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Elevated aerobic glycolysis in renal tubular epithelial cells influences the proliferation and differentiation of podocytes and promotes renal interstitial fibrosis

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Abstract. – OBJECTIVE: The aim of this study was to elaborate the influence of changing energy metabolism pattern of renal tubular epithelial cells in the process of renal interstitial fibrosis on podocytes. Meanwhile, we also investigated the relationship between energy metabolism pattern and the development of renal interstitial fibrosis.

MATERIALS AND METHODS: We established a model of renal interstitial fibrosis by unilateral ureteral obstruction (UUO). The protein and messenger RNA (mRNA) expression of fibrosis signs, such as α-smooth muscle actin (α-SMA) and fibronectin (FN) were detected. We also measured the protein and mRNA expression of key glycolytic enzymes, including pyruvate kinase muscle isozyme 2 (PKM2) and human glandular kallikrein 2 (HK2). The proliferation and differentiation of podocytes during fibrosis were observed by monitoring the expression of nephrin and myocardin. In vitro experiments, primary podocytes were extracted, cultured, and stimulated with lactate. Then the alterations during the process were observed. Finally, PKM2 expression was inhibited by intravenous infusion of the plasmid. The link between the expression of marker protein as well as differentiation protein in podocytes and renal interstitial fibrosis was analyzed.

RESULTS: During the process of renal interstitial fibrosis, phenotypic changes and enhanced expression of fibrosis and proliferation markers were found in fibroblasts. Meanwhile, in renal tubular epithelial cells, increased expression of key enzymes of glycolysis, the level of glycolysis as well as lactate metabolites cooperatively led to hypoxic and acidic environment, eventually inhibiting the proliferation and differentiation of podocytes and aggravating fibrosis. When the level of glycolysis in renal tubular epithelial cells was reduced, the number and function of podocytes were partially restored, and renal interstitial fibrosis was alleviated.

CONCLUSIONS: During renal interstitial fibrosis, glycolysis of renal tubular epithelial cell was increased, leading to the recodification of energy metabolism. This process affected the number and function of podocytes and aggravated renal interstitial fibrosis.

Key Words:
Glycolysis, Energy reprogramming, Renal tubular epithelial cells, Podocytes, Renal interstitial fibrosis.

Abbreviations

UUO: unilateral ureteral obstruction, mRNA: messenger RNA, α-SMA: α-smooth muscle actin, FN: fibronectin, PKM2: pyruvate kinase muscle isozyme 2, p-PKM2: phosphorylational pyruvate kinase muscle isozyme 2, HK2: human glandular kallikrein 2, CKD: Chronic kidney disease, ECM: extracellular matrix, SFP: specific-pathogen-free, DMEM: Dulbecco's Modified Eagle Medium, FBS: fetal bovine serum, qRT-PCR: quantitative Real Time-Polymerase Chain Reaction, cDNA: Complementary deoxyribose nucleic acid, SDS: sodium dodecyl sulphate, BCA: bicinchoninic acidt, SPSS: Statistical Product and Service Solutions, TGF-β1: transforming growth factor-β, NRK-52E: Renal tubular epithelial cells.

Introduction

Chronic kidney disease (CKD) refers to chronic kidney injury in structure and function resulted from various causes for more than 3 months. With

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the development of the disease, CKD eventually progresses to end-stage renal failure, bringing a heavy economic burden to the society and families. The incidence of CKD is increasing year by year over the world[1]. Studying the mechanism of the occurrence and development of CKD is of great importance in delaying renal failure.

Renal interstitial fibrosis, which is mainly caused by the deposition of extracellular matrix (ECM)[2,3], is the final pathological outcome of CKD resulted from different reasons[4,5]. Scholars[6,7] have shown that myofibroblasts, the most important source of ECM, are primarily derived from fibroblasts, renal tubular epithelial cells, endothelial cells, mesenchymal stem cells, peripheral cells, and others.

