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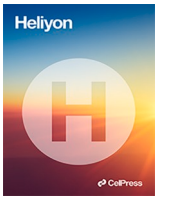

Research article

Myricetin alleviates renal tubular epithelial-mesenchymal transition via NOX4/NF-κB/snail axis in diabetic nephropathy based on network pharmacology analysis

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ABSTRACT

Diabetic nephropathy (DN), a leading cause of end-stage renal disease, remains a formidable challenge in diabetes management due to the complex nature of its pathogenesis, particularly the epithelial-mesenchymal transition (EMT) process. Our innovative study leverages network pharmacology to explore the therapeutic potentials of Myricetin, a natural flavonoid, focusing on its effects against NOX4, a critical mediator in DN progression. This investigation marks a pioneering approach by integrating network pharmacology to predict and elucidate the inhibitory relationship between Myricetin and NOX4. Utilizing a high-fat diet/streptozotocin (HFD/STZ) induced DN mouse model, we delved into the effects of Myricetin on renal EMT processes. Through network pharmacology analyses coupled with molecular docking studies, we identified and confirmed Myricetin's binding efficacy to NOX4. Extensive in vitro and in vivo experiments further established Myricetin's significant impact on mitigating EMT by modulating the NOX4-NF-κB-Snail signaling pathway. Results from our research demonstrated notable improvements in renal function and reductions in tissue fibrosis among treated HFD/STZ mice. By curtailing NOX4 expression, Myricetin effectively reduced reactive oxygen species (ROS) production, thereby inhibiting NF-κB activation and subsequent Snail expression, crucial steps in the EMT pathway. Supported by both theoretical predictions and empirical validations, this study unveils the mechanism underlying Myricetin's modulation of EMT in DN through disrupting the NOX4-NF-

Abbreviations: ANOVA, One-way analysis of variance; BUN, blood urea nitrogen; DN, Diabetic nephropathy; DAPI, 4′,6-diamidino-2-phenylindole; ESRD, end-stage renal disease; HE, Hematoxylin and eosin; HFD, high fat diet; HG, high glucose; HK2, human renal proximal tubule epithelial cell line; IHC, immunohistochemistry; MYR, Myricetin; qRT-PCR, Quantitative real-time polymerase chain reaction; STZ, Streptozotocin; PAS, Periodic acid-Schiff's; Scr, serum creatinine; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; BP, Biological Process; CC, Cellular Component; FBG, Fasting blood glucose; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; MF, Molecular Function; PPI, Protein-protein interaction; ROS, Reactive oxygen species.

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κB-Snail axis. These findings not only contribute a new therapeutic avenue for DN treatment but also underscore the utility of network pharmacology in advancing drug discovery processes.

1. Introduction

Diabetic nephropathy (DN) represents one of the most severe complications associated with diabetes mellitus, leading to a significant increase in the risk of end-stage renal disease (ESRD) globally [1,2]. As the prevalence of diabetes continues to surge, DN emerges as a critical public health challenge [3], underscoring the urgent need for innovative therapeutic strategies. The pathogenesis of DN is multifaceted, involving complex interactions between metabolic, hemodynamic, and inflammatory pathways [4]. Central to the progression of DN is the phenomenon of epithelial-mesenchymal transition (EMT) in renal tubular cells [5], a process whereby epithelial cells lose their characteristic polarity and adhesion, acquiring mesenchymal features that contribute to the fibrosis and sclerosis of renal tissue [6].

The activation of the EMT process in DN is influenced by various factors, including hyperglycemia-induced oxidative stress, inflammation, and the dysregulation of signaling pathways such as TGF-β, Wnt/β-catenin, and Notch [7–9]. Among these, the role of oxidative stress mediated by NADPH oxidase 4 (NOX4) and the resultant reactive oxygen species (ROS) production is pivotal [10]. ROS not only directly damages cellular components [11] but also acts as a signaling molecule that activates downstream pathways, including NF-κB, culminating in the transcriptional activation of genes that promote EMT, such as Snail [12]. Snail, a key transcriptional regulator [13], orchestrates the repression of epithelial marker genes like E-cadherin and the induction of mesenchymal markers, facilitating the EMT process and renal fibrogenesis [14].

Despite the advances in understanding the molecular mechanisms underlying DN, the translation of this knowledge into effective therapeutic interventions has been limited. Cur-rent treatments primarily focus on controlling blood glucose levels and managing hypertension [15] but fail to directly address the underlying cellular and molecular changes, such as EMT, driving the progression of DN.

In this context, flavonoids, a diverse group of polyphenolic compounds with potent an-tioxidative, anti-inflammatory, and anti-fibrotic properties [16,17], have attracted attention for their potential therapeutic benefits in chronic diseases, including DN [18]. Myricetin, a naturally occurring flavonoid, has been widely studied for its health benefits, encompassing anti-tumor, antioxidative, and antiviral activities [19–21]. However, the specific impact of Myricetin on the EMT process in DN, particularly through the modulation of the NOX4-ROS–NF–κB-Snail axis, remains largely unexplored.

This study leverages network pharmacology to identify the potential inhibitory effect of Myricetin on NOX4, a novel finding that sheds light on the molecular basis of its therapeutic action in DN. Through molecular docking and extensive in vitro and in vivo experiments, we aim to validate the role of Myricetin in modulating the NOX4-ROS–NF–κB-Snail signaling axis, offering new insights into its potential as a therapeutic agent for halting the progression of DN. By elucidating the mechanistic pathways through which Myricetin exerts its effects, this research contributes to the broader effort of developing targeted and effective treatments for diabetic nephropathy.

2. Materials and methods

2.1. Cell culture and chemicals

HK-2 human proximal tubular epithelial cells were obtained from Procell [22–24] (CL-0109; Wuhan, China) and grown in DF12 (DMEM/F12 1:1 mixture) culture medium (Hyclone, UT, USA) containing 10 % fetal bovine serum (FBS; Hyclone) in a regular CO_2 incubator at 37 °C. HK-2 cells were used in all experiments described here, which were authenticated by STR profiling conducted on April 28, 2024 and tested negative for mycoplasma.

Following subculturing and the subsequent attainment of an approximate 80 % confluence, the cells were subjected to treatment. The experimental design entailed culturing the cells with glucose at two distinct concentrations: 5.5 mmol/L, serving as the control condition (CT), and 35 mmol/L, representing the high-glucose environment (HG, model group). In addition to these conditions, a treatment group was established, wherein cells were exposed to a combination of high glucose and Myricetin (Myricetin + HG) for a period of 24 h.

