

# Enhanced glycolysis in the process of renal fibrosis aggravated the development of chronic kidney disease

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**Abstract. – OBJECTIVE:** To explore the effect of glycometabolism on renal fibrosis and its underlying mechanism.

**MATERIALS AND METHODS:** For *in vivo* experiments, unilateral ureteral obstruction (UUO) mouse model was constructed to induce renal interstitial fibrosis. Fibrosis and proliferation indicators in renal tissues were detected to observe the fibroblast phenotype changes during the process of renal fibrosis. Moreover, mRNA and protein levels of key enzymes in glycometabolism were also detected. For *in vitro* experiments, plasmid transfection was performed to overexpress pyruvate kinase M2 (PKM2) to explore the relationship between PKM2 and renal interstitial fibrosis. Energy metabolism monitoring was performed to detect changes in aerobic glycolysis and oxidative phosphorylation during the process of TGF- $\beta$ 1-induced fibroblast phenotype changes.

**RESULTS:** Fibroblast phenotype was changed. Both fibrosis and proliferation indicators were upregulated during renal fibrosis. Meanwhile, elevated expressions of key enzymes in glycometabolism and metabolic reprogramming of fibroblasts were observed. Overexpressed PKM2 activated fibroblasts and induced renal interstitial fibrosis, accompanied by increased glycometabolism level.

**CONCLUSIONS:** Metabolic reprogramming promoted renal interstitial fibrosis, which leads to alteration of cell energy metabolism model.

*Key Words:*

Aerobic glycolysis, Metabolic reprogramming, Fibroblast, Renal interstitial fibrosis.

## Introduction

Chronic kidney disease (CKD) is a common disease that poses a serious threat to human he-

alth after cardiovascular disease, cancer, and diabetes. The incidence of CKD is about 10-14% worldwide<sup>1</sup>, which has been rising annually<sup>2</sup>. In recent years, the number of CKD patients have been sharply increasing, which brings a huge burden to the patients and their families.

It has been confirmed that renal fibrosis is a common cause of end-stage renal failure caused by various pathological changes, which is the key factor in the progress of renal failure<sup>3,4</sup>. Numerous evidence<sup>5,6</sup> has demonstrated that fibroblasts are activated and aggregated in the renal interstitium and produce large amounts of extracellular matrix, which are directly related to CKD progression. Sources of renal interstitial fibroblasts include activation of renal intrinsic fibroblasts, pericyte differentiation, and trans-differentiation of tubular epithelium cells, endothelial cells and bone marrow-derived mesenchymal stem cells<sup>7-11</sup>. Recent studies have shown that the activation of renal intrinsic fibroblasts is the main source of renal interstitial fibroblasts, accounting for about 50% of total cases<sup>12</sup>.

Previous studies were focused on the effect of cytokines, growth factors, inflammatory mediators, renal tubular epithelial cells and stromal fibroblasts on the development of renal interstitial fibrosis. Energy metabolism and renal interstitial fibrosis, however, are not fully elucidated. Energy metabolism is capable of maintaining the basis of the organism, tissue structure and function, abnormalities of which may directly lead to pathological changes. Current researches<sup>13-16</sup> have demonstrated that metabolic reprogramming is involved in the development of cancer, aging, diabetes, cardiovascular disease, polycystic kidney disease, focal segmental glomerulosclerosis, and renal ischemia-reperfusion injury.

Kidneys belong to highly energy-consuming organs<sup>17</sup>. Therefore, a normal and orderly energy metabolism system is a biochemical basis for maintaining the renal-specific structure and its physiological function. Glycometabolism, as the core of energy metabolism, generates adenosine triphosphate (ATP) through two metabolic pathways under normal circumstances, that is, glycolysis and aerobic metabolism. However, glycometabolism alters under the disease state. In 1927, Otto Warburg<sup>18,19</sup> first found that malignant cells predominantly produce their energy through a high rate of glycolysis followed by lactic acid fermentation even in the presence of abundant oxygen, which is called the Warburg effect. Although aerobic glycolysis was originally found in malignant cells, activation and proliferation of fibroblasts during renal interstitial fibrosis are similar to those of tumor cells. So far, there is still no relevant research on the glycolytic metabolism in renal interstitial fibrosis.

Therefore, we hypothesized that metabolic reprogramming of renal interstitial fibroblasts occurs during renal interstitial fibrosis. Here, we used UUO mouse model and NRK-49F cells to explore the mechanism of metabolic reprogramming during renal interstitial fibrosis, which provides a new direction in treating CKD.

## Materials and Methods

### Experimental Animals

Male CD1 mice weighing 18-20 g were purchased from the Experimental Animal Center, the First Affiliated Hospital of Kunming Medical University. All mice were maintained in the specific pathogen free (SPF) environment with feeding temperature of 23±2°C and relative humidity of 55±10%. This investigation was approved by the Institutional Animal Care and Use Committee of the First Affiliated Hospital of Kunming Medical University.