There has been a growing concern regarding the relationship between energy metabolism and diseases. It has been confirmed that energy metabolism reprogramming in cells is involved in the development of tumor, cardiovascular diseases and polycystic kidney diseases[8-10]. Consequently, the change of energy metabolism is of considerable importance for the development of CKD. Additionally, normal and ordered energy metabolism is a prerequisite for stable renal activity, with the reason that kidney, heart and brain are highly energy-demanding organs[11]. Previous researchers have found that malignant tumors can still consume a large amount of glucose through metabolic pattern of glycolysis when oxygen is abundant. This may result in the accumulation of lactic acid, rapidly generating energy for the proliferation of tumor cells. This metabolic pattern converting from oxidative phosphorylation to glycolysis is known as the Warburg effect[12]. We investigate whether there is a similar change in energy metabolism during the process of renal interstitial fibrosis. Authors[13] have shown that the changes in energy metabolism pattern of renal interstitial fibroblasts can promote renal interstitial fibrosis. Renal tubular cells are the main parenchyma cells of the kidney. Therefore, we speculate that the level of glycolysis in renal tubular epithelial cells increases during the process of renal interstitial fibrosis. Podocytes and the split diaphragm between them are part of the glomerular capillary wall, which plays an important role in renal filtration function[14]. Previous studies[15,16] of podocytes have mainly focused on molecular signals, charge barriers, and high-glucose environment. However, few researches have explored related energy metabolism. Changes in renal structure and function can alter the internal environment of podocytes, which in turn affect the proliferation and differentiation of podocytes. Therefore, we suggested that in the process of renal interstitial fibrosis, reprogramming of cellular energy metabolism in renal tubular cells could influence the proliferation and differentiation of podocytes, and might further aggravate renal impairment and renal interstitial fibrosis.

Materials and Methods

Experimental Animals

Male CD1 mice weighing 18-20 g were bought from the Experimental Animal Center of Nanjing University. According to the feeding standard of experimental animals, the mice were kept in specific-pathogen-free (SFP) environment with allowed access to food and water. All mice were fed in an environment with 55±10% relative humidity and 23±2°C temperature. All the experiments were performed in accordance with the institutional guidelines of the Third Affiliated Hospital of Soochow University. This study was approved by the Animal Ethics Committee of the Animal Center of Soochow University.

Construction of a Unilateral Ureteral Obstruction (UUO) Mouse Model

All experimental mice were anesthetized with sodium pentobarbital (45 mg/kg). The left ureter was bluntly dissected after laparotomy under aseptic conditions, and was ligated in triplicates with a 4-0-monofilament suture at ureteropelvic junction. Sham-operated animals were treated with blunt dissection without ligation. Finally, the abdomen was closed by layer. Animals were sacrificed at 1, 3, and 7 days after operation. Subsequently, urine and renal tissues of the surgical side were collected.

Extraction and Culture of Primary Podocytes

We collected the kidney samples from sterile mice weighing 18-20 g. The cortex was separated and dissected, followed by syringe trituration and filtration through a 100-mm mesh cell strainer with Dulbecco's Modified Eagle Medium (DMEM) culture medium (Gibco, Rockville, MD, USA). Before seeded into plates, the cell filtrate was collected, filtered through a 200-mm mesh cell strainer and centrifuged at 1,000 rpm for 5 min. Cells harvested from 4 mice were cultured with 1640 culture medium containing 10%

fetal bovine serum (FBS, Gibco, Rockville, MD, USA) and 50 U/mL interferon. All cells were grown at 37°C, 5% CO_2 incubator.

Pyruvate Kinase Muscle Isozyme 2 (PKM2) Low Expression Mouse Model

Male CD-1 mice weighing 18-20 g were chosen to construct the PKM2 low expression model. The plasmid was intravenously injected through the tail veins, while the littermates were injected with pcDNA3. Plasmid was prepared as follows: the experimental group: 1 mg/kg PKM2 expression plasmid in 20 mL 0.9% saline; the control group: 1 mg/kg cDNA3.1 in 20 mL 0.9% saline. The plasmids were rapidly injected within 10 s one day before the construction of the UUO model.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

QRT-PCR was performed according to the instructions of previous researches[14]. Totally mRNA was extracted from tissues or cells with TRIzol (Invitrogen, Carlsbad, CA, USA). Complementary deoxyribose nucleic acid (cDNA) was then synthesized by using the miScriptRT II Kit (15596-026, Life Technology, Gaithersburg, MD, USA), and was stored at -20°C. Extracted cDNA was used as a template for amplification with the miScript SYBR Green PCR Kit (28073, Qiagen, Hilden, Germany). All samples were repeated for 3 times, and CT values were measured by ABI7300 (Applied Biosystems, Foster City, CA, USA). Finally, the expression of target genes was calculated by the relative quantitative method.

