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MiRNA-145 is involved in the development of resistin-induced insulin resistance in HepG2 cells

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ABSTRACT

Background: Resistin is associated with insulin resistance, and determining its developmental and molecular mechanisms may help the development of novel treatments. MicroRNAs (miRNAs) are involved in many physiological and pathological processes as negative regulators. However, it remains unclear whether miRNAs play a role in resistin-induced insulin resistance. We performed mouse liver miRNA microarrays to analyze the differences in expression between resistin-treated and control mice. Resistin upregulated miR-145 both *in vivo* and *in vitro*. Therefore, we aimed to study whether miR-145 played a role in resistin-induced insulin resistance.

Methods and results: We transfected HepG2 cells, and used miR-145 mimics and inhibitors to assess the role of miR-145 in resistin-induced insulin resistance. The overexpression of miR-145 inhibited glucose uptake in HepG2 cells, diminished the phosphorylation of Akt and IRS-1, and induced insulin resistance in hepatocytes. Next, a study of transcriptional regulation revealed that p65 was essential for the upregulation of miR-145 by resistin, and chromatin immunoprecipitation (ChIP) confirmed that p65 could bind to the promoter region of miR-145.

Conclusion: miR-145 plays a role in the development of resistin-induced insulin resistance via the p65 pathway.

1. Introduction

Insulin resistance refers to the reduced responsiveness of target tissues to ordinary levels of insulin, which is regarded as the key factor of Type 2 diabetes mellitus (T2DM) [1,2]. T2DM is increasingly common worldwide [3], and only a complete understanding of the pathophysiological reasons for the occurrence of diabetes can provide novel methods for successful prevention and treatment.

Resistin is an adipocyte-derived cytokine discovered in 2001. It was named "resistin" due to its role in the development of insulin resistance [4]. Resistin is upregulated during adipocyte differentiation and lipid deposition [5]. It is expressed at higher levels in obese humans and mice compared with lean controls [6,7], but levels decrease significantly upon weight loss [8]. Increased serum resistin levels are associated with insulin resistance [9,10], atherosclerosis [11,12], non-alcoholic liver disease [13], inflammatory conditions [14,15], and metabolic syndrome [16]. Although many studies have attempted to clarify the mechanism of resistin-induced insulin resistance, knowledge of the relationship between resistin and insulin remains unclear.

Micro-RNAs (miRNAs) are short, endogenously produced non-coding RNA molecules [17] that regulate the expression of target genes by pairing with sites in the 3′ untranslated region (3′-UTR) [18]. Increasingly evidence has suggested that miRNAs have an important function in many physiological and pathological processes such as cell growth and differentiation, proliferation [19,20], vascular angiogenesis [21–23], embryonic development [24,25], and apoptosis [26]. MiRNAs also play critical roles in cellular signaling pathways [27] and cross-species gene expression variations; they can also co-regulate with transcription factors [28]. Consequently, dysfunction of miRNAs and their target genes can lead to a variety of disorders. In this study, we found that resistin upregulated miR-145 in HepG2 cells. We then investigated whether miR-145 plays a role in the development of resistin-induced insulin resistance in hepatocytes.

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2. Materials and methods

2.1. Materials

TRIzol reagent was purchased from TaKaRa (Dalian, China), Lipofectamine™ 2000 was from Invitrogen (Carlsbad, CA, USA), and a mammalian cell protein extraction kit was from Beyotime (Jiangsu, China). The antibody against NF-κB p65 subunit was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); antibodies against Akt, pAkt, IRS-1, pIRS-1, IRS-2, and β-Actin were supplied by Cell Signaling Technology (Danvers, MA, USA). Recombinant human and mouse resistin were purchased from PeproTech, Inc. (Rochy Hill, NJ, USA). A glucose assay kit was purchased from Applygen Technologies Co., Ltd. (Beijing, China). PDTC (Pyrrolidinedithiocarbamic acid) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Small interfering RNAs (siRNAs) were synthesized by IBS Bio (Shanghai, China). MiRNA mimics and inhibitors were purchased from GenePharma (Shanghai, China). The miRcute miRNA isolation kit was obtained from Tiangen Biotech Co. Ltd. (Beijing, China). A dual Luciferase Assay kit was purchased from Promega (WI, USA). The chromatin immunoprecipitation (ChIP) assay kit was from Millipore (Massachusetts, USA).

