Aus dem Experimental and Clinical Research Center der
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D I S S E R T A T I O N

Human *KCNQ1* mutations cause neonatal diabetes and hyperinsulinemic hypoglycemia: Clinical and functional characterization

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Abbreviation

α	alpha
β	beta
bHLH.	basic helix–loop–helix
bp	base pair
BSA	bovine serum albumin
BCA	bicinchoninic acid
CaM	calmodulin
CHI	congenital hyperinsulinism
CNS	central nervous system
cDNA	complementary deoxyribonucleic acid
Ct	threshold cycle
dpc	days post-conception
δ	delta
DM	diabetes mellitus
DKA	diabetic ketoacidosis
DAPI	Vectashield HardSet Mounting Medium
ED	embryonic day
ε	epsilon
EDTA	ethylenediamine-N,N,N',N –tetraacetate
FBS	fetal bovine serum
GDM	gestational diabetes mellitus
GAD	glutamic acid decarboxylase
GWAS	genome-wide association studies
GLUT2	glucose transporter 2
HCl	hydrochloric acid
hk	house-keeping
IGT	impaired glucose tolerance
ICA	islet cell autoantibodies
IAA	insulin autoantibodies
IgG	immunoglobulin G
ID	identity number
I_{ks}	delayed outwardly rectifier potassium current
I_{Kr}	inwardly rectifier potassium current
K_v	voltage-gated potassium
K_{ATP}	ATP-sensitive potassium

KO	knock out
LADA	latent autoimmune diabetes of adults
LQTS	long QT syndrome
MODY	maturity-onset diabetes of the young children
NGS	next-generation sequencing
ND	neonatal diabetes
NDS	normal donkey serum
oGTT	oral glucose tolerance test
PP	pancreatic polypeptide
PND	permanent neonatal diabetes
PD	pore domains
PaO2	average arterial oxygen tension
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PAGE	polyacrylamide gel electrophoresis
PFA	paraformaldehyde
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
PBMC	peripheral blood mononuclear cell
PH	pancreatic hypoplasia
RIPA	radio immunoprecipitation assay buffer
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
SNPs	single nucleotide polymorphisms
SUR1	sulfonylurea receptor 1
TAE	tris-acetate-EDTA
TND	transient neonatal diabetes
TBS buffer	tris-buffered solution
T1D	type 1 diabetes
T2D	type 2 diabetes
T1DA	autoimmunity related type 1 diabetes
T1DB	non-autoimmunity related type 1 diabetes
VSD	voltage-sensing domains
WT	wild type

Abstract

The morbidity of diabetes mellitus (DM) is increasing all over the word. Diabetes mellitus is usually classified into type 1 diabetes (T1D), type 2 diabetes (T2D), gestational diabetes mellitus (GDM), and other specific types of insulin deficiency with developmental syndromes. In contrast, congenital hyperinsulinism (CHI) is the most common cause of persistent or recurrent hypoglycemia in infancy. The underlying pathogenesis of the defective insulin secretion involved in the two disorders remains obscure. Our research group mainly focuses on human inherited disorders of pancreatic β cell dysfunction, namely monogenic diabetes and hyperinsulinism. We set up a database of patients with β cell dysfunction, including sporadic and familial diabetes mellitus and congenital hyperinsulinism. We have performed molecular screening in this cohort, and detected mutations in 30% of the patients with known candidate genes, mutations in 30% of the patients with novel candidate genes, which are important in the regulation of insulin secretion. From that, we detected three *KCNQ1* gene mutations in one neonatal diabetes patient (R397W, homozygous) and two congenital hyperinsulinism patients (CHI, heterozygous of G292D & E13 del), respectively. *KCNQ1* encodes a potassium channel protein, $K_v7.1$, which is a voltage-gated potassium channel present in the cell membranes of cardiac tissue, pancreas, and in inner ear neurons, among other tissues. *KCNQ1* was shown to be closely related to long QT syndrome (LQTS) and type 2 diabetes. We performed functional analysis of the three mutations detected in mouse pancreatic beta cells, and investigated the functional changing of the pancreas in *Kcnq1* knock out mice. We detected defective insulin secretion caused by the three *Kcqn1* mutations: decreased insulin secretion in the diabetic cellular models, and increased insulin secretion in the two CHI cellular models, which is consistent with the human phenotypes. The diabetic mutation (R397W) down-regulates the expression of *Foxa2* and induces the conversion from beta cells to alpha cells, which is very likely the molecular mechanism involved in defective insulin secretion. By contrast, the beta cell maintenance and function are enhanced by the two CHI mutations (G292D & E13 del) through up-regulation of Pdx1 expression. In *Kcnq1* KO mice, we found an obvious reduction of β cell mass and disrupted differentiation of both beta and alpha cells in the islets. Further deep investigation is necessary for better understanding the detail molecular pathways caused by the *KCNQ1* mutations in pancreatic beta cell dysfunction.

Zusammenfassung

Beim Menschen sind zwei wesentliche Störungen der Insulinausschüttung mit schwerem, klinischen Erkrankungsbild bekannt – Diabetes mellitus, charakterisiert durch erhöhten Blutzucker und relativen Insulinmangel, und hyperinsulinämische Hypoglykämie, verursacht durch eine fehlende Hemmung der Insulinsekretion bei niedrigen Bltrzuckerwerten und klinisch schweren Hypoglykämien mit schweren, neurologischen Schäden. Die Diabetesprävalenz und die Mortalität durch Diabetes-assoziierte Folgeerkrankungen nehmen weltweit zu. Diabeteserkrankungen werden in Diabetes mellitus Typ-1 (T1D), Typ-2 (T2D), Gestationsdiabetes (GDM), und andere, spezifische Diabetestypen (Typ-3), wie bekannte, monogene Diabetesformen oder Diabetessyndrome eingeteilt. Im Gegensatz dazu ist der angeborene Hyperinsulinismus (Congenital Hyperinsulinism, CHI) die häufigste Ursache für schwere und anhaltende Hypoglykämien im Neugeborenen- und Säuglingsalter. In einem Teil der Kinder mit seltenen Diabeteserkrankungen oder mit angeborenem Hyperinsulinismus ist die Pathogenese bisher nicht geklärt. Unsere Forschungsgruppe untersucht familiäre Störungen der Betazellfunktion beim Menschen, im Detail monogene Diabetesformen und Hyperinsulinismus unklarer Pathogenese. Wir haben eine Datenbank mit klinischen und anamnestischen Daten von Patienten mit ungeklärter Betazelldysfunktion etabliert, genauer mit sporadischem oder familiärem Diabetes, sowie mit hyperinsulinämischem Hyperinsulinismus. Wir hatten in dieser Kohorte mittels Sequenzierung von Kandidatengenen und anschliessend Next-Generation Sequenzierung in 30% der Patienten Mutationen in bekannten Diabetes- oder CHI-Genen gefunden und in weiteren 30% Mutationen in Genen, die sehr wahrscheinlich eine Schlüsselkrolle in der humanen Betazellfunktion oder Anlage endokriner Pankreaszellen spielen. Unter diesen neuen Mutationen hatten wir drei KCNQ1-Genmutationen in einem Kind mit neonatalem Diabetes ohne jede Insulinsekretion (R397W, homozygot) und in zwei Kindern mit Hyperinsulinämischer Hypoglykämie identifiziert (heterozygot, G292D & E13 del). *KCNQ1* kodiert ein Kaliumkanalprotein (K_v7.1), das als spannungsabhängiger Kaliumkanal die Repolarisation an der Zellmembran u.a. von Herzmuskelzellen, Pankreaszellen und Kochlearzellen reguliert. Mutationen in *KCNQ1* sind bisher heterozygot beim Long-QT-Syndrom (LQTS) und homozygot Jervell and Lange-Nielsen-Syndrom (LQT, Taubheit) beschrieben. Wir hatten unsere drei neuen Mutationen in Glukose-sensitiven Betazellen (Maus) überexprimiert und funktionell untersucht sowie Pankreasschnitte von Kcnq1-Knockout-Mäusen histopathologisch ausgewertet. Die Insulinsekretion aller drei Mutationen war, verglichen mit dem Wildtyp, gestört: Die basale und stimulierte Insulinsekretion der Diabetes-Mutation (R397W) war reduziert, die der Hyperinsulinismusmutationen (G292D & E13 del) erhöht. Die diabetesassoziierte Mutation R397W supprimiert die Genexpression von Foxa2 und erhöht die Expression von Alphazell-

versus Betazellmakern, ein Hinweis auf den molekularen Mechnismus. Im Gegensatz dazu waren war Pdx1, als Marker differenzierter Betazellen, bei Transfektion mit den Hyperinsulinismusmutationen G292D & E13 del erhöht exprimiert. In den *Kcnq1* KO Mäusen war die Betazellmasse deutlich reduziert und gibt einen Hinweis auf eine gestörte Differenzierung von endokrinen Pankreaszellen, zuallererst der Betazellen. Weitere Untersuchungen werden Aufschluss darüber geben, über welche Signalwege der Kaliumkanal KCNQ1 die Betazellmasse und die Insulinsekretion reguliert und Mutationen Betazelldysfunktionen beim Menschen auslösen.