Immunohistochemistry

Renal tissues were embedded in paraffin wax, cut into 3 um slices, deparaffinized in xylene, and rehydrated in ethanol and pure water. Next, the sections were blocked in the blocking buffer at room temperature for 30 min. Subsequently, the sections were incubated with primary antibody at 4°C overnight, followed by the incubation with secondary antibody at room temperature for 1 hour. Finally, the sections were photographed by using an Eclipse 80i microscope.

Western Blot

Lysis liquid was used to grind tissues on ice, and cell scrape was used for cell collection. After centrifugation at 16,000 rpm for 30 min, the supernatant was collected and the protein concentration was detected by the bicinchoninic acid kit (BCA) (Pierce, Rockford, IL, USA). Totally, 10-20 ug proteins were added to each well after adjusting to the same concentration with sodium dodecyl sulphate and de-ionized water. Primary antibodies used in this experiment were as follows: anti-α-smooth muscle actin antibody (α-SMA, cat: 5691), anti-tubulin (α-Tubulin, cat: ab7291, Abcam, Cambridge, MA, USA), anti- pyruvate kinase muscle isozyme 2 (PKM2, cat: ab38237, Abcam, Cambridge, MA, USA), anti-phosphorylational pyruvate kinase muscle isozyme 2 (p-PKM2, cat: ab156856, Abcam, Cambridge, MA, USA), anti-nephrin (nephrin, cat: ab58968, Abcam, Cambridge, MA, USA), anti-vascular smooth muscle cell differentiation factor (myocardin, cat: ab107301, Abcam, Cambridge, MA, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) version 16.1 (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis. Data were represented as mean ± standard error (mean ± SD). Student's *t*-test was performed for comparing the difference between two groups. However, one-way ANOVA was used for comparison among 3 or more groups, followed by the Post-Hoc Test (Least Significant Difference). $p < 0.05$ was considered statistically significant.

Results

Elevated Expression of Key Glycolytic Enzymes in the UUO Model

All CD1 mice were randomly divided into 4 groups with 5 mice in each group (n=5). Sham-operation and UUO operation were performed for the control group and the experimental group, respectively. Renal tissues were collected from sacrificed mice on 1, 3, and 7 days after operation. Time-dependent fibrosis was observed in postoperative renal tissues by detecting the protein expression of a-SMA, which was a fibrosis marker. Concomitantly, the protein level of key glycolytic enzymes, p-PKM2 and HK2 increased during the renal fibrosis process in a time-dependent manner (Figure 1A). In addition, the same trend of PKM2 and HK2 was observed on the mRNA level (Figure 1B, 1C). The immune-histochemical results revealed that a large amount of PKM2 deposited predominantly in renal tissues of the UUO model (Figure 1D). The concentration of lactic acid produced by glycoly-

