มหาวิทยาลัยมหิดล
Mahidol University

Final Report

Project title

Functional studies of Paired box 4 in

pancreatic beta-cells

By

Jatuporn Sujjitjjoon

Nov 2018

Contract no. TRG5980017

Final Report

Project title

Functional studies of Paired box 4 in pancreatic beta-cells

Jatuporn Sujjitjjoon

Mahidol University

The project granted by Thailand Research Fund and Mahidol University

(ความเห็นในรายงานนี้เป็นของผู้วิจัย

สกว.และต้นสังกัดไม่จำเป็นต้องเห็นด้วยเสมอไป)

Abstract

Project Code : TRG5980017

Project Title : Functional studies of Paired box 4 (Pax4) in pancreatic beta-cells

Investigator : Jatuporn Sujjitjoon

E-mail Address : jatuporn.suj@mahidol.ac.th, tangnina19@gmail.com

Project Period : June 2016 - Nov 2018

Polymorphisms of *Paired box 4 (PAX4*) were associated with type 2 diabetes (T2D) in East Asian population and mutations of *PAX4* caused maturity onset diabetes of the young, type 9 (MODY9). The role of Pax4 in pancreatic beta-cell survival and/or proliferation has been reported and a decrease in beta-cell number contributes to the pathogenesis of T2D and MODY. To better understand the critical role that Pax4 exerts in pancreatic beta-cells, this study generated *Pax4* knockdown INS-1 832/13 cells using specific siRNA and identified transcriptional regulatory network of genes involved in beta-cell survival. *PAX4* knockdown (KD) cells displayed a 2.5-fold increase in beta-cell apoptosis and reduce in beta-cell viability. Down-regulation of *Wnt3a*, *Bcl2l1*, and *Bcl2l2* gene and protein were confirmed in *PAX4* KD cells. *Wnt3a* and *Bcl2l2* (Bcl-w) were firstly described as target genes regulated by Pax4. Overexpression of PAX4 did not show any significant increase transcriptional activity on the *Wnt3a* and *Bcl2l2* promoters, while it had significantly increased transcriptional activity on the *Bcl2l1* promoter. The cells overexpressing HNF1A or HNF4A had significantly increased *Wnt3a* promoter activity, and those cells expressing HNF1A can also stimulate *Bcl2l2* gene promoter. Decreased expression of *Hnf1a,* but not *Hnf4a,* was observed in *Pax4* KD cells. Furthermore, we show that Pax4 may play roles in the regulation of *Hnf1a* gene expression. This finding suggests that decreased *Wnt3a* gene expression may be partly controlled by HNF4A and HNF1A, which is regulated by PAX4. For *Bcl2l1* gene promoter, we demonstrate that overexpression of PAX4, HNF1A**,** and HNF1A can activate the promoter activity. In addition, we confirm the crucial role of PAX4 in maintaining beta-cell number and survival of human pancreatic beta-cell line (1.1B4). In conclusion, Pax4 is a crucial transcription factor regulating beta-cell survival via Wnt3a signaling and anti-apoptotic pathways. The understanding of PAX4 function and its downstream target may offer a promising approach for endogenous replenishment of the lost beta-cell mass for DM treatment.

Keywords: Paired box 4, PAX4, MODY9, Beta-cell survival, Anti-apoptosis

บทคัดย่อ

รหัสโครงการ : TRG5980017

ชื่อโครงการ : การศึกษาหน้าที่ของโปรตีน Paired box 4 ในเซลล์เบต้าจากตับอ่อน

ชื่อนักวิจัย : จตุพร สุจจิตร์จูล/ มหาวิทยาลัยมหิดล

E-mail Address : jatuporn.suj@mahidol.ac.th, tangnina19@gmail.com

ระยะเวลาโครงการ : มิถุนายน 2559 ถึง พฤศจิกายน 2561

ความแปรผันของยีน *Paired box 4* หรือ *PAX4* มีความสัมพันธ์กับการเกิดโรคเบาหวานชนิดที่สองในประชากรชาวเอเชียตะวันออก และการกลายพันธุ์ของยีนยังเป็นสาเหตุของการเกิดโรคเบาหวานชนิดโมดีที่ 9 อีกด้วย โปรตีนนี้มีบทบาทสำคัญในเซลล์เบต้าจากตับอ่อน ช่วยสนับสนุนการเพิ่มจำนวนเซลล์และช่วยป้องกันการตายของเซลล์เบต้าในสภาวะต่างๆ ซึ่งการที่เซลล์เบต้ามีจำนวนลดลงนับเป็นพยาธิกำเนิดหนึ่งของโรคเบาหวานทั้งชนิดที่สองและโมดี ดังนั้น เพื่อเพิ่มองค์ความรู้ในเรื่องหน้าที่ของโปรตีน Pax4 ในเซลล์เบต้าในการควบคุมการอยู่รอดของเซลลี การศึกษานี้จึงทำการลดการแสดงออกของยีนในเซลล์เบต้าด้วยการใช้ siRNA และศึกษาการแสดงออกของยีนที่เกี่ยวข้องในกระบวนการอยู่รอดของเซลล์ รวมถึงกลไกระดับโมเลกุลของ Pax4 ในการควบคุมยีนเหล่านี้ด้วย ผลการศึกษาพบว่า เมื่อลดการแสดงออกของ Pax4 พบอัตราการตายของเซลล์เบต้าของหนูสูงขึ้นถึง 2.5 เท่า และมีอัตราการรอดของเซลล์ที่ลดลง นอกจากนี้ ยังพบการแสดงออกของยีนและโปรตีน *Wnt3a*, *Bcl2l1 (Bcl-xL)*, และ *Bcl2l2* (Bcl-w) ลดลงในเซลล์ที่ถูกลดการแสดงออกของ Pax4 ซึ่งการศึกษานี้เป็นการรายงานแรกที่แสดงว่า Pax4 น่าจะมีบทบาทในการควบคุมการแสดงออกของยีน *Wnt3a* และ *Bcl2l2* การศึกษานี้ยังพบว่า PAX4 ไม่สามารถกระตุ้น *Wnt3a* และ *Bcl2l2* ได้โดยตรง แต่สามารถกระตุ้น *Bcl2l1* ได้ เมื่อทดสอบด้วยวิธี luciferase promoter assay นอกจากนี้ ยังแสดงให้เห็นว่าเมื่อเพิ่มการแสดงออกของโปรตีน HNF1A หรือ HNF4A สามารถกระตุ้นการแสดงออกของ *Wnt3a* promoter activity ได้ นอกจากนี้ยังพบว่าโปรตีน HNF1A สามารถกระตุ้นการแสดงออกของ *Bcl2l2* promoter นอกจากนี้ Pax4 อาจมีบทบาทสำคัญในการควบคุมการแสดงออกของยีน *Hnf1a* อีกด้วย เซลล์เบต้าที่ถูกลดการแสดงออกของ *Pax4* พบระดับการแสดงออกของยีน *Hnf1a* ลดลงแต่ไม่มีผลต่อยีน *Hnf4a* ดังนั้น การแสดงออกของยีน *Wnt3a* น่าจะถูกควบคุมด้วย HNF4A และ HNF1A ซึ่งถูกควบคุมด้วยโปรตีน PAX4 สำหรับยีน *Bcl2l1* นั้นน่าจะถูกควบคุมด้วยโปรตีน PAX4 และ HNF-1A และ HNF-4A นอกจากการศึกษาในเซลล์เบต้าจากหนูแล้ว ผู้วิจัยได้แสดงให้เห็นความสำคัญของ PAX4 ที่ช่วยควบคุมการอยู่รอดของเซลล์เบต้าจากตับอ่อนของมนุษย์อีกด้วย โดยสรุป Pax4 เป็นโปรตีนสำคัญที่ทำหน้าที่ควบคุมการอยู่รอดของเซลล์เบต้าจากตับอ่อน โดยอาศัยการทำงานผ่าน Wnt3a signaling ที่น่าจะช่วยกระตุ้นการเพิ่มจำนวนเซลล์และผ่านทางการสร้างโปรตีนที่ช่วยต่อต้านการตายของเซลล์ ความรู้ความเข้าใจเกี่ยวกับบทบาทของ PAX4 และยีนเป้าหมายที่ถูกควบคุมโดย PAX4 นี้อาจจะนำไปสู่การค้นหาแนวทางการรักษาที่ดีขึ้นในการหาวิธีสร้างเซลล์เบต้าทดแทนเซลล์เบต้าที่ลดลง เพื่อใช้สำหรับการรักษาโรคเบาหวานในอนาคต

คำหลัก : Paired box 4, PAX4, โรคเบาหวานชนิดโมดี, การอยู่รอดของเซลล์เบต้าจากตับอ่อน, ภาวะต้านการตายของเซลล์

EXECUTIVE SUMMARY

Diabetes mellitus (DM) is a group of metabolic disorder characterized by hyperglycemia resulted from defects in insulin secretion, in insulin action, or the combination of both defects. Type 2 diabetes (T2D) is characterized by hyperglycemia resulted from the presence of insulin resistance and impaired insulin secretion. Maturity-onset diabetes of the young (MODY) is a monogenic form of diabetes, characterized by autosomal dominant inheritance, early age at onset, and pancreatic beta-cell dysfunction. Reduction of beta-cell function and beta-cell number can contribute to insulin deficiency in T2D and in MODY. Moreover, no controlled strategy for restoration of beta-cell mass has been identified. At the molecular level, pancreatic beta-cell loss by apoptosis appears to play an important role in the development of insulin deficiency and the onset and/or progression of the disease. Therefore, a more complete understanding of the mechanisms that control beta-cell proliferation, survival, and function is required in order to develop more effective therapies for diabetes. Our group discovered that mutations of *Paired box 4* (*PAX4*) gene causing MODY, type 9 (MODY9). Genetic variations of *PAX4* have been identified in various types of diabetes including type 1 diabetes (T1D), T2D, and MODY. We had demonstrated that PAX4 R164W mutant and R192H variant protein had decreased repression activity on human *insulin* and *glucagon* promoters. *Pax4* encodes a transcription factor that promotes pancreatic beta-cell development and survival. Since the survival or anti-apoptotic role of PAX4 protein has not totally been clear, we had identified potential target-gene of Pax4 in rat insulinoma cell line (INS-1 832/13). We demonstrate that suppression of *Pax4* gene in INS-1 832/13 cells increased cell apoptosis. *Pax4* knockdown (KD) cells had decreased expression of *Wnt3a*, *Bcl2l1*, and *Bcl2l2* genes, which encoded a growth-promoting factor or anti-apoptotic proteins. Our study originally reports that *Bcl2l2* (Bcl-w) and *Wnt3a* are newly identified genes regulated by Pax4. The molecular mechanism underlying that Pax4 protects beta-cell survival might be mediated by up-regulating anti-apoptotic genes, *Bcl2l1* (Bcl-xL) and *Bcl2l2* (Bcl-w), and/or *Wnt3a* gene.

In this project, we explore the exact molecular mechanism of Pax4 in regulating these identified target-genes including *Wnt3a*, *Bcl2l1*, and *Bcl2l2*, and confirm the role of PAX4 in human pancreatic beta-cell line (1.1B4). Molecular mechanism underlying Pax4 regulation on these target-genes were determined using luciferase promoter assay. Overexpression of PAX4 did not show any significant increase transcriptional activity on the *Wnt3a* and *Bcl2l2* (Bcl-w) gene promoters, while had significantly increased transcriptional activity on the *Bcl2l1* (Bcl-xL) gene promoter. The cells overexpressing HNF1A or HNF4A had significantly increased transactivation on the *Wnt3a* gene promoter, and those expressing HNF1A can also stimulate *Bcl2l2* gene promoter. Furthermore, we demonstrate the decreased expression of *Hnf1a,* but not *Hnf4a,* gene in *Pax4* KD cells while increased expression of these genes were observed in PAX4 overexpressing cells. Our results suggest that the decreased *Wnt3a* gene expression in *Pax4* KD cells may be partly controlled by other transcription factors, such as HNF1A and HNF4A, and Pax4 may play roles in the regulation of *Hnf1a* gene expression. For *Bcl2l1* gene promoter, we show that overexpression of PAX4, HNF-1**α,** and HNF-4**α** can activate the promoter activity. However, studying of exact binding site of PAX4 on this gene promoter is still needed to clarify further. In addition, we confirm a crucial role of PAX4 in human pancreatic beta-cell line (1.1B4) which is required to maintain cell survival and cell number. Human PAX4 might control Wnt3a and Bcl-w protein expression that are essential for promoting beta-cell proliferation and protecting beta-cells from stress-induced apoptosis. In conclusion, Pax4 is a crucial transcription factor regulating beta-cell survival via Wnt3a signaling and anti-apoptotic pathways. The understanding of PAX4 function and its downstream target may offer a promising approach for endogenous replenishment of the lost beta-cell mass for DM treatment.

