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LncRNA XIST promotes insulin resistance in gestational diabetes mellitus *via* the microRNA-181b-5p/NDRG2 axis

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Abstract. Many studies have explored the role of lncRNA X inactivation-specific transcript (XIST) in diabetes. This study was designed to unravel the regulatory mechanism of XIST on animal models of gestational diabetes mellitus (GDM) progression *via* the microRNA (miR)-181b-5p/N-myc downstream-regulated gene 2 (NDRG2) axis. XIST, miR-181b-5p, and NDRG2 expression levels in GDM mice were detected. The GDM mice were subjected to gain- and loss-of-function assays to examine the change of glucose metabolism indices (fasting blood glucose (FBG), fasting insulin (FINS) and homeostasis model assessment of insulin resistance (HOMA-IR)), serum oxidative stress factors (glutathione (GSH), superoxide dismutase (SOD), and malondialdehyde (MDA)), serum inflammatory factors (interleukin-1 β (IL-1β), IL-6, and tumor necrosis factor α (TNF-α)), pathological changes of pancreatic tissues, and apoptotic cells in pancreatic islets in GDM mice. XIST and NDRG2 expression were elevated while miR-181b-5p expression was depleted in GDM mice. Down-regulated XIST or NDRG2 or up-regulated miR-181b-5p reduced the FBG level, HOMA-IR, and serum IL-1β, IL-6, and TNF-α, and MDA contents, elevated the FINS, GSH, and SOD level, mitigated pathological changes in pancreatic tissues, and decelerated apoptotic cells in pancreatic islets in GDM mice. Silenced XIST dampens insulin resistance in GDM mice *via* the modulation of the miR-181b-5p/NDRG2 axis.

Key words: Gestational diabetes mellitus — lncRNA X inactivation-specific transcript — miRNA-181b-5p — N-myc downstream-regulated gene 2 — Insulin resistance — Apoptosis

Abbreviations: FBG, fasting blood glucose; FINS, fasting insulin; GDM, gestational diabetes mellitus; GSH, glutathione; HDL-C, density lipoprotein cholesterol; HG, high glucose; HOMA-IR, homeostasis model assessment of insulin resistance; IL, interleukin; lncRNAs, long non-coding RNAs; MDA, malondialdehyde; NDRG2, N-myc downstream-regulated gene 2; SOD, superoxide dismutase; TC, total cholesterol; TG, triglyceride; TNF-α, tumor necrosis factor α; XIST, X inactivation-specific transcript.

Introduction

Gestational diabetes mellitus (GDM), termed as the glucose intolerance during pregnancy, is featured by potential maternal inability in the β-cell response to insulin (Moon et al. 2017). Generally, the occurrence of GDM is mainly associated with ethnicity, obesity, and family history of diabetes (Farahvar et al. 2019). It has been reported that women with GDM history have the higher risk of developing classical type 2 diabetes, and the risk rate usually ranges from 20 to 50% (Chen et al. 2015). Glucose monitoring and lifestyle change are generally initial managements for GDM. If glucose levels remains higher than the normal values, pharmacologic therapy with metformin, glyburide, or insulin would be further adopted (Garrison 2015). Even

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though, optimal treatment for GDM patients during long-term follow-up still encounters great obstacles, with poorly implemented preventive strategies for GDM worldwide (McIntyre et al. 2019). Therefore, the coordinated and well-implemented focus for GDM treatment still requires further exploration.

Long non-coding RNAs (lncRNAs) critically participate in metabolic processes in GMD, and their expression has been uncovered to be implicated in insulin resistance and β-cell dysfunction in GDM (Filardi et al. 2020). For instance, silenced lncRNA metastasis-associated lung adenocarcinoma transcript 1 has been revealed to mitigate inflammatory response in GDM patients (Zhang et al. 2020). The high-expressed lncRNA maternally expressed gene 8 is attributed for inducing high incidence rate of GDM (Zhang et al. 2021). As for lncRNA X inactivation-specific transcript (XIST), it has been validated that patients with type 2 diabetes mellitus exhibits a high level of XIST, furthermore, the altered expression of XIST is positively correlated with insulin resistance, transcriptional markers of senescence, and inflammation in type 2 diabetes mellitus (Sathishkumar et al. 2018). Besides, Li et al. have validated the importance of XIST in HTR-8/SVneo cell models induced by high glucose and GDM patients (Sathishkumar et al. 2018). Nevertheless, the detailed role of XIST in animal models of GDM remained obscure. It was predicated through the bioinformatic website that XIST had binding sites with microRNA (miR)-181b-5p. miRs have emerged as metabolic and developmental regulators in GDM (Poirier et al. 2017). miR-181 has been found to be decreased in patients with gestational obesity that related to GDM, and its expression is associated with maternal metabolic parameters like density lipoprotein cholesterol (HDL-C) and the insulin homeostasis model assessment insulin resistance (HOMA-IR) (Carreras-Badosa et al. 2017). miR-181b-5p is validated to be lowly expressed in livers of diabetic mice, and the hepatic repression of miR-181b-5p results in insulin resistance in diabetic mice (Wang et al. 2019), yet the studies for probing the function of miR-181b-5p in GDM are extremely rare. In addition, N-myc downstream-regulated gene 2 (NDRG2) was predicated to be targeted by miR-181b-5p in our paper. NDRG2 has been elucidated to be up-regulated in diabetic mice (Wang et al. 2020), and functions as a key molecule in beta-cell survival (Shao et al. 2013), whose dysfunction is responsible for inducing GDM (Alejandro et al. 2020). As stated above, the regulatory mechanism of XIST on GDM *via* the miR-181b-5p/NDRG2 axis remained largely unknown. Thereby, this research was conducted to unravel the function of the XIST/miR-181b-5p/NDRG2 axis in GDM, updating novel therapeutic targets for GDM treatment.

Materials and Methods

Ethics statement

The protocol of animal experiments was approved by the Institutional Animal Care and Use Committee of Beijing Ditan Hospital Affiliated Capital Medical University (approval number: 20200602).

Experimental animals

The specific pathogen-free grade 4-week-old female and male C57BL/6J mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The housing unit was maintained at constant temperature (22–25°C) and relative humidity (45 ± 5%), with a 12/12-hour light/dark cycle (6:00 a.m. lights on; 6:00 p.m. lights off), with free access to tap water. The subsequent experiments were carried out after 1-week adaptive feeding.

The establishment of GDM mouse models

After 4 weeks on a high-fat diet, the animals were caged 2:1 male to female, and the following morning at 7:00 a.m., the clitoral plugs (white or creamy yellow) were examined and found to be a successful mating, and were designated as gestational day zero (GD0). Streptozotocin (STZ) was prepared in citric acid-sodium citrate buffer to a concentration of 1% at pH 4.2–4.5. The animals were fasted for 12 h. The STZ solution was injected intraperitoneally at 40 mg/kg/*per* mouse (Li et al. 2017; Huang et al. 2018) on GD6, GD7 and GD8 for 3 consecutive days, and blood was collected from the tail vein for blood glucose measurement. The GDM mouse modeling was completed when the fasting blood glucose (FBG) within 72 h ≥11.1 mmol/l (Chen et al. 2019). Mice in the control group were injected intraperitoneally with equal amounts of citric acid/sodium citrate buffer. Meanwhile, the GDM mice were maintained on high-fat chow at all times. In contrast, the control mice were maintained on normal chow.

