

## Original Article

# Piwi2 gene transfection changes the autophagy status in a rat model of diabetic nephropathy

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**Abstract:** This study aims to investigate effects of Piwi2 on autophagy in a DN rat model. Sixty health SD rats were selected and divided into four groups, including normal group, control, DN and Piwi2 therapy group. DN model (DN group) was established by injecting the streptozotocin (50 mg/kg) into rats. Piwi2 therapy group was injected with viral plasmid carrying Piwi2 mRNA to DN rats. The urinary protein concentrations were determined by placing the animals in individual metabolic cages for a timed urine collection every 8 weeks. Blood and soleus muscle samples were collected after animals were sacrificed. Blood glucose was examined by using commercial detection kits. Western blot assay was employed to examine expression of Beclin 1 and LC3 (LC3 I and LC3 II) protein. Results indicated that urinary protein levels were remarkably higher in DN group compared to Normal and Control group ( $P < 0.05$ ). Blood glucose values were also increased in DN group compared to Normal and Control group ( $P < 0.05$ ). Body weights decreased significantly in DN rats compared to Normal group and Control group ( $P < 0.05$ ). Expression of Beclin 1 protein and LC3 proteins was significantly decreased in DN group compared to Normal and Control group ( $P < 0.05$ ). However, Piwi2 transfection could enhance level of Beclin 1 and LC3 protein significantly compared to DN group. In conclusion, the Piwi2 mRNA transfection could obviously enhance the autophagy biomarker, including Beclin 1 and LC3 protein, which indicates that the Piwi2 treatment has improved the autophagy in diabetic nephropathy rats.

**Keywords:** Diabetic nephropathy, Piwi2 gene, autophagy, Beclin 1, LC3 protein

## Introduction

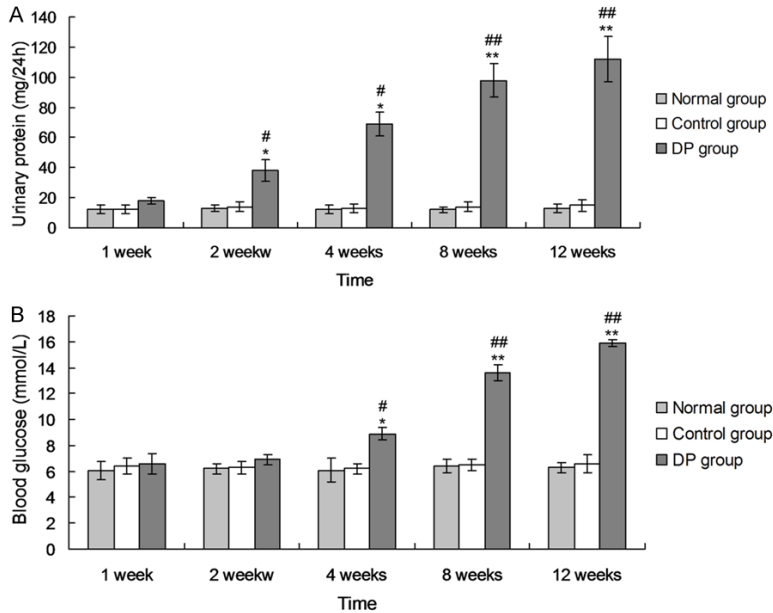
During the recent years, the diabetes mellitus prevalence has been increasing worldwide year by year [1-3]. Diabetic nephropathy [DN] is a serious complication of diabetes mellitus, which is also the most prevalent reason for the end stage renal disease (ESRD) [4]. Nowadays, the DN has also become the major health problem all over the world. Therefore, it is an urgent requirement to explore new therapeutic methods or molecules that treat the DN.