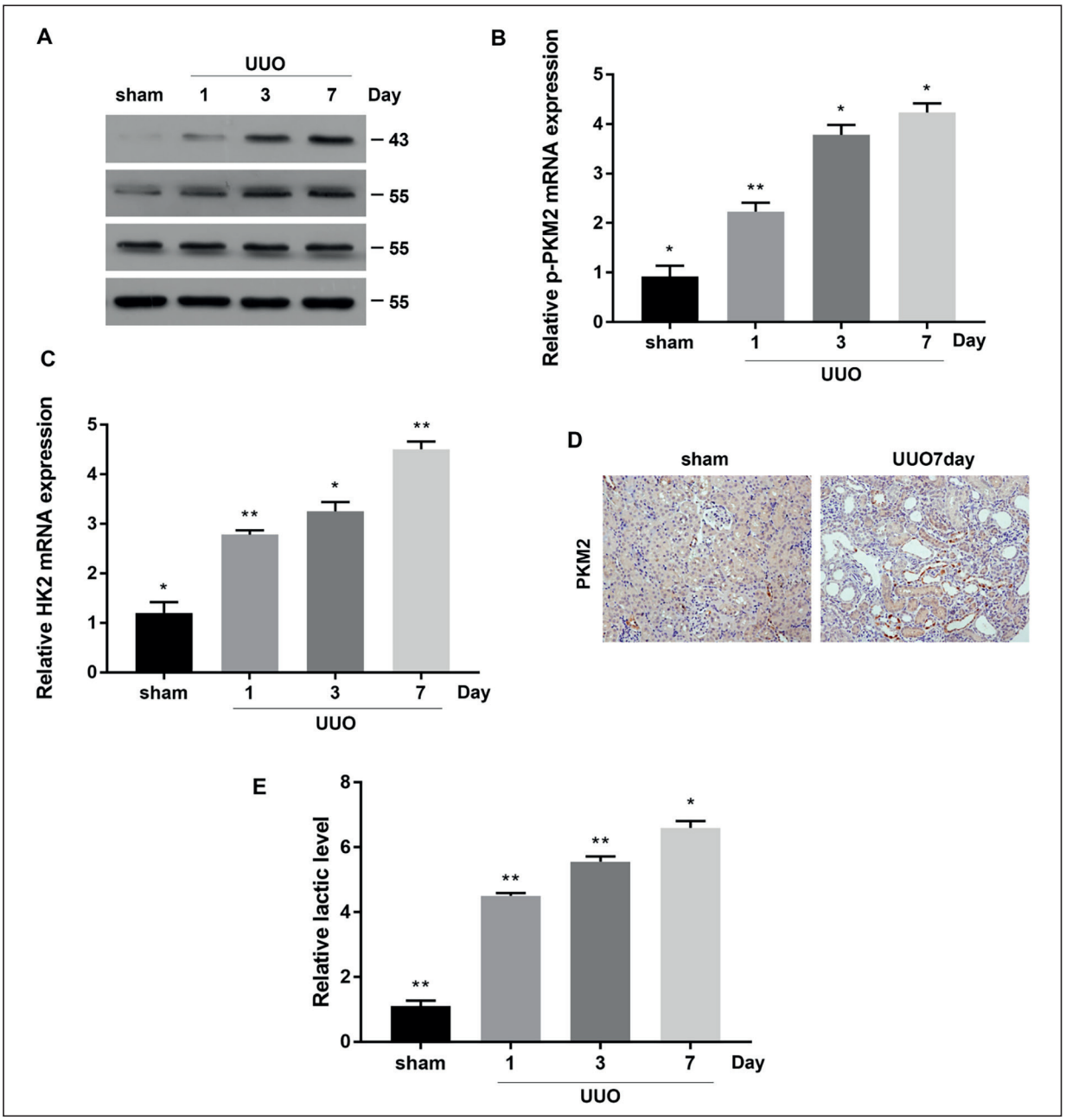

Figure 1. Elevated expression of key glycolytic enzymes in the UUO model. **A,** The protein expression of a-SMA, p-PKM2 and HK2 in renal tissues of each group. **B,** The mRNA expression of p-PKM2 in renal tissues of each group. ***C,*** The mRNA expression of HK2 in renal tissues of each group (*$p < 0.05$, statistical significance). ***D,*** Immuno-histochemical staining of PKM2. ***E*,** The contents of lactic acid in renal tissues of each group (40×) (*$p < 0.05$, statistical significance).

sis increased significantly in postoperative renal tissues (Figure 1E), which could indirectly indicate that the energy metabolism pattern of tubular epithelial cells was changed in the process of renal interstitial fibrosis in UUO mice. Meanwhile, the specific manifestation of the changed pattern was mainly enhanced glycolysis level.

Decreased Proliferation and Differentiation of Podocytes During the Process of Renal Interstitial Fibrosis in the UUO Model

Nephrin, a maker protein and indirect representation of the number of podocytes, decreased from the first day after operation. Meanwhile,

significant reduction was observed on day 7 postoperatively on both the protein level (Figure 2A) and mRNA level (Figure 2B). These findings suggested that the number of podocytes reduced in the process of renal interstitial fibrosis. Myocardin is a vascular smooth muscle cell differentiation factor that is widely existed in cells. Its expression can reflect the degree of cell differentiation. Therefore, we examined the expression of myocardin in kidney tissues in the UUO model. It was found that the UUO treatment reduced the protein (Figure 2C) and mRNA expression (Figure 2D) of myocardin in a time-dependent manner, indicating that podocyte differentiation was inhibited and the function was impaired in the process of renal interstitial fibrosis.