Animal grouping and treatment

In addition to control mice (control group), other GDM mice were randomly classified into GDM group, short hairpin RNA (sh)-negative control (NC) group, sh-XIST group, agomir NC group, miR-181b-5p agomir group, sh-NC group, sh-NDRG2 group, sh-XIST+antagomir NC group, sh-XIST+miR-181b-5p antagomir group, miR-181b-5p agomir+oe-NC group and miR-181b-5p agomir+oe-NDRG2 group (10 mice/group). XIST shRNA, miR-181b-5p agomir, NDRG2 shRNA, miR-181b-5p antagomir, oe-NDRG2, and corresponding NCs (GenePharma,

Shanghai, China) were packaged with lentivirus, and then injected rapidly into the caudal vein of the mice at 2×10^7 thousand units (TU) according to the group setting. On GD18, mice in each group were fasted for 12 h without water in advance, and blood samples were collected through the tail vein for detecting FBG, fasting insulin (FINS). The mice were then anesthetized, and blood was collected from the abdominal aorta and centrifuged to obtain serum for the subsequent cytokine assays. Then, mice were anesthetized and fixed in the supine position, and then the pancreatic tissues were obtained by opening the abdomen for pathological histological examination (Tang et al. 2019).

Detection of glucose metabolism index levels

FBG levels in mice were measured with a blood glucose meter, and the FINS levels in serum were examined with a mouse insulin ELISA kit (Beyotime, Shanghai, China). According to the levels of FBG, FINS, the insulin HOMA was conducted to examine HOMA-IR. HOMA-IR = FBG (mmol/l) × FINS (mU/l)/22.5 (Tang et al. 2021). The high level of HOMA-IR indicated the lower insulin sensitivity and higher insulin resistance in peripheral tissues (Tang et al. 2019).

Determination of blood lipid contents

Serum concentrations of triglyceride (TG) and total cholesterol (TC) were measured with ELISA kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The standard samples, samples, and the horseradish peroxidase (HRP) labeled antibodies (TG and TC antibodies) were added to the microplate to generate the complex. Then, the tetramethylbenzidine substrate was added to develop the color. The optical density value was assessed at 450 nm by a microplate reader. The levels of TG and TC in the sample were calculated through the standard curve (Huang et al. 2018).

Serum levels of oxidative stress factors

The serum contents of oxidative stress factors were examined by the glutathione (GSH) kit, malondialdehyde (MDA) kit, and superoxide dismutase (SOD) kit as *per* manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute) (Lu et al. 2019).

Detection of serum inflammatory factors

The concentrations in serum of interleukin (IL)-1β, IL-6, and tumor necrosis factor α (TNF-α) in mice were measured with ELISA kits (Beyotime Institute of Biotechnology, Nanjing, China) (Wu et al. 2020).

Hematoxylin-eosin (HE) staining

Pancreatic tissue was fixed with 4% paraformaldehyde, decalcified, embedded with paraffin, cut into 5 μm sections and stained with HE. The morphological structure of pancreatic tissue was observed under a light microscope and different visual fields were photographed (Wang et al. 2019).

TUNEL staining

The pancreatic islets' cells apoptosis were analyzed using a TUNEL assay kit (Roche, Mannheim, Germany) conforming to the manufacturer's instructions. The cell nucleus in brown was defined as apoptotic cells. Five visual fields were selected and the average apoptotic cells *per* 200 cells were counted. Apoptosis index = (number of positive cells/number of total cells) × 100% (Sun et al. 2019).

RT-qPCR

Total RNA was isolated using Trizol LS reagent (Invitrogen, CA, USA) with reference to instructions. An ultraviolet spectrophotometer (Thermo Fisher Scientific, MA, USA) was adopted to test the purity. RNA was reversely transcribed into cDNA by different reagents (for XIST and NDRG2 mRNA: SuperScriptTM II reverse transcriptase (Invitrogen, Carlsbad, CA); for miR-181b-5p: a miScript reverse transcription kit (Qiagen, Hilden, Germany)). The PCR analysis was conducted by the use of SYBR@ Premix Ex TaqTM (TaKaRa, Shiga, Japan). The levels XIST, miR-181b-5p, and NDRG2 was evaluated using the $2^{-\Delta\Delta Ct}$ method, U6 and β-actin were set as endogenous references. The related sequences were listed in Table 1 (Wu et al. 2019).

Table 1. Primer sequences for genes used in our study

Gene	Primer sequences (5'– 3')
XIST	F: GTCAGCAAGAGCCTTGAATTG
	R: TTTGCTGAGTCTTGAGGAGAATC
miR-181b-5p	F: AACATTCATTGCTGTCGGTGGGTT
	R: Common reverse primer provided in the kit
NDRG2	F: TCACTCTGTGGAGACACCTTATG
	R: GTGGCTGGAAGCAAGACTTATAG
U6	F: CGCTTCGGCAGCACATATACTA
	R: CGCTTCACGAATTTGCGTGTCA
β-actin	F: GCACCACACCTTCTACAATGAGC
	R: TCGTTGCCAATAGTGATGACC

F, forward; R, reverse; XIST, Long noncoding RNA X inactivation-specific transcript; miR-181b-5p, microRNA-181b-5p; NDRG2, N-myc downstream-regulated gene 2.

Western blot analysis

Total protein was harvested using radio-immunoprecipitation assay (RIPA) cell lysis buffer (Sigma-Aldrich). Protein concentration was determined by the Bradford method. Thereafter, the proteins were isolated on 10% gel through sodium dodecyl sulphate polyacrylamide gel electrophoresis, and transferred to polyvinylidene fluoride membrane and sealed with 5% skim milk powder at 4°C for 1 h. The membrane was subjected to incubation with the primary antibodies NDRG2 (1:1000; Abcam, MA, USA) and β-actin (1:1000; Abcam) for 2 h. Thereafter, the goat anti-rabbit secondary antibody (1:10000; Abcam) that coupled with HRP for 1 h was used for incubation with membrane. The enhanced chemiluminescence reagent (Thermo Fisher Scientific) was added to react with protein bands for 1 min. The results were assessed through the ImageJ software (version 1.46r; National Institutes of Health, Maryland, USA) (Tang et al. 2019).

Dual luciferase reporter gene assay

The wild-type (Wt) or mutant (Mut) sequences of XIST or NDRG2 3'UTR containing the putative miR-181b-5p binding sites were cloned into the pmirGLO vector (Promega, Madison, WI, USA), respectively. 293T cells (American type culture collection, Maryland, USA) were seeded into a 24-well plate. At 70% cell confluence, Wt or Mut reporter genes of XIST and NDRG2 were co-transfected with miR-181b-5p agomir or agomir NC through the Lipofectamine 3000 reagent (Invitrogen). After 48-h transfection, the dual luciferase reporter assay system (Promega) was adopted to test the luciferase activity (Wang et al. 2018).