### Unilateral Ureteral Obstruction (UUO) Mouse Model

Male CD1 mice weighing 18-20 g were anesthetized with 45 mg/kg pentobarbital sodium. The left ureter was dissected and subsequently ligated with 4-0 silk for three times through the abdominal incision. Mice in sham group received blunt dissection of the left ureter without the subsequent ligation. The abdomen was closed layer by layer. All mice were sacrificed 1, 3, and

7 days after the surgery, and blood, urine, and renal tissues of the surgical side were collected.

### Cell Culture

Rat renal fibroblast cell line NRK-49F was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle medium (DMEM, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 µg/mL streptomycin, and maintained in a 5% CO<sub>2</sub> incubator at 37°C. Serum-free medium with 2 ng/mL TGF-β1 (Sigma-Aldrich, St. Louis, MO, USA) was replaced when cell confluence was up to 80%. Glycolysis inhibitor was added half an hour prior to TGF-β1.

### Immunohistochemistry

Kidneys were sliced into 3-µm thickness and embedded in paraffin. Kidney slices were deparaffinized, hydrated in ethyl alcohol, and blocked in blocking solution for 30 min. Then, the kidney slices were incubated with the primary antibody at 4°C overnight, followed by the incubation of secondary antibody at room temperature for 1 h. Immunohistochemistry results were captured using Nikon Eclipse 80i microscope (Tokyo, Japan).

### Western Blot

The total protein of the transfected cells or kidney tissues was extracted. The concentration of each protein sample was determined by a bicinchoninic acid (BCA) kit. Briefly, 50 µg of total protein was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 5% skimmed milk, followed by the incubation of specific primary antibodies (α-SMA, α-Tubulin, PCND, PKM2, and p-pkm2; Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight. Membranes were then incubated with the secondary antibody (Cell Signaling Technology, Danvers, MA, USA) at room temperature for 1 h. Immunoreactive bands were exposed by enhanced chemiluminescence method.

### Cell Transfection

Cells were seeded in a 6-well plate and cultured in serum-containing medium. 1.5 mL of serum-free medium was replaced in each well

when cell confluence was up to 90-95%. Briefly, 2.5  $\mu\text{g}$  of plasmid DNA and 5  $\mu\text{L}$  of Liposomes 2000 were diluted with 250  $\mu\text{L}$  of Opti-MEM (serum-reducing medium), respectively, and incubated for 5 min at room temperature. The two transfection mixture were mixed for 20-min incubation at room temperature. 500  $\mu\text{L}$  of the mixture was added to each well and cells were incubated for the following experiments.

### **Real-Time Measurement of Cell Energy Metabolism**

(1) Cell preparation: NRK-49F cells were digested with trypsin digestion after treatment of TGF- $\beta$ 1 for 48 h. Cells were centrifuged at 1000 rpm for 5 min and resuspended for cell counting.  $1 \times 10^4$  cells/100  $\mu\text{L}$  of NRK-49F cells were seeded into the XF24 well plates (Seahorse Bioscience, Billerica, MA, USA) and cultured overnight.

(2) Measurement of extracellular acidification rate (ECAR): Cells were sequentially treated with glucose, oligomycin and 2-deoxyglucose (2-DG). The detection process was divided into four stages, with 3 instantaneous values recorded in each stage. Aerobic glycolysis was evaluated by basic glycolysis, maximum glycolysis, and glycolysis reserves. The calculation formulas were as follows: Basic glycolysis = maximum ECAR after stimulation with saturated glucose medium – the third measurement of ECAR after adding glucose-free medium; Maximum glycolysis = the third measurement of ECAR after adding oligomycin; Glycolysis reserve = maximum glycolysis – basic glycolysis.

(3) Measurement of oxygen consumption rate (OCR): Cells were sequentially treated with oligomycin, carbonyl cyanide-P-trifluoromethoxy phenylhydrazone (FCCP), rotenone, and antimycin A. Mitochondrial function was assessed by basic respiration level, coupling efficiency, and residual respiratory capacity. The calculation formulas were as follows: Basic respiration level = the third measurement of baseline OCR; Coupling efficiency = 1- the third measurement of baseline OCR after adding oligomycin / basic respiration level; Residual respiratory capacity = the third measurement of OCR after adding FCCP / basic respiration level.

### **Statistical Analysis**

Statistical Product and Service Solutions (SPSS) 16.1 statistical software (Chicago, IL, USA) was used for data analysis. Measurement data were expressed as mean  $\pm$  standard deviation

( $\bar{x} \pm s$ ). Comparison of measurement data was conducted using *t*-test. Comparison of differences among each group was conducted using one-way ANOVA followed by Post-Hoc Test (Least Significant Difference).  $p < 0.05$  was considered statistically significant.