The previous studies have reported that the programmed cell death (PCD) plays an important role in the pathogenesis of the DN [5]. The PCD mainly composed of apoptosis and autophagy [6]. The autophagy dysfunction of autophagy in DN patients may lead to the degradation obstacle for abnormal proteins. Autophagy is an evolutionarily conserved homeostatic cellular process that has garnered widespread interest as an important pathway in

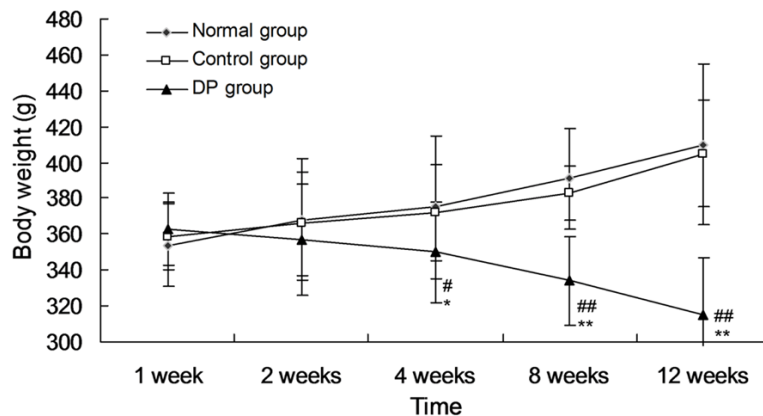
many biological functions [7]. The autophagy plays important roles in normal and abnormal states of human, including immunity, inflammation, adaptation to stress, metabolic and neurodegenerative diseases, development and aging, and tumor or cancers [8].

Recently, Piwi family proteins have come to light as a new set of players in transcriptional and post-transcriptional regulation of gene expression [9]. Especially, Piwi2 has been repeatedly proposed as a potential marker for cancers [10], by regulating the neoplasia through different signaling pathways [11]. Recent reported indicates that the Piwi2 could combine with signal transducer and activator of transcription 3 (STAT3), and inhibits the apoptosis by regulating the p53 signaling pathway [12]. Though there is no direct evidence proving the Piwi2 participates in the autophagy, the STAT3 and its down-stream factors are closely related to the regulation of autophagy. Therefore, this study aims to investigate the effects

## Piwi2 gene changes autophagy status in DN rats



**Figure 1.** Urinary protein and blood glucose in the experimental groups. Urinary protein (A), blood glucose (B) in normal SD rats and the DN model rats. Data are expressed as the mean  $\pm$  SD, \* $P$ <0.05, \*\* $P$ <0.01 represent the Urinary protein and blood glucose levels in DN group compared to the Normal group. # $P$ <0.05, ## $P$ <0.01 represent the Urinary protein and blood glucose levels in DN group compared to the Control group. DN, diabetic nephropathy.



**Figure 2.** Body weight loss in the experimental groups. Data are expressed as the mean  $\pm$  SD, \* $P$ <0.05, \*\* $P$ <0.01 represent the body weight in DN group compared to the Normal group. # $P$ <0.05, ## $P$ <0.01 represent the body weight in DN group compared to the Control group. DN, diabetic nephropathy.

of the Piwi2 on the autophagy in the DN rat model.

### Materials and methods

#### Animals

Male SD rats, aged 20 weeks and weighing  $350 \pm 10$  g, were purchased from the Chinese Academy of Sciences, Shanghai, China. The rats were individually housed for four weeks

with a standard diet before the model establishment. The animals were maintained in cages at 22°C under a 12-hours light/12-hours dark cycle and were allowed free access to water.

#### Establishment of DN model and trial grouping

This study selected 60 health SD rats and divided into four groups, including normal group, control group, DN group and Piwi2 therapy group. The Normal group treated without any other materials except for the normal diet. The control group was injected with the PBS solution. The DN group was injected with the streptozotocin (50 mg/kg). The Piwi2 therapy group was injected the viral plasmid carrying the Piwi2 mRNA to DN rats.

Establishment of the DN model was identified by detecting the blood glucose level at least 3 times, 72 hours after streptozotocin injection. The criteria was as the following: blood glucose level >16.7 mmol/L continuously for 3 times examination, urinary production higher than 1.5 times of original urinary, excretion of urine protein >30 mg/24 h.