Myricetin, procured with a purity exceeding 95 % from Topscience Co., Ltd. (Shanghai, China), was dissolved in dimethyl sulfoxide (DMSO) and administered to the culture at concentrations of 40 μM or 60 μM. This strategic approach allowed for the investigation of cellular dynamics under normoglycemic conditions, hyperglycemic stress, and the mitigating influence of Myricetin within a hyperglycemic setting, providing insights into the potential therapeutic efficacy of Myricetin in counteracting the deleterious effects of elevated glucose levels.

2.2. Animal model and experimental design

Animal housing and initial procedures: Male C57BL/6J mice (4 weeks old), a widely recognized model for type 2 diabetes research, were maintained under specific pathogen free (SPF) conditions. The mice were kept at an ambient temperature ranging from 18 to 23 °C, with a relative humidity of 40–70 %. They were provided with a standard clean diet and water. All animal experiments

were conducted in accordance with established animal welfare guidelines and complied with all regulations. This study was reviewed and approved by the Animal Ethics Committee of Southern Medical University with the approval number: SMUL2022007.

Diabetic model induction: The mice were subjected to a high-fat diet (HFD, with 60 % of kilocalories from fat) for a duration of one month. This was followed by intraperitoneal injections of streptozotocin (STZ; 40 mg/kg dissolved in 0.1 mol/L citrate buffer, pH adjusted to 4.5) administered daily for five consecutive days. Successful induction of the diabetic nephropathy (DN) phase was confirmed in mice that consistently displayed fasting blood glucose levels exceeding 11.1 mmol/L over three days, accompanied by positive 24-h urine protein tests.

Grouping and treatment: Subsequent to the model confirmation, the mice were randomized into four distinct groups using a random number table method, each comprising six mice:

Control Group (Con): Healthy mice without any signs of DN.

Diabetic Nephropathy Group: Mice with STZ/HFD-induced diabetes, given equivalent volumes of double-distilled water as placebo.

Myricetin-Low Dose Group: DN mice administered Myricetin at a dose of 200 mg/kg/day.

Myricetin-High Dose Group: DN mice administered Myricetin at a dose of 300 mg/kg/day.

All treatments were dispensed intragastrically on a daily regimen spanning 11 weeks.

Tissue collection and preservation: Post 11 weeks of treatment, mice were anesthetized using pentobarbital sodium. Blood samples and kidney tissues were promptly harvested. A fraction of the kidney tissue samples was preserved in 4 % paraformaldehyde, suitable for histological evaluations. The remaining kidney tissue was snap-frozen and stored at −80°C, awaiting subsequent biochemical assessments.

2.3. Histological examination of kidney tissues

Tissue preparation: Kidney tissues embedded in paraffin were sectioned to a thickness of 4 μm. Subsequent to deparaffinization, sections were prepared for various histological staining procedures.

Staining processes: Hematoxylin and Eosin (H&E) Staining; Periodic Acid Schiff (PAS) Staining; Sirius Red Staining; Masson's Trichrome Staining. All staining kits were sourced from Beijing Solarbio Science & Technology Co. Ltd.

Microscopic evaluation: Blinded microscopic analysis was executed to ensure unbiased results. Random fields from each slide were imaged using an OLYMPUS microscope (Japan).

2.4. Biochemical analysis of renal function markers

The levels of 24H-Urine Protein, Serum Creatinine (SCr) and Blood Urea Nitrogen (BUN) were quantitatively analyzed using commercial assay kits sourced from Nanjing Jiancheng (Jiangsu, China). The assays were meticulously executed as per the detailed guidelines and protocols provided by the manufacturer, ensuring accuracy and reproducibility in the results.

2.5. Potential targets identification of myricetin, epithelial-mesenchymal transition (EMT) and diabetic nephropathy (DN)

Pharmacological target prediction of myricetin database: Myricetin's potential biological targets were predicted using the SwissTargetPrediction database (available at http://www.swisstargetprediction.ch/www.swisstargetprediction.ch/). The molecular structure of Myricetin was inputted into the SwissTargetPrediction platform. The database rendered its predictions based on the structural similarity of Myricetin to known ligands, proposing a list of prospective protein targets that Myricetin might interact with.

Identification of targets pertinent to DN and EMT Database: To discern the targets specifically relevant to Diabetic Nephropathy (DN) and Epithelial-mesenchymal Transition (EMT), the GeneCards disease database was employed (accessible at https://www.genecards.org/). Keywords "Diabetic Nephropathy (DN)" and "Epithelial-mesenchymal Transition (EMT)" were utilized to systematically comb through the GeneCards database. This integrative database consolidates gene-centric information derived from a myriad of sources, encapsulating both annotated and predicted human genes.

2.6. Construction of component-target-disease (CTD) and protein-protein interaction (PPI) networks

Target overlap analysis for drug and disease: To discern the potential targets through which Myricetin might alleviate EMT and subsequently treat DN, we utilized the jvenn tool (https://jvenn.toulouse.inrae.fr/app/example.html). This facilitated the identification of shared targets between Myricetin and both DN and EMT.

Construction of PPI network for overlapping targets: With the shortlisted disease targets in hand, we employed the STRING database (http://string-db.org) to construct the Protein-Protein Interaction (PPI) network. In this, we set the organism as '*Homo sapiens*' and the minimum required interaction score was medium confidence. For enhanced visualization and analysis, the results from STRING, saved in "tsv" format, were imported into Cytoscape 3.9.1. This approach led to the creation of a network termed "MYR-DN-EMT-target". It's noteworthy that within this network, the size and color of each target were modulated based on its Degree Centrality (DC) value, as calculated by the CytoNCA plugin in Cytoscape 3.9.1. These DC values provided insights into the relative importance of each target within the overall network.

2.7. Functional enrichment analyses

GO and KEGG pathway analysis: Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were performed on the shared genes of Myricetin, DN and EMT using the Metascape online analysis platform (https://metascape.org/gp/index.html#/main/step1). The GO enrichment analysis included cellular components (CC), molecular functions (MF) and biological processes (BP). The results of the first nineteen pathways from KEGG enrichment analysis were visualized using an online bioinformatics visualization platform (http://www.bioinformatics.com.cn/), and the data were presented via information bubble plots. Statistically significant results were identified using a threshold of $P < 0.05$.

Disease-Target-Pathway network construction: To construct the disease-target-pathway network, the top 20 pathways from the previous analysis and their corresponding 24 key targets were imported into Cytoscape 3.9.1 software. The visualization of the network was based on the BC (betweenness) values calculated using the CytoNCA plugin.

2.8. Molecular docking

AutoDock Vina and Pymol software were used for molecular docking. The NOX4 crystal structure (PDB:2ETR) was downloaded from the PDB (https://www.rcsb.org), and the molecular structures were downloaded from Pubchem (https://pubchem.ncbi.nlm.nih.gov/).

