁻¹⁶ However, current biomarkers remain insufficient for early diagnosis and prognostication. Given the emerging link between disulfidptosis and glucose metabolism, we explored the association between disulfidptosis-related genes and DKD using public databases.

In this study, we analysed multiple transcriptomic datasets and identified 2,283 DEGs. Integrating WGCNA with the transcriptomic datasets yielded 4,414 genes. By integrating WGCNA and disulfidptosis-related genes, we identified 11 key genes and predicted 10 genes from the PPI network. Functional enrichment analysis showed that actin cytoskeleton organisation, enzyme activity, and cell division were prominent, with regulation of actin cytoskeleton, cAMP signaling pathway and leukocyte transendothelial migration emerging as key pathways. These results indicate that inflammation and cytoskeletal dynamics are central to DKD pathogenesis.

Through the application of two machine-learning models, we ultimately identified DSTN, RAC1, ACTB, and PDLIM1 as the four most critical genes associated with diabetic nephropathy, and all exhibited upregulated expression. ROC analyses revealed AUC values exceeding 0.60 for these genes, suggesting favorable diagnostic utility. Moreover,

calibration plots and decision curve analysis (DCA) indicated that the predictive model offers both high accuracy and clinical benefit. In patient samples, all four genes showed inverse correlations with GFR, while PDLIM1 was positively associated with serum creatinine levels. Functionally, these genes are involved in inflammation, apoptosis, oxidative stress, and cell-cycle regulation—processes tightly linked to DKD pathogenesis. Single-cell transcriptomic data confirmed that the expression patterns of these four genes were consistent with those from bulk RNA sequencing. Collectively, these findings suggest that the four genes could serve as novel candidate biomarkers for DKD, meriting further exploration.

RAC1, a Rho GTPase family member,²² is upregulated under high-glucose conditions, activating downstream signaling pathways involved in glomerular cell injury and apoptosis.²³ It mediates crosstalk between AGE-RAGE and mineralocorticoid receptor, promoting ROS production and inflammation²⁴. RAC1 inhibition alleviates podocyte injury and tubulointerstitial fibrosis in DKD²⁵. Ying et al. subsequently reported that suppression of RAC1 results in NLRP3 inflammasome inhibition in the context of diabetic kidney disease.²⁶ ACTB, encoding the cytoskeletal housekeeping protein β -actin, regulates cell growth and migration.²⁷ Its malonylation at K61 is decreased in ESKD patients, suggesting its potential as a biomarker for disease progression.²⁸ DSTN,

an F-actin depolymerizing factor²⁹, modulates the ratio of monomeric actin to filamentous actin fragments by binding to F-actin^{30,31}, thereby regulating cell shape, motility, and sarcomere function in myocytes, as well as influencing nuclear size, shape, and polarity³². Targeting SNRK-DSTN interaction may reverse pathological cardiac hypertrophy³³. PDLIM1, a member of the actin-associated LIM protein (ALP) subfamily within the PDZ-LIM protein family³⁴, contains both a PDZ domain and a LIM domain³⁵. Recruits diverse proteins and participates in cytoskeletal assembly, adhesion, and migration.^{36,37} The transcription factor NF- κ B comprises dimeric proteins consisting of p65 (RelA), NF- κ B1 (p50/p105), NF- κ B2 (p52/p100), c-Rel, and RelB.³⁸ Following the breakdown of its suppressor I κ B, NF- κ B migrates into the nucleus to trigger the transcription of target genes that mediate inflammatory reactions. Serving as an essential mediator of p65 nuclear translocation, PDLIM1 thus modulates the NF- κ B signaling pathway.³⁹ Mice deficient in PDLIM1 exhibit elevated nuclear p65 levels, resulting in upregulated production of proinflammatory cytokines and chemokines (e.g., IL-6, TNF α , IL-18, and CXCL2), suggesting that PDLIM1 negatively modulates NF- κ B-mediated inflammatory pathways.⁴⁰ PDLIM1 is upregulated in fibrotic liver and TGF- β -treated hepatic stellate cells (HSCs), and its knockdown downregulates inflammation-related genes⁴¹. Overall, the present data indicate that

PDLIM1 may play a bifunctional regulatory role in inflammatory responses. However, contributions of PDLIM1 and DSTN to DKD have not been specifically elucidated, and further investigation into their precise functions and mechanisms is warranted.

Metabolic dysfunction, chronic inflammation, and immune responses are now widely regarded as contributing factors to the multifactorial disease DKD.⁴² While previous studies focused on podocyte injury, recent evidence highlights innate and adaptive immunity.⁴³ In diabetic patients, activated macrophages infiltrate the kidneys and trigger cytokine storms that cause tubular injury and fibrosis.^{44,45} Disulfidptosis is also linked to immune effects.⁴⁶ Our immune infiltration analysis showed that monocytes, M2 macrophages, and Tfh cells were significantly altered in DKD, consistent with previous reports.^{47,48} All four genes were negatively correlated with Tfh cells. Among these genes, DSTN, ACTB, and RAC1 were positively correlated with monocytes and M2 macrophages, while PDLIM1 was positively correlated with M2 macrophages. PDLIM1 accelerates the progression of liver fibrosis by promoting inflammation in hepatic stellate cells⁴¹. DSTN expression in alcoholic liver disease is positively correlated with macrophages.⁴⁹ ACTB serves as a molecular hub that coordinates cytoskeletal dynamics and participates in regulating the Rho/NF- κ B signaling axis.⁵⁰ Through

modulation of RhoA and Rac1, Pcdh7 governs monocyte migration.⁵¹ Disulfidptosis in macrophages shows marked activation of metabolic pathways, including sulphur metabolism, the pentose phosphate pathway, oxidative phosphorylation, glycolysis, and gluconeogenesis.⁵² Thus, hub genes may influence DKD by modulating these immune subsets.

