

## Original Article

# Diesel exhaust particles induce toxicity to beta cells by suppressing miR-140-5p

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**Abstract:** The toxicologic effects of diesel exhaust particles (DEPs) on lung cells and function have been heavily studied. However, it remains largely unknown how DEPs affect the function of pancreatic beta cells. In this study, we demonstrated that DEP extract (DPE) exposure significantly reduces cell viability, insulin secretion, and ATP and GSH production of rat pancreatic beta cells. Also, DPEs induce the accumulation of ROS, p53 expression, and DNA damage in beta cells. In addition, the expression level of miR-140-5p was downregulated in beta cells following DPE exposure, and ectopic expression of miR-140-5p could partly attenuate the toxic effects of DPEs. Mechanistically, HDCA4 and HDCA7 were downstream targets of miR-140-5p. In conclusion, our findings demonstrate that DPE exposure impairs the normal functions of beta cells by downregulating miR-140-5p. Further studies are warranted to explore the toxic effects of circulating DEPs on the pancreas.

**Keywords:** Beta cell, diesel exhaust particle, toxicity, miR-140-5p

## Introduction

Diesel exhaust generated during combustion may contain hundreds of organic and inorganic compounds in either gaseous or particulate phases. Therefore, diesel exhaust particles (DEPs) have become one of the dominant pollutants in ambient air. Due to DEP's inert carbonaceous cores with large surface areas, they are ideal for the adsorption of trace amounts of metals and various organic substances, such as polycyclic aromatic hydrocarbons (PAHs), quinoline, acids and nitroaromatic hydrocarbons. Since these molecules are highly toxic, DEP exposure has been demonstrated to cause DNA and chromosomal damage to cells *in vitro* and *in vivo*, which may lead to a broad spectrum of mutations [1-3]. The small size of DEPs makes them highly respirable and they may penetrate deeply into the lung. In particular, studies have shown that the ultrasmall carbon particles present in DEPs, after deposition in the lung, largely escape alveolar macrophage surveillance and gain access to the pulmonary interstitium [4]. In fact, DEPs may be carcinogenic due to the presence of toxic chemicals such as PAH and nitroaromatic hydrocarbons.

MicroRNAs (miRNAs) are a class of endogenous non-coding RNAs approximately 21-25 nucleotides in length [5, 6]. miRNAs negatively regulate gene expression by binding to the 3'-untranslated region of target mRNA, leading to mRNA degradation or translational suppression. These small molecules have been demonstrated to be actively involved in almost all biologic processes including, but not limited to, proliferation, differentiation, inflammation and apoptosis [7]. Deregulation of miRNA is closely associated with the pathogenesis of many human diseases [8]. Emerging evidence has shown that miRNAs play an important role in protecting cells from the toxicity generated by outside substances. For instance, miR-181c significantly suppressed the inflammatory responses, neutrophil infiltration and reactive oxygen species (ROS) generation induced by cigarette smoke, and opposite results were found when miR-181c was overexpressed [9].

The toxicologic effects of DEPs on lung cells and function have been heavily studied. However, it is largely unknown how DEPs affect pancreatic beta cells. In this study, we have demonstrated that DEP extracts (DPEs) induce cytotoxicity and DNA damage to pancreatic be-

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ta cells, which suggests that circulating DEPs may cause toxicity to the pancreas. In addition, we found that the expression level of miR-140-5p was downregulated in beta cells following DPE exposure, and ectopic expression of miR-140-5p could partially attenuate the toxic effects of DPEs. Mechanistically, HDCA4 and HDCA7 were downstream targets of miR-140-5p.

### Materials and methods

#### *Cell culture and transfection*

Both RIN-m5F and INS-1 (rat insulinoma) cell lines were cultured in a humidified atmosphere of 5% CO<sub>2</sub>, 95% air at 37°C. The culture medium for RIN-m5F was ATCC-formulated RPMI-1640 (ATCC 30-2001) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. The culture medium for INS-1 was RPMI-1640 (Gibco BRL, Grand Island, NY, USA), which contains 11.2 mmol/l glucose and 2 mmol/l l-glutamine, and is supplemented with 10% fetal bovine serum (FBS), 1 mmol/l pyruvate, 10 mmol/l HEPES, 50 µmol/l 2-mercaptoethanol, 100 units/ml penicillin, and 100 µg/ml streptomycin. When the cell density reached 60% confluence, the miR-140-5p mimic and the negative control were transfected into the cells with the Lipofectamine® RNAiMAX Transfection Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction.