2.2. Animal experiments

Male C57BL/6J mice (8 weeks old) were purchased from Huafukang Biotech (Beijing, China) and housed in individual plastic cages at room temperature with a 12:12 h light–dark cycle with free access to water and food. Mice were given standard chow and water, and were given a daily vena caudalis injection for 6 days with or without resistin (400 ng/day) [29]. Mice were then sacrificed on day 7. The Hubei Province Committee on Laboratory Animal Care approved all procedures.

2.3. Cell culture and treatment

HepG2 and HEK293A cells were maintained in DMEM medium (HyClone, Thermo Scientific, USA) supplemented with 10% fetal bovine serum (FBS) (Tianhang, Zhejiang, China) and cultured at 37 °C in a humidified chamber containing 5% CO_2. Cells were stimulated with human recombinant resistin at different concentrations (0, 10, 25, 50 and 100 ng/μL) for 24 h.

2.4. RNA isolation

Total RNA was extracted using Tiangen miRcute miRNA isolation kit following the manufacturer's instructions. RNA quality was assessed using UV–vis spectrophotometry (SMA4000). RNA was generally of high quality (average 260/280 ratio of 2.02, and an average 260/230 ratio of 1.79).

2.5. Real-time PCR (qRT-PCR)

Total RNA extracted from tissues or cells was reverse transcribed into cDNA using M-MLV reverse transcriptase (Promega) and gene-specific stem-loop primers or oligo(dT)18. The sequences of the primers used are listed in Table 1. Real-time PCR was performed using SYBR Green PCR Mix (ABI, CA, USA) with CFX96™ real timer PCR system (Bio-Rad). PCR cycles were as follows: initial denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 15 s and 60 °C for 30 s. The average threshold (Ct) was determined for each gene, and normalized to GAPDH mRNA or snRu6 miRNA as internal normalization controls. Fold changes in relative expression were calculated using the $2^{-\Delta\Delta Ct}$ method.

2.6. Transfection

Unless otherwise indicated, all experiments were performed in triplicate in three independent experiments. All cells were grown and maintained in DMEM supplemented with 10% FBS, and the media were replaced with DMEM without serum for 6 h before stimulation or transfection. The vectors and siRNAs were transfected into cells in serum-free medium; media were changed to DMEM with 10% FBS 6 h later. Cells were harvested 24 h after transfection to isolate RNA or protein.

2.7. Western blotting

Cells were harvested and lysed in RIPA lysis buffer (Beyotime, P0013C) with or without phosSTOP phosphatase inhibitor cocktail tablets (Roche Applied Science). Protein concentrations were determined using a BCA kit (Beyotime). Protein samples were separated by electrophoresis on 10% or 12% SDS–polyacrylamide gels, and then transferred to PVDF membranes (Bio-Rad). Membranes were blocked with 5% non-fat dry milk in TBS-Tween. Target proteins were detected using specific polyclonal antibodies; β-actin served as an internal control.

2.8. Luciferase assays

Cells were seeded into 24 well plates and allowed to grow overnight. Cells were then incubated in serum-free DMEM for 6 h before transfection. Recombinant vectors or empty vector (pGL3-Basic) were co-transfected into the cells using Lipofectamine™ 2000 (Invitrogen) following the manufacturer's protocol. pGL3-Basic vector, containing the Firefly luciferase reporter, was used for normalization. After 24 h, firefly and Renilla luciferase activities were measured consecutively using the Dual Luciferase Assay. Three independent experiments were performed in triplicate.

2.9. Measurement of glucose

The glucose concentration in medium was assayed using the Glucose Assay Kit (Applygen). Absorbance was measured at 500 nm using a Beckman Coulter DU 800 UV–visible spectrophotometer. All samples were normalized to total amount of protein.

