E3 ubiquitin ligase FBW7-mediated ubiquitination of EZH2 prevents type I diabetes in NOD mice

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Research

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Abstract

Background

Type I diabetes (T1D) is a heritable autoimmune disease targeting pancreatic beta cells. This study explores the molecular mechanism of E3 ubiquitin ligase FBW7 in reducing the occurrence of T1D.

Methods

The protein expression of FBW7, EZH2, and ZBTB16 was quantified in non-obese diabetic (NOD) mouse model of streptozotocin-induced T1D. Next, splenocytes and pancreatic beta cells were isolated to measure production of pro-inflammatory cytokines in splenocytes, as well as islet beta-cell apoptosis. Besides, the stability of EZH2 induced by FBW7 was analyzed by cycloheximide chase assay. The binding affinity of FBW7 and EZH2 and the consequence of ubiquitination were monitored by immunoprecipitation (IP) assay. Last, chromatin immunoprecipitation (ChIP) assay was employed to determine the accumulation of EZH2 and H3K27me3 at the ZBTB16 promoter region.

Results

We found downregulated FBW7 and ZBTB16, and upregulated EZH2 in diabetic NOD mice. Overexpression of FBW7 in the NOD mice inhibited the production of pro-inflammatory cytokines in the splenocytes and the apoptosis of islets beta cells. FBW7 destabilized EZH2 and accelerated ubiquitin-dependent degradation. EZH2 and H3K27me3 downregulated the ZBTB16 expression by accumulating in the ZBTB16 promoter and methylation.

Conclusion

FBW7 upregulates the expression of ZBTB16 by targeting histone methyltransferase EZH2 thus reducing the occurrence of T1D.

Background

Type I diabetes (T1D) is a heritable autoimmune disease [1]. Caused by autoimmune response against pancreatic beta cells, T1D results beta cell destruction and may accompanies with other autoimmune disease [2]. T1D is generally considered as a juvenile onset disease but it can occur at any age of life. Patients have to administrate insulin for survival causing tremendous burden to individuals and the society psychologically and economically [3]. T1D is a chronic autoimmune disease, which seriously impairs the pancreatic beta cells [4]. Healthcare provider are struggling for economic and standardized therapies.

F-box and WD repeat domain-containing 7 (FBW7, also known as FBXW7), an E3 ubiquitin ligase, is found to be a pancreatic beta cell apoptosis inhibitor *in vivo* [5]. Of note, a recent study has suggested that FBW7 prevents the development of insulin resistance in the T2D mice models [6]. Despite that a previous genome wide association studies (GWAS) showed FBW7 contributed to the risk for type 2 diabetes (T2D) [7], evidence for the association of FBW7 and T1D is limited. These results suggest that FBW7 may play a role in diabetes and encourage us to investigate the functional relevance of FBW7 in T1D.

As a potential substrate of FBW7, enhancer of zeste homolog 2 (EZH2) transfers a methyl group from S-adenosyl-L-methionine (SAM) to the lysine 27 on histone H3 [8]. Methylation by EZH2 assists the heterochromatin formation and suppresses the respective gene [9]. Interestingly, prior evidence has suggested that EZH2 was able to modulate pancreatic beta cell proliferation and regeneration in diabetes mellitus [10]. Recent report has documented that EZH2 can be suppressed by FBW7 in pancreatic cancer cells, thus restraining tumor cell migration and invasion [11]. Taken together, we reasonably propose EZH2 interacts with FBW7 and pancreatic cells in diabetes, which would fill the gap of FBW7 and T1D

Zinc finger and BTB domain containing 16 (ZBTB16, also known as PLZF), initially discovered as a cause of human retinoic acid-resistant acute promyelocytic leukemia, is a DNA sequence-specific transcriptional repressor [12]. Increasing studies have highlighted that ZBTB16 expression correlates with diabetes through the adaptive thermogenesis response, mitochondrial respiration promotion [13] and regulation of the insulin signaling pathway [14]. Importantly, ZBTB16 is found to be a possible candidate therapeutic target for T1D [15].

Here, we report that a regulatory axis of the three factors, FBW7, EZH2, and ZBTB16 contributes to the reduction of T1D occurrence. In this pathway, FBW7 degrades EZH2 and upregulates ZBTB16. We show that FBW7 is an emergent new therapeutic target for T1D treatment.

Methods

Ethics statement

The animal experimental processes were approved by the Ethnic Committee of Linyi People's Hospital and conducted in strict accordance to *the Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health.

Establishment of mouse model

A total of 70 male NOD mice aged 4 weeks were obtained from Shanghai Eason Biotech Co., Ltd. (Shanghai, China). The mice were randomized into the normal group (n = 10) and T1D model group (n = 60). For the streptozotocin (STZ)-induced T1D group, STZ (Sigma-Aldrich, Shanghai, China) at 40 mg/kg dose was administered intraperitoneally for 6 successive days. Nonfasting morning blood glucose concentrations were measured by tail vein prick using a OneTouch Ultra 2 blood glucose meter (LifeScan

Inc., Milpitas, CA, USA) every other day. When two successive blood glucose readings were more than 250 mg/dL, the mouse was considered as diabetic [16]. Afterwards, the occurrence was recorded every 6 days. The presented data were cumulative incidence of T1D collected from 3 independent experiments.