The Number and Function of Podocytes was Affected by Lactic Acid

Our above experiments demonstrated that in the process of renal interstitial fibrosis, the glycolysis level of proximal tubular epithelial cells was increased. This process was accompanied by a large accumulation of glycolytic products such as lactic acid, resulting in changes in pH values of the intracellular environment. Therefore, we wondered whether changed pH values could affect the proliferation and differentiation of podocytes. Experimental results showed that the expression of α-smooth muscle actin (α-SMA) and phosphorylational pyruvate kinase muscle isozyme 2 (p-PKM2) in renal tubular epithelial cells (NRK-52E) cells increased significantly after the treatment with transforming growth factor-β (TGF-β1) for different time (Figure 3A). In addition, the lactate content in the cell supernatant culture fluid was also significantly increased in the experimental group (Figure 3B). Subsequently, we used lactic acid (10 mM) to stimulate the podocytes for different time. Interestingly, we found that the protein levels of nephrin and myocardin were gradually reduced (Figure 3C). Similarly, we also found that the mRNA level of both molecules decreased after lactate stimulation (Figure 3D, 3E), indicating that the production of adjusted energy metabolism affected podocytes in the process of renal interstitial fibrosis.

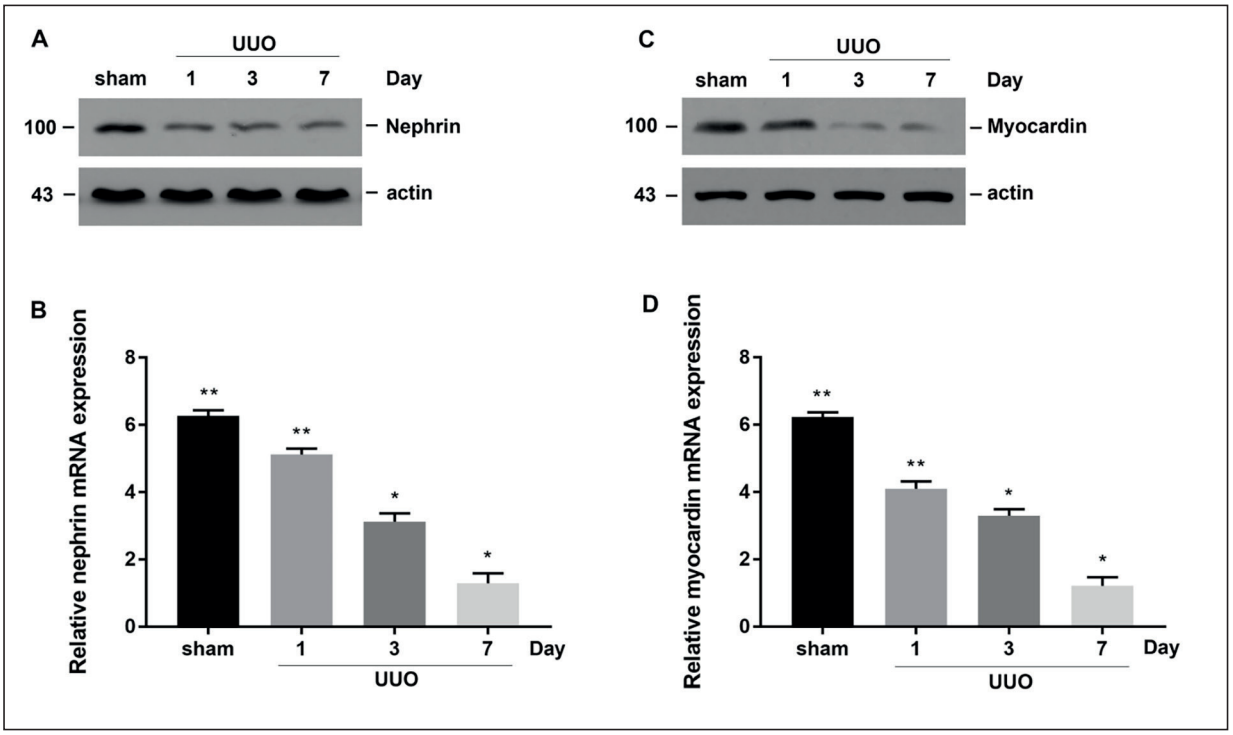

Figure 2. Decreased proliferation and differentiation of podocytes during the process of renal interstitial fibrosis in the UUO model. ***A***, The protein expression of nephrin in renal tissues of each group. ***B,*** The mRNA expression of nephrin in renal tissues of each group. (*$p < 0.05$, statistical significance). ***C,*** The protein expression of myocardin in renal tissues of each group. **D,** The mRNA expression of myocardin in renal tissues of each group (*$p < 0.05$, statistical significance).

