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Resistin Inhibits the Synthesis of Insulin by miR-494 through Traget on CREB1

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Authors' contributions

This work was carried out in collaboration between all authors. Authors FW and ZY designed the study and wrote the first draft of the manuscript. Author YY wrote the protocol, performed the statistical analysis and managed the analyses of the study. Authors HF, LN, LL, YC, YL, QW and YY performed the experiments and managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Resistin was first described as an adipokine and found to impair the pancreatic beta cells. The reason that it was named "resistin" is because it related with insulin resistance. Our previous study showed that miR-494 were significantly downregulated by resistin, while it is unknown that whether miR-494 involved in the regulation of insulin synthesis induced by resistin. The current study investigated the effects of miR-494 in the insulin synthesis reduced by resistin. MIN6 cells were treated with resistin and insulin synthesis was measured, the results showed that the insulin synthesis were significantly reduced by resistin. The over-expression of miR-494 inhibited the insulin synthesis both in diet culture and high glucose medium. Further, we discovered that miR-494 down-regulated the protein level of CREB1 by pairing with sites in the 3'UTR, which suggested that CREB1 is one of a target genes of miR-494. In conclusion, resistin inhibit the synthesis partly by miR-494 through target on CREB1 gene.

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Keywords: Resistin; insulin; miR-494; CREB1.

1. INTRODUCTION

Diabetes is a metabolic syndrome that can be caused by insufficient insulin secretion [1] or by insulin resistance [2]. It is characterized mainly by hyperglycemia and metabolic disorders involving lipids and proteins. Type II diabetes is the most common and most damaging class of diabetes [3]. It is closely related to age [4], obesity [5], diet [6], and lifestyle [7], and it has a strong genetic component as well [8]. Currently, no therapy can completely cure diabetes and the fundamental reason for this lies in the fact that its pathogenesis is not entirely clear. Insulin is critical to the progression of diabetes. How much insulin to produce, and the body's sensitivity to insulin secretion, directly determine whether diabetes will develop.

Resistin was originally defined as "adipose tissue-specific secretory factor" [9], but because of its ability to resist insulin, it was renamed resistin [10]. Adipocytes treated with thiazolidinedione (TZD) can express resistin during differentiation, but this expression becomes less pronounced once the adipocytes are fully differentiated [11]. Serum resistin levels are significantly higher in congenitally obese and diet-induced obese mice than in normal mice [10, 12]. This suggests that resistin is an important hormone and may be a key link between obesity and diabetes. Resistin was found to be negatively regulated by rosiglitazone and other anti-diabetic drugs in mice [13], and results have shown that resistin is associated with insulin resistance. Resistin levels decreased in plasma of mice after fasting, but it returned to normal levels after they were again allowed to eat [14]. In diet-induced obese mice, immunoneutralization of resistin led to a drop in blood glucose and improved insulin sensitivity, however, recombinant resistin impairs insulin-stimulated glucose uptake [15], thus confirming that resistin is indeed associated with diet-induced insulin resistance.

Minn et al. [16] found that resistin was expressed in human and mouse islets and up-regulated in insulin resistance model. Insulin secretion stimulated by glucose was less pronounced in resistin-overexpressing mice. Both the resistance of islet β cells to insulin and the ability of glucose to stimulate insulin were reduced after treatment with resistin *in vitro* for one day. In islet β cells, resistin at least in part inhibited insulin-induced phosphorylation of Akt, and increased the expression of SOCS3 resulted in reduced insulin secretion stimulated by glucose [17]. Other studies also confirmed that resistin down-regulated mRNA and protein levels of insulin receptor in pancreatic β cells [18]. These findings suggest that resistin plays a role in the regulation of pancreatic β cell function. However, it is not clear whether resistin can affect the synthesis of insulin. In this study, we aimed to investigate the effect of resistin on the insulin synthesis in MIN6 cells and the role of miR-494.