1. Introduction

1.1 Pancreatic β cell function, origination

1.1.1. The role of pancreas and pancreatic α and β cells in glucose metabolism

Pancreas plays its significant roles in nutrient digestion, absorption and utilization of body, and it is made up of exocrine (99%) and endocrine(1%) components [1]. Pancreatic islet consists mainly of β (beta) cells, which are the only source of insulin resources [2, 3], together with α (alpha), δ (delta), ε(epsilon), and PP (pancreatic polypeptide) cells, secreting glucagon, somatostatin, ghrelin and PP separately [3, 4].

Glucagon and insulin, secreted by pancreatic α and β cells respectively, exert counter-regulatory actions on glucose metabolism. Glucagon increases circulating glucose levels by stimulating hepatic glycogenolysis and gluconeogenesis. Conversely, insulin decreases glucose levels by eliciting cellular influx and storage of glucose while dampening hepatic glucose production. In the pancreatic islet, α and β cells are physically juxtaposed; in order to modulate secretory activities (i.e. cell function), each cell type receives and responds to secreted signals from the other cell types. For instance, insulin represses glucagon expression and release from the α cell, whereas glucagon stimulates β cell insulin secretion (Figure 1) [5].

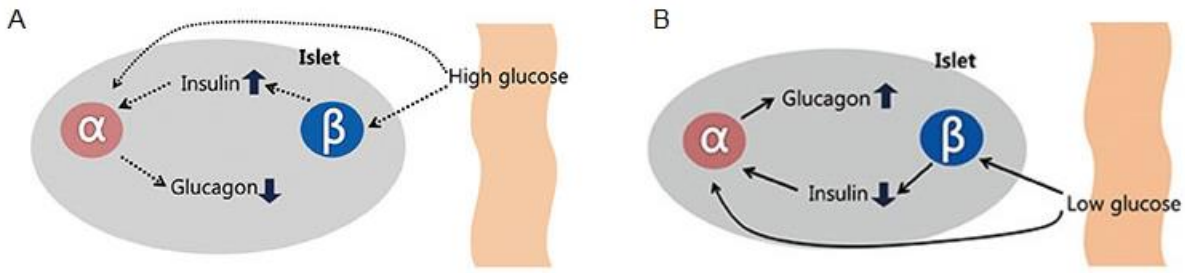

Figure 1: Intra-islet insulin & glucagon secretion. (A) An increase in plasma glucose level results in an increase β-cell insulin secretion that decreases in α-cell glucagon secretion to reduce the plasma glucose level. (B) A decrease in plasma glucose level causes a decrease in β-cell insulin secretion that signals an increase in α-cell glucagon secretion during hypoglycemia adapted from [6].

Pancreatic β cell dysfunction could manifest as either hyperglycemia (diabetes mellitus) with a relative deficiency of insulin or hypoglycemia due to a failure to adequately stop insulin secretion at low glucose levels (congenital hyperinsulinism, CHI). Therefore, understanding the molecular mechanism of β cells will provide a helpful knowledge for dealing with all types of diabetes and hyperinsulinism. Abnormal differentiation and regeneration in β cells and/or apoptosis in β cells is related to inappropriate insulin secretion, which cannot fulfil the body requirements resulting in hyperglycemia or diabetes. However, enhanced β cells function resulting in over-secretion of insulin, which then eventually cause hypoglycemia. Hence, it's important to understand the origin, development, and apoptosis of β cells. Beta cell mass is increased by β cell proliferation and hypertrophy, and is decreased by β cell apoptosis and

atrophy. Beta cell proliferation proceeds at a fast rate in late stage of gestation [7], and the ability of β cell proliferation decreases with age [8]. The evidence from mouse models shows that the factors to regulate β cell proliferation may vary at different periods of fetal gestation and after birth [9]. Decreased β cell proliferation rate could be one of the reasons leading to a decreased β cell mass. After postnatal period, β cells continue to proliferate, but during this time this is accompanied with apoptosis [10]. Beta cell apoptosis occurs at a very low rate in embryonic development period and infant stage, it increases with age in the adult [10]. Increased β cell apoptosis causes insufficient β cell mass, which results in loss of insulin secretion, and then causes hyperglycemia or diabetes.

Pancreatic α cell dysregulation and excessive glucagon secretion contribute to both type 1 (T1D) and type 2 diabetes (T2D). In type 1 diabetes, islets contain mostly hyperplastic α-cells, which produce an unregulated and excessive amount of glucagon. In the absence of opposing action of insulin, this results in unrestricted hepatic gluconeogenesis and glycogenolysis, resulting in hyperglycaemia and contributing to diabetic ketoacidosis [11].

1.1.2 Development of the pancreas

The embryonic endoderm develops into a primitive gut, which will then develop into the digestive system. The pancreas derives from two parts of epithelium that bud dorsally and ventrally from the foregut/mid-gut around embryonic day 9(E9) in mouse (Figure 2 [12]) [13]. The bud parts fuse together approximately at E12.5 [14] and then epithelial cells differentiate into endocrine and exocrine cells [15]. Development of dorsal and ventral are out of step, development of the ventral bud takes place somewhat later compared to the ventral bud [16]. The required signals for development show a substantial difference between dorsal bud and ventral bud. For example, the homeobox 9 (Hb9) transcription factor is required for the formation of the dorsal, but not ventral bud [16].

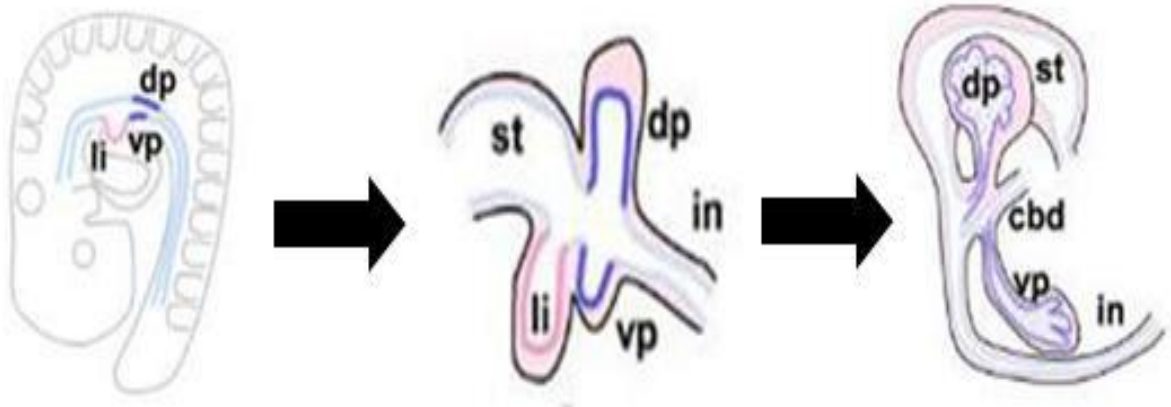

Figure 2: Pancreatic anatomy and development. The dorsal pancreas (dp) and ventral pancreas (vp) were found approximately at E9 in mouse (left) from gut endoderm that are located close to forming liver (li) during the development of gut endoderm. The pancreatic dorsal and ventral bud in the surrounding mesenchyme and locate between stomach and intestine at approximately E10.5 (middle). At approximately E12.5 (right), the dorsal and ventral pancreatic bud fuse together and through original ductal connect to the intestine and/or common bile duct (cbd) (adapted from [12]).

1.1.2.1. The regulation factors in pancreatic β cell development and maintenance of mature β cell function

The embryonic development of the endocrine pancreas is regulated by extracellular signals especially by transcription factor (Figure 3 adapted from [17]). Transcription factors such as neurogenin 3 (*Ngn3*), Pancreatic-duodenal homeobox1 (*PDX1*), and regulatory factor X6 (*Rfx6*) play crucial roles in regulating the differentiation of endodermal cells into β cells [18]. For maintenance of the mature β cell function, *PDX1*, *Foxa2*, and *MafA* play crucial roles by regulating several β-cell-related genes.

PDX1 plays a crucial role in the early stage of pancreas development [19], which is induced at E8.5 in the foregut endoderm and is expressed in all the pancreatic epithelium at E9.5 in mice (29-31 days post-conception (dpc) in human) [16]. *PDX1* is required for the growth of the pancreatic buds and pancreatic bud arrest at the early stage of development in the *Pdx1* KO (Knock out) mice [16]. *Pdx1* does not only affect the development of the whole pancreas but also affects the differentiation of β cells [20] and maintains the mature β cells function by regulating several related β cell genes [21]. In addition, *Pdx1* affects exocrine cells in the pancreas as well (Figure 3).

Another important molecular factor for pancreas and β cells is *NeuroD1*. *NeuroD1* is expressed in pancreatic and intestinal endocrine cells as well as neural tissues. Its high expression is maintained till the mature endocrine cells in pancreas. It was reported that null mutation in *NeuroD1* obviously decreased in number of β cells in mice [22], and humans with heterozygous mutations in *NeuroD1* have MODY6 [23].