Objectives

1. To investigate the molecular mechanism by which PAX4 regulates the Wnt signaling pathway and/or anti-apoptotic gene expressions
2. To examine the role of PAX4 in human beta-cell line (1.1B4) and to evaluate the function of human PAX4 on the newly identified target-genes

Research Methodology

1. Cell lines and reagents

1.1 Rat and human insulinoma cell lines

Rat insulinoma cell line (INS-1 82/13) and human pancreatic beta-cell line (1.1B4) cells are used in this study. These two cell lines are maintained in Roswell Park Memorial Institute (RPMI) 1640 (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 100 units/ml penicillin and 100 µg/ml streptomycin in a humidified atmosphere at 37°c and 5% CO_2. For INS-1 832/13 cells, the medium is supplemented with 1 mM sodium pyruvate, 50 µM β-mercaptoethanol, and 10 mM HEPES. The cells are sub-cultured twice per week following a standard trypsinization protocol.

1.2 Purified Wnt3a protein

Purified full-length mouse Wnt3a protein was purchased from R&D systems.

1.3 Antibodies

Antibodies against Pax4 was purchased from Abcam, CA, USA. Antibodies against Wnt3a, Bcl-xL, Bcl-w, beta-catenin were purchased from Cell Signaling Technology, MA, USA.

1.4 Small interfering RNA (siRNA)

Universal negative control siRNA and Stealth siRNA targeting rat *Pax4* were purchased from Invitrogen (CA, USA). siGENOME SMARTpool siRNA targeting human PAX4 and ON-TARGETplus Non-targeting Pool (negative control siRNA) were purchased from Dharmacon company (Lafayette, CO, USA).

1.5 Reagents and materials for RNA extraction, real-time PCR and Western blotting

- TRIzol® reagent and SuperScript® III Reverse Transcriptase kits were obtained from Life Technologies, Carlsbad, USA.

- Transcriptor First Strand cDNA Synthesis kit LightCycler® 480 SYBR Green I reagent were purchased from Roche Applied Science, IN, USA.

- Lipofectamine 2000™ transfection reagent was purchased from Invitrogen, CA, USA.

- Dual-Luciferase® reporter assay system was purchased from Promega, WI, USA.

- Protran nitrocellulose transfer membrane was obtained from Schleicher & Schuell, NH, USA.

- SuperSignal West pico Chemiluminescent Substrate was obtained from PIERCE, IL USA.

- FITC AnnexinV apoptosis detection kit was obtained from BD Biosciences, MD, USA.

1.6 Oligonucleotide primers

Primer name	Orientation	Nucleotide sequences (5'⟶3')	Length (bp)	Annealing Ta (°C)	PCR product size (bp)
rPax4-134-F	Forward	GGAGCCTTAAGGTATCTAACG	21	60	238
rPax4-371-R	Reverse	ATAGAGGATACACTGGGAGC	20	60	
rPax6-F	Forward	CCAGCATGCAGAACAGTCAC	20	60	207
rPax6-R	Reverse	GTCTGATGGAGCCAGTCTCG	20	60	
rWnt3a-F	Forward	TGAATTTGGAGGAATGGTCTCT	22	60	323
rWnt3a-R	Reverse	TGGGCACCTTGAAGTATGTGTA	22	60	
rCycD1-F	Forward	TGGCCTCTAAGATGAAGGAGA	21	56	170
rCycD1-R	Reverse	AGGAAGTGTTCGATGAAATCGT	22	56	
rBcl2l1-F	Forward	GAGCAGACCCAGTGAGTGAG	20	56	170
rBcl2l1-F	Reverse	GTCCTGTTCTCTTCGACATCG	21	56	
rBcl2l2-F	Forward	GAGTTTGAGACCCGCTTCC	19	56	362
rBcl2l2-R	Reverse	GTCCTCACTGATGCCCAGTT	20	56	
rBcl2l11-F	Forward	AGAGATACGGATCGCACAGG	20	56	100
rBcl2l11-R	Reverse	GTCTTCCGCCTCTCGGTAAT	20	56	
Beta-Actin-F	Forward	TGAAGATCCTGACCGAGC	18	60	387
Beta-Actin-R	Reverse	CAGTAATCTCCTTCTGCATCC	21	60	

2. Restoration of β-cell apoptosis in Pax4 KD cells upon Wnt3a treatment

To prove that increased cell apoptosis in the *Pax4* knockdown cells was mediated through the decreased Wnt3a protein, purified Wnt3a protein (100 ng/ml) was added into *Pax4* knockdown cells and examined for cell apoptosis. If the treatment with purified Wnt3a can reduce the cell apoptosis, it suggests that the anti-apoptotic role of Pax4 is mediated by promoting expression of Wnt3a protein. If this does not alter cell apoptosis, it is possible that anti-apoptotic role of Pax4 is not mediated through Wnt3a protein, but it may be mediated through others anti-apoptotic protein.

3. *In silico* prediction of potential PAX4-binding site on target-gene promoter

Potential Pax4-binding site within *Wnt3a*, *Bcl2l1*, and *Bcl2l2* promoters were predicted by using computational programs such as PROMO, TRANSFAC®, ContraV2, and MAPPER2.

4. Cloning of target-gene promoter in luciferase reporter plasmid

Wnt3a promoter region (-1000 to +50 relative to transcription start site) was cloned into a luciferase reporter plasmid, pGL3-*luc*+ vector. The pGL3-*luc*+ vector is a gift from Associate Professor Dr. Hiroto Furuta from Wakayama University, Wakayama, Japan. The *Bcl2l1* gene promoter (-227 to +562 relative to transcription start site) and *Bcl2l2* promoter (-796 to +236 relative to transcription start site) were inserted into the *Xho*I and *Kpn*I at the 5′ and 3′ site of pGL3-*Luc+* vector. Promoter region of human HNF1A encoding gene at position -685 to +285 relative to transcriptional start site was also cloned into pGL3-*Luc*+ vector, named as pGL3-*HNF1A* promoter plasmid. All constructs were verified the correct sequences by using automated DNA sequencing.

5. Analyses of Pax4 transactivation activity on target-gene promoter

Luciferase plasmid harbouring *Wnt3a, Bcl-xL, Bcl-w,* or *HNF1A* promoter was co-transfected with an expression plasmid containing *PAX4* wild-type cDNA, HNF1A, or HNF4A, together with a pRL-SV40 plasmid that encoding a renilla luciferase enzyme using for normalization. After transfection and cultured for 24 h, cells were harvested and lysed in a lysis buffer. Lysed supernatant was quantified for *firefly* and *renilla* luciferase enzymatic activities by using dual-luciferase assay in a luminometer machine (LumatLB9507). Calculation of normalized luciferase activity is followed this formula:

$$\frac{\text{[Firefly luciferase activity (Sample)/ Renilla Luciferase activity (Sample)]}}{\text{[Firefly luciferase activity (Basal control)/ Renilla Luciferase activity (Basal control)]}}$$

6. SiRNA-mediated knockdown of *PAX4* gene expression in human β-cell (1.1B4)

Small interfering RNA (siRNA) fragments against rat *Pax4* and human *PAX4* were purchased from Invitrogen and Dharmacon company, respectively. The optimal condition to suppress endogenous *Pax4* gene and protein expression were determined by varying a concentration of siRNAs and time of cell collection. Specific siRNA targeting *Pax4* and negative control siRNAs were transfected into INS-1 832/13 or 1.1B4 cells using lipofectamine 2000 reagents (Invitrogen).

7. Examination of Wnt3a and anti-apoptotic gene expressions in *PAX4* KD 1.1B4 cells by using polymerase chain reaction (PCR) and real-time PCR

To examine mRNA expression of the *Wnt3a* and *Bcl2l2* genes in *PAX4* KD cells, total RNA was prepared and was reverse transcribed into complementary DNA (cDNA) by using reverse-transcription PCR. Total RNA was extracted by using Trizol reagent (Invitrogen). RNA samples were reverse transcribed then cDNA templates were assayed for level of transcription using a pair of specific primers. For level of transcription of the genes in PAX4 KD or negative control cells, LightCycler® 480 SYBR Green I Master (Roche) was used and the reactions were recorded and analyzed using LightCycler® 480 Instrument equipped with a 96-well or 384-well thermal cycler (Roche).

8. Examination of Wnt3a and anti-apoptotic protein expressions in *PAX4* KD 1.1B4 cells by Western blot analysis

The expression of PAX4, Wnt3a, Bcl-xL, Bcl-w proteins were determined by using western blotting. PAX4 KD or negative control cells were collected by standard trypsinization, washed with PBS, and then lysed with lysis buffer. Protein content was measured by the Bradford assay (Bio-Rad), and the protein samples were separated on an 10-12% sodium dodesyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrically transferred onto nitrocellulose membrane (Bio-Rad). The target proteins on the blots were probed with the specific antibodies against Pax4, Wnt3a, beta-catenin, Bcl-xL, and Bcl-w, followed by horseradish peroxidase (HRP) conjugated antibodies for subsequence detection by SuperSignal West Pico Chemiluminescent Substrate (Pierce).

9. Analyses of human rat insulinoma cell survivals in *PAX4* knockdown cells

9.1 Cell viability assay

Cell viability of *Pax4* KD or negative control cells were determined by using PrestoBlue® Reagent (Invitrogen). INS-1 832/13 or 1.1B4 cells was cultured in 96-well plates (Costar) then after 24 h incubation, cells were transfected with siRNA targeting rat Pax4 or human PAX4 at optimal conditions. Then, PrestoBlue® dye was added to the plate and incubated for further 2-4 h then the absorbance of the color complex was read at 570 nm with a reference wavelength set at 600 nm using a BIO-TEK Power-Wave XS multiwell plate reader.

9.2 DNA synthesis using BrdU-incorporation assay

Proliferation of PAX4 KD or negative control cells with or without Wnt3a treatment was evaluated by using a 5'-Bromo-2'-deoxy-uridine (BrdU) labeling and detection kit (Roche Diagnostics). The cells were grown onto sterile coverglass in 24-well plate. After siRNA transfection and cultured for 24 h, the cells were incubated with a medium containing BrdU and incubated for 4-8 h. Then, cells were fixed and incubated with anti-BrdU antibody for 1 h followed by detecting with a fluorescein-conjugated IgG secondary antibody. Nucleus was counterstained with Hoechst33342 dye (Molecular Probe). Immunofluorescent stained cells were monitored under fluorescence microscope. Proliferation was quantified by counting cells positive for BrdU staining in total cell count.

9.3 Cell apoptosis by using AnnexinV/PI staining

Detection of cell apoptosis was performed by using Annexin-V/PI staining and flow cytometry. Pax4 KD and negative control cells were trypsinized and washed with ice-cold PBS. The cell pellet was washed in an Annexin-V buffer and resuspended in Annexin V-FITC/PI solution (BD Bioscience) and incubated on ice for 15 min. At least 10,000 cells were counted by FACSort^{TM} and FACSCalibur flow cytometers (Becton Dickinson) and data were analyzed by using Cell Quest software (Becton Dickinson) and Flowjo software (LLC, USA)

10. Statistical analysis

Statistical difference between means of two compared groups were determined by Student's t-test using SPSS software version 21.0 (SPSS Inc, NY, USA) and a GraphPad Prism5 software (GraphPad, San Diego, USA). *p*-value <0.05 was considered statistically significant.

Results

1. Examination of INS-1 cell viability in *Pax4* knockdown cells by using Prestoblue viability assay

INS-1 832/13 cells were cultured in 96-well plate and transfected with universal negative control siRNA and siRNAs targeting Pax4 using Dharmafect transfection reagent. After transfection and culture for 24, 48, and 72 h, cells were incubated with Prestoblue cell viability dye for 2 h and then absorbance were measured at 570 nm and 600 nm (reference wavelength). The results showed that *Pax4* knockdown cells had a significantly decreased cell viability when compared to that of negative control cells in every time points of measurement (Fig 1). The results were confirmed in three independent experiments.

