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MicroRNA-19b downregulates insulin 1 through targeting transcription factor NeuroD1

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ABSTRACT

MiR-17-92 cluster miRNAs are disclosed to contribute to the development of multiple organs and tumorigenesis, but their roles in pancreas development remains unclear. In this study, we found that miR-19b, a member of miR-17-92, was highly expressed in the pancreatic progenitor cells, and miR-19b could target the 3′ UTR of NeuroD1 mRNA to decrease its protein and mRNA levels. Functional analysis showed that miR-19b exerted little effect on the proliferation of pancreatic progenitors, whereas it inhibited the expression of insulin 1, but not insulin 2 in MIN6 cells. These results suggest that miR-19b can downregulate insulin 1 expression through targeting transcription factor NeuroD1, and thus regulate the differentiation and function of β-cells.

1. Introduction

Pancreatic islet is comprised of endocrine cells, which control multiple homeostatic functions. β-cell, the predominant islet cell type, responds to glucose by synthesizing and secreting insulin, thus maintains physiological glucose homeostasis [1,2]. Functional disturbance of β-cells and insufficient insulin secretion lead to diabetes [3,4]. During the development of mouse pancreas, endocrine cells occur from e13.5 to e15.5, a stage termed the "secondary transition" [5]. In this stage, the proliferation of pancreatic progenitor cells steps down, and their differentiation into endocrine cells, particularly β-cells, increases rapidly [5]. It has been demonstrated that the proliferation and differentiation of pancreatic progenitor cells involve a cascade of inhibition and activation of specific transcription factors, such as pancreatic and duodenal homeobox factor-1 (Pdx-1), Neurogenin 3 (Ngn3) and Neurogenic differentiation factor 1 (NeuroD1) [6].

In recent years, several studies have shown that microRNAs (miRNAs), a class of non-coding RNAs, contribute to pancreatic endocrine development. In Dicer (an essential enzyme for miRNAs formation) null mutation mice, the blockage of miRNAs formation led to gross defects in all pancreatic lineages, and the differentiation of endocrine cells especially the insulin-producing β-cells, was inhibited [7,8]. MiR-375 was found to be involved in regulating insulin secretion and maintaining α and β cell mass [9,10]. MiR-15, miR-16 and miR-195 were reported to target Ngn3 in the regenerating pancreas [11], and miR-124a could repress Foxa2 expression in MIN6 cells [12]. However, the function of miRNAs in the proliferation or differentiation of pancreatic progenitor cells is largely unknown.

It has been reported that the miR-17-92 cluster, which encodes miR-17-5p, miR-18a, miR-19a, miR-20a, miR-19b and miR-92, contributes to the development of heart, lung, blood vessel or immune system, and plays an important role in tumorigenesis [13–16]. MiR-19b, a key oncogenic component of miR-17-92 [17], was found to regulate cell cycle progression by negatively regulating tumor suppressor Pten and pro-apoptotic protein Bim to induce lymphomas [17–19]. In the present study, we explored the function of miR-19b in the β-cell proliferation and differentiation.

2. Materials and methods

2.1. Experimental animals

Outbred strain CD1 mice were obtained from Beijing Laboratory Animals Research Centre, and kept in compliance with the

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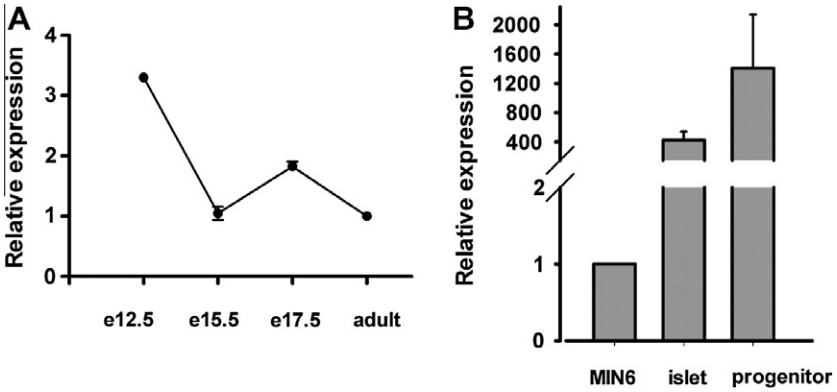