2.10. Chromatin immunoprecipitation (ChIP)

ChIP assays were performed using Chromatin Immunoprecipitation Kits (Millipore) according to the manufacturer's instructions. Briefly, cultured cells were harvested and treated with 1% formaldehyde for 15–20 min to crosslink the chromatin; the reaction was then stopped by adding glycine to a final concentration of 0.125 M. The cells were disaggregated by homogenizing, passed through a 200 μm pore filter and centrifuged at 1000 rpm for 5 min at 4 °C. The cell pellets were resuspended in cell lysis buffer, and samples were aliquotted at 1 mL/tube. Aliquots were incubated with p65 antibody at 4 °C overnight with constant rotation. Subsequently, DNA was extracted from the immunoprecipitated DNA–protein complexes and purified. Purified DNA was used as the template for PCR with the following conditions: 95 °C for 5 min followed by 30 cycles of 20 s at 94 °C, 30 s at 60 °C, and 30 s at 72 °C, and a 5 min final extension at 72 °C. The primers used are shown in Table 1.

2.11. Statistical analysis

Data are presented as means ± standard deviation (SD). Statistical analyses were performed using unpaired two-tailed t-test (for two groups) and analysis of variance (ANOVA; for multiple groups). P-values <0.05 were considered statistically significant.

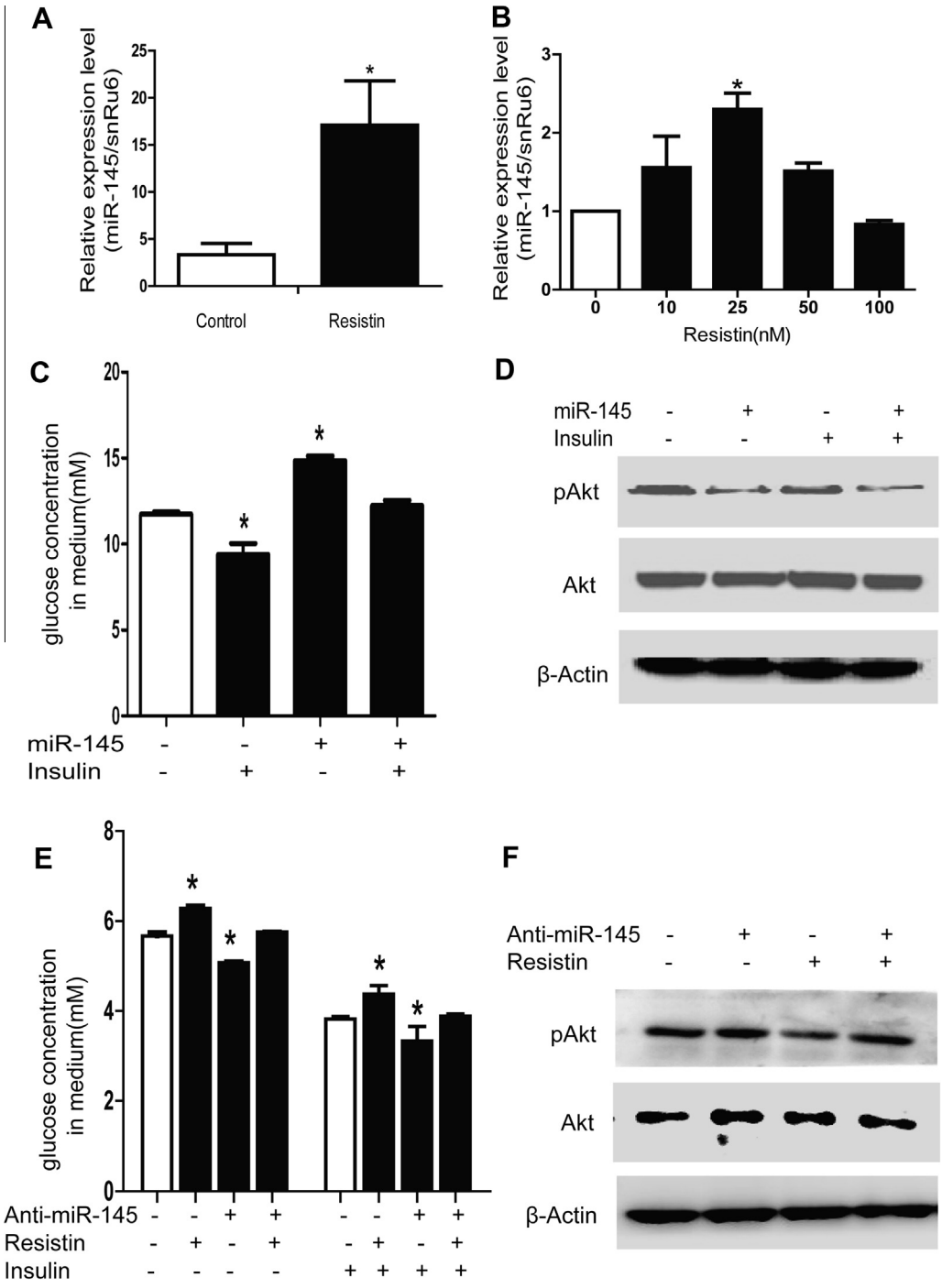

Fig. 1. MiR-145 was upregulated by resistin *in vivo* and *in vitro* and inhibited insulin signaling. (A) Male C57BL/6J mice were given a daily vena caudalis injection of PBS or resistin (400 ng/day) for 6 days. Mice were sacrificed on day 7, and miR-145 expression levels were then determined relative to snRu6 by real-time PCR (qRT-PCR). (B) HepG2 cells were treated with different doses of resistin (10, 25, 50, and 100 ng/mL) for 24 h. The expression of miR-145 relative to snRu6w as then determined using qRT-PCR. HepG2 cells were transfected with miR-145 mimics or inhibitors (anti-miR-145) (50 nM), or negative control for 24 h. The glucose concentrations in the media were determined using a glucose assay kit, and the expression of insulin signaling intermediates (Akt, IRS-1) and their phosphorylation (pAkt, pIRS-1) were analyzed by western blotting. (C) MiR-145 overexpression inhibited insulin-stimulated glucose uptake. (D) MiR-145 overexpression reduced insulin-stimulated Akt phosphorylation. (E) Anti-miR-145 rescued insulin-stimulated glucose uptake. (F) Anti-miR-145 inhibited the reduction in insulin-stimulated Akt and IRS-1 phosphorylation. Data are presented as mean ± SD. $^{*}p < 0.05$ and $^{**}p < 0.01$.