RNA immunoprecipitation (RIP) assay

The RIP assay was conducted using the EZMagna RIP RNA binding protein immunoprecipitate kit (Millipore, MA, USA). Cells were lysed with the RIP lysis buffer. Thereafter, 100 μl lysis buffer was incubated with the RIP immunoprecipitate buffer with magnetic beads, which were treated with conjugation human anti-argonaute 2 (Ago2) (Millipore) or NC normal immunoglobulin G (IgG; Millipore). The protein was digested by adding the Protease K buffer, and the purified RNA was extracted by RT-qPCR (Wang et al. 2020).

Statistical methods

Statistical analysis was performed using SPSS 21.0 (IBM Corp., Armonk, NY, USA). The measurement data was expressed as mean ± standard deviation. The Student's *t*-test was used for the comparison between two groups, and one-way analysis of variance (ANOVA) with Tukey's post hoc test was used for multiple-groups comparison. $p < 0.05$ was regarded to be indicative of statistical significance.

Results

GDM mouse model is successfully established

To investigate the insulin resistance in GDM, we firstly constructed the GDM mouse model. As reflected by glucose metabolism index detection, the levels of FBG, and HOMA-IR were high in GDM mice, and the decreased levels of FINS were detected in mice with GDM (Fig. 1A–C). The detection of serum lipid implied that TC and TG were enriched in GDM mice (Fig. 1D). Next, we detected serum oxidative stress factor and serum inflammatory factor concentrations in mice. The results showed a reduction of serum GSH level and SOD activity in GDM mice (Fig. 1E,F); and an elevation in MDA content (Fig. 1G); serum IL-1β, IL-6 and TNF-α also displayed high levels in GDM mice (Fig. 1H).

As indicated in HE staining, the exocrine part was composed of serous acinar that were stained deeply, whereas the endocrine part was consisted of scattered islets and light-stained. In control mice, the islets were regular in morphology, with round or oval shape; the boundary was obvious without membrane, and numerous islets and cells in islets were observed. In GDM mice, the islet morphology was irregular; the degree of atrophy was aggravated; islet cells were lessened, and inflammatory lesions were exacerbated (Fig. 1I). The TUNEL staining uncovered that the number of apoptotic cells in pancreatic islets was increased and apoptotic index in pancreatic islets was high in GDM mice (Fig. 1J).

These above experimental results demonstrated that GDM mouse model was established successfully.

XIST silencing mitigates insulin resistance in GDM mice

Previous studies have reported that XIST is robustly expressed in type 2 diabetes (Jiao et al. 2019). We first examined XIST expression in control and GDM mice by RT-qPCR, which indicated that XIST exhibits a high level in GDM mice (Fig. 2A).

To further explore the effects of XIST on insulin resistance of GDM mice, we injected lentiviral vectors containing sh-NC or sh-XIST into GDM mice, the outcomes of RT-qPCR implied that XIST expression was depleted in GDM mice after treated with sh-XIST (Fig. 2B).

As suggested by the glucose metabolism index detection, affected by the down-regulated XIST, the serum levels of FBG and HOMA-IR were decreased, and FINS levels were

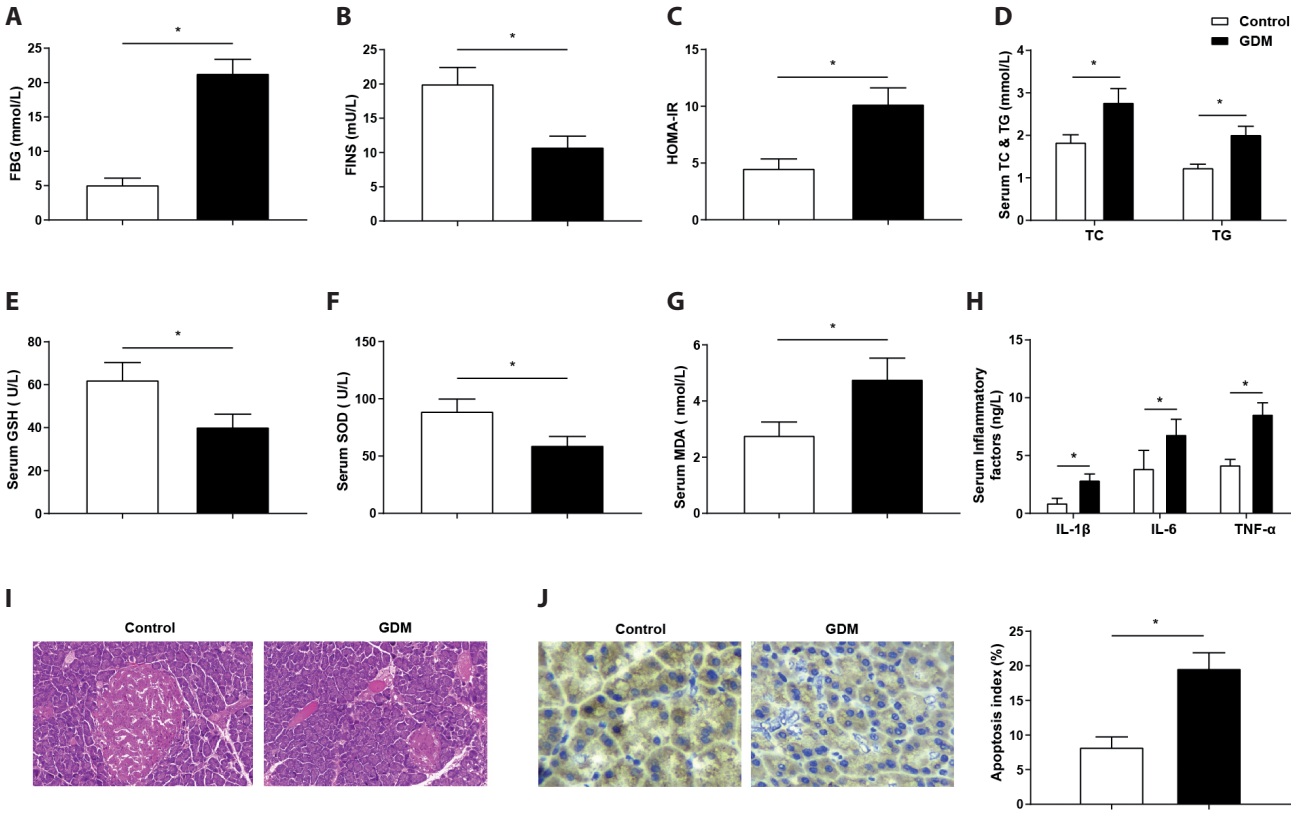

Figure 1. Serum levels of FBG (**A**), FINS (**B**), HOMA-IR (**C**), TC and TG (**D**), serum levels of oxidative stress factors GSH (**E**), SOD (**F**) and MDA (**G**) and inflammatory factors IL-1β, IL-6, and TNF-α (**H**) were measured in Control and GDM mice. **I.** Histological changes of pancreatic tissue in control and GDM mice were monitored by HE staining. **J.** Apoptosis cells in pancreatic islets in Control and GDM mice were detected by TUNEL staining. * $p < 0.05$. Comparisons between two groups were done by Student's *t*-test.

elevated (Fig. 2C–E); serum lipid levels of TC and TG were reduced (Fig. 2F); GSH content was enhanced and SOD activity were accelerated (Fig. 2G,H); MDA content was significantly depleted (Fig. 2I); and the serum contents of IL-1β,IL-6, and TNF-α were also suppressed in GDM mice (Fig. 2J).