2.9. Western blotting

The tissues of the renal cortex or HK2 cells were lysed with lysis buffer a protease inhibitor cocktail and a phosphate inhibitor cocktail (Sigma-Aldrich). The quantification of total protein content was determined using the bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific). The protein samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and subsequently transferred onto a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA). To block non-specific binding, the membrane was treated with 5 % milk at room temperature for 2 h, followed by overnight incubation with specific primary antibodies: NOX4 Polyclonal antibody (14347- 1-AP; Proteintech), E-cadherin Antibody (G-10) (sc-8426; Santa Cruz Biotechnology), Snail (C15D3) Rabbit mAb (3879T; CellSignalingTechnology), Vimentin Antibody (AF7013; Affinity), Beta Actin Monoclonal antibody (66009-1-Ig; Proteintech), NF-κB p65 (D14E12) XP® Rabbit mAb (8242T; CellSignalingTechnology), Phospho–NF–κB p65 (Ser536) (93H1) Rabbit mAb (3033T; CellSignalingTechnology), GAPDH Antibody (AF7021; Affinity). After washing with TBS-T for three times, the membrane was incubated with corresponding secondary antibodies (1:10000) at 4 °C for 2 h. The signals were detected with the enhanced chemiluminescence (ECL) method via the FluorChem E™ system (ProteinSimple, San Francisco, CA, USA).

2.10. Statistical analysis

The statistical analyses were conducted using GraphPad Prism version 9.4.0 (GraphPad Software Inc, San Diego, CA, USA). One-way or Two-way analysis of variance (ANOVA) and appropriate post hoc test were used to compare multiple groups. The Mean ± SD was used to represent the results. Significance was defined as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$ in all statistical analyses.

2.11. Immunohistochemical and immunocytochemical staining

Mouse kidneys were prepared for paraffin embedding according to conventional tech-niques after being fixed in 4 % PFA (Biosharp Life Science, China) at 4 °C for at least 24 h. Paraffin sections were prepared at 4-μm-thickness and deparaffinized and hydrated in graded ethanol series. Antigen retrieval was performed on the tissue sections by microwaving tissue sections in 10 mmol/L sodium citrate buffer (pH 6.0) for 10 min. Endogenous peroxidase was inhibited by incubating for 10 min with 3 % hydrogen peroxide. Then, sections were blocked with 5 % normal goat serum solution for 1 h at RT. Primary antibodies were applied to the sections and left on them at 4°C overnight. The appropriate secondary antibodies were applied to the sections and incubated. Diaminobenzidine (DAB) (ZSGB-BIO, China) was used as the chromogen. Sections were lightly counterstained with hematoxylin and were dehydrated and cover slipped. Image acquisition was performed on the KF-PRO-005-EX scanner.

For immunocytochemical staining of NOX4 and NF-κB, HK2 (2×10^4 cells/well) were seeded in a 4-well chamber, cells were treated with 35 μM glucose in the presence or absence of 10 μM MYR for 24 h. Then cells were fixed, permeabilized, blocked, and incubated overnight at 4°C with NOX4/NF-κB, and subsequently incubated with an anti-rabbit Alexa Fluor 488 secondary antibody (A11010; Invitrogen). The nuclei were stained with DAPI, mounted, and observed under a FV3000 confocal scanning microscope.

2.12. ROS determination

Reactive oxygen species (ROS) in tissue sections were detected using Dihydroethidium (DHE, Beyotime, S0063). This ROS-sensitive red fluorescent dye was applied to the tissue sections following standard protocols. After appropriate incubation, the sections were examined under a fluorescence microscope (Nikon Eclipse C1). The intensity and localization of the red fluorescence emitted by DHE were used to assess ROS levels in the tissues.

2.13. MDA determination

An appropriate amount of mouse kidney tissue was taken and nine times the volume of saline was added and then the tissue was ground into a homogenate. The supernatant of each tissue was collected after centrifugation and its content was measured using Malondialdehyde (MDA) assay kit (A003-1-1; Nanjing, China).

3. Results

3.1. Myricetin enhances kidney function and suppresses renal fibrosis in STZ/HFD mice-induced diabetic mice

To probe the potential therapeutic efficacy of MYR in ameliorating renal impairment in diabetic nephropathy (DN), we instituted high-fat diet/streptozotocin (HFD/STZ) paradigm to emulate DN in mice. Following this, the mice received oral doses of either MYR (at concentrations of 200 or 300 mg/kg) or a control vehicle for a duration of 11 weeks (Fig. 1 A).

Weight assessments across cohorts revealed no significant deviations, attesting to Myricetin's safety (Fig. 1 B). A granular analysis of fasting blood glucose (FBG) and renal function evinced pronounced alterations in salient markers—embracing urine protein metrics, serum creatinine (SCr), and blood urea nitrogen (BUN) values—within the purview of the HFD/STZ ensemble (Fig. 1C–F). Contrarily, these metrics exhibited significant remedia-tion in a dose-responsive paradigm within the Myricetin-administered cohorts. Histopathol-ogy, using H&E staining, highlighted Myricetin's counteractive effects on hyperglycemia-induced renal anomalies (Fig. 1 G). Periodic Acid-Schiff (PAS) staining indicated increased glycogen in the renal cortex of the model group, predominantly within the tubular basement and renal interstitium (Fig. 1 G). This was reduced with Myricetin intervention. Sirius red and Masson's trichrome staining revealed exacerbated renal fibrosis in diabetic mice, indicating increased collagen in the renal interstices. However, Myricetin administration moderated this collagen accumulation (Fig. 1 G). In essence, findings indicate Myricetin's potential in counteracting diabetic-induced renal fibrosis.

3.2. Myricetin alleviates renal tubular epithelial-mesenchymal transition in vivo

Building on the foundational results of Myricetin's renal protective effects, we delved into its specific impact on the process of epithelial-mesenchymal transition (EMT) within renal tubular cells—a critical event in the progression of renal fibrosis in diabetic nephropathy. Through quantitative RT-PCR, we observed a significant upregulation of E-cadherin, an epithelial marker typically suppressed in diabetic nephropathy, alongside a downregulation of mesenchymal markers Snail and Vimentin, in a dose-dependent manner following Myricetin treatment (Fig. 2 A).

Further reinforcing these observations, Western blot analysis (Fig. 2B and C) provided additional evidence of the dose-responsive effect of Myricetin on these EMT markers. The protein expression levels of E-cadherin increased while snail and vimentin decreased, aligning with the qRT-PCR findings.

