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ORIGINAL ARTICLE

Basic Study

cNPAS2 induced β cell dysfunction by regulating KANK1 expression in type 2 diabetes

Yan-Bin Yin, Wei Ji, Ying-Lan Liu, Qian-Hao Gao, Dong-Dong He, Shi-Lin Xu, Jing-Xin Fan, Li-Hai Zhang

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Yan-Bin Yin, Shi-Lin Xu, Li-Hai Zhang, Department of General Surgery, The First Affiliated Hospital of Jiamusi University, Jiamusi 154000, Heilongjiang Province, China

Wei Ji, Department of Anesthesiology, Yantai Affiliated Hospital of Binzhou Medical University, Yantai 264199, Shandong Province, China

Ying-Lan Liu, Operating Room, The First Affiliated Hospital of Jiamusi University, Jiamusi 154000, Heilongjiang Province, China

Qian-Hao Gao, Department of Anesthesiology, Huazhong University of Science and Technology Union Jiangbei Hospital, Wuhan 430100, Hubei Province, China

Dong-Dong He, Jing-Xin Fan, Department of Endocrinology, The First Affiliated Hospital of Jiamusi University, Jiamusi 154000, Heilongjiang Province, China

Co-first authors: Yan-Bin Yin and Wei Ji.

Corresponding author: Li-Hai Zhang, MSc, Associate Chief Physician, Department of General Surgery, The First Affiliated Hospital of Jiamusi University, No. 348 Dexiang Street, Xiangyang District, Jiamusi 154000, Heilongjiang Province, China. zhanglihai@jmsu.edu.cn

Abstract

BACKGROUND

Diabetes mellitus type 2 (T2DM) is formed by defective insulin secretion with the addition of peripheral tissue resistance of insulin action. It has been affecting over 400 million people all over the world.

AIM

To explore the pathogenesis of T2DM and to develop and implement new prevention and treatment strategies for T2DM.

METHODS

Receiver operating characteristic (ROC) curve analysis was used to conduct diagnostic markers. The expression level of genes was determined by reverse transcription-PCR as well as Western blot. Cell proliferation assays were performed by cell counting kit-8 (CCK-8) tests. At last, T2DM mice underwent Roux-en-Y gastric bypass surgery.

RESULTS
We found that *NPAS2* was significantly up-regulated in islet β cell apoptosis of T2DM. The ROC curve revealed that *NPAS2* was capable of accurately diagnosing T2DM. *NPAS2* overexpression did increase the level of *KANK1*. In addition, the CCK-8 test revealed knocking down NPAS2 and KANK1 increased the proliferation of MIN6 cells. At last, we found that gastric bypass may treat type 2 diabetes by down-regulating *NPAS2* and *KANK1*.

CONCLUSION
This study demonstrated that *NPAS2* induced β cell dysfunction by regulating *KANK1* expression in type 2 diabetes, and it may be an underlying therapy target of T2DM.

Key Words: Diabetes mellitus type 2; KANK1; NPAS2; Gastric bypass

Core Tip: Diabetes mellitus type 2 (T2DM) is formed by defective insulin secretion with the addition of peripheral tissue resistance of insulin action. This study demonstrated that *NPAS2* induced β cell dysfunction by regulating *KANK1* expression in type 2 diabetes, and it may be an underlying therapy target of T2DM.

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INTRODUCTION

Diabetes mellitus is mainly divided into 2 categories, type 1 diabetes is caused by autoimmune destruction of beta cells, and diabetes mellitus type 2 (T2DM) is formed by errors of insulin secretion and the addition of peripheral tissue resistance of insulin action. Both of the two types are polygenic. There are also 2%-5% of diabetes cases caused by mutations in single genes which results in uncommon early-onset monogenic diabetes[1]. Some studies indicated that T2DM has affected approximately 500 million people around the world. And the high incidence of this disease is a serious risk factor of human beings[2]. Monogenic diabetes is classified as Neonatal Diabetes Mellitus and Maturity Onset Diabetes of the Young. Monogenic diabetes is a heterogeneous disease caused by variants in the single genes that play a key role in beta cell development, functional performance, including transcription factors like PTF1A, HNF1B, PDX1, RFX6, NEUROG3[3].

Proteins are crucial in human different biological processes, about 2500 proteins are regarded as binding to chromatin, such as DNA transcription, DNA replication, DNA repair. Moreover, there are approximately 1500 transcription factors among these proteins. Transcription factor is a specific kind of proteins which combine DNA helix at regulatory sequences, thereby activating or repressing transcription through trans-activation or trans-repression domains. In addition, ribonucleic acids, such as mRNA, rRNA, lnc-RNA, were expressed in the processes of transcription in living organisms.