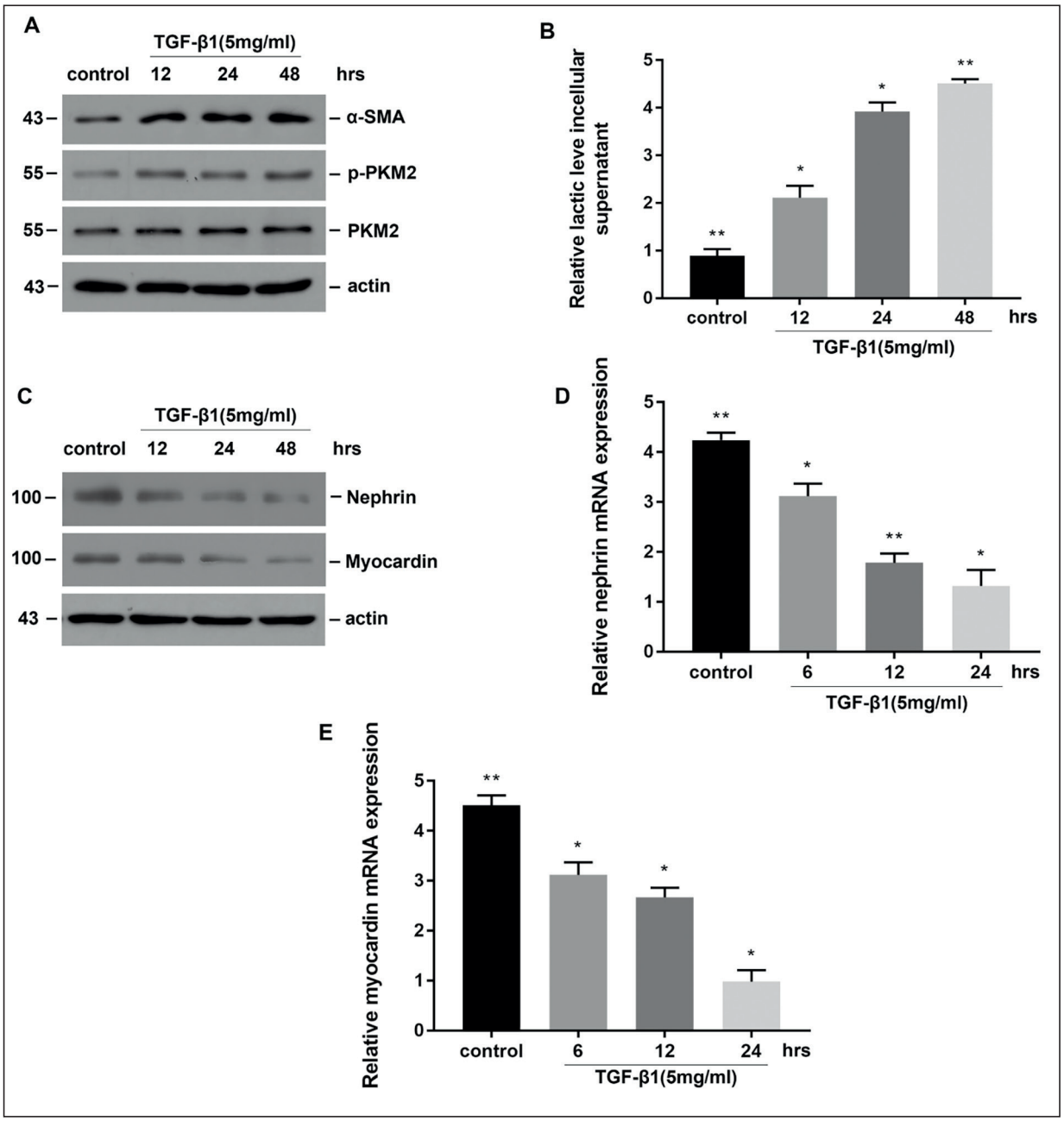

Figure 3. The number and function of podocytes was affected by lactic acid. ***A,*** The protein expression of a-SMA and p-PKM2 in cells of each group. ***B,*** The content of lactic acid in the supernatant culture medium of each group (*$p < 0.05$, statistical significance). ***C,*** The protein expression of nephrin and myocardin in cells of each group. ***D-E,*** The mRNA expression of nephrin and myocardin in each group (*$p < 0.05$, statistical significance).

