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Silence of HOTAIR inhibits insulin secretion and proliferation in pancreatic β cells

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Abstract. – OBJECTIVE: To examine the expression level of HOTAIR in pancreatic β cells. Moreover, regulatory effects of HOTAIR on insulin secretion, proliferation, cell cycle, and apoptosis in β cells are determined.

MATERIALS AND METHODS: HOTAIR levels in mouse primary pancreatic cells and MIN6 cell line were determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Its level was significantly observed in MIN6 cells treated with different doses of glucose. After the knockdown of HOTAIR, insulin secretion, cell cycle distribution, proliferation, and apoptosis in pancreatic β cells were assessed.

RESULTS: HOTAIR was abundantly expressed in pancreatic islets. HOTAIR level in pancreatic tissues of db/db mice was downregulated and influenced by glucose level. Knockdown of HOTAIR attenuated insulin secretion and synthesis capacities in both MIN6 cells and primary pancreatic cells, which may be related by the downregulation of MafA, Pdx1, and NeuroD. Moreover, the silence of HOTAIR suppressed proliferation, arrested cell cycle, and stimulated apoptosis in pancreatic β cells.

CONCLUSIONS: HOTAIR is highly expressed in pancreatic tissues. The silence of HOTAIR inhibits insulin secretion by downregulating insulin transcription-related genes. In addition, the silence of HOTAIR suppresses proliferation, arrests cell cycle progression, and induces apoptosis in pancreatic β cells.

Key Words:
HOTAIR, Pancreatic β cells, Insulin secretion.

Introduction

With the economic development, the incidence of type 2 diabetes mellitus (T2DM) rapidly increases. Currently, the number of T2DM patients in China ranks first worldwide, and has become the third prevalent chronic disease secondary to tumors and cardiovascular diseases[1]. Pancreatic β cells are the only type of cells capable of synthesizing and secreting insulin. They can regulate insulin secretion according to the organism needs and maintain the blood glucose balance[2]. The dysfunction of pancreatic β cells leads to insulin deficiency or resistance, which are the two major steps responsible for the pathogenesis of T2DM[3]. Dysfunction of pancreatic β cells is particularly critical in triggering T2DM[4].

Long non-coding RNAs (lncRNAs) are a type of functional RNAs with a transcript of more than 200 nucleotides in length, lacking the protein-encoding ability[5]. LncRNAs can regulate the protein-coding genes at different levels by stimulating mRNA degradation or inhibiting mRNA translation[6,7]. They are extensively involved in physiological and pathological processes. Abnormally expressed lncRNAs are closely related to human diseases[8-10]. Reports have shown that differentially expressed lncRNAs in islets are generally located near the regions where islet-specific protein-encoding genes belong to. They are co-expressed with T2DM susceptibility loci such as PDX1 and FOXA2, thus regulating islet differentiation, development, and maturation[11].

HOTAIR is located within the HOXC gene cluster on chromosome 12 and it is co-expressed with the HOXC genes[12,13]. It is proved that HOTAIR could inhibit tumor-suppressor genes (i.e., HOXD10 and PGR) and protocadherin genes in breast cancer cells[14,15]. HOTAIR is upregulated in cervical cancer tissues and peripheral blood of cervical cancer patients[16]. It is a risk factor leading to metastasis and death of cervical cancer by influencing corresponding miRNAs and Notch protein[17,18]. High level of HOTAIR is linked to unsatisfactory detective rate in cervical cancer patients[19,20]. Moreover, HOTAIR is found to be upregulated in many other types of malignancies[21,22]. In this paper, we focused on the biological function of HOTAIR in influencing pancreatic β cells, which may provide new directions for the clinical treatment of T2DM.

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Materials and Methods

Experimental Animals

8-week-old and 12-week-old C57BL/KsJ mice and db/db mice were provided by the Model Animal Research Center of Nanjing University. The animal study was approved by the Model Animal Ethics Committee of Shenzhen Mindray Biomedical Electronics Co., Ltd., Shenzhen, China.