The transcription factors are organized in various families reflecting homologies in their DNA-binding domains and, so are DNA-binding sequences[4,5]. They could be organized into 71 different families, some of which have more members than others, such as zing-finger C2H2 (more than 600 members), homeobox (more than 200 members) and helix-loop-helix (more than 80 members) families that represent more than half of the total number of transcription factors[6]. The significance of transcription factors in the study of human pathology is now increasingly mentioned in the literature. Furthermore, a more recent study found that a bigger number of transcription factors are involved in human disorders. In fact, Zhang *et al*[7] identified that there are 164 transcription factors (approximately 12%) directly associated with 277 diseases. But the role of transcription factors in type 2 diabetes has rarely been studied.

Here, we found that transcription factor (*NPAS2)* made the expression of *KANK1* significantly increase in the pancreas of T2DM rats. We also explore the role of *KANK1*and *NPAS2* in β-cell dysfunction in T2DM, which could lead to a novel strategy for the mechanism of T2DM.

MATERIALS AND METHODS

Animal studies

In this study, we used C57BL/6J male mice (weighing 22 + 2 g, Institute of laboratory animal sciences, Beijing, China) to established the animal model. The animal investigation described in this report was approved by the Biological and Medical Research Ethics Committee of Jiamusi University (2021-0330).

We put the male mice in a temperature-controlled room with a 12-hour light/dark cycle. The high-fat diet as well as low-dose intraperitoneal injection of streptozocin (STZ) was used to create the T2DM model, as previously described. The mice were fed adaptively for one week before being randomly divided into two groups. We chose 5 10% kcal% fat diet mice(D12450B) as the normal control group and 5 60% kcal% high-fat diet mice(D12492i) as T2DM model group.

After 28 days, T2DM group was given 100 mg/kg STZ. Meanwhile, the control group also received 100 mg/kg citrate buffer. After 7 days, the tail cutting was used to test the fasting blood glucose (FBG) of mice and FBG between 7.8 and 15 mmol/L were included for analysis as diabetic cases. After feeding for 16 weeks, the mice were subjected to glucose-stimulated insulin secretion test (GSIS) and the glucose tolerance test (OGTT). Afterwards, mice were anesthetized using 100 mg/kg pentobarbital injection. The serum which was from orbital venous plexus was centrifuged for 15 minutes at 3500 g. Total cholesterol (TC), serum FBG, triglyceride (TG), and insulin, which were form Abcam, United Kingdom, were determined. The pancreas tissues from the mice was washed and half-fixed with 10% formalin, then stored at minus 80 degrees Celsius in liquid nitrogen until needed.

The procedure of Roux-en-Y gastric bypass (RYGB) surgery has been described in detail by Hao *et al*[8]. Briefly, created little gastric pouch with around 5 percent of the total stomach content and remained the Roux and biliopancreatic limbs 6-7 cm long. The jejunum was sliced open it's distal end was anastomosed end-to-end with the stomach pouch. Mice in the control group performed laparotomy without severing jejunum and stomach transection. On the stomach's front wall, a simple continuous suture was employed. After surgery, all mice fasted only with tap water for one day, and for liquid food freely for 2 days.

OGTT and GSIS

In order to perform OGTT, mice were fasted for half a day, and took 2 g/kg of glucose dissolved in water orally. The concentration of blood glucose, which was from tail tip, was determined every 30 minutes for 2 hours. In order to perform GSIS, blood was taken from mice's ocular venous sinuses for 0 min. The stomach was then infused with 40 percent (w/v) glucose. Blood samples were taken every 15 minutes for 1 hour. Insulin ELISA kit (Merck Millipore Corporation, Germany) was used to detect insulin in serum, and we recorded and calculated the area under curve.

Culture and transfection of MIN6 cell injury model

Islet β-cell MIN6 cells (ATCC, United States) were cultivated in DMEM (25 mmol/L glucose) containing 1% penicillin-streptomycin (PS), 10% fetal bovine serum (FBS) as well as 1% β-mercaptoethanol at 37 degrees Celsius in a 5% CO_2 environment. The MIN6 cell injury model was grown in 24 well plates with 2×10^4 cells per well or in six well plates with 10^5 cells per well. The cells were cultivated in DMEM which contained 10% FBS as well as 1% β-mercaptoethanol. Transfection started after the cells had reached 60%-70% confluence. For the first 5 minutes, we diluted plasmid, transfected mimic, as well as Lipofectamine 2000 in Opti-MEM. After that, we added the diluted plasmid and mimic to the transfection reagent for double mixing for 20 minutes. In the end, we added the double mixed solution into the culture plate well, cultivated for 4 hours in Opti-MEM without FBS, and then changed for normal DMEM (with 25 mmol/L glucose, 1% β-mercaptoethanol, 1% PS, 10% FBS) culture. All reagents were from Gibco (United States).

Injury model of MIN6 cells induced by palmitic acid

We seeded MIN6 cells in 96 well plates. Then we set up the blank control group as well as the treatment group with various concentrations of palmitic acid (0.1-1.0 mmol/L), as soon as the fusion degree of cells reached about 80%. Cell counting kit-8 (CCK-8) tests was used to determine the cell survival rate after 24 hours of treatment, and the ideal concentration of palmitic acid was chosen to create the MIN6 cell damage model.