Immunofluorescence staining (Fig. 2D and E) offered visual confirmation of these molecular alterations. In renal tubules affected by DN, a marked reduction in E-cadherin expression was evident, which was effectively reversed post-Myricetin intervention. Similarly, the elevated expression levels of snail and vimentin in DN conditions were substantially diminished in response to Myricetin, further validating its therapeutic efficacy.

3.3. DN-component-EMT target and PPI network analysis

To elucidate the underlying molecular targets and pathways through which Myricetin exerts its anti-EMT effects, we employed a network pharmacology approach. Utilizing the SwissTargetPrediction database, we initially identified 100 potential Myricetin targets. To discern targets specifically relevant to DN and EMT, we employed the GeneCards disease database. This led to the integration of drug targets with disease targets through a Venn diagram approach. Ultimately, 35 targets specifically associated with the treatment of EMT in DN by Myricetin were selected (Fig. 3 A). Constructing a Protein-Protein Interaction (PPI) network from these targets illuminated the intricate web of potential interactions modulated by Myricetin, with GO and KEGG enrichment analyses pinpointing ROS-related signaling pathways among the most significantly affected (Fig. 3B–D).

3.4. Myricetin inhibits ROS in DN mice

Given the pivotal role of reactive oxygen species (ROS) in the pathogenesis of diabetic nephropathy and its contribution to EMT, we quantitatively assessed the impact of Myricetin on ROS production in kidney tissues from DN mice. A notable reduction in ROS and MDA levels was observed in a dose-dependent manner in Myricetin-treated groups compared to diabetic controls (Fig. 4 A and 4 D). Molecular docking studies provided structural insights into the interaction between Myricetin and NOX4, a key enzyme involved in ROS generation, suggesting a direct inhibitory effect of Myricetin on NOX4 activity (Fig. 4B and C).

3.5. Inhibition of EMT in HK2 cells by myricetin through NOX4 expression

The production of ROS is closely linked to the activity of NOX4, a pivotal player that is upregulated in DN and contributes to increased oxidative stress. This oxidative stress, in turn, activates NF-κB, a key transcription factor known to regulate the expression of

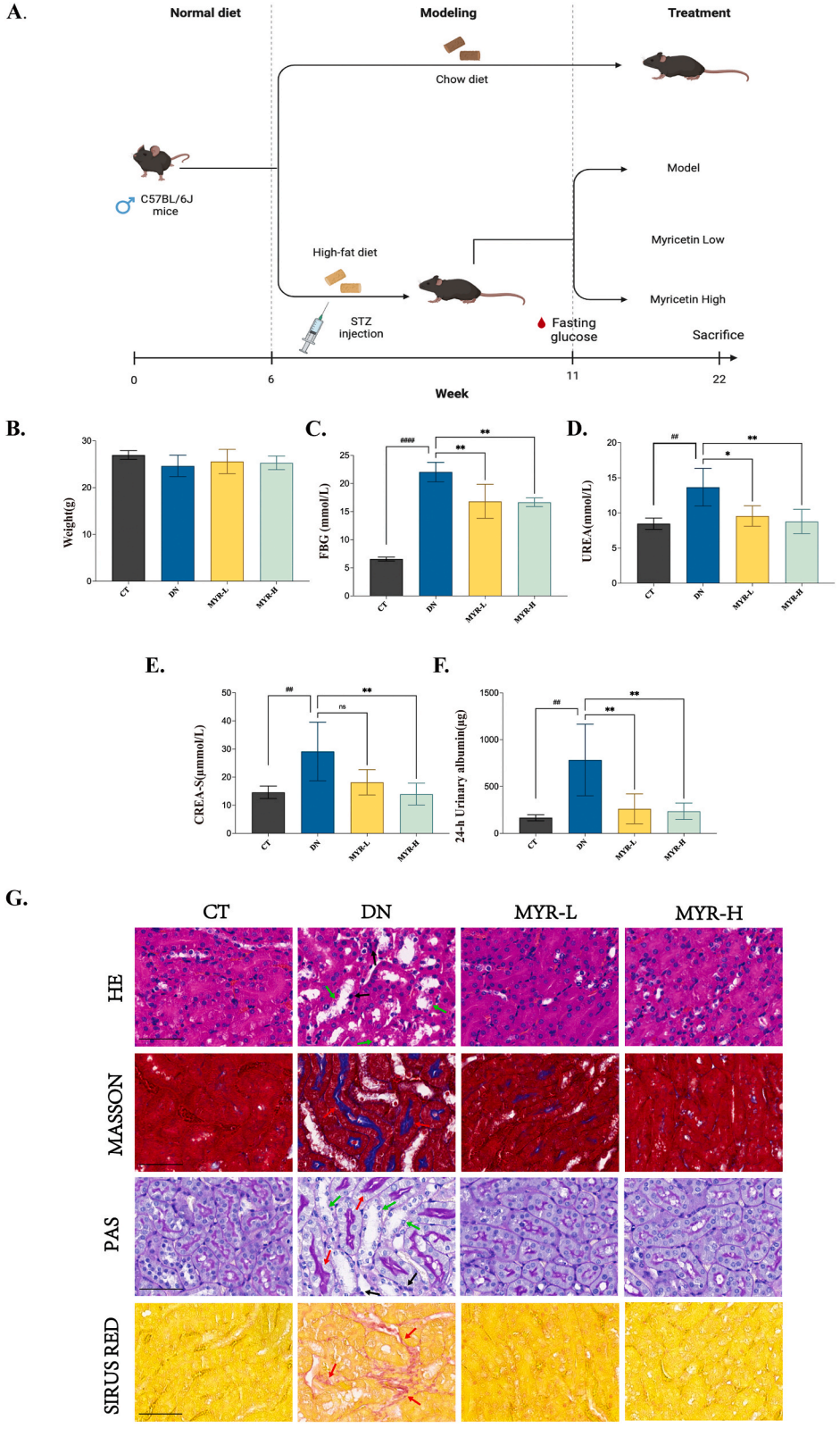

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Fig. 1. Protective efficacy of myricetin in STZ/HFD-induced renal injury.
A. Schematic representation of the Myricetin administration protocol in STZ/HFD mice is illustrated. B. Evaluation of the impact of Myricetin treatment over 11 weeks on the body mass of mice. C–F. Assessment of fasting blood glucose, blood urea nitrogen, serum creatinine, and 24-h urinary albumin levels post 11 weeks of Myricetin intervention. G. Histological examination of renal tissues post Myricetin treatment, visualized through hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), and Masson's trichrome staining. (×200 magnification, Bar = 40 μm) The red arrows: renal interstitial fibrosis; the green arrows: swollen tubular epithelial cells and vacuolar degeneration; the black arrows: lymphocytic or plasma cell infiltration in the renal interstitium. Statistical representation is depicted as mean ± standard deviation (n = 5/group); significance denoted by #p < 0.05, ##p < 0.01, ###p < 0.001, and ####p < 0.0001, ns = no significant, versus control (Ctrl); *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns = no significant, versus diabetic nephropathy (DN).

