Tohoku J. Exp. Med., 2025, **266**, 209-217

Mechanism of Hippo/YAP Axis Mediating High Glucose-Induced Ferroptosis in HK-2 Cells

Yifan Zhang,[1] Zhaoyu Lin,[2] Zhoutao Xie[1] and Yingxue He[1]

[1]Division of Nephrology, Wenzhou Central Hospital, The Dingli Clinical College of Wenzhou Medical University, The Second Affiliated Hospital of Shanghai University, Wenzhou, P.R. China
[2]Division of Endocrinology, Wenzhou Central Hospital, The Dingli Clinical College of Wenzhou Medical University, The Second Affiliated Hospital of Shanghai University, Wenzhou, P.R. China

The current study focused on the molecular mechanisms behind high glucose (HG)-induced ferroptosis in HK-2 cells. HG-induced epithelial-to-mesenchymal transition (EMT) HK-2 cell model displayed elevated Vimentin, α-SMA, Fe^{2+} and MDA expression, suppressed cell viability and proliferation capabilities, decreased E-cadherin, GPX4 and GSH levels, and increased cell death. Additionally, knockdown of Vimentin or α-SMA caused the opposite outcomes. Restaint of ferroptosis partially reversed the promotion role of knockdown of Vimentin or α-SMA in HK-2 cell proliferation. Further inhibition of the Hippo/YAP pathway could partly regulate the effects of Vimentin or α-SMA on HG-induced ferroptosis and proliferation in HK-2 cells. Conclusively, HG could increase the expression levels of Vimentin and α-SMA in HK-2 cells, and induce EMT, thereby inhibiting the activation of the Hippo/YAP signaling axis and cell proliferation, and promoting cell ferroptosis of HK-2 cells.

Keywords: epithelial-to-mesenchymal transition; ferroptosis; high glucose; hippo/yes-associated protein; HK-2 cells
Tohoku J. Exp. Med., 2025 July, **266** (3), 209-217.
doi: 10.1620/tjem.2024.J148

Introduction

Diabetic kidney disease (DKD) stands as a clinical syndrome marked by persistent proteinuria and progressive renal function decline, which constitutes one of the most prevailing and severe complications of diabetes mellitus (Samsu 2021; Limonte et al. 2022). Notably, angiotensin-converting enzyme inhibitors, angiotensin II receptor blockers, and sodium-glucose cotransporter 2 inhibitors have demonstrated benefits in renal protection (Liu et al. 2023a). Nevertheless, these treatment options fail to reverse the progression of DKD completely, and cardiovascular mortality among DKD patients in China is still on the increase (Samsu 2021). Accordingly, there is a pressing need to further investigate the pathogenesis of DKD, with the view of providing novel insights and strategies for its treatment.

The complex pathogenesis of DKD encompasses a diverse range of pathways, which can be generally classified into inflammatory, metabolic, fibrotic and hemodynamic factors (Watanabe et al. 2022). Notably, fibrosis is featured by myofibroblast (the collagen-depositing cells) accumulation. Apart from bone marrow-sourced myofibroblasts, epithelial-to-mesenchymal transition (EMT)-produced resident fibroblasts play a pivotal role in the progression of renal fibrosis (Lovisa et al. 2015). A lineage tracing analysis has indicated that EMT in tubular epithelial cells plays a crucial role in the development of renal fibrosis (LeBleu et al. 2013). Rather than transforming into myofibroblasts directly, the renal epithelial cells undergo a partial EMT and dedifferentiation to produce chemokines and cytokines that stimulate inflammation and fibrogenesis, thus prompting fibrosis development (Liu et al. 2023b). Importantly, α-smooth muscle actin (α-SMA) is currently recognized as a marker for myofibroblasts (Zhao et al. 2018), and Vimentin is deemed to be an intermediate product during the transformation of renal tubular epithelial cells into myofibroblasts (Bob et al. 2014). Under hyperglycemic conditions, the levels of Vimentin and α-SMA are proven to be elevated, which further contributes to the complex pathology of DKD-involved renal fibrosis (Li et al. 2024). The underlying mechanisms of DKD-invloved renal fibrosis are intricate, which warrants further exploration.

Received July 26, 2024; revised and accepted December 19, 2024; J-STAGE Advance online publication December 26, 2024
Correspondence: Yifan Zhang, Division of Nephrology, Wenzhou Central Hospital, The Dingli Clinical College of Wenzhou Medical University, The Second Affiliated Hospital of Shanghai University, No. 252, Baili East Road, Wenzhou, Zhejiang province 325000, P.R. China.
e-mail: Yzfan252wz@163.com

Yes-associated protein (YAP) is perceived as a critical downstream transcriptional coactivator within the Hippo signaling pathway (Wei et al. 2023). The Hippo/YAP pathway plays a pivotal role in regulating various biological processes comprising cell proliferation, survival, and differentiation, which is also correlated with fibrogenesis (Gokey et al. 2021). During the development of fibrosis, activated YAP demonstrates the ability to accelerate the formation of myofibroblasts and stimulates their accumulation (Hu et al. 2021). Importantly, high-density cell contact-induced overexpression of E-cadherin has demonstrated the role of activating the tumor suppressor NF2 (merlin), which subsequently restrains cysteine deprivation or elastin-caused activation of the Hippo/YAP pathway and ferroptosis (Sun and Chi 2021). Furthermore, activation of the Hippo pathway is confirmed to induce ferroptosis in atherosclerosis and lung injury (Wang et al. 2021b; Wang et al. 2022). However, whether the Hippo/YAP pathway mediates ferroptosis in DKD remains obscure.

Currently, there are no domestic or international reports regarding the mediation of HG-triggered ferroptosis of renal tubular epithelial cells via the Hippo/YAP signaling axis and its impact on the progression of DKD, which attracts our attention to provide theoretical foundation for DKD treatment.

Materials and Methods

Cell culture

Human renal tubular epithelial cells (HK-2, FH0228) were cultured in HK-2 complete medium (FH-HK-2) at 37°C in a humidified atmosphere with 5% CO_2. Both the cells and medium were sourced from FuHeng BioLogy (Shanghai, China).