Western blot

We used western blot to test the protein concentration as previously reported[9]. Briefly, total protein which from MIN6 cells or pancreatic tissue was tested by BCA protein assay kit (Thermo Fisher Scientific, United States). Each specimen was isolated from twelve alkyl sulfonate polyacrylamide gel, transferred to a polyvinylidene fluoride membrane (Bio-Rad, China), then sealed with 5 percent (w/V) BSA for 2 hours. Next, membranes were detected overnight at 4 degrees Celsius with suitable primary antibodies (Caspase-3, SIRT1, β-actin). The membranes were cultured with secondary antibodies at room temperature for 2 hours after washing three times with TBST [150 mmol/L NaCl, 10 mm Tris-HCl, as well as 0.1 percent (V/V) Tween-20]. Protein bands were shown using enhanced chemiluminescence, and pictures were created using GENE Imaging system (Tannon, China).

Detection of accumulated insulin secretion by MIN6 cells

We seeded MIN6 cells in 24 well plates. The cells were divided into groups and treated respectively as soon as the fusion degree of cells reached about 80%. After collecting the supernatant and cell protein, the insulin content in the supernatant was detected by insulin ELISA kit (Merck, Germany).

RNA quantification

The expression levels of gene was detected by reverse transcription-PCR (RT-PCR) as previously reported[10]. Briefly, TRIzol (Merck, Germany) was used to extract the total RNAs, followed the instruction by the manufacturer. The primer was used to detect the mRNA level. One μg extracted RNA was reverse transcribed to cDNA, using the kit from Madison, United States. On an ABI 7300, RT-PCR was carried out using Fast Start universal SYBR Green Master (Roche, United States). $2^{-\triangle\triangle CT}$ was used to evaluate the expression levels in comparison to the control.

Cell proliferation assay

Cell viability was detected by the CCK-8 assay as indicated by the protocol from the manufacturer. Briefly, the cells were grown in 96-well plates with appropriate amount of tumor cells. Then the varied amounts of temozolomide or a dimethyl sulfoxide control was incubated with the cells. After that, the CCK-8 solution was supplemented into the wells. Cells were found in a microplate reader at 450 nm of absorbance.

Statistical analysis

For statistical analysis, we utilized SPSS 23.0 (SPSS, United States). The final data were presented as the average SD of three separate studies. To compare two or three groups, the Student's *t*-test or ANOVA were utilized, accordingly. A value of *P* less than 0.05 was regarded statistically significant.

RESULTS

Establishment of type 2 diabetic mice

Table 1 showed the amount of change in lipid-related parameters in T2DM mice. The findings showed, compared with the control group, the levels of TG ($P < 0.05$), low-density lipoprotein cholesterol ($P < 0.05$), TC (*P* less than 0.01) as well as FBG ($P < 0.01$) were remarkably increase, but the levels of fasting insulin ($P < 0.05$) as well as high-density lipoprotein cholesterol ($P < 0.05$) was significantly decreased in T2DM mice.

The area under the glucose concentration curve of the OGTT ($P < 0.0001$) as well as GSIS ($P < 0.0001$) showed that the glucose tolerance was damaged in the model group, and insulin secretion sensitivity was markedly reduced following glucose stimulation (Figure 1).

NPAS2 was significantly up-regulated in islet β cell of T2DM

To verify if the above results were connected with *NPAS2* in T2DM mice or not, the mRNA of *NPAS2* in islet tissue was determined with RT-PCR. The expression of *NPAS2* in β-cell of T2DM mice was notably increase compare with that in control group ($P < 0.0001$; Figure 2A). And western blotting showed the overexpression of *NPAS2* in T2DM groups ($P < 0.0001$; Figure 2B). The above results show that NPAS2 was significantly up-regulated in islet β cell of T2DM.

KANK1 was the potential target gene of NPAS2

Using the R package "Dorothea" to look for *NPAS2* downstream target genes, we discovered that *NPAS2* may influence *KANK1* expression. In T2DM, a scatter plot revealed a favorable connection between *KANK1* and *NPAS2* ($r = 0.659$, $P = 5.01e-06$; Figure 3A).

To see whether *KANK1* is expressed in T2DM. We observed *KANK1* mRNA and protein levels in β-cells of T2DM mice and normal mice. According to the results, the mRNA level of *KANK1* in T2DM group was higher than that in control group ($P < 0.0001$; Figure 3B and C).

NPAS2 positively regulates KANK1 expression

We created an *NPAS2* overexpression construct and transfected it into a MIN6 cell injury model to further establish the regulatory link between NPAS2 and KANK1. MIN6 cell injury model were transfected using overexpression plasmids of *KANK1* for 2 days and then for Western blotting and quantitative reverse transcriptase PCR. The findings showed that overexpression of *NPAS2* did enhance *KANK1* mRNA and protein levels ($P < 0.001$; Figure 4A and B), On the contrary, knocking down *NPAS2* with shRNA had the opposite effect (Figure 4C and D), demonstrating that *NPAS2* controls *KANK1* expression. Above results showed that *NPAS2* positively regulates *KANK1* expression.