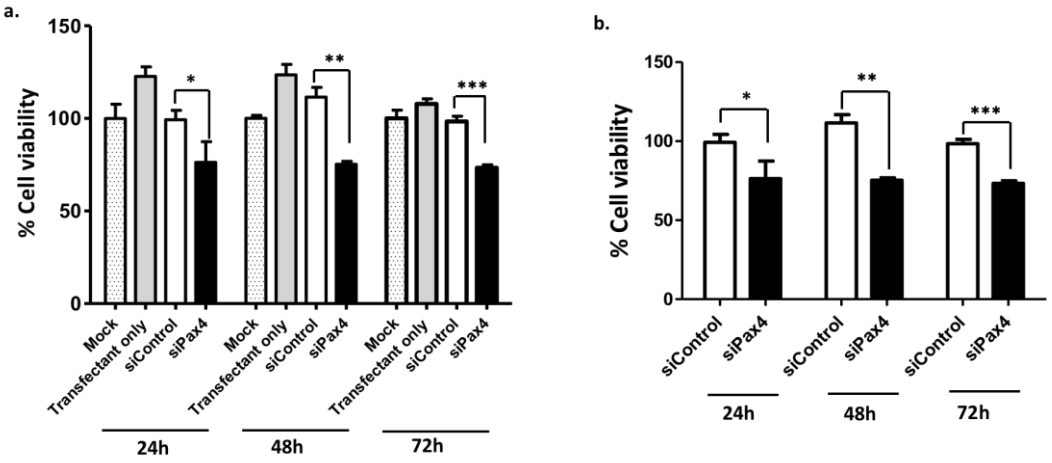


Figure 1. Cell viability assay of INS-1 832/13 cells. INS-1 832/13 cells transfected with siRNA targeting Pax4 and negative control a. Viability of INS-1 cells in various conditions including non-transfection control (Mock), cells with transfection only, cells transfected with siControl, and those cells with siPax4. b. Viability of INS-1 cells transfected with siControl and siPax4 at 24, 48, and 72h. Results are mean $\pm$ standard error of the mean (SEM) [n= 4]. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

2. Examination of INS-1 cell proliferation/DNA synthesis in *Pax4* knockdown cells by using BrdU-incorporation assay

INS-1 832/13 cells were transfected with negative control and Pax4 siRNAs by using Dharmafect transfection reagent. Two days after transfection, complete media containing BrdU was added into the cells and incubated for 6 or 24h in CO_2 incubator. BrdU-labelling cells were fixed and stained with anti-BrdU antibody followed by incubating with fluorescein-conjugated secondary antibody. Stained cells were monitored under fluorescence microscope. The results were derived from two independent experiments. There is no difference in proliferation were detected in *Pax4* KD INS-1 cells as compared to negative control INS-1 cells (Fig 2).

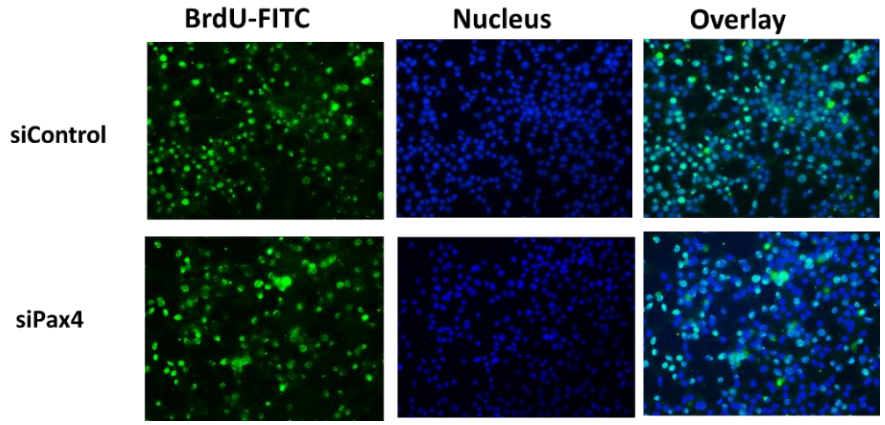


Figure 2. BrdU-incorporation assay of INS-1 832/13 cells. INS-1 832/13 cells were transfected with siControl and siPax4. After transfection and culture for 48 h, cells were incubated with BrdU-labelling medium for 6 h and immunostained with anti-BrdU followed by fluorescein-conjugated antibody. Nucleus was counterstained with Hoechst33342. Stained cells were monitored under fluorescence microscope.

3. Determination of molecular mechanism underlying Pax4 regulation on the Wnt signaling pathway

To explore whether addition of exogenous Wnt3a protein can rescue cell apoptosis, knockdown cells were treated with recombinant Wnt3a protein and examined using Prestoblue viability assay and AnnexinVPI staining. INS-1 832/13 cells

were cultured in 96-well plate and transfected with negative control and Pax4 siRNAs. The cells were co-incubated with recombinant Wnt3a protein 50 μg/ml or 100 μg/ml for 48 h and then measured viability of cells by using Prestoblue viability reagent (Fig 3a) and cell apoptosis by AnnexinV/PI staining (Fig 3b). The results showed that *Pax4* knockdown cells had a significantly decreased cell viability (Fig 3a) and increased cell apoptosis (Fig 3b) when compared to that of negative control cells. Addition of recombinant Wnt3a protein 50 μg/ml and 100 μg/ml did not increase cell viability (Fig 3a) and/or reduce cell apoptosis (Fig 3b). However, the results were obtained from only one or two experiments. Thus, we need to do more experiments to make a conclusive data.

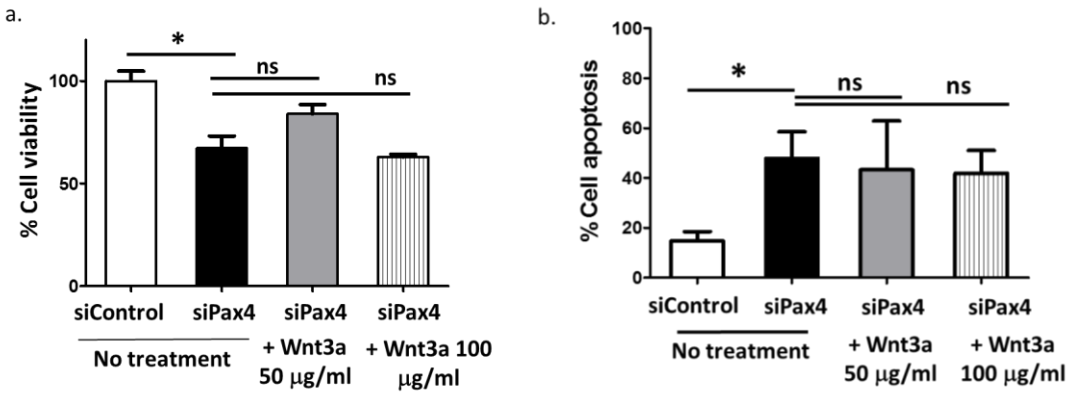


Figure 3. Cell viability and apoptosis of Pax4 knockdown cells after treated with recombinant Wnt3a protein.

INS-1 832/13 cells were transfected with negative control and Pax4 siRNAs. After transfection, cells were incubated with or without recombinant mouse Wnt3a protein 50 μg/ml and 100 μg/ml for 48 h. Cell viability and apoptosis were examined by using Prestoblue viability reagent (a) and AnnexinV/PI staining (b), respectively. Results are mean ± SEM (n= 3). * $P < 0.05$.

4. Determination of transactivation activities of PAX4 on its downstream targets

4.1 Analyses of *Wnt3a* promoter activity

Function of Pax4 to activate gene expression was demonstrated in mature pancreatic beta-cells. Over-expression of mouse Pax4 in rat islets increased expression of *c-myc*, *Id2*, and *Bcl-xL* genes; however, the exact Pax4-binding sites on these two promoters are still undefined. Overexpression of *Pax4* resulted in increased beta-cell proliferation and in protection of the cells from cytokine-induced apoptosis may mediate by up-regulation of *c-myc* and *Bcl-xL* expression. Therefore, this study aims to determine the exact molecular mechanism by which Pax4 regulation on either *Wnt3a*, *Bcl2l1* (Bcl-xL), and *Bcl2l2* (Bcl-w) gene promoters by using dual-luciferase reporter assay. We constructed a luciferase reporter plasmid harbouring a 1000 bp region of *Wnt3a* promoter, approximately 800 bp region of *Bcl2l1* gene promoter and a 1000 bp region of *Bcl2l2* gene promoters by molecular cloning techniques. We have analyzed the presence of potential PAX4-binding site on the *Wnt3a* gene promoter by using *in silico* programme. We did not find any potential PAX4-binding site on the 1000 bp upstream promoter region (Fig 4a), however, there is a potential site for PAX6 which can bind the same *cis*-acting sequences as well as PAX4, and HNF within the promoter region. Therefore, we constructed a 1000 bp upstream promoter into the luciferase reporter plasmid and examined its activity in the presence of exogenous PAX4, HNF4A and HNF1A. Results showed that overexpression of human PAX4 did not increase *Wnt3a* luciferase activity in HeLa cell line (Fig 4b). In contrast, overexpression of HNF4A and HNF1A proteins exerted the transactivation on *Wnt3a* gene promoter. Transactivation activity of *Wnt3a* gene promoter showed a very high in the presence of HNF1A indicating that HNF1A may play crucial role in regulation of *Wnt3a* transcription. However, the molecular mechanism by which HNF1A protein on *Wnt3a* gene promoter is still unknown. By these evidences, we speculated that Pax4 may not directly bind on the *Wnt3a* gene promoter but it might indirectly regulate the gene expression via other transcription factors, for example, HNF1A and HNF4A. Therefore, further study will propose to investigate the exact molecular mechanism of these transcription factors within the *Wnt3a* gene promoter.

a.

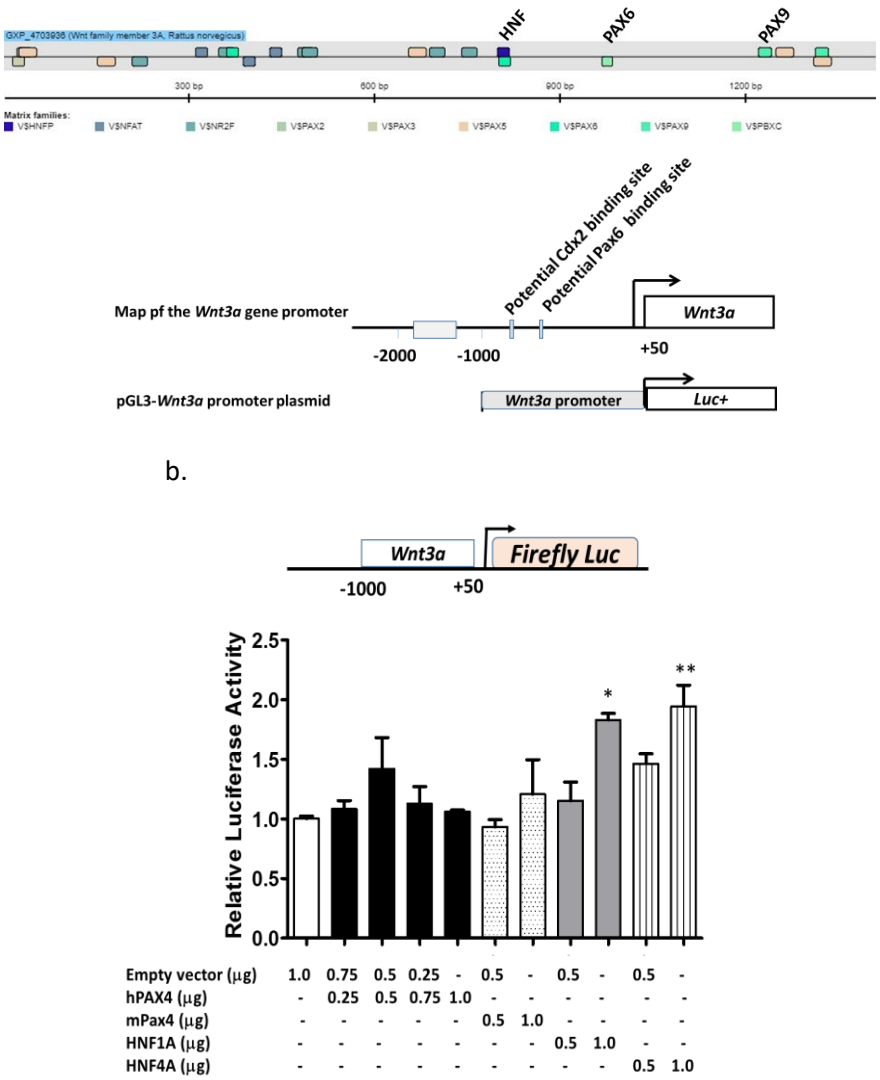


Figure 4. Transcriptional activities of PAX4, HNF1A, and HNF4A on *Wnt3a* gene promoter.

a. Map of *Wnt3a* gene promoter region and the prediction of potential transcription factor-binding sites. b. Relative luciferase activity of cells overexpressing PAX4, HNF4A, and HNF1A in the presence of *Wnt3a* luciferase plasmid. After transfection and culture for 24h, luciferase activities were measured by using luminometer. Values correspond to the mean±SEM of at least four individual transfections. Results are presented as fold induction of the control sample obtained from cells transfected with empty expression vector, pcDNA3.1/hisB. * $P < 0.05$, ** $P < 0.01$.

4.2. Analyses of *Bcl2l1* (Bcl-xL) promoter activity

To find the potential binding site of PAX4 within the Bcl2l1 gene promoter, we used genomatrix software for analysis. We found the presence of one potential PAX4-binding site within the 800 bp upstream of the gene (Fig 5a). In addition, there are other

potential binding sites of PAX6, HNF1, Nkx6, and PDX1 within this promoter region. The expression plasmid harbouring human *PAX4*, *HNF4A* or *HNF1A* gene, encoding beta-cell transcription factors, were transfected into the cells together with the *Bcl2l1* promoter construct to investigate whether these transcription factors can transactivate the *Bcl2l1* promoter transcription. The results showed that PAX4, HNF4A, and HNF1A can activate the *Bcl2l1* promoter transcription in human beta cell line (1.1B4) and HeLa cell lines (Fig 5b). Thus, the pancreatic transcription factor PAX4, HNF4A, and HNF1A play roles in the transactivation of *Bcl2l1* gene promoter. However, the molecular mechanism by which these transcription factors transactivate on the promoter is still undefined. Then, this study has further explored the potential regulation of *Bcl2l1* gene expression mediated by these transcription factors.