3. Results

3.1. MiR-145 was upregulated by resistin both in vivo and in vitro and overexpression of miR-145 impaired insulin signaling

Analysis of miR-145 expression in C57BL/6 mice and HepG2 cells showed that resistin markedly increased miR-145 levels (Fig. 1A and B). Male C57BL/6 mice were injected in the vena caudalis with or without resistin for 6 days. qRT-PCR was then used to determine the expression of miR-145 in the liver. The results demonstrated that miR-145 was significantly upregulated after treatment with resistin (Fig. 1A). Subsequently, HepG2 cells were treated with different concentrations of resistin for 24 h; miR-145 expression levels were then analyzed using qRT-PCR.

Consistent with the *in vivo* study, miR-145 expression was increased significantly after resistin treatment compared with control in a dose-dependent manner (Fig. 1B).

MiR-145 can target the 3′-UTR of insulin receptor substrate-1 (IRS-1) and inhibit the growth of human cancer cells [30]. IRS-1 is a major substrate of the insulin receptor. To determine whether miR-145 was involved in the development of insulin resistance, we assessed insulin-induced glucose uptake in HepG2 cells that overexpressed miR-145 (40 nM miR-145 mimic). MiR-145 decreased glucose uptake significantly compared with the control group after treatment with 100 nM insulin for 24 h (Fig. 1C). However, miR-145 inhibition using antisense-miR-145 partially but significantly restored glucose uptake (Fig. 1D). These findings clearly suggest that miR-145 inhibits insulin-stimulated glucose uptake in HepG2 cells.

We next analyzed the expression and insulin-stimulated phosphorylation of Akt in hepatocytes transfected with miR-145 mimics. The overexpression of miR-145 significantly reduced insulin-stimulated Akt phosphorylation, whereas total Akt was unaffected (Fig. 1E). Treating hepatocytes with anti-miR-145 partially but significantly restored the expression of phosphorylated Akt (Fig. 1F). These data suggest that elevated miR-145 impaired insulin signaling and caused insulin resistance in hepatocytes.