Knocking down NPAS2 and KANK1 increased the proliferation of MIN6 β-cell

This study investigated if *NPAS2* and *KANK1* deceased β-cell survival or not. Using sh-strategy, we were able to knockdown gene expression in MIN6 cell damage model. CCK-8 tests were used to assess cell proliferation. The results showed that knocking down *NPAS2 and KANK1* increased the proliferation of MIN6 cells ($P < 0.01$; Figure 5).

NPAS2 deceased β-cell survival by regulating KANK1 expression

These findings proved that *KANK1* and *NPAS2* are important in inducing β cell dysfunction. According to the above assays, we conjectured that *KANK1* was an effector of *NPAS2*. To verify this conjecture, *KANK1* was knocked down in *NPAS2* overexpressing MIN6 cell injury model. The CCK-8 test revealed that cell proliferation rate was higher in the sh-*NPAS2* + empty vector MIN6 cell injury model compared to the sh-Control + empty vector model ($P < 0.01$). However, it was reversed by *KANK1* overexpression (sh-*NPAS2* + OE-*KANK1*), showing that *NPAS2* suppressed MIN6 cell proliferation through *KANK1* ($P < 0.01$; Figure 6). These results suggested that *NPAS2* deceased β-cell survival by regulating *KANK1* expression.

Gastric bypass may treat type 2 diabetes by down-regulating NPAS2 and KANK1

RYGB surgery is a usual method for T2DM. To investigate the role of *NPAS2* and *KANK1* in this surgery, T2DM mice underwent RYGB surgery. In the RYGB group, the mRNA content of *KANK1*and *NPAS2* in the β-cell of mice was much lower than that in control group ($P < 0.0001$; Figure 7A). Meanwhile western blotting showed the same result. These

Table 1 Serum biochemical parameters of Con and diabetes mellitus type 2 C57BL/6J mice

Parameter	Con	T2DM
FBG (mM)	3.16 ± 0.25	11.77 ± 1.71[b]
LDL-C (mM)	0.26 ± 0.02	0.53 ± 0.10[a]
TG (mM)	-0.035, 1.40 ± 0.04	0.080, 2.23 ± 0.24[a]
TC (mM)	0.972, 5.03 ± 0.23	0.013, 7.04 ± 0.42[b]
FINS (mU/L)	13.01 ± 3.36	5.85 ± 0.20[a]
HDL-C (mM)	2.75 ± 0.15	2.20 ± 0.14[a]

[a]$P < 0.01$.
[b]$P < 0.001$.
LDL-C: Low-density lipoprotein cholesterol; T2DM: Diabetes mellitus type 2; FBG: Fasting blood glucose; TG: Triglyceride; TC: Total cholesterol; FINS: Fasting insulin; HDL-C: High-density lipoprotein cholesterol.

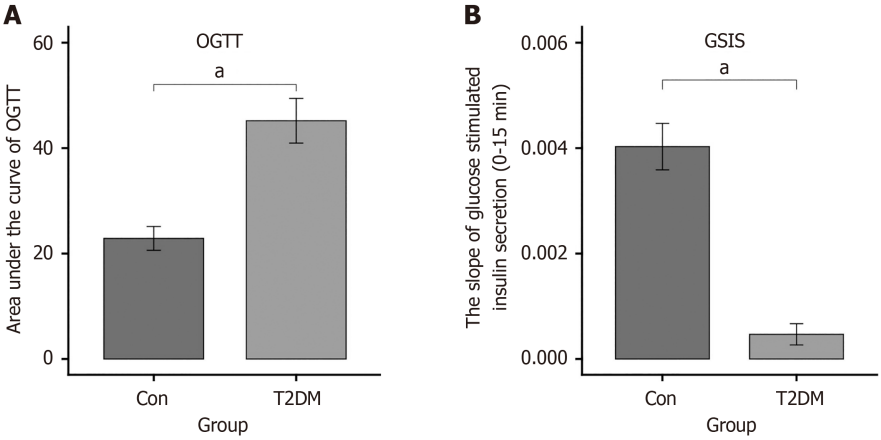

Figure 1 Changes of the glucose tolerance test, glucose-stimulated insulin secretion test in diabetes mellitus type 2 mice. A: In the control group and diabetes mellitus type 2 (T2DM) groups, the glucose tolerance test curve and the area under the curve were assessed after orally gavage with glucose; B: After stimulation of glucose, plasma insulin concentrations were measured in different phases during glucose-stimulated insulin secretion test in control group and T2DM group. [a]$P < 0.0001$. T2DM: Diabetes mellitus type 2; OGTT: Glucose tolerance test; GSIS: Glucose-stimulated insulin secretion test.