Immunohistochemistry (IHC)

The antigens were retrieved in a citrate repair solution. The tissue sections were subjected to blocking in a TBS solution containing 10% normal serum and 1% BSA for 2 hrs at room temperature. Each section was treated with antibodies at 4 °C overnight. Normal serum was employed as a negative control instead of primary antibody. Then, 50 µl of 3% H₂O₂ was added to each section, followed by incubation at room temperature for 20 min to eliminate endogenous peroxidase activity. Secondary antibody was added dropwise to each section, and incubated at 37 °C for 30 min. The sections were exposed to DAB reagent, and hematoxylin counterstaining. The brown color represented positive expression.

Vector construction and cell transfection

FBW7 and EZH2 coding region of the gene were ligated into pLVX-DsRed2-3FLAG vector. GFP Lentivirus control plasmid (Heyuan, Shanghai, China) was applied in this study. FBW7 shRNA, ZBTB16 shRNA, and negative control shRNA were obtained from Shanghai Gene Pharma Co., Ltd. (Shanghai, China) 293T cell was cultured and inoculated into petri dish 1 hr prior to transfection. Lentiviral vectors containing pLVX-FBW7 and pLVX-EZH2 were transfected to 293T cells.

After 24 hrs, the cell medium was changed to Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% feta bovine serum (FBS). After 48 hrs, the cells were centrifuged and the supernatant was collected, flited using a 0.45 µm PVDF filter, and kept at -80°C. The plasmids were classified as shControl group, shFBW7-1 group, OE-NC group, OE-FBW7, OE-FBW7 + OE-NC group, OE-FBW7 + sh-NC group, OE-FBW7 + OE-EZH2 group, and OE-FBW7 + sh-ZBTB16 group.

Lentiviral vectors with FBW7 overexpression, ZBTB16 interference, and control vectors were transduced into T1D mice via tail vein injection. The occurrence of T1D was determined after 24 hrs. The mice were sacrificed by cervical dislocation after 30 days. The pancreatic tissue, splenocyte, and pancreatic islet beta cells of mice were isolated [17]. Islet autoantigen glutamic acid decarboxylase (GAD65, 20 µg/ mL) was added to the splenocytes *in vitro* in order to induce T1D-specific T-cell response.

Isolation of mouse islets

Islets were isolated from the pancreas as follows. After occlusion of the distal end proximal to the duodenum, 4 mL collagenase P solution with the concentration of 1 mg/mL was slowly injected into the common bile duct. The distended pancreas was excised. The digestion was performed in a water bath at 37 °C for 30 min. The digest was subjected to a Ficoll gradient separation to facilitate the harvesting of islets, which was then picked by hand and counted under an inverted microscope.

Enzyme-linked immunosorbent assay (ELISA)

To determine the cytokine levels, splenocytes were cultured in DMEM supplemented with 10% FBS (0.2 mL) at a final concentration of 1.5×10^6 cells/ well. The supernatant was collected after 48 hrs and cytokines were assessed using quantitative ELISA kit (BD Bioscience, San Jose, CA, USA) [18].

Real-time polymerase chain reaction (RT-PCR)

Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA) and reverse transcribed to cDNA using PrimeScript™ RT Reagent Kit with gDNA Eraser (RR047A, Takara, Kusatsu, Japan). After a reverse transcription step at 37°C for 15 min followed by denaturation at 85°C for 5 sec, cDNA was mixed with TB Green Premix Ex Taq kit (RR420A, Takara, Kusatsu, Japan). Real-time PCR was conducted in ABI7500 system (Applied Biosystems, Foster City, CA, USA). Each sample was analyzed in triplicate. FBW7, EZH2, and ZBTB16 primer sequences were synthesized by Shanghai Sangon Biotech Co., Ltd (see Table 1 for primer sequence). CT values were recorded and GAPDH was served as the internal reference for FBW7, EZH2, and ZBTB16. The $2^{-\Delta\Delta CT}$ method was applied to calculate the relative expression of genes.