Fig. 1. Expression pattern of miR-19b during mouse pancreatic development. (A) The expression of mature miR-19b in e12.5, e15.5, e17.5 and adult pancreata were determined by qRT-PCR, the mixed samples of e12.5 pancreata (550), e15.5 pancreata (150), e17.5 pancreata (5), and an adult mouse pancreas have been analyzed in the test. The expression of miR-19b in adult pancreata was defined as 1 and the Y-axis represented relative expression levels of miR-19b compared with adult pancreata. Expression of miR-19b was normalized to U6. (B) qRT-PCR detected the relative levels of miR-19b in pancreatic progenitor cells, pancreatic islets and MIN6 cells. Expression of miR-19b was normalized to U6.

protocols established and approved by the Animal Care and Ethics Committee of Northeast Forestry University.

2.2. Cell culture

3T3 and 293GP cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 25 mmol/l glucose supplemented with 10% heat-inactivated fetal bovine serum (FBS). The MIN6 insulinoma cell line was a kind gift of Yiming Mu (General Hospital of PLA, China), it was maintained in DMEM containing 25 mmol/l glucose supplemented with 15% FBS and 50 μmol/l 2-mercaptoethanol (Sigma-Aldrich).

2.3. MiR-19b target gene prediction

MiR-19b target genes were predicted by the PicTar miRNA target gene prediction website (http://pictar.mdc-berlin.de/) and the TargetScan algorithm from TargetScan Version 5.1 (http://www.targetscan.org/).

2.4. DNA construct

A 417-bp fragment from the 3′ untranslated region (3′ UTR) of NeuroD1 (position 1777–2193, NM_010894) containing the predicted miR-19b binding sites was cloned into the XbaI and FseI sites of pGL3 control vector. The corresponding mutant constructs were created by mutating the seed region of the miR-19b binding sites. To construct enhanced green fluorescent protein-NeuroD1 (EGFP-NeuroD1) fusion protein, the full length of NeuroD1 which contains coding sequence and 3′ UTR, but without start codon, was inserted into the XhoI and EcoRI sites of pEGFP-C1 vector. All constructs were confirmed by DNA sequencing.

2.5. Cells transfection

Mimic miRNA, miRNA inhibitor (2′O-Me modified antisense oligonucleotide) (GenePharma) and plasmids were transfected into MIN6, 293GP, 3T3 or pancreatic progenitor cells using Lipofectamine 2000 (Invitrogen). 80 nmol/l (final concentration) mimic miRNA or miRNA inhibitor was transfected into MIN6 cells, total RNA was isolated for real time quantitative PCR (qRT-PCR) 48 h after transfection, immunofluorescent staining of insulin and western blot analysis of NeuroD1 were performed 72 h after transfection. 300 ng pEGFP-C1-NeuroD1, 60 nmol/l (final concentration) mimic miRNA and 120 nmol/l (final concentration) miRNA inhibitor were co-transfected into 293GP or 3T3 cells, NeuroD1 mRNA or protein expression was measured at 48 h or 72 h after transfection, respectively. For cell proliferation assay, 80 nmol/l (final concentration) mimic miRNA was transfected into MIN6 cells or pancreatic progenitor cells.

2.6. Dual luciferase reporter assay

3T3 cells were plated into 24-well plates 24 h before transfection, 200 ng pGL3-control containing the wild type or mutated NeuroD1 3′ UTRs, 50 ng pRL-TK and 30 nmol/l mimic miRNAs were co-transfected into 3T3 cells by using Lipofectamine 2000. Relative firefly luciferase activity (normalized to Renilla luciferase activity) was measured 24 h after transfection by the Dual-Luciferase Reporter Assay Kit (Promega) on GloMaxTM 20/20 Luminometer (Promega).