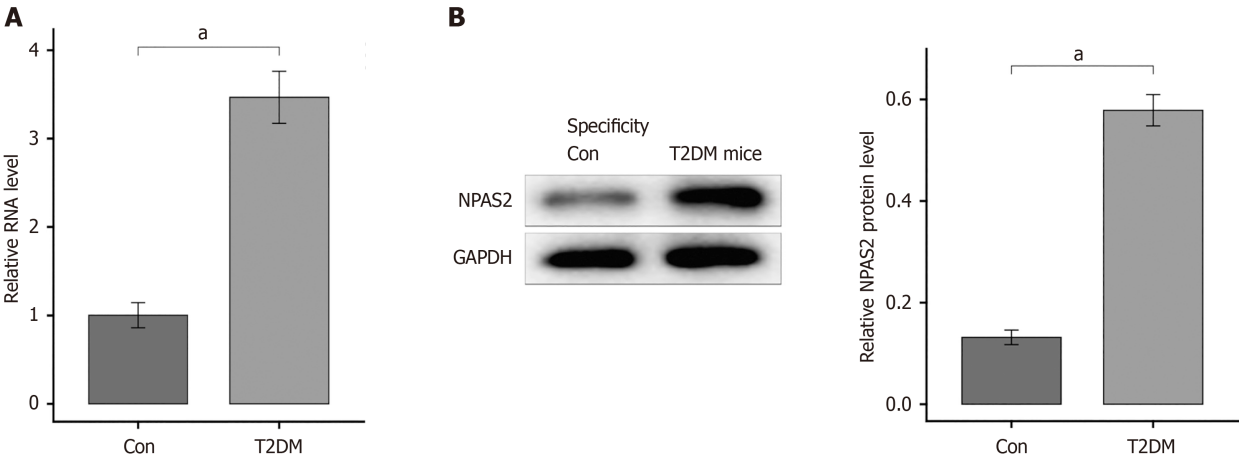

Figure 2 *NPAS2* is highly expressed in diabetes mellitus type 2 samples. A: *NPAS2* levels in islet tissues of control group and diabetes mellitus type 2 (T2DM) group were detected by reverse transcription-PCR; B: Western blotting showed the overexpression of NPAS2 in T2DM. [a]$P < 0.0001$. T2DM: Diabetes mellitus type 2.

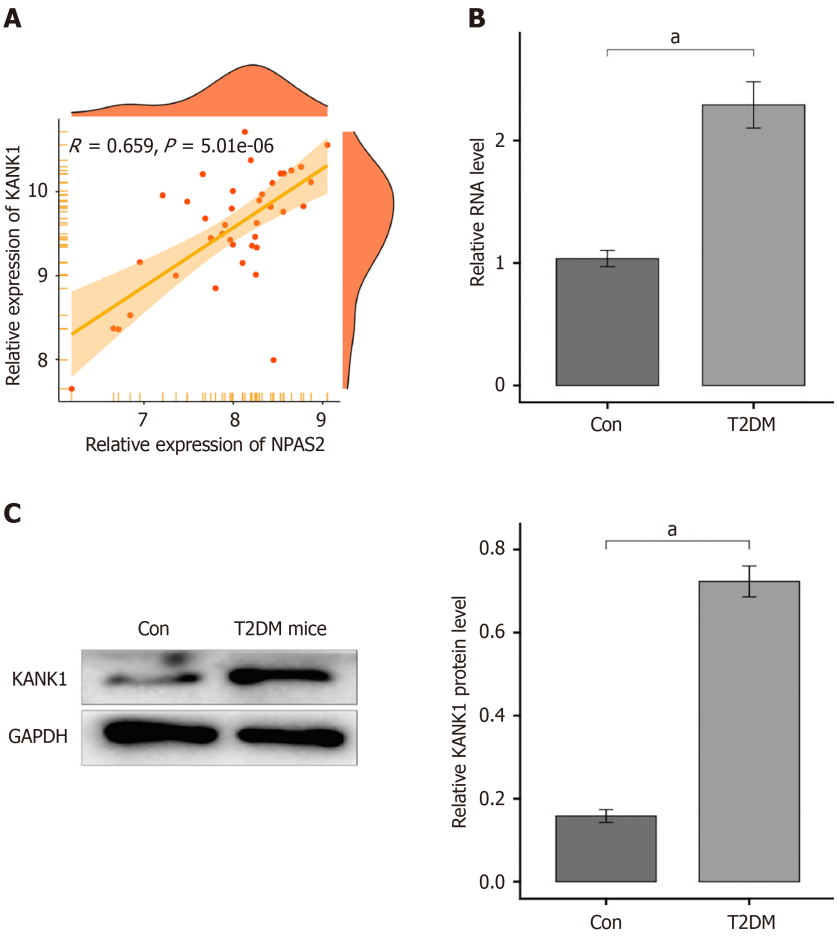

Figure 3 *KANK1* was the potential target gene of *NPAS2*. A: Scatter plot revealed the favorable connection between *KANK1* and *NPAS2* in diabetes mellitus type 2 (T2DM); B and C: The expression of *KANK1* in T2DM group was higher than that in control group. [a]*P* < 0.0001. T2DM: Diabetes mellitus type 2.

findings implied that RYGB surgery, by inhibiting the *NPAS2*/*KANK1* signaling pathway, may prevent β-cell dysfunction ($P < 0.0001$; Figure 7B).