Cell transfection and grouping

HK-2 cells were transfected with Vimentin siRNA (si-Vimentin), *α*-SMA siRNA (si-*α*-SMA), YAP siRNA (si-YAP), and scramble siRNA (si-NC) (all from GenePharma, Shanghai, China) at a final concentration of 100 ng/μL utilizing Lipofectamine® 2000 (Invitrogen, Carlsbad, CA, USA). Cells were allocated into the two groups: Blank (cultured with normal 5.5 mM D-glucose) and HG (induced with 30 mM D-glucose) groups (Lin et al. 2020). After HG treatment, cells were assigned into the following groups: si-NC (transfected with si-NC), si-Vim (transfected with si-Vimentin), si-*α*-SMA (transfected with si-*α*-SMA), si-Vim + Lip-1 (HK-2 cells were treated with si-Vimentin and 1 μM liproxatin-1 [a ferroptosis inhibitor, SML1414, sigma]) (Li et al. 2023a), si-Vim + vehicle (HK-2 cells were treated with si-Vimentin and solvents [50% PEG300 and 50% Saline] equal to liproxstatin-1), si-*α*-SMA + Lip-1 (HK-2 cells were treated with si-*α*-SMA and 1 μM liproxatin-1), si-*α*-SMA + vehicle (HK-2 cells were treated with si-*α*-SMA and the same amount of solvents [50% PEG300 and 50% Saline] as liproxatin-1), si-Vim + si-NC (HK-2 cells were co-transfected with si-Vimentin and si-NC), si-Vim + si-YAP (HK-2 cells were co-transfected with si-Vimentin and si-YAP), si-*α*-SMA + si-NC (HK-2 cells were co-transfected with si-*α*-SMA and si-NC), and si-*α*-SMA + si-YAP (HK-2 cells were co-transfected with si-*α*-SMA and si-YAP). Experiments were conducted independently in triplicate. Following 48 hours, cells were gathered for further analysis.

Western blot analysis

After trypsin detachment, cells were lysed using lysis buffer (AR0107, Boster Biological Technology, Wuhan, China). This was followed by determination of total protein concentration using a bicinchoninic acid kit (AR1189, Boster Biological Technology). Proteins were separated by SDS-PAGE, transferred to PVDF membranes, and then blocked with 3% bovine serum albumin (AR0184, Boster Biological Technology) for 2 hours. The samples were added and incubated with primary antibodies (Table 1) overnight at 4°C, and cultured with goat anti-rabbit IgG H&L (HRP) secondary antibody for 1 hour at room temperature in the dark. Protein bands were visualized using electrochemiluminescence solution (AR1191, Boster Biological Technology). Finally, band density quantification were enforced using Image Pro Plus 6.0 software (Media Cybernetics, USA), with glyceraldehyde-3-phospate dehydrogenase acting as an internal parameter. The experiment was repeated thrice (Ali 2021).

Cell counting kit-8 (CCK-8) assay

Differently-treated cells were seeded onto a 96-well plate at a density of 5×10^3 cells per well. Cell viability at 0, 12, 24, and 48 hours was assessed using the CCK-8 assay kit (CA1210, Solarbio). The samples were added with CCK-8 solution (100 μL/well), followed by incubation at 37°C for 2 hours. A microplate reader (Thermo Fisher Scientific, USA) was employed for measuring optical density at 450 nm (Lin et al. 2020).

Table 1. Primer sequences.

	Article number and company	Dilution rate
Vimentin	Ab92547, Abcam (Rabbit)	1/1,000
α-SMA	Ab5694, Abcam (Rabbit)	1 μg/ml
E-cadherin	Ab314063, Abcam (Rabbit)	1/1,000
GPX4	Ab125066, Abcam (Rabbit)	1/2,000
Lats1	#3477, Cell Signaling (Rabbit)	1/1,000
p-Lats1	#8654, Cell Signaling (Rabbit)	1/1,000
YAP	#14074, Cell Signaling (Rabbit)	1/1,000
p-YAP	#13008, Cell Signaling (Rabbit)	1/1,000
GAPDH	Ab181602, Abcam (Rabbit)	1/1,000
IgG H&L (HRP)	Ab6721, Abcam (Goat anti-rabbit)	1/2,000

Colony formation assay

Cells were seeded into 6-well plates (500 cells/well), and subjected to incubation for two weeks. When colony formation was visible, the culture medium was removed, and the cells were fixed with 2 mL of methanol for 30 minutes. After discarding the methanol, colonies were stained with 0.1% crystal violet (2 mL/well; 548-62-9, MACKLIN, Shanghai, China) for 3 minutes. Colonies were monitored and counted under a microscope (Olympus, Tokyo, Japan) (Zhu et al. 2020). The formula was as hereafter: colony number = average colony number × dilution multiple.

Determination of Fe^{2+}, malondialdehyde (MDA) and glutathione (GSH) levels

Levels of GSH, MDA and Fe^{2+} were measured using the reduced GSH Assay kit (Ab138881, Abcam, Cambridge, MA, USA), MDA Assay kit (Ab118970, Abcam) and Fe^{2+} Assay kit (Ab83366, Abcam), respectively (Wang et al. 2021a).

Lactate dehydrogenase (LDH) release assay

As per the instructions, cell death was evaluated using a LDH Assay kit (A020-2-2, Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China) (Li et al. 2022).

Statistical analysis

Data were analyzed using GraphPad Prism software version 8.01 (GraphPad Software Inc). Normality of data distribution was confirmed by the Kolmogorov-Smirnov test. Results were signified as mean ± standard deviation. For comparisons between two groups, the independent sample t-test was adopted, while comparisons among multiple groups were conducted using one-way ANOVA, followed by Tukey's multiple comparisons test. Differences were reported as statistically significant at $P < 0.05$.

Results

HG induces EMT in HK-2 cells and promotes E-cadherin-mediated ferroptosis

Referring to previously established methodologies, we cultured HK-2 cells and induced them with HG (30 mM D-glucose) for 48 hours (Lin et al. 2020). Western blot analysis detecting the expression of fibroblast markers