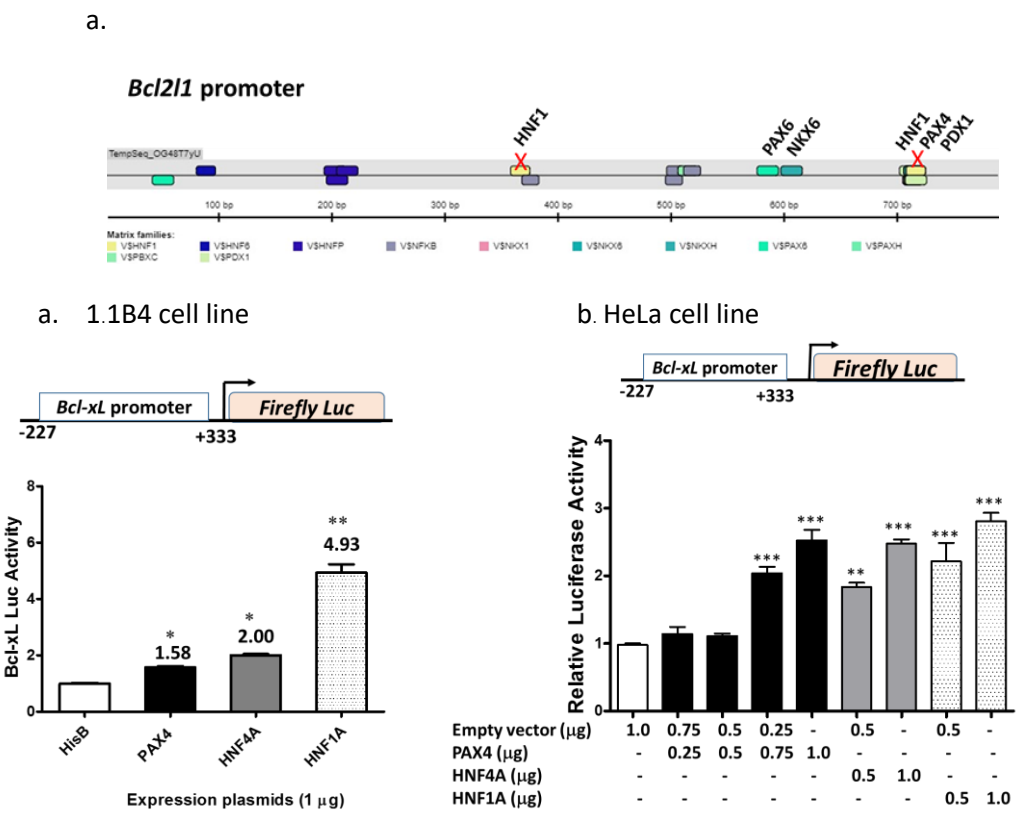


Figure 5. Transcriptional activities of PAX4, HNF4A, and HNF1A on *Bcl-xL* gene promoter.

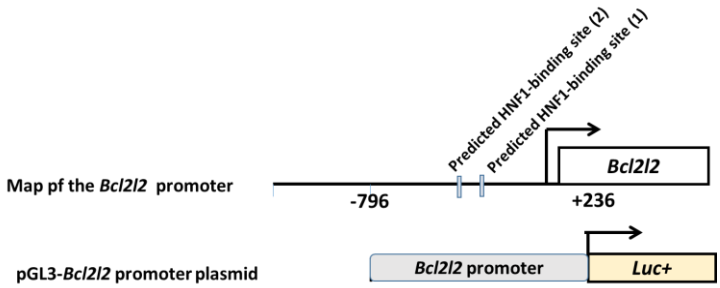
a. Prediction of the potential binding sites of pancreatic transcription factors within the Bcl2l1 promoter. b,c. Relative luciferase activity of cells overexpressing PAX4, HNF4A, and HNF1A in the presence of *Bcl2l1* luciferase plasmid. Human pancreatic beta-cell line (b) and HeLa cell line (c) were transfected with one microgram each of

expression plasmid encoding for PAX4 (hPAX4), HNF4A, or HNF1A together with pGL3-*Bcl2l1* luciferase plasmid and internal control plasmid. Values correspond to the mean±SEM of at least three individual transfections. Results are presented as fold induction of the control sample obtained from cells transfected with empty expression vector, pcDNA3.1/hisB. ** $P < 0.01$, *** $P < 0.001$.

3. Analyses of *Bcl2l2* (Bcl-w) promoter activity

Approximately 1000 bp region of human *Bcl2l2* gene promoter was analyzed for the presence of potential PAX4-binding site and other transcription factors by using *in silico* program such as AliBaba2.1 and PROMO. The result showed that there is no potential binding of PAX4 within the promoter region, however, there are two potential binding sites of HNF1 within this promoter (Fig 6a). We cloned the promoter region into a luciferase reporter vector and named as pGL3-*Bcl2l2* promoter plasmid. Expression vector encoded PAX4, HNF4A, and HNF1A was co-transfected with the pGL3-Bcl2l2 promoter plasmid into Huh7 and 1.1B4 cell lines. We have found that over-expression of PAX4 and HNF4A did not activate transcription of the *Bcl2l2* luciferase expression, while those cells overexpressing HNF1A exerted a high transactivation activity on Bcl2l2 promoter in both cell lines (Fig 6b and 6c). This result suggested that regulation of *Bcl2l2* gene expression might not be directly controlled via PAX4 and HNF4A, however, it may be controlled by HNF1A.

a.



b. Huh7 cell line **c. 1.1B4 cell line**

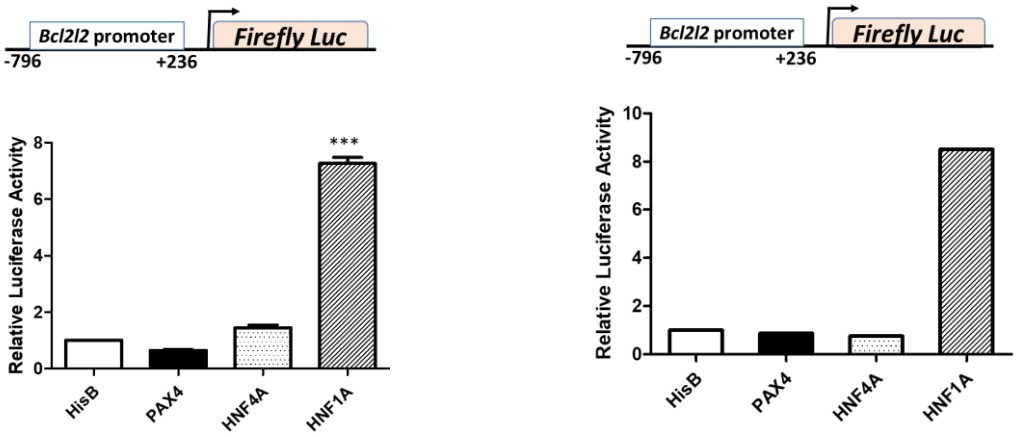


Figure 6. Transcriptional activities of PAX4 on *Bcl2l2* gene promoter in HeLa cell line

a. Prediction of the potential binding sites of pancreatic transcription factors within the *Bcl2l2* promoter. b and c. Relative luciferase activity of cells overexpressing PAX4, HNF4A, and HNF1A in the presence of *Bcl2l2* luciferase plasmid. Huh7 cell line (b) and 1.1B4 cell lines (c) were transfected with expression plasmid encoding PAX4, HNF4A, or HNF1A together with pGL3-*Bcl2l1* luciferase plasmid and internal control plasmid, pRL-SV40. Values correspond to the mean±SEM of at least two individual transfections. Results are presented as fold induction of the control sample obtained from cells transfected with empty expression vector, pcDNA3.1/hisB. *** $P < 0.001$.

5. Examination of altered gene expression in *Pax4* knockdown cells

To find out the molecular mechanism by which regulation of Pax4 on the *Wnt3a*, *Bcl2l1*, and *Bcl2l2* gene promoters, we have examined the expression of possible genes encoding transcription factors that might act interplay in this regulation. From our data, we showed that HNF4A and HNF1A might play role in the transcription network with Pax4 to regulate *Wnt3a* gene transcription. Expression level of beta-cell genes that play roles in regulation of beta-cell survival and/or proliferation were examined by real-time PCR using specific primer pairs. Data represents fold change of gene expression by normalizing with negative control siRNA transfected sample. The result showed decreased expression of *Wnt3a*, *Bcl2l1* and *Hnf1a* levels in *Pax4* knockdown cells (Fig 7), indicating that Pax4 may regulates *Wnt3a* and *Bcl2l1* gene expression and *Hnf1a* may involve in the transcription regulatory network.

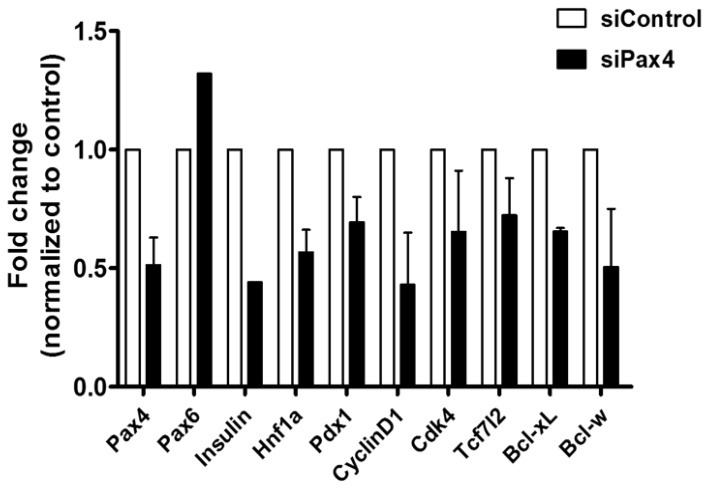


Figure 7. Altered gene expression in *Pax4* knockdown cells.

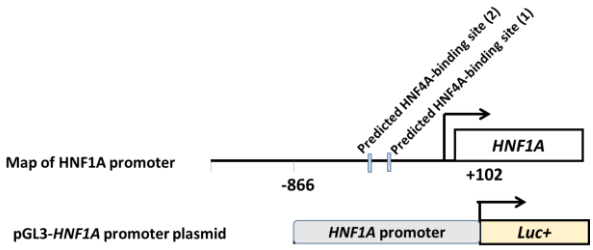
INS1 832/13 cells transfected with siControl or siPax4 were examined for gene expression by real-time RT-PCR. After transfection and culture for 48 h, total RNA was extracted and used as a template in a synthesis of complementary DNA (cDNA). PCR reactions were performed by using cDNA template, gene-specific primers, PCR reaction cocktails, and a SYBR green I dye for measuring gene expression level. Beta-actin gene was amplified for normalization.

6. Investigation of transcriptional activation of PAX4 on *HNF1A* gene promoter

Our data showed that several pancreatic transcription factors including PAX4, HNF4A, and HNF1A play roles in the regulation of *Wnt3a* and *Bcl-xL* gene expressions. PAX4 might control *Wnt3a* gene expression via other transcription factor such as HNF1A due to the decrease expression of *Hnf1a* gene was also found in Pax4 knockdown cells. Therefore, we had further investigated whether human PAX4 can transactivate the *HNF1A* gene promoter by using luciferase reporter assay. Previous study had showed that HNF1A gene transactivation is partly controlled by the transcription factors HNF4A and Nkx6.1 at a proximal and distal promoter region, respectively. Prediction of potential PAX4-binding site was not discovered in this promoter region, but there are two potential HNF4A-binding sites within the promoter. (Fig 8a). Huh7 cell line was transfected with the luciferase reporter plasmid and expression vector. Here, we showed that overexpression of PAX4 and HNF4A, which is a known transcriptional regulator of HNF1A,

had an increased luciferase activity in Huh7 cells (Fig 8b). This result indicated that HNF1A gene expression might be controlled via PAX4 and HNF4A transcription factors.

a.



b. Huh7 cell line

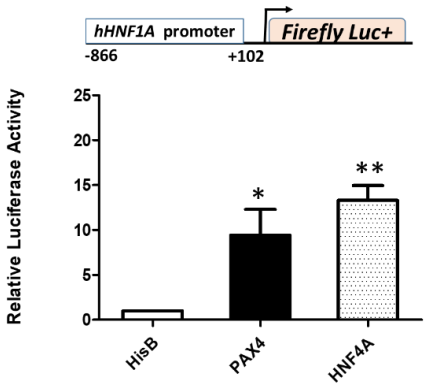


Figure 8. Transcriptional activities of PAX4 and HNF4A on *HNF1A* gene promoter.

a. Map of a promoter region of HNF1A gene cloned into luciferase reporter constructs. b. Transcriptional activities of PAX and HNF4A on HNF1A gene promoter in Huh7 cell. Values correspond to the mean ± SEM. Results are presented as normalized luciferase activity.