Foxa2 (Forkhead box a2) is one of winged helix/forkhead Foxa family of transcription factors, which regulates hepatic and/or pancreatic gene expression [24]. In embryonic development, targeted disruption of *Foxa2* results in the embryo's death after gastrulation [24]. *Foxa2* is an upstream transcription factor of the Pdx1, Nkx6.1 (Figure 3) [25], therefore transcription factor Foxa2 can affect β cell differentiation and insulin secretion. *Foxa2* is involved in multiple pathways of insulin secretion and plays an important role in regulating the expression of genes [25]. Deletion of *Foxa2* in β cells resulted in hyperinsulinemic hypoglycemia [25], this accounts for *Foxa2*'s vital role in insulin secretion and glucose hemostasis.

V-mafmusculoaponeurotic fibrosarcoma oncogene homologue A (*MafA*) has also recently been identified as an important regulator of β cell function and a marker of mature β cells [26]. In development phase, *MafA* is expressed after specification of insulin$^+$ cells that are expressing another Maf factor, *MafB*; after birth, these insulin$^+$ *MafA*$^+$ cells stop MafB expression and gain glucose responsiveness. *MafA* controls the specificity expression of the

insulin gene of β cells and function with a potent transactivator for insulin gene [27]. *Mafa* KO mice have a normal development of pancreas but develop diabetes after birth [16]. In all transcription factors of affect pancreatic development, transcription factors *Pax4*, *Pax6*, *Nkx6.1* and *Nkx2.2* also play an important role in differentiation of β cells [28].

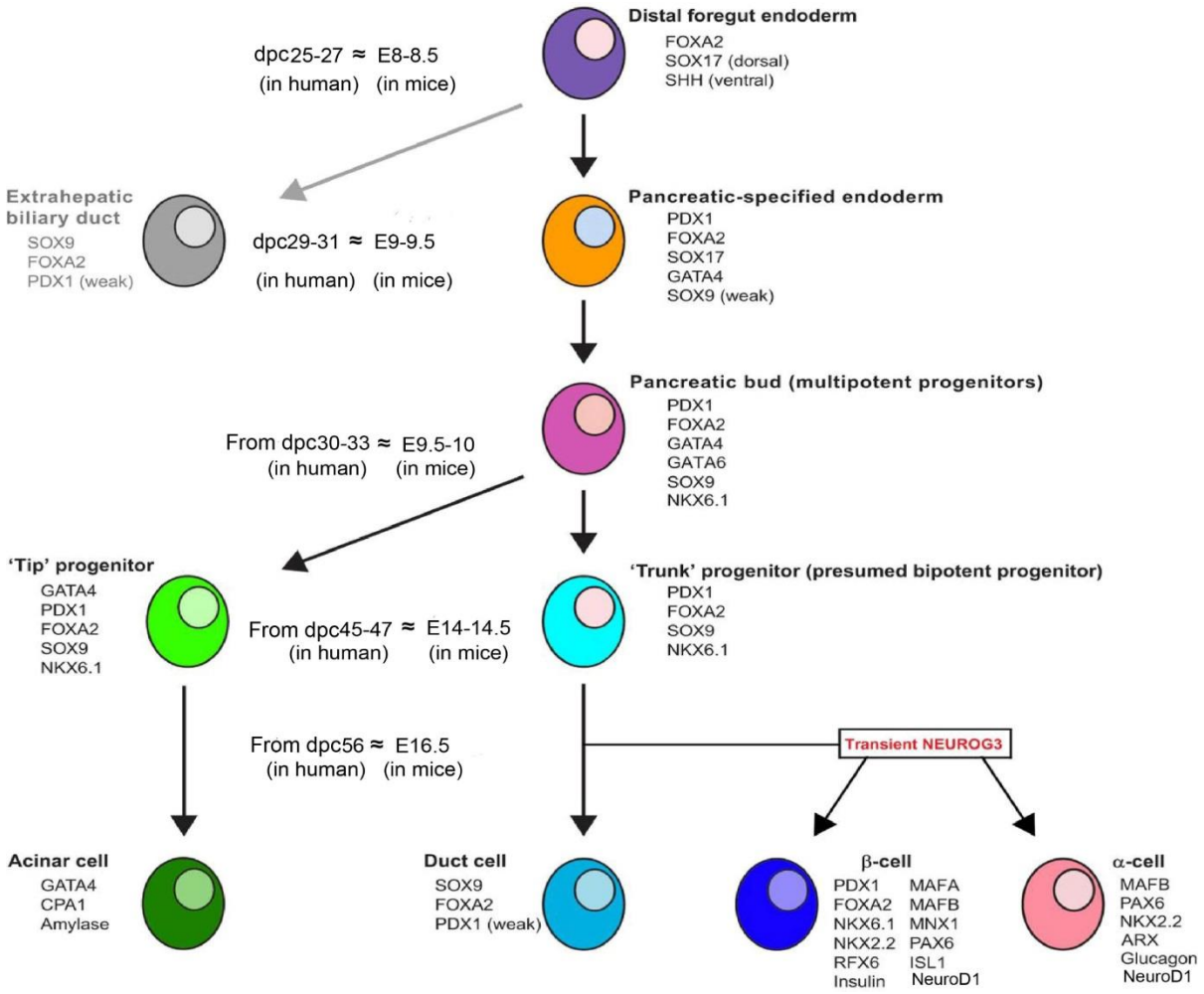

Figure 3: Transcription factor network of human pancreas development. Transcription factors and other key markers which identify different cell types and stages during early pancreas specification and subsequent lineage commitment. Pancreatic development starts from distal foregut endoderm approximately at 25-27 days post-conception (dpc) in human (corresponding to approximately E8-E8.5 in mouse). At this stage, *Foxa2*, *SOX17* and sonic hedgehog (SHH) expressed in embryo. Parts of distal foregut endoderm develops into extrahepatic biliary duct (gray color), the left of distal foregut endoderm develop to the pancreatic-specified endoderm at dpc 29-31 (corresponding to approximately E9-E9.5 in mouse), *PDX1*, *FoxA2*, *SOX17*, GATA4 and *SOX9* find expression at this stage. Pancreatic-specified endoderm continues to develop into pancreatic bud from dpc30-33 (corresponding to approximately E9.5-E10 in mouse), *PDX1*, *FoxA2*, *GATA6*, *SOX9* and *NKX6.1* find expression at this stage. Pancreatic bud continue to develop, parts of them develop to peripheral cluttered cell ('tip') and finally develop to acinar cells, left parts develop to central duct-like structures ('trunks') progenitor cells. At dpc45-47(corresponding to approximately E14-E14.5 in mouse), pancreatic progenitor cells are differentiated into duct cells and endocrine cells, *PDX1*, *FoxA2*, *SOX9* and *NKX6.1* find expression in this stage. From dpc56 (corresponding to approximately E16.5 in mouse), progenitor cells are further differentiated into α, β cells by Neurog 3 (adapted from [17]).

The transcription factors attribute to the pancreatic development and β cell fate are closely relative to dysfunction of mature β cell as well. Understanding pancreatic development and β

cell fate helps improve the therapies of dysfunction of β cell.

1.1.2.2. The regulation factors in pancreatic α cell development and mature α cell cell function maintenance

Proper differentiation and function of α- and β-cells are critical for blood glucose homeostasis. The differentiation of α-cells and the maintenance of α-cell function can be influenced at several stages during development and in the maturing islet. Similar to the development of pancreatic β cells, *Ngn3*, *Pdx1*, and *Rfx6*, play their critical roles in the determination of the endocrine cell fate, while other transcription factors, such as aristaless-related homeobox (*Arx*) and forkhead box a2 (*Foxa2*), are implicated in the initial or terminal differentiation of α-cells (Figure 3) [11].

The paired box homeodomain (*Pax6*) is crucial for endocrine cell development and function and plays an essential role in glucose homeostasis. Homozygous mutant mice for *Pax6* are characterized by markedly decreased β and δ cells and absent α cells. *Pax6* regulates directly or indirectly the transcription of the Proglucagon, *PC2*, *MafB*, *cMaf*, and *NeuroD1*/*Beta2* genes, which are critical for α cell development and differentiation as well as glucagon biosynthesis. This provides a potential explanation for the dramatic absence of pancreatic α cells in *Pax6* knock-out mice [29].

MafB and *MafA* bind Maf response elements, and most *MafA*-regulated genes are first regulated by *MafB* during embryonic development. *MafA* and *MafB* are distinct from others in regard to temporal and islet cell expression pattern, with β-cells affected by *MafB* only during development and exclusively by *MafA* in the adult [30], *MafB* was restricted to α-cells soon after birth (Figure 4). Therefore, *MafA* was found to activate insulin expression, whereas *MafB* specifically stimulated glucagon in experiments performed in endocrine cells [31].

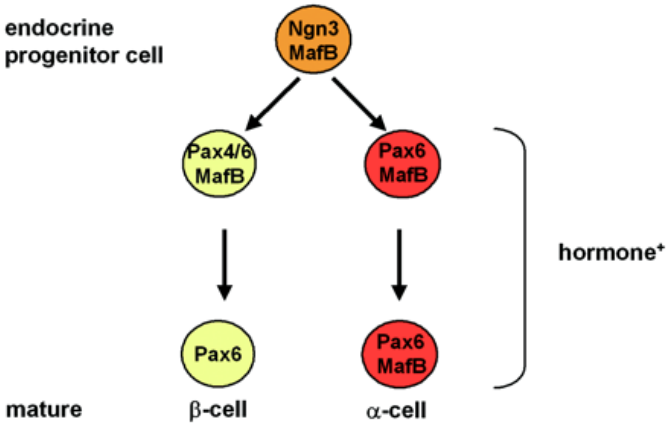

Figure 4: Expression pattern of *MafB* during α- and β-cell differentiation. *MafB* is expressed in ngn3^{+} cells (orange) insulin^{+} (yellow), and glucagon^{+} (red) cells. Developing β-cells produce both *Pax4* and *Pax6*, whereas α-cells only express *Pax6*. *MafB* expression persists only in α-cells after birth [31].