3.2. MiR-145 downregulated IRS-1, but not IRS-2, protein and mRNA

Although miRNAs usually inhibit the protein levels of target genes, they can also decrease mRNA levels. We next assessed IRS-1 mRNA and protein levels in HepG2 cells transfected with miR-145 mimics. The results of repeated experiments using western blotting or qRT-PCR are summarized. As expected, miR-145 decreased IRS-1 protein levels (Fig. 2A), as well as *irs1* mRNA (Fig. 2B). Consequently, IRS-1 phosphorylation was also decreased by miR-145 (Fig. 2A). The changes in expression of IRS-1 and pIRS-1 were reversed significantly in HepG2 cells treated with anti-miR-145 (Fig. 2C). IRS-2 also plays a role in insulin signaling, but its protein and mRNA levels were unaffected by miR-145 (Fig. 2A and D). Therefore, miR-145 decreased IRS-1 levels at the translational level, and attenuated insulin signaling by decreasing insulin-dependent phosphorylation of insulin receptor substrate-1; this caused a subsequent reduction in the downstream signals.

3.3. p65 directly binds to the miR-145 promoter

We next searched for factors that might be responsible for regulating miR-145 expression to clarify the mechanism by which resistin upregulates miR-145. We performed bioinformatics analysis using the Genomatix MatInspector, and found that p65, which was upregulated by resistin [29,31], is one of the transcription factors that might bind to the miR-145 promoter (Fig. 3A). Therefore, we generated three miR-145 promoter luciferase reporters: wild-type (pGL-miR-145p), and those carrying mutations in the two putative p65 binding sites (pGL-miR-145m1p and pGL-miR-145m2p) (Fig. 3B). ChIP assays confirmed that p65 interacted directly with the miR-145 promoter at both binding sites (Fig. 3D). However, experiments assessing the ectopic expression

