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Thesis Title: Dissecting the role of diabetes-associated PAX4 polymorphisms in modulating pancreatic beta cell development and function

Diabetes is a leading health problem affecting over 537 million individuals worldwide, incurring a huge healthcare burden on society. Populations living in Asia have the highest prevalence of diabetes among all ethnicities and often have a younger age of onset with a lower body mass index (BMI). To develop novel therapeutic strategies, numerous genome-wide studies have been performed across different ancestries to identify genetic variants that can predispose carriers to elevated risks for diabetes. A missense variant within the coding region of the *PAX4* gene (rs2233580) is associated with T2D in East Asians. The variant is common in East Asians (MAF 10 %) but rare or absent in other ancestry groups. Carriers of the R192H allele have a dose-

dependent earlier age of diabetes-onset and have a lower C-peptide level, suggesting a defect in pancreatic beta cell function.

To elucidate the mechanisms underlying the associations between *PAX4* variants and the risk of diabetes, our study included detailed clinical and *in vitro* studies on two distinct *PAX4* coding variants. We recruited donors carrying the East Asian-specific R192H variant and a novel protein-truncating variant Y186X identified in a Singapore family for assessment. We demonstrated carriers of the *PAX4* R192H variant to have reduced beta cell function, reflected as elevated blood glucose and insufficient insulin secretion. The two carriers of the *PAX4* Y186X variants had poor beta cell function as reflected by low disposition index.

Our *in silico* and *in vitro* molecular studies predicted that proteins derived from PAX4 R192H and Y186X variants have a loss of function due polymorphism within the DNA binding domain and protein truncation respectively, possibly resulting in reduced beta cell function. In mice, *Pax4* is essential for beta cell formation, but neither the role of diabetes-associated variants in *PAX4* nor *PAX4* itself on human beta cell development and/or function are known. To study the consequence of *PAX4* variants in human beta cell development, we generated three independent human induced pluripotent stem cell (hiPSC) models. First, a *PAX4*-knockout hiPSC model was generated using CRISPR-Cas9-mediated genome editing to investigate the role of *PAX4* in human pancreatic beta cell development. Second, we generated donor-derived hiPSCs carrying $PAX4^{+/+}$, $PAX4^{+/R192H}$, $PAX4^{R192H/R192H}$ and $PAX4^{+/Y186X}$ genotypes to study the consequences of gene variants in beta cell development. Finally, we utilized gene-corrected donor-derived hiPSCs to confirm the association of phenotype with the *PAX4* variant via a rescue study. Contrary to the observation in rodent models, we found that *PAX4* is not required for insulin-expressing beta cell formation from human