Extraction of Mouse Primary Pancreatic Cells

After anesthesia by intraperitoneal injection of 10% chloral hydrate, the mouse was placed on the surgical table in a supine position. After skin disinfection, the abdominal cavity was exposed to find the common bile duct. Ligation was performed at duodenal papillary and proximal hepatic portal using 1-0 suture. Mice were sacrificed by femoral artery bloodletting. A 0.45-mm scalp needle was inserted into the common bile duct near the hilar, followed by injection of 2 ml of collagenase V (1 g/L) into the pancreas until the full expansion of pancreas. The pancreas was immediately harvested and placed in 37°C water bath for 20-25 min. Pancreas tissues were digested and washed in pre-cold Hank's buffer on ice. The mixture was applied in a culture dish and islets were manually selected under a stereo microscope.

Cell Culture and Transfection

MIN6 cells and primary pancreatic cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA). Cells were inoculated in 6-well plates with 4×10^5 cells per well and transfected using Lipofectamine 2000 at 60% confluence. The fresh medium was replaced at 6 h. Transfected cells for 48 h were collected for functional experiments.

Glucose Treatment

MIN6 cells were inoculated in a 24-well plate. Cells were treated with 5.5, 11.1, 25, or 33.3 mM glucose, respectively, for 24 h.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Cellular RNA was isolated using TRIzol method (Invitrogen, Carlsbad, CA, USA) and the extracted RNAs were subjected to reverse transcription according to the instructions of PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan). The RNA concentration was detected using a spectrometer. QRT-PCR was performed based on the instructions of SYBR Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). The relative level was calculated using the $2^{-\Delta\Delta Ct}$ method. Primer sequences were listed as follows: LncRNA HOTAIR: forward: GCGAGGTGCAAGAAAGCC; LncRNA HOTAIR: reverse: ACATGCCGCGTGATCCTA; Ins1: forward: CAATCATAGACCATCAGCAAG; Ins1: reverse: AGAAACCACGTTCCCCAC; Ins2: forward: CCCAGGCTTTTGTCAAACAG; Ins2: reverse: GTGCCAAGGTCTGAAGGTC; Pdx1: forward: AGCTCCCTTTCCCGTGGATGAAAT; Pdx1: reverse: TAGGCAGTACGGGTCCTCTTGTTT; MafA: forward: ATCATCACTCTGCCCACCAT: MafA: reverse: AGTCGGATGACCTCCTCCTT; NeuroD: forward: CAGGGTTATGAGATCGTC; NeuroD: reverse: GTTTCTGGGTCTTGGAGT; GLUT2: forward: ATCGCCCTCTGCTTCCAGTAC; GLUT2: reverse: GAACACGTAAGGCCCAAGGA; Nkx6.1: forward: CCGGTCGGACGCCCATC; Nkx6.1: reverse: GAGGCTGCCACCGCTCGATTT; CyclinD1: forward: CAGAGAGCTACAGACTCCGC; CyclinD1: reverse: CACAGGAGCTGGTGTTCCAT; CyclinD2: forward: AAGGAGGTAAGGGAAGCACTC; CyclinD2: reverse: CCTCGATGGTCAACAGGTTCT; CyclinD3: forward: AATGATGGCAGTGGATGGAGC; CyclinD3: reverse: CCTTTTGCACGCACTGGAAG; CyclinE1: forward: TCCACGCATGCTGAATTATC; CyclinE1: reverse: TTGCAAGACCCAGATGAAGA; CyclinE2: forward: AAAAAGTCTTGGGCAAGGTAAA; CyclinE2: reverse: GCATTCTGACCTGGAACCAC; β-actin: forward: TCCTGTGGCATCCACGAAACTACA; β-actin: reverse: ACCAGACAGCACTGTGTTGGCATA; glyceraldehyde 3-phosphate dehydrogenase (GAPDH): forward: GACTCATGACCACAGTCCATGC; GAPDH: reverse: AGAGGCAGGGATGATGTTCTG.

GSIS (Glucose-Stimulated Insulin Secretion)

Cells were seeded into a 24-well plate with 5×10^4 cells per well. On the next day, glucose-free KRH loading buffer was replaced. After 30-min incubation, cells were subjected to 1-h induction of 5.6 mmol/L glucose, followed by 1-h induction of 16.7 mmol/L glucose. The supernatant was collected and intracellular insulin was extracted by overnight incubation with acid-ethanol (0.15 M HCl in 75% ethanol). At last, supernatant and

intracellular insulin contents were measured by enzyme-linked immunosorbent assay (ELISA).