#### *DPE preparation*

SRM 1975 (standard reference material 1975) was purchased from the National Institute of Standards and Technology (NIST; Gaithersburg, MD, USA). It is a dichloromethane extract of the diesel particulate matter SRM 2975, which was generated by an industrial diesel-powered forklift truck and collected under specifically designed heavy-duty conditions (NIST 2000) [10]. DPEs were transferred to the biocompatible solvent dimethyl sulfoxide (DMSO) following widely accepted protocols [11-13], and DPE prepared in complete serum-containing growth media was used to maintain cells for 24 or 48 h exposure time.

#### *RNA isolation and quantitative real-time PCR (qRT-PCR)*

Total RNA was isolated from cells using TRIzol (Invitrogen) according to the manufacturer's instruction. A total of 300 ng of RNA was used

to synthesize the first strand complementary DNA using a PrimeScript RT Reagent Kit (Takara, Dalian, China). Quantitative real-time PCR was performed on a 7500 Real-time PCR System (Applied Biosystems, Carlsbad, CA, USA) with SYBR Premix Ex Taq (TaKaRa). U6 snRNA and GAPDH were used as the endogenous controls for miRNA and mRNA respectively. The relative expression of each gene was calculated and normalized using the 2<sup>-ΔΔCt</sup> method and all the reactions were run in triplicate.

#### *Cell viability measurement*

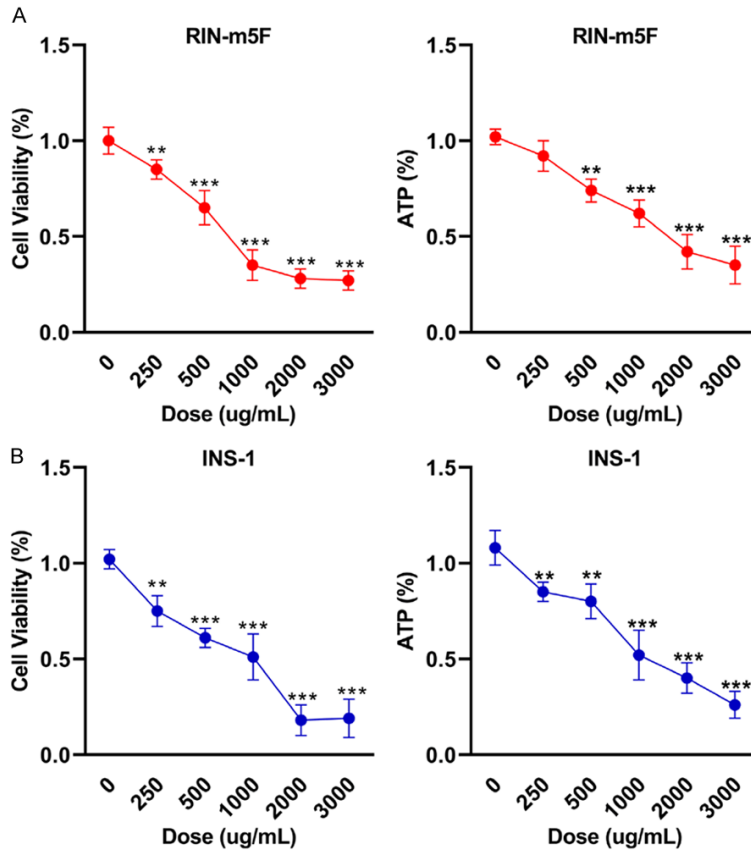
Cell viability was determined using an MTS assay, which was performed with a CellTiter 96 AQueous kit (Promega Corporation). In brief, 1 × 10<sup>4</sup> cells in 100 µL of the culture medium were plated in each well for overnight culture. The medium was then removed and cells treated for 24 h with DPE. Subsequently, the cell culture medium was removed and the plates washed three times with PBS. Afterwards, 100 µL of culture medium containing 16.7% of the MTS stock solution was added to each well for an hour at 37°C in a humidified 5% CO<sub>2</sub> incubator, and 80 µL of the supernatant was removed from each well and transferred into a new 96-well plate for readout at 490 nm on a Synergy HTX BioTek microplate reader (BioTek Instruments).

#### *ATP assay*

Cells grown in 96 well plates were treated with DPEs at different doses for 24 h. The plates were washed three times with PBS and 25 µL/well of ATPLite (Perkin Elmer, MA, USA) mixture was added. After 10 mins, the plates were shaken for 5 sec and placed on a microwell plate reader (BioTek), and the luminescence intensity was recorded.