1.2 Current studying of diabetes mellitus

Diabetes mellitus is considered to be one of the most common and non-infective chronic disease worldwide. Over the last two decades, DM has become one of the main health challenges in the world. It is estimated that approximately 366 million patients with diabetes

were diagnosed in 2011, and the figure could reach 552 million by 2030 [32]. Current statistics indicate that 382 million patients suffered from diabetes in 2013 worldwide and this figure is expected to reach 592 million by 2035 [33]. Therefore, it's important to understand the pathogenic factor and aetiology of diabetes which might contribute either to preventing it or delaying its occurrence.

1.2.1 Definition and symptoms of diabetes

It is commonly known that diabetes is a group of metabolic diseases characterized by hyperglycemia resulting from inadequate production or utilization of insulin. The chronic hyperglycemia of diabetes is a kind of disease causing chronic health damage, dysfunction, and failure of different organs such as eyes, kidneys, nerves, heart, and blood vessels [34]. Diabetic patients show classic symptoms such as polyuria, polydipsia, polyphagia, and weight loss [35]. In addition to the symptoms described above, blurred vision, impairment of growth, slow healing of cuts, itchy skin, susceptibility to certain infections accompany diabetes. These patients may also experience some symptoms of acute and life-threatening in diabetic ketoacidosis (DKA) or nonketotic hyperosmolar syndrome including nausea, vomiting and abdominal pain, the smell of acetone on the breath, Kussmaul's respiration and so on [36], they are considered to be important signs in diagnosing diabetes mellitus. Further, diabetes is associated with multisystem complications, such as retinopathy, nephropathy, neuropathy and cardiovascular disease, and it was reported that complications of cardiovascular system are 2- to 4-fold higher than others system complications [37].

1.2.2 Etiology of diabetes and the role of pancreas secret hormones

The causes of diabetes are due either to the pancreas's inability to produce the amount of insulin required or to the body cells' inability to respond properly to the insulin produced. Even though the other hormones like glucagon, adrenalin and others can also regulate the glucose levels in the body. Insulin is the principal hormone that regulates the uptake of glucose from blood into adipose tissue, liver, muscle, and other organs in the body. Insulin restrains not only the breakdown of glycogen or the process of gluconeogenesis, but also stimulates the transport of glucose into fat and muscle cells, and it can stimulate the storage of glucose in the form of glycogen as well (Figure 1 Chapter 1.1.1).

Insulin levels will decrease when there is a dysfunction of β cells or deficiency in β cells, causing increased blood glucose levels, and elevated glucose in the body reach a threshold of reabsorption in the kidneys, failure of absorption of glucose are excreted in the urine. The osmotic pressure of the urine is raised, and inhibiting reabsorption of water by the kidney, and resulting in polyuria and increased fluid loss, decreased blood volume causes patients who have symptoms with dehydration and polydipsia. Therefore, pancreatic β cells play a key

role in regulating glucose homeostasis by secreting insulin to maintain glucose levels in balance in the body.

1.2.3 Classification of diabetes mellitus based on its etiology

The classification of diabetes is divided into type 1 diabetes (T1D), which results from absolute deficiency of insulin secretion; type 2 diabetes (T2D), which results from a relative insulin deficiency and insulin resistance; gestational diabetes mellitus (GDM); and other specific types, including monogenic diabetes, neonatal diabetes (ND), disease of the exocrine pancreas, endocrinopathies, and drug or chemical- induced diabetes [34](Figure 5).

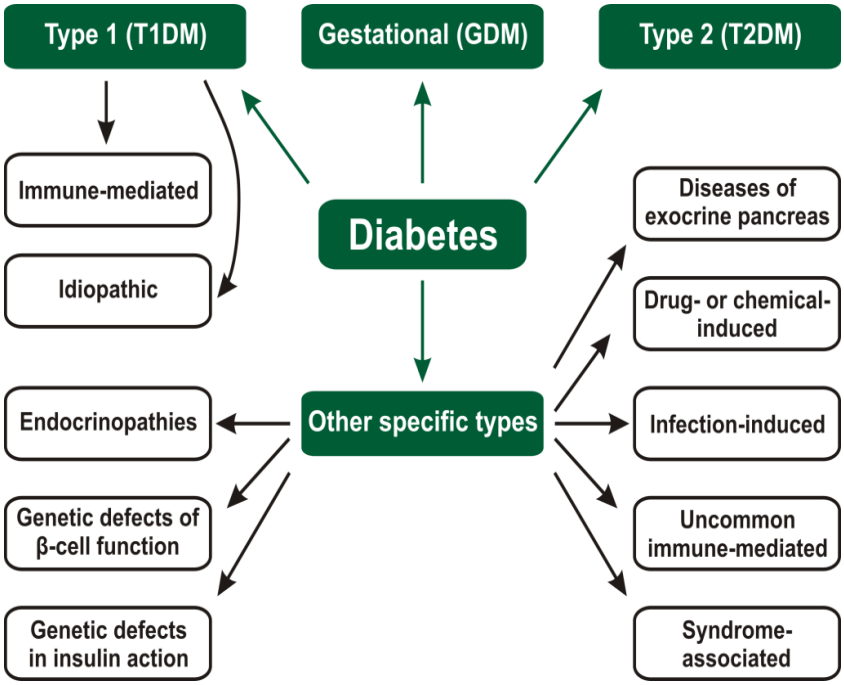

Figure 5: Classification of diabetes based on the mechanism of development. Three main types: type 1, gestational and type 2 diabetes mellitus account for ~ 95% of all diabetes cases. Other specific types of diabetes comprise rare types of disease having heterogeneous mechanisms of development. Monogenic diabetes is caused by mutations in one of the several genes required for proper pancreatic β-cell function or insulin action. The figure is summarized by Dr. Šimaitė based on the review of [34].

T1D can be further classified as immune-mediated or idiopathic diabetes. In T1D, immune-mediated by T cell was considered to be as the main cause of β cells failure [38]. Signals of the immune destruction of the β cells include islet cell autoantibodies (ICA), insulin autoantibodies (IAA), autoantibodies to glutamic acid decarboxylase (GAD), and autoantibodies to the tyrosine phosphatases IA-2 and IA-2b. This form of diabetes is also closely associated with certain HLA. T1D accounts for only 5–10% of diabetes sufferers [34], and the disease can happen at any age, but morbidity is highest between birth and 14 years old [33]. T1D can develop rapidly in infant or children. Sometimes, diabetic ketoacidosis as the first manifestation of the disease are found in children. Of course, rapid development of the disease occurs in situations of infection, stress and so on. Latent autoimmune diabetes of adults (LADA) is a condition in which T1D develops in adults, and patients with LADA were often initially diagnosed as T2D, based on age rather than etiology. This kind of patients with T1D have a rather slow progress and maybe residual β cells function sufficient to prevent diabetic ketoacidosis [34, 39]. This is because T1D is mainly immune-mediated, the patients with T1D may also have other autoimmune-disorders, such as Graves' disease, Hashimoto's

thyroiditis, Addison's disease and so on [34]. This kind of autoimmunity related type 1 diabetes is usually called T1DA.

The second form of T1D has no known etiologies, is thus called idiopathic diabetes. These patients have varying degree of insulin deficiency but no evidence of autoimmunity. This form of diabetes has strongly hereditary, and usually is called T1DB [34] or monogenic diabetes.