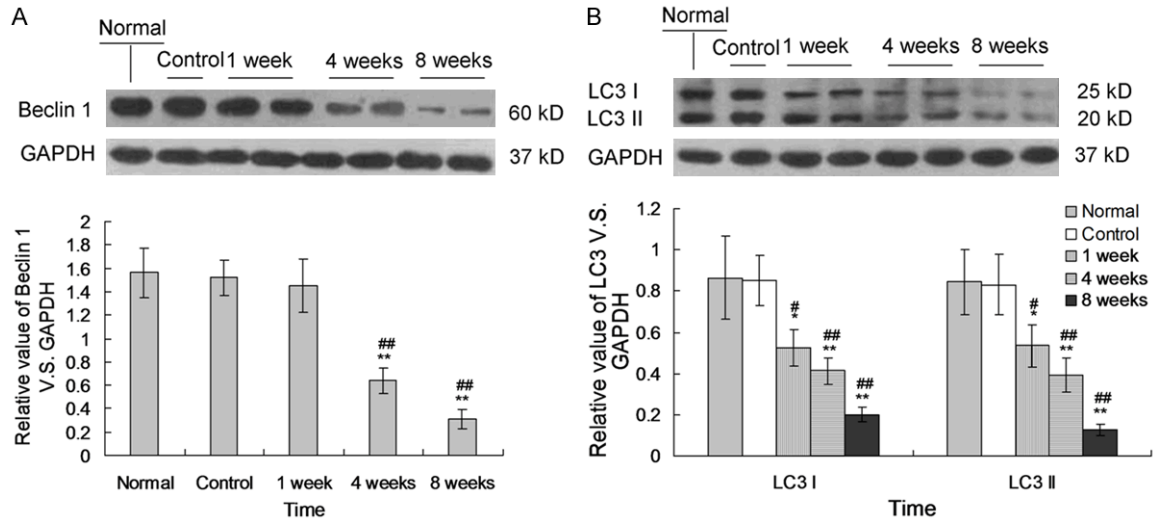
#### Blood glucose, urinary protein level and body weight change measurement

The urinary protein concentrations were determined by placing the animals in individual metabolic cages for a timed urine collection every 8 weeks. Blood and soleus muscle samples were collected after the animals were sacrificed. The blood glucose was detected by using the methods in the previous study [13].

#### Western blot assay

Tissue lysates were prepared from samples frozen in liquid nitrogen. The samples were pulver-

## Piwil2 gene changes autophagy status in DN rats



**Figure 3.** Observation of the autophagy markers expression in the experimental groups. Representative Western blot assay and group data of Beclin-1 (A) and LC3 (B) in the soleus muscle of the experimental groups. Data are expressed as the mean  $\pm$  SD, \* $P < 0.05$ , \*\* $P < 0.01$  represent the relative expression of Beclin 1 or LC3 (LC3 I or LC3 II) in DN group compared to the Normal group. # $P < 0.05$ , ## $P < 0.01$  represent the relative expression of Beclin 1 or LC3 (LC3 I or LC3 II) in DN group compared to the Control group. DN, diabetic nephropathy.

ized and lysed in RIPA buffer for 2 hours at 4°C. The lysates were centrifuged at 10,000 g for 10 min at 4°C, and the obtained supernatants were transferred into separate tubes. The proteins were separated using the SDS-PAGE and transferred onto the nitrocellulose membranes. The membranes were incubated with primary antibodies (including mouse anti-rat Beclin 1 monoclonal antibody and mouse anti-rat LC3 I and LC3 II monoclonal antibodies) at 4°C in 5% skim milk overnight. Following, the membranes were incubated with the secondary antibody (goat anti-mouse IgG) conjugated with horseradish peroxidase. Band visualization was performed using an ECL Western bolt substrate kit (Millipore, USA).

### Statistical analysis

Statistical analysis was performed using SPSS 19.0 software. The data were analyzed by Students t test. A  $P$  value of less than 0.05 was considered statistically significant.