Using scRNA-seq datasets, we mapped hub gene expression patterns. CellChat analysis showed significantly higher SPP1–CD44 communication between PT cells and monocytes in DKD samples than in controls, and immune infiltration analysis also confirmed monocyte involvement in DKD. Wu et al. reported that macrophages contribute to sustained chronic low-grade inflammation and renal fibrosis associated with aging.⁵³ Previous studies have shown that ligamentum flavum hypertrophy (LFH) stimulates SPP1+ macrophages, which regulate fibroblast death via the SPP1–CD44 axis, leading to LFH fibrosis.⁵⁴ Collectively, the present results imply that these four hub genes might contribute to DKD pathogenesis and evolution through regulation of the SPP1–CD44 axis, which mediates crosstalk between PT cells and monocytes.

To validate the reliability of the above bioinformatics findings, we further performed in vitro experiments. HK-2 cells were exposed to high glucose for 48 h to establish a DKD model, and subsequent

RT-qPCR and Western blot analyses revealed that the mRNA and protein levels of DSTN, RAC1, and PDLIM1 were upregulated relative to the control group, whereas ACTB expression remained unchanged, suggesting a potential correlation between these genes' expression and DKD progression. Although aberrant expression of ACTB and RAC1 in DKD has been previously reported,^{55,56} the specific contributions of PDLIM1 and DSTN remain to be elucidated. Given that PDLIM1 exhibited the best clinical diagnostic performance among the four core genes and exerted dual roles in inflammation regulation, we selected it as a representative gene for further functional investigation. CCK-8 cell viability assay revealed that high glucose significantly suppressed HK-2 cell viability, while PDLIM1 knockdown reversed this reduction. Western blotting further demonstrated that high glucose upregulated the expression of SLC7A11 and disulfide-crosslinked proteins, whereas PDLIM1 knockdown suppressed their expression. Additionally, treatment with the disulfide-reducing agent DTT rescued high-glucose-induced cell viability loss. Collectively, these results indicate that PDLIM1 plays a pro-injury role in high-glucose-induced renal tubular epithelial cell injury, and its upregulation may participate in DKD-related pathological processes through modulating SLC7A11-mediated disulfide stress responses.

This study has several limitations that warrant emphasis. Firstly, our findings are currently based on comprehensive bioinformatics analyses and validation via RT-PCR and Western blot, and lack further confirmation through animal experiments and clinical trials. Secondly, the RT-PCR and Western blot results indicated no significant change in ACTB. We speculate this may be because disulfide bond-crosslinked actin polymers cannot dissociate under non-reducing conditions, forming high-molecular-weight complexes. Consequently, a band above 250kDa was observed in disulfidptosis samples (whereas normal actin is 42kDa). Thirdly, Third, validation of the SPP1-CD44 axis and causal relationships between hub genes and cell-cell communication requires future functional experiments, including ligand-receptor blockade and conditional gene knockout.

5. Conclusion

In summary, this study uses single-cell transcriptomics and machine learning to characterize disulfidptosis-related genes in DKD and their impact on the immune microenvironment. Our findings demonstrate a strong association between disulfidptosis-related genes and DKD. Four hub genes—DSTN, RAC1, ACTB, and PDLIM1—were pinpointed as potentially crucial modulators of immune responses in DKD. In view of the bifunctional involvement of PDLIM1 in inflammatory regulation, additional experiments were performed,

revealing that its upregulation likely contributes to DKD pathology by modulating SLC7A11-mediated disulfide stress, ultimately promoting cellular injury.

Collectively, this study highlights a novel perspective on the pathogenesis of DKD, in which DSTN, RAC1, ACTB, and PDLIM1 emerge as potential diagnostic and therapeutic candidates.

Declarations

- Ethics approval and consent to participate

Not applicable

- Consent for publication

Not applicable

- Availability of data and material

All data generated or analyzed in this study are included in the published article and its supplementary information files.

- Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

- Funding

The author declares that no financial support was received for the research, authorship, and publication of this article.

- Author contributions

Y.P: conceptualization, analyzed data, and writing the first manuscript.

S.L: conceptualization, analyzed data, and performed experiments.

Y.Z: Review and editing, data organization, methodology, software.

Y.T: Financial management, project administration, resource provision, verified the accuracy of the draft.

- Acknowledgements

Not applicable

- Clinical trial number

Not applicable

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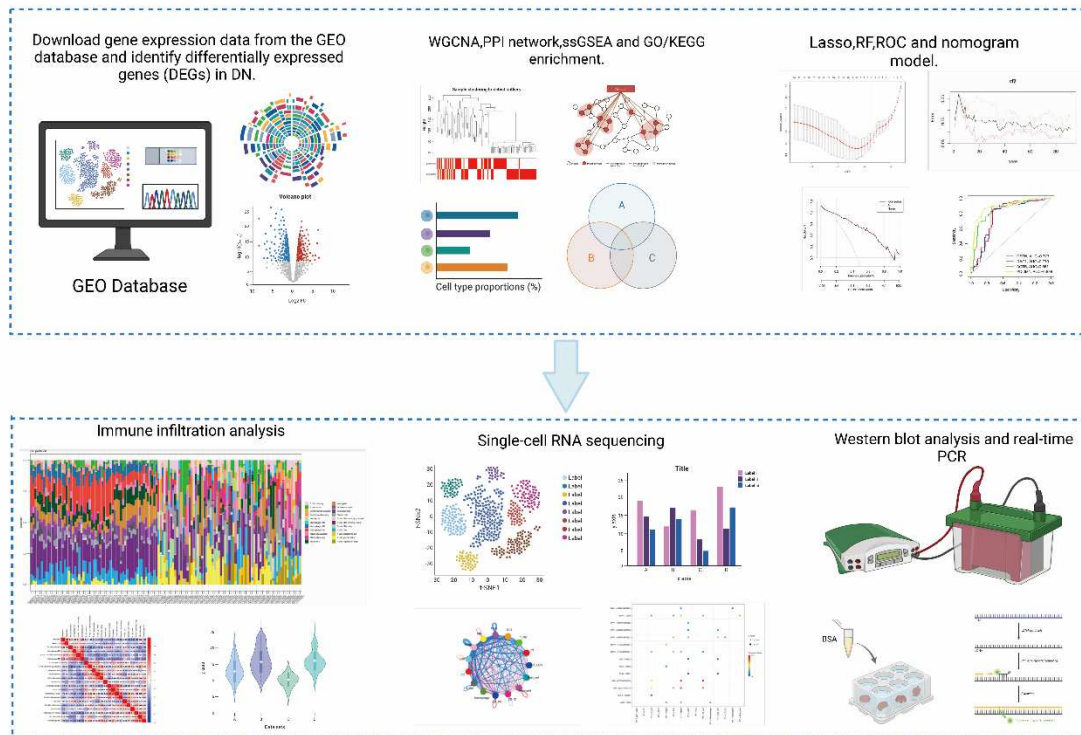