Monogenic diabetes is believed to be caused by inheriting a mutation or mutations in a single gene [40]. It affects 1~4% of pediatric diabetic patients [41, 42]. Most of the mutations in monogenic diabetes cause the insulin secretion defect to fulfil the body's requirements. Genetic defects of β cells include maturity-onset diabetes of young children (MODY), neonatal diabetes (ND), mitochondrial DNA defects, and other syndromic diabetes disorders. MODY and ND are the two mainly monogenic forms of diabetes. MODY is considered as the more common type of monogenic diabetes than ND, accounting for 2-5% of all patients with diabetes [43]. In the past, MODY was related to family history, absence of insulin dependence, and early onset of symptoms (<25 years), MODY can be diagnosed recently by molecular genetic testing [44]. At present, known subtypes of MODY result from dominantly acting heterozygous mutations in important genes which affect the function of β cells [45](Figure 6). In the MODY family, MODY3 is the most common form, its mutations in the gene encoding the transcription factor hepatocyte nuclear factor (*HNF*)-1A on chromosome 12 [34]. MODY2 is the second most common form, which is caused by a mutation in glucokinase gene on chromosome 7 [34]. The less frequent forms in the MODY family are: MODY1 (*HNF4A*), MODY4 (*PDX1),* MODY5 (*HNF1B*), MODY6 (*NEUROD1*), MODY7 (*KLE11*), MODY8 (*CEL*), MODY9 (*PAX4)*, MODY10 (*INS*), MODY11 (*BLK*), MODY12 (*ABCC8*), and MODY13 (*KCNJ11*) (OMIM: #606391). About 5–20% of patients with MODYs can be correctly diagnosed [46]. However, some patients with MODY are often misdiagnosed as having T1D and treated with insulin, because they are frequently young and non-obese [44, 47]. Since most patients with MODY can be treated with oral antidiabetic agents, it is important that the patients are correctly diagnosed [44]. ND is defined as insulin-requiring hyperglycemia that diagnosed in the first 6 months of life [48]. Nonetheless, it is a rare disorder with an incidence of approximately 1.43-1.96 in 100.000 infants [49]. Patients with ND are usually classified as having either transient or permanent ND (PND). Transient ND (TND) patients typically have hyperglycemia that resolves by 18 months, hyperglycemia in patients with PND typically does not resolve. Many patients with ND are transient but nearly half of them develop into PND [50]. The most common genetic defect in patients with TND is detected on ZAC/HYAMI imprinting on chromosome 6q [34, 47, 48]. Whereas *KCNJ11* gene mutations are the most common known gene that can cause PND, which encoding the Kir6.2 subunit of the β-cell K_{ATP} (ATP-sensitive potassium) channel [51]. Point mutations in

mitochondrial DNA result in inherited diabetes, and are commonly associated with sensorineural deafness [34]. In general, a correct diagnosis is very important so that the appropriate treatment can be selected to ensure a better control of glucose, reduce complications and improve the quality of life in the long term. Gene tests are especially important in diabetic patients with early onset, family history, glucose level uncontrolled by conventional therapy.

Type 2 diabetes mellitus (T2D) is the most common type, accounting for the majority (>85%) of total diabetes [34]. This form of diabetes was previously referred to as non-insulin-dependent diabetes or adult onset diabetes. In fact, nearly 250 million people were diagnosed as having T2D in 2010, and it is assumed that this figure may as much as double by 2030 [34]. There are many different reasons result in T2D, however autoimmune destruction of β cells does not happen in T2D. T2D is a multifactorial caused disease, in which genetic and environment factors are crucial for its development [52]. It was reported that about 70 candidate genes are associated with T2D; nevertheless, presumably these confirmed variants account only for 10% of the heritability of T2D [53]. Patients with T2D have insulin resistance and relative insulin deficiency, which usually, is not acute or life threatening unless under the pressure of other diseases triggering diabetic ketoacidosis. T2D has a later onset and the development of hyperglycemia is a gradual process, therefore, the earlier period of T2D is often too mild for the patients to be noticed for therapy. This group of patients with T2D is often at high risk of developing complications of vascular lesion. The risk of developing into this form of diabetes increases with age, lack of physical activity, obesity, especially abdominal obesity, visceral adiposity, and family history of T2D. [33]. The individual who had gestational diabetes is also at a high risk progressing to T2D in future.

Gestational diabetes mellitus (GDM) is defined as diabetes without previously diagnosed existing hyperglycemia state during pregnancy [34]. In the past, GDM has been categorized by severity of glucose impairment, with less severe cases referred to as impaired glucose tolerance, and more severe cases as GDM [54]. GDM is a strong predictor for the disease developing into T2D in the future [55, 56]. For these GDM patients, the risk of developing into T2D in the first 10 years after delivery is almost 20% [55, 57], and the lifetime risk of this may be higher than 70% [58]. The prevalence of gestational diabetes is increasing as a result of increasing maternal age and obesity [59]. Reducing risk factors for developing GDM or its severity by eating a healthy diet (food), maintaining a normal weight and exercising before and during pregnancy, but the evidence to sustain this argument seems to be limited [60]. Due to maternal glucose crosses the placenta and stimulates fetal insulin secretion which can increase the risk of the baby being born with neonatal hypoglycemia, hyperbilirubinemia,

hypocalcemia, respiratory distress syndrome and polycythemia [55, 56]. Therefore, it is important to detect and interpose early GDM for pregnant women and fetus.

Other specific types of diabetes include genetic defects of the β cells, genetic defected in insulin action, diseases of the exocrine pancreas, endocrinopathies, drugs or chemically-induced diabetes, infection [34] (Figure 5).

1.3 Congenital hyperinsulinemia

1.3.1 Definition and treatment of congenital hyperinsulinemia

It is well known that congenital hyperinsulinemia is a very rare disease with inadequate insulin secretion by β cells of the islets of Langerhans [61]. CHI is the most common cause of hypoglycaemia in the neonate and infancy period [62].

The pancreatic histopathology could be a diffuse type, with abnormal, hyperactive β cells throughout the pancreas; or a focal form with single focus or foci with isolated hypersecretion; a condition that could be healed by resection of abnormal tissue. Plasma insulin levels are only of diagnostic value if detectable insulin levels are still present during a clear-cut low glucose value of 40 mg/dl or less. Supporting evidence are low ketone bodies, as lipolysis as well as ketogenesis are both suppressed if inadequately high insulin is present.

Current treatment strategies are divided into dietary, medical and surgical therapy. Dietary therapies include hypertonic glucose infusion, corn-starch, glycogen storage disorder formula and enteral feeding [62]. Medical therapies include diazoxide, nifedipine, octreotide, glucagon [62] and recently, low-dose Sirolimus [63] if diazoxide treatment fails. Finally, if all medical treatments fail to resolve severe hypoglycaemia, pancreatectomy is being performed [62].

1.3.2. Classification of congenital hyperinsulinemia and its possible aetiology

Congenital hyperinsulinism (CHI) is classified into transient and persistent CHI. Transient CHI usually develops soon after birth and could spontaneously solve within the first 3-4 weeks of life [64]. Persistent CHI can develop over a long time, it could persist over the whole neonatal and infant period and even persist beyond that period [64].

The aetiologies of transient and persistent CHI vary. Transient CHI is mainly due to non-genetic factors, such as premature birth, stressful perinatal condition and etc. By contrast, persistent CHI is mainly due to genetic factors, the known genes of cause of persistent CHI are *KCNJ11, ABCC8, GLUD1, HNAF4A, HNAF1A, UCP2, GCK, HADH* and *SLC16A1* [64] (Figure 6). Mutations in *GLUD1* and *HADH* lead to leucine-induced HH (hyperinsulinaemic hypoglycaemia), and these two genes encode the key enzymes glutamate dehydrogenase and short chain 3-hydroxyacyl-CoA dehydrogenase which play a key role in amino acid and fatty acid regulation of insulin secretion, respectively. Autosomal recessive and dominant mutations in *ABCC8/KCNJ11* are the commonest cause of medically unresponsive CHI.

Finally, showing the duality of genetic abnormalities; *HNF4A* and *HNF1A* mutations can lead to a phenotype of HH in the neonatal period and maturity onset-diabetes later in life.