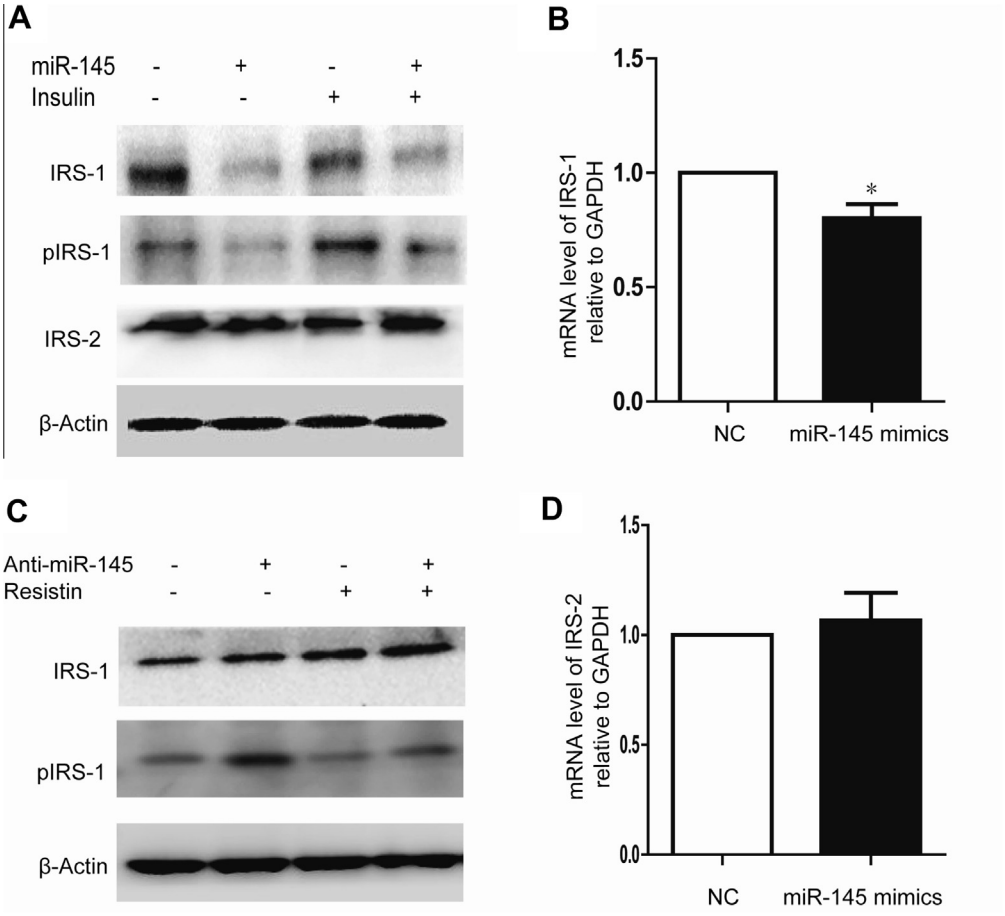

Fig. 2. MiR-145 downregulated IRS-1, but not IRS-2, protein and mRNA levels. MiR-145 mimics or inhibitors were transiently transfected into HepG2 cells as indicated. The protein levels of IRS-1, pIRS-1, and IRS-2 were determined by western blotting 24 h after transfection, and mRNA levels were determined using real-time PCR. β-Actin was used for normalization. MiR-145 overexpression decreased IRS-1 and pIRS-1 levels significantly at both the protein (A) and mRNA level (B). IRS-2 protein (A) and mRNA levels (C) were unaffected.

of p65 together with the three luciferase vectors revealed that p65 was able to enhance the promoter activity of both the miR-145 wild type and site 2 mutant, but not the site 1 mutant (Fig. 3C). This suggests that p65 regulates the transcription of miR-145, and that the binding of p65 to site 1 is non-specific.

3.4. Resistin induced miR-145 at least partially via p65

We found that resistin stimulated p65 (Fig. 4A) in HepG2 cells, which was consistent with previous results [29]. The overexpression of p65 increased miR-145 expression significantly (Fig. 4B). These results suggest that p65 is a positive regulator of miR-145. We hypothesized that resistin induces miR-145 via the upregulation of p65. To determine the role of p65 in the induction of miR-145 by resistin, we treated HepG2 cells with resistin (25 ng/mL) after inhibiting the expression or nuclear localization of p65 using siRNA (Fig. 4C) or PDTC (a kind of p65 inhibitor), respectively. Treatment with either of these inhibitors restored miR-145 expression (Fig. 4D and E). This suggests that resistin upregulates miR-145 via the p65 pathway.

4. Discussion

Steppan et al. [4] described resistin for the first time in 2001 as a novel peptide that was synthesized and secreted from murine adipocytes. Accumulated evidence in both animal models and humans has supported the notion that elevated serum resistin levels correlate with insulin resistance [9,10,32]. MiRNAs are involved in a variety of biological processes, and act as negative regulators of genes. However, it remains unclear whether miRNAs play a role in the development of resistin-induced insulin resistance. In this study, miR-145 was upregulated in C57BL/6 mice and HepG2 cells that were treated with resistin. Although resistin upregulated miR-145 in a dose-dependent manner, it remains unclear why resistin is not effective at miR-145 at higher concentrations. One possible explanation that the impact of resistin was higher concentration resistin affect the cell condition at the same time. Another possible explanation is the existence of a feedback loop between resistin and miR-145, which is yet to be verified.

Previous reports have identified several miRNAs that are involved in insulin resistance in hepatocytes [33–36]. However, the exact mechanisms underlying these processes remain unclear. MiR-145 has been linked to various human cancers including breast [37], prostate [38,39], and colon cancers [30,40]. MiR-145 also regulates the chondrogenic differentiation of mesenchymal stem cells by targeting *sox9* [41,42], plays a critical role in the differentiation of human embryonic stem cells into vascular cells [43], and regulates human corneal epithelial differentiation [44]. In the present study, we found that overexpressing miR-145 inhibited insulin-stimulated glucose uptake and significantly reduced Akt phosphorylation; total Akt was unaffected. Moreover, treating hepatocytes with a miR-145 inhibitor restored resistin-induced Akt phosphorylation and glucose uptake. Therefore, miR-145 induced insulin resistance by inhibiting glucose uptake and insulin signaling.