Figure 1: Study workflow diagram.

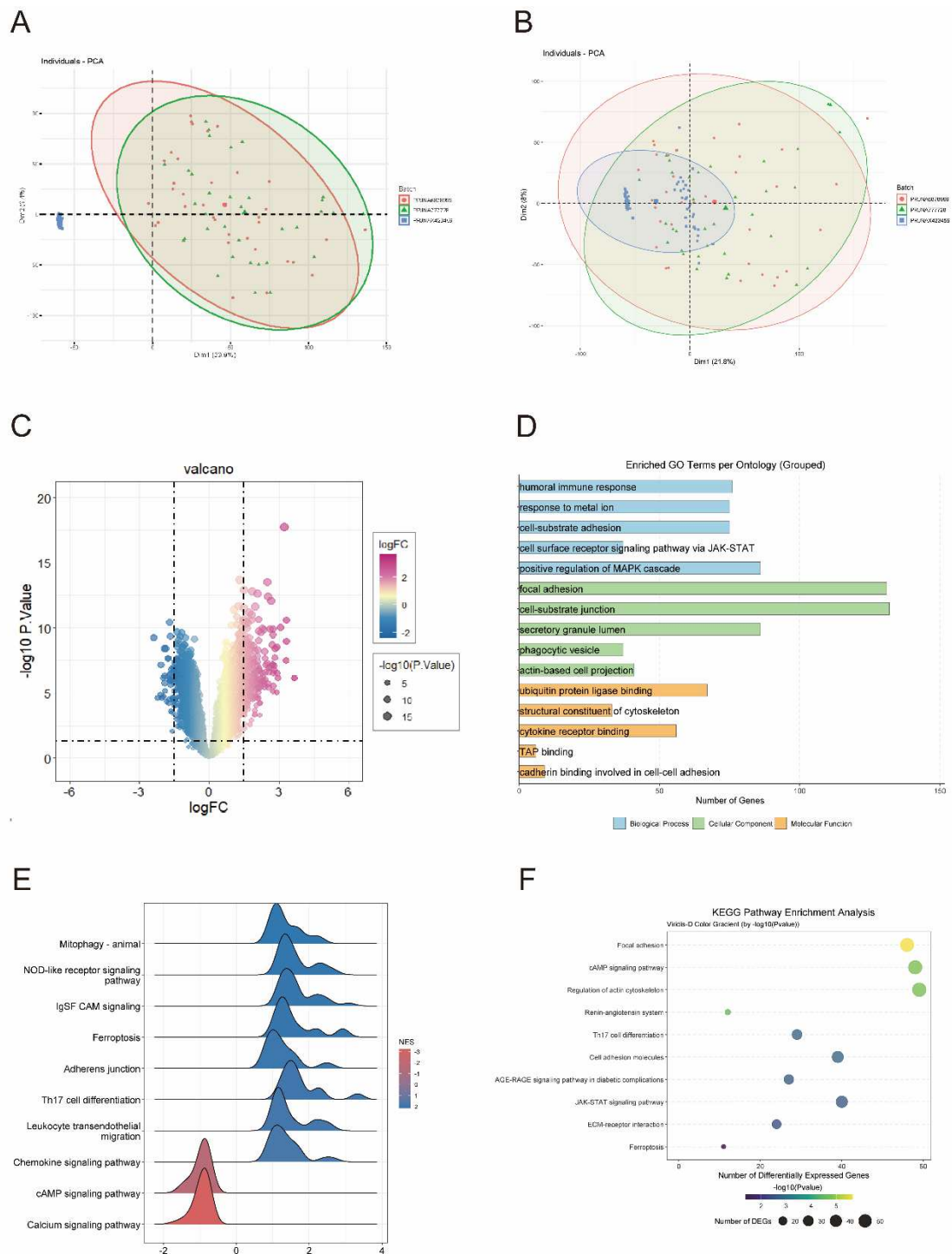


Figure 2. Screening of DEGs and Functional Enrichment Analysis

A: The sample distribution before batch effect correction

B: The sample distribution after batch effect correction

C: The DEGs results are presented as a volcano plot.

D: GO enrichment analysis of DEGs (containing subsections of biological processes, cellular components, and molecular functions).

E: GSEA enrichment of DEGs.

F: KEGG enrichment of DEGs.