2.7. RT-PCR and qRT-PCR

Total RNAs were isolated by using TRIzol reagent (Invitrogen) or RNAprep Micro Kit (Tiangen, Beijing), then treated with DNase I (RNase free) (Takara). To detect miRNA expression, total RNAs were polyadenylated with ATP by *Escherichia coli* poly (A) polymerase (Biolabs, New England). The polyadenylated total RNAs were reverse transcribed with M-MLV Reverse Transcription Reagents (Invitrogen) and a poly (T) primer ligated with a adapter for miRNA quantitative assays. The RNAs without polyadenylation were reverse transcribed with oligod (T) for mRNA assay.

qRT-PCR was performed using the ABI 7500 sequence detection system (Applied Biosystems). U6 small nuclear RNA was used as an endogenous control for miRNAs and β-actin was used for mRNAs. All reactions were run in triplicate and included no template controls for each gene. The relative amount of each mRNA to β-actin or miRNA to U6 RNA was calculated using the $2^{-\Delta\Delta CT}$ method. The level of significance was determined by one-way analysis of variance (ANOVA) with SPSS Statistics 17.0. All quantitative data presented were the mean ± SEM. Sequences of primers used for RT-PCR and qRT-PCR in this study were as described in Supplementary Table 1.

2.8. Western blotting analysis

Cellular total proteins were extracted with cell lysis buffer. Protein extracts were then centrifuged at 12 000×*g* for 5 min at 4 °C and the supernatants were used for the experiments. Proteins were separated on 10% SDS–PAGE gels and electroblotted onto a

nitrocellulose membrane. The membranes were probed with anti-NeuroD1 (Santa Cruz) and anti-β-actin (Biosynthesis). The bands were quantified using the ImageJ 1.42 software after densitometric scanning of the films.

2.9. Immunofluorescent staining

Cells were fixed in 4% fresh paraformaldehyde for 30 min followed by permeabilization using 0.3% triton X-100 for 30 min. After blocking for 1 h, cells were incubated in primary antibody rabbit anti-insulin (1:100, Santa Cruz), goat anti-c-peptide (1:100, Linco), rabbit anti-glucagon (1:200, Zymed) or rabbit anti-somatostatin (1:500, DAKO) overnight at 4 °C. Incubation with secondary antibody FITC-conjugated IgG (1:150, Jackson Immunoresearch) or Rhodamine-conjugated IgG (1:150, Jackson Immunoresearch) was conducted for 1 h. Images were acquired using a fluorescent microscope equipped with a cooled three-chip charge-coupled-device camera (Carl Zeiss).

2.10. Cell proliferation assay

MIN6 and pancreatic progenitor cells proliferation were determined by CCK-8 assay and bromodeoxyuridine (BrdU) incorporation assay. For CCK-8 (Dojindo Molecular Technologies) assay, cells were incubated in CCK-8 solution for 2 h at 37 °C. The amount of formazan dye was measured by absorbance at 450 nm with a microplate reader. For the BrdU incorporation assay, 10 μmol/l BrdU was first added into the culture medium for 1 h, and then cells were fixed, acid-treated and followed by immunostaining analysis with BrdU antibody (Sigma) and HRP-conjugated secondary antibody (Biosynthesis). BrdU incorporation was observed under a light microscope. At least 2000 cells were counted for each sample.

2.11. Isolation of adult pancreatic progenitor cells and islets

The adult pancreatic progenitor cells were screened as the following procedure. Pancreata from 4–6-week-old mice were minced into 8-mm^3 fragments to perform the digestion with type IV collagenase (Invitrogen) at 37 °C for 20 min. Single cells were collected and seeded at a density of 1.5×10^4 cells/cm^2 in proliferating media (DMEM/F12 medium containing 2% FBS and supplemented with 1 × B27 (Invitrogen), 2 ng/ml EGF (R&D), 10 ug/ml insulin (Sigma-Aldrich) and 50 μmol/l 2-mercaptoethanol). The fibroblasts were removed through deserting the attached cells after seeding for 40 min, the epithelial cells attached at a low density (<300 cells per square cm). 7–10 days after culturing, single-clone epithelial cells were picked out for detection, or to subculture for cell proliferation assays. Islet isolation was conducted as reported by Gotoh, M. et al [20].

3. Results

3.1. Expression pattern of miR-19b in the developing mouse pancreas

At e12.5, the mouse pancreatic bud is composed of a common pool of multipotent progenitor cells, while at e15.5, most of progenitor cells begin to differentiate toward endocrine and exocrine cell lineages [5,21]. To determine which miRNAs participate in the proliferation and differentiation of pancreatic progenitors, we carried out qRT-PCR assays at four distinct developmental stages: e12.5, e15.5, e17.5 and adult (6 weeks old). The results showed that the expression of miR-19b was highest at e12.5, but lowest at e15.5, comparing with the other stages (Fig. 1A).