A positive correlation between resistin and inflammatory disorders has been described. Human resistin stimulates the pro-inflammatory cytokines TNF-α and IL-12 in macrophages via the

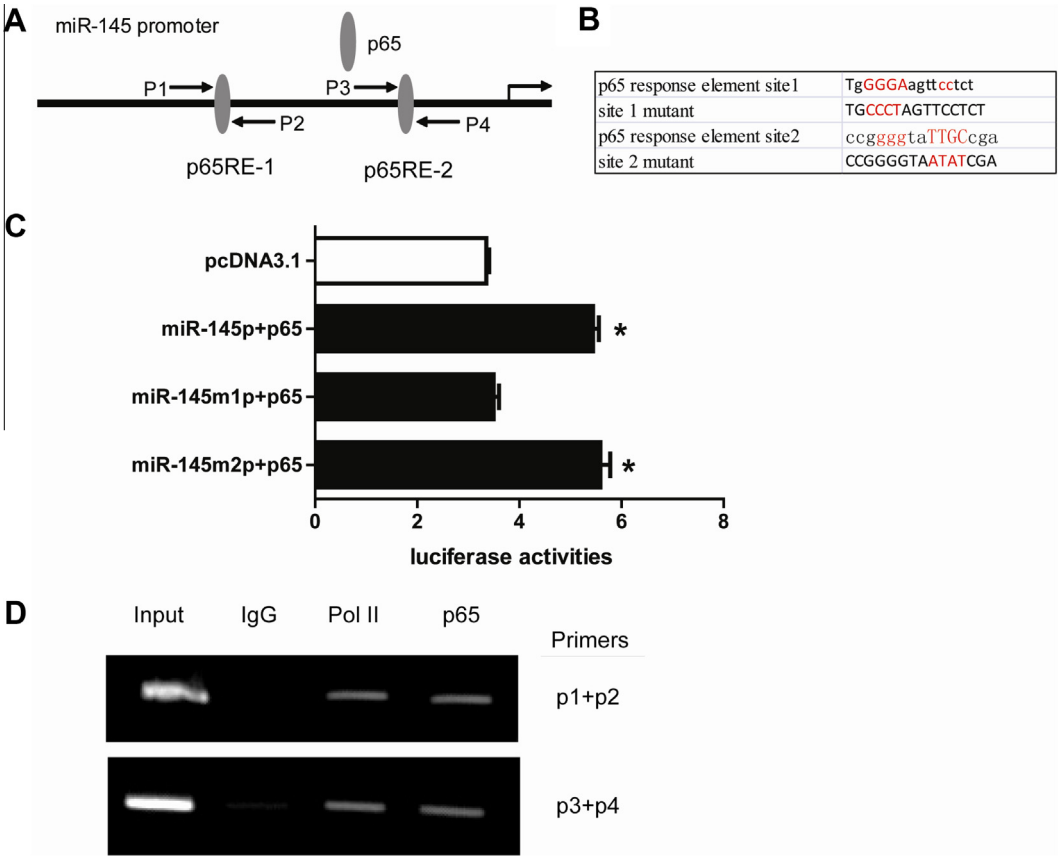

Fig. 3. p65 induced miR-145 promoter activity by binding to the p65RE-1. (A) Schematic diagram of the putative miR-145 promoter with two potential p65 response elements (p65RE-1 and p65RE-2). (B) The site mutagenesis strategies for p65RE-1 and p65RE-2. Small letters denote deviations from the consensus sequence. The conserved nucleotides are highlighted in red. (C) Luciferase assays in HEK293A cells. Site-directed mutagenesis analysis identified the importance of the miR-145 p65RE-1 for the p65-mediated induction of luciferase activity. (D) Chromatin immunoprecipitation assays revealed that p65 interacts specifically with p65RE-1 and p65RE-2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