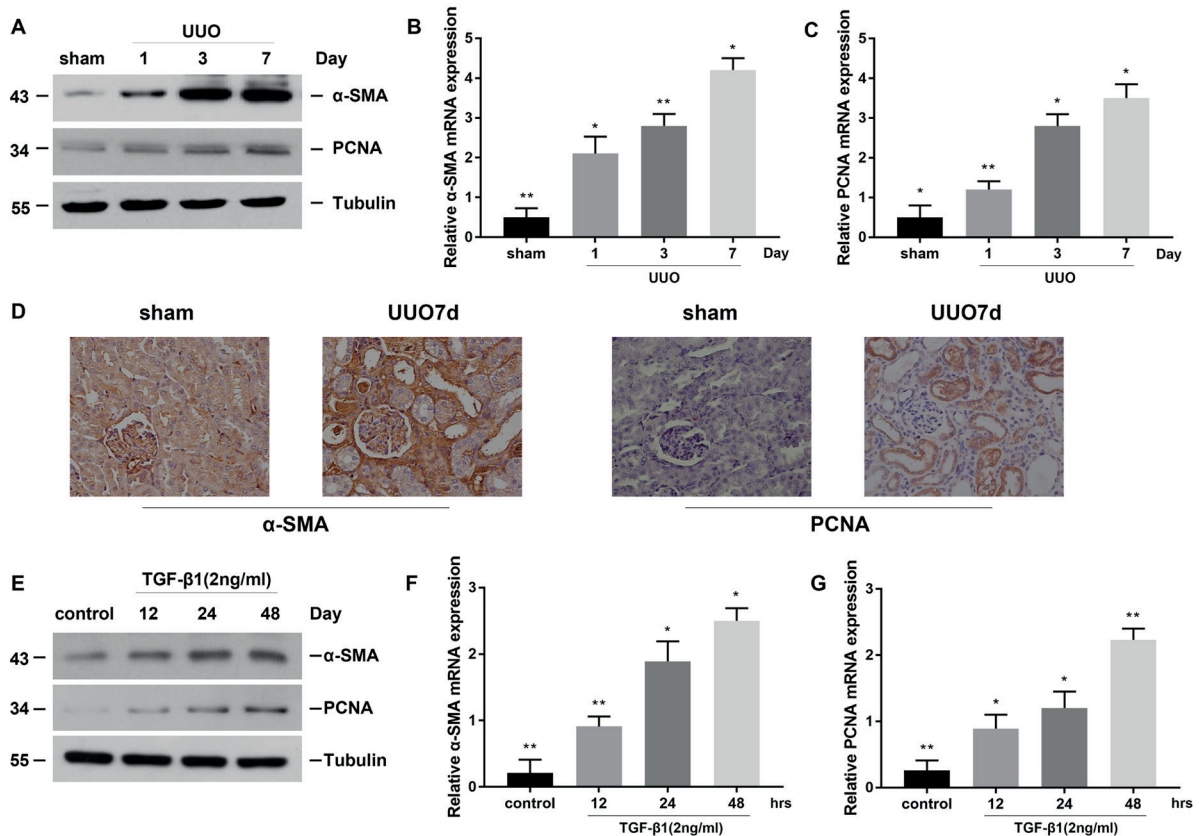
## **Results**

### **Activation and Proliferation of Fibroblasts Participated in Renal Interstitial Fibrosis**

To investigate the activation of mesangial fibroblasts in fibrotic kidneys, we constructed the UUO mouse model. Our results demonstrated that protein expressions of  $\alpha$ -SMA and PCNA in renal tissues on the 1<sup>st</sup>, 3<sup>rd</sup>, and 7<sup>th</sup> day after UUO were significantly increased in a time-dependent manner (Figure 1A). Detection of mRNA levels of  $\alpha$ -SMA and PCNA in renal tissues obtained the same results (Figure 1B, 1C). Immunohistochemical staining showed that there was a large number of  $\alpha$ -SMA-positive and PCNA-positive fibroblasts in renal tissues on the 7<sup>th</sup> day after UUO, indicating the proliferation of activated fibroblasts during renal interstitial fibrosis (Figure 1D). TGF- $\beta$ 1 is a crucial fibrogenic cytokine. In the present study, NRK-49F cells were treated with 2 ng/mL TGF- $\beta$ 1 for different time points. Results showed that TGF- $\beta$ 1 can significantly increase protein expressions of  $\alpha$ -SMA and PCNA in a time-dependent manner, indicating the activation and proliferation of fibroblasts (Figure 1E). In addition, mRNA expressions of  $\alpha$ -SMA and PCNA in NRK-49F cells were also increased, which were consistent with those of *in vivo* results (Figure 1F, 1G). The above experiments demonstrated that proliferative fibroblasts are involved in renal interstitial fibrosis.