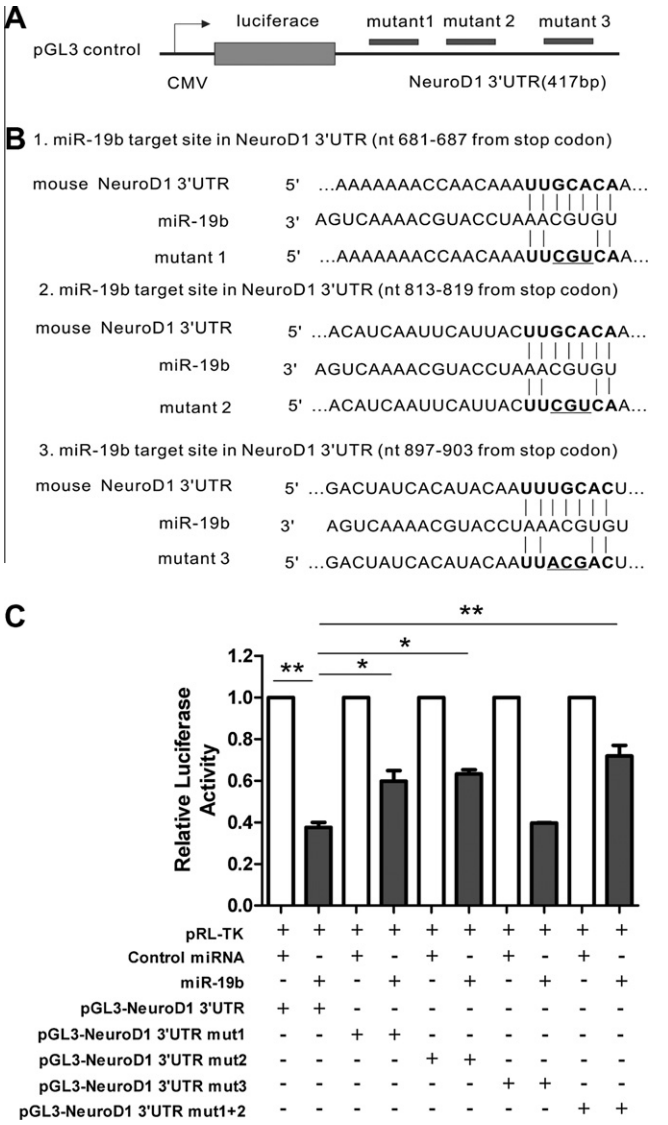

Fig. 2. Identification of NeuroD1 as the target gene of miR-19b. (A) A schematic representation of the pGL3-luciferase reporter constructs used in this study. (B) Location and sequences of the three miR-19b target sites (nominated here as 1, 2 and 3) in the NeuroD1 3′ UTR; the sequence of mouse miR-19b is indicated, alongside the mutations introduced in the different target sites (underlined nucleotides) for generating mutated reporter constructs. (C) Luciferase activity was determined 24 h after transfection. All luciferase values were normalized to Renilla luciferase. Single and double stars indicate $P < 0.05$ and $P < 0.01$, respectively.

To further explore the expression of miR-19b in pancreatic progenitor cells, we isolated a population of epithelia-morphological cells with colony-forming capability from adult mouse pancreata. RT-PCR analysis showed that these cells did not express endocrine markers *insulin 1*, *insulin 2*, *glucagon*, *somatostatin*, and *pancreatic polypeptide* or the exocrine markers *amylase 2* and *elastase 1*, but expressed *pancreas specific transcription factor-1a (ptf1a)*, *c-Met*, *CD133*, and *Hes1* (Fig. S1A), which are candidate molecular markers of pancreatic progenitor cells [22–24]. The differentiation potential of these cells was analyzed by culturing them under the endocrine differentiation condition. After inducing the cells for 15 days, Pdx1, Ngn3, NeuroD, Nkx2.2, Nkx6.1, Pax6, and glucose transporter 2 (Glut2) were detected by RT-PCR (Fig. S1B). In addition, the endocrine hormone genes insulin, glucagon and somatostatin were expressed, but pancreatic polypeptide mRNA was not detected (Fig. S1B). Immunofluorescent staining results showed that