Cell Counting Kit (CCK-8) Assay

Cells were seeded into a 96-well plate with 1.5×10^3 cells per well. At the established time points, 10 μl of CCK-8 solution (Dojindo Laboratories, Kumamoto, Japan) was added in each well. After 2-h incubation, the absorbance at 450 nm of each sample was recorded.

Cell Cycle Determination

Cells were collected and incubated in pre-cold 70% ethanol for overnight fixation. On the next day, cells were centrifuged, resuspended in 50 μL of RNaseA, and subjected to 37°C water bath for 30 min. Subsequently, they were incubated with 450 μL of Propidium Iodide (PI) at 4°C for 30 min. The cell cycle distribution was analyzed by flow cytometry (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA).

Apoptosis Determination

Cells were collected and adjusted to 1×10^6 cells/mL. They were incubated with AV solution in the dark for 10 min, followed by incubation with 10 μL of PI for another 10 min. At last, the apoptotic percentage was determined by flow cytometry.

5-Ethynyl-2′-Deoxyuridine (EdU)

Cells seeded in a 96-well plate with 1×10^5 cells per well were labeled with EdU solution in dark for 30 min and stained with Hoechst 33342 for another 30 min. Images of EdU-labeled, Hoechst-labeled cells, and their merged images were taken under a fluorescence microscopy (magnification 40×).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 18.0 (SPSS Inc., Chicago, IL, USA) was used for data analysis. Data were expressed as mean ± standard deviation ($\bar{x}\pm$ S.D.). The *t*-test was utilized for analyzing differences between the two groups. $p<0.05$ considered the difference was statistically significant.

Results

HOTAIR Enrichment in Pancreatic Islet

HOTAIR levels were first determined in different organs of mice, including the lung, liver, heart, pancreas, spleen, muscle, and brain. QRT-PCR data showed a remarkable upregulation of HOTAIR level in mouse pancreas than that of other organs (Figure 1A). Furthermore, HOTAIR levels were examined in exocrine gland and islet, which was highly expressed in the latter (Figure 1B). Compared with control mice, 8-week-old, and 12-week-old db/db mice presented much higher blood glucose level, confirming the presence of hyperglycemia (Figure 1C, 1E). Relative levels of HOTAIR, Ins1, and Ins2 were lowly expressed in db/db mice compared to those of controls (Figure 1D, 1F). Subsequently, an *in vitro* hyperglycemia environment was established by glucose induction in MIN6 cells. HOTAIR, MafA, and Ins2 were dose-dependently regulated after treatment of different doses of glucose (Figure 1G). It is noteworthy that under the induction of 33.3 mM glucose, HOTAIR level was unexpectedly downregulated.

Silence of HOTAIR Suppressed Insulin Secretion in MIN6 Cells

To uncover the *in vitro* effects of HOTAIR on regulating pancreatic cells, three HOTAIR siRNAs were constructed. Transfection of either si-HOTAIR-2 or si-HOTAIR-3 could markedly downregulate HOTAIR2 level in MIN6 cells, while si-HOTAIR-1 failed (Figure 2A). In particular, si-HOTAIR-2 exhibited the most pronounced transfection efficacy and was utilized for the subsequent experiments. GSIS results demonstrated that under 20 mM glucose induction, insulin secretion in MIN6 cells with HOTAIR knockdown was remarkably attenuated (Figure 2B). Such a decline was not observed under 2 mM glucose induction. Knockdown of HOTAIR also decreased insulin content in MIN6 cells (Figure 2C). Relative levels of Ins1 and Ins2 were downregulated after the silence of HOTAIR (Figure 2D). Furthermore, key transcription factors involved in insulin regulation were determined. MafA, Pdx1, and NeuroD were downregulated, whereas Nkx6.1 and Glut2 were unchangeable in MIN6 cells transfected with si-HOTAIR-2 compared with those of controls (Figure 2E).