### **Elevated Expressions of Glycometabolism-Related Enzymes in Renal Tissues**

In recent years, researches have shown that there is a significant metabolic disorder in CKD. In our study, renal tissues on the 1<sup>st</sup>, 3<sup>rd</sup>, and 7<sup>th</sup> day after UUO were collected as experimental group and those in sham operation were collected as control group. The mRNA expressions of glycometabolism-related enzymes in renal tissues were detected, including hexokinase (HK) and pyruvate kinase muscle isozyme type 2 (PKM2). Results found that mRNA expressions of HK and PKM2 were significantly increased (Figure 2A, 2B). A large number of studies



**Figure 1.** Activation and proliferation of fibroblasts participated in renal interstitial fibrosis. **A**, Western blot results of  $\alpha$ -SMA and PCNA in renal tissues of UUU mice on the 1<sup>st</sup>, 3<sup>rd</sup>, and 7<sup>th</sup> day after UUU. **B**, The mRNA level of  $\alpha$ -SMA in renal tissues of UUU mice on the 1<sup>st</sup>, 3<sup>rd</sup>, and 7<sup>th</sup> day after UUU ( $*p < 0.05$ ). **C**, The mRNA level of PCNA in renal tissues of UUU mice on the 1<sup>st</sup>, 3<sup>rd</sup>, and 7<sup>th</sup> day after UUU ( $*p < 0.05$ ). **D**, Immunohistochemical staining (400 $\times$ ) showed a large number of  $\alpha$ -SMA-positive and PCNA-positive fibroblasts in renal tissues on the 7<sup>th</sup> day after UUU. **E**, Western blot results of  $\alpha$ -SMA and PCNA in NRK-49F cells treated with TGF- $\beta$ 1 (2 ng/mL). **F**, **G**, The mRNA level of  $\alpha$ -SMA (**F**) and PCNA (**G**) in NRK-49F cells treated with TGF- $\beta$ 1 (2 ng/mL).

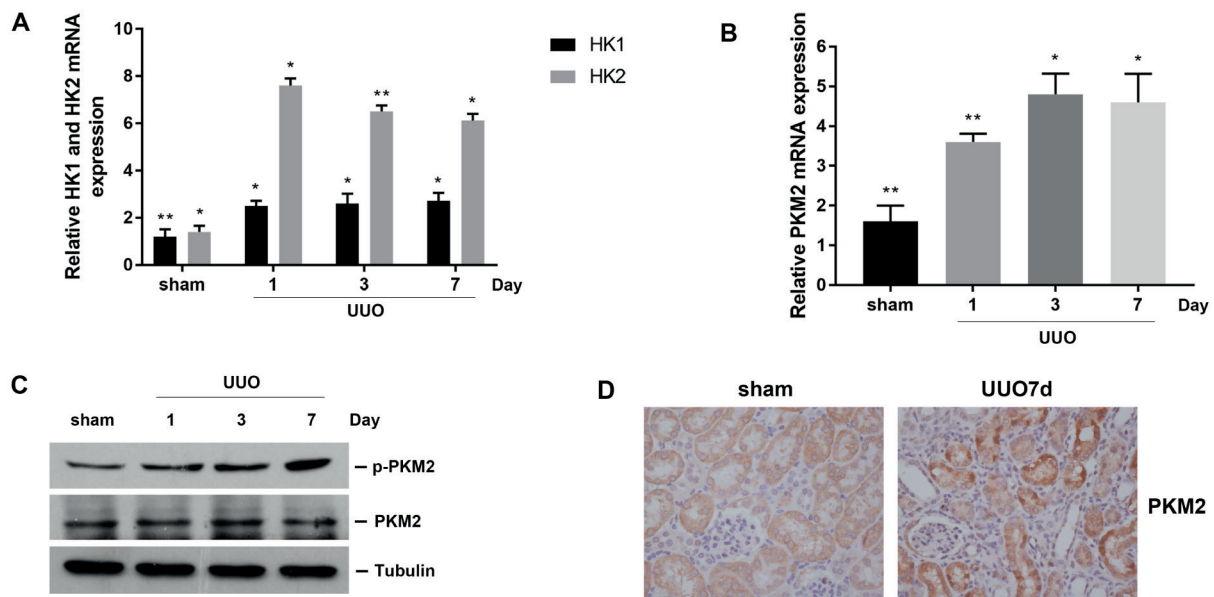
have reported that PKM2 exerts a crucial role in the aerobic glycolysis of tumor cells. Therefore, we detected protein expressions of PKM2 and p-PKM2 in renal tissues of UUU mice. Upregulated PKM2 and p-PKM2 were found in renal tissues on the 1<sup>st</sup>, 3<sup>rd</sup>, and 7<sup>th</sup> day after UUU (Figure 2C). PKM2-positive fibroblasts were observed in renal interstitium of UUU mice (Figure 2D). The above data demonstrated that expressions of glycometabolism-related enzymes in renal tissues are significantly increased, indicating the metabolic reprogramming in UUU mice.