Table 1
Primer sequences for RT-qPCR

Gene of interest	Sequence
FBW7 (human)	Forward: 5'-CCACTGGGCTTGTACCATGTT-3'
	Reverse: 5'-CAGATGTAATTCGGCGTCGTT-3'
FBW7 (mice)	Forward: 5'-CGAGACTTCATCTCCTTGCTTCC-3'
	Reverse: 5'-CCAGAGAAGGTTATCCTCAGCC-3'
EZH2 (human)	Forward: 5'-GCCAGACTGGGAAGAAATCTG-3'
	Reverse: 5'-TGTGCTGGAAAATCCAAGTCA-3'
EZH2 (mice)	Forward: 5'-CAACCCGAAAGGGCAACAAA-3'
	Reverse: 5'-TCACCAGTCTGGATAGCCCT-3'
ZBTB16 (human)	Forward: 5'-GAGCTTCCTGATAACGAGGCTG-3'
	Reverse: 5'-AGCCGCAAACCTATCCAGGAACC-3'
ZBTB16 (mice)	Forward: 5'-ACATACGGGTGTGAACTCTGCG-3'
	Reverse: 5'-TGAGAACTGGGCACCGCATTGA-3'
GAPDH (human)	Forward: 5'-CACCCACTCCTCCACCTTTG-3'
	Reverse: 5'-CCACCACCCTGTTGCTGTAG-3'
GAPDH (mice)	Forward: 5'-CATCACTGCCACCCAGAAGACTG-3'
	Reverse: 5'-ATGCCAGTGAGCTTCCCGTTTCA-3'

Western blot assay

Tissue or cell total protein was extracted using RIPA lysis buffer (R0010, Solarbio, Beijing, China) at 4°C for 15 min followed by centrifugation at 15000 r/min for 15 min according to the user manual. The supernatant was collected, and the protein concentration was measured and normalized using bicinchoninic acid (BCA) kit (20201ES76, Yeasen, Shanghai, China). The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto polyvinylidene fluoride (PVDF) membrane, blocked in 5% BSA for 1 hr. The membrane was washed with TBST buffer for 5 min three times, and incubated at 4°C overnight with diluted primary antibodies: Fbxw7 (ab109617, 1:200), EZH2 (ab186006, 1:500), ZBTB16 (ab39354, 1:1000), FLAG (ab1162, 1:200), HA (ab9110, 1:2000), Myc (ab9106, 1:500), Ub (ab7780, 1:500). All the rabbit polyclonal antibodies were purchased from Abcam, Cambridge, UK unless otherwise noted.

After incubation, the membrane was washed with TBST buffer for 5 min three times. HRP labeled anti-Rabbit IgG H&L (ab205718, 1:10000) was added at room temperature. The membrane was incubated for 1 hr at room temperature followed by TBST wash for 10 min three times. The blot was visualized with enhanced chemiluminescence (ECL) reagents. The images were captured using FUSION FX5 (Vilber Lourmar, France), and analyzed by ImageJ 1.48u (National Institutes of Health, USA). GAPDH (ab9485, 1:500) was served as the internal reference. Each experiment was conducted in triplicate [18].

Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed to determine the accumulation of EZH2 and H3K27me3 in the ZBTB16 promoter region using the EZ-Magna ChIP kit (17-10086, Millipore, Billerica, MA, USA). Airway smooth muscle cells (ASMCs) in the logarithmic growth phase were crosslinked with formaldehyde at the final concentration of 1% for 10 min. Crosslinking was quenched by the addition of 125 mM glycine at room temperature for 5 min. Cells were washed with pre-cooled PBS buffer twice, collected after centrifuging for 5 min at 2000 rpm, and suspended in the lysis buffer with the concentration of 150 mM NaCl, 50 mM Tris (pH = 7.5), 5 mM EDTA, 0.005% NP40, 0.01% Triton X-100. The final concentration of cells was 2×10^6 per 200 mL. After adding the protease inhibitors, the mixture was centrifuged at 5000 rpm for 5 min. The cell pellet was resuspended in nuclear isolation buffer, lysed on ice for 10 min, sheared to 200–1,000 bp by sonication, and centrifuged at $14000 \times g$ at 4°C for 10 min. The supernatant (100 μ L), ChIP dilution buffer (900 μ L), protease inhibitor cocktail (PIC, 50 $\times$, 20 μ L), and Protein A-Agarose/Salmon Sperm DNA (60 μ L, Sigma-Aldrich, Shanghai, China) were mixed at 4°C for 1 hr and allowed to stand still at same temperature for 10 min. The mixture was centrifuged at 700 rpm for 1 min. The supernatant (20 μ L) from experimental group was mixed with EZH2 Ab (1 μ L, ab186006, 1:200), H3K27me3 Ab (1 μ L, ab192985, 1:200), and Protein A-Agarose/Salmon Sperm DNA (60 μ L). For the control experiments, rabbit IgG (1 μ L, zb186006, 1:200) was used. The system was incubated at 4°C for 2 hrs and allowed to stand still at the same temperature for 10 min followed by centrifugation at 700 rpm for 1 min. The supernatant was discarded. The precipitate was washed with low salt buffer (1 mL), high salt buffer (1 mL), LiCl aq. (1 mL), TE buffer (1 mL, 2 $\times$). ChIP wash buffer (250 μ L, 2 $\times$) was added to elute the complex. DNA was de-crosslinked and recycled by adding NaCl aq. (5 M, 20 μ L) in which RT-PCR was performed to quantify the LRP5 promoter [19].

Protein stability assay

Vector shControl and shFBW7 were transfected into Min6 cells per calcium phosphate method. After 24 hrs, the cells were treated with cycloheximide (CHX, CalBiochem, Gibbstown, NJ, USA) at a dose of 40 µg/mL. The cells were harvest and lyzed at designed time points. The lysate was analyzed by Western blot, imaged and quantified by Licor Odyssey and Image Quant software [20, 21].