7. Examination of DNA-binding activity of PAX4 protein on target-gene promoter by using Electrophoretic-mobility shift assay (EMSA)

To examine the DNA-binding activity of PAX4 on its target-gene promoter region, electrophoretic-mobility shift assay (EMSA) was performed. First, optimal condition in a DNA and protein binding was investigated by using biotinylated double-stranded oligonucleotides of the rat insulin C2 and SMS-UE that contains the binding sequences of the PAX4 protein. In addition to the PAX4 WT, we also used a mutant PAX4 protein (PAX4 R192H) in the experiment. Optimal condition and the system to test DNA-binding activity of PAX4 protein on DNA probe was successfully performed. Then, we aim to test

the activity of PAX4 to bind on the *Bcl2l1* promoter and/or *HNF1A* promoter region as well as other candidate target-gene of PAX4.

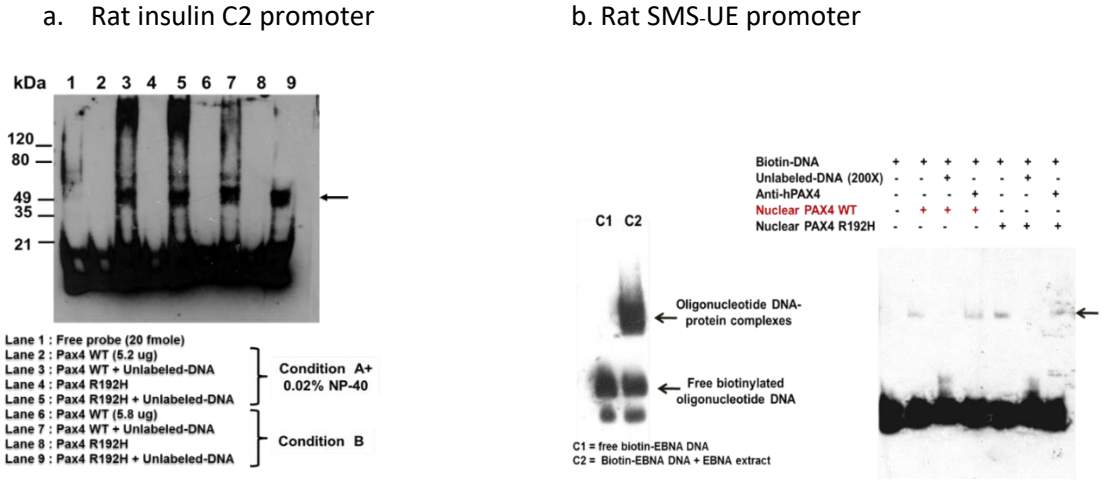


Figure 9. Analyses of DNA-binding activity of PAX4 on rat insulin C2 and SMS-UE promoter region by using EMSA

Nuclear extracts from HeLa-overexpressing PAX4 WT or mutant was prepared and subjected into the binding reaction of the rat insulin C2 promoter (a) or rat SMS-UE promoter (b). Protein-DNA binding reactions are loaded onto 5% native acrylamide gel and detected by using streptavidin conjugated horseradish peroxidase (HRP) followed by exposure onto an X-ray film.

8. Optimization of siRNA-mediated *PAX4* suppression in 1.1B4 cell line

8.1 Detection of human *PAX4* mRNA isoform in various human cell lines

Human *PAX4* mRNA has at least four isoforms that expressed in various tissue cell types. *PAX4* mRNA isoforms are PAX4 isoform 3, isoform 4, isoform4v, and the truncated isoform identified in Huh7 cells. PAX4 mRNA isoform 3 is an isoform that had well-characterized function in pancreatic beta-cells. First, we examined the mRNA isoforms of *PAX4* in 1.1B4 and other human cell lines by using reverse-transcription PCR (RT-PCR). *PAX4* isoform-specific primer pairs are designed and used in PCR reaction to amplify each PAX4 isoform (Fig 10). The results showed that 1.1B4 expressed an isoform 3 of human PAX4 while other cell lines expressed isoform 4 or Pax4v.

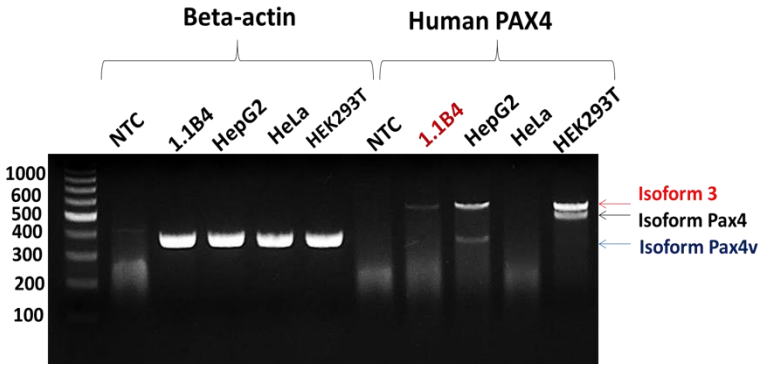


Figure 10. Expression of human *PAX4* mRNA isoforms in human cell lines

Total RNA was extracted from human cell lines including 1.1B4, HepG2, HeLa, and HEK293T using Trizol reagent. First-strand synthesis was performed using oligo(dT) primer and subjected to PCR amplification using specific primer pairs against each isoform of PAX4. Beta-actin amplification is used as control for RT-PCR reaction.

8.2 Detection of human PAX4 protein in various human cell lines

We have examined an expression of human PAX4 protein in various human cell lines, such as Huh7, HEK293T, HeLa, and 1.1B4 (human pancreatic beta-cell line). Human PAX4 isoform 3 mRNA encoded 343 amino acids protein that has approximately 37 kDa. PAX4 variant protein (short) has approximately 26 kDa. However, recent paper has demonstrated that human PAX4 protein has post-translational modification, such as, sumolyation. The results showed the presence of various PAX4 protein variants in human cell lines (Fig 11). Human pancreatic beta-cell line (1.1B4) contained both short form of protein (26 kDa) and isoform 3 (37 kDa), and also high molecular-weight size protein that was post-translation modification.

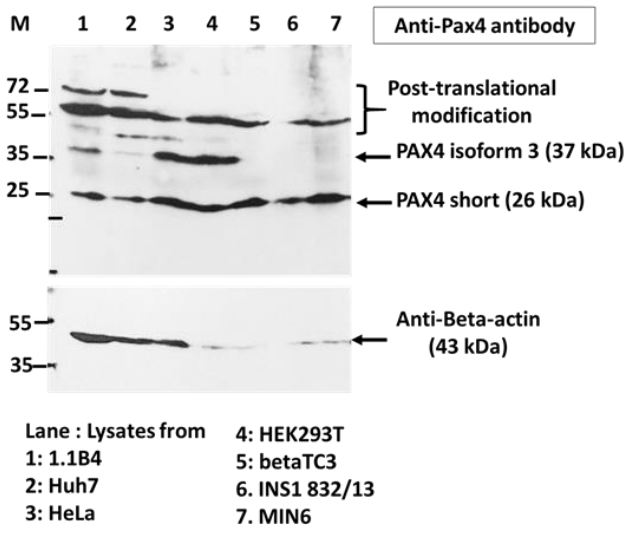


Figure 11. Expression of PAX4 protein in various human cell lines.

Total protein was extracted from human cell lines including 1.1B4, Huh7, HeLa, and HEK293T, and murine insulinoma cell lines including betaTC3, INS1 832/13 and MIN6 cells. Protein lysate was loaded onto 12% SDS-PAGE and transferred onto nitrocellulose membrane. Human PAX4 and beta-actin were detected by using antibody against PAX4 and beta-actin, respectively.

9. Optimization of SMARTpool siRNA targeting PAX4 concentration and determination of knockdown efficiency

To investigate the role of human *PAX4* in human pancreatic beta-cell line (1.1B4), endogenous human *PAX4* gene expression was suppressed by using the SMARTpool siRNA targeting human *PAX4* mRNA (Dharmacon). For negative control sample, siGENOME non-targeting siRNA control pools were used in the experiment. The siGENOME non-targeting siRNA control pools and SMARTpool siRNA targeting *PAX4* at 100 μM, 200 μM, and 300 μM were transfected into the cells using Dharmafect Duo transfection reagent. After siRNA transfection, equal amounts of total proteins from negative control and *Pax4* knockdown cell lysates were separated on 12% SDS-PAGE, followed by electroblotting and immunodetection of Pax4 using anti-Pax4 antibody. The results showed that Pax4 protein level was significantly decreased in *Pax4* knockdown cells compared to that of the negative control cells (Fig 12a and 12b). Expected size of PAX4 protein is 37 kDa. However, the observed bands showed higher molecular weight size than the expected one. It is possible that human PAX4 protein has post-translational modification (PTM) in modulating PAX4 activity, however, its evidences of PTMs yet little is shown. One of

the PTMs have been reported is SUMOylation that regulates transcription factors function. Nevertheless, this issue is still need to study in the future. In summary, optimization of PAX4 knockdown in 1.1B4 is still required to investigate its role in pancreatic beta-cells.

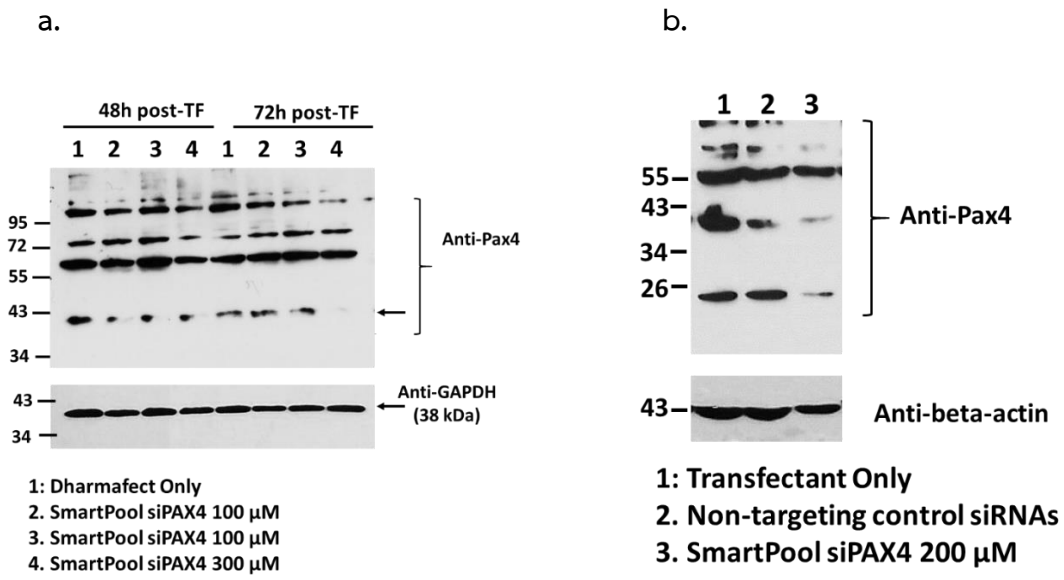


Figure 12. Western blot analyses of PAX4 protein in lysates from siRNA transfected 1.1B4

siGENOME non-targeting siRNA control pools and SMARTpool siRNA targeting *PAX4* at concentration of 100 µM, 200 µM, and 300 µM were transfected into 1.1B4 cells using Dharmafect Duo transfection reagent. Expected size of PAX4 protein is 38 kDa, however, the observed band size had higher molecular weight size than expected one.

10. Examination of cell number and cell apoptosis after PAX4 knockdown in 1.1B4 cells

To investigate the role of human *PAX4* in human pancreatic beta-cells (1.1B4), beta-cell number and cell apoptosis were investigated from non-targeting control siRNA and PAX4 knockdown cells by using trypan blue exclusion assay and AnnexinV/PI staining, respectively. The result showed that PAX4 knockdown 1.1B4 cells had significantly decreased cell number (Fig 13) and increased cell apoptosis (Fig 14) when compared to those negative control cells. Our finding indicated that PAX4 may play role in regulating cell number and cell survival of human pancreatic beta-cells.

a. b.

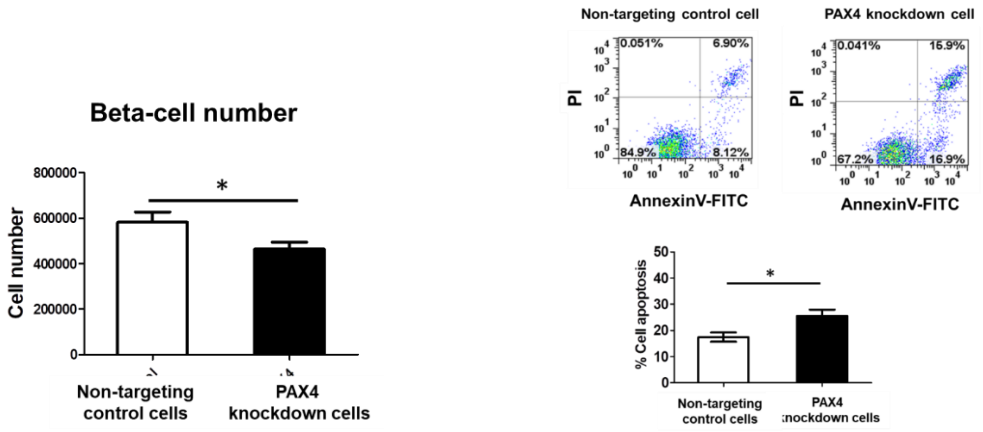


Figure 13. Analyses of cell number and cell apoptosis of *PAX4* knockdown 1.1B4 cells.