HE staining disclosed that, after the down-regulation of XIST, the islet structure was restored, the number of islet cells was increased, the inflammatory lesions were alleviated, and the islet regeneration was promoted (Fig. 2K); TUNEL staining showed a significant reduction in apoptotic index in pancreatic islets in GDM mice (Fig. 2L).

To sum up, XIST silencing mitigated insulin resistance in GDM mice.

XIST binds to miR-181b-5p

To unravel the relation between XIST and miR-181b-5p, the presence of binding sites between XIST and miR-181b-5p was predicted *via* the Starbase website (http://starbase.sysu.edu.cn/), which validated that binding sites were existed between XIST and miR-181b-5p (Fig. 3A).

It was demonstrated that the luciferase activity was impaired after co-transfection with miR-181b-5p agomir and XIST-Wt in dual luciferase reporter gene assay (Fig. 3B). The result of RIP assay uncovered that the enrichment levels of XIST and miR-181b-5p were increased after treated with Ago2 (Fig. 3C), reflecting that XIST bound to miR-181b-5p.

It has been elucidated that miR-181b-5p is down-regulated in the plasma of diabetic cardiomyopathy mice (Copier et al. 2017). In our research, the outcomes of RT-qPCR unearthed that miR-181b-5p was also lowly expressed in GDM mice (Fig. 3D).

To examine the regulatory relation between XIST and miR-181b-5p, RT-qPCR was conducted, which showed that miR-181b-5p level was augmented after down-regulation of XIST (Fig. 3E).

It was concluded that XIST bound to miR-181b-5p and regulated miR-181b-5p expression.

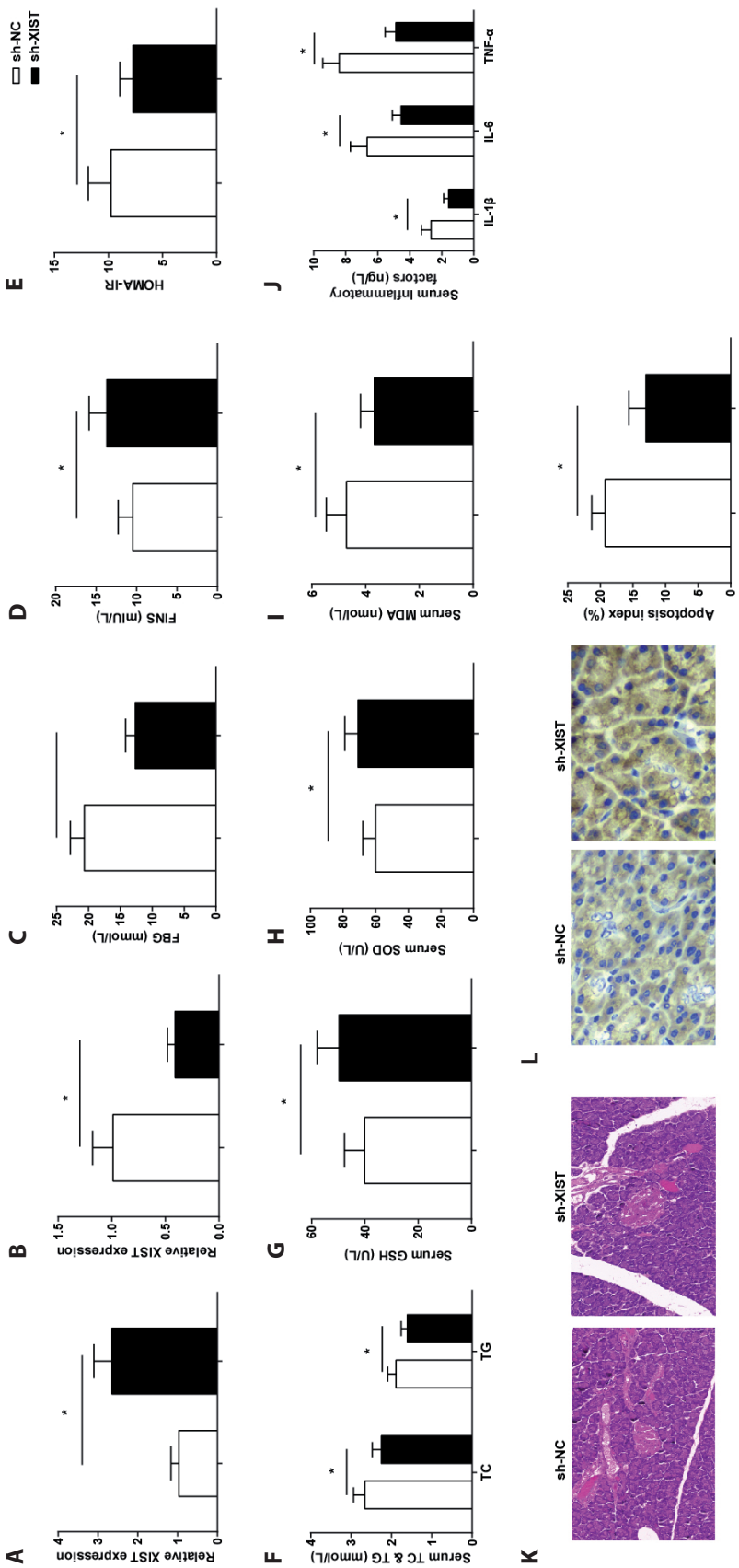

Figure 2. XIST silencing mitigates insulin resistance in GDM mice. **A.** XIST expression in Control and GDM mice. **B.** XIST expression after the down-regulation of XIST. Serum levels of FBG (**C**), FINS (**D**), and HOMA-IR (**E**), TC and TG (**F**), serum levels of oxidative stress factors GSH (**G**), SOD (**H**) and MDA (**I**) and inflammatory factors IL-1β, IL-6, and TNF-α (**J**) after the down-regulation of XIST. **K.** Histological changes of pancreatic tissue after the down-regulation of XIST in GDM mice was monitored by HE staining. **L.** Apoptosis cells in pancreatic islets in mice after the down-regulation of XIST were detected by TUNEL staining. * $p < 0.05$. Comparisons between two groups were done by Student's *t*-test.