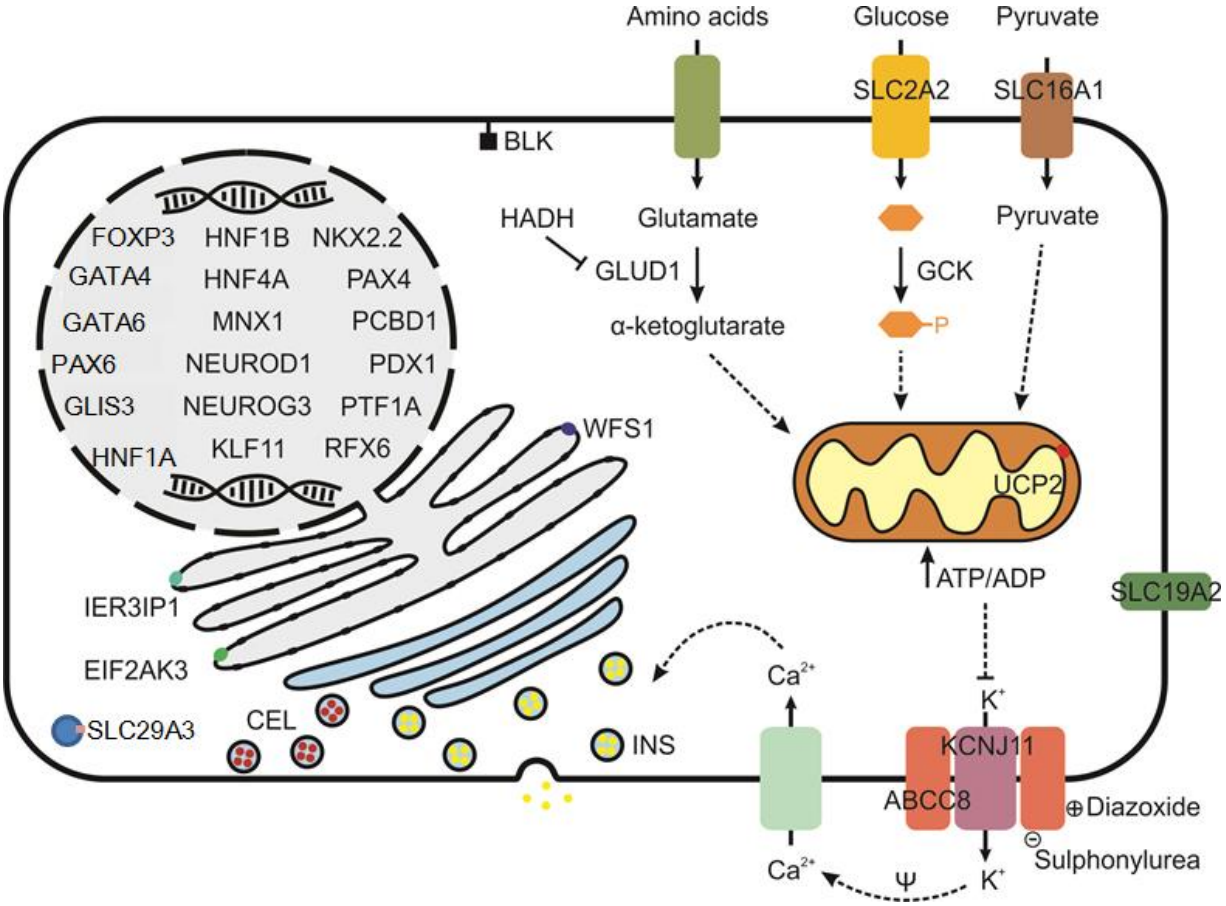

Figure 6: Insulin secretion pathway and genes involved in diabetes and CHI. Glucose is imported via GLUT2 transporter, encoded by *SLC2A2* gene and phosphorylated by *GCK (Glucokinase*) which acts as a glucose sensor. Mitochondria further metabolize glucose into ATP which binds to and closes ATP-sensitive potassium channel comprised of *KCNJ11* and *ABCC8* proteins. The rise in plasma membrane potential opens voltage-gated Ca^{2+} channels. Calcium influx into the cell eventually results in insulin secretion. All the named genes are implicated in the development of monogenic diabetes or CHI.

1.4 Potassium channels and insulin secretion in pancreatic beta cells

1.4.1 Insulin secretion and the K_{ATP} channel (*KCNJ11* and *ABCC8*)

Ion fluxes constitute a major integrative signal in beta cells that leads to insulin secretion and regulation of gene expression. K_{ATP} channels are best studied so far; they act as a key link in glucose-induced insulin secretion [65]. In these cells, fluctuations in the [ATP]/ [ADP] ratio, brought about by changes in blood glucose levels, push the equilibrium of K_{ATP} channels toward the closed or open state. Thus, when blood glucose levels rise, the intracellular [ATP]/ [ADP] ratio also rises, blocking K^+ efflux through K_{ATP} channels. This depolarizes the β-cell and opens voltage-gated Ca^{2+} channels; the subsequent Ca^{2+} influx then triggers exocytosis of insulin secretory granules. When blood glucose levels fall, the intracellular [ATP]/[ADP] ratio will fall, pushing the equilibrium toward open K_{ATP} channels, repolarizing the β-cell and blocking further insulin release (Figure 7) [66, 67].

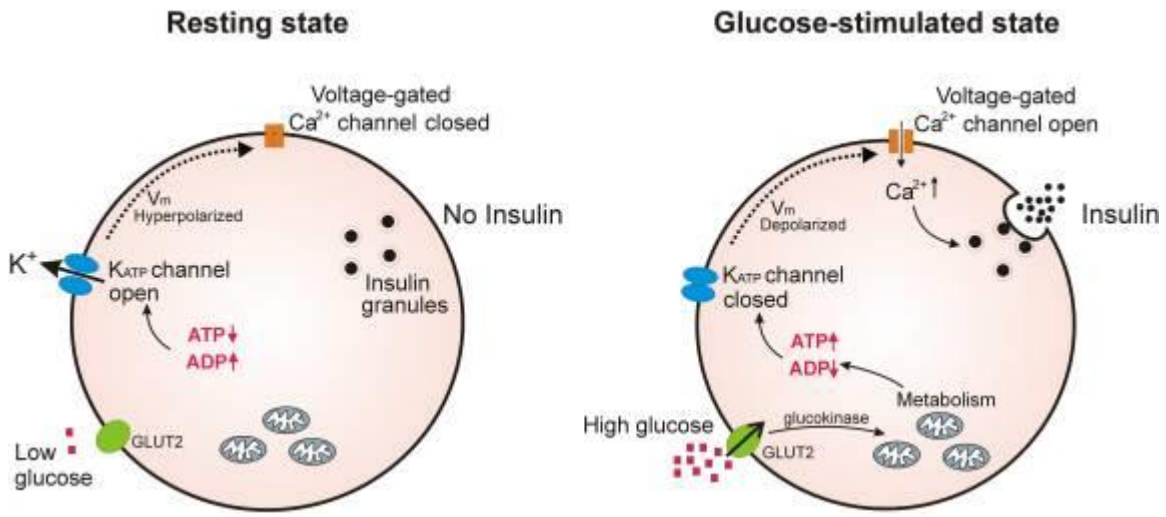

Figure 7: **K_{ATP} channels regulate insulin secretion in pancreatic β-cells**. Under conditions of low blood glucose, the relatively low ATP/ADP ratio in the β-cell promotes opening of K_{ATP} channels, keeping β-cell membrane potential at a hyperpolarized state to prevent Ca^{2+} influx and insulin release. Upon an increase in blood glucose, β-cells increase glucose uptake through *GLUT2* transporters; glycolysis and respiration then elevate the intracellular ATP/ADP ratio and close K_{ATP} channels. This causes depolarization of the plasma membrane potential which opens voltage-gated Ca^{2+} channels; the subsequent influx of Ca^{2+} initiates fusion of insulin secretory granules with the plasma membrane

KCNJ11 and *ABCC8* locate at the 11p15.1 chromosome region [68], encoding the Kir6.2 and sulfonylurea receptor 1 (SUR1) proteins separately. *ABCC8* or *KCNJ11* gene mutations alter the function of K_{ATP} channels and further affect depolarization of β cell membrane which can lead to delayed insulin secretion in diabetes or failure to completely switch off in hyperinsulinemic hypoglycemia [69]. In detail, *KCNJ11* and *ABCC8* genetic variations are associated with the development of all types of insulin secretion defects from neonatal diabetes to T2D [70] and CHI [71]. On the molecular level, loss-of-function mutations of the K_{ATP} channel cause frequent depolarization of the beta cell membrane and hyperinsulinism, while gain-of-function mutations cause permanent potassium leakage, failure to depolarize and as consequence neonatal diabetes [72]. Recessive inactivating mutations in the *KCNJ11* and *ABCC8* genes are the most comment cause of CHI, which accounted for approximately 36.3% of all CHI cases [73].

Potassium channel inhibitor such as sulfonylurea can promote closure of channel. Conversely, potassium channel openers such as diazoxide can increase the opening time of the channel [74] and prevent insulin secretion. As mentioned above, diazoxide is used to inhibit hyperinsulinism in CHI, while sulfonylurea can stimulate insulin secretion by closing K_{ATP} channels; this drug is often used for patients with neonatal diabetes and T2D [74].

1.4.2 Kv7.1 (KCNQ1) channel and insulin secretion

1.4.2.1. The *KCNQ1* gene is associated with type 2 diabetes mellitus

KCNQ1 is also expressed in pancreas. Genome-wide association studies (GWAS) showed single nucleotide polymorphisms (SNPs) locating at the *KCNQ1* intron 15 (e.g. rs2237892,

rs2237895 and rs2237897) are significantly associated with T2D [75] and impaired β cells function [76]. The risk alleles of the *KCNQ1* gene are associated with impaired insulin secretion [75, 77] and were found with reduced depolarization-evoked insulin exocytosis [78]. Chromanol 293B, an inhibition of KCNQ1 channel activity, inhibits whole cell outward K^+ current, alters action potential duration, and significantly increased glucose-stimulated insulin secretion [66] and exocytosis in mice [78]. *Kcnq1* knockdown with small interfering RNA similarly increases insulin exocytosis and secretion (Figure 8) [78]. Furthermore, increased expression of *Kcnq1* in the MIN6 mouse β-cell line resulted in impairment of insulin secretion induced by glucose, pyruvate, or tolbutamide [79]. In brief, gain-of-function in *KCNQ1* impairs insulin secretion by inducing premature repolarization of the action potential in pancreatic β cells [79]. Moreover, the risk allele of *KCNQ1* may increase the expression of *KCNQ1* in pancreatic β cells and thereby decreased insulin exocytosis and secretion, and then contribute to the development of T2D [78, 79]. Insulin exocytosis and secretion relies on electrical activity of β cell membrane which is regulated by ionic signaling pathways. It was reported that β cell proliferation rate and β cell mass were both reduced in mouse with *Kcnq1* gene mutations [80].