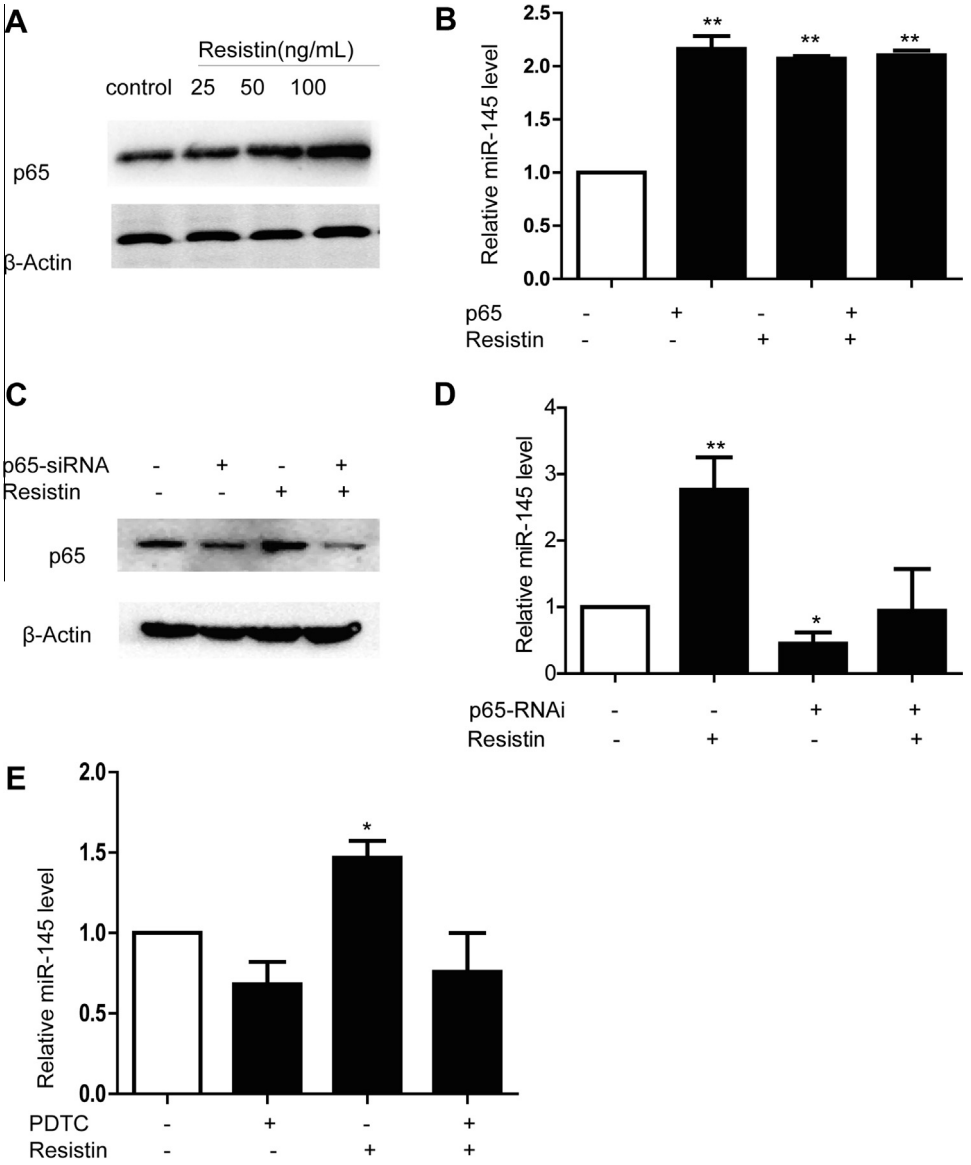

Fig. 4. Resistin upregulated miR-145 through p65. (A) Resistin stimulated the protein levels of p65. (B) p65 overexpression increased miR-145 levels. (C) The inhibition efficiency of p65 (p65-RNAi) was determined by western blotting. (D) Interfering with p65 by siRNA inhibited the effect of resistin on miR-145. (E) The NF-κB inhibitor PDTC (10 μM) blocked the effect of resistin on miR-145.

NF-κB pathway, and exogenous resistin stimulates p50/p65 (NF-κB) heterodimeric complex formation and localization into the nucleus [31]. Resistin also increases p65 RNA and protein levels in HepG2 cells [29]. In the present study, we also found that p65 was significantly upregulated by resistin in HepG2 cells in a dose-dependent manner. Treating cells with either siRNA against p65 or PDTC (a NF-κB antagonist) abrogated the effects of resistin on miR-145. Therefore, resistin induced hepatic insulin resistance by upregulating miR-145 via the p65 pathway.

In conclusions, resistin upregulated miR-145 via p65, and stimulated hepatic insulin resistance. These data link resistin to inflammation and insulin resistance, and provide a novel therapeutic target (miR-145) for hepatic insulin resistance.

Disclosure summary

The authors have nothing to disclose.

Acknowledgments

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