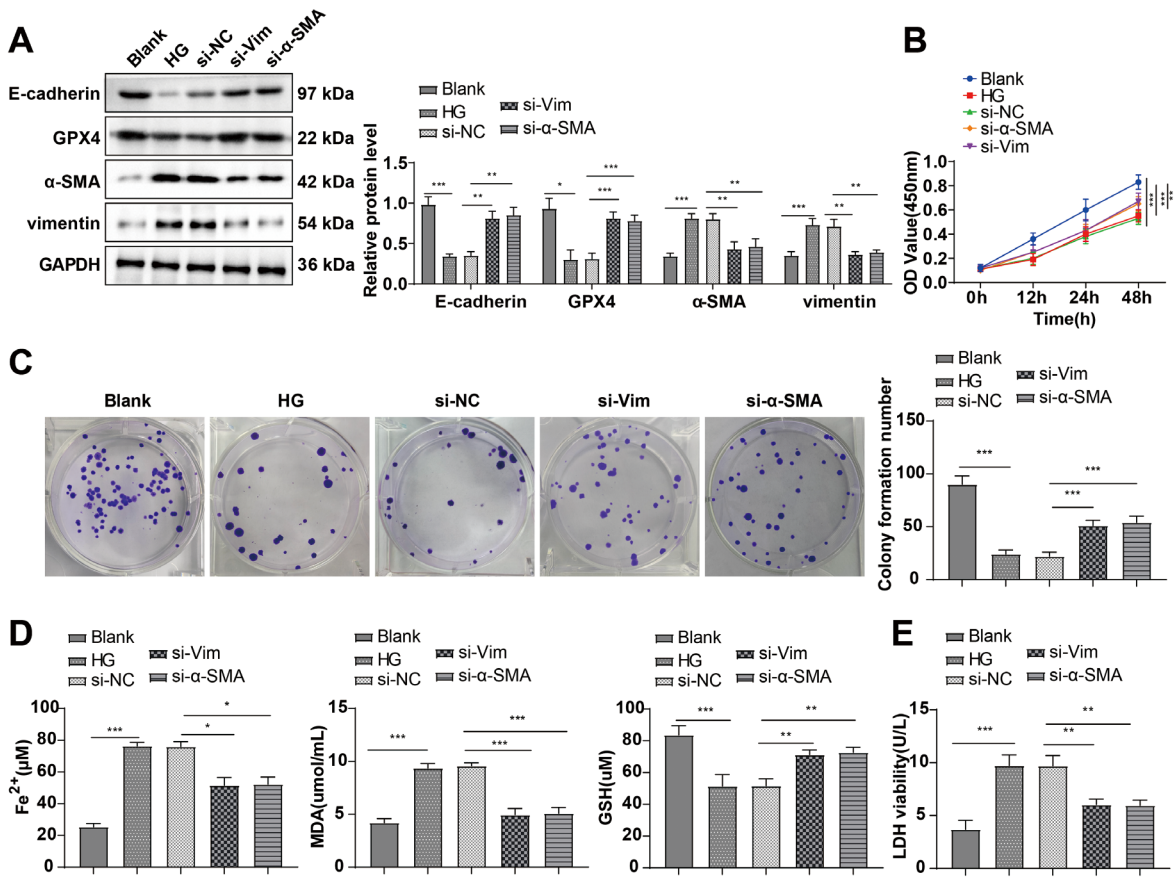

Fig. 1. HG induces EMT in HK-2 cells, suppresses proliferation, and promotes ferroptosis.
(A) Western blot analysis of GPX4, Vimentin, α-SMA, and E-cadherin protein expression levels in cells; (B) Cell proliferation was assessed by CCK-8 assay; (C) Cell proliferative ability was evaluated through colony formation assay; (D) Levels of Fe^{2+}, MDA, and GSH were measured using ELISA; (E) Cell death was determined by LDH assay. Experiments were independently repeated three times, and data are presented as mean ± standard deviation. Comparisons between two groups were performed using an independent sample t-test, with Tukey's multiple comparisons test used for post-hoc analysis. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Vimentin and α-SMA revealed that the HG group represented elevations in Vimentin and α-SMA expression levels ($P < 0.001$) (Fig. 1A). Cell viability and proliferation were assessed through CCK-8 and colony formation assays. It was indicative of that in contrast to the Blank group, the HG group exhibited marked decreases in cell viability and proliferation ($P < 0.01$) (Fig. 1B,C). Additionally, HK-2 cells exposed to a HG environment underwent ferroptosis (Lu et al. 2023). Western blot analysis was further used to determine E-cadherin and the ferroptosis-related protein GPX4. It was revealed that in comparison with the Blank group, E-cadherin ($P < 0.01$) and GPX4 ($P < 0.05$) expression dropped in the HG group (Fig. 1A). The kit detection demonstrated that relative to the Blank group, levels of Fe^{2+} and MDA were elevated while GSH levels were diminished in the HG group ($P < 0.05$) (Fig. 1D). LDH assays assessing cell death identified an increase in cell death in the HG group versus the Blank group ($P < 0.05$) (Fig. 1E). Furthermore, we transfected HK-2 cells with si-Vimentin or si-α-SMA during HG induction. Outcomes unraveled that in comparison with the si-NC group, the si-Vim and si-α-SMA groups exhibited decreased expression patterns of Vimentin and α-SMA, and heightened expression of E-cadherin ($P < 0.01$), significantly strengthened cell viability and proliferation ($P < 0.001$), markedly higher GPX4 expression and GSH levels ($P < 0.01$), and notably, lower levels of Fe^{2+} and MDA ($P < 0.01$), thus resulting in an attenuation in cell death ($P < 0.01$) (Fig. 1A-E). These findings suggest that HG induction promotes the EMT of HK-2 cells, constrains cell proliferation and enhances ferroptosis.

Inhibition of ferroptosis affects the functional regulation of HK-2 cells mediated by Vimentin or α-SMA

To ascertain whether Vimentin or α-SMA can influence ferroptosis in HK-2 cells, we co-treated HK-2 cells with HG, si-Vimentin or si-α-SMA and the ferroptosis inhibitor liproxstatin-1. Our findings manifested that relative to the si-Vim + vehicle group, the si-Vim + Lip-1 group exhibited a promotion in cell viability and proliferation, reductions in levels of Fe^{2+} and MDA, enhancements in levels of GPX4 and GSH, and an abatement in cell death ($P < 0.01$) (Fig. 2A-E). Moreover, compared to the si-α-SMA + vehicle group, the si-α-SMA + Lip group showed a facilitation in cell viability and proliferation, decreases in Fe^{2+} and