Silence of HOTAIR Suppressed Proliferation and Induced Apoptosis in MIN6 Cells

Flow cytometry data illustrated that MIN6 cells were mainly arrested in G0/G1 phase after the transfection of si-HOTAIR-2 (Figure 3A). Moreover, cell cycle-related genes were determined. CyclinD1, CyclinD2, CyclinD3, CyclinE1, and

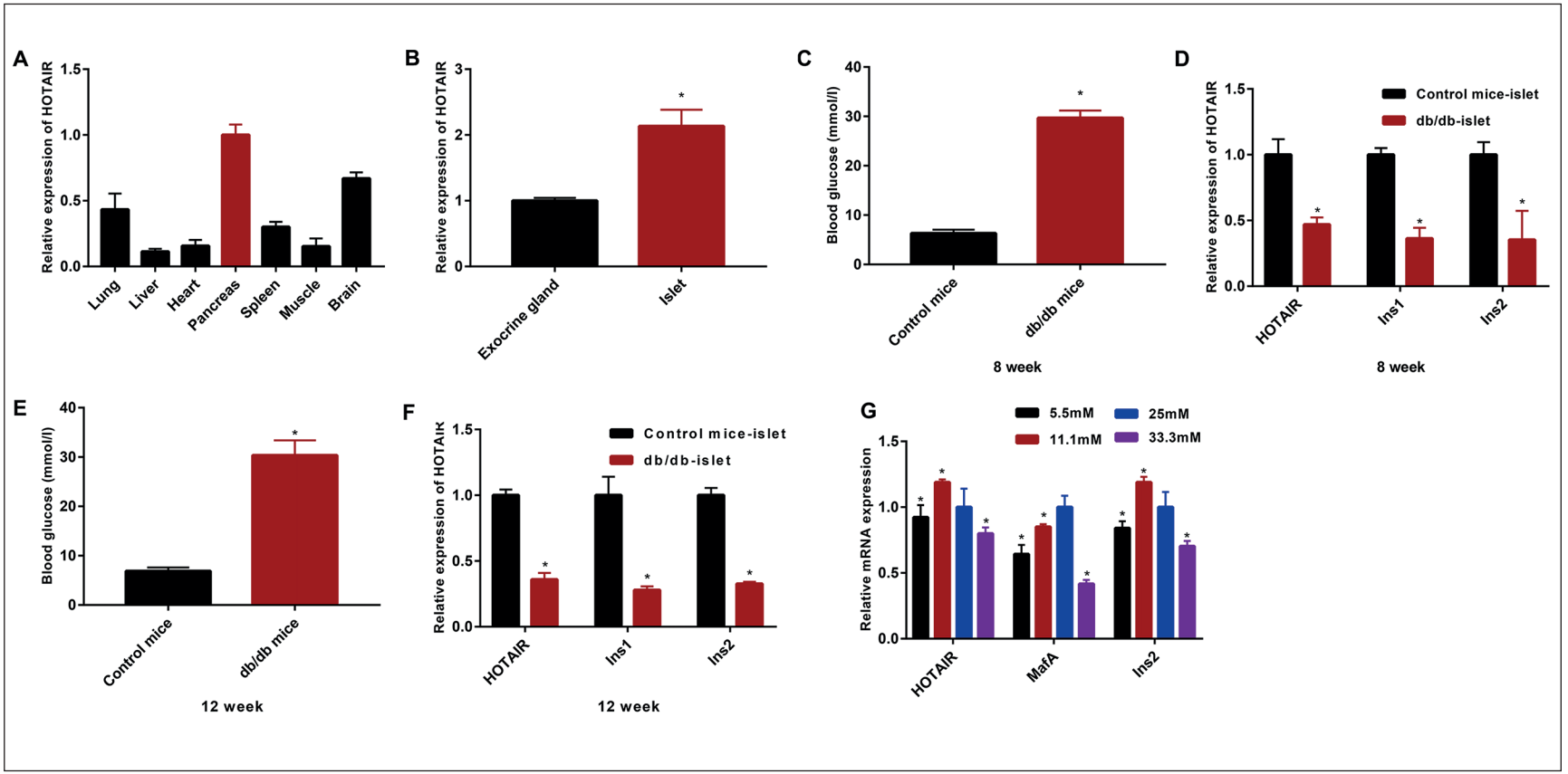

Figure 1. HOTAIR enrichment in pancreatic islet. **A,** HOTAIR levels in mouse lung, liver, heart, pancreas, spleen, muscle and brain. **B,** HOTAIR in mouse exocrine gland and islet. **C,** Blood glucose in control mice and db/db mice aged 8 weeks old. **D,** Relative levels of HOTAIR, Ins1 and Ins2 in islet tissues of control mice and db/db mice aged 8 weeks old. **E,** Blood glucose in control mice and db/db mice aged 12 weeks old. **F,** Relative levels of HOTAIR, Ins1 and Ins2 in islet