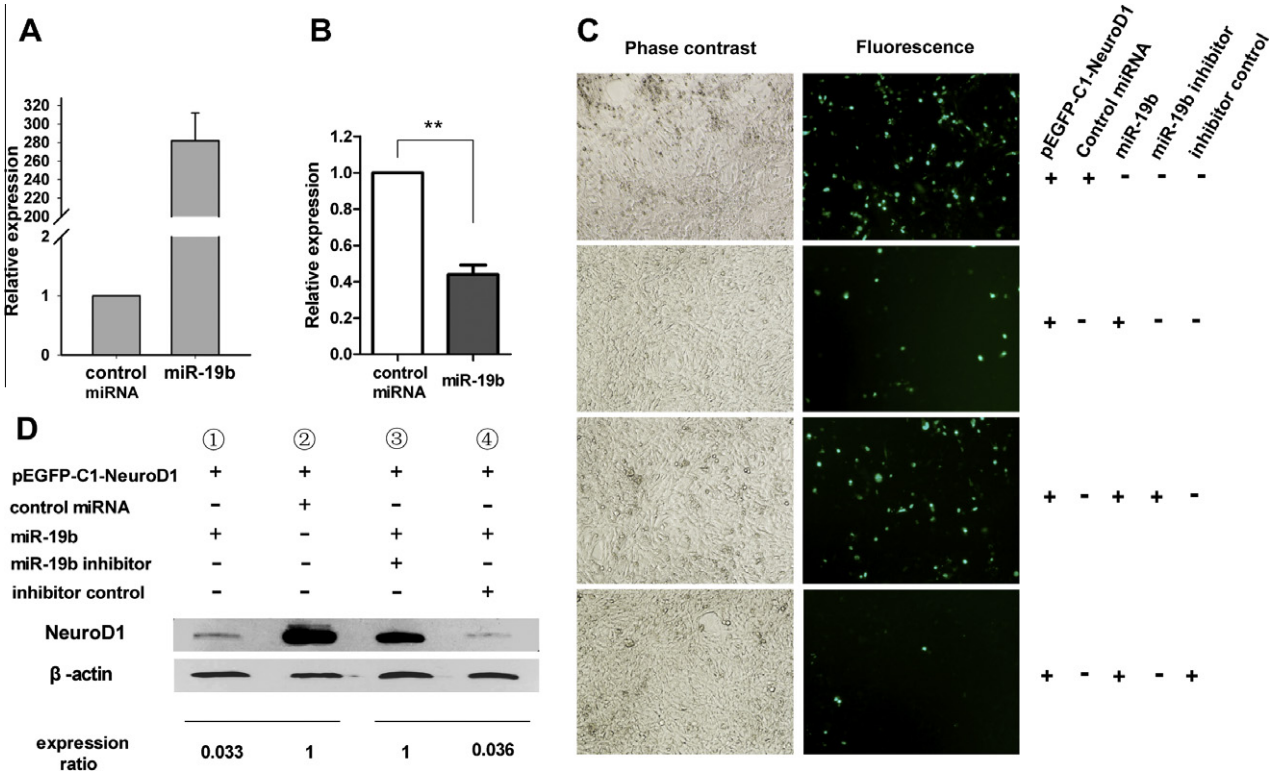

Fig. 3. Overexpression of miR-19b reduced NeuroD1 protein and mRNA in vitro. 3T3 cells were co-transfected with pEGFP-C1-NeuroD1, mimic miRNA and/or miRNA inhibitor. 48 h after transfection, RNA was extracted and the overexpression of miR-19b was measured by qRT-PCR, expression of miR-19b was normalized to U6 (A); the expression of NeuroD1 mRNA was analyzed by qRT-PCR, expression of NeuroD1 was normalized to β-actin (B); 72 h after transfection, EGFP-NeuroD1 fusion protein expression in 3T3 cells was analyzed under a fluorescence microscope (C) (100×); The total proteins were obtained from the lysis cells and the expression of NeuroD1 was analyzed by western blotting, expression ratio represented relative expression levels of NeuroD1 in group ① compared with ② or ④ compared with ③, expression of NeuroD1 in group ② or ③ was defined as 1, respectively (D).

c-peptide-positive, glucagon-positive and somatostatin-positive cells could be detected among the differentiated cells (Fig. S1C). The results indicated that these cells are pancreatic progenitor cells. The expression of miR-19b in the isolated pancreatic progenitor cells, islets and MIN6 cells was assessed by qRT-PCR. The result showed that the expression of miR-19b was higher in the pancreatic progenitors than the pancreatic islets and MIN6 cells (Fig. 1B).