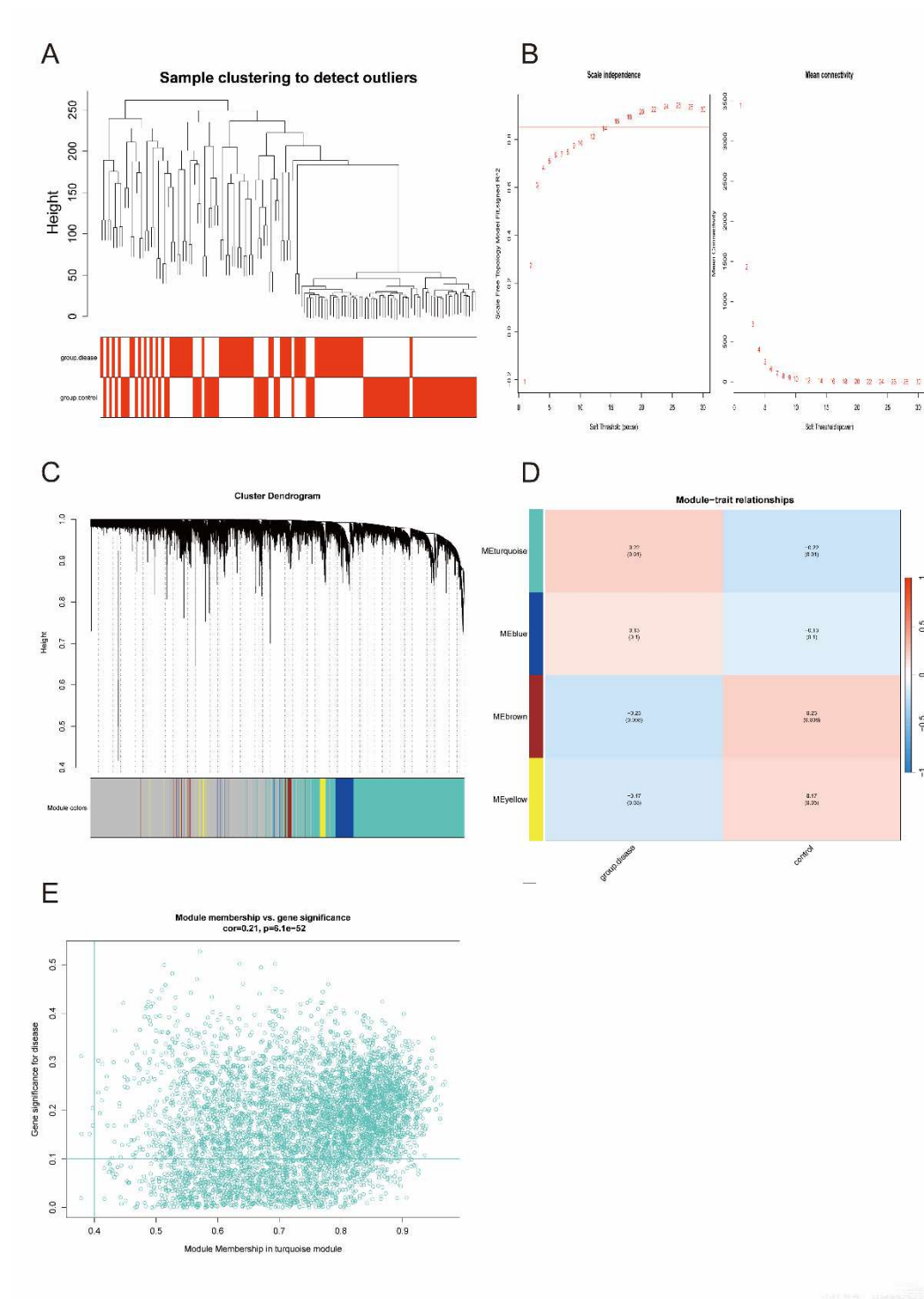


Figure 3. Co-Expression Network of DKD-Related Genes

A: Hierarchical clustering of the merged dataset.

B: Soft thresholds and average connectivity of WGCNA.

C: Dynamic module identification.

D: A total of 8 modules were identified.

E: Scatterplot between gene significance and module membership in the turquoise module.