tissues of control mice and db/db mice aged 12 weeks old. **G,** Relative levels of HOTAIR, Ins1 and Ins2 in MIN6 cells treated with 5.5, 11.1, 25- or 33.3 mM glucose.

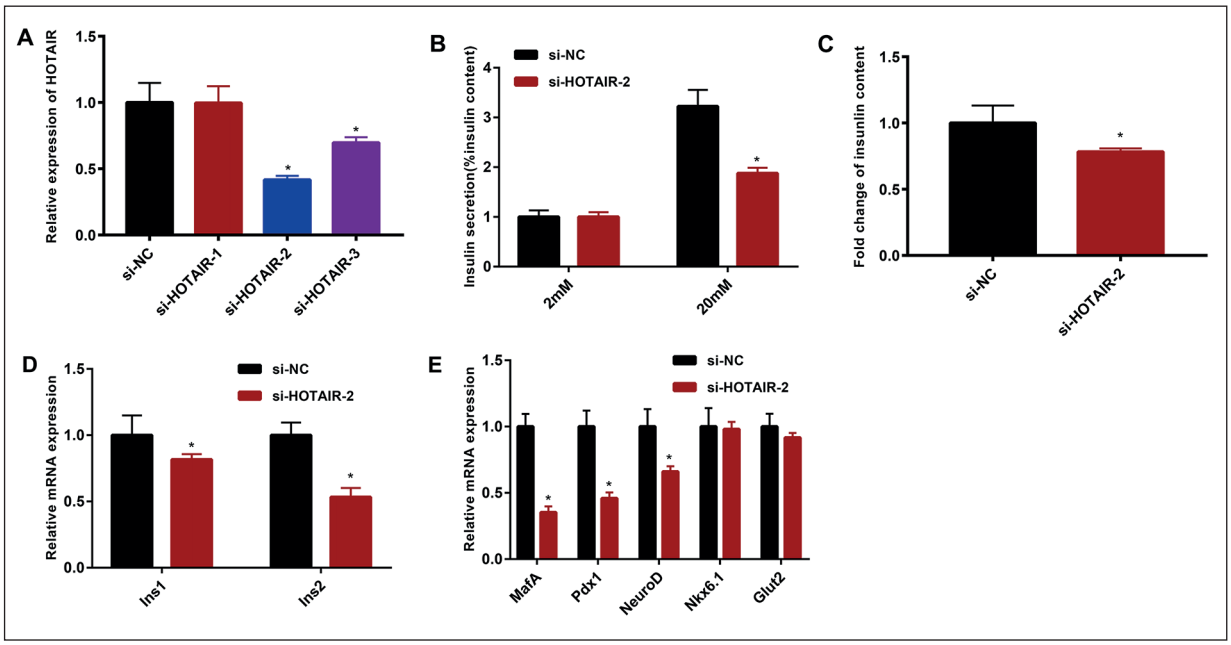

Figure 2. Silence of HOTAIR suppressed insulin secretion in MIN6 cells. **A,** Transfection efficacy of si-HOTAIR-1, si-HOTAIR-2 and si-HOTAIR-3. **B,** Insulin secretion in MIN6 cells transfected with si-NC or si-HOTAIR-2 under 2 or 20 mM glucose treatment. **C,** Insulin content in MIN6 cells transfected with si-NC or si-HOTAIR-2 under 20 mM glucose treatment. **D,** Relative levels of Ins1 and Ins2 in MIN6 cells transfected with si-NC or si-HOTAIR-2 under 20 mM glucose treatment. **E,** Relative levels of MafA, Pdx1, NeuroD, Nkx6.1, and Glut2 in MIN6 cells transfected with si-NC or si-HOTAIR-2 under 20mM glucose treatment.