Snail. Snail is a crucial transcription factor in the EMT process, exerting its influence by suppressing the expression of E-cadherin and promoting the expression of mesenchymal markers such as vimentin, thereby driving the transition of epithelial cells into mesenchymal cells.

Complementing the in vivo findings, in vitro experiments conducted on high glucose-induced HK2 cells, a human renal proximal tubule epithelial cell line. We observed that high glucose conditions led to an increase in NOX4 fluorescence and nuclear translocation of NF-κB, indicative of elevated oxidative stress and NF-κB activation. However, the introduction of Myricetin treatment effectively attenuated these changes, providing compelling evidence of its ability to reduce oxidative stress (Figs. 5 A,6 A). Western blot analysis further corroborated these findings by revealing dose-dependent alterations in the expression levels of NOX4, phosphorylated NF-κB, and key EMT markers, including Snail, E-cadherin and vimentin (Fig. 5B and C). These changes were consistent with the reversal of EMT processes observed in our in vivo experiments, highlighting the ability of Myricetin to modulate NOX4 and the NF-κB signaling pathway, ultimately inhibiting EMT.

3.6. Myricetin modulates NF-κB activation and NOX4 expression in DN contexts

To confirm the mechanisms underlying the therapeutic effects of Myricetin in diabetic nephropathy (DN), we conducted Western blot analysis on kidney tissues from DN mice. This analysis revealed elevated levels of NOX4 and phosphorylated NF-κB, indicative of increased oxidative stress and NF-κB activation in the DN context. However, the introduction of Myricetin treatment resulted in a dose-dependent reversal of these effects (Fig. 6B and C).

Furthermore, we employed immunohistochemistry to assess the expressions of Nox4 and phosphorylated NF-κB in the renal cortex of DN mice. Consistently, we observed increased expressions of both Nox4 and phosphorylated NF-κB in DN mice compared to the control group (Fig. 6D and E). Notably, Myricetin treatment markedly attenuated these expressions, highlighting its potential in mitigating NF-κB-mediated inflammatory pathways in the context of diabetic nephropathy (Fig. 6 G).

4. Discussion

Diabetic nephropathy (DN), a formidable complication of diabetes mellitus, is a principal contributor to the global prevalence of end-stage renal disease [25,26] While primarily affecting the kidneys, diabetes can also exacerbate risks of hepatotoxicity, complicating the overall management of the disease [27]. The pathophysiological progression of DN, characterized by the epithelial-mesenchymal transition (EMT) of renal tubular cells [28,29], presents a complex therapeutic challenge, underscoring the urgent need for innovative treatment strategies. In this context, our comprehensive study on Myricetin, a flavonoid with well-documented pharmacological properties [30], marks a significant advancement in understanding potential therapeutic interventions for DN, particularly in modulating the pivotal EMT process.

The role of flavonoids in influencing cellular processes is widely recognized within the scientific community [31]. However, the specific mechanisms by which Myricetin affects the EMT process in DN have remained largely unexplored. Our investigation aimed to fill this critical gap by elucidating the effects of Myricetin on DN's pathogenesis. We observed that Myricetin administration significantly ameliorates renal dysfunction and fibrosis in an HFD/STZ-induced model of DN [32]. This effect is manifested through Myricetin's modulation of key EMT biomarkers, including a marked downregulation of Snail, a central transcription factor in EMT induction, and Vimentin, a mesenchymal marker [33], coupled with an up-regulation of E-cadherin, an epithelial hallmark [34]. These molecular alterations underscore Myricetin's capacity to directly counteract the EMT process, providing a plausible therapeutic mechanism for its action against DN.

Delving deeper into the molecular underpinnings of Myricetin's action, our utilization of network pharmacology and molecular docking approaches revealed a notable interaction between Myricetin and NADPH oxidase 4 (Nox4). This interaction suggests Myricetin's potential role in modulating Nox4 expression and ROS generation, which plays a critical role in exacerbating DN. Furthermore, our findings extend to the identification of Myricetin's inhibitory effects on the NF-κB signaling pathway, a key regulator of inflammatory and fibrotic responses. The suppression of NF-κB activity by Myricetin likely mediates the observed downregulation of Snail, further impeding the EMT process and its contribution to renal fibrosis in DN.

Our research identifies Myricetin as a potent candidate for treating DN [35], primarily by inhibiting Nox4 expression, reducing ROS levels, and suppressing the NF-κB signaling pathway. These mechanisms work together to weaken Snail expression and reverse the epithelial-mesenchymal transition (EMT) process, presenting a comprehensive strategy against the challenges of DN. Additionally, as a natural compound, Myricetin offers a cost-effective alternative to synthetic drugs, potentially improving patient accessibility.

However, the clinical application of Myricetin is limited by its low bioavailability in the body, a challenge that can be mitigated