Flow cytometry

Apoptosis of pancreatic beta cells was detected by flow cytometry. Pancreatic beta cells were isolated from T1D mice and cultured in the medium supplemented with TNF-α (10 ng/mL) for 3 days to simulate the insulin-dependent T1D [17]. The medium was discard and the cells were washed with PBS buffer twice and centrifuged. The cell pellet was resuspended in the binding buffer to give a single cell suspension with a concentration of 5×10^5 cell/mL. Annexin V (5 µL) and 7-aminoactinomycin D (7AAD, 5 µL) were added to the suspension at room temperature protected from light. The mixture was incubated for 30 min before determined by flow cytometry (BD Bioscience, San Jose, CA, USA).

Assessment of cytotoxicity by CCK-8 assays

Cells were treated with the same protocol as stated in the flow cytometry. Cytotoxicity was checked using the CCK-8 kit (Yisen, Shanghai, China). First, the cell suspension was placed in a 96-well plate with a concentration of 5×10^4 cell/well, and pre-warmed in the incubator (37°C, 5% CO₂). Next, CCK-8 (10 µL) was added to each well and the plate was incubated for 1–4 hrs. Finally, the absorbance at 450 nm was recorded on a microplate reader (Bio-Rad, Hercules, CA, USA) [18].

Assessment of insulin secretion

Min6 and pancreatic beta cells were inoculated on a 24-well plate at the concentration of 5×10^4 cell/well pretreated with or without Notoginsenoside R1 (NGR1). Next, the pancreatic beta cells were placed in the medium supplemented with TNF-α (20 ng/mL) for 3 days to simulate the islet inflammation in T1D. The supernatant was collected and the insulin concentration was determined by radioimmunoassay [17, 22].

Statistical analysis

Statistical and mathematical data were processed by SPSS 21.0. Values were reported as mean ± standard deviation. Data comparisons between two groups were analyzed by unpaired *t*-test. Comparisons among multiple groups were performed by one-way analysis of variance (ANOVA). Statistical analysis in relation to time-based measurements within each group was realized using repeated measures ANOVA. A value of $p < 0.05$ indicated significant difference.

Results

FBW7 is under-expressed in mouse model with T1D

The correlation between FBW7 and diabetic nephropathy has been previously established [23]. T1D mouse model was established using STZ. After 15 days of STZ administration, averaged blood glucose levels of the mice models exceeded 250 mg/dL, which confirmed the T1D model has been successfully established (Fig. 1A). FBW7 expression levels in the pancreatic islets of mice were examined by Western blot assay and IHC. A significant decrease in the FBW7 expression level was observed in the T1D model group (Fig. 1B and C). The above results concluded that FBW7 was down-regulated in T1D mouse model.

FBW7 suppresses the occurrence of T1D *in vivo* and *in vitro*

To further illustrate the impact of FBW7 on T1D, we overexpressed FBW7 in the T1D mouse model and analyzed the incidence of T1D in both groups. Compared to the normal group, T1D incidence was increased in the model group. Compared to the overexpression negative control (OE-NC) group, T1D incidence declined noticeably in the overexpression FBW7 (OE-FBW7) group (Fig. 2A). The size of the islet was measured after isolation. Results showed that mice islets were remarkably shrunken in the model group in comparison with the normal group while islets were expanded in the OE-FBW7 group as to the OE-NC group (Fig. 2B). FBW7 expression was identified using Western blot (Fig. 2C). Compared to the normal group, FBW7 expression in the mice islet from the model group was negatively regulated. The expression level in the mice islets of the OE-FBW7 group was positively regulated as to the OE-NC group. Therefore, we showed FBW7 decreased the T1D incidence *in vivo*.

Autoreactive T cells are considered as the major factor in the beta cell destruction. Therefore, we simulated the T1D-induced isletitis by mixing splenocytes and T1D-specific T cells and induced Min6 apoptosis in beta cells using inflammatory factor TNF- α . FBW7 was over-expressed in a part of cells from the model group. FBW7 expression in the splenocytes and pancreatic beta cells were checked by Western blot. We observed that FBW7 in both cell types from the model group was down-regulated compared to the normal group. Compared to the OE-NC group, FBW7 was reasonably up-regulated in both cell types from the OE-FBW7 group (Fig. 2D). ELISA was applied to measure the IL-17, IL-2, IFN- γ , IL-4 level in the cell supernatant (Fig. 2E). All four cytokines production has been elevated in the model group compared to the normal group while all have been depressed in the OE-FBW7 group with reference to the OE-NC group. These results demonstrated FBW7 can eliminate the activation of autoreactive T cells thus preventing T1D.

Additionally, pancreatic beta cell apoptosis was verified by flow cytometry (Fig. 2F). Apoptosis was accelerated in the model group relative to the normal group. Refer to the OE-NC group, apoptosis in the OE-FBW7 group decreased considerably. Assessment of glucose-stimulated insulin secretion disclosed that insulin secretion was lessened in the model group compared to the normal group while enhanced secretion was observed in the OE-FBW7 group in reference to the OE-NC group (Fig. 2G). Here, we have shown FBW7 decreased the T1D incidence both *in vitro* and *in vivo*.