#### *Measurement of cellular GSH*

Briefly, 1 × 10<sup>4</sup> cells were plated in 100 µL of the appropriate culture medium into 96-well plates (Costar, Corning, NY) overnight. The medium was then removed and cells were treated with DPE for 24 h. The culture medium was then removed and each well washed three times with PBS. Afterwards, 100 µL of the GSH-Glo reagent (Promega Corporation) was added to each well and incubated for 0.5 h at room temperature, and 100 µL of the Luciferin Detection Reagent was added for an additional 15 min at room temperature. The luminescence intensity



**Figure 1.** DPE exposure led to reduced cell viability and ATP production in pancreatic beta cell lines RIN-5mF and INS-1. Each experiment was performed in triplicate and the data are expressed as mean  $\pm$  standard deviation. \*\* $P < 0.01$  compared to control; \*\*\* $P < 0.001$  compared to control.

of each well was then measured with a microplate reader.

#### Reactive oxygen species (ROS)

Intracellular ROS formation was measured using the cell-permeant fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (H2-DCFDA, Cat. K936, BioVision) following the manufacturer's protocol. Cells were loaded with 10  $\mu$ M H2DCFDA and incubated for 45 min at 37°C in the dark. After incubation, cells were treated with a range of DEP extract concentrations for 150 min. Intracellular ROS content was then measured by BioTek fluorescent microplate reader. Assays were carried out in triplicate, and a minimum of 10,000 cell events were collected for each analysis.

#### Western blotting

Cells were lysed in a rehydration buffer containing 7 M urea, 2 M thiourea, 50 mM DTT, 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-pr

opanesulfonate (CHAPS), 5% glycerol, and 10% isopropanol. The total concentration of protein was quantified using a Bradford assay. Lysates were separated on a 12% Bis-Tris gel, electrophoretically transferred to nitrocellulose membrane, and blocked with 5% nonfat milk (Santa Cruz Biotech) in TBS-Tween (1  $\times$ ). The membranes were then incubated with a rabbit polyclonal antibody against human p53 (GeneTex) at a dilution of 1:1000 overnight, followed by incubation with HRP-linked anti-rabbit IgG (1:7500; GE Healthcare, Marlborough, MA). Detection was performed with the ECL western blotting reagent kit (GE Healthcare).

#### Glucose-stimulated insulin secretion and measurement

The cells were pre-incubated with Krebs-Ringer-HEPES buffer (KRHB) buffer containing 2.8 mM glucose for 1 h. Then the supernatant was discarded and cells were cultured in the above medium for 1 h. Afterwards, the cells were in-

cubated in KRHB supplemented with 28 mM glucose for 1 h. All the supernatants were collected and the insulin concentration was determined by a rat insulin ELISA (Mercodia, Sweden) according to the manufacturer's instruction.