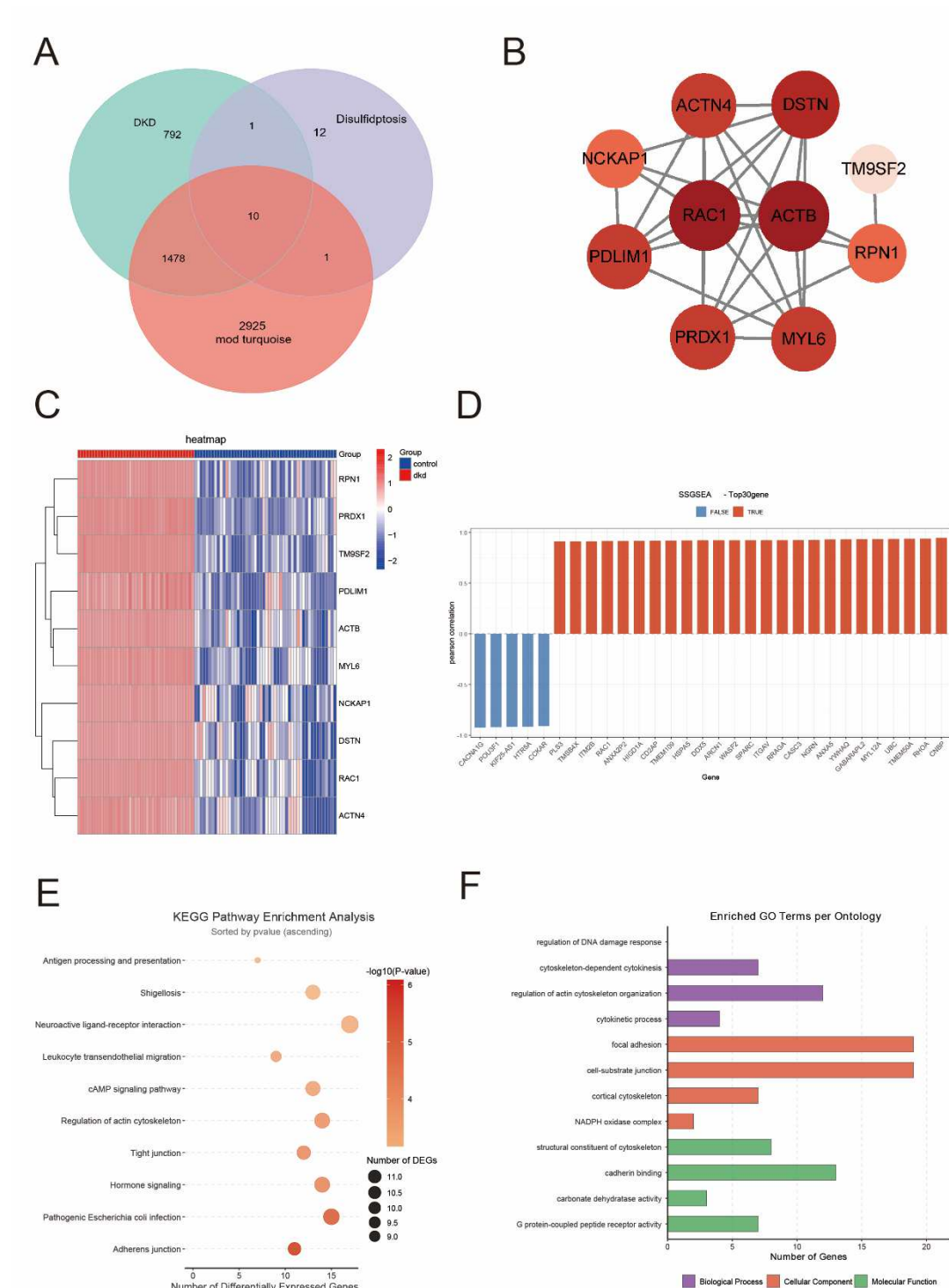


Figure 4: Identification and functional enrichment of key genes

A: The intersection of the potential DKD-related genes with the DEGs and

disulfidptosis-related genes yielded 11 core genes.

B: Predicting 10 genes from the PPI network.

C: The 10 genes are displayed as a heatmap.

D: Based on these 10 genes, ssGSEA was performed.

E: KEGG enrichment analysis of 10 genes.

F: GO enrichment analysis (the 10 genes of BP, CC, and MF enrichment analysis).

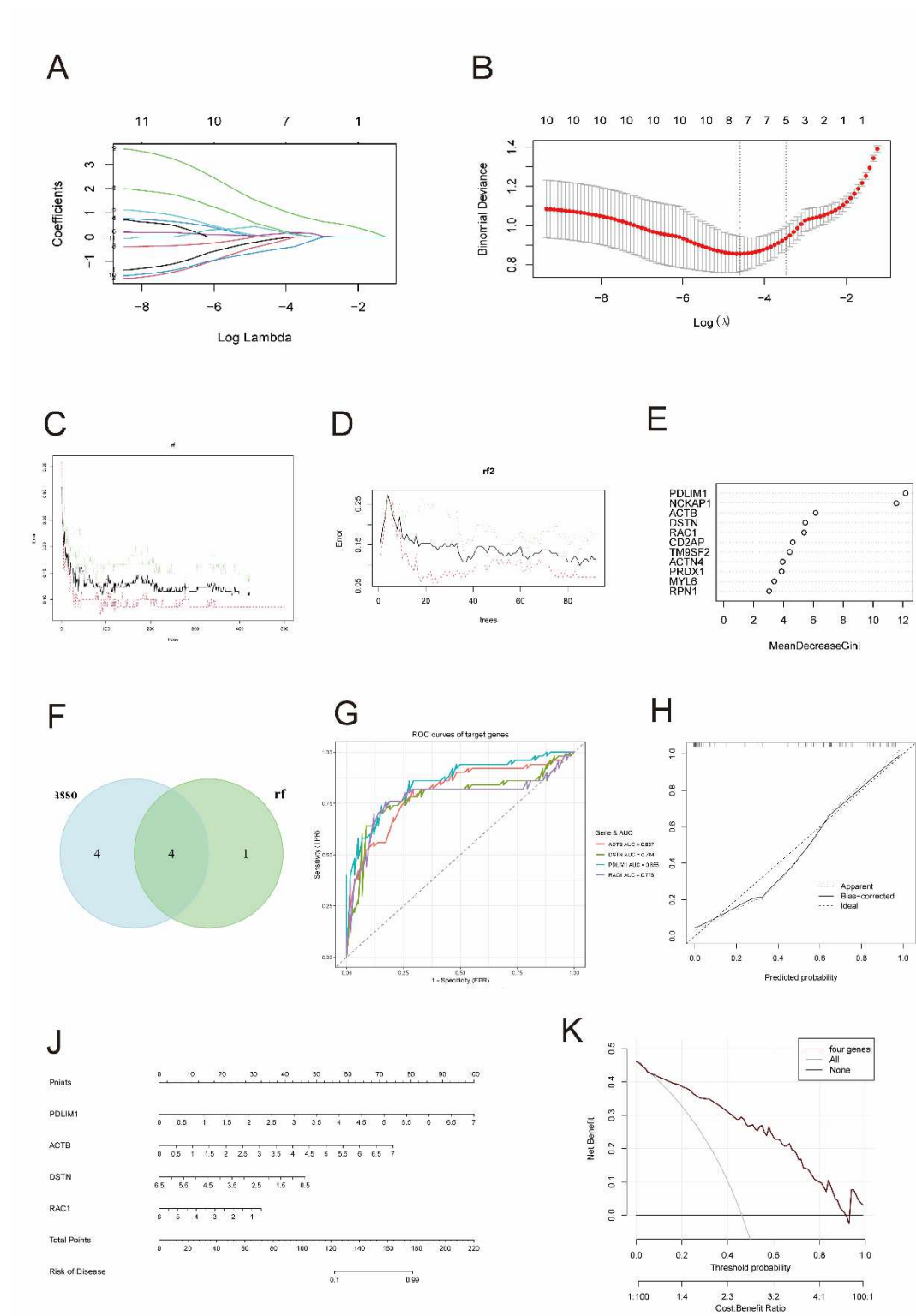


Figure 5. Identification and Validation of DKD Hub Genes

A-B: Lasso identified 8 genes.