#### TGF- $\beta$ 1 Induced Metabolic Reprogramming in NRK-49F Cells

For *in vitro* experiments, NRK-49F cells were treated with TGF- $\beta$ 1 for 12, 24, and 48 h,

respectively. It was found that mRNA expression of glycometabolism-related enzymes in NRK-49F cells treated with TGF- $\beta$ 1 were remarkably increased (Figure 3A, 3B). Moreover, protein expression of p-PKM2 was remarkably increased 0.5 h after TGF- $\beta$ 1 treatment (Figure 3C). Protein expression of PKM2, however, did not change. The culture medium of NRK-49F cells treated with TGF- $\beta$ 1 was then collected to detect its pH value. Our data illustrated that pH value of TGF- $\beta$ 1-treated culture medium was decreased (Figure 3D), while lactate concentration (Figure 3E) and glucose consumption (Figure 3F) were increased in a time-dependent manner. The above *in vitro* experiments demonstrated that glycometabolism level is elevated, indicating the metabolic reprogramming during renal fibrosis.





**Figure 2.** Elevated expressions of glycometabolism-related enzymes in renal tissues. **A**, The mRNA expressions of HK and PK were significantly increased in renal tissues of UUO mice on the 1<sup>st</sup>, 3<sup>rd</sup>, and 7<sup>th</sup> day after UUO ( $*p < 0.05$ ). **B**, The mRNA expression of pyruvate dehydrogenase kinase in renal tissues of UUO mice on the 1<sup>st</sup>, 3<sup>rd</sup>, and 7<sup>th</sup> day after UUO ( $*p < 0.05$ ). **C**, Western blot results of p-PKM2 and PKM2 in renal tissues of UUO mice on the 1<sup>st</sup>, 3<sup>rd</sup>, and 7<sup>th</sup> day after UUO ( $*p < 0.05$ ). **D**, Immunohistochemical staining (400 $\times$ ) showed a large number of PKM2-positive fibroblasts in renal tissues on the 7<sup>th</sup> day after UUO.

### Metabolic Reprogramming Induced the Activation of NRK-49F Cells

The above-mentioned experiments demonstrated metabolic reprogramming in renal interstitial fibroblasts both *in vitro* and *in vivo*. To further verify whether PKM2 can directly induce fibroblast activation, we first constructed an overexpression plasmid of PKM2 and transfected it into NRK-49F cells. 30  $\mu$ g of PKM2 overexpression plasmid showed the highest transfection efficiency (Figure 4A). Protein expressions of  $\alpha$ -SMA and PCNA were remarkably elevated in NRK-49F cells transfected with PKM2 overexpression plasmid (Figure 4B). In addition, mRNA expressions of  $\alpha$ -SMA and PCNA were also remarkably increased (Figure 4C, 4D). Higher concentration of lactic acid in the cell supernatant transfected with PKM2 overexpression plasmid was observed compared to that of the negative control (Figure 4E). It is demonstrated that overexpressed PKM2 can effectively induce the activation of NRK-49F cells and increase cellular glycometabolism level.