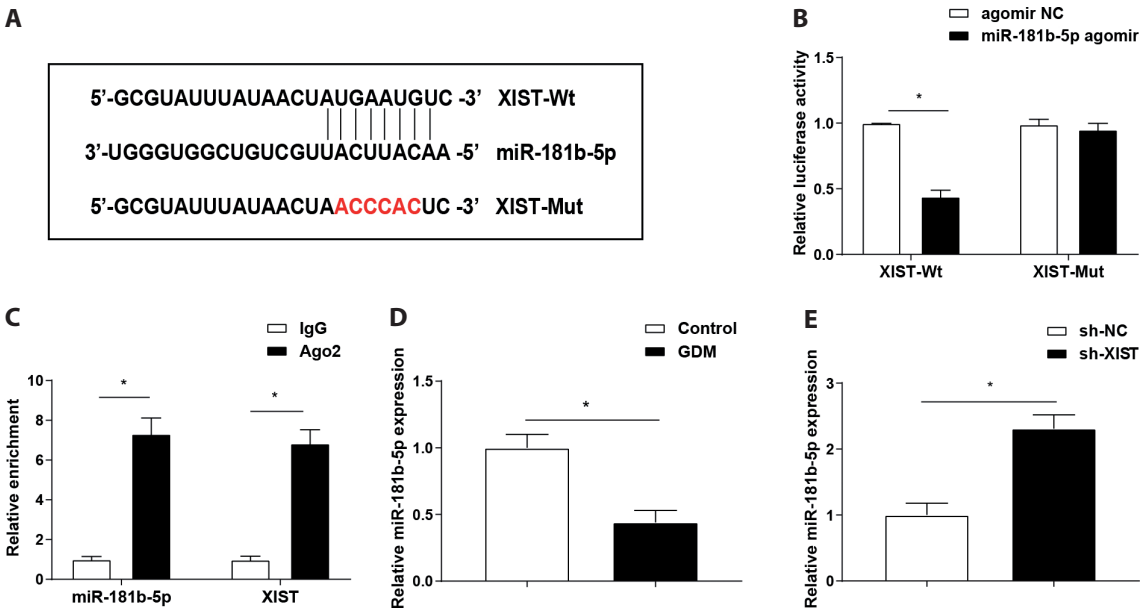

Figure 3. XIST binds to miR-181b-5p. **A.** The binding sites between XIST and miR-181b-5p were predicated through the Starbase website. **B**. The binding relation between XIST and miR-181b-5p was validated through the dual luciferase reporter gene assay. **C.** The enrichment level of XIST and miR-181b-5p were detected by RIP assay. miR-181b-5p expression before (**D**) and after (**E**) the silencing of XIST was tested by RT-qPCR. * $p < 0.05$. Comparisons between two groups were done by Student's *t*-test.

Overexpression of miR-181b-5p relieves insulin resistance in GDM mice

To probe the effects of miR-181b-5p on GDM mice, we injected the high-expressed miR-181b-5p lentiviral vector or its NC into GDM mice, the result of RT-qPCR indicated that miR-181b-5p was amplified after the up-regulation of miR-181b-5p (Fig. 4A).

In response to the up-regulated miR-181b-5p, levels of FBG, HOMA-IR, TC, and TG were reduced (Fig. 4B,D,E), FINS levels were elevated, and the concentrations of GSH and SOD were increased (Fig. 4C,F,G), while MDA content was decreased (Fig. 4H); the levels of IL-1β, IL-6, and TNF-α were also significantly depleted (Fig. 4I). Moreover, it was observed that the number of islet cells was amplified while the inflammatory lesions were relieved; the apoptotic index in pancreatic islets was also decelerated in GDM mice (Fig. 4J,K).

To conclude, overexpression of miR-181b-5p relieved insulin resistance in GDM mice.

miR-181b-5p targets NDRG2

We first predicted through the bioinformatic website Starbase that the miR-181b-5p sequence contained binding sites with NDRG2 (Fig. 5A), suggesting that NDRG2 might directly be targeted by miR-181b-5p. To confirm the prediction, the dual luciferase reporter gene assay was performed, which implied that the luciferase activity was dampened after being co-transfected with miR-181b-5p agomir and NDRG2-Wt (Fig. 5B). The results of the RIP assay showed that Ago2 could immunoprecipitate miR-181b-5p and NDRG2 at high levels, such finding was in consistent with the outcomes of bioinformatics analysis and the dual luciferase reporter gene assay (Fig. 5C).

NDRG2 expression in GDM mice was tested through RT-qPCR and Western blot analysis, suggesting a salient elevation in NDRG2 expression in GDM mice (Fig. 5D). In addition, after treatment with miR-181b-5p agomir, NDRG2 expression was diminished in GDM mice (Fig. 5E). These discoveries evidenced that miR-181b-5p targeted NDRG2.

Silenced NDRG2 attenuates insulin resistance in GDM mice

To unravel the impacts of NDRG2 on insulin resistance in GDM mice, we injected sh-NC and sh-NDRG2 lentiviral vectors into GDM mice, and the outcomes of RT-qPCR and Western blot analysis unveiled that NDRG2 expression was depleted after injected with sh-NDRG2 lentiviral vectors (Fig. 6A,B).

In response to the down-regulated NDRG2, it was found that FBG, HOMA-IR, TC, and TG levels were reduced (Fig. 6C,E,F), FINS, GSH, and SOD levels were accelerated (Fig.