3.2. Analysis of the target genes for miR-19b

We searched for the potential target genes of miR-19b by PicTar [25] and Targetscan [26], and focused on the genes known to be involved in the differentiation of β-cells. Our immediate attention was drawn to NeuroD1, a transcription factor that regulates the differentiation of pancreatic progenitor cells into β-cells and plays an essential role in maintaining normal β-cells function [27].

After comparing the results given by the two miRNA prediction programs, we found that miR-19b has three target sites in the NeuroD1 mRNA 3′ UTR (Fig. 2A), which are highly conserved in vertebrate. To test the effect of miR-19b on the NeuroD1 expression, a fragment of NeuroD1 3′ UTR containing the three putative target sites was cloned into the downstream of the firefly luciferase stop codon of pGL3 control expression vector. After the recombinant vector was co-transfected with synthetic miR-19b into 3T3 cells, the luciferase activity was significantly inhibited than that co-transfected with the control miRNA (Fig. 2C).

Then we mutated the targeting sites of NeuroD1 3′ UTR in the pGL3-control vector (Fig. 2B). Mutational analysis showed that the first and second site in the fragment were important in mediating the inhibition of miR-19b (Fig. 2C). When the first site was mutated, luciferase activity was less efficiently repressed by miR-19b than that in the wild-type construct. This result indicates that the first site is functional. In addition, the mutation of the second site also modified miR-19b-induced repression of luciferase, which indicates that the second site is also necessary for miR-19b activity. However, the mutation of the third site did not rescue the luciferase activity. Furthermore, when the first site and the second site were both mutated, the rescue of luciferase activity was stronger than either mutation of them, these results demonstrate that the two target sites exert cooperative functions.

3.3. miR-19b inhibited the expression of NeuroD1 at both mRNA and protein level

In order to validate whether miR-19b is capable of regulating NeuroD1 expression, we cloned the DNA sequence corresponding to the entire NeuroD1 mRNA into pEGFP-C1 to construct EGFP-NeuroD1 fusion protein expression vector (pEGFP-C1-NeuroD1), then the vector was co-transfected with either synthesized miR-19b or miR-19b inhibitor into 3T3 cells. The overexpression of miR-19b was confirmed by qRT-PCR (Fig. 3A). Also, qRT-PCR results showed that overexpression of miR-19b decreased *NeuroD1* mRNA level (Fig. 3B). Western blotting results showed that the NeuroD1 protein was significantly inhibited by miR-19b (Fig. 3D). Meanwhile, the expression of EGFP protein was repressed because of the reducing NeuroD1 (Fig. 3C). Similar results were observed in 293GP cells which were co-transfected with pEGFP-C1-NeuroD1, miR-19b and/or miR-19b inhibitor (Fig. S3).

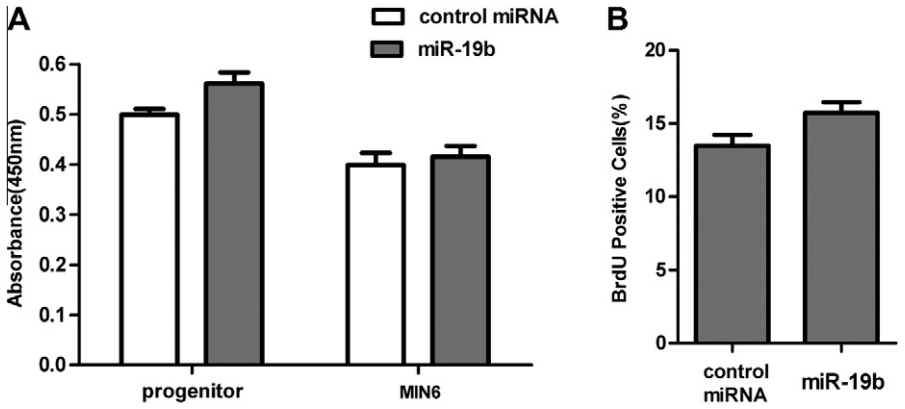

Fig. 4. Overexpression of miR-19b had no effect on pancreatic cells proliferation. (A) MIN6 or pancreatic progenitor cells were transfected with miR-19b or control miRNA. 72 h after transfection, cells proliferation was evaluated by CCK-8 assay; (B) MIN6 cells proliferation was further confirmed by BrdU incorporation assay.