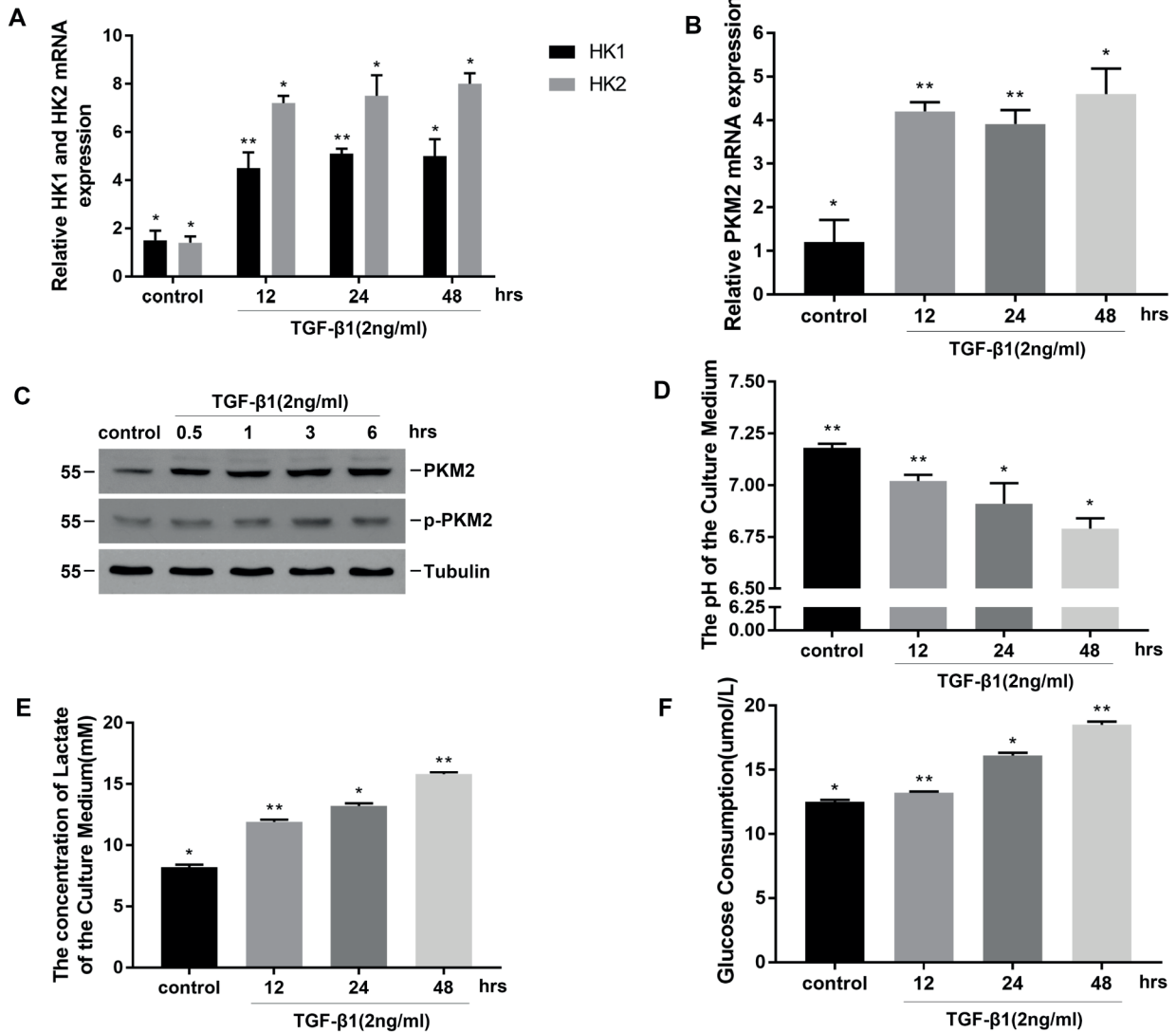
### TGF- $\beta$ 1 Induced the Metabolism Conversion of NRK-49F Cells From Oxidative Phosphorylation to Aerobic Glycolysis

Subsequently, we used the energy metabolism monitor to observe the metabolism of NRK-49F

cells. As shown in Figure 5A, basic glycolysis level and maximum glycolysis capacity of cells treated with TGF- $\beta$ 1 after 48 h were found to be higher than those of the negative control group. NRK-49F cells treated with TGF- $\beta$ 1 exhibited decreased mitochondrial basal oxygen consumption, including decreased maximum respiratory capacity and ATP production (Figure 5B and 5C). In addition, ECAR and OCR were elevated in cells treated with TGF- $\beta$ 1 after 48 h, indicating the metabolic reprogramming in NRK-49F cells. These data confirmed that the metabolic mode is converted from oxidative phosphorylation to aerobic glycolysis after fibroblast activation, that is, the metabolic reprogramming.

## Discussion

Majority of CKD cases will eventually progress to irreversible chronic renal interstitial fibrosis regardless of the primary diseases. The mechanism of CKD, however, is still not fully elucidated. Normal and orderly energy metabolism system maintains the renal-specific structure and basic physiological functions<sup>20-22</sup>. Abnormalities of renal structure and physiological function would lead to energy metabolism