DISCUSSION

As a matter of fact, T2DM has been affecting over 400 million people all over the world[11]. In addition, the incidence of T2DM may keep growing and, it is projected to influence approximately 33 percent people of the United States by 2050 [12]. The Global Burden of Diseases study showed T2DM as well as its complications was the reason for the 22% increase in disability in the past decade[13]. For the past few years, tremendous advance has been made in the prevention and therapy of diabetes[14]. It is well known that T2DM is a kind of multifactorial endocrine illness. At present, the major treatment in T2DM involved lifestyle intervention, the use of anti-diabetic drugs and monitoring of arterial pressure and lipid profile[15,16]. However, if the patients with T2DM have poor glycemic control, long-term hyperglycemia will cause great damage to the blood vessels and nerves. While studies on molecular mechanisms could suggest a new therapeutic option. As a result, there is an urgent need to explore the molecules that can be used as diagnostic marker in order to develop the novel preventative and treatment strategies. In the present study, we found that NPAS2 was capable of accurately diagnosing T2DM. Furthermore, our results revealed that *NPAS2* induced β cell dysfunction by regulating *KANK1* expression in type 2 diabetes.

Recent studies have revealed that the *NPAS2* can play an vital role in oncogenesis as an oncogene or tumor suppressor in tumor and endocrine diseases[17-20]. To study the function of *NPAS2* in T2DM, we first created a T2DM model. The t-test results indicated that *NPAS2* was considerably elevated in islet tissues of T2DM patients. In addition, the ROC curve revealed that *NPAS2* was capable of accurately diagnosing T2DM. What's more, *NPAS2* is link to multiple endocrine diseases such as obesity, thyroid gland, osteoporosis as well as gout[21,22]. Kovanen *et al*[23] elevated that *NPAS2* mRNA amounts are strongly related to higher disease free survival and overall rate in thyroid gland. Englund *et al*[18] found that *NPAS2* has a closely relationship with hypertension. Not only fetal liver metabolism, but also non-alcoholic fatty liver disease were linked to *NPAS2*[24]. The above studies illustrated the importance of *NPAS2* in endocrine diseases.

Diabetes mellitus is a kind of endocrine diseases. But studies on the relationship between *NPAS2* and T2DM have not been carried out in depth. This paper is to further investigate the impact of *NPAS2* on T2DM. In the study we discovered that *NPAS2* may influence *KANK1* expression. Previous study demonstrated that *KANK1* was not only related to cir-