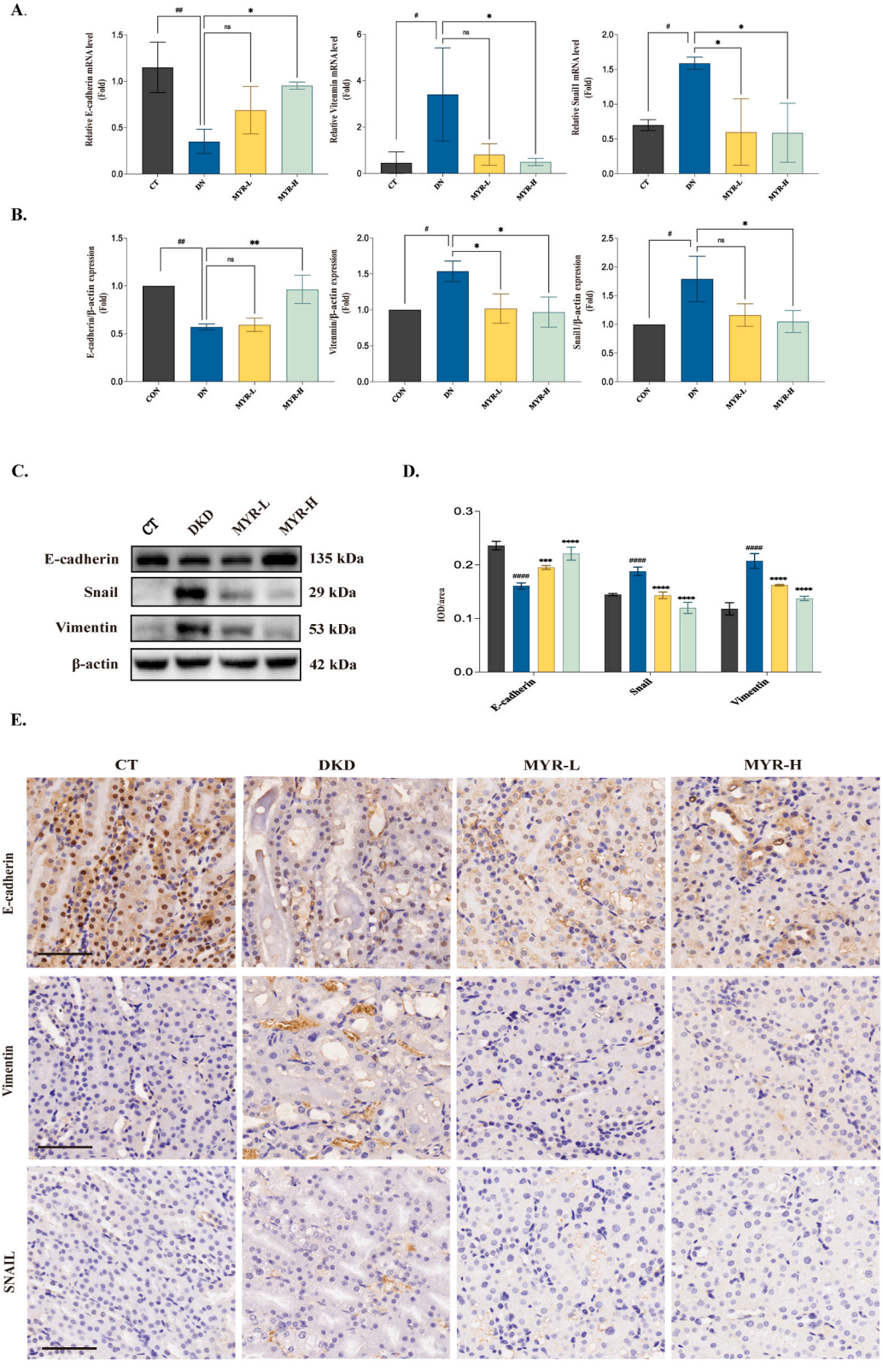

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Fig. 2. Mitigation of epithelial-mesenchymal transition in renal tubule cells by myricetin In vivo.
A. RT-PCR quantification of mRNA levels of E-cadherin, Vimentin, and Snail in renal tissues (n = 3). B–C. Verification of E-cadherin, Vimentin, and Snail protein expression in renal tissues via Western blot analysis (n = 3), with β-actin serving as the internal standard. D-E. Immunohistochemical analysis of E-cadherin, Vimentin, and Snail in renal tissues. (×200 magnification, Scale bar = 60 μM) Data presented as means ± SD, (n = 3/group). Significance indicated by #p < 0.05, ##p < 0.01, ###p < 0.001, and ####p < 0.0001, ns = no significant, versus control (Ctrl); *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns = no significant, versus diabetic nephropathy (DN).

Fig. 3. Network pharmacology analysis and target identification for DN-Component-EMT Interface.
A. Correlation of DN with Myricetin and EMT target genes illustrated. B. Shared PPI network among DN, Myricetin, and EMT delineated. C. Enriched GO Terms across Biological Processes (BP), Cellular Component (CC), and Molecular Function (MF) for Myricetin's Potential Targets. D. Visualization of predominant KEGG pathways via bubble maps, highlighting Myricetin's action spectrum.

through advanced delivery systems such as nanomaterials. For instance, a recent study demonstrated that Myricetin-loaded solid lipid nanoparticles (SLNs) significantly alleviate DN pathology more effectively than Myricetin in its natural form by reducing oxidative stress and enhancing antioxidant enzyme levels [36]. This highlights that SLN encapsulation successfully addresses the bioavailability issues of Myricetin, underscoring its potential as a valuable natural compound for future clinical needs.

5. Conclusions

Through an integrative approach that combines network pharmacology with advanced bioinformatics, our study provides detailed insights into the therapeutic mechanisms and key molecular targets of Myricetin in the modulation of EMT in diabetic nephropathy.