C-D: RF selected the optimal fitting model from 154 models

E: Based on importance scores, identified 5 genes.

F: Intersecting these results yielded 4 key genes.

G: ROC analysis of the four hub genes in the training cohort.

H: Calibration curve to evaluate prediction efficiency of nomogram model.

I: A nomogram model was developed to predict DKD diagnosis.

J: The results of the DCA curve indicated a high net benefit of the model.

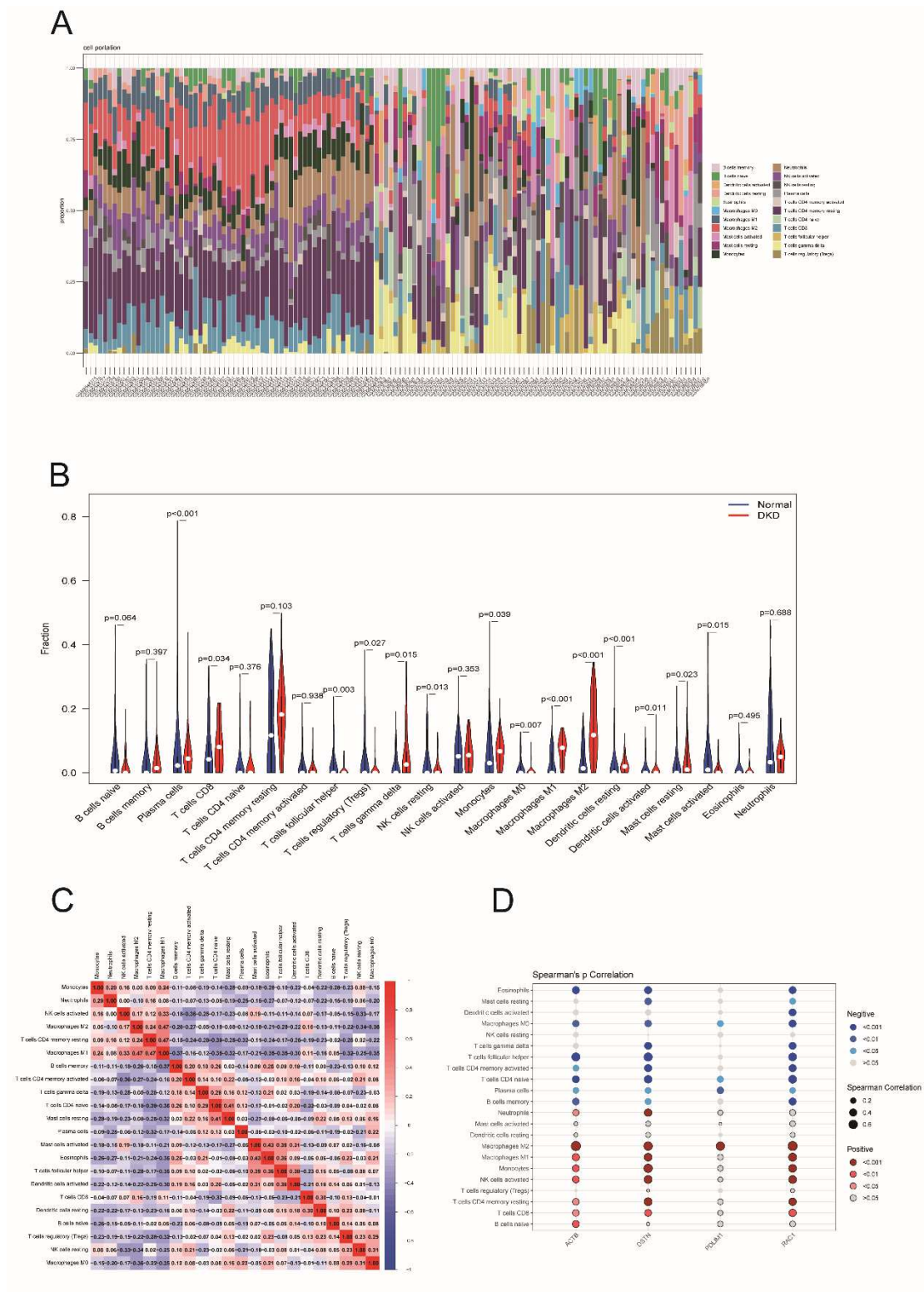


Figure 6. Immune infiltration analysis.

A: An immune infiltration analysis showed significant differences between the two groups.

B: The violin diagram further illustrates the specific differences in immune cells between the two groups.

C: A correlation analysis was conducted to examine the relationships between immune cells.

D: The hub genes are associated with specific immune cells.