Knockdown of PKM2 in Renal Tubular Epithelial Cells Enhanced the Proliferation and Differentiation of Podocytes and Alleviated Interstitial Fibrosis

Increased glycolysis of renal tubular epithelial cells could affect the proliferation and differentiation of podocytes. Then, we speculated that reduced glycolysis level might improve the functional status of podocytes. We successfully constructed a PKM2 low expression mice model by intravenously injecting the plasmid through tail veins one day before the UUO operation (Figure 4A). Inhibition of PKM2 expression decreased the expression of a-SMA (Figure 4B), whereas increased the protein (Figure 4C) and mRNA

expression of nephrin and myocardin (Figure 4D, 4E). In addition, we collected urine samples from the obstructed side of the kidney and measured the protein content in the urine samples. We found that the protein content was reduced in the urine of the PKM2 low expression group (Figure 4F). The above results suggested that after reducing the glycolytic level of renal tubular epithelial cells, the proliferation and differentiation of podocytes were improved, urinary albumin was decreased, and renal interstitial fibrosis was also alleviated.

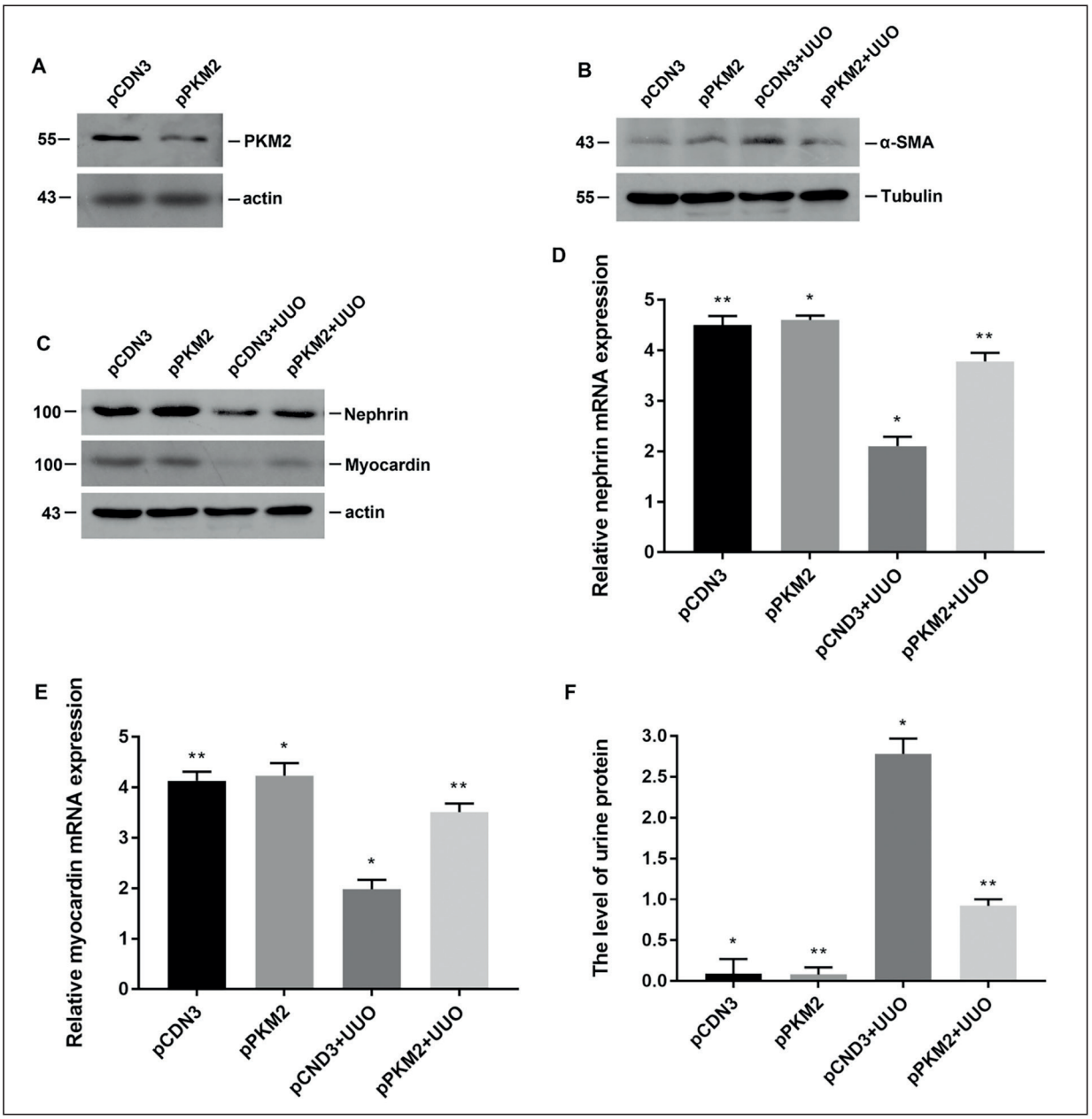

Figure 4. Knockdown of PKM2 in renal tubular epithelial cells enhanced the proliferation and differentiation of podocytes and alleviated interstitial fibrosis. ***A,*** The protein expression of PKM2 in renal tissues of the control group and the PKM2 low expression group. ***B,*** The protein expression of a-SMA in renal tissues of each group. ***C,*** The protein expression of nephrin and myocardin in renal tissues of each group. ***D-E,*** The mRNA expression of nephrin and myocardin in renal tissues of each group (*$p < 0.05$, statistical significance). ***F,*** The protein content in urine sample collected from the operative side in each group (*$p < 0.05$, statistical significance).

Discussion

Renal interstitial fibrosis is the final pathological outcome of CKD. It is mainly characterized by the production of a large number of extracellular matrix (ECM) after the myofibroblasts activation. Once occurs, this process is irreversible. This is a main cause of the progression of kidney diseases, which is also an important indicator of the severity of CKD. Therefore, it is particularly important to detect the disease to slow down its progression at an early stage.

In recent years, energy metabolism has become a hot topic in various fields. Glucose metabolism is the main source of energy for living organisms. Previous studies have shown that tumor cells rapidly generate large amounts of energy in a short time through glycolysis, which is known as the "Warburg effect". In recent years, researches have shown that the "Warburg effect" also occurs in renal interstitial fibroblasts. We found that the expression of key enzymes in glycolysis, such as PKM2 and HK2, was significantly increased in the UUO mouse model and in TGF-β1-stimulated NRK-52E cells. This suggested that energy metabolism recoding existed in renal tubular epithelial cells during renal interstitial fibrosis.