1.1B4 cells were transfected with non-targeting control siRNA and SMARTPool siRNA targeting human PAX4. After transfection and culture for 48 hr, cells were collected and counted by using Trypan blue exclusion assay (a) and stained with AnnexinV /PI dye followed by flow cytometry (b). Results are presented as mean ± SEM. * $P < 0.05$.

11. Examination of Wnt3a, Bcl-xL, and Bcl-w protein expression in PAX4 knockdown 1.1B4 cells

To determine whether suppression of PAX4 affect the identified target genes or not, Wnt3a, Bcl-xL, and Bcl-w protein expression level were analyzed from lysates of negative control and SMARTPool siPAX4 transfected cells. The result showed the decreased human PAX4 protein expression level in SMARTPool siPAX4 transfected cells when compared to those cells transfected with non-targeting negative control siRNAs (Fig 14). In addition, we found that expression of Wnt3a and Bcl-w, but not Bcl-xL protein, were reduced in PAX4 knockdown 1.1B4 cells. This finding indicated that PAX4 may also involve in the regulation of Wnt3a and Bcl-w expression in human pancreatic beta-cell similar to those observed in rat INS-1 832/13 cells.

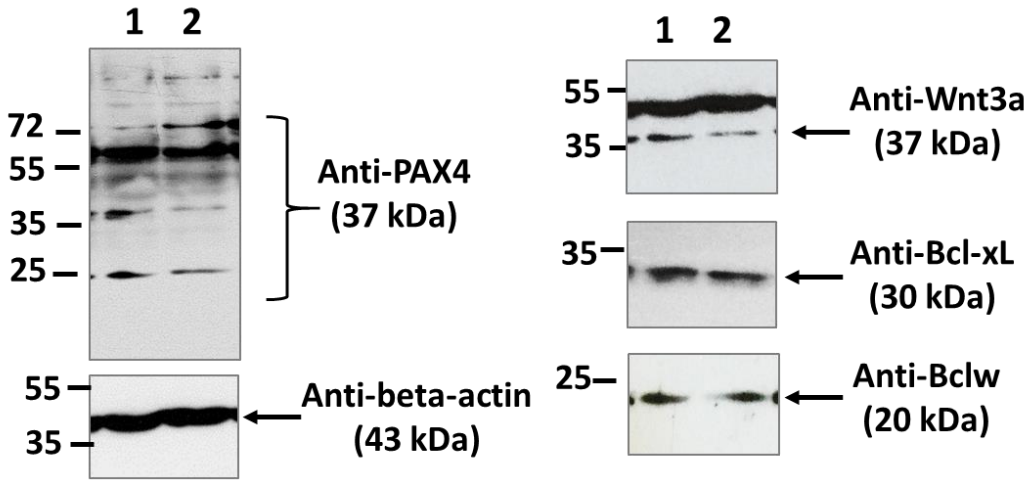


Figure 14. Analyses of PAX4 and its target protein expression in *PAX4* knockdown 1.1B4

1.1B4 cells were transfected with non-targeting control siRNA and SMARTPool siRNA targeting human *PAX4*. After transfection and culture for 48 h, cells were collected, lysed and separated on SDS-PAGE, followed by detecting specific protein using specific antibody.

Proposed model of molecular mechanism by which PAX4 regulation on its target genes including *Wnt3a*, *Bcl2l1*, and *Bcl2l2*, for maintaining beta-cell survival (Fig 15). Under the physiological condition, Pax4 is required to maintain pancreatic beta-cell survival by regulating a wide variety of genes involved in anti-apoptosis and Wnt signaling pathways. This study demonstrates that Pax4 may directly controlled expression of *Bcl2l1* (Bcl-xL) and the *HNF1A* gene promoter, while it did not directly regulate on the *Wnt3a* and *Bcl2l2* (Bcl-w) gene promoters. Increased expression of anti-apoptotic proteins will protect beta-cells from apoptosis. In addition, the secreted Wnt3a protein acts via its binding to receptor, leading to inactivation of GSK3β that can be modified beta-catenin; consequently, free beta-catenin can translocate into the nucleus to acts in combination with T-cell factor (TCF), e.g. Tcf7l2, to stimulate transcription of Wnt-responsive genes, many of which (such as *Cyclin D* or *c-myc*) stimulate cell proliferation. Thus, the reduction of Pax4 expression results in imbalance of cell proliferation and apoptosis, leading to reduction of beta-cell mass and pathogenesis of diabetes.

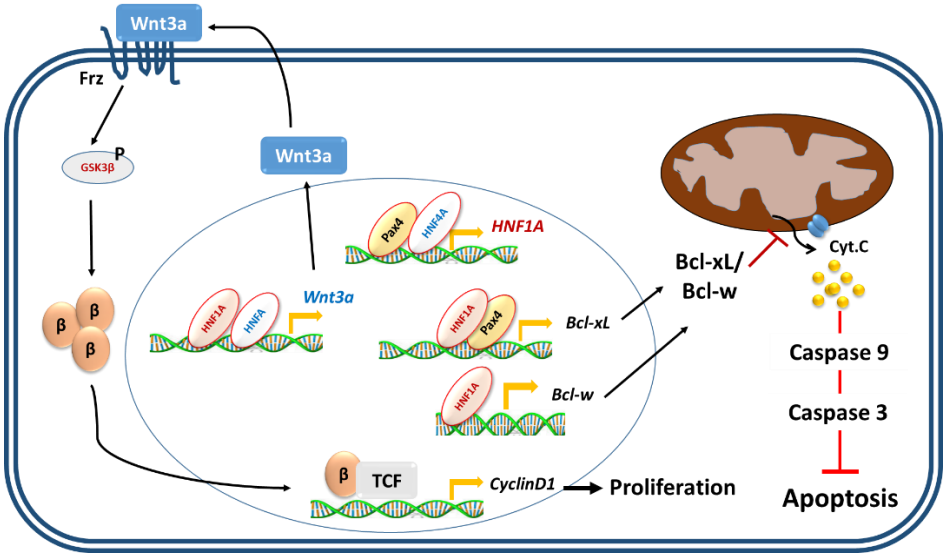


Figure 15. Schematic illustration of the proposed mechanism of how Pax4 may regulate beta-cell survival.

Conclusion and discussion

Diabetes mellitus (DM) is a group of metabolic disorder characterized by hyperglycemia resulted from defects in insulin secretion, in insulin action, or the combination of both defects. T2D is the most common form of diabetes. T2D is characterized by hyperglycemia resulted from the presence of insulin resistance and impaired insulin secretion. Maturity-onset diabetes of the young (MODY) is a monogenic form of diabetes, characterized by autosomal dominant inheritance, early age at onset, and pancreatic beta-cell dysfunction (1). Reduction of pancreatic beta-cell function and beta-cell number can contribute to insulin deficiency in T2D and in MODY. Moreover, no controlled strategy for restoration of beta-cell mass has been identified. At the molecular level, pancreatic beta-cell loss by apoptosis appears to play an important role in the development of insulin deficiency and the onset and/or progression of the disease. Therefore, a more complete understanding of the mechanisms that control beta-cell proliferation, survival, and function is required in order to develop more effective therapies for diabetes.

Previous study by Siriraj Center of Research Excellence-Diabetes and Obesity (Si-CORE-DO) discovered that mutations of *Paired box 4* (*PAX4*) gene causing MODY, type 9 (MODY9) (2). Genetic variations of *PAX4* have been identified in various types of diabetes including type 1 diabetes (T1D) (3), T2D (4-6), and MODY (2, 7-9). Our group identified two novel mutations of PAX4 including *PAX4* R164W and IVS7-1G>A in two Thai families with MODY (2). Our group demonstrated that PAX4 R164W mutant (2) and R192H variant protein had decreased repression activity on human *insulin* and *glucagon* promoters (10). *Pax4* encodes a transcription factor that promotes pancreatic beta-cell development (11, 12), differentiation (13-15) and survival (16, 17). PAX4 plays dual roles in the regulation of target-gene promoters: transrepression of *glucagon*, *insulin*, *somatostatin*, *IAPP*, and *ghrelin* promoters (18, 19) and transactivation on *c-myc* and *Bcl-xL* promoters (16), which promotes islet proliferation and protects cells from stress-induced apoptosis, respectively. Several lines of evidence support the role of PAX4 in promoting proliferation of human embryonic kidney cell line (HEK293) (20), rat insulinoma cell line (INS-1E) (17), human insulinoma cells (21), and murine and human islets (22). These data suggested that *Pax4* acts as survival gene in these cells. Recent study elucidated that targeted overexpression of wild-type Pax4 in mice that targeted

induction of wild-type Pax4 can protect against streptozotocin-induced hyperglycemia, while those mice overexpressing Pax4 R129W mutant were not (23). These data strongly indicated that Pax4 plays implicated role in regulating beta-cell survival under stress conditions. *Pax4* knockdown INS-1E cells resulted in down-regulation of *Bcl-xL* expression (17). Since the survival or anti-apoptotic role of PAX4 protein has not totally been clear, our preliminary experiment was performed to identify potential target-gene of Pax4. We have found that suppression of *Pax4* gene expression in INS-1 832/13 cells increased cell apoptosis. *Pax4* knockdown cells had decreased expression of *Wnt3a*, *Bcl2l1*, and *Bcl2l2* genes, which encoded a growth-promoting factor or anti-apoptotic proteins. We originally report that *Wnt3a* and *Bcl2l2* (Bcl-w) are newly identified genes regulated by Pax4. The molecular mechanism underlying that Pax4 protects beta-cell survival might be mediated by up-regulating anti-apoptotic genes, *Bcl2l1* (Bcl-xL) and *Bcl2l2* (Bcl-w), and/or *Wnt3a* gene. However, the exact mechanism of Pax4 in the regulation of these genes is still unknown.

Wnt3a encodes Wnt3a protein that belongs to the Wnt family (24). It is a secreted protein and plays implicated role in cell proliferation (25, 26), differentiation (27), and oncogenesis (28, 29). Activation of the Wnt signaling is mediated by the interaction of Wnt protein with the receptors, Frizzled receptor and LRP co-receptors. Expression of components of the Wnt signaling pathway, including Wnt ligand family members and the receptors, had been documented in the adult human pancreas (30). The Wnt signaling pathway is shown to play important modulator in several biological processes, such as in adipogenesis (31), pancreas development (32), insulin secretion (33), beta-cell proliferation and survival (34). An endogenous activation of Wnt signaling had been illustrated in INS-1 cells transfected with the TOPflash construct, a Lef/Tcf-sensitive reporter assay (33). Addition of Wnt3a or fat-cell conditioned medium (FCCM) into the INS-1 cells transfected TOPflash reporter plasmid increased activation of this reporter construct in this cells, indicating that Wnt3a protein or Wnt protein secreted from adipocyte can activate the Wnt signaling pathway (33). In addition, mouse pancreatic beta-cell line, NIT-1 cells, treated with purified Wnt3a protein had been shown to promote NIT-1 cells proliferation, decrease cell apoptosis in the presence of cytokine, and enhance glucose-stimulated insulin secretion (34). The mechanism underlying the effect of Wnt3a protein in promoting NIT-1 cell proliferation has been shown to be mediated by the activation of IRS2/PI3K/Akt pathway (34). The NIT-1 cells treated with purified Wnt3a protein increased *cyclin D2* and *Bcl-2* mRNA levels (34). This evidence

supports the results of the present study that decreased Wnt3a expression was correlated with the reduction of Bcl-xL and Bcl-w expression levels. The evidence to support anti-apoptosis function of Wnt3a was illustrated in the mouse embryonic liver stem cells, the mechanism of this event involved up-regulating Bcl-2 and Mcl-1 and down-regulating pro-apoptotic factors (35).