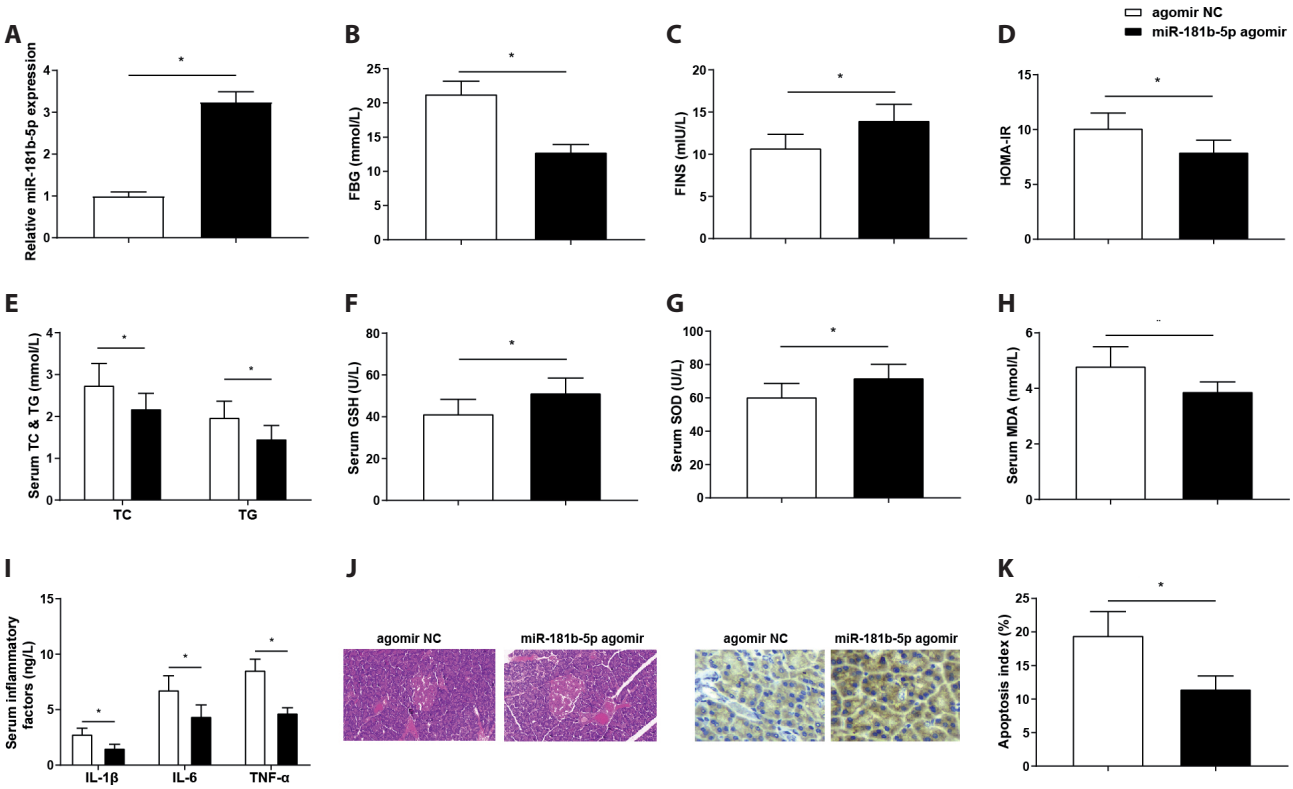

Figure 4. Overexpression of miR-181b-5p relieves insulin resistance in GDM mice. **A.** miR-181b-5p expression after the up-regulation of miR-181b-5p. Serum levels of FBG (**B**), FINS (**C**), and HOMA-IR (**D**), TC and TG (**E**), serum levels of oxidative stress factors GSH (**F**), SOD (**G**) and MDA (**H**) and inflammatory factors IL-1β, IL-6, and TNF-α (**I**) after the up-regulation of miR-181b-5p were assessed. **J.** Histological changes of pancreatic tissue after the up-regulation of miR-181b-5p in GDM mice was monitored by HE staining. **K.** Apoptosis cells in pancreatic islets in mice after the up-regulation of miR-181b-5p were detected by TUNEL staining. * $p < 0.05$. Comparisons between two groups were done by Student's *t*-test.

6C,G,H); MDA, IL-1β, IL-6, and TNF-α levels were restrainеed (Fig. 6I,J). Additionally, NDRG2 down-regulation promoted the islet regeneration and relieved the inflammatory lesions; the decreased apoptotic index in pancreatic islets was also observed in GDM mice (Fig. 6K,L).

These discoveries above disclosed that the down-regulation of NDRG2 could alleviate insulin resistance in GDM mice.

Silenced XIST alleviates insulin resistance in GMD mice via modulating the miR-181b-5p/NDRG2 axis

Finally, to further explore the mechanisms of XIST in insulin resistance in GDM mice *via* the miR-181b-5p/NDRG2 axis, we injected sh-XIST+antagomir NC, sh-XIST+miR-181b-5p antagomir, miR-181b-5p agomir+oe-NC, and miR-181b-5p agomir+oe-NDRG2 into GDM mice *via* lentiviral packaging (Fig. 7A,B). As reflected by the experimental results, down-regulation of miR-181b-5p reversed the ameliorative effect of XIST silencing on insulin resistance in GDM mice; whereas up-regulation of NDRG2 abrogated the therapeutic effect of miR-181b-5p overexpression on insulin resistance in GDM mice (Fig. 7C–L).

Discussion

GDM is the most common medical pregnant complication that related to maternal and neonatal adverse outcomes (Alfadhli 2015). This study focused on the regulatory mechanism of XIST in GDM. It was manifested that down-regulated XIST could relieve the insulin resistance induced by GDM *via* regulating miR-181b-5p to target NDRG2.

Initially, it was verified that XIST exhibited a high level in GDM mice, and XIST deletion could hinder the insulin resistance in GDM mice *via* decreasing the levels of glucose metabolism indices and that of inflammatory factors, relieving the oxidative response, histological change, and apoptotic cells in pancreatic islets. Many studies have deciphered the role of XIST in diabetes or diabetes-related diseases. For

instance, the robustly expressed XIST is found in diabetic cardiomyopathy (Chen et al. 2019) and coronary artery disease patients with type 2 diabetes (Sohrabifar et al. 2022). In consistent with such finding, Sathishkumar et al. have also reported that patients with type 2 diabetes display a high level of XIST, whose altered expression is related to insulin resistance and accelerated inflammation (Sathishkumar et al. 2018). Functionally, in diabetic nephropathy, the silenced XIST makes contribution to alleviate the inflammatory response of human mesangial cells (Wang 2022). Furthermore, Yang et al. (2019) have illustrated that XIST is also amplified in kidney tissue of diabetic nephropathy mice; the fibrosis in diabetic nephropathy is attenuated after XIST decrement. Especially, another article has revealed that XIST expression is elevated in the GDM patients and high glucose-induced trophoblastic cell models, and silenced XIST suppressed the function of high glucose on trophoblastic cell viability (Sathishkumar et al. 2018). Unlike this article, our article highlights the role of XIST in animal models of GDM, which is a highlight of our paper.

Thereafter, it was predicated thorough the bioinformatics website that there were binding sites between XIST and miR-181b-5p. Then, we manifested the deficient expression of miR-181b-5p in GDM mice, and the up-regulated miR-181b-5p could attenuate the insulin resistance in GDM mice. In line with our finding, the suppressed miR-181b-5p level has also been confirmed in myocardium during diabetic cardiomyopathy progression, serving as a potential biomarker for diabetic cardiomyopathy (Copier et al. 2017). Similarly, miR-181b-5p has also been validated to display the low expression in livers of diabetic mice and patients; and the miR-181b-5p overexpression accelerates hepatic glycogenesis *in vivo*, leading to suppressed insulin resistance (Wang et al. 2019). In children with obesity born small for gestational age, silenced miR-181b-5p has been verified by Marzano et al., who have disclosed that miR-181b-5p expression is implicated in insulin signaling, glucose transport, insulin resistance, cholesterol and lipid metabolism (Marzano et al. 2018). In addition, the elevated miR-181b-5p functions as a therapeutic intervention in the management of type 2