2. MATERIALS AND METHODS

2.1 Materials

TRIzol reagent was purchased from TaKaRa (Dalian, China), LipofectamineTM 2000 was from Invitrogen (Carlsbad, CA, USA). Recombinant mouse resistin were purchased from PeproTech, Inc. (Rochy Hill, NJ, USA). MiRNA mimics and inhibitors were purchased from GenePharma (Shanghai, China). The miRcute miRNA isolation kit was obtained from Tiangen Biotech Co. Ltd. (Beijing, China). CREB1 antibody was purchased from Proteintec (Chicago, USA) and β-actin was from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2 Place and Duration of Study

Key Laboratory of Agricultural Animal Genetics, Breeding and Reproduction of Ministry of Education, College of Life Science and Technology, Huazhong Agricultural University, Wuhan during Sep 2013 to Aug 2014; College of Animal Science and Technology, Henan University of Science and Technology, Luoyang during Sep 2014 to Jun 2015.

2.3 Cell Culture and Treatment

MIN6 cells (pancreatic β cells) were maintained in DMEM medium (HyClone, Thermo Scientific, USA) supplemented with 20% fetal bovine serum (FBS) (Gibco, Rockville, USA) containing 100 U/mL penicillin and 100 μg/mL streptomycin. The cells were grown at 37℃ in a humidified chamber containing 5% CO2.

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℃ for 5 min, followed by 35 cycles of 95℃ for 15 sec and 60℃ for 30 sec. The average threshold (*Ct*) was determined for each gene, and normalized to 36B4 mRNA or snRu6 miRNA as internal normalization controls. Fold changes in relative expression were calculated using the $2^{-\Delta\Delta Ct}$ method.

Table 1. Primers for real-time RT-PCR

Gene name	Sequences (5'-3')
Mus INS1	F: GTTATCTCTGTCTTTGTCTTACC
	R: TTCCTGTTGCTGTGACTC
Mus INS2	F: CTCTCTACCTGGTGTGTG
	R: GTAGTGGTGGGTCTAGTTG
Mus CREB1	F: CCAAACAGTTCAGATTTCAAC
	R: GCATCAGAAGATAAGTCATTC
Mus Stxbp5	F: CGGTGCACAATCTCTCGAT
	R: CACCTTCGATCCCACCAG
Mus Vamp2	F: TGCACCTCCTCCAAACCTTA
	R: AGCTCCGACAACTTCTGGTC
Mus Stxbp1	F: ATATCAACAAGCGCCGAGAG
	R: ATGGGGTGATGAGGTACACG
Mus Stx1a	F: AAGAAGGCCGTCAAGTACCA
	R: CCAGAATCACACAGCAAATGA
Mus Snap25	F: GGCTCCTCCACTCTTGCTAC
	R: CAGCAAGTCAGTGGTGCTTC
Mus Pdx1	F: ATGAACAGTGAGGAGCAG
	R: ACTTCGTATGGGGAGATG
Mus NeuroD1	F: AGTTATTGCGTTGCCTTAG
	R: GTGTTATGGGTCTGGTTTC
Mus Mafa	F: CACCATCACCACCATCAC
	R: ATGACCTCCTCCTTGCTG
Mus 36B4	F: TCGCTTTCTGGAGGGTGTC
	R: CAGATGGATCAGCCAGGAAGG

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 20% FBS. The miRNA mimics and inhibitors were transfected into cells in serum-free medium; media were changed to DMEM with 20% FBS 6 h later. Cells or culture medium were harvested 24h after transfection to isolate RNA or 48h to extract 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 LipofectamineTM 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 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.

3. RESULTS

3.1 Resistin and Overexpression of miR-494 Inhibits the Synthesis of Insulin

To elucidate the effect of resistin on intracellular synthesis of insulin, in this study, MIN6 cells were treated with different concentrations of resistin for 24 h, and collected for examination of

intracellular insulin by western blot. Results showed that resistin can inhibit the synthesis of insulin in a concentration-dependent manner (Fig. 1A).