In this project, we explore the molecular mechanism of Pax4 in regulating these identified target-genes including *Wnt3a*, *Bcl2l1*, and *Bcl2l2*, and confirm the role of PAX4 in human pancreatic beta-cell line (1.1B4). Molecular mechanism underlying Pax4 regulation on these target-genes were determined using luciferase promoter assay. Promoter region of *Wnt3a*, *Bcl2l1*, or *Bcl2l2* was cloned into a luciferase reporter plasmid, that were co-transfected with expression plasmid containing human *PAX4*, *HNF4A*, or *HNF1A*. Overexpression of HNF1A or HNF4A, but not PAX4, had significant increase transcriptional activity on the *Wnt3a*. This finding suggests that PAX4 did not directly stimulate *Wnt3a* promoter activity, however, it may be partly controlled via HNF1A and HNF4A. The cells overexpressing PAX4, HNF1A or HNF4A had significantly increased transactivation on the *Bcl2l1* (Bcl-xL) gene promoter. The finding of this study supports the results of the previous studies that anti-apoptotic gene – *Bcl2l2* (Bcl-xL) was regulated by Pax4 (16). However, our study provides additional evidence on the decreased Bcl-xL protein, which was not demonstrated in the previous studies (16). Identification of functional Pax4 binding sites on *Bcl2l1* gene promoter is still under investigation. We try to figure out potential binding site of PAX4 on the gene promoter by using *in silico* program. There is only one potential PAX4-binding site on the *Bcl2l1* gene promoter from the prediction program. Luciferase plasmid containing mutation at this potential PAX4-binding site was constructed and transfected into the cells together with PAX4 expression plasmid. However, we did not observe any change in transactivation activity on the promoter. We thus hypothesize that there might be other position of PAX4-binding site on the promoter. We suggest to serially delete region of the promoter and then examine luciferase promoter assay. In addition to PAX4, we have found that HNF1A transcription factor may also regulate *Bcl2l1* gene expression, and overexpression of HNF1A mutant had significantly decreased transactivation on the *Bcl2l1* gene promoter (unpublished data). For *Bcl2l2* (Bcl-w) gene promoter, overexpression of HNF1A had significantly increased transcriptional activity on the gene promoter, while those cells overexpressing PAX4 did not. This finding indicates that

regulation of *Bcl2l2* gene expression may be mediated through HNF1A, but not PAX4 transcription factor.

Furthermore, we demonstrate the decreased expression of *Hnf1a,* but not *Hnf4a,* gene in *Pax4* KD cells while increased expression of these genes were observed in PAX4 overexpressing cells. We have shown by transfection studies that PAX4 may indirectly regulate *Wnt3a* gene expression through the activation of the *HNF1A* gene promoter. Our results suggest that the decreased *Wnt3a* gene expression in *Pax4* KD cells may be partly controlled by other transcription factors, such as HNF1A and HNF4A, and Pax4 may play roles in the regulation of *Hnf1a* gene expression. We now report that Pax4 regulates directly or indirectly the transcription of the *Wnt3a*, *Bcl2l1*, and *Bcl2l2* genes, which are critical for cell proliferation and survival in rat insulinoma cells.

In addition, we confirm a crucial role of PAX4 in human pancreatic beta-cell line (1.1B4) which is required to maintain cell survival and cell number. Human PAX4 might control Wnt3a and Bcl-w protein expression that are essential for promoting beta-cell proliferation and protecting beta-cells from stress-induced apoptosis. In conclusion, Pax4 is a crucial transcription factor regulating beta-cell survival via Wnt3a signaling and anti-apoptotic pathways. The understanding of PAX4 function and its downstream target may offer a promising approach for endogenous replenishment of the lost beta-cell mass for DM treatment.

References

1. Fajans SS. Maturity-onset diabetes of the young (MODY). Diabetes/metabolism reviews. 1989;5(7):579-606. Epub 1989/11/01.
2. Plengvidhya N, Kooptiwut S, Songtawee N, Doi A, Furuta H, Nishi M, et al. PAX4 mutations in Thais with maturity onset diabetes of the young. The Journal of clinical endocrinology and metabolism. 2007;92(7):2821-6. Epub 2007/04/12.
3. Biason-Lauber A, Boehm B, Lang-Muritano M, Gauthier BR, Brun T, Wollheim CB, et al. Association of childhood type 1 diabetes mellitus with a variant of PAX4: possible link to beta cell regenerative capacity. Diabetologia. 2005;48(5):900-5. Epub 2005/04/19.
4. Shimajiri Y, Sanke T, Furuta H, Hanabusa T, Nakagawa T, Fujitani Y, et al. A missense mutation of Pax4 gene (R121W) is associated with type 2 diabetes in Japanese. Diabetes. 2001;50(12):2864-9. Epub 2001/11/28.
5. Shimajiri Y, Shimabukuro M, Tomoyose T, Yogi H, Komiya I, Takasu N. PAX4 mutation (R121W) as a prodiabetic variant in Okinawans. Biochemical and biophysical research communications. 2003;302(2):342-4. Epub 2003/02/27.
6. Sujjitjoon J, Kooptiwut S, Chongjaroen N, Semprasert N, Hanchang W, Chanprasert K, et al. PAX4 R192H and P321H polymorphisms in type 2 diabetes and their functional defects. Journal of human genetics. 2016;61(11):943-9. Epub 2016/06/24.
7. Sujjitjoon J, Kooptiwut S, Chongjaroen N, Tangjittipokin W, Plengvidhya N, Yenchitsomanus PT. Aberrant mRNA splicing of paired box 4 (PAX4) IVS7-1G>A mutation causing maturity-onset diabetes of the young, type 9. Acta diabetologica. 2016;53(2):205-16. Epub 2015/05/09.
8. Chapla A, Mruthyunjaya MD, Asha HS, Varghese D, Varshney M, Vasan SK, et al. Maturity onset diabetes of the young in India - a distinctive mutation pattern identified through targeted next-generation sequencing. Clinical endocrinology. 2015;82(4):533-42. Epub 2014/07/22.
9. Jo W, Endo M, Ishizu K, Nakamura A, Tajima T. A novel PAX4 mutation in a Japanese patient with maturity-onset diabetes of the young. The Tohoku journal of experimental medicine. 2011;223(2):113-8. Epub 2011/01/26.
10. Kooptiwut S, Plengvidhya N, Chukijrungroat T, Sujjitjoon J, Semprasert N, Furuta H, et al. Defective PAX4 R192H transcriptional repressor activities associated

with maturity onset diabetes of the young and early onset-age of type 2 diabetes. Journal of diabetes and its complications. 2012;26(4):343-7. Epub 2012/04/24.

11. Sosa-Pineda B, Chowdhury K, Torres M, Oliver G, Gruss P. The Pax4 gene is essential for differentiation of insulin-producing beta cells in the mammalian pancreas. Nature. 1997;386(6623):399-402. Epub 1997/03/27.
12. Sosa-Pineda B. The gene Pax4 is an essential regulator of pancreatic beta-cell development. Molecules and cells. 2004;18(3):289-94. Epub 2005/01/15.
13. Blyszczuk P, Czyz J, Kania G, Wagner M, Roll U, St-Onge L, et al. Expression of Pax4 in embryonic stem cells promotes differentiation of nestin-positive progenitor and insulin-producing cells. Proceedings of the National Academy of Sciences of the United States of America. 2003;100(3):998-1003. Epub 2003/01/15.
14. Liew CG, Shah NN, Briston SJ, Shepherd RM, Khoo CP, Dunne MJ, et al. PAX4 enhances beta-cell differentiation of human embryonic stem cells. PloS one. 2008;3(3):e1783. Epub 2008/03/13.
15. Lin HT, Kao CL, Lee KH, Chang YL, Chiou SH, Tsai FT, et al. Enhancement of insulin-producing cell differentiation from embryonic stem cells using pax4-nucleofection method. World journal of gastroenterology. 2007;13(11):1672-9. Epub 2007/04/28.
16. Brun T, Franklin I, St-Onge L, Biason-Lauber A, Schoenle EJ, Wollheim CB, et al. The diabetes-linked transcription factor PAX4 promotes {beta}-cell proliferation and survival in rat and human islets. The Journal of cell biology. 2004;167(6):1123-35. Epub 2004/12/15.
17. Brun T, Duhamel DL, Hu He KH, Wollheim CB, Gauthier BR. The transcription factor PAX4 acts as a survival gene in INS-1E insulinoma cells. Oncogene. 2007;26(29):4261-71. Epub 2007/01/30.
18. Smith SB, Ee HC, Conners JR, German MS. Paired-homeodomain transcription factor PAX4 acts as a transcriptional repressor in early pancreatic development. Molecular and cellular biology. 1999;19(12):8272-80. Epub 1999/11/24.
19. Smith SB, Watada H, Scheel DW, Mrejen C, German MS. Autoregulation and maturity onset diabetes of the young transcription factors control the human PAX4 promoter. The Journal of biological chemistry. 2000;275(47):36910-9. Epub 2000/09/01.

20. Li Y, Nagai H, Ohno T, Ohashi H, Murohara T, Saito H, et al. Aberrant DNA demethylation in promoter region and aberrant expression of mRNA of PAX4 gene in hematologic malignancies. Leukemia research. 2006;30(12):1547-53. Epub 2006/05/17.
21. Miyamoto T, Kakizawa T, Ichikawa K, Nishio S, Kajikawa S, Hashizume K. Expression of dominant negative form of PAX4 in human insulinoma. Biochemical and biophysical research communications. 2001;282(1):34-40. Epub 2001/03/27.
22. Brun T, Hu He KH, Lupi R, Boehm B, Wojtusciszyn A, Sauter N, et al. The diabetes-linked transcription factor Pax4 is expressed in human pancreatic islets and is activated by mitogens and GLP-1. Human molecular genetics. 2008;17(4):478-89. Epub 2007/11/09.
23. Hu He KH, Lorenzo PI, Brun T, Jimenez Moreno CM, Aeberhard D, Vallejo Ortega J, et al. In vivo conditional Pax4 overexpression in mature islet beta-cells prevents stress-induced hyperglycemia in mice. Diabetes. 2011;60(6):1705-15. Epub 2011/04/28.
24. Katoh M. WNT3-WNT14B and WNT3A-WNT14 gene clusters (Review). International journal of molecular medicine. 2002;9(6):579-84.
25. Verras M, Brown J, Li X, Nusse R, Sun Z. Wnt3a growth factor induces androgen receptor-mediated transcription and enhances cell growth in human prostate cancer cells. Cancer research. 2004;64(24):8860-6.
26. Davidson KC, Jamshidi P, Daly R, Hearn MT, Pera MF, Dottori M. Wnt3a regulates survival, expansion, and maintenance of neural progenitors derived from human embryonic stem cells. Molecular and cellular neurosciences. 2007;36(3):408-15.
27. Aulehla A, Wehrle C, Brand-Saberi B, Kemler R, Gossler A, Kanzler B, et al. Wnt3a plays a major role in the segmentation clock controlling somitogenesis. Developmental cell. 2003;4(3):395-406.
28. Nygren MK, Dosen G, Hystad ME, Stubberud H, Funderud S, Rian E. Wnt3A activates canonical Wnt signalling in acute lymphoblastic leukaemia (ALL) cells and inhibits the proliferation of B-ALL cell lines. British journal of haematology. 2007;136(3):400-13.
29. Qiang YW, Shaughnessy JD, Jr., Yaccoby S. Wnt3a signaling within bone inhibits multiple myeloma bone disease and tumor growth. Blood. 2008;112(2):374-82.

30. Heller RS, Dichmann DS, Jensen J, Miller C, Wong G, Madsen OD, et al. Expression patterns of Wnts, Frizzleds, sFRPs, and misexpression in transgenic mice suggesting a role for Wnts in pancreas and foregut pattern formation. Developmental dynamics : an official publication of the American Association of Anatomists. 2002;225(3):260-70.
31. Prestwich TC, Macdougald OA. Wnt/beta-catenin signaling in adipogenesis and metabolism. Current opinion in cell biology. 2007;19(6):612-7.
32. Papadopoulou S, Edlund H. Attenuated Wnt signaling perturbs pancreatic growth but not pancreatic function. Diabetes. 2005;54(10):2844-51.
33. Schinner S, Ulgen F, Papewalis C, Schott M, Woelk A, Vidal-Puig A, et al. Regulation of insulin secretion, glucokinase gene transcription and beta cell proliferation by adipocyte-derived Wnt signalling molecules. Diabetologia. 2008;51(1):147-54.
34. Gui S, Yuan G, Wang L, Zhou L, Xue Y, Yu Y, et al. Wnt3a regulates proliferation, apoptosis and function of pancreatic NIT-1 beta cells via activation of IRS2/PI3K signaling. Journal of cellular biochemistry. 2013;114(7):1488-97.
35. Zhang X, Hu D, Lu Y, Feng T, Huang J. [Overexpression of Wnt3a inhibited the apoptosis of mouse embryonic liver stem cells]. Xi bao yu fen zi mian yi xue za zhi = Chinese journal of cellular and molecular immunology. 2013;29(12):1277-80.

Output

1. International Journal Publication

1.1 Project-related outputs

- Manuscript will be submitted to publish in "Molecular and Cellular Endocrinology" with title "*PAX4 is crucial for β-cell survival via the activation of Wnt signaling and anti-apoptotic pathways*".
- Manuscript will be submitted to publish in "Biochemical and Biophysical Research Communications" with title "*Functional defect of hepatocyte nuclear factor-1A mutations causing maturity-onset diabetes of the young, type 3 in Thais*".