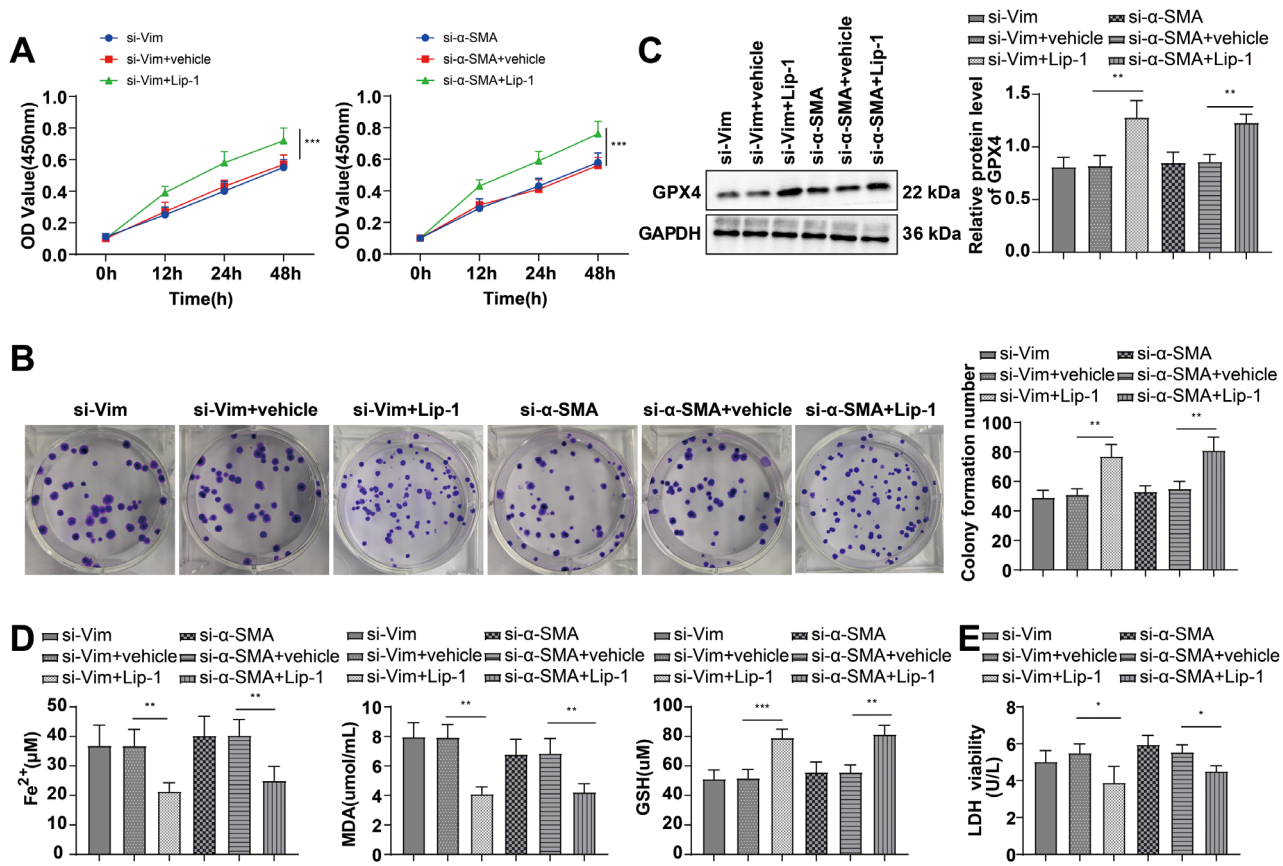

Fig. 2. Inhibition of ferroptosis impacts the functional modulation of HK-2 cells mediated by Vimentin or α-SMA.
(A) Cell proliferation was assessed by CCK-8 assay; (B) Cell proliferative ability in each group was evaluated through colony formation assay; (C) Western blot analysis of the ferroptosis-related protein GPX4 expression; (D) Expression levels of Fe^{2+}, MDA, and GSH were measured using ELISA; (E) Cell death was determined by LDH assay. Experiments were conducted three times independently, and data are shown as mean ± standard deviation. Comparisons among multiple groups were performed using one-way ANOVA with Tukey's multiple comparisons test for post-hoc analysis. $^{**}P < 0.01$, $^{***}P < 0.001$.

MDA levels, and augmentations in GPX4 and GSH levels, with an alleviation in cell death ($P < 0.01$) (Fig. 2A-E). The aforementioned performances indicate that suppression of ferroptosis produces the effects on functional modulation of HK-2 cells through Vimentin or α-SMA.

Suppression of HG-induced EMT in HK-2 cells further suppresses the activation of Hippo/YAP signaling pathway

Activation of the Hippo pathway has been identified to induce ferroptosis (Wang et al. 2021b; Wang et al. 2022). Consequently, we hypothesized that HG-induced overexpression of Vimentin or α-SMA could activate the Hippo/YAP signaling pathway. Western blot was applied for assessing the levels of the Hippo pathway-related proteins Lats1, p-Lats1, and the levels of YAP and p-YAP proteins. Relative to the Blank group, apparent elevations in p-Lats1/Lats1 and p-YAP/YAP ratios were noticed in the HG group (all $P < 0.001$). Conversely, in the si-Vim and si-α-SMA groups, significant declines in p-Lats1/Lats1 and p-YAP/YAP ratios were observed versus the si-NC group (all $P < 0.001$) (Fig. 3). These results suggest that silence of Vimentin or α-SMA curbs the Hippo/YAP signaling pathway.

Restraint of the Hippo/YAP signaling pathway partly regulates the effects of Vimentin or α-SMA on ferroptosis and proliferation in HK-2 cells

Lastly, we administered the simultaneous treatment of si-Vimentin or si-α-SMA and si-YAP on HK-2 cells during HG induction. As reflected by the results, compared to the si-Vim + si-NC group, the si-Vim + si-YAP group exhibited reduced p-YAP/YAP ratio, enhanced cell viability and proliferation, decreased levels of Fe^{2+} and MDA, increased levels of GPX4 and GSH, and dampened cell death (all $P < 0.05$). In a similar light, relative to the si-α-SMA + si-NC group, si-α-SMA + si-YAP group showed decreased p-YAP/YAP ratio, increased cell viability and proliferation, lowered Fe^{2+} and MDA levels, and raised GPX4 and GSH levels, and diminished cell death (all $P < 0.05$) (Fig. 4A-E). These findings suggest that containment of the Hippo/YAP signaling pathway curbs HG-caused ferroptosis in HK-2 cells.

Discussion

The pathogenesis of DKD is multifaceted and has not yet been fully elucidated. Among the relevant mechanisms, hyperglycemia-induced EMT in renal tubular epithelial cells, is believed to be a process associated with renal fibrosis (Wang et al. 2023b). In the current study, we focused on HG-caused ferroptosis of HK-2 cells. Through a series of *in vitro* experiments, this study highlighted that HG could induce EMT of HK-2 cells, and increase the expression of Vimentin and α-SMA, thereby inactivating the Hippo/YAP signaling axis, limiting cell proliferation and bolstering cell ferroptosis of HK-2 cells. This study provided a comprehensive understanding of the possible mechanism of Hippo/YAP axis on ferroptosis and proliferation of HG-induced HK-2 cells, with the aim of offering the basis for the development of new therapeutic targets and effective drugs for DKD.