According to Tang et al. [19] miR-494 is differentially expressed in pancreatic β-cells after glucose stimulation. Previous studies in our lab showed miR-494 to be significantly down-regulated in liver of mice treated with resistin [20]. In order to determine whether miR-494 is involved in the regulation of resistin in the synthesis of insulin, MIN6 cells conditioned to overexpress or underexpress miR-494 (Fig. 1B) were treated with resistin, and the expression of insulin and insulin biological synthesis-related genes were examined by using real-time fluorescence quantitative PCR. Results showed that mRNA levels of INS1 and transcription factors NeuroD1 and MafA were significantly down-regulated in MIN6 cells that overexpressed miR-494 when treated with resistin (Fig. 1C). However, the inhibition of miR-494 expression was found to restore the mRNA levels in INS 1 and NeuroD1 genes reduced by resistin partially (Fig. 1D). These results suggest that miR-494 is involved in the regulation of resistin during the synthesis of insulin.

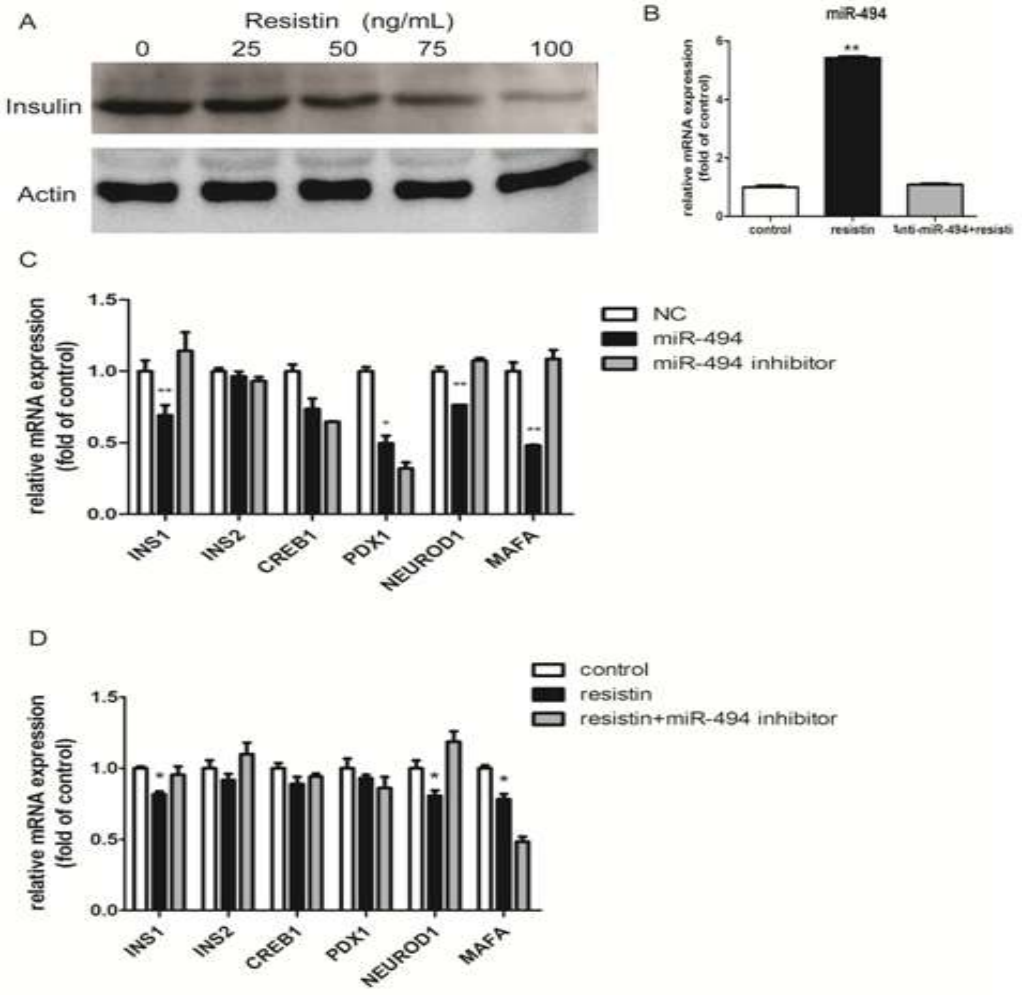

Fig. 1. Resistin and overexpression of miR-494 inhibits insulin synthesis in MIN6 cells