1.2 Non project-related outputs

- **Sujjitjoon J**, Kooptiwut S, Chongjaroen N, SemprasertN, Hanchang W, Chanprasert K. et al. PAX4 R192H and P321H polymorphisms in type 2 diabetes and their functional defects. Journal of Human Genetics. 2016; 61(11):943-9.
- Kulanuwat S, Tangjittipokin W, Jungtrakoon P, Chanprasert C, **Sujjitjjjon J**, Binnima N., et al. DNAJC3 mutation in Thai familial type 2 diabetes mellitus. International Journal of Molecular Medicine. 2018;42(2):1064-73.
- Panya A, Thepmalee C, Sawasdee N, **Sujjitjoon J**, Phanthaphol N, Junking M, et al. Cytotoxic activity of effector T cells against cholangiocarcinoma is enhanced by self-differentiated monocyte-derived dendritic cells. Cancer Immunology, Immunotherapy. 2018;67(10):1579-88.

- Kooptiwut S, Kaewin S, Semprasert N, **Sujjitjoon J**, Junking M, Suksri K, et al. Estradiol prevents high glucose-induced beta-cell apoptosis by decreased BTG2 expression. Scientific Reports. 2018;8(1):12256.

2. Application

   -

3. Others e.g. national journal publication, proceeding, international conference, book chapter, patent

   Poster presentation

   Jatuporn Sujjitjoon, Suwattanee Kooptiwut, Watip Tangjittipokin, Prapaporn Jungtrakoon, Nattachet Plengvidhya, Pa-thai Yenchitsomanus [abstract]. Paired box 4 regulates pancreatic beta-cell survival via the Wnt signaling and anti-apoptosis pathways [abstract]. In: Proceedings of the TRF-OHEC Annual Congress 2018 (TOAC2018); 2018 Jan 10-12; Phetchaburi, Thailand.

APPENDIX

Paired box 4 regulates pancreatic beta-cell survival via the Wnt signaling and anti-apoptosis pathways

Jatuporn Sujjitjoon[1*], Suwattanee Kooptiwut[2], Watip Tangjittipokin[3], Prapaporn Jungtrakoon[1], Nattachet Plengvidhya[4], Pa-thai Yenchitsomanus[1]

[1]*Division of Molecular Medicine, Department of Research and Development, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand*

[2]*Department of Physiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand*

[3]*Department of Immunology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand*

[4]*Division of Endocrinology and Metabolism, Department of Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand*

Abstract

Genetic variations of *Paired box 4* (*PAX4*) were reported to be associated with type 2 diabetes (T2D) and caused maturity onset diabetes of the young type 9 (MODY9). A decrease in β-cell number contributes to the pathogenesis of T2D and MODY. Role of PAX4 in β-cell survival has been reported, however, the molecular mechanism by which PAX4 controls β-cell survival is not clearly understood. We aimed to explore how PAX4 regulates β-cell survival by using siRNA-mediated *PAX4* knockdown (KD) in rat insulinoma cell line. *PAX4* KD cells displayed a 2.5-fold increase in β-cell apoptosis, while did not alter β-cell proliferation. Down-regulation of *Wnt3a*, *Bcl2l1* (*Bcl-xL*) and *Bcl2l2* (*Bcl-w*) were demonstrated in *PAX4* KD cells. Overexpression of PAX4 did not show any significant increase transcriptional activity on the *Wnt3a* gene promoter. In contrast, the cells overexpressing HNF1A or HNF4A had significantly increased transactivation on the promoter. Decreased expression of *Hnf1a*, but not *Hnf4a*, gene was found in *Pax4* KD cells. Our results suggest that the decreased *Wnt3a* gene expression in *Pax4* KD cells may be partly controlled by other transcription factors, such as HNF1A and HNF4A, and Pax4 may play roles in the regulation of *Hnf1a* gene expression. Pax4 is a crucial transcription factor regulating β-cell survival via Wnt3a signaling and anti-apoptotic pathways. The understanding of PAX4 function in association with β-cell development, proliferation, and apoptosis may lead to a novel therapeutic strategy for diabetes.

Keywords: Paired box 4, Pax4, beta-cell survival, Wnt signaling, anti-apoptosis

[*]Corresponding author.

Tel.: 0-2419-7000 ext. 2774; Fax: 0-2411-0169

E-mail: jatuporn.suj@mahidol.ac.th, tangnina19@gmail.com

Paired Box 4 Regulates Beta-Cell Survival via Wnt Signaling and Anti-Apoptosis Pathways

Jatuporn Sujjitjoon[1,2], Nattachet Plengvidhya[2,3], Suwattanee Kooptiwut[4], Prapaporn Jungtrakoon[1,2], Watip Tangjittipokin[5], Pa-thai Yenchitsomanus[1,2]

[1]Siriraj Center of Research Excellence for Molecular Medicine, [2]Siriraj Center of Research Excellence for Diabetes and Obesity, [3]Division of Endocrinology and Metabolism, Department of Medicine, [4]Department of Physiology, [5]Department of Immunology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand. E-mail: tangnina19@gmail.com

Background

Maturity-onset diabetes of the young (MODY) is characterized by autosomal dominant inheritance, early age at onset, and pancreatic β-cell dysfunction. To date, mutations in at least fourteen distinct genes causing MODY types 1-14 have been discovered (1; Figure 1).

MODY9 is caused by mutations of Paired box 4 (Pax4) which encodes a transcription factor promoting pancreatic β-cell development, differentiation (2) and survival (3). *Pax4* expression is controlled by various transcription factors encoded from other MODY genes such as *HNF1α*, *HNF4α*, *Pdx1*, and *NeuroD1* (Figure 1).

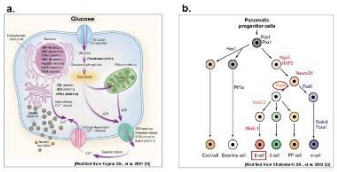


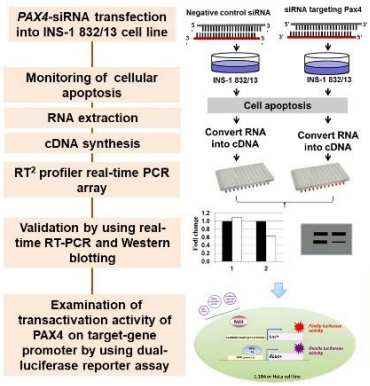
Figure 1. Model of proteins implicated in MODY1-14 (a), and the proteins required for beta-cell development and differentiation (b).

Pax4 plays dual roles in the regulation of target-gene promoters: trans-repression of *glucagon*, *insulin*, and *ghrelin* promoters and trans-activation on *c-myc* and *Bcl-xL* promoters. Pax4 controls INS-1E cell survival is at least mediated through *Bcl-xL* gene up-regulation (3). However, there may be other unknown apoptotic and signaling pathways controlled by Pax4.

Objectives

To identify other genes regulated by Pax4 and to examine the molecular mechanism of Pax4 in regulating pancreatic beta-cell survival and/or apoptosis, which may guide to the development of new strategies for treatment of diabetes.

Experimental design



Results

Silencing of *Pax4* increased INS-1 832/13 cell apoptosis and decreased cell viability, while did not alter cell proliferation rate.

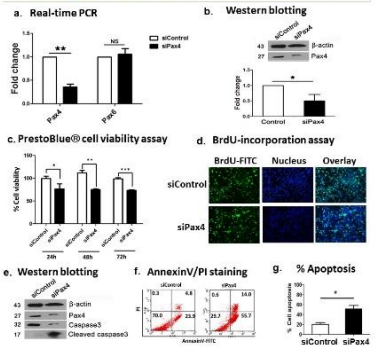


Figure 2. Silencing of *Pax4* gene increased INS-1 832/13 apoptosis without altered cell proliferation rate.

siControl and siPax4 were transfected into INS-1 832/13 cells by using lipofectamine 2000. After transfection and culture for 48 h, mRNA and protein expression of Pax4 were examined by using real-time RT-PCR (a) and Western blot analyses (b), respectively. *Pax4* knockdown (KD) cells were examined for viability, proliferation, cleaved caspase 3 protein level, and apoptosis by using PrestoBlue® viability assay (c), BrdU-incorporation assay (d), Western blotting (e), and AnnexinV/PI staining (f and g), respectively. The statistical differences between groups were analyzed by the unpaired Student's *t* test. (*; $p<0.05$, **; $p<0.01$, NS; no statistical significance difference).

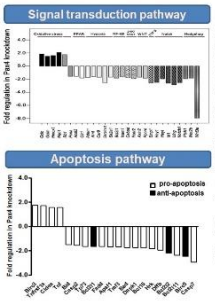


Figure 3. Differential gene expression in signal transduction (a) and apoptosis (b) pathways in *Pax4* KD cells.

INS-1 832/13 cells were transfected with siControl or siPax4 for two consecutive days. After transfection and culture for 48 h, total RNAs were extracted and cDNAs were prepared. The cDNAs were subjected to the RT² Profiler™ PCR array screening;
a. signal transduction pathway finder array;
b. apoptosis array.
The up-regulated and down-regulated gene expressions were illustrated.

Silencing of *Pax4* leads to decreased expressions of *Wnt3a*, *Bcl2l1* (Bcl-xL), and *Bcl2l2* (Bcl-w).

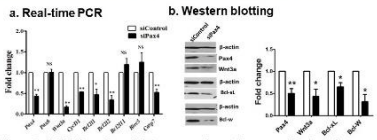


Figure 4. Validation of candidate gene and protein expression by using real-time RT-PCR and Western blotting.

After transfection of siControl or siPax4 into INS-1 832/13 cells and cultured for 48 h, RNAs were isolated and cDNAs were prepared. The cDNAs were used as templates for gene-specific amplifications by real-time PCR (a). Total cell lysate were extracted and separated onto SDS-PAGE Each protein was detected by specific antibody (b). The statistical differences between groups were analyzed by the unpaired Student's *t* test (*; $p<0.05$, **; $p<0.01$, NS; no statistical significance difference).

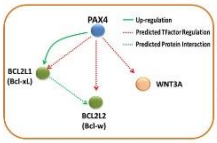
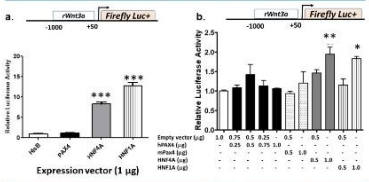


Figure 5. A proposed model of Pax4 regulation on target genes by using a Gene Network Central Pro software.

The *Wnt3a*, *Bcl2l1*, and *Bcl2l2* gene expressions are predicted to be regulated by PAX4. The promoter of these target-genes were predicted to harbour *cis*-regulatory elements for PAX4.

Results (cont.)

***Wnt3a* promoter activity in (a) 1.1B4 and (b) HeLa cells**



***Bcl-xL* promoter activity in (c) 1.1B4 and (d) HeLa cells**

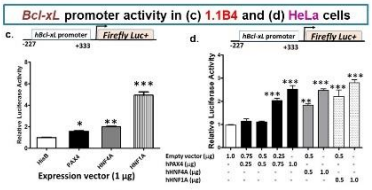


Figure 6. Transactivation activities of TFs on *Wnt3a* and *Bcl-xL* gene promoters using luciferase reporter assay.

Human pancreatic beta-cell line (1.1B4) and HeLa cells were transfected with expression vector encoding PAX4, or HNF1A, or HNF4A, together with *Wnt3a* gene promoter or *Bcl-xL* gene promoter, and pRL-SV40 internal control plasmid. Firefly and renilla luciferase activities were measured by using luminometer. The statistical differences between groups were analyzed by the unpaired Student's *t* test. (*; $p<0.05$, **; $p<0.01$, ***; $p<0.001$).

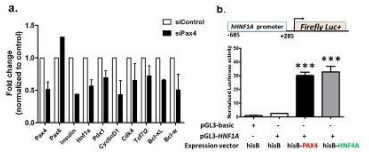


Figure 7. Relative mRNA expression levels of genes involved in regulating beta-cell function and/or survival (a) and the transactivation activities of PAX4 and HNF4A on human *HNF1A* gene promoter (b)

Discussion and conclusion

Pax4 plays a crucial role in regulating β-cell survival. The might be mediated by up-regulating anti-apoptotic genes, *Bcl2l1* (Bcl-xL) and *Bcl2l2* (Bcl-w), and/or *Wnt3a* gene that involved in promoting cell proliferation.

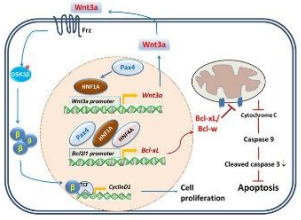


Figure 8. Schematic illustration of the proposed mechanism of how Pax4 may regulate beta-cell survival.

References

1. Fajans SS., *et al.* N Engl J Med. 2001;345(13):971-80.
2. Chakrabrati SK., *et al.* Trends Endocrinol Metab. 2003;14(2):78-84.
3. Brun T., *et al.* J Cell Biol. 2004.;167(6):1123-35.

Acknowledgements

This work was supported by Siriraj Research Development Grant, Faculty of Medicine Siriraj Hospital, Mahidol University Grant, and Research Career Development Grant from Thailand Research Fund (TRF). JS was supported by Thailand Research Fund (Grant Code: TRG5980017).