## Results

### Urinary protein and blood glucose

The urinary protein levels were remarkably higher in the DN group compared to the normal and control groups (**Figure 1A**,  $P < 0.05$ ) from 2

weeks to 12 weeks after the DN establishment. Blood glucose values were also increased in the DN group compared to the normal and control groups (**Figure 1B**,  $P < 0.05$ ) from 4 weeks to 12 weeks after the DN model establishment. The above results indicated that the DN model has been successfully established.

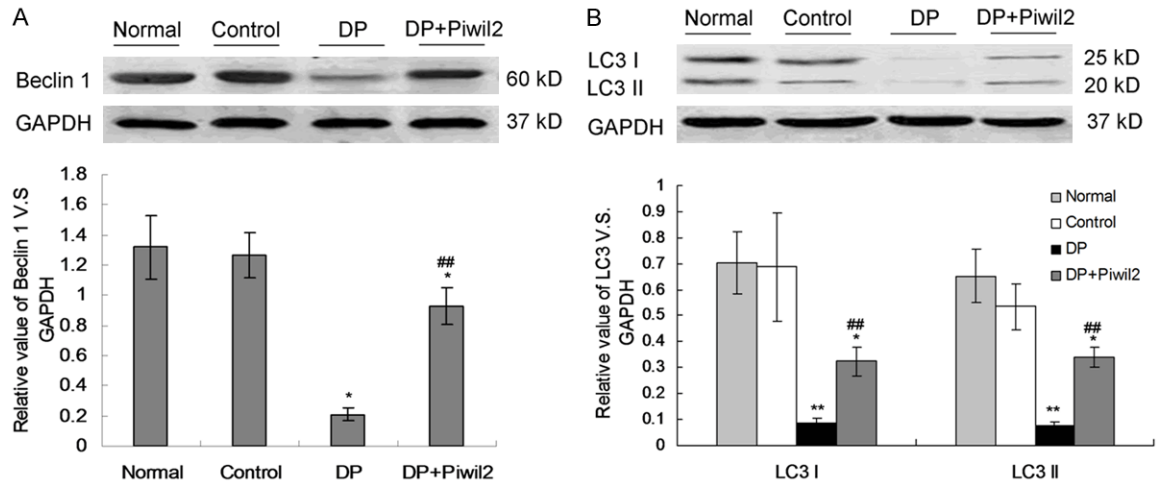
### Body weight

In normal SD rats, body weight was progressively increased from 4 weeks to 12 weeks after DN model establishment (**Figure 2**). Contrary, the body weights decreased significantly in DN rats compared to the normal group and control group from 4 weeks to 12 weeks after DN model establishment (**Figure 2**,  $P < 0.05$ ). Through the body weight was lower in the DN group compared to the normal and control groups at 2 weeks, no significant difference was observed.

### Autophagy biomarkers were decreased in PD model rats

In order to investigate the relationship between the autophagy and PD, the autophagy biomarkers, Beclin 1 and LC3 protein (LC3 I and LC3 II), were detected by using the Western blot assay. The results indicated that the levels of Beclin 1 protein was significantly decreased from the 4

## Piwi2 gene changes autophagy status in DN rats



**Figure 4.** Piwi2 mRNA transfection enhances the expression of Beclin 1 and LC3 proteins examined by western blot assay. A. Expression and statistical analysis of the Beclin 1 protein. B. Expression and statistical analysis of the LC3 proteins. \* $P < 0.05$ , \*\* $P < 0.01$  represent the relative expression of Beclin 1 or LC3 (LC3 I or LC3 II) in DN group compared to the Normal group. # $P < 0.05$ , ## $P < 0.01$  represent the relative expression of Beclin 1 or LC3 (LC3 I or LC3 II) in DN + Piwi2 group compared to the DN group. DN, diabetic nephropathy.

weeks to 8 weeks in DN group compared to the normal and control groups (**Figure 3A**,  $P < 0.05$ ). Meanwhile, the LC3 I and LC3 II protein were also decreased significantly in DN group compared to normal and control groups from 1 week to 8 weeks (**Figure 3B**,  $P < 0.05$ ).