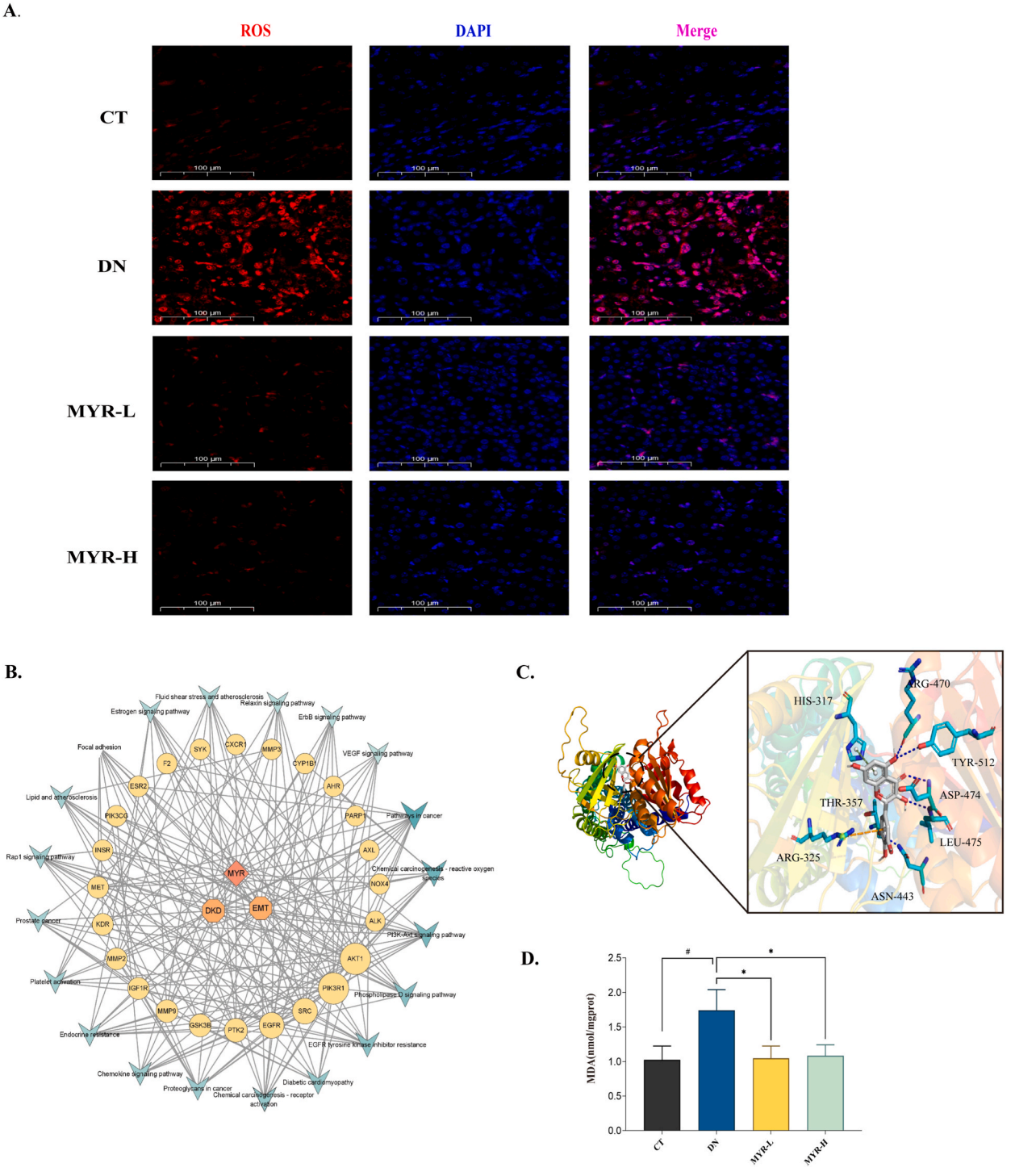

Fig. 4. Myricetin's suppressive effect on reactive oxygen species in DN mice.
A. Visualization of ROS in renal tissue sections through fluorescent staining, with ROS intensity mirrored by red fluorescence and nuclei depicted in blue via DAPI staining. (Scale bar = 100 μm) B. Construction of a target-pathway map, featuring top KEGG pathways and key targets, with visual adjustments based on betweenness centrality (BC) values. C. Illustration of Myricetin's interaction with NOX4, depicting the ligand and protein structure interactions, with hydrogen bonds marked by dark blue dashed lines. D. The levels of MDA (Malonaldehyde) in kidney tissues of mice.

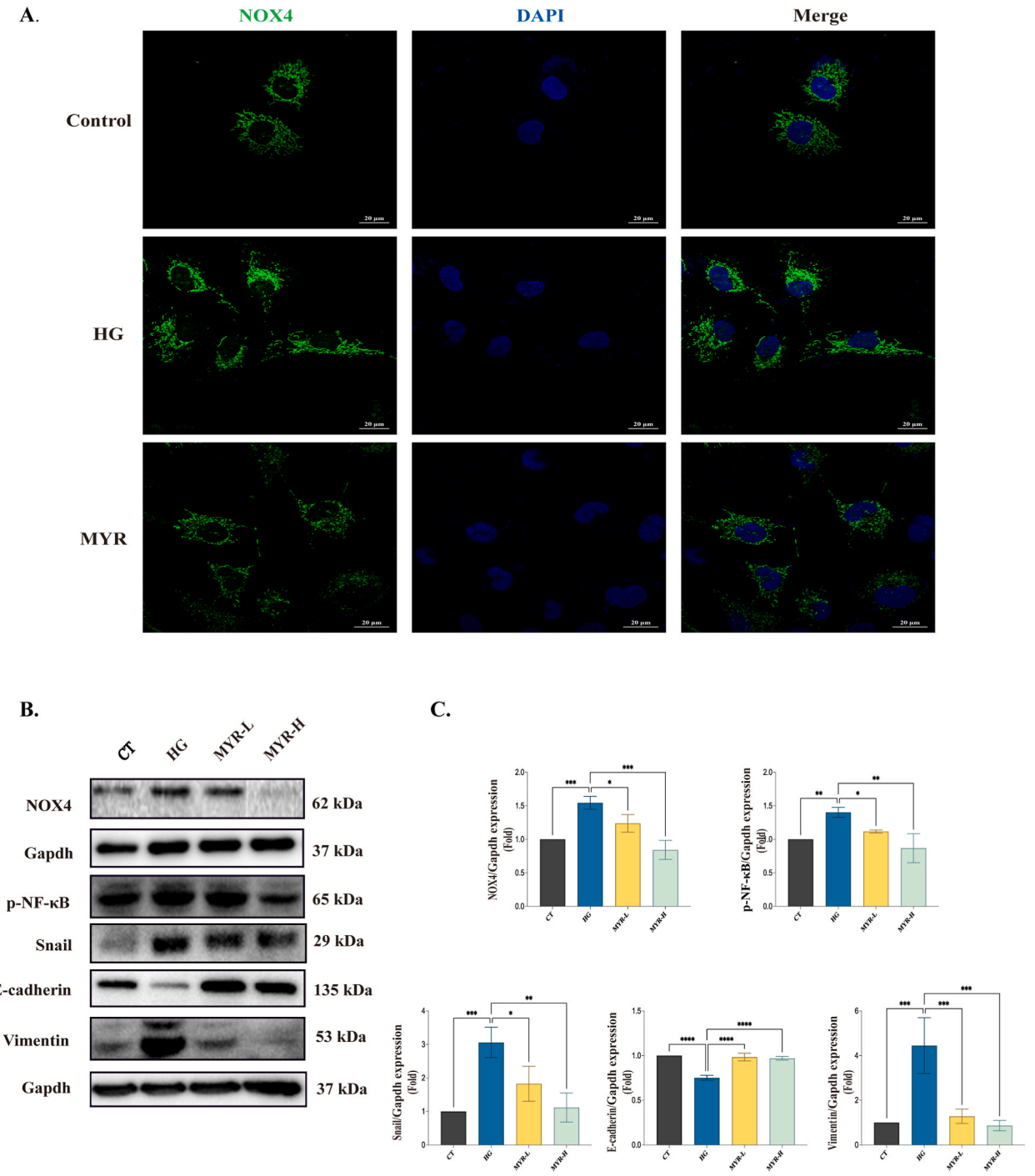

Fig. 5. Myricetin attenuates EMT in HK2 cells by downregulating NOX4 expression.
A. HK-2 cells cultured and treated with glucose ± Myricetin for 24 h, followed by immunocytochemical analysis using NOX4-specific primary antibody and Alexa Fluor 488-conjugated secondary antibody. Nuclei stained with DAPI (blue). (Scale bar = 20 μM) B–C. Protein expression analysis of Snail, E-cadherin, Vimentin, NOX4, and P–NF-κB in HK-2 cells post-treatment, evaluated via Western blot (n = 3). Quantification conducted with ImageJ, normalized to GAPDH. Data represented as mean ± standard deviation (n = 3/group), indicating significance with ##p < 0.01, ###p < 0.001, and ####p < 0.0001, ns = no significant, versus control (Ctrl); *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns = no significant, versus 35 mM glucose (HG).