FBW7 inhibits TNF- α -induced pancreatic beta cell apoptosis and dysfunction through ubiquitin degradation of EZH2

T1D downstream pathway affected by FBW7 was further investigated. Researchers reported that EZH2, highly expressed in T1D rat [24], was a substrate of FBW7 and was negatively regulated [11].

To clarify whether FBW7 acts as E3 ubiquitin ligase of EZH2, we transfected the FBW7 vector with Myc tag in gradually increased concentration, or empty vector, into Min6 cells, together with Flag-tagged EZH2 vector at a constant concentration. After 24 hrs, the cells were incubated with MG132, a proteasome inhibitor, or dimethyl sulfoxide (DMSO) for 16 hrs. Western blot revealed that ectopically expressed EZH2 was co-downregulated by FBW7 while under-expression of EZH2 was blocked by MG132 (Fig. 3A). We have tested the effect of FBW7 in endogenous EZH2 in pancreatic beta cells. Knocking down the endogenous FBW7 by two independent *Fbw7*-specific shRNAs enhanced the EZH2 expression level (Fig. 3B), leaving EZH2 mRNA expression unchanged (Fig. 3C). Moreover, Knocking down *Fbw7* increased the stability of endogenous EZH2 as shown in Western blot (Fig. 3D). Results obtained from immunoprecipitation demonstrated EZH2 polyubiquitination was diminished as *Fbw7* was knocked down (Fig. 3E). The results above indicate FBW7 boosted EZH2 ubiquitination and proteasome degradation.

Next, we explained the effect of the FBW7 and EZH2 on the TNF- α -induced pancreatic beta cells. FBW7 alone, or together with EZH2, was overexpressed in Min6 cells. As shown in Western blot, FBW7 was upregulated impressively while EZH2 was downregulated, which was then remedied by EZH2 overexpression (Fig. 3F). Cytotoxicity assay with CCK-8 and flow cytometry revealed that the OE-FBW7 + OE-NC group displayed higher cell viability and less apoptosis as to the OE-NC group while overexpression EZH2 simultaneously caused more cytotoxicity and accelerated apoptosis (Fig. 3G and 3H). Assessment of glucose-induced insulin secretion proved that more insulin was secreted from the OE-FBW7 + OE-NC group as to the OE-NC group while secretion from OE-FBW7 + OE-EZH2 group was uprooted (Fig. 3I). In short, we settled down with the conclusion that TNF- α -induced beta cells apoptosis and dysfunction was inhibited by FBW7 through ubiquitin degradation of EZH2.

EZH2 enhances TNF- α -induced pancreatic beta cells apoptosis and dysfunction through ZBTB16 methylation

Bioinformatic analysis revealed ZBTB16 was down-regulated in T1D patients and EZH2 suppressed ZBTB16 expression [19]. Min6 cell model was established by EZH2 overexpression, or with ZBTB16 overexpression simultaneously, and TNF- α induction. Western blot convinced the positive regulation of EZH2 and negative regulation of ZBTB16 in EZH2-overexpressed Min6 cells. Overexpression of ZBTB16 reversed its expression (Fig. 4A).

Considering the possibility of *Zbtb16* methylation, ChIP assay was performed to testify the enrichment of EZH2 and H3K27me3 on *Zbtb16* (Fig. 4B and C). EZH2 and EZH2-mediated H3K27me3 were accumulated in the *Zbtb16* promoter region.

Furthermore, cell viability has been tested using the CCK-8 kit. OE-EZH2 + OE-NC group showed worse cell viability compared to both the OE-NC group and the OE-EZH2 + OE-ZBTB16 group (Fig. 4D). Apoptosis was monitored by flow cytometry (Fig. 4E). The bivariate histogram displayed the apoptosis was

accelerated in the OE-EZH2 + OE-NC group compared to the OE-NC group. Apoptosis had been retarded in the OE-EZH2 + OE-ZBTB16 group as to the OE-EZH2 + OE-NC group. Glucose-induced insulin secretion assay proved that insulin secreted more in the OE-EZH2 + OE-NC group as to the OE-NC group while insulin secretion from OE-EZH2 + OE-ZBTB16 group was eliminated refer to the OE-EZH2 + OE-NC group (Fig. 4F). Therefore, we were confident to conclude that EZH2 was anti-correlated to ZBTB16. EZH2 mediated *Zbtb16* methylation thus enhancing TNF- α -induced pancreatic beta cells apoptosis and dysfunction.

FBW7 inhibits T1D occurrence via EZH2/ZBTB16 in vivo and in vitro

To verify the EZH2/ZBTB16 pathway in the T1D inhibition by FBW7, we transfected the shZBTB16 vector to the Min6 cells. The following experiments were conducted using sh-ZBTB16-1 cells selected by qRT-PCR (Fig. 5A).