A. MIN6 cells were treated with resistin at doses of 25, 50, 75, and 100ng/mL for 24 h. Cells insulin synthesis was measured by western blot. B. The efficiency of overexpression or inhibitoion of miR-494 in MIN6 cells were identified. C. The expression of insulin and insulin biological synthesis-related genes were examined by qRT-PCR on the condition of miR-494 overexpression or inhibition. D. The miR-494 inhibition restored the mRNA levels in INS1 and Neurod1 genes reduced by resistin. Data were presented as means ± SD of percentage of controls. (,p<0.05significant; **, p<0.01very significant; ns, not significant)*

3.2 CREB1 as a Target Gene of miR-494

In order to define the molecular mechanism by which miR-494 is involved in the regulation of insulin synthesis in MIN6 cells by resistin, bioinformatics online software was used to identify possible target genes in miR-494, and results showed there to be a conserved site at mRNA 3'UTR in cAMP response element binding (CREB1) gene that can complementally bind miR-494 (Fig. 2A).

To verify the interaction between miR-494 and 3'UTR of CREB1 gene, a CREB1 mRNA 3'UTR dual luciferase enzyme reporter vector and a reporter vector in which miR-494 binding site was mutated in CREB1 within mRNA 3'UTR sequence were constructed. The effects of miR-494 overexpression on luciferase enzyme activity were examined, and the results are shown in Fig. 2B. MiR-494 rendered the luciferase activity of CREB1 mRNA 3'UTR significantly lower in the reporter vector than in the mutated vector, but luciferase activity was not affected in the mutated vector (Fig. 2B). These results suggest that miR-494 can directly binding on the 3'UTR region of CREB1 mRNA.

To further validate CREB1 as a target gene in miR-494 at the cellular level, the expression of CREB1 protein was examined under the condition of overexpression of miR-494. Previous data showed that overexpression of miR-494 did not have much effect on CREB1 mRNA levels (Fig. 1D). Because miRNA regulation of the target gene occurs mainly at the post-transcriptional level, CREB1 protein levels were examined and the results are shown in Fig. 2C. There was significantly less CREB1 protein during overexpression of miR-494. These result suggests that miR-494 directly combines CREB1 3'UTR, thereby inhibiting the expression of CREB1 at the translational level.