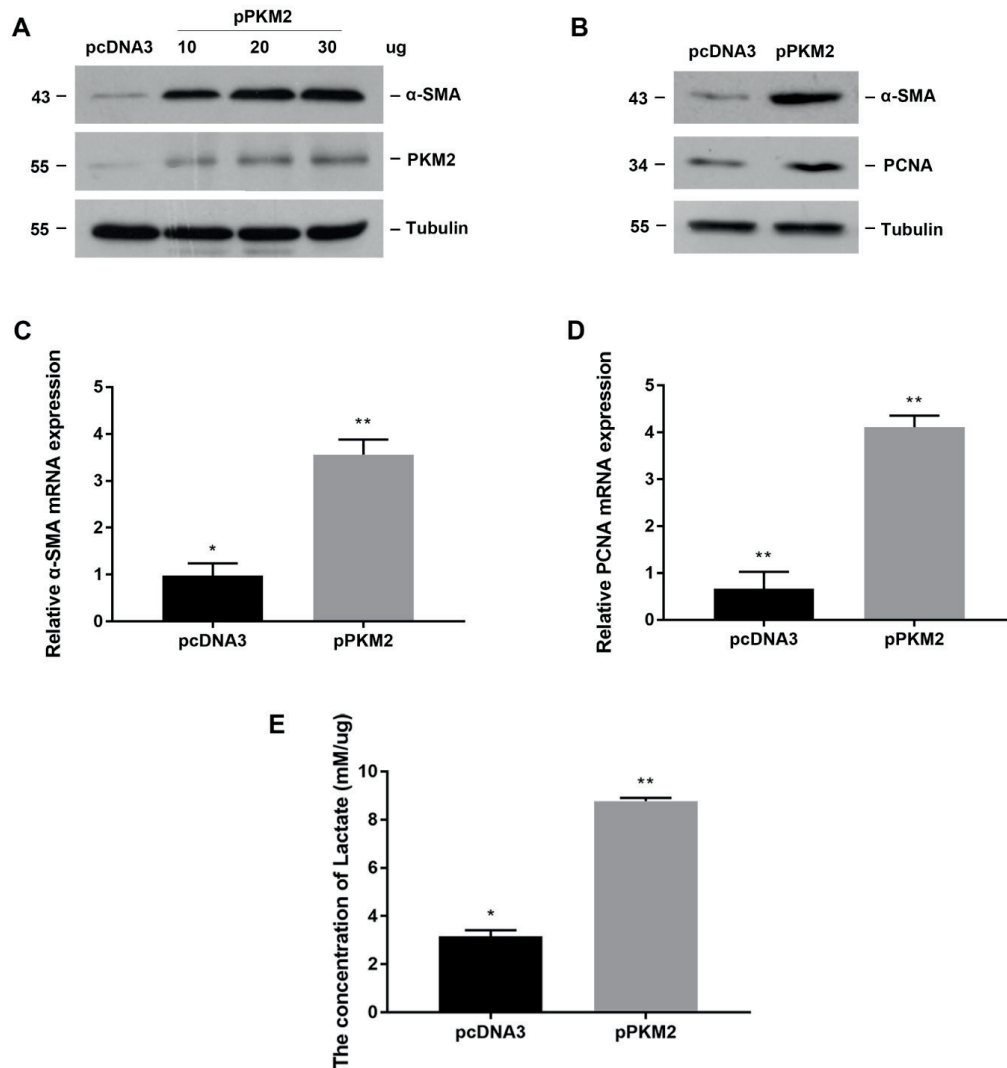


**Figure 3.** TGF- $\beta$ 1 induced metabolic reprogramming in NRK-49F cells. **A**, The mRNA expressions of HK and PK in NRK-49F cells treated with 2 ng/mL TGF- $\beta$ 1 ( $*p < 0.05$ ). **B**, The mRNA expression of pyruvate dehydrogenase kinase in NRK-49F cells treated with 2 ng/mL TGF- $\beta$ 1 ( $*p < 0.05$ ). **C**, Western blot results of p-PKM2 and PKM2 in NRK-49F cells treated with 2 ng/mL TGF- $\beta$ 1 ( $*p < 0.05$ ). **D**, **E**, PH value (**D**) lactic acid concentration (**E**) and glucose consumption (**F**) in the cell medium treated with 2 ng/mL TGF- $\beta$ 1 ( $*p < 0.05$ ).

disorders. In 1988, Harris et al<sup>23</sup> demonstrated a phenomenon of high metabolism in the residual 1/6 kidneys of nephrectomized rats. Compared with normal kidneys, kidneys with unilateral ureteral obstruction manifested increasingly incomplete catabolism of glucose (glycolytic pathway and pentose phosphate pathway) and decreased gluconeogenesis ability. Meanwhile, the uptake of fatty acids and oxidation ability was significantly reduced, thus leading to less ammonia production. We therefore hypothesized

that metabolic reprogramming of interstitial fibroblasts promotes renal fibrosis.