CyclinE2 levels were all downregulated in MIN6 cells transfected with si-HOTAIR-2, whereas significant differences were only observed in the former three genes (Figure 3B). The proliferative change in MIN6 cells influenced by HOTAIR was evaluated as well. Reduced viability and EdU-positive ratio after transfection of si-HOTAIR-2 proved that HOTAIR accelerated MIN6 cells to proliferate (Figure 3C, 3D). Besides, the apoptotic rate was found to increase after silencing of HOTAIR (Figure 3E).

Silence of HOTAIR Suppressed Insulin Secretion in Mouse Primary Pancreatic Cells

To further validate our findings, primary pancreatic cells were isolated from db/db mice and cultured for experimental usage. Transfection of si-HOTAIR-2 effectively downregulated HOTAIR level in mouse primary pancreatic cells (Figure 4A). Similarly, under high-level glucose treatment, the insulin secretion ability was attenuated and insulin content was reduced in primary pancreatic cells transfected with si-HOTAIR-2, verifying the impaired insulin secretion capacity (Figure 4B, 4C). Relative levels of Ins1, Ins2, MafA, Pdx1, and NeuroD were all downregulated in primary pancreatic cells transfected with si-HOTAIR-2 (Figure 4D, 4E).

Discussion

LncRNAs are closely related to development, proliferation, and insulin production in pancreatic β cells. By detecting the lncRNA profile of human pancreatic β-cells, 1128 islet-specific lncRNAs have been discovered, and most of them were responsible for islet differentiation[23]. High-throughput sequencing analysis demonstrated that the classification of islet-specific lncRNAs is based on cell types. Some of them are distributed in α cells, while others are expressed in β cells[24]. A relevant work[25] uncovered a specific lncRNA expressed in β cells, which is 1600-fold higher than that of non-β cells. LncRNA-XLOC-019089 is only expressed in β cells, which could bind Pdx1 loci and further participate in insulin secretion. LincRNA-DYNL-RB2-2 is able to upregulate GRP119, a vital regulator for metabolic homeostasis maintenance and insulin secretion stimulation[26]. By targeting the insulin transcription