Notably, HG treatment induces the accumulation of extracellular matrix (ECM), secretion of inflammatory cytokines, cytotoxicity (Lin et al. 2020), and interaction between ferroptosis and E-cadherin (Wu et al. 2019). Li et. al. (2023b) have confirmed that under HG environment, the death of HK-2 cells is increased. Following 30 mM D-glucose induction for 48 hours, we discovered that HG

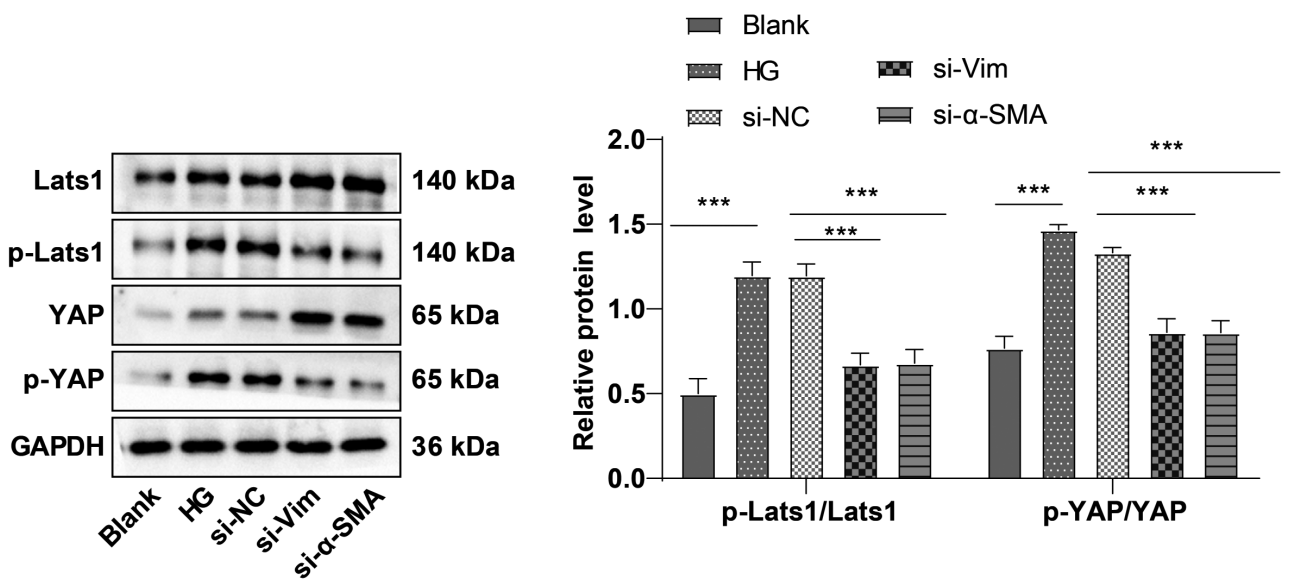

Fig. 3. Silence of Vimentin or α-SMA inhibits the Hippo/YAP signaling pathway.
Western blot analysis for Lats1, p-Lats1, YAP and p-YAP proteins related to Hippo/YAP signaling pathway. Experiments were independently repeated three times, and data are presented as mean ± standard deviation. Comparisons among multiple groups were made using one-way ANOVA, with Tukey's multiple comparisons test for post-hoc analysis. $^{**}P < 0.01$, $^{***}P < 0.001$.