We used UO mice models and NRK-49F cells to explore the activation and proliferation of renal interstitial fibroblasts during renal fibrosis. Elevated expressions of HK and PKM2 were observed in renal tissues and fibroblasts during renal fibrosis. Pyruvate kinase (PK) is the last rate-limiting enzyme in the glycolysis pathway, cancer cell metabolism and cell cycle progression. Abnormal regulation of PK may

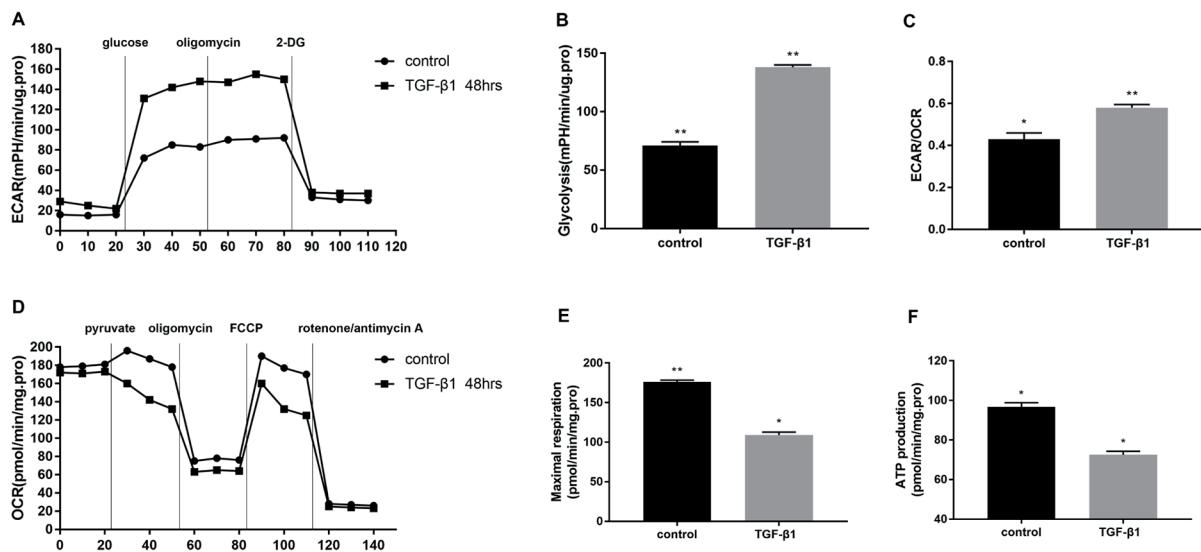


**Figure 4.** Metabolic reprogramming induced activation of NRK-49F cells. **A**, Western blot results of p-PKM2 and PKM2 in NRK-49F cells transfected with 10, 20, and 30  $\mu$ g of PKM2 overexpression plasmid, respectively. **B**, Western blot results of p-PKM2 and PKM2 in NRK-49F cells transfected with 30  $\mu$ g of PKM2 overexpression plasmid. **C**, The mRNA level of  $\alpha$ -SMA in NRK-49F cells transfected with 30  $\mu$ g of PKM2 overexpression plasmid. **D**, The mRNA level of PCNA in NRK-49F cells transfected with 30  $\mu$ g of PKM2 overexpression plasmid. **E**, Lactic acid concentration in the cell medium treated with 30  $\mu$ g of PKM2 overexpression plasmid.

lead to the continuous progress of renal interstitial fibrosis. We demonstrated metabolic reprogramming of fibroblasts during renal interstitial fibrosis both *in vitro* and *in vivo*. The specific mechanism of PKM2 in promoting renal interstitial fibrosis, however, deserves further investigations. By up-regulating PKM2 expression, we subsequently confirmed that metabolic reprogramming itself can induce fibroblast activation. Finally, Real-time monitoring of energy metabolism indicated that glycometabolism and

oxidative phosphorylation level were decreased during fibroblast activation.

Taken together, our results demonstrated metabolic reprogramming of renal interstitial fibroblasts during renal interstitial fibrosis, characterized as the conversion from oxidative phosphorylation to aerobic fermentation. Metabolic reprogramming was highly correlated with the occurrence and development of renal interstitial fibrosis, which may be the key to the continuous progress of CKD.



**Figure 5.** TGF- $\beta$ 1 induced the metabolism of NRK-49F cells from oxidative phosphorylation to aerobic glycolysis. **A**, Basic glycolysis level of cells treated with 2 ng/mL TGF- $\beta$ 1 after 48 h ( $*p < 0.05$ ). **B**, Maximum glycolysis capacity of cells treated with 2 ng/mL TGF- $\beta$ 1 after 48 h ( $*p < 0.05$ ). **C**, **D**, ECAR/OCAR of cells treated with 2 ng/mL TGF- $\beta$ 1 after 48 h ( $*p < 0.05$ ). **E**, **F**, Decreased maximum respiratory capacity and ATP production after cells were treated with 2 ng/mL TGF- $\beta$ 1 for 48 h ( $*p < 0.05$ ).

## Conclusions

We observed that upregulated PKM2 in renal fibrosis may serve as a specific marker of CKD for evaluating the severity of renal fibrosis, which provides a new direction in diagnosing and treating CKD.

## Conflict of Interest

The Authors declare that they have no conflict of interest.

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