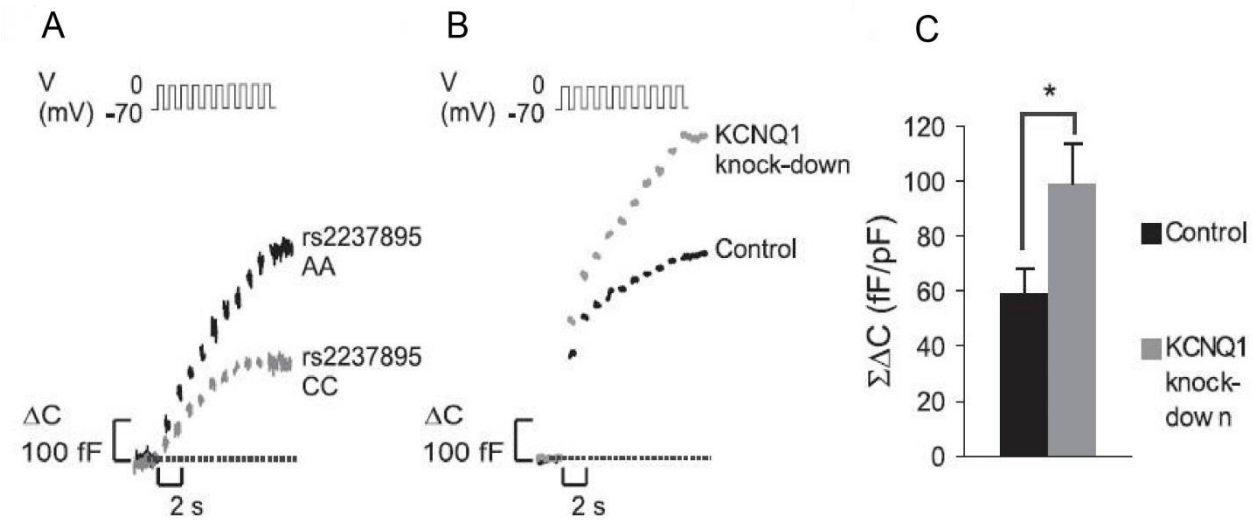

Figure 8: The disease allele of *KCNQ1* cause reduced insulin secretion. A**:** Depolarization-evoked increase in cell capacitance (ΔC) in β-cells representative for individuals carrying risk or nonrisk alleles for rs2237895 (*KCNQ1*). B and C: Exocytosis response in human β cells treated with control siRNA or siRNA targeting *KCNQ1* [78].

1.4.2.2. The protein structure of KCNQ1

KCNQ1, located on 11p15.5, encodes for the pore-forming α -subunit of a voltage-gated potassium (K_V) channel. Each channel has four subunits and each subunit consists of six transmembrane segments that are named S1 through S6 (Figure 9 adapted from [81]). S1-S4 forming one voltage sensor module, and S5 and S6 contributing to each unit forming one quarter of a pore structure. Four voltage-sensing domains (VSD) are made from S1-S4, S5 and S6 form central pore domains (PD), 4 VSD surround the PD and control its gate [82].

Different regions have been suggested responsible for different current amplitudes. An assembly domain in the C-terminus is responsible for assembling four KCNQ1 subunits into a functional K_v channel [83]. The N-terminus plays a role in influences channel trafficking [84]. The opening or closure of the intracellular channel gate is directly controlled by the movement of the VSD via an electromechanical coupling. This electromechanical coupling is realized by an interaction between the S4-S5 linker and the C-terminal end of S6 [85].

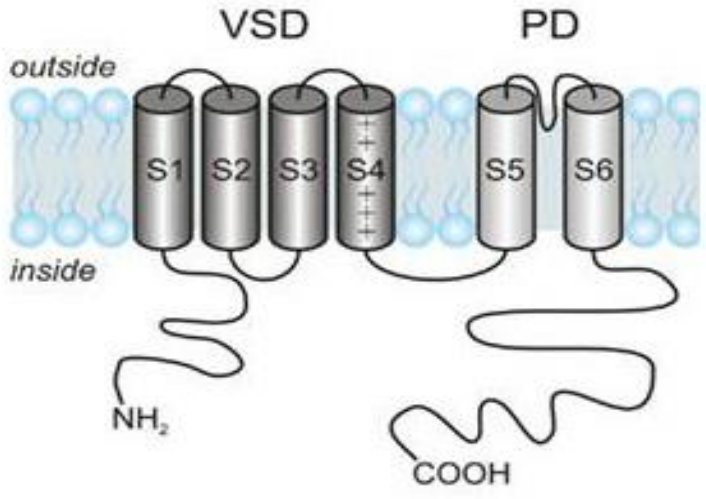

Figure 9: Schematic topology of a single KCNQ1 channel subunit

1.4.2.3 Molecular genetic study of *KCNQ1* and other human disorders

KCNQ1 is expressed in several tissues in the body, and predominantly in the myocardium, stomach, kidney, inner ear, intestine, and pancreas, in which KCNQ1 mainly contributes to maintaining K^+ homeostasis or to the regulation of electric activity [86]. Mutations in *KCNQ1* can cause several human disease syndromes.

In cardiac myocytes, action potential is produced by a balance of inward and outward current in repolarization duration. K^+ plays a significant role in repolarization duration in the human heart and thereby contributes to the termination of the cardiac action potential (Figure 10). KCNQ1 and the regulatory subunit KCNE1 assemble to form a slowly activating cardiac potassium channel [87]. This channel contributes to the delayed outwardly rectifying potassium current (I_{Ks}), and functional mutations in *KCNQ1* lead to dysfunction of the channel, which causes *KCNQ1* long QT syndrome (LQTS). The LQTS was characterized by the reduction of the repolarizing potassium cardiac current I_{Ks} [88]. LQTS was predicted in population prevalence of 1:2,000 and it was diagnosed by prolonged QT interval, which prolongs the cardiac action potential duration (Figure 10, red curve). On the electrocardiogram—reflecting the time from initiation of ventricular depolarization to the end of ventricular repolarization—is prolonged, it causes syncope, malignant arrhythmias, and sudden death [89]. Whereas heterozygous dominant mutations in *KCNQ1* can cause LQTS symptoms ranging from mild to severe arrhythmia, homozygous mutations inherited by an

autosomal recessive trait always cause severe arrhythmia combined with hearing loss, which is defined as a separate disease entity named Jervell & Lange-Nielsen syndrome [83].

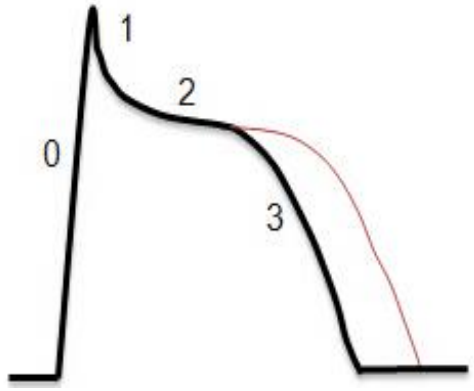

Figure 10: Action potential in human ventricular cardiomyocytes. Influx of Na^+ causes cardiomyocyte depolarization (0). Notch is caused by the repolarization of transient outward K^+ current (1). The plateau phase is a balance between simultaneous influx of Ca2+, residual Na^+ and efflux of K^+ (2). Cardiomyocyte repolarization is mainly achieved by K^+ efflux though the I_{Ks} channel and the inwardly rectifying potassium current (I_{Kr}) channel (3). Loss-of-function of the I_{Ks} channel tends to impair cardiomyocyte repolarization and prolong the cardiomyocyte action potential duration (red curve).

Except for the autosomal dominant LQT syndrome, *KCNQ1* homozygous mutations cause hearing impairment and irregular heart rhythm like Jervell & Lange-Nielsen syndrome (OMIM #220400). *KCNQ1* are co-expressed with *KCNE1* subunits in the apical membrane of marginal cells of the stria vascularis in cochlea in inner ear [90]. Strial marginal cells and vestibular dark cells secrete K^+ into the endolymph via the K^+ channel KCNQ1/KCNE1 [91]. Mutations in these genes alter the usual structure and function of potassium channels or prevent the assembly of normal channels. These changes disrupt the flow of potassium ions in the inner ear and in cardiac muscle, leading to hearing loss and an irregular heart rhythm characteristic of Jervell and Lange-Nielsen syndrome. In this case, the capacity of KCNQ1/KCNE1 is to sustain current for long periods without inactivating may be important for function.

KCNQ1 plays a critical role in gastrointestinal tract as well. It was reported that gastrin can modulate *KCNQ1* gene expression [92], and mouse with mutated *Kcnq1* develop atrophic gastritis, metaplasia, and adenomatous hyperplasia [93]. *KCNQ1* mainly located in parietal cell in the stomach, and it has an essential function in controlling gastric secretion. The process of gastric acid secretion requires one H^+ to be exchanged for one K^+ at the expense of one ATP molecule [94]. The K^+ efflux pathway in gastric parietal cells is thought to be formed by potassium channels. Gastric acid secretion was decreased in vivo by inhibiting the KCNQ1 activity, it was certificated that the K^+ channel is a key in control gastric acid secretion [95].

In kidney, KCNQ1 channels expressed together with KCNE1 subunits can be found in proximal tubule and distal tubule of the nephron[96]. *Kcnq1* and *Kcne1* K^+ channels have been located on the brush border of mid to late proximal tubule and basolaterally in distal tubule in mouse, where they maintain the proximal tubule brush border membrane potential, and loss of function in KCNQ1/KCNE1 leads to urinary loss of Na^+ and glucose [97]. The function of KCNQ1 K^+ channels in kidney has not been definitively established, but *Kcnq1* knockout mice were found to suffer from lower potassium sera level, urinary and fecal salt

wasting, together with volume depletion, thereby indicating its important role for the I_{Ks} channel in renal function[98].