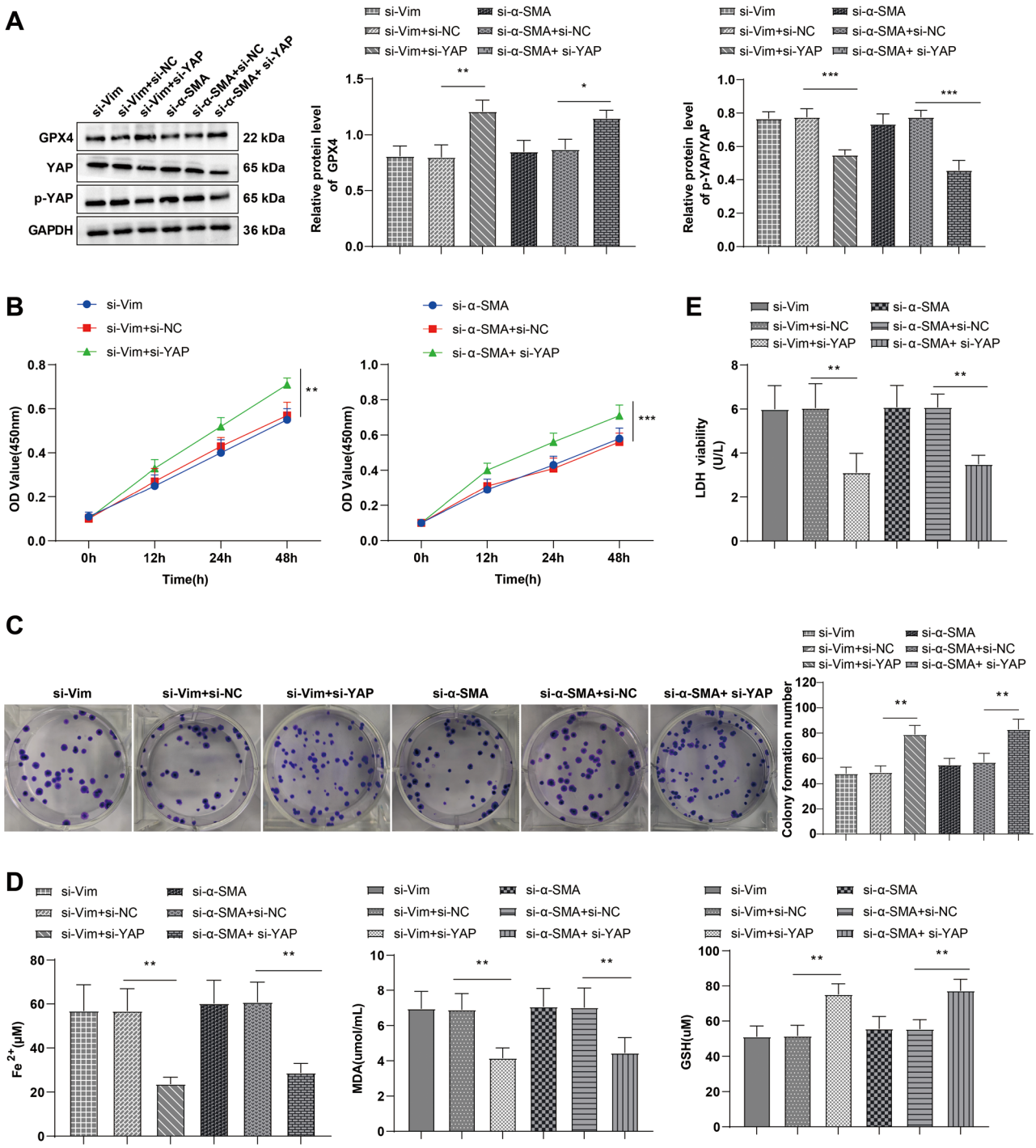

Fig. 4. Inhibition of the Hippo/YAP signaling pathway partly reverses the effects of knockdown of Vimentin or α-SMA on ferroptosis and proliferation in HK-2 cells.
(A) Western blot analysis for GPX4, YAP and p-YAP protein expression levels; (B) Cell viability was assessed by CCK-8 assay; (C) Cell proliferative ability was evaluated through colony formation assay; (D) Levels of Fe^{2+}, MDA, and GSH were measured using ELISA assay kits; (E) Cell death was determined by LDH assay. Experiments were independently repeated three times, with data represented as mean ± standard deviation. Comparisons among multiple groups were made using one-way ANOVA, with Tukey's multiple comparisons test used for post-hoc analysis. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

may induce the EMT in HK-2 cells to promote E-cadherin-mediated ferroptosis. Specifically, we identified raised expression levels of Vimentin and α-SMA, subdued E-cadherin, elevated Fe^{2+} and MDA, and reduced GSH level in HG-treated HK-2 cells. Previous *in vitro* study has indicated that HG environments stimulate fibroblasts to produce both fibrillary and non-fibrillar collagens, along with specialized ECM proteins including fibronectin and

matricellular macromolecules (Tuleta and Frangogiannis 2021). Importantly, renal tubular epithelial cells have been demonstrated to transform into myofibroblasts in diabetes mellitus under specific conditions, subsequently increasing ECM production. This transformation typically involves an increase in mesenchymal markers comprising Vimentin and α-SMA and a decrease in the epithelial marker E-cadherin (Gu et al. 2013). Of note, ferroptosis is documented to be non-cell autonomously modulated by cadherin-mediated intercellular interaction (Wu et al. 2019). The E-cadherin-mediated interaction dampens ferroptosis in epithelial cells via the activation of intracellular NF2 (merlin) and the Hippo pathways (Bao et al. 2021; Minikes et al. 2023). In a similar light, Huang et al. (2022) have also elicited that HG distinctly enhances LDH activity and lipid reactive oxygen species production, raises Fe^{2+} content and MDA levels, and subdues GSH and GPX4 levels. Ferroptosis is considered a unique form of non-apoptotic regulated cell death in 2012, which is characterized by its occurrence independent of caspases that is involved in apoptosis (Jiang et al. 2021). Since its discovery, ferroptosis has been implicated in various disease processes, encompassing those affecting immune, neuronal and renal cells (Sha et al. 2021). Furthermore, HK-2 cells in established HG-induced models exhibit signs of ferroptosis (Lu et al. 2023). Taken together, HG led to EMT in HK-2 cells, restraint cell proliferation and contributed to ferroptosis.

Next, we shift our focus to Vimentin and α-SMA to further investigate whether Vimentin or α-SMA can influence ferroptosis in HK-2 cells. Our findings revealed that ferroptosis suppression can influence the functional regulation of HK-2 cells via Vimentin or α-SMA. Accumulating evidence underscores the pivotal role of ferroptosis in the initiation and advancement of DKD, and the protective role of ferroptosis inhibition in DKD models (Wang et al. 2020; Wang et al. 2023a). In addition, Ren et al. (2023) have reported that the occurrence of EMT may result in greater sensitivity to ferroptosis. Importantly, EMT serves as a primary pathological mechanism in DKD toward end-stage renal disease, and its progression is related to endoplasmic reticulum stress (ERS) activation, which in turn drives ferroptosis. By inhibiting ERS, one recent study has found not only a reversal of EMT-associated changes but also a reduction in HG-caused ferroptosis (Liu et al. 2023b). Altogether, ferroptosis repression impacted functional regulatory role of Vimentin or α-SMA in HK2 cells.

Furthermore, this study also elicited that silencing of Vimentin or α-SMA could suppress the activation of the Hippo/YAP signaling pathway. The Hippo-YAP signaling is acknowledged as an integrator of cellular signals that plays a pivotal role in governing proliferation and differentiation processes (Ibar and Irvine 2020). Meanwhile, Patel et. al. (2019) have proposed that Rac-GTPase contributes to fibrotic TGF-β1 signal transduction and chronic kidney disease via the Hippo/YAP/TAZ, epidermal growth factor receptor and p53 pathways. Activation of the Hippo pathway can trigger ferroptosis, which highlights its potential role in cellular regulation and disease pathogenesis (Wang et al. 2021b; Wang et al. 2022). YAP, known as a critical transcriptional regulator in this pathway, is notably influenced by HG conditions. Specifically, elevated glucose levels possess the ability to activate YAP/TAZ in cultured mesangial cells via the canonical Hippo pathway. This activation leads to the binding and stabilization of N-Myc proteins from the oncogene Myc family, thereby causing MC damage and the pathogenesis of DKD (Choi et al. 2023). Furthermore, a previous study has identified the Hippo/YAP signaling pathway as a potential therapeutic target for addressing diabetic angiopathy (Wei et al. 2023). Furthermore, we also observed that suppression of the Hippo/YAP pathway partially controlled the effects of Vimentin or α-SMA on ferroptosis and proliferation in HK-2 cells.

Collectively, our current study affirms that HD-induced elevation of Vimentin and α-SMA blocks the Hippo/YAP signaling axis to advance HG-caused ferroptosis of HK-2 cells, thus affecting the progression of DKD. However, our investigation is confined to the impact of HG-caused ferroptosis in HK-2 cells mediated by the Hippo/YAP signaling axis on the development of DKD, without extending to animal models or clinical studies. In addition, this study only conducts the experiment in a single cell line. As a consequence, future research will delve deeper into the effects of downstream target genes mediated by the Hippo/YAP signaling pathway and the regulation of processes including necroptosis and autophagy on the progression of DKD. Furthermore, we will continue the research on animal models or more cell lines, or conduct clinical studies. Additionally, broader and more in-depth studies are essential to develop new therapeutic targets and effective drugs for DKD.