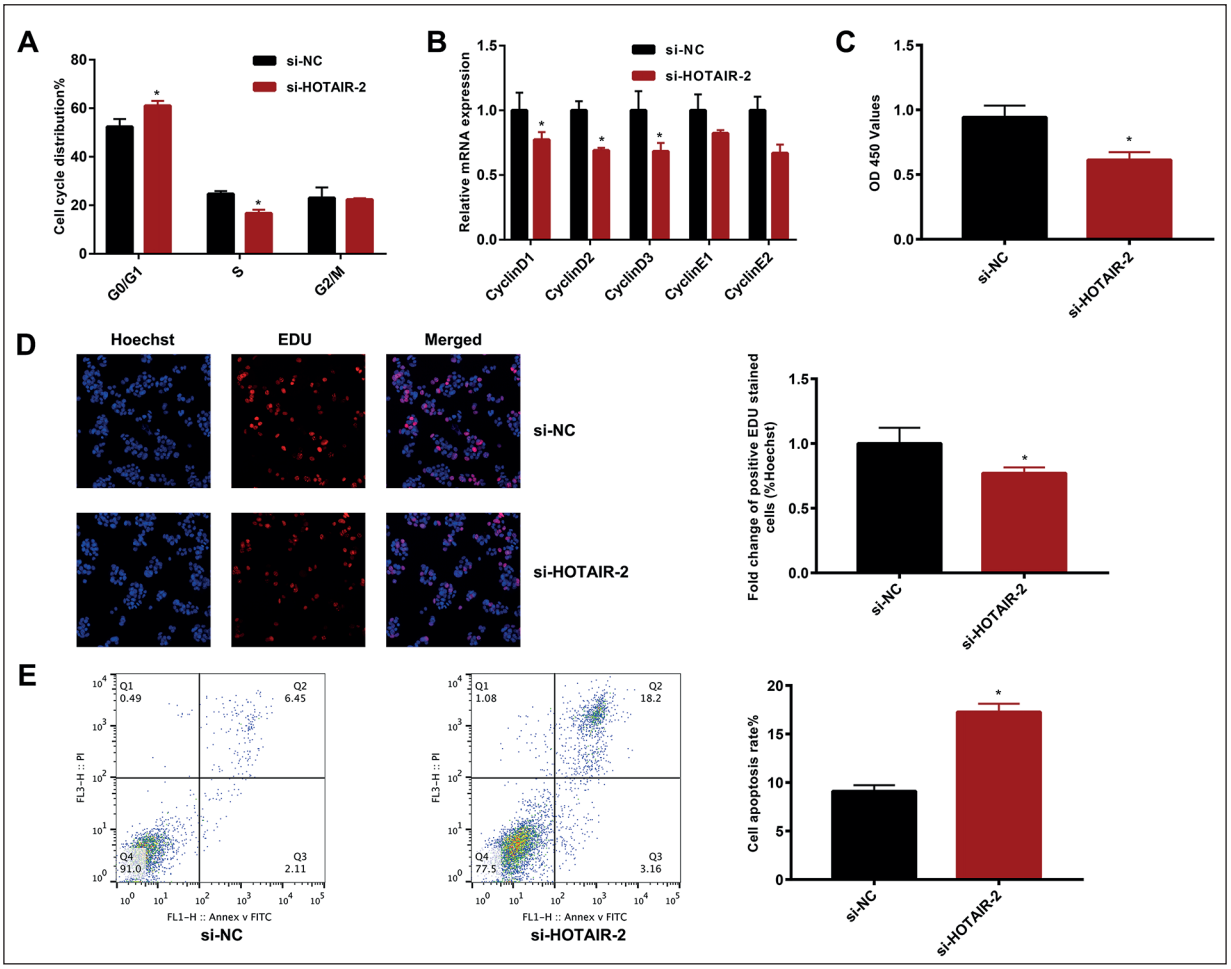

Figure 3. Silence of HOTAIR suppressed proliferation and induced apoptosis in MIN6 cells. **A,** Cell cycle distribution in MIN6 cells transfected with si-NC or si-HOTAIR-2. **B,** Relative levels of CyclinD1, CyclinD2, CyclinD3, CyclinE1, and CyclinE2 in MIN6 cells transfected with si-NC or si-HOTAIR-2. **C,** OD450 value in MIN6 cells transfected with si-NC or si-HOTAIR-2. **D,** EdU-positive ratio in MIN6 cells transfected with si-NC or si-HOTAIR-2 (magnification 40×). **E,** Cell apoptosis rate in MIN6 cells transfected with si-NC or si-HOTAIR-2.

factors, HI-LNC25 is specifically expressed in β cells and contributes to balance insulin secretion[23]. However, the knockdown of HI-LNC25 fails to decrease insulin secretion, suggesting the involvement of other important lncRNAs in regulating β cell functions[23].

In this paper, HOTAIR was found to be mainly expressed in islet tissues. Additionally, HOTAIR level was dynamically regulated by blood glucose levels. The silence of HOTAIR resulted in remarkable reductions in insulin synthesis and secreted levels. Relative levels of MafA, Pdx1, and NeuroD were downregulated by transfection of si-HOTAIR. MafA is necessary for the maturation of mammalian β cells, which becomes functional after birth[27]. MafA is a transcription factor and its downstream targets are vital mediators for insulin synthesis and secretion, including Pdx1, Glut2, and GLP1 receptors[28]. The maturation of insulin$^+$ cells is a well-ordered process, manifesting as the first observed MafB$^+$insulin$^+$ cells, followed by cells overexpressing Pdx1, and finally MafA$^+$insulin$^+$ cells[29]. In MafA$^{-/-}$ mice, multiple biological processes are altered, such as glucose transport, insulin transcription, glucose sensing, and insulin secretion. It is reported that β cell ratio remains to be normal in MafA$^{-/-}$ mice immediately after birth. However, the ratio of β cells to α cells gradually decreases, leading to the occurrence of glucose intolerance and T2DM[30].