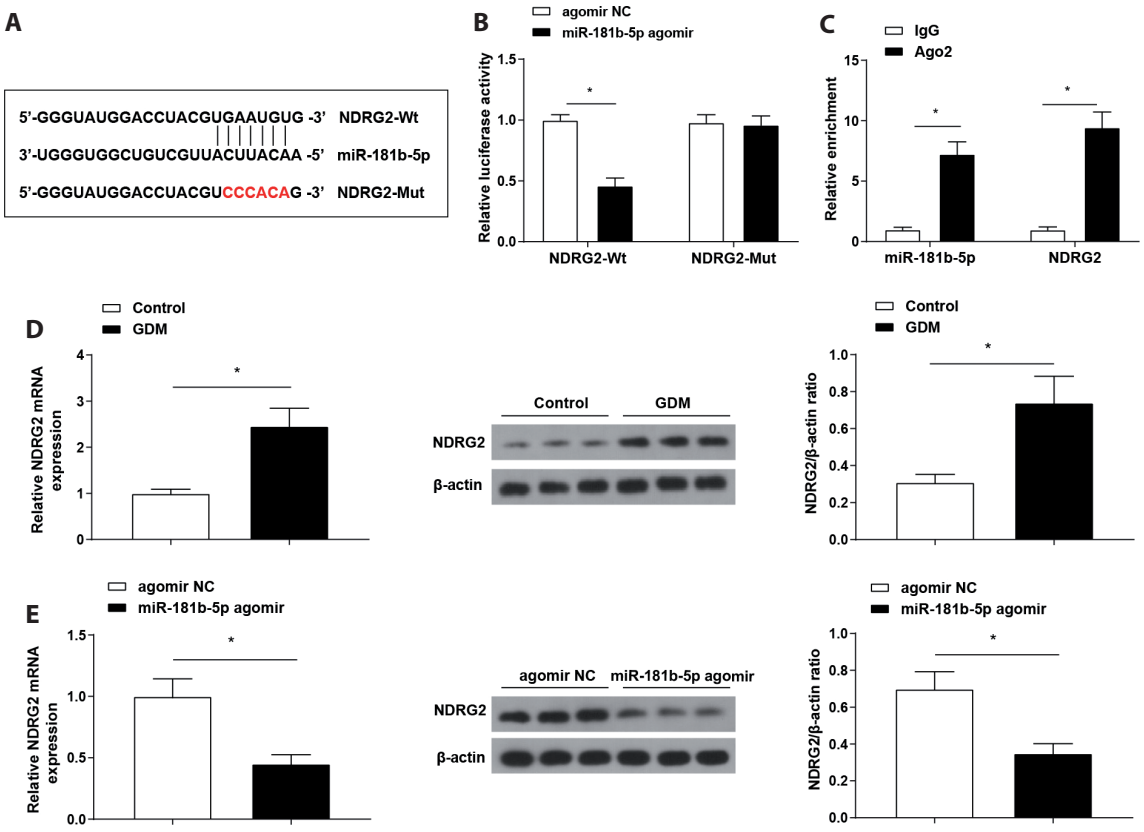

Figure 5. miR-181b-5p targets NDRG2. **A.** The binding site between miR-181b-5p and NDRG2 were predicated by Starbase. **B.** The targeting relationship between miR-181b-5p and NDRG2 was validated by dual luciferase reporter gene assay. **C.** The enrichment levels of miR-181b-5p and NDRG2 were tested by RIP assay. NDRG2 expression before (**D**) and (**E**) after the up-regulation of miR-181b-5p was detected by RT-qPCR and Western blot assay. * $p < 0.05$. Comparisons between two groups were done by Student's *t*-test.

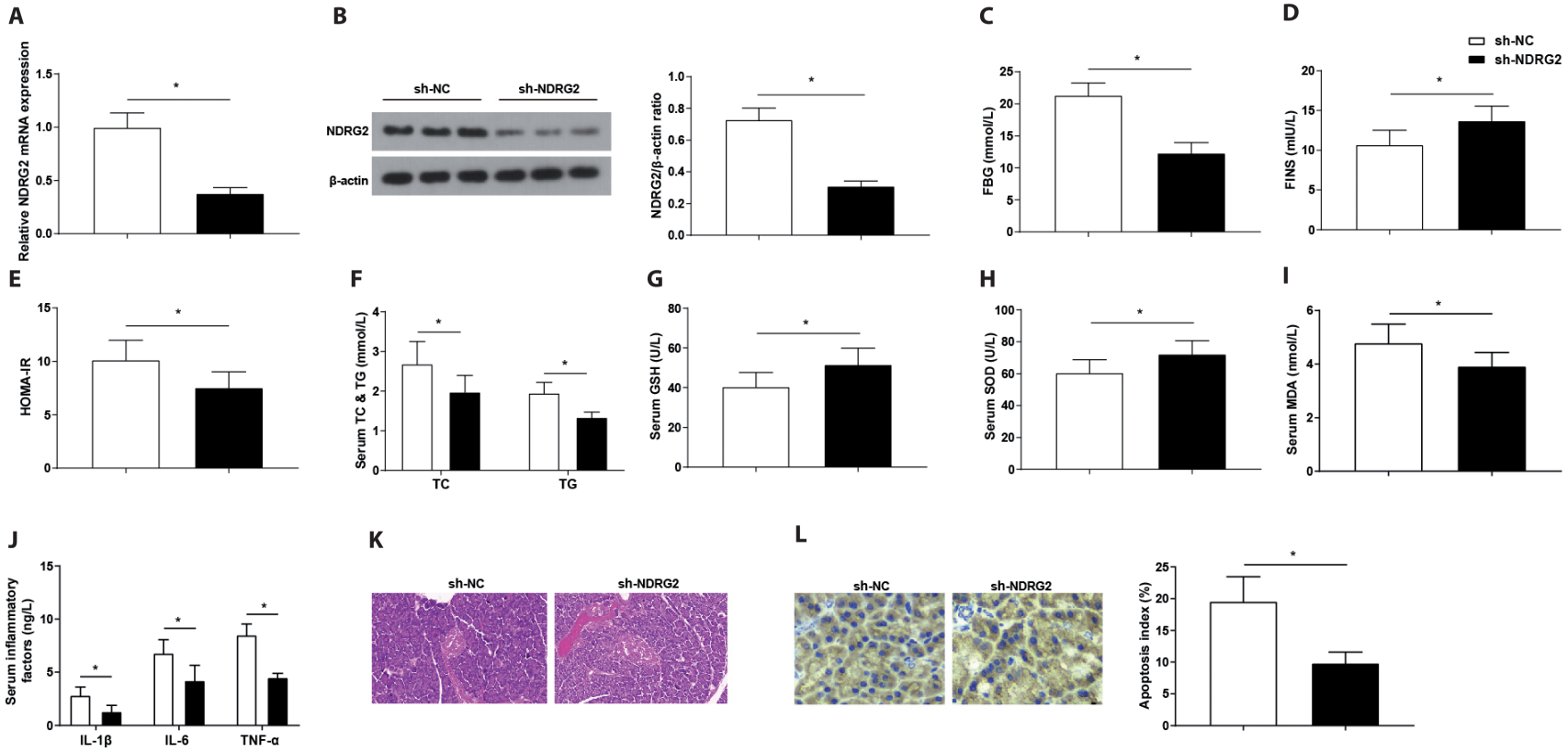

Figure 6. Silenced NDRG2 alleviates insulin resistance in GDM mice. NDRG2 expression after the down-regulation of NDRG2 was detected by RT-qPCR (**A**) and Western blot analysis (**B**). Serum levels of FBG (**C**), FINS (**D**), and HOMA-IR (**E**), TC and TG (**F**), serum levels of oxidative stress factors GSH (**G**), SOD (**H**) and MDA (**I**) and inflammatory factors IL-1β, IL-6, and TNF-α (**J**) after the down-regulation of NDRG2 were assessed. **K.** Histological changes of pancreatic tissue after the down-regulation of NDRG2 were monitored by HE staining. **L.** Apoptosis cells in pancreatic islets of GDM mice after the down-regulation of NDRG2 were detected by TUNEL staining. * $p < 0.05$. Comparisons between two groups were done by Student's t-test.

diabetes *via* hindering the biological functions in human umbilical vein endothelial cells (Sun et al. 2020).