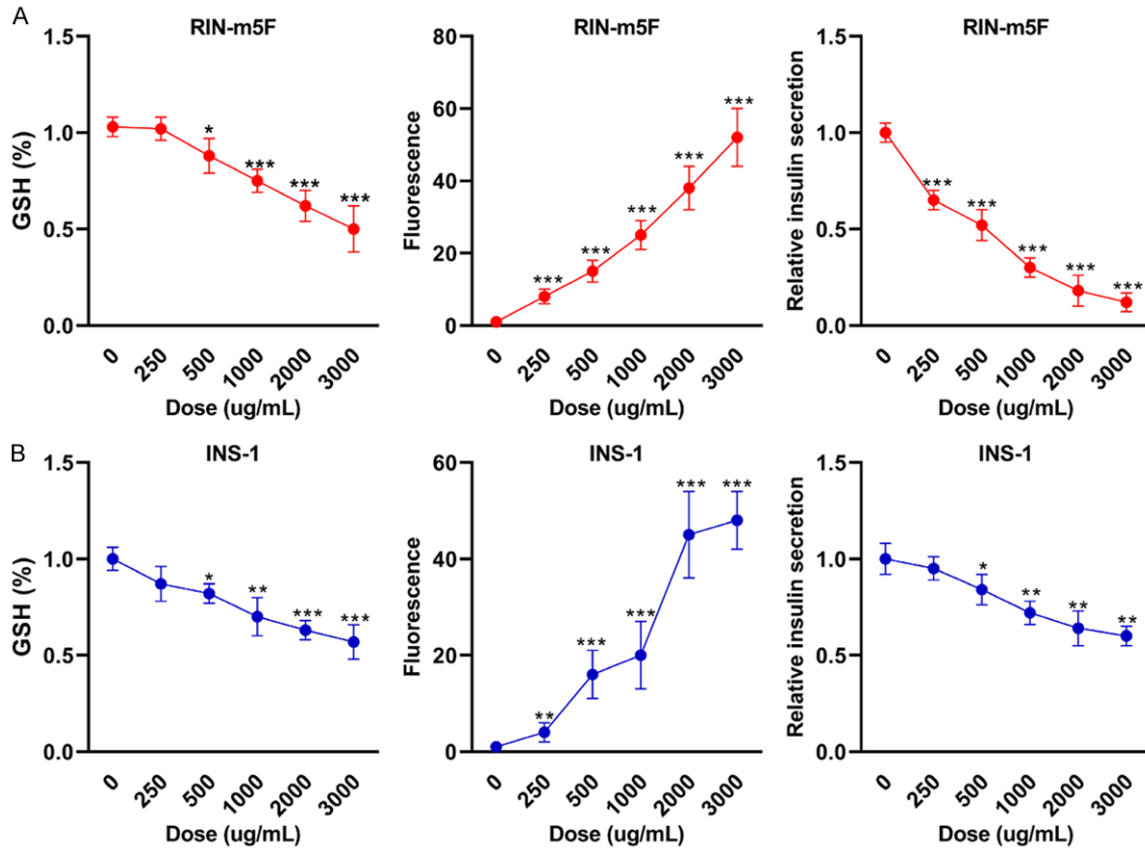
#### Statistical analysis

All data are presented as mean  $\pm$  SD. Statistical analysis was carried out using Student's t-test. The downstream targets of miR-140-5p were obtained from Targetscan7.0 and protein-protein interaction was analyzed using the String database. A difference between groups was considered significant if  $P < 0.05$ .

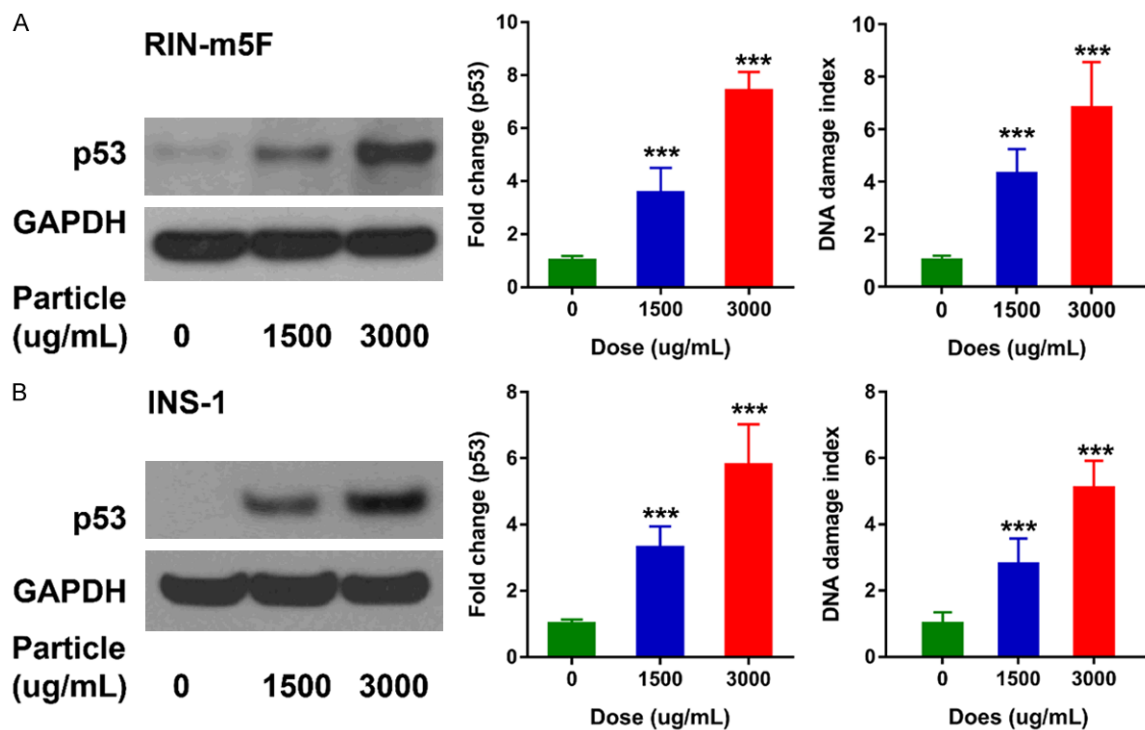
## Results

#### Cytotoxicity of pancreatic beta cells exposed to DPEs

As shown in **Figure 1A, 1B**, DPE exposure had substantial effect on the viability of pancreatic beta cells. Statistical analysis of the data indi-