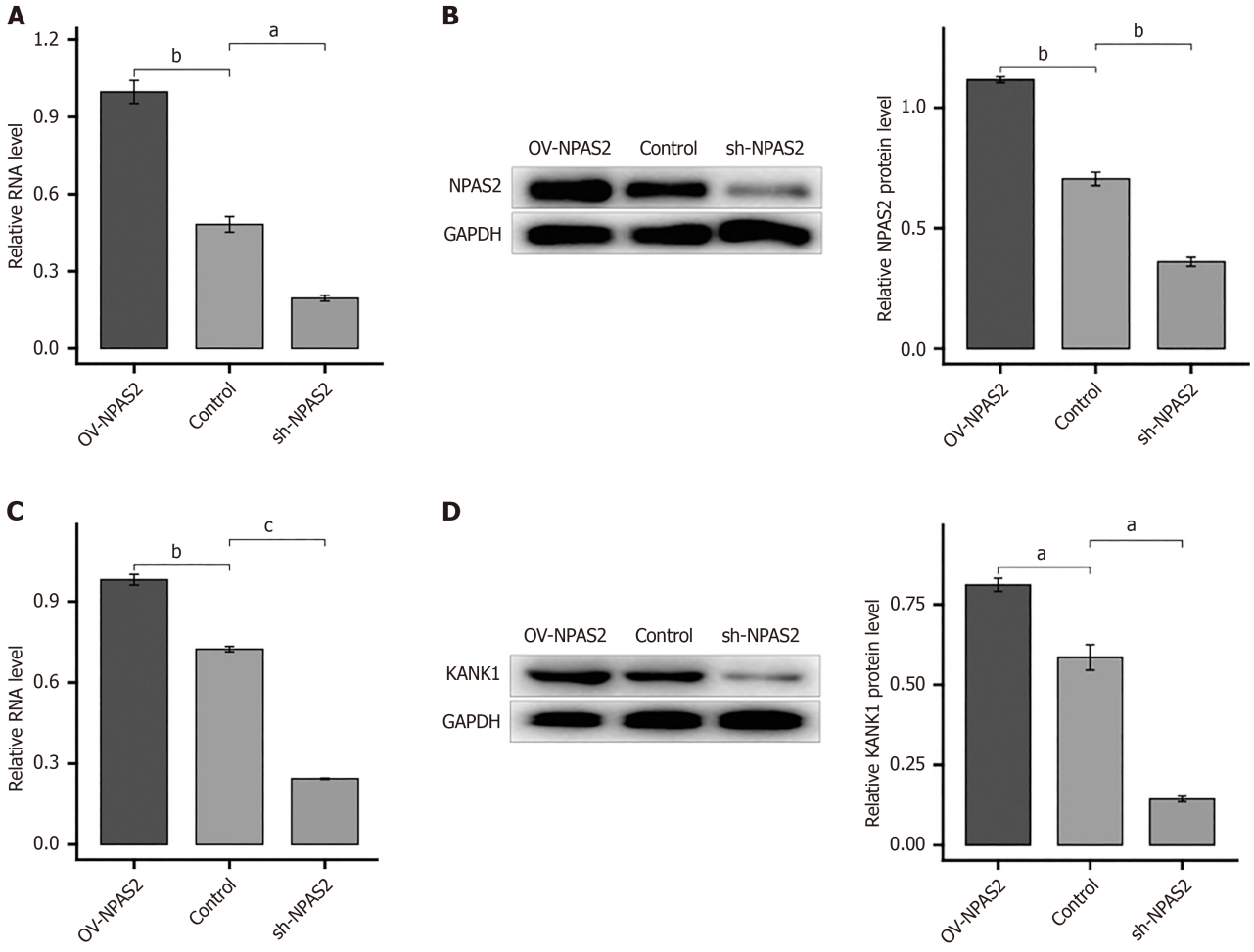

Figure 4 *NPAS2* regulates *KANK1*expression. A: Quantitative reverse transcriptase PCR (qRT-PCR) showed that NPAS2 overexpression increased the mRNA level of KANK1; B: Western blotting showed that NPAS2 overexpression enhanced the protein of KANK1; C: qRT-PCR showed knocking down *NPAS2* with shRNA decreased the mRNA level of KANK1; D: Western blotting showed knocking down *NPAS2* with shRNA decreased the protein of KANK1. $^{a}P < 0.01$, $^{b}P < 0.001$, $^{c}P < 0.0001$. T2DM: Diabetes mellitus type 2.

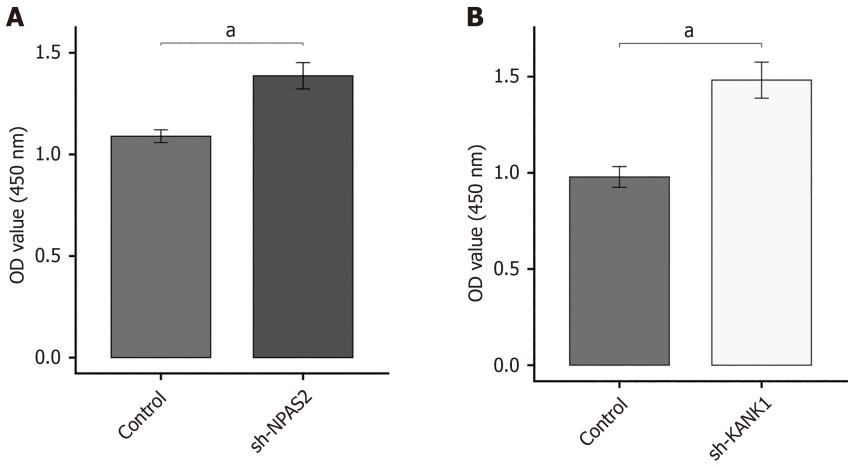

Figure 5 *NPAS2* and *KANK1* deceased β-cell survival. A and B: CCK-8 tests showed that knocking down *NPAS2 and KANK1* increased the proliferation of MIN6 cells. $^{a}P < 0.01$.

culating proinsulin levels but also connected with insulin processing as well as secretion traits[25]. To see whether *KANK1* is the target of *NPAS2*, we knocked down *KANK1* in *NPAS2* overexpressing MIN6 cell injury model. The CCK8 test revealed that knocking down NPAS2 and KANK1 increased the proliferation of MIN6 cells. The above results indicated that *NPAS2* induced β cell dysfunction by regulating *KANK1* expression in type 2 diabetes. Meanwhile, we also found that RYGB surgery prevent β-cell dysfunction by inhibiting the NPAS2/KANK1 signaling pathway. This project