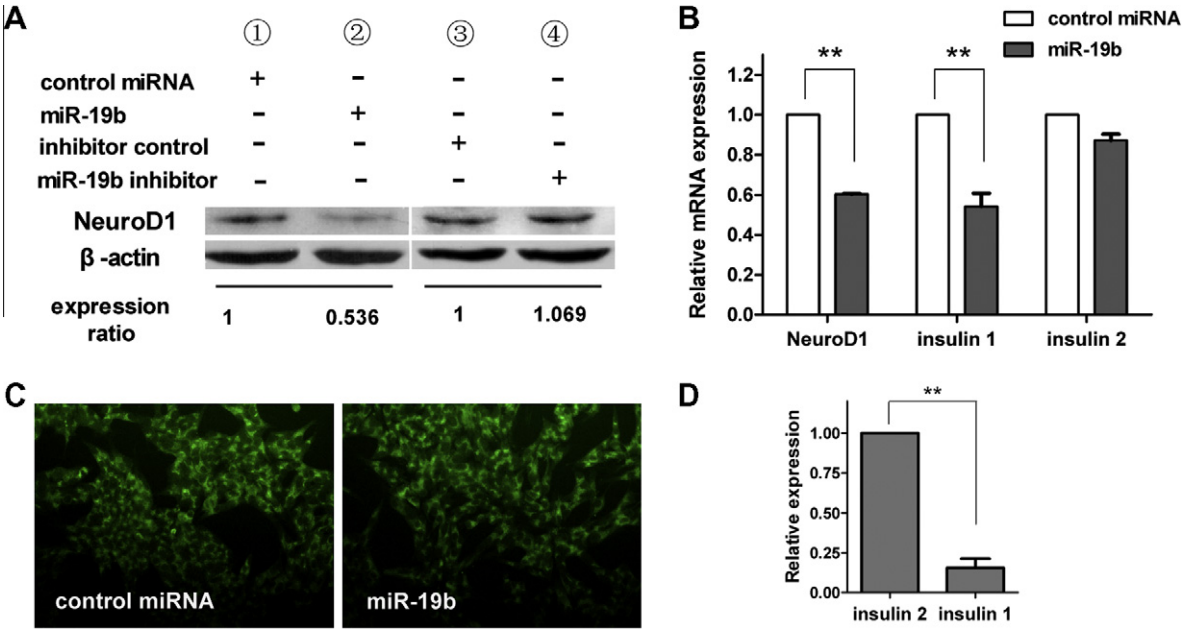

Fig. 5. Effect of miR-19b repressed NeuroD1 and insulin 1 expression in MIN6 cells. MIN6 cells were transfected with mimic miRNA or miRNA inhibitor, (A) 72 h after transfection, the cells were homogenized and the expression of NeuroD1 was analyzed by western blotting, expression ratio represented relative expression levels of NeuroD1 in group ② compared with ① or ③ compared with ④, expression of NeuroD1 in group ① or ③ was defined as 1, respectively. (B) 48 h after transfection, NeuroD1, insulin 1 and insulin 2 mRNA levels were measured by qRT-PCR; (C) 72 h after transfection, cells were fixed and insulin content (green) was detected by immunostaining (200×). (D) MIN6 insulin 1 and insulin 2 mRNA levels were measure by qRT-PCR. Expression of mRNA was normalized to β-actin. Double stars indicate $P < 0.01$.

3.4. miR-19b did not influence significantly the proliferation of MIN6 and pancreatic progenitor cells

Previous studies indicated that miR-17-92 promoted lung epithelial progenitor cells and leukemia stem cells proliferation [15,28]. Here we explore whether miR-19b can influence the proliferation of pancreatic cells. To address this question, miR-19b or control miRNA was transfected into pancreatic progenitor or MIN6 cells. Overexpression of miR-19b was confirmed by qRT-PCR (Fig. S2). CCK8-assay results showed that the proliferation of pancreatic progenitor cells or MIN6 was not interfered significantly by miR-19b (Fig. 4A). Also, BrdU incorporation study confirmed that miR-19b could hardly influence MIN6 cells proliferation (Fig. 4B).