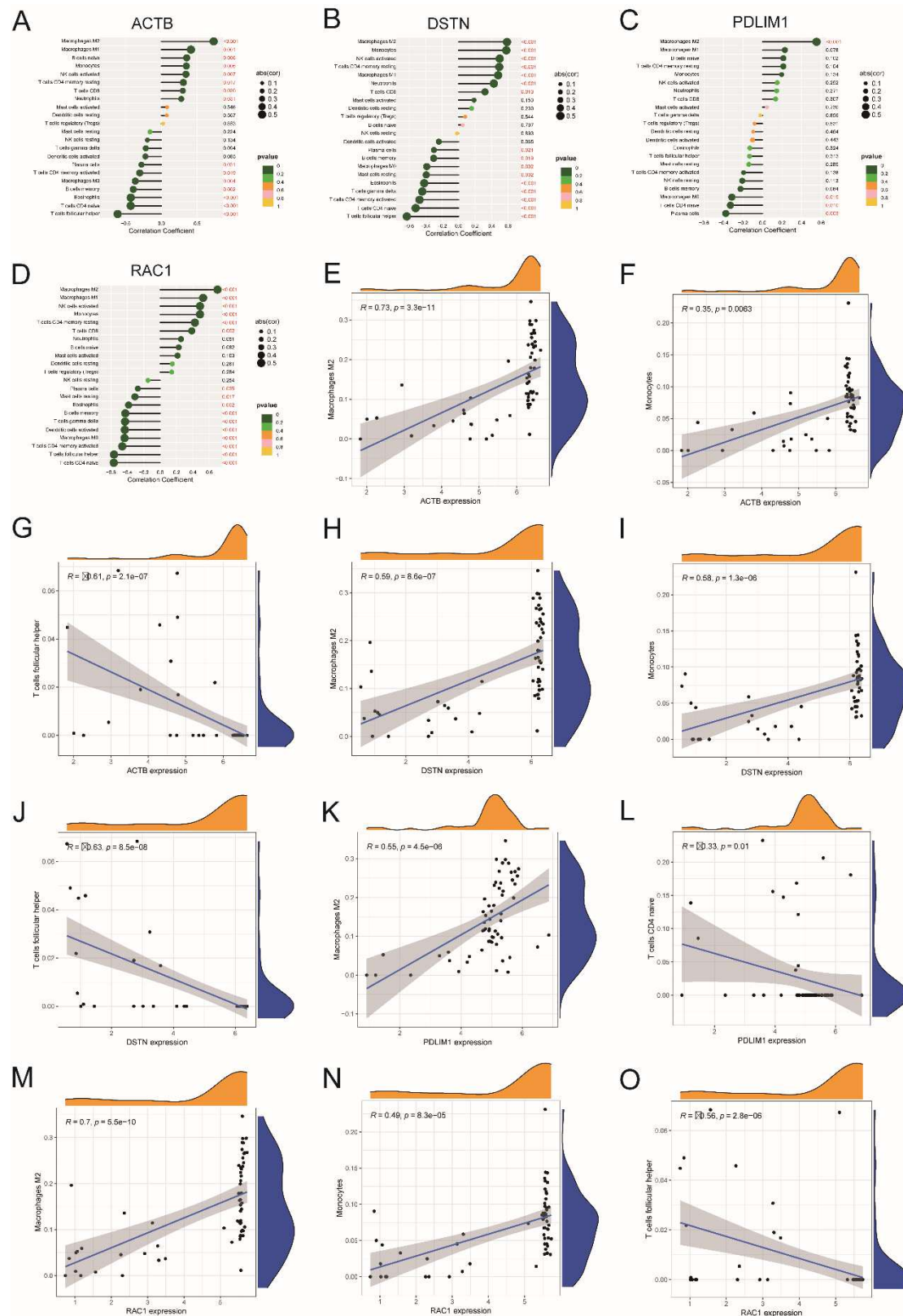


Figure 7. Correlation of hub genes with infiltrating immune cells.

A-O: Association between diagnostic signatures and infiltrating immune cells.

subtypes.

B:The marker genes are shown.

C:Circle plot showed the frequency and strength of cell–cell communication across all subtypes.

D:The number and intensity of intercellular communications between two groups.

E-F:Receptor–ligand communication weights between DKD and control samples.

G:The distribution of the hub genes in control group.

H:The distribution of the hub genes in DKD group.

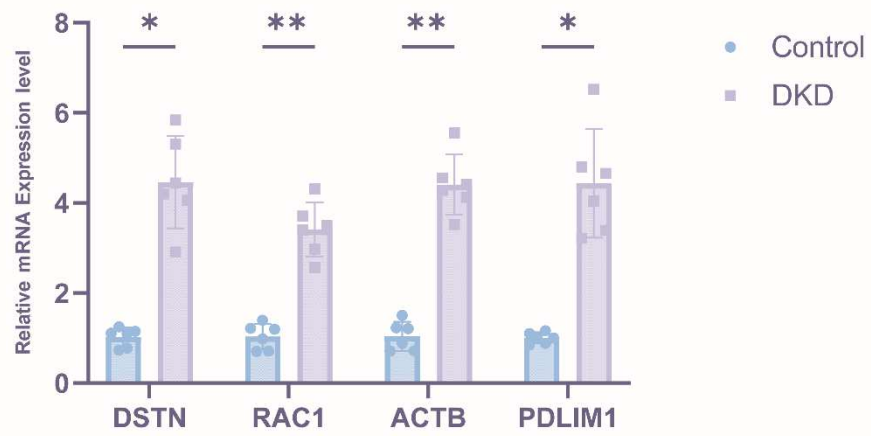
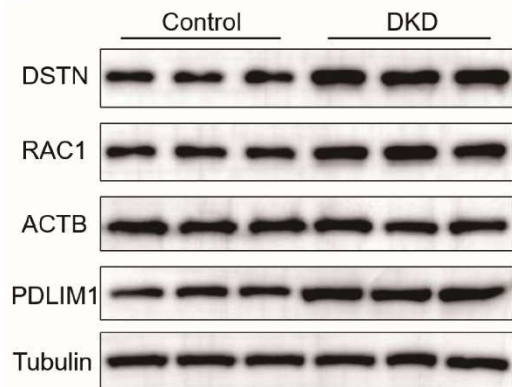
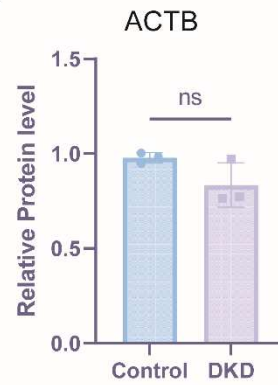
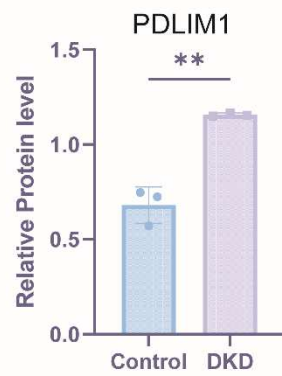
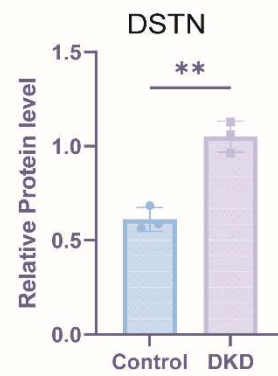
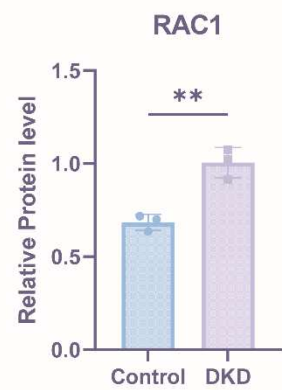
A**B****C****D****E****F**