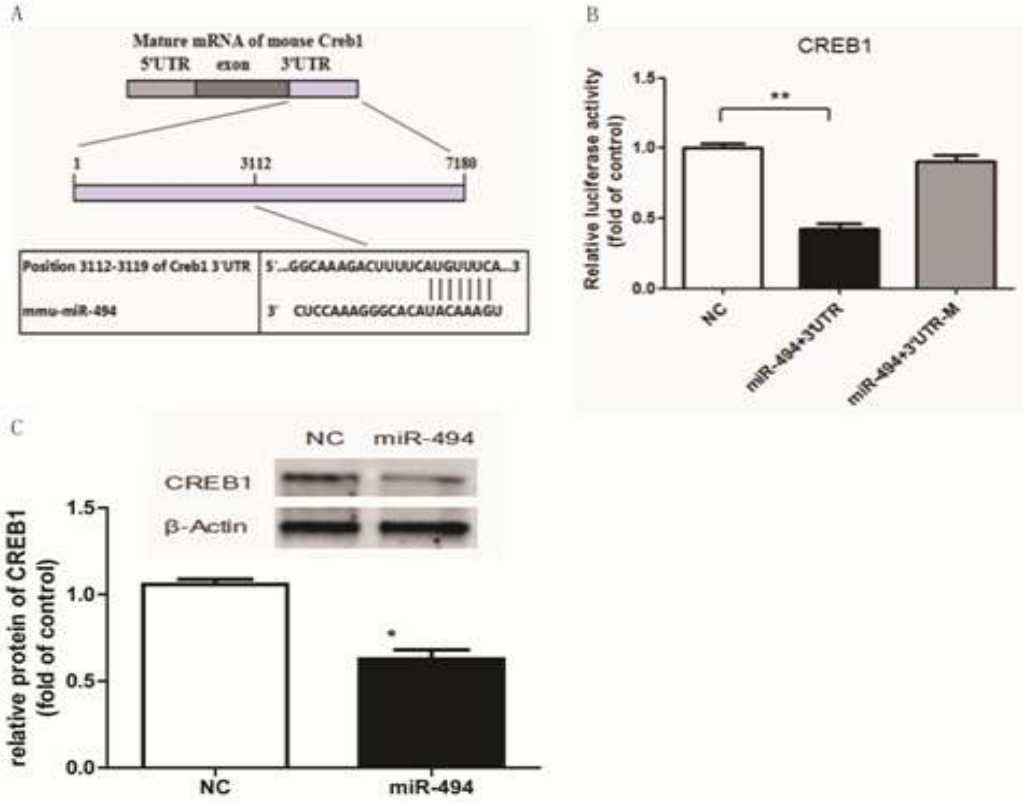

Fig. 2. CREB1 as a target gene of miR-494

(A) The result of bioinformatics prediction shows that miR-494 can binding on the 3112-3119bp sites of CREB1 mRNA 3'UTR region. (B) miR-494 and CREB1 3'UTR report vector or binding site mutant vector (Stxbp5 - 3'UTR-M) were co-transfected into NIH-3T3 cells and luciferase activity were detected according to the instruction of Dual Luciferase Reporter Assay System after 24h. (C) Effect on CREB1 protein level of miR-494 by Western blot. Data were presented as means ± SD of percentage of controls. (,p<0.05significant; **, p<0.01very significant; ns, not significant)*

3.3 MiR-494 Suppresses CREB1 during the Promotion of Insulin Synthesis

CREB1 was involved in the transcriptional regulation stimulated by glucose, incretin, and other hormones [21], and it has extremely important functions in glucose sensors, insulin secretion, insulin gene transcription, and β cell survival [22]. The results reported by Dalle et al. [22] showed that miR-494 inhibited CREB1 expression at the translational level through direct combination of CREB1 at the 3'UTR. In the current work, in order to study the effect of miR-494 on the synthesis of insulin in islet β cells, the expression of insulin protein was examined in MIN6 cells overexpressing miR-494 with or without CREB1. The results are shown in Fig. 3A, B, and the overexpression of miR-494 had no significant effect on the synthesis of insulin, but it was interesting that when co-transfected miR-494 and CREB1 eukaryotic expression vectors into MIN6 cells, the overexpression of miR-494 can inhibit the promotive effect of CREB1 on the synthesis of insulin (Fig. 3A,B).