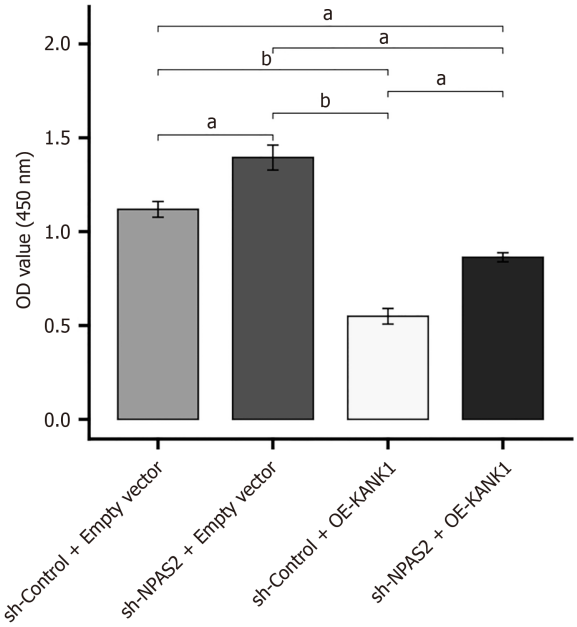

Figure 6 *NPAS2* deceased β-cell survival by regulating *KANK1* expression. The cell proliferation rate in sh-Control + Empty vector, sh-NPAS2 + Empty vector, sh-Control + OE-KANK1 and sh-NPAS2 + OE-KANK1, MIN6 cell injury model and the Empty vector + sh-Control model by CCK8 test. [a]*P* < 0.01, [b]*P* < 0.001.

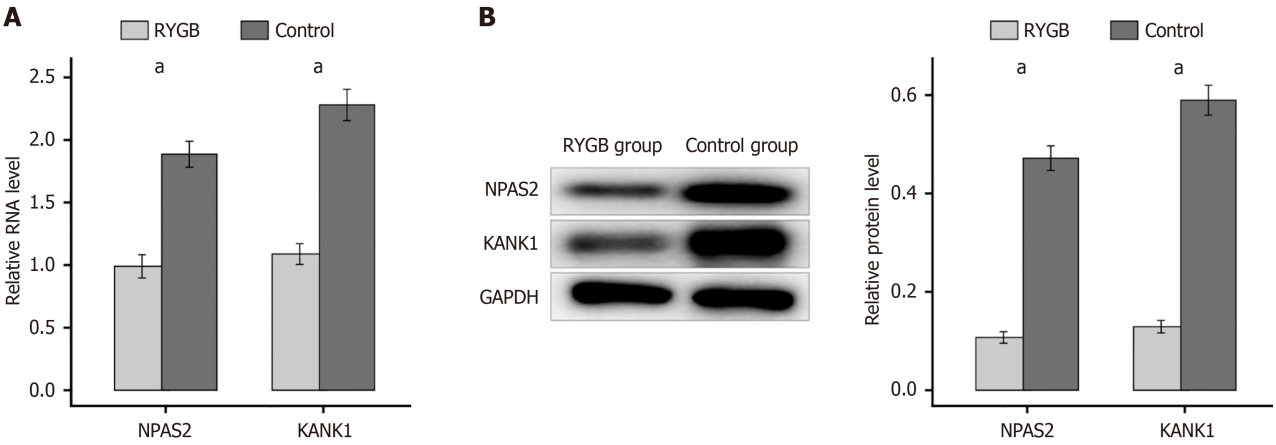

Figure 7 Gastric bypass may treat type 2 diabetes by down-regulating *NPAS2* and *KANK1*. A: Quantitative reverse transcriptase PCR showed the mRNA content of *KANK1* and *NPAS2* in the β-cell of mice; B: Western blotting indicated the protein of *KANK1* and *NPAS2* in the β-cell of mice. [a]*P* < 0.0001.

aims to make the β cell dysfunction regulatory network more complete, and lay theoretical foundation for revealing the dependent conditions of T2DM.

However, there is still room for further improvement in future experiments. For example, using more advanced sequencing technology, we can determine the cellular origin of NPAS2 and the mode of cell interaction. It is also a good treatment method to analyze the susceptibility of T2DM based on a large clinical sample, so as to target therapeutic drugs for susceptible genes. The high incidence of T2DM makes it more and more important to explore the depth and breadth of its mechanism.

CONCLUSION

This study demonstrated that NPAS2 induced β cell dysfunction by regulating KANK1 expression in type 2 diabetes, and it may be an underlying therapy target of T2DM.

FOOTNOTES

Author contributions: Yin YB, Zhang LH and Ji W were responsible for conception and design; He DD, Fan JX were responsible for

administrative support; Xu SL, Gao QH, He DD and Fan JX were responsible for provision of study materials or patients; Zhang LH and Gao QH were responsible for collection and assembly of data; Zhang LH and Yin YB were responsible for data analysis and interpretation; all authors were responsible for manuscript writing; all authors were responsible for final approval of manuscript.

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Institutional animal care and use committee statement: The animal investigation described in this report was approved by the Biological and Medical Research Ethics Committee of Jiamusi University (No. 2021-0330).

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ORCID number: Li-Hai Zhang 0009-0000-3658-8983.

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