T1D mouse model with *Fbw7* overexpression, or simultaneously with *Zbtb16* suppression using shRNA, were established and checked for diabetes incidence (Fig. 5B). Compared to the OE-FBW7 + sh-NC group, the OE-FBW7 + sh-ZBTB16 group showed a significantly higher occurrence of T1D. Islets were isolated and their sizes were measured and plotted (Fig. 5C). Islets from the OE-FBW7 + sh-ZBTB16 group shrank considerably referred to the OE-FBW7 + sh-NC group. Western blot has been done to determine the expression of FBW7, EZH2, and ZBTB16 in the islet (Fig. 5D). Expression levels of FBW7 and ZBTB16 were escalated while EZH2 declined in the OE-FBW7 + sh-NC group as to the OE-NC group. In comparison to the OE-FBW7 + sh-NC group, no significant change in FBW7 and EZH2 expression but a reduction in ZBTB16 expression was observed. From the above, we have shown the EZH2/ZBTB16 pathway played a role in T1D inhibition of FBW7 *in vivo*.

Meanwhile, mice splenocytes were reacted with T1D-specific T cells. A part of model cells overexpressed FBW7, or simultaneously suppressed *Zbtb16*. Pancreatic beta cell Min6 was induced to apoptosis by inflammatory factor TNF- α to simulate the isletitis T1D. Expression of FBW7, EZH2, and ZBTB16 in the splenocytes and pancreatic beta cells were revealed by Western blot (Fig. 5E). Compared to the OE-NC + sh-NC group, FBW7 and ZBTB16 were positively regulated while EZH2 was negatively regulated in the OE-FBW7 + sh-NC group. Refer to the OE-FBW7 + sh-NC group, expression of FBW7 and EZH2 stayed consistent while ZBTB16 decreased in the OE-FBW7 + sh-ZBTB16 group. Quantification of IL-17, IL-2, IFN- γ , IL-4 in the cell supernatant was achieved by ELISA (Fig. 5F). All four cytokines were boosted in *Zbtb16*-silenced cells compared to the OE-FBW7 + sh-NC group. Determination of pancreatic beta cell apoptosis by flow cytometry indicated expedited apoptosis occurred in the OE-FBW7 + sh-ZBTB16 group refer to OE-FBW7 + sh-NC group (Fig. 5G). Assessment of glucose-induced insulin secretion confirmed that less insulin was secreted in the OE-FBW7 + sh-ZBTB16 group as to OE-FBW7 + sh-NC (Fig. 5H). In conclusion, sufficient evidence is found to claim that FBW7 inhibited T1D occurrence via the EZH2/ZBTB16 pathway both *in vivo* and *in vitro*.

Discussion

In the past decades, our knowledge on T1D increased rapidly, but insufficient to satisfy the demands on standardized clinical treatment and contracting disease-associated burdens. Researches on T1D were concentrated on the mechanism of autoimmunity [25] and genetically relevant loci in human leukocyte antigen (HLA) [26, 27], which might benefit for T1D prediction and prevention [28]. Here in the present study, the mechanism of EZH2/ZBTB16 in T1D inhibition by FBW7 is clarified, suggesting that FBW7 is a promising target for T1D therapy.

Previous GWAS showed that more than 40 loci link to the risk of T1D [29], a few of which are non-HLA loci, e.g. INS, CTLA4, and PTPN22. We are interested in these non-HLA loci as HLA loci possibly associated with other autoimmune diseases. We started our further investigation from FBW7 located on chromosome 4, encoding an E3 ubiquitin ligase that targets key regulators of cell division and growth for ubiquitylation and subsequent degradation by the proteasome [30]. FBW7 suppresses colorectal cancer [31, 32], gastric cancer [33], and pancreatic cancer [34] through ubiquitin degradation of varied components in the pathway. The experimental data obtained from our study validated that FBW7 was downregulated both in T1D mouse model. Growing evidence has implied that F-box protein protects pancreatic beta cells and negatively regulates the cytokines in renal mesangial cells [23, 35]. Results in the present study confirmed that FBW7 impeded the T1D incurrence, corresponding to down-regulated cytokines expression and inhibited pancreatic beta cell apoptosis, indicating its significance in autoimmune diseases.

A wide variety of oncogenic proteins are found to be the substrates of FBW7 for ubiquitination including c-Myc [36], SREBP [37], and HIF-1 α [32], many of which play key roles in metabolic pathways [38]. We found EZH2 was the FBW7 substrate as we investigated the downstream pathway. Similarly, FBW7-dependent ubiquitination and degradation of EZH2 is found in the pancreatic cancer cells and prevents cancer migration and invasion [11]. EZH2 degradation by FBW7 is meanwhile reported in macrophage [39]. These results strongly suggest the important function of FBW7 in the regulation of the EZH2 protein level.