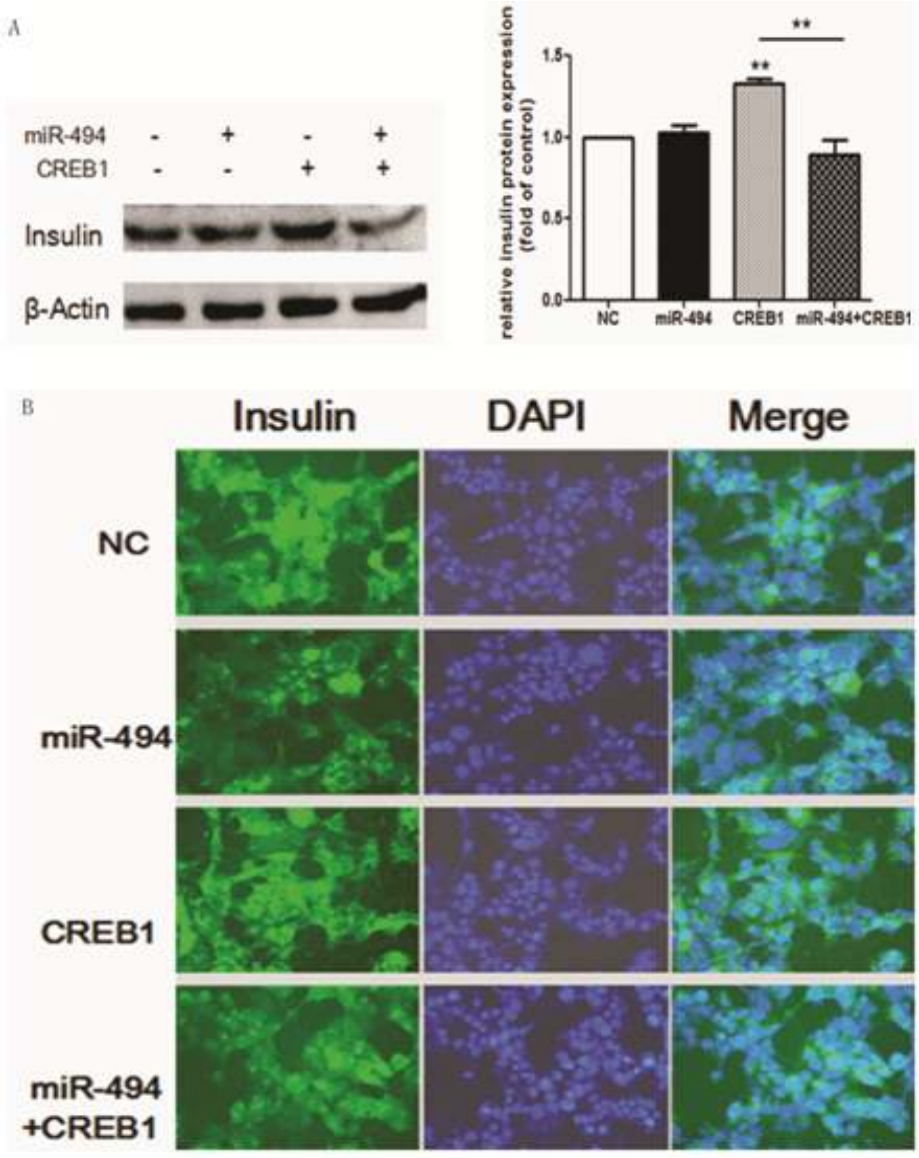

Fig. 3. MiR-494 suppresses CREB1 during the promotion of insulin synthesis

MIN6 cells were co-transfected with miR-494 and CREB1. The insulin synthesis was determined by western blot (A) or immunofluorescence (B). Data were presented as means ± SD of percentage of controls. (,p<0.05significant; **, p<0.01very significant; ns, not significant)*

4. DISCUSSION

Resistin is an important factor in adipocytes. In recent years, it was discovered to affect the regulation of glucose, lipid metabolism, and insulin sensitivity. Because of its importance in metabolic regulation and its potential value in insulin resistance, resistin is now considered a candidate of great concern. In 2003, the expression of resistin was observed in pancreatic islets in humans and mice, and it was found to be up-regulated in insulin resistance model [16]. Studies have confirmed that resistin down-regulated the mRNA and protein of insulin receptor in pancreatic β cells [18]. These findings suggest that resistin may play a role in the regulation of pancreatic β cell function. In this study, we found that resistin can inhibit the synthesis of insulin in a concentration-dependent manner (Fig. 1A). Resistin is secreted by adipose tissue specifically in human, Hence, insulin diminution occurs during the rising of resistin implying that resistin impair the function of pancreas and maybe a reason of type I diabetes. The point is that cell work may not be translate to humans, so it needs further study to clarify.