3.5. miR-19b decreased insulin 1 expression via negative regulation of NeuroD1

After miR-19b was overexpressed in MIN6 cells, it was found that the endogenous NeuroD1 protein was reduced, while inhibition of the endogenous miR-19b has little effect on the expression of NeuroD1 (Fig. 5A). Previous studies have shown that NeuroD1 is a crucial factor for insulin gene transcription [29,30]. To test whether miR-19b can inhibit insulin transcription by negatively regulating NeuroD1, miR-19b or control miRNA was transfected into insulin-producing MIN6 cells, then the expression of *NeuroD1*, *insulin 1* and *insulin 2* mRNAs were measured by qRT-PCR. The results showed that the expression levels of *NeuroD1* and *insulin 1* mRNA were reduced by miR-19b, whereas *insulin 2* transcripts

were not influenced significantly, comparing with the control (Fig. 5B). Moreover, the total insulin protein was not significantly reduced by immunofluoresent analysis (Fig. 5C), since the expression of *insulin 2* mRNA was about 9 times more than that of *insulin 1* mRNA in MIN6 cells (Fig. 5D).

4. Discussion

In this study, we have found that miR-19b is highly expressed in the developing pancreas and adult pancreatic progenitor cells, but not in the differentiated cells. MiR-17-92 has been reported to promote lung epithelial progenitor cells and leukemia stem cells proliferation [15,28]. However, our results show that miR-19b exerts little effects on the proliferation of both MIN6 and pancreatic progenitor cells. This result is consistent with previous studies that overexpression of miR-19 does not enhance cell proliferation but reduces apoptosis in lymphoma [17,19].

Through bioinformatics prediction and experimental assays, we have noticed that NeuroD1 is a target gene of miR-19b. NeuroD1 as one of downstream genes of endocrine progenitor maker Ngn3, a key transcription factor in regulating differentiation of endocrine cells, plays an important role in endocrine development [31,32]. NeuroD1 null mice died postnatally from severe diabetes due to a dramatic loss of insulin-producing β-cells, moreover, the number of glucagon-producing α-cells and somatostatin-producing δ-cells were reduced modestly [27]. In this study, we showed that miR-19b could target the 3′ UTR sequence of NeuroD1 mRNA to inhibit its expression, combined with that miR-19b was expressed highly in pancreatic progenitor cells, lowly in pancreatic islets and MIN6 cells, these results indicate that miR-19b involves in the regulation of pancreatic endocrine differentiation from progenitor cells.

Although overexpression of miR-19b repressed the level of NeuroD1 mRNA and protein, transfection of miR-19b inhibitor into MIN6 cells did not increase the level of NeuroD1, this may cause by the low level of miR-19b in MIN6 cells (Fig. 1B). On the other hand, it has been reported that multiple miRNAs bind the same target mRNA, they act cooperatively to reduce mRNA translation by more than the sum of their individual effects [33]. When predicting the miRNAs which can regulate NeuroD1 using bioinformation softwares, we found that NeuroD1 was the potential target of 64 miRNAs using Targetscan (http://www.targetscan.org/), or 44 miRNAs using PicTar (http://pictar.mdc-berlin.de/). Otherwise, it has been confirmed that NeuroD1 is the target gene of miR-124 in neural cells [34], and miR-124 is highly expressed in MIN6 cells [9]. Thus, miR-19b, miR-124 and other miRNAs might act combinatorially NeuroD1 in MIN6 cells.

Several previous in vitro studies have shown that NeuroD1 is capable of activating both insulin 1 and insulin 2 through binding their conserved E box elements to function as an important transcription factor in pancreatic β-cell differentiation and mature β-cell function [31,35,36]. In this study, overexpression of miR-19b in MIN6 cells exactly reduced *insulin 1* transcription, but *insulin 2* transcription was not influenced. These results are consistent with the published observation that *insulin 1* was almost extinguished and *insulin 2* was unaffected in the mutant mice with β-cell-specific knockout of NeuroD1 [37]. The molecular mechanism of miR-19b in regulating the expression of insulin genes should be further explored by performing complementary in vitro and in vivo studies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2011.06.039.

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