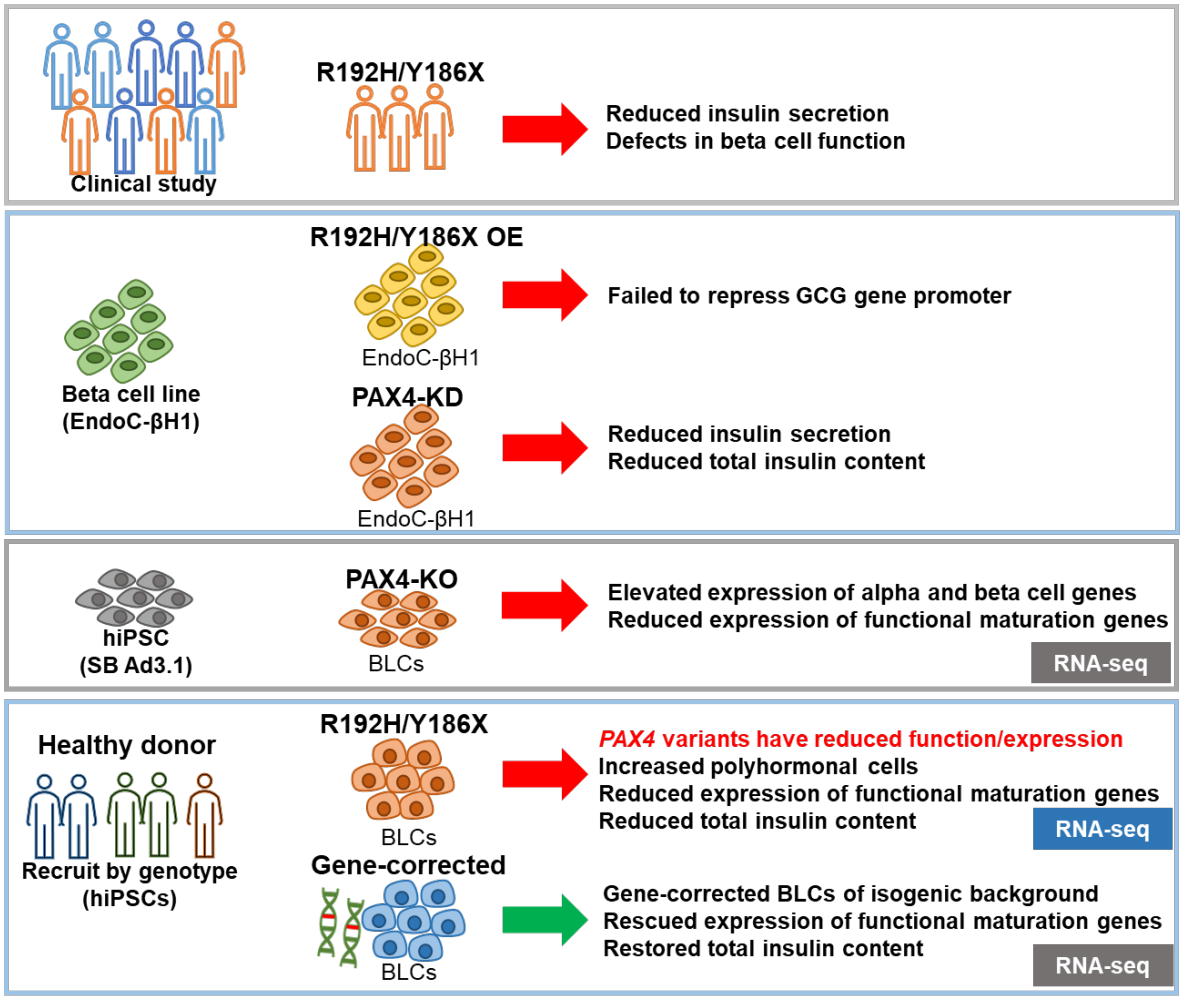
hiPSCs. We found that, in beta cells derived from hiPSCs that were deficient in *PAX4* or carried *PAX4* variants exhibited derepression of genes associated with alpha cells. These cells were more likely to be polyhormonal and demonstrated to coexpress GCG^+/C-PEP^+ in immunostaining assays. These cells also had reduced total insulin content, contributing to decreased functionally. This phenotype was reversed in the donor-derived hiPSC lines through correction of the *PAX4* variant allele(s).

Using the human beta cell line EndoC-βH as a model, we demonstrated that PAX4 variant proteins had aberrant transcriptional regulatory activities on *INS* and *GCG* gene promoters. The loss of repression of the *GCG* gene promoter in beta cells possibly explained the coexpression of GCG^+ in C-PEP^+ endocrine cells carrying *PAX4* gene variants. Gene silencing of *PAX4* in EndoC-βH1 cells also resulted in elevated *GCG* expression, reduced total insulin content and impaired glucose-stimulated insulin secretion (GSIS) function.

Collectively, we made use of clinical *in vivo* studies, hiPSC models (including *PAX4*-knockout, donor-derived hiPSCs and gene-corrected hiPSCs) and mature beta cell line models to sequentially interrogate the role of *PAX4* in human beta cell development and function. Our study i) does not support a role of *PAX4* variant in causing maturity-onset diabetes of the young (MODY); ii) demonstrated that unlike the mouse, *PAX4* is not essential in the differentiation and formation of beta cells in hiPSC models; iii) supports a role of *PAX4* deficiency or gene variant in causing the formation of polyhormonal endocrine cells with reduced insulin content and impaired insulin secretion, uncovering a role of human *PAX4* in modulating mature beta cell function. Our study therefore facilitates a better understanding of the effects of *PAX4* gene variants contributing to the risk of T2D. We conclude that *PAX4* R192H (loss of function) and Y186X (haploinsufficiency) variants contribute to the formation of

polyhormonal endocrine cells with impaired insulin secretion function, predisposing East Asian carriers to higher risks of developing T2D.

Supervisors: Dr Adrian Teo and Assoc Prof Tan Nguan Soon



Graphical summary: PAX4 R192H and Y186X contribute to the formation of polyhormonal endocrine cells with impaired insulin secretion function, predisposing carriers to higher risks of T2D. This work contributes to Lau, H. H., et al. (2022). "PAX4 loss of function alters human endocrine cell development and influences diabetes risk." bioRxiv: 2022.2005.2015.491987.