Figure 9. Validation of hub gene expression by RT-qPCR and Western blot

A: mRNA expression of ACTB, DSTN, PDLIM1 and RAC1 by RT-qPCR.

B-F: Detection and statistical analysis of key disulfidptosis-related genes and marker protein expression in DKD group and control group.

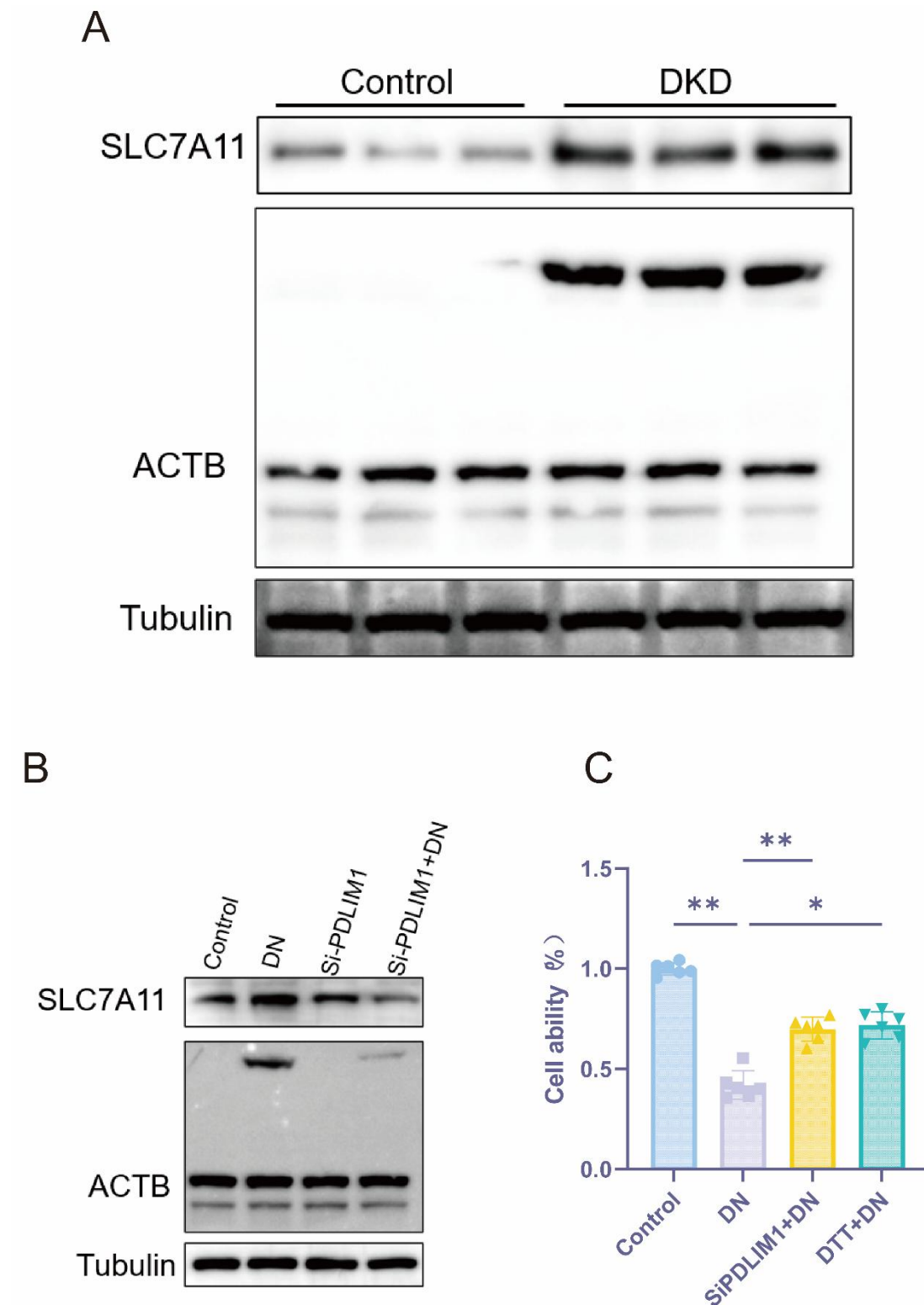


Figure 10 Knockdown experiments and cell viability assays among groups

A: Expression of SLC7A11 and disulfide-crosslinked proteins between the control and disease groups.

B: Expression of SLC7A11 and disulfide-crosslinked proteins among groups in knockdown experiments.

C: Comparison of cell viability among groups

Supplementary Files

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