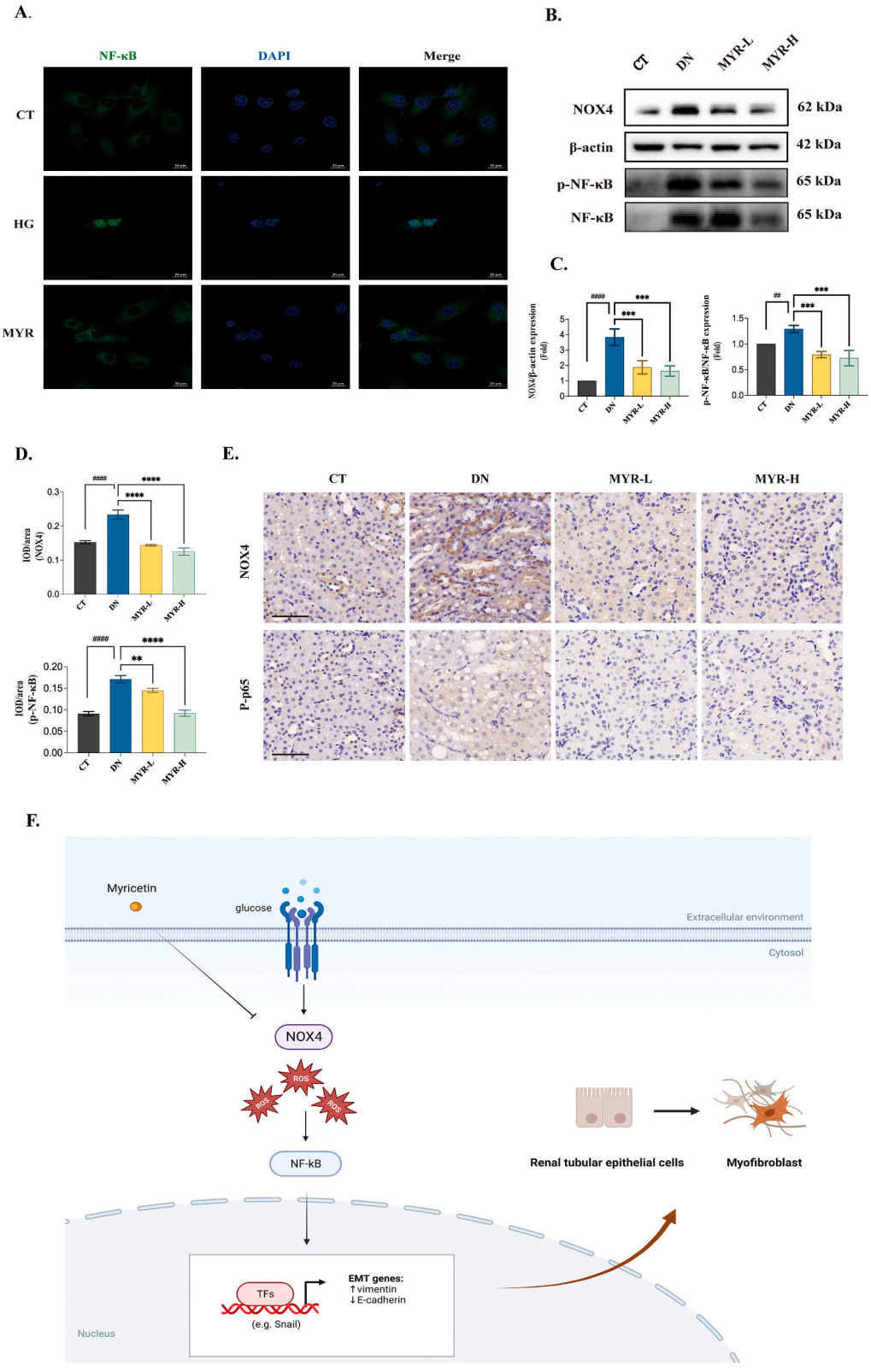

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Fig. 6. Reduction of NF-κB activation and NOX4 expression in DN mice via myricetin.
A. NF-κB staining in HK-2 cells treated with glucose ± Myricetin for 24 h, using NF-κB-specific primary antibody and Alexa Fluor 488-conjugated secondary antibody. Nuclei counterstained with DAPI (blue). (Scale bar = 20 μm) B–C. Validation of NF-κB and NOX4 protein levels in renal tissues by Western blot (n = 3), with β-actin as internal reference. D-E. Immunohistochemical assessment of P–NF-κB and NOX4 expression in the renal cortex of DN mice, accompanied by statistical analysis. (×200 magnification, Scale bar = 60 μm) G. Elucidation of Myricetin's mechanism in mitigating EMT in diabetic nephropathy via inhibition of the NOX4-NF-κB-Snail signaling pathway. Data depicted as mean ± standard deviation (n = 3/group), with significance denoted by ##$p < 0.01$, ###$p < 0.001$, and ####$p < 0.0001$, ns = no significant, versus control (Ctrl); *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, ns = no significant, versus diabetic nephropathy (DN).

Our findings illuminate Myricetin's action through the modulation of the NOX4/NF-κB/Snail signaling pathways, alongside a significant reduction in ROS production, delineating a multi-targeted, safe therapeutic strategy for DN. This investigation not only highlights Myricetin as a potent therapeutic agent with the potential to ameliorate DN but also sets a foundation for further clinical research to exploit its therapeutic benefits fully. The insights gained here advocate for Myricetin's inclusion in future treatment paradigms for diabetic nephropathy, emphasizing the critical role of targeted molecular interventions in overcoming this complex disease.

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Institutional review board statement

This study was reviewed and approved by the Animal Ethics Committee of Southern Medical University with the approval number: SMUL2022007. The date of resolution: March 10, 2022.

Data availability statement

Data are contained within the article.

CRediT authorship contribution statement

Ningning Yuan: Writing – original draft, Visualization, Validation, Project administration, Methodology, Data curation. **Yuchi Chen:** Writing – original draft, Visualization, Software, Data curation, Conceptualization. **Yangtian Yan:** Software, Resources, Investigation, Formal analysis, Data curation. **Fujing Wang:** Methodology. **Xinyao Xu:** Data curation. **Mingqing Wang:** Validation, Project administration, Funding acquisition. **Jianxin Diao:** Validation, Resources, Project administration. **Wei Xiao:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e35234.

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