Author Contributions

Conceptualization, Yifan Zhang; Methodology, Yifan Zhang; Software, Yingxue He; Validation, Zhaoyu Lin and Zhoutao Xie; Formal Analysis, Zhaoyu Lin and Yingxue He; Investigation, Zhoutao Xie; Resources, Yingxue He; Data Curation, Zhaoyu Lin; Writing – Original Draft Preparation, Yifan Zhang; Writing – Review & Editing, Yifan Zhang; Visualization, Zhoutao Xie; Supervision, Zhaoyu Lin; Project Administration, Yifan Zhang; Funding Acquisition, Yifan Zhang.

Funding

This research was supported by grants from Wenzhou Basic Scientific Research Project in 2023 (Y2023115), Zhejiang Province Traditional Chinese Medicine Science and Technology Plan Project (2024ZF142), “ShangDa Translational Medicine Funding” supported by Wenzhou Institute of Shanghai University and 2021 Zhejiang Health Science and Technology Plan Project (2021KY340).

Conflict of Interest

The authors declare no conflict of interest.

References

Ali, D. (2021) Reasons for Extraction of Permanent Teeth in a University Dental Clinic Setting. *Clin. Cosmet. Investig. Dent.*, **13**, 51-57.

Bao, Z., Hua, L., Ye, Y., Wang, D., Li, C., Xie, Q., Wakimoto, H., Gong, Y. & Ji, J. (2021) MEF2C silencing downregulates NF2 and E-cadherin and enhances Erastin-induced ferroptosis in meningioma. *Neuro. Oncol.*, **23**, 2014-2027.

Bob, F., Gluhovschi, G., Herman, D., Petrica, L., Bozdog, G., Gluhovschi, C., Velciov, S., Gadalean, F., Timar, R., Potencz, E., Dema, A. & Schiller, A. (2014) Immunohistochemical study of tubular epithelial cells and vascular endothelial cells in glomerulonephritis. *Ren. Fail.*, **36**, 1208-1214.

Choi, S., Hong, S.P., Bae, J.H., Suh, S.H., Bae, H., Kang, K.P., Lee, H.J. & Koh, G.Y. (2023) Hyperactivation of YAP/TAZ Drives Alterations in Mesangial Cells through Stabilization of N-Myc in Diabetic Nephropathy. *J. Am. Soc. Nephrol.*, **34**, 809-828.

Gokey, J.J., Patel, S.D. & Kropski, J.A. (2021) The Role of Hippo/YAP Signaling in Alveolar Repair and Pulmonary Fibrosis. *Front. Med.* (*Lausanne*), **8**, 752316.

Gu, L., Gao, Q., Ni, L., Wang, M. & Shen, F. (2013) Fasudil inhibits epithelial-myofibroblast transdifferentiation of human renal tubular epithelial HK-2 cells induced by high glucose. *Chem. Pharm. Bull.* (*Tokyo*), **61**, 688-694.

Hu, D., Jiang, J., Ding, B., Xue, K., Sun, X. & Qian, S. (2021) Mechanical Strain Regulates Myofibroblast Differentiation of Human Scleral Fibroblasts by YAP. *Front. Physiol.*, **12**, 712509.

Huang, J., Chen, G., Wang, J., Liu, S. & Su, J. (2022) Platycodin D regulates high glucose-induced ferroptosis of HK-2 cells through glutathione peroxidase 4 (GPX4). *Bioengineered*, **13**, 6627-6637.

Ibar, C. & Irvine, K.D. (2020) Integration of Hippo-YAP Signaling with Metabolism. *Dev. Cell*, **54**, 256-267.

Jiang, X., Stockwell, B.R. & Conrad, M. (2021) Ferroptosis: mechanisms, biology and role in disease. *Nat. Rev. Mol. Cell Biol.*, **22**, 266-282.

LeBleu, V.S., Taduri, G., O'Connell, J., Teng, Y., Cooke, V.G., Woda, C., Sugimoto, H. & Kalluri, R. (2013) Origin and function of myofibroblasts in kidney fibrosis. *Nat. Med.*, **19**, 1047-1053.

Li, D., Wang, Y., Dong, C., Chen, T., Dong, A., Ren, J., Li, W., Shu, G., Yang, J., Shen, W., Qin, L., Hu, L. & Zhou, J. (2023a) CST1 inhibits ferroptosis and promotes gastric cancer metastasis by regulating GPX4 protein stability via OTUB1. *Oncogene*, **42**, 83-98.

Li, J., Pang, Q., Huang, X., Jiang, H., Tang, G., Yan, H., Guo, Y., Yan, X., Li, L. & Zhang, H. (2024) 2-Dodecyl-6-Methoxycyclohexa-2, 5-Diene-1, 4-Dione isolated from Averrhoa carambola L. root inhibits high glucose-induced EMT in HK-2 cells through targeting the regulation of miR-21-5p/Smad7 signaling pathway. *Biomed. Pharmacother.*, **172**, 116280.

Li, S., Feng, L., Li, G., Liu, R., Ma, C., Wang, L., Gao, A., Liu, C., Cui, Y., Jiang, Z., Xie, Y., Wu, Q., Wang, X., Yang, L., Qi, Z., et al. (2023b) GSDME-dependent pyroptosis signaling pathway in diabetic nephropathy. *Cell Death Discov.*, **9**, 156.

Li, Y., Sun, X., Zhuang, J., Wang, J. & Yang, C. (2022) Donepezil ameliorates oxygen-glucose deprivation/reoxygenation-induced cardiac microvascular endothelial cell dysfunction through PARP1/NF-kappaB signaling. *Mol. Med. Rep.*, **25**, 121.

Limonte, C.P., Kretzler, M., Pennathur, S., Pop-Busui, R. & de Boer, I.H. (2022) Present and future directions in diabetic kidney disease. *J. Diabetes Complications*, **36**, 108357.

Lin, Y., Zhong, L., Li, H., Xu, Y., Li, X. & Zheng, D. (2020) Psoralen alleviates high glucose-induced HK-2 cell injury by inhibition of Smad 2 signaling via upregulation of microRNA 874. *BMC Pharmacol. Toxicol.*, **21**, 52.