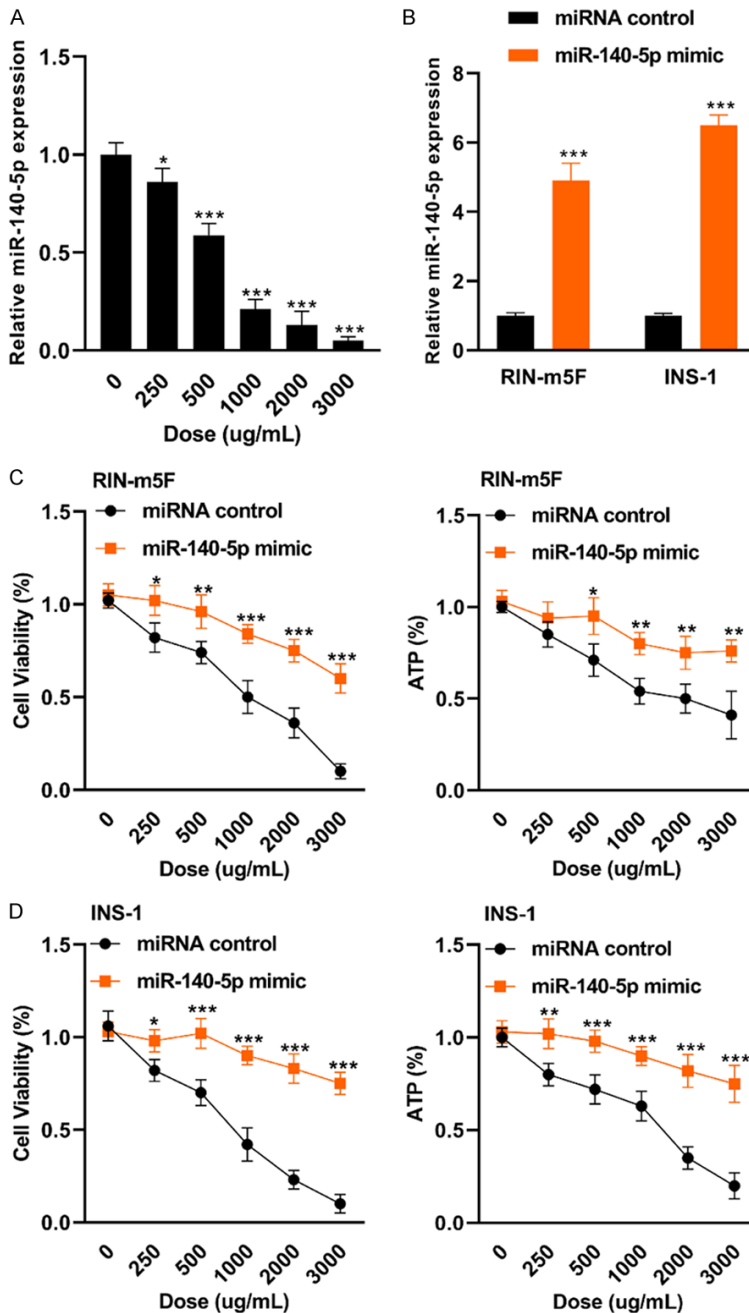


**Figure 2.** DPE exposure caused significant accumulation of ROS and reduced production of GSH and insulin in RIN-5mF and INS-1 beta cells. Each experiment was performed in triplicate and the data are presented as mean  $\pm$  standard deviation. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  when compared to control.



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**Figure 3.** DPEs induce the expression of p53 and DNA damage in pancreatic beta cells. The expression of p53 was measured by western blotting, and DNA damage analysis was performed by the alkaline Comet assay.  $**P < 0.01$ ,  $***P < 0.001$  when compared to control.



**Figure 4.** The expression level of miR-140-5p was significantly downregulated following DPE exposure. Overexpression of miR-140-5p partially rescued the reduced cell viability and ATP production caused by DPEs.  $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$  when compared to control.

cated significantly reduced cell viability after exposure to DPEs at all concentrations examined when compared to controls. There was a

dose-dependent effect of DPEs on cell viability. In addition, cells exposed to DPEs showed significant reduction of ATP levels ( $**P < 0.01$ ,  $***P < 0.001$ ).