As the enzymatic subunit of Polycomb Repressive Complex 2 (PRC2), EZH2 epigenetically silences the genes in target promoters by histone H3 lysine 27 trimethylation [40]. In addition to H3K27me₃, EZH2 can also bind DNMT1 thus methylating DNA [41]. Kowluru et al. reported that DNMT1 alters the methylation status of MMP-9 promoter in diabetes [42, 43]. Studies on the parallel possible methylation pathway, as well as determination of 5mC and 5hmC levels at the ZBTB16 promoter, are currently ongoing in our lab.

ZBTB16 has been proved to be beneficial for tumor suppression as it represses c-myc oncogene [44]. ZBTB16 is critical in diabetes progression. Activation of ZBTB16 reduces serum advanced glycation end (AGE) products in STZ-induced diabetic mice and further inhibits AGE-induced apoptosis [45]. Furthermore, ZBTB16 involves in insulin signaling pathway regulation by reduction of phosphorylation levels of IRS1, Ake, and FoxO1 in the mouse model [46]. In our study, we described ZBTB16 negatively correlated to pancreatic beta cell apoptosis and contributed to beta cell viability. Therefore, the downstream pathway of T1D inhibition by FBW7 has been established.

Conclusions

Taken together, this study suggests the role of FBW7 (Fig. 6), which sits outside the HLA region, in the prevention of T1D occurrence. We established the FBW7/EZH2/ZBTB16 axis in the T1D pathogenesis and provided a new therapeutic target for T1D treatment. Further research should focus on other T1D-related genes and the detailed interaction of ZBTB16 and pancreatic beta cell apoptosis and dysfunction.

Abbreviations

T1D: Type I diabetes; NOD: non-obese diabetic; IP: immunoprecipitation; ChIP: chromatin immunoprecipitation; GWAS: genome wide association studies; EZH2: enhancer of zeste homolog 2; SAM: S-adenosyl-L-methionine; BCA: bicinchoninic acid; PVDF: polyvinylidene fluoride.

Declarations

Authors' contributions

JMT and YYZ designed the study. JMT, YYZ, SSL performed the experiment. YYZ and SSL analysed the data and wrote the manuscript. JMT, SSL participated in the design of the study and helped to draft the manuscript. YYZ and SSL helped to draft the manuscript and participated in the data analysis. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests

Availability of data and materials

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

Ethics approval and consent to participate

The animal experimental processes were approved by the Ethnic Committee of Linyi People's Hospital and conducted in strict accordance to *the Guide for the Care and Use of Laboratory Animals* published by

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Figures

Figure 1

FBW7 is under-expressed in T1D mouse model. A) blood glucose level in the mouse model; B) Western blot results of FBW7 expression in islets from T1D mice (n = 10) and control mice (n = 10), *, p < 0.05, vs. normal group; C) Representative images of IHC for FBW7 expression in islets from T1D mice and control mice (bar = 50 μ m). Measurement data are presented. Data comparisons between two groups were analyzed by unpaired t-test. Statistical analysis in relation to time-based measurements within each group was realized using repeated measures ANOVA.

Figure 2

FBW7 decreases the occurrence of T1D in vivo and in vitro. A) diabetes incidence of each group, *, $p < 0.05$, vs. normal group, #, $p < 0.05$, vs. OE-NC group; B) islet size of each group, *, $p < 0.05$, vs. normal group, #, $p < 0.05$, vs. OE-NC group; C) FBW7 expression in mice islet of each group determined by Western blot, *, $p < 0.05$, vs. normal group, #, $p < 0.05$, vs. OE-NC group; D) FBW7 expression in induced splenocytes and pancreatic beta cells of each group determined by Western blot, *, $p < 0.05$, vs. normal group, #, $p < 0.05$, vs. OE-NC group; E) level of IL-17A, IL-2, IFN- γ , IL-4 in cell supernatant of each group determined by ELISA, *, $p < 0.05$, vs. normal group, #, $p < 0.05$, vs. OE-NC group; F) pancreatic beta cell apoptosis of each group determined by flow cytometry, *, $p < 0.05$, vs. normal group, #, $p < 0.05$, vs. OE-NC group; G) insulin secretion of each group, *, $p < 0.05$, vs. normal group, #, $p < 0.05$, vs. OE-NC group. All experiments were conducted in triplicate. Data comparisons between two groups were analyzed by unpaired t-test. Comparisons among multiple groups were performed by one-way ANOVA. Statistical analysis in relation to time-based measurements within each group was realized using repeated measures ANOVA.