Liu, Z., Liu, J., Wang, W., An, X., Luo, L., Yu, D. & Sun, W. (2023a) Epigenetic modification in diabetic kidney disease. *Front. Endocrinol.* (*Lausanne*), **14**, 1133970.

Liu, Z., Nan, P., Gong, Y., Tian, L., Zheng, Y. & Wu, Z. (2023b) Endoplasmic reticulum stress-triggered ferroptosis via the XBP1-Hrd1-Nrf2 pathway induces EMT progression in diabetic nephropathy. *Biomed. Pharmacother.*, **164**, 114897.

Lovisa, S., LeBleu, V.S., Tampe, B., Sugimoto, H., Vadnagara, K., Carstens, J.L., Wu, C.C., Hagos, Y., Burckhardt, B.C., Pentcheva-Hoang, T., Nischal, H., Allison, J.P., Zeisberg, M. & Kalluri, R. (2015) Epithelial-to-mesenchymal transition induces cell cycle arrest and parenchymal damage in renal fibrosis. *Nat. Med.*, **21**, 998-1009.

Lu, Q., Yang, L., Xiao, J.J., Liu, Q., Ni, L., Hu, J.W., Yu, H., Wu, X. & Zhang, B.F. (2023) Empagliflozin attenuates the renal tubular ferroptosis in diabetic kidney disease through AMPK/NRF2 pathway. *Free Radic. Biol. Med.*, **195**, 89-102.

Minikes, A.M., Song, Y., Feng, Y., Yoon, C., Yoon, S.S. & Jiang, X. (2023) E-cadherin is a biomarker for ferroptosis sensitivity in diffuse gastric cancer. *Oncogene*, **42**, 848-857.

Patel, S., Tang, J., Overstreet, J.M., Anorga, S., Lian, F., Arnouk, A., Goldschmeding, R., Higgins, P.J. & Samarakoon, R. (2019) Rac-GTPase promotes fibrotic TGF-beta1 signaling and chronic kidney disease via EGFR, p53, and Hippo/YAP/TAZ pathways. *FASEB J.*, **33**, 9797-9810.

Ren, Y., Mao, X., Xu, H., Dang, Q., Weng, S., Zhang, Y., Chen, S., Liu, S., Ba, Y., Zhou, Z., Han, X., Liu, Z. & Zhang, G. (2023) Ferroptosis and EMT: key targets for combating cancer progression and therapy resistance. *Cell. Mol. Life Sci.*, **80**, 263.

Samsu, N. (2021) Diabetic Nephropathy: Challenges in Pathogenesis, Diagnosis, and Treatment. *Biomed. Res. Int.*, **2021**, 1497449.

Sha, W., Hu, F., Xi, Y., Chu, Y. & Bu, S. (2021) Mechanism of Ferroptosis and Its Role in Type 2 Diabetes Mellitus. *J. Diabetes Res.*, **2021**, 9999612.

Sun, T. & Chi, J.T. (2021) Regulation of ferroptosis in cancer cells by YAP/TAZ and Hippo pathways: The therapeutic implications. *Genes Dis.*, **8**, 241-249.

Tuleta, I. & Frangogiannis, N.G. (2021) Diabetic fibrosis. *Biochimica Et Biophysica Acta-Molecular Basis of Disease*, **1867**, 166044.

Wang, H., Liu, D., Zheng, B., Yang, Y., Qiao, Y., Li, S., Pan, S., Liu, Y., Feng, Q. & Liu, Z. (2023a) Emerging Role of Ferroptosis in Diabetic Kidney Disease: Molecular Mechanisms and Therapeutic Opportunities. *Int. J. Biol. Sci.*, **19**, 2678-2694.

Wang, W., Zhu, L., Li, H., Ren, W., Zhuo, R., Feng, C., He, Y., Hu, Y. & Ye, C. (2022) Alveolar macrophage-derived exosomal tRF-22-8BWS7K092 activates Hippo signaling pathway to induce ferroptosis in acute lung injury. *Int. Immunopharmacol.*, **107**, 108690.

Wang, Y., Bi, R., Quan, F., Cao, Q., Lin, Y., Yue, C., Cui, X., Yang, H., Gao, X. & Zhang, D. (2020) Ferroptosis involves in renal tubular cell death in diabetic nephropathy. *Eur. J. Pharmacol.*, **888**, 173574.

Wang, Y., Jin, M., Cheng, C.K. & Li, Q. (2023b) Tubular injury in diabetic kidney disease: molecular mechanisms and potential therapeutic perspectives. *Front. Endocrinol.* (*Lausanne*), **14**, 1238927.

Wang, Y., Quan, F., Cao, Q., Lin, Y., Yue, C., Bi, R., Cui, X., Yang, H., Yang, Y., Birnbaumer, L., Li, X. & Gao, X. (2021a) Quercetin alleviates acute kidney injury by inhibiting ferroptosis. *J. Adv. Res.*, **28**, 231-243.

Wang, Y., Zhao, Y., Ye, T., Yang, L., Shen, Y. & Li, H. (2021b) Ferroptosis Signaling and Regulators in Atherosclerosis. *Front. Cell Dev. Biol.*, **9**, 809457.

Watanabe, K., Sato, E., Mishima, E., Miyazaki, M. & Tanaka, T. (2022) What's New in the Molecular Mechanisms of Diabetic Kidney Disease: Recent Advances. *Int. J. Mol. Sci.*, **24**, 570.

Wei, L., Gao, J., Wang, L., Tao, Q. & Tu, C. (2023) Hippo/YAP signaling pathway: a new therapeutic target for diabetes mellitus and vascular complications. *Ther. Adv. Endocrinol. Metab.*, **14**, 20420188231220134.

Wu, J., Minikes, A.M., Gao, M., Bian, H., Li, Y., Stockwell, B.R., Chen, Z.N. & Jiang, X. (2019) Intercellular interaction dictates cancer cell ferroptosis via NF2-YAP signalling. *Nature*, **572**, 402-406.

Zhao, W., Wang, X., Sun, K.H. & Zhou, L. (2018) alpha-smooth muscle actin is not a marker of fibrogenic cell activity in skeletal muscle fibrosis. *PLoS One*, **13**, e0191031.

Zhu, G., Cheng, Z., Huang, Y., Zheng, W., Yang, S., Lin, C. & Ye, J. (2020) MyD88 mediates colorectal cancer cell proliferation, migration and invasion via NF-kappaB/AP-1 signaling pathway. *Int. J. Mol. Med.*, **45**, 131-140.