Thereafter, it was predicated that NDRG2 was targeted by miR-181b-5p. NDRG2 expression was upregulated in the pancreatic tissues of GDM mice. Silencing miR-181b-5p could effectively inhibit the insulin resistance in GDM mice. Regarding NDRG2 expression, Wang et al. have reported that NDRG2 level is elevated in rats with diabetic retinopathy; moreover, NDRG2 decrement can block oxidative stress, mitigate inflammatory cell infiltration, thereby exerting pro-

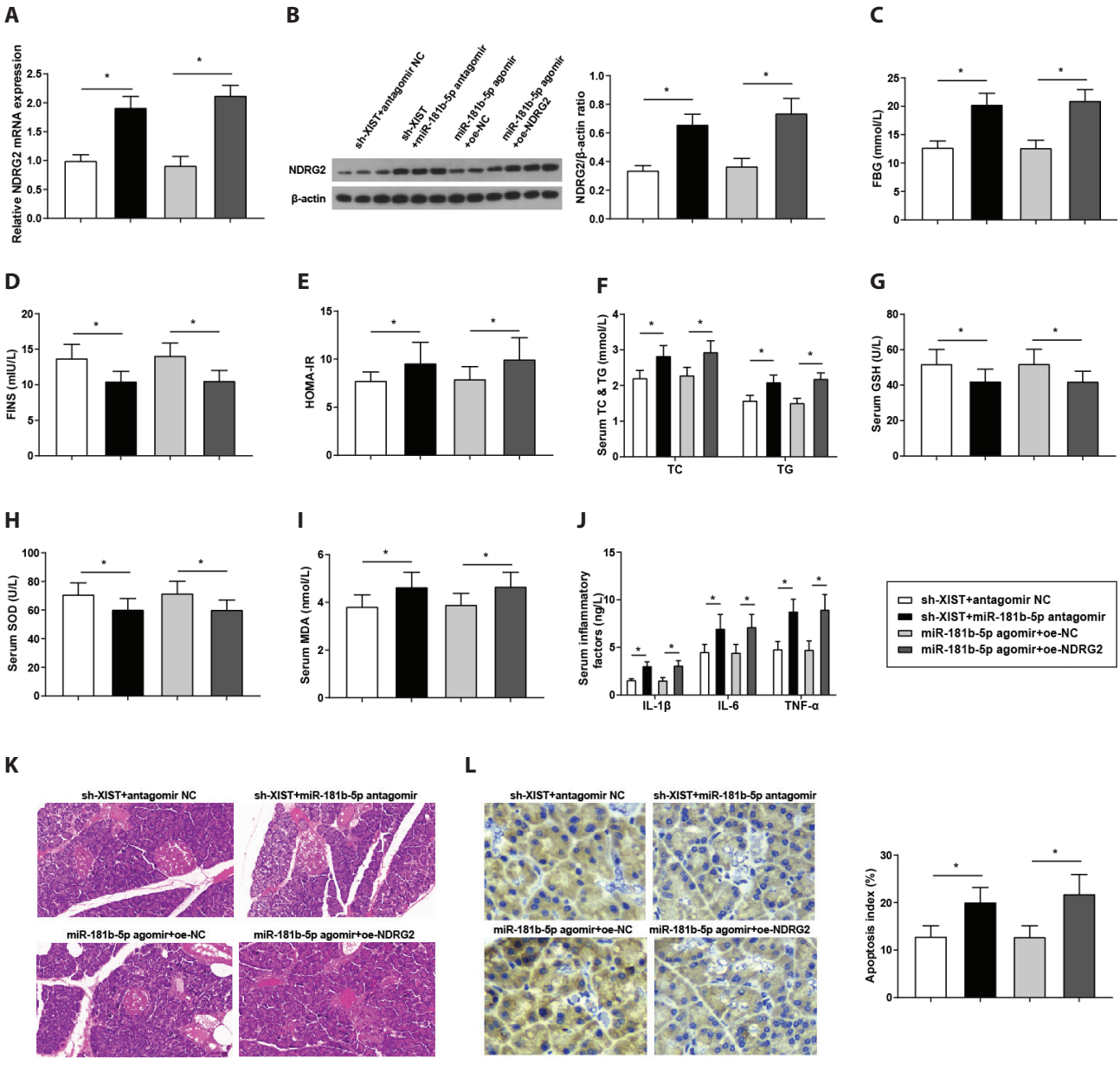

Figure 7. Silenced XIST attenuates insulin resistance in GMD mice *via* modulating the miR-181b-5p/NDRG2 axis. NDRG2 expression after treated with sh-XIST+miR-181b-5p antagomir or miR-181b-5p agomir+oe-NDRG2 was detected by RT-qPCR (**A**) and Western blot analysis (**B**). Serum levels of FBG (**C**), FINS (**D**), and HOMA-IR (**E**), TC and TG (**F**), serum levels of oxidative stress factors GSH (**G**), SOD (**H**) and MDA (**I**) and inflammatory factors IL-1β, IL-6, and TNF-α (**J**) after treated with sh-XIST+miR-181b-5p antagomir or miR-181b-5p agomir+oe-NDRG2 was examined. **K.** Histological changes of pancreatic tissue after treated with sh-XIST+miR-181b-5p antagomir or miR-181b-5p agomir+oe-NDRG2 were monitored by HE staining. **L.** Apoptosis cells in pancreatic islets after treated with sh-XIST+miR-181b-5p antagomir or miR-181b-5p agomir+oe-NDRG2 were detected by TUNEL staining. * $p < 0.05$. Comparisons among different groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test.

tective effects against retinal injury in diabetes rats (Wang et al. 2020). Such finding has also been evidenced by Chen et al. (2015), who have also confirmed the reduced expression of NDRG2 in diabetic retinopathy, and they further manifested that NDRG2 silencing restrains the apoptosis of ganglion cells as well as the oxidative stress damage and oxidation reaction (Pu et al. 2020). Furthermore, the diabetic rats with mechanical allodynia also display increased NDRG2 levels, contributing to glucocorticoid-regulated astrocyte reactivity (Zuo et al. 2015).

In conclusion, this study manifests that silencing XIST relieves the insulin resistance in GDM mice *via* the modulation of the miR-181b-5p/NDRG2 axis. This current study advances the understanding of the therapeutic strategies of GDM, thereby affording novel insights for the clinical treatment of GDM. However, more study samples and grouping should be supplemented to diminish the potential data errors in the experimental results. Additionally, we will measure plasma C-peptide levels to clearly see the effect of streptozotocin in future research.

Conflict of interest. The authors declare no conflicts of interest directly related to the contents of this article.

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