### Piwi2 treatment changes the autophagy in PD rats

To identify the effects of Piwi2 on the autophagy, we treated the DN rats by injecting the Piwi2 mRNA into the rat tails. The results showed that the Beclin 1 level was significantly enhanced in Piwi2 treatment group compared to the DN group (**Figure 4A**,  $P < 0.01$ ), but also lower compared to the normal and control groups ( $P < 0.05$ ). Furthermore, the LC3 I and LC3 II protein levels were also increased in Piwi2 treatment group compared to the normal and control groups (**Figure 4B**,  $P < 0.01$ ).

### Discussion

The chronic kidney diseases, such as ESRD, DN, are associated with a loss in body protein mass and fuel reserves. In this study, we firstly established the DN rat model, and sought to determine the effect of the Tiwil 2 treatment on autophagy in rats with diabetic nephropathy. In the present research, we provide two novel findings. Firstly, our results indicated that autophagy was down-regulated in the rats with diabetic nephropathy. Secondary, the injection

of Tiwil 2 mRNA to PD rats increased the autophagy compared to Normal rats.

Recent years, protein restriction has been utilized to alleviate or inhibit the uremic symptoms, in order to protect the function of kidneys, as well as to prevent the complications, such as abnormal glucose metabolism and weight changes in chronic kidney disease [14, 15]. The previous studies confirmed that the autophagy could degrade the abnormal proteins in the kidney tissues or the abundant proteins in the urine [16, 17], which process could protect the kidney from the chronic kidney disease in some extents. Therefore, we observed the changes of autophagy in the PD rats by examining the autophagy associated protein Beclin 1 and LC3 proteins.

Autophagy begins with the production of double-membrane vacuoles (named autophagosomes) that entrap the materials to be degraded and eventually fuse with lysosomes [18]. The autophagosomes are characteristically marked by the presence of protein LC3 protein on their membranes [19]. Among the many proteins that directly or indirectly regulate the autophagy process, Beclin 1 seems to be of particular relevance in ovarian carcinogenesis. Beclin 1 was initially isolated as an interactor of the oncogenic antiapoptotic protein Bcl-2, and it was reported to be deleted in up to 75% of human ovarian cancers [20, 21]. Qu et al. [22] also reported that the Beclin 1 was associated

with the autophagy. Therefore, we examined the Beclin 1 and LC3 protein in kidney tissues. The results indicated that the Bclin 1 and LC3 (LC3 I and LC3 II) were significantly decreased in the PD rats, which suggest that the autophagy was inhibited in the PD rats. Therefore, we speculated that the decreased autophagy might cause the accumulation of the abundant proteins in the kidney tubules or urine in DN rats.

The previous studies have demonstrated some therapeutic method for the diabetic nephropathy. Gembaradt et al. [23] found that the SGLT2 inhibitor, empagliflozin, can ameliorate the early symptoms of diabetic nephropathy in type 2 diabetic mice. Chen et al. [24] also proved that the astragaloside V could ameliorate the diabetic nephropathy. However, most of the anti-diabetic nephropathy drugs were synthesized, which always triggers some side effects [25, 26]. In the present study, we established the no-toxicity and safe plasmid (Tiwil 2 mRNA) to the PD rats, and observed the effects of the Tiwil 2 on the autopahgy. The results indicated that the treatment of Tiwil 2 mRNA could significantly enhance the Beclin 1 protein and LC3 proteins.

Totally, the autophagy status was significantly changed in the diabetic nephropathy rats. The Tiwil 2 mRNA transfection could obviously enhance the autophagy biomarker, including Beclin 1 and LC3 protein, which indicates the changes of autophagy in diabetic nephropathy rats.

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#### Disclosure of conflict of interest

None.

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## Piwil2 gene changes autophagy status in DN rats

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## Piwil2 gene changes autophagy status in DN rats

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