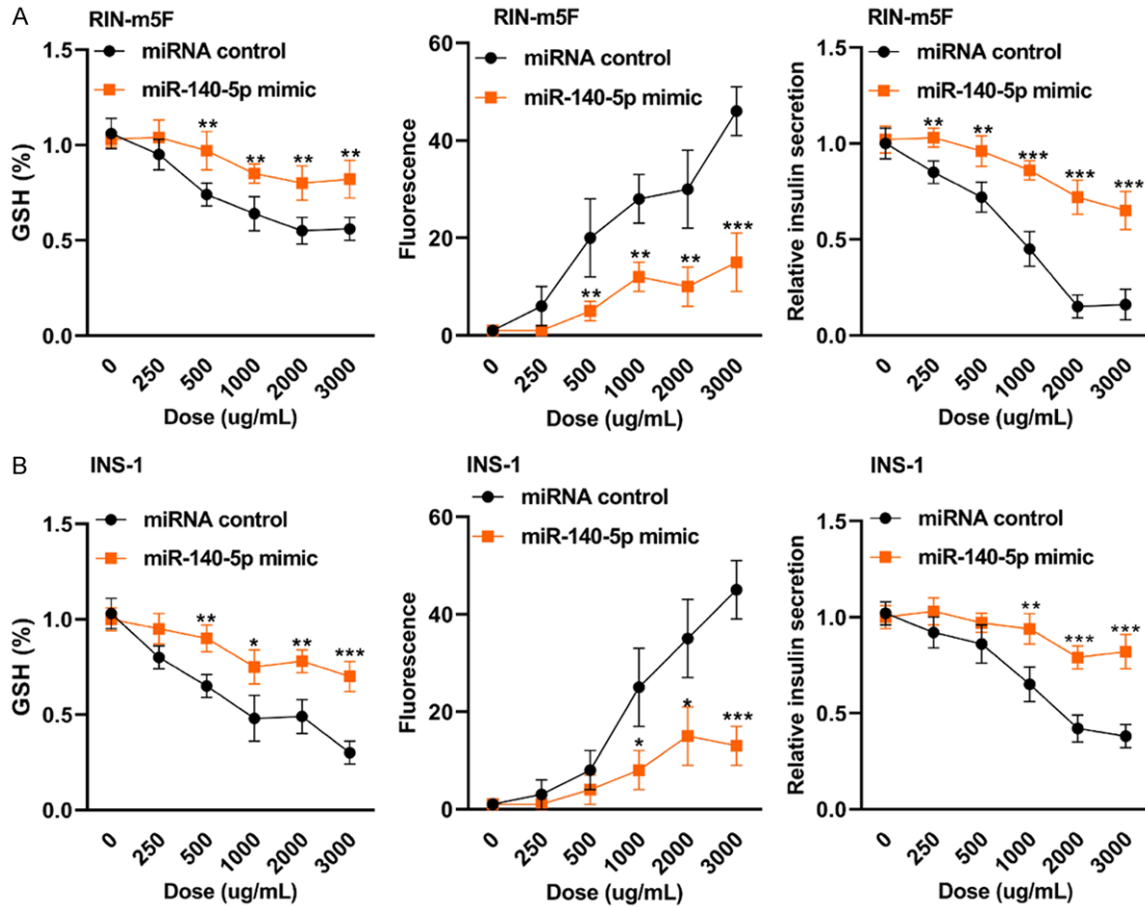
### ROS accumulation

Both lines of beta cells exposed to DPEs exhibited large dose-dependent increases in ROS. Statistical analysis showed significant increases over controls for all concentrations examined. Additionally, increases in response to DPE concentrations above 1000  $\mu\text{g}/\text{ml}$  led to significant increases in ROS production when compared to all lower doses. These data suggest that equivalent doses of DPEs have much higher potential to induce ROS production in beta cells. On the other hand, the levels of GSH in both types of beta cells were significantly down-regulated by exposure to DPEs in a dose dependent manner (Figure 2A, 2B) ( $**P < 0.01$ ,  $***P < 0.001$ ).

### DPE exposure induces p53 expression

p53 accumulation in both cell lines was determined, after exposing log-phase cells to DEP extract. As shown in Figure 3, exposure to DPEs induced significantly higher levels of p53 (compared to control) after exposure to the DPE concentrations tested. In addition to findings from cytotoxicity measurements, accumulation of p53 in cells further suggests cell stress responses, which may include DNA damage, in these beta cells exposed to DPEs ( $***P < 0.001$ ).

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**Figure 5.** Overexpression of miR-140-5p partially rescued the increased oxidative stress and reduced insulin production caused by DPEs. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  when compared to control.

*miR-140-5p is gradually downregulated following DPE exposure*

Our qRT-PCR results showed that the expression level of miR-140-5p was significantly decreased after DPE exposure in both cell lines. In addition, the effect of DPEs on the expression level of miR-140-5p was closely associated with the concentration of DPEs, and higher DPEs led to lower miR-140-5p levels (Figure 4A) (\* $P < 0.05$ , \*\*\* $P < 0.001$ ).