There are still some shortcomings in this experiment. Previous studies[31] have found that

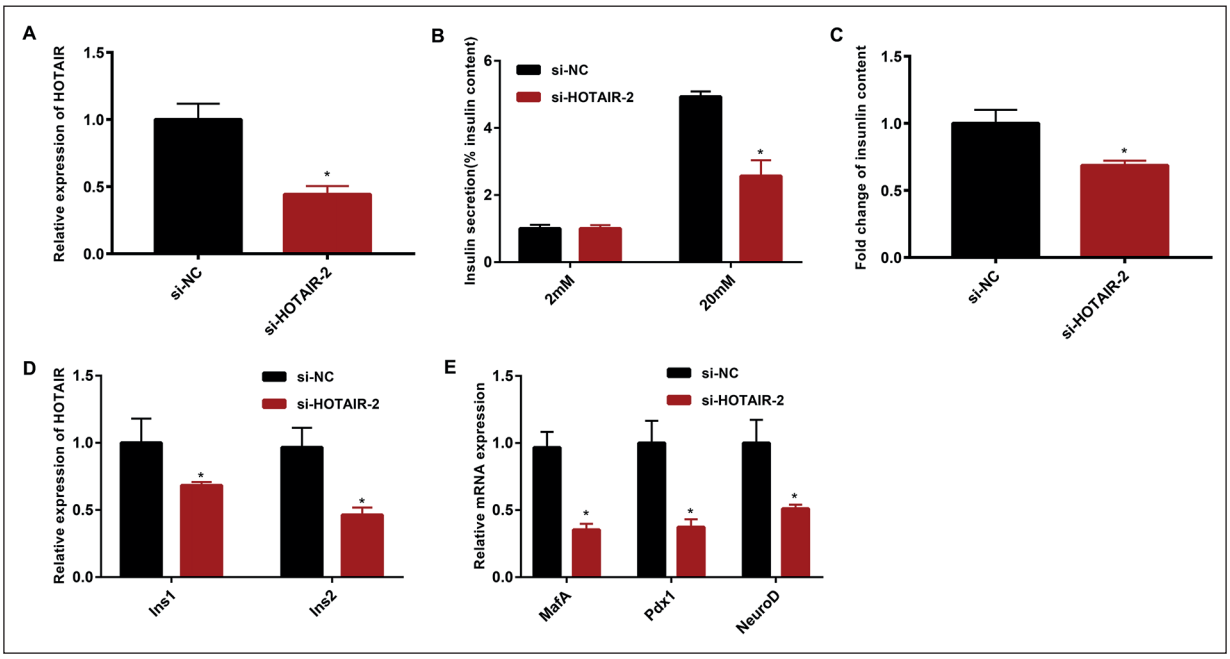

Figure 4. Silence of HOTAIR suppressed insulin secretion in mouse primary pancreatic cells. **A,** Transfection efficacy of si-HOTAIR-2 in primary pancreatic cells. **B,** Insulin secretion in primary pancreatic cells transfected with si-NC or si-HOTAIR-2 under 2 or 20 mM glucose treatment. **C,** Insulin content in primary pancreatic cells transfected with si-NC or si-HOTAIR-2 under 20 mM glucose treatment. **D,** Relative levels of Ins1 and Ins2 in primary pancreatic cells transfected with si-NC or si-HOTAIR-2 under 20 mM glucose treatment. **E,** Relative levels of MafA, Pdx1 and NeuroD in primary pancreatic cells transfected with si-NC or si-HOTAIR-2 under 20 mM glucose treatment.

PRC2 mediated by EZH2, SUZ12, and EDD (embryonic ectoderm development) could interact with HOTAIR to further influence target gene expressions. In tumor diseases, HOTAIR is capable of mediating oncogenes or tumor suppressors by targeting certain miRNAs[32,33]. Therefore, we speculated that HOTAIR may regulate expressions of Ins1 and Ins2 by targeting certain miRNAs, which are required for further validation.

Conclusions

We first found confirmed that HOTAIR is enriched in pancreatic tissues. The silence of HOTAIR inhibits insulin secretion by downregulating insulin transcription-related genes. In addition, the silence of HOTAIR suppresses proliferation and induces apoptosis in pancreatic β cells.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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