MiRNA is a widely distributed, endogenous, non-coding RNA of about 22 nt in size [23], and it is involved in a variety of life processes, such as embryonic development [24], cell growth and differentiation, proliferation [25, 26], cell apoptosis [27], and diseases such as diabetes [28] and cancer [29]. Using miRNA microarray technology, Tang et al. [19] found miR-494 to be significantly up-regulated after glucose stimulation in MIN6 cells. In our previous studies, results showed miR-494 to be significantly down-regulated in the livers of mice treated with resistin [20]. Another study reported that miR-494 was up-regulated in the cardiac microvascular endothelial cells in diabetic GK rats [30]. These results showed that miR-494 is likely to play a role in the occurrence or development of diabetes. In this study, INS1 and transcription factors NeuroD1 and MafA were significantly down-regulated by miR-494 when treated with resistin (Fig. 1C), while miR-494 inhibitor restore the mRNA levels in INS 1 and NeuroD1 genes reduced by resistin partially(Fig. 1D). Based on these data, we presumed that the regulation of insulins synthesis at the post-transcriptional level at least partially.

Nuclear transcription factor CREB was first discovered in 1957 as an intracellular second messenger mediating the effects of glucagon and epinephrine in the cAMP signaling pathway and named cAMP response element binding protein[31]. It was later discovered that CREB may be involved in a variety of other signaling pathways activated by many stimuli. Once CREB is activated, it recognizes the CRE sequence in genes through its N-terminal basic region and forms dimers through its conserved C-terminal leucine zipper motifs; it then binds target gene CREs and enhances gene transcription [32]. In islet β cells, CREB was involved in the transcriptional regulation stimulated by glucose, incretin, and other hormones [21], and it has an extremely important effect on glucose sensors, insulin secretion, insulin gene transcription, and β cell survival. The result of this study showed that CREB1, a target gene of miR-494, may be regulated by miR-494 at the post-transcription level, thereby affecting the synthesis of insulin which tell us that miR-494 involve in the regulation of insulin synthesis through CREB1.

The potential avenues of insulin synthesis regulation through miR-494 are shown in Fig. 4. Resistin upregulates miR-494 through an unknown transcriptional factor in pancreatic β cells, and then miR-494 inhibit the expression of CREB1, consequently, insulin synthesis was inhibited.

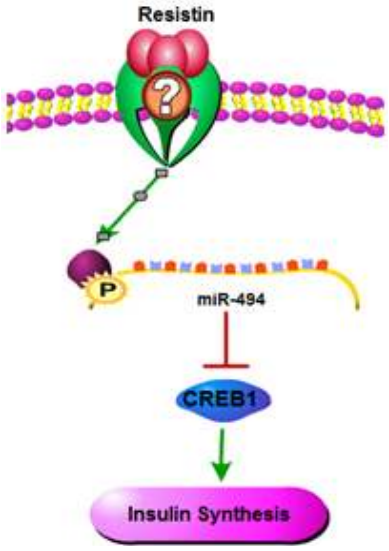

Fig. 4. The proposed pathway of miR-494 effects in the insulin synthesis

The scheme showed the potential avenues of the insulin synthesis regulation through CREB1 by miR-494.Resistin upregulates miR-494 through an unknown transcriptional factor in pancreatic β cells, and then miR-494 inhibit the expression of CREB1, consequently, insulin synthesis was inhibited.

5. CONCLUSION

Resistin can inhibit the expression of CREB1 through up-regulation of miR-494, thereby reducing the biosynthesis of insulin in MIN6 cells. Our data link resistin to type I diabetes via miR-494 and provide some novel targets (such as CREB1, INS 1 and NeuroD1) to regulate insulin synthesis. Further study should focus on the mechanism of resistin on miR-494 and verify the results of this study in living level.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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