Figure 3

FBW7 inhibits TNF- α -induced pancreatic beta cells apoptosis and dysfunction through ubiquitin degradation of EZH2 A) Western blot assay of Min6 cells treated with MG132; B) Western blot assay of Min6 cells transfected with shFBW7, *, $p < 0.05$, vs. shControl group in FBW7 blotting, #, $p < 0.05$, vs. shControl group in EZH2 blotting; C) RT-PCR quantification of FBW7 and EZH2 mRNA in Min6 cell transfected with shFBW7, *, $p < 0.05$, vs. shControl group; D) Western blot of EZH2 after CHX treatment at different time points, EZH2 was first normalized to GAPDH then normalized to time 0 at each time point,

*, $p < 0.05$, vs. shControl group; E) After 48 hrs of transfection of designed plasmid, the Min6 cells was incubated with the MG132 (20 μM) for 8 hrs, Western blot analysis of the EZH2 ubiquitination following with IP assay; F) Western blot assay of the FBW7 and EZH2 proteins in the FBW7-, or with EZH2-, overexpressed Min6 cells, *, $p < 0.05$, vs. normal group, #, $p < 0.05$, vs. OE-EZH2 + OE-NC group, &, $p < 0.05$, vs. OE-EZH2 + OE-FBW7 group; G) CCK-8 assay for cell viability in each group, *, $p < 0.05$, vs. normal group, #, $p < 0.05$, vs. OE-EZH2 + OE-NC group, &, $p < 0.05$, vs. OE-EZH2 + OE-FBW7 group; H) flow cytometry determination for apoptosis in each group, *, $p < 0.05$, vs. normal group, #, $p < 0.05$, vs. OE-EZH2 + OE-NC group, &, $p < 0.05$, vs. OE-EZH2 + OE-FBW7 group; I) determination of insulin secretion, *, $p < 0.05$, vs. normal group, #, $p < 0.05$, vs. OE-EZH2 + OE-NC group, &, $p < 0.05$, vs. OE-EZH2 + OE-FBW7 group. All experiments were conducted in triplicate. Data comparisons between two groups were analyzed by unpaired t-test. Comparisons among multiple groups were performed by one-way ANOVA. Statistical analysis in relation to time-based measurements within each group was realized using repeated measures ANOVA.

Figure 4

EZH2 enhances TNF- α -induced pancreatic beta cells apoptosis and dysfunction through ZBTB16 methylation A) Western blot assay of the EZH2 and ZBTB16 proteins in the EZH2-, or with ZBTB16-, overexpressed Min6 cells, *, $p < 0.05$, vs. normal group, #, $p < 0.05$, vs. OE-EZH2 + OE-NC group, &, $p < 0.05$, vs. OE-EZH2 + OE-ZBTB16 group; B) ChIP assay for detecting EZH2 accumulation in ZBTB16 promoter, *, $p < 0.05$, vs. OE-NC group; D) ChIP assay for detecting H3K27me3 accumulation in ZBTB16 promoter, *, $p < 0.05$, vs. OE-NC group; D) CCK-8 assay for cell variability, *, $p < 0.05$, vs. normal group, #, $p < 0.05$, vs. OE-EZH2 + OE-NC group, &, $p < 0.05$, vs. OE-EZH2 + OE-ZBTB16 group; E) flow cytometry assay for apoptosis, *, $p < 0.05$, vs. normal group, #, $p < 0.05$, vs. OE-EZH2 + OE-NC group, &, $p < 0.05$, vs. OE-EZH2 + OE-ZBTB16 group; F) determination of insulin secretion, *, $p < 0.05$, vs. normal group, #, $p < 0.05$, vs. OE-EZH2 + OE-NC group, &, $p < 0.05$, vs. OE-EZH2 + OE-ZBTB16 group. All experiments were conducted in triplicate. Data comparisons between two groups were analyzed by unpaired t-test. Comparisons among multiple groups were performed by one-way ANOVA.

Figure 5

FBW7 inhibits T1D occurrence via EZH2/ZBTB16 in vivo and in vitro A) RT-PCR detection of ZBTB16 mRNA level in each group, *, $p < 0.05$, vs. OE-NC group; B) diabetes incurrence in each mouse model group, *, $p < 0.05$, vs. OE-FBW7 + sh-NC group; C) islet size in each mice model group, *, $p < 0.05$, vs. OE-NC group, *, $p < 0.05$, vs. OE-FBW7 + sh-NC group; D) Western blot detection of FBW7, EZH2, and ZBTB16 expression in islet, *, $p < 0.05$, vs. OE-FBW7 + sh-NC group, #, $p < 0.05$, vs. OE-FBW7 + sh-NC group; E) Western blot detection of FBW7, EZH2, and ZBTB16 expression in splenocytes and pancreatic beta cells, *, $p < 0.05$, vs. OE-FBW7 + sh-NC group, #, $p < 0.05$, vs. OE-FBW7 + sh-NC group; F) ELISA detection of IL-17A, IL-2, IFN- γ , IL-4 in cell supernatant, *, $p < 0.05$, vs. OE-FBW7 + sh-NC group; G) flow cytometry detection of pancreatic beta cell apoptosis in each group, *, $p < 0.05$, vs. OE-FBW7 + sh-NC group; H) determination of insulin secretion, *, $p < 0.05$, vs. OE-FBW7 + sh-NC group. All experiments were conducted in triplicate. Comparisons among multiple groups were performed by one-way ANOVA. Statistical analysis in relation to time-based measurements within each group was realized using repeated measures ANOVA.

Figure 6

The mechanism graph of the regulatory network and function of FBW7. E3 ubiquitin ligase FBW7 prevents type I diabetes by upregulating ZBTB16 expression via histone methyltransferase EZH2.