*Ectopic expression of miR-140-5p decreases the toxic effects of DPEs*

The cells were transfected with miR-140-5p mimic and miRNA control respectively. qRT-PCR results showed that the expression level of miR-140-5p was dramatically increased in the miR-140-5p group compared to the miRNA control group (Figure 4B) (\*\*\* $P < 0.001$ ). Following DPE exposure, MTS assay revealed that the

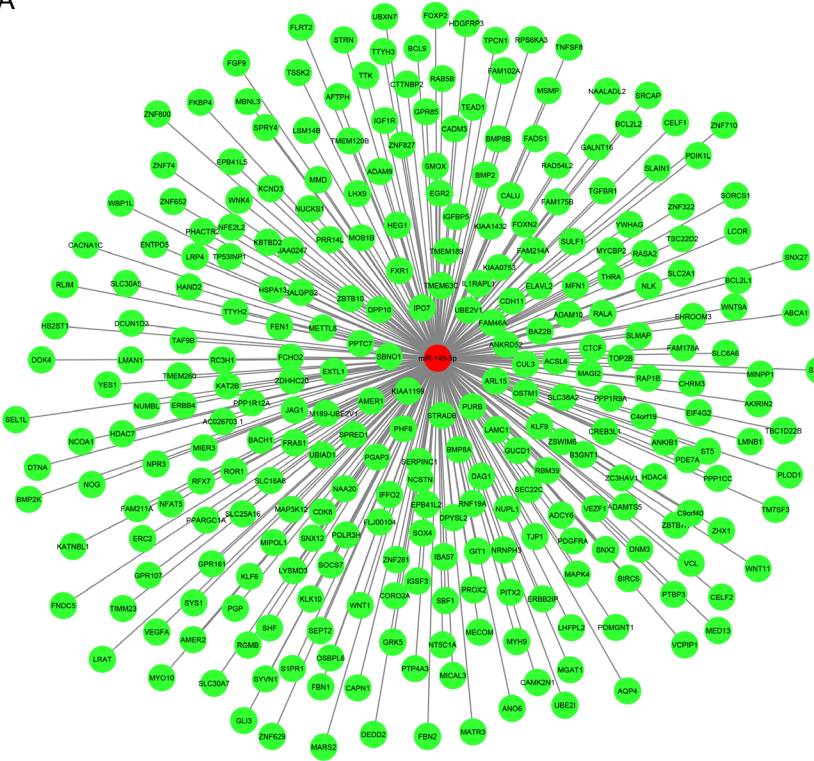
percentage of viable cells and the relative ATP levels were higher in the cells transfected with miR-140-5p mimic compared to those transfected with miRNA control (Figure 4C, 4D) (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ). Interestingly, miR-140-5p overexpression in both lines of beta cells partially decreased the ROS levels and increased GSH levels. In addition, upregulation of miR-140-5p also partially rescued the insulin secretion function suppressed by the DPEs (Figure 5A, 5B) (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).

*HDACs are downstream targets of miR-140-5p in beta cells*

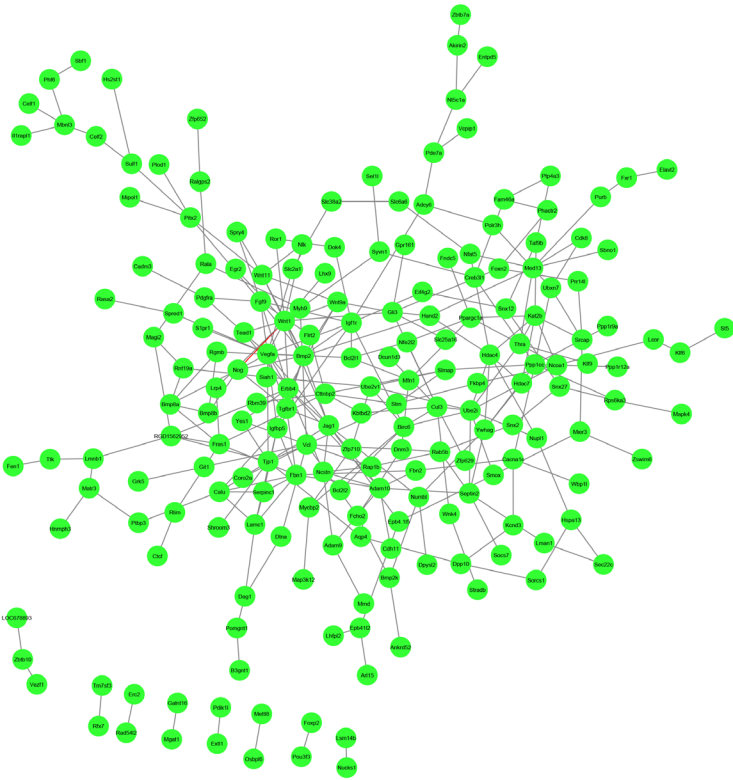
The potential downstream targets of miR-140-5p were obtained from Targetscan7.0 (Figure 6A). Our protein-protein analysis showed that HDCA4 was the central gene of the network (Figure 6B). In addition, qRT-PCR showed that the expression levels of HDAC4 and HDAC7