In the process of renal interstitial fibrosis, pathological changes also occur in the glomeruli. Podocytes are the main cells that make up the glomerular basement membrane, which are also involved in maintaining the material basis of the glomerular filtration membrane charge barrier. After podocyte injury, foot process fusion, retraction, disappearance, cell body shrinkage, pseudo-capsule formation, and anionic charge reduction may occur. Podocytes are detached from the basement membrane and the fissure membrane is destroyed. A large amount of proteins are filtered from this, eventually making the kidney smaller. The ball forms "high filtration, high perfusion, and high transmembrane pressure", eventually resulting in glomerulosclerosis and the progressive loss of renal function. Nephrin is the main marker protein of podocytes and plays a key role in maintaining the structural integrity of the septum membrane[17,18]. The abnormal expression of nephrin can result in the loss of proteins and the disruption of the filtration barrier, leading to massive proteinuria[19,20].

We hypothesized that the function of podocytes could be affected by the reprogramming of energy metabolism in renal tubular epithelial cells. Our results indicated that in the process of renal interstitial fibrosis, both the expression of podocyte marker protein and differentiation protein were reduced. However, when glycolysis level was reduced, the proliferation and differentiation of podocytes were improved, whereas the degree of renal interstitial fibrosis was reduced. However, the specific mechanism still remains unclear and further studies are needed. The pathogenesis of renal interstitial fibrosis has been proposed from a completely new perspective.

Conclusions

During renal interstitial fibrosis, phenotypic changes of renal tubular epithelial cells occurred, and energy metabolism was recoded at the same time. This was mainly manifested by elevated levels of glycolysis, the accumulation of lactic acid and other products that might cause intracellular environmental disorders. This process affected the proliferation and differentiation of podocytes and further aggravated renal interstitial fibrosis. However, the podocyte function was partially restored and interstitial fibrosis was alleviated by reducing the glycolysis level of renal tubular epithelial cells.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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