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A



B



C

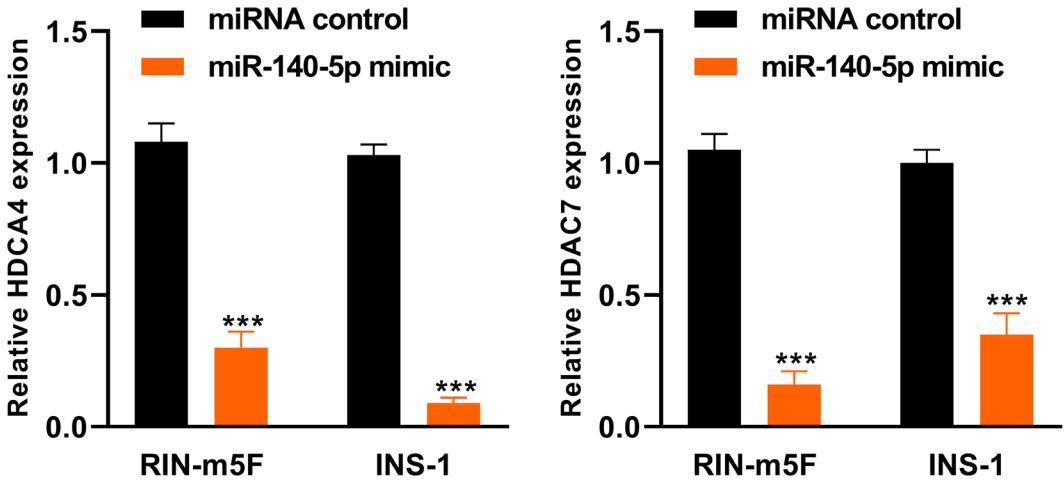


Figure 6. HDAC4 and HDAC7 were identified as downstream targets of miR-140-5p in beta cells. \*\*\*P < 0.001 when compared to control.

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were remarkably reduced following miR-140-5p transfection (**Figure 6C**) ( $***P < 0.001$ ).

## Discussion

To the best of our knowledge, this was the first study to report the toxic effects of DPEs on beta cells. Our results showed that DPEs led to significant increases in oxidative stress and cell death in beta cells. In addition, the expression level of miR-140-5p in beta cells was gradually decreased following DPE exposure. Ectopic expression of miR-140-5p reduced the oxidative stress levels, promoted cell survival, and protected the insulin secretion function of beta cells. Mechanistically, miR-140-5p might improve the insulin secretion function of beta cells by suppressing HDACs.

miRNAs have been demonstrated to be important regulators of diabetes pathogenesis, beta cell dysfunction and insulin production. Consistent with our findings, Delic *et al* compared the serum miRNAs profile between type 2 diabetes (T2D) rats and controls. The results showed that the expression level of miR-140 was significantly reduced in T2D rats, indicating that downregulation of miR-140 might promote the progression of T2D [14]. Pro-inflammatory cytokines could cause slight upregulation of miR-140-5p in beta cells. We speculated that beta cells needed to increase miR-140-5p levels to protect from destructive effects of cytokines [15]. Upregulation of miR-140-5p could protect HK2 cells from Cisplatin-induced oxidative stress through decreasing ROS level and increasing the expression of manganese superoxide dismutase, and Nrf2 was demonstrated to be the direct target of miR-140-5p [16]. Therefore, it is possible that miR-140-5p exerts its anti-oxidative stress effect by downregulating Nrf2 in beta cells subjected to DPE exposure. HDACs play an important role in regulating epigenetic modifications and thus are crucial for gene expression and cellular function modulation. Current evidence has indicated that HDACs are indispensable for maintaining glucose homeostasis and normal function of beta cells [17, 18]. For instance, the expression level of HDAC7 was significantly upregulated in human diabetic islets, and ectopic expression of HDAC7 resulted in beta cell dysfunction in an animal model [19]. Upregulation of HDAC4 and HDAC5 could reduce the number of insulin-producing  $\beta$ -cells [20]. These data

suggest that HDACs negatively regulates the normal function of beta cells. Therefore, it is not surprising that miR-140-5p could restore the insulin secretion of beta cells as both HDAC4 and HDAC7 are downstream targets of miR-140-5p.

In conclusion, our results have demonstrated that DPEs can significantly affect the normal function of beta cells, including reduced cell viability, ATP, and GSH levels, and insulin production. On the other hand, DPE exposure enhances ROS production, p53 expression and DNA damage in beta cells. In addition, upregulation of miR-140-5p partially rescues the toxic effects of DPEs on beta cells by inhibiting HDAC4 and HDAC7.

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## Disclosure of conflict of interest

None.

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