2. Materials and Methods

2.1 Materials

2.1.1 Antibodies

anti-insulin guinea pig pAb	Abcam, UK
anti- Glucagon rabbit pAb	ImmunoStar, USA
β-*Actin* Rabbit Ab	Cell signaling, Switzerland
Foxo1 Rabbit mAb	Cell signaling, Switzerland
Neurod(D35G2) Rabbit mAb	Cell signaling, Switzerland
anti-Vinculin V284	GeneTex, USA
KCNQ1(H-130):sc-20816	Santa Cruz Biotechnology, Texas, USA
Anti-rabbit IgG HRP-linked Antibody	Cell signaling, Switzerland
PDX1 Antibody (A-17)	Santa Cruz Biotechnology, Texas, USA
Pax6 antibody	GeneTex, USA
Foxa2 (M-20): sc-6554	Santa Cruz Biotechnology, Texas, USA
Goat Anti-Mouse IgG, HRP conjugate	Merck Millipore ,Darmstadt, Germany
FITC-conjugated donkey guinea pig IgG	Jackson ImmunoResearch, Suffolk**,** UK
FITC-conjugated donkey anti-rabbit IgG	Jackson ImmunoResearch, Suffolk**,** UK
Donkey anti-goat IgG-HRP	Santa Cruz Biotechnology, Texas, USA

2.1.2 Kits and molecular weight markers

QIAamp DNA blood mini kit (250)	QIAGEN, GmbH, Hilden, Germany
BCA Protein Assay Kit	Thermo Scientific, Rockford, USA
QIAGENE Gel extraction kit	QIAGEN, GmbH, Hilden, Germany
Nuclo Spin PCR purification kit	Macherey-Nagel GmbH Dueren, Germany
Bigdye™ Terminator Cycle Sequencing Ready reaction kit (V1)	Applied Biosystems, Wrington, UK
QuikChange II Site-Directed Mutagenesis Kit	Agilent Technologies, CA, USA
Amaxa™ Cell Line Nucleofector™ Kit V	Lonza, Basel, Switzerland
Mouse High Range Insulin ELISA	Alpco, Salem, USA
QIAfilter™ Plasmid Maxi Kit	QIAGEN, GmbH, Hilden, Germany
QIAprep® Spin Miniprep Kit	QIAGEN, GmbH, Hilden, Germany
50 bp DNA-ladder	Fermentas, St. Leon-Rot, Germany
PageRuler Plus Prestained Protein Ladder	Thermo Scientific, Bonn, Germany

2.1.3 h*KCNQ1* mutagenesis primers

Variant	Forward primer	Reverse primer
R397W	ccacctggaagatctacatctggaaggccccccggagccaca	tgtggctccgggggggccttccagatgtagatcttccaggtgg
G292D	cgaactccacgcggtctgactcgttcacc	ggtgaacgagtcagaccgcgtggagttcg
E13 del	caatggactggtccagcctggaatttcttcttgg	ccaagaagaaattccaggctggaccagtccattg

2.1.4 Quantitive mouse RT-PCR and human *KCNQ1* RT-PCR primers

Gene	Exons	Forward primer	Reverse primer
m*Ppib*	E3-E4	GGAGATGGCACAGGAGGAAA	GTAGTGCTTCAGCTTGAAGTTC
m*Pdx1*	E1-E2	TGGATGAAATCCACCAAAGCT	GGGTTCCGCTGTGTAAGCA
m*Neurod1*	E1	CTCCAGGGTTATGAGATCGTC	ACAGACAACAGGTCAAGGCAT
m*Foxa2*	E2-E3	TGGGAGCCGTGAAGATGGAA	TGTGTTCATGCCATTCATCCCC
m*Gcg*	E3-E4	ACCAGCGACTACAGCAAATAC	TGTTGTTCCGGTTCCTCTTG
m*Arx1*	E2-E3	CTTTCCAGAAGACGCACTACC	TCTGGAACCACACCTGGA
m*Irx1*	E1-E2	GCACCACGTTCACCAGTTA	GGGTTGTCCTTCAGTTCATACT
m*Pcbd1*	E2-E3	GCAGTTCCATTTTAAAGACTTCAAC A	GATGGTGGTCCAGCTTTTCAG
m*Pcbd2*	E1-E2	AGCAGGGCGGCCATGT	CTTTCAAGCCAGGAATAAGTTGGT
m*Hnf1a*	E1-E2	GCCGTGGTGGAGTCACTTCT	TGCAAGTACGACTTGACCATCTTC
m*Hnf1b*	E4-E5	TCCTCTCCACCCAACAAGATG	GACTGATTGTCGAAGAGGAAGTGA
m*Hnf4a*	E3-E4	GTTACTGCAGGCTTAAGAAGTGCTT	GGTCCCGCTCATTTTGGA
m*Gck*	E4-E5	CTTCACCTTCTCCTTCCCTGTAAG	GGCCTTGAAGCCCTTGGT
m*Ins1*	E2	GAAGTGGAGGACCCACAAGTG	CAACGCCAAGGTCTGAAGGT
m*Ins2*	E2	GCTGGCCCTGCTCTTCCT	AACCACAAAGGTGCTGCTTGA
m*Slc2a2*	E9-E10	TGTTTTTCTGCACCATCTTCATG	CGTAACTCATCCAGGCGAATTT
m*MafA*	E1	GCCACCACGTGCGCTTG	CCGCTTCTGTTTCAGTCGGAT
m*Ghrl*	E2-E3	CTTCCTGAGCCCAGAGCAC	CCTGACAGCTTGATGCCAAC
m*Pp*	E2-E3	CCCCTGGAGCCAATGTACC	GAGCAGGGAATCAAGCCAAC
m*Sst*	E1-E2	TGCCACCGGGAAACAGGAAC	TTCTTGCAGCCAGCTTTGCG
m*Gast*	E2-E3	TGCTGGCTCTAGCTACCTTC	TGGCCTCTGCTTCTTGGAC
m*Nkx2.2*	E1-E2	GCCTGCCCCTTAAGAGCC	TCATTGTCCGGTGACTCGT
Nkx6.1	E1-E2	CCATCTTCTGGCCCGGAGT	CTCCAGGGCGAAGATTTGCT
m*MafB*	3 UTR	AATCCCCACCTGAATTTGCT	CGATGCAGGACGAATATCCAC
m*Abcc8*	E11-E12	TGCCGTCCTCATTACCTTTG	GGATGTGGAAGAGAGAGAGAGA
m*Kcnj11*	E1	GGACCTGGAGATCATTGTCATC	TAGAATCTCGTCAGCTAGGTAGG
m*Kcnq1*_iso1	E13-E15	AGAACTACAGAGAAGGCTGGA	GTGTTACTGCCACGGTCTTT
m*Kcnq1*_iso7	E10-E12	GACAGCTCAGTAAGGAAGAG	CACTGTGACACATGGGTG
h*Kcnq1*_1	E11-E13	GACACCCATCACCCACATC	GCGCTTGCTGGAATTTCTTC
h*Kcnq1*_2	E11& e12-E14	GCCAAGAAGAAATTCCAGCAAG	GATGAACAGTGAGGGCTTCC
h*Kcnq1*_3	E12&e13-E14	GCCAAGAAGAAATTCCAGCAAG	GATGAACAGTGAGGGCTTCC
h*Kcnq1*_4	E11-E14	CACCCATCACCCACATCTC	ATGAACAGTGAGGGCTTCC

E: exon; Iso: Isoform. m: mouse, h: human; E11 & e12, junction of e11 and e12; E12&e13: junction of E12 and e13.

2.2 Methods

2.2.1 Establishing the database with β-cell disorders

We set up a database of patients with pancreatic β cell dysfunction and their family members. The database includes personal data (name, gender, date of birth, family position, ethnicity, consanguinity), disease-related data (onset, symptoms and current medical treatment, associated organ failures), laboratory testing (glucose, HbA1c, acidosis), molecular diagnosis (gene, genomic position, nucleotide aberration, protein alteration) and bioinformatics evaluation. Our institutional review boards approved the studies and a written consent was obtained from the participants or their guardians. Our institution cooperates with institutions from United Kingdom, Turkey, and Egypt. These patients' ethnicities are Turkish, Caucasian, Ashkenazi Jewish, Arab, Vietnamese, and Chinese. In total, more than 520 cases and family members (patients: 225 cases) come from above mentioned countries. Onset age from 0 to18 years, mean age of these patients was 6.7±5.95 years old, the gender ratio is 1.2:1 (152 male :127 female) .These patients were separately diagnosed as T1DB, T2D, MODY, neonatal diabetes, mitochondrial diabetes [34], congenital hyperinsulinism (CHI) [99] and pancreatic hypoplasia. Their parents and sibling(s) with family history were also included.

2.2.2 Molecular screening of the patients (working procedure in the RG RAILE)

As shown in figure 11, we have screened our samples through a series of working procedures to finally identify the disease-causing variants for the patients. Initially, we started with a candidate gene approach in all samples, excluding known MODY-genes, *ABCC8* and *KCNJ11*, both causing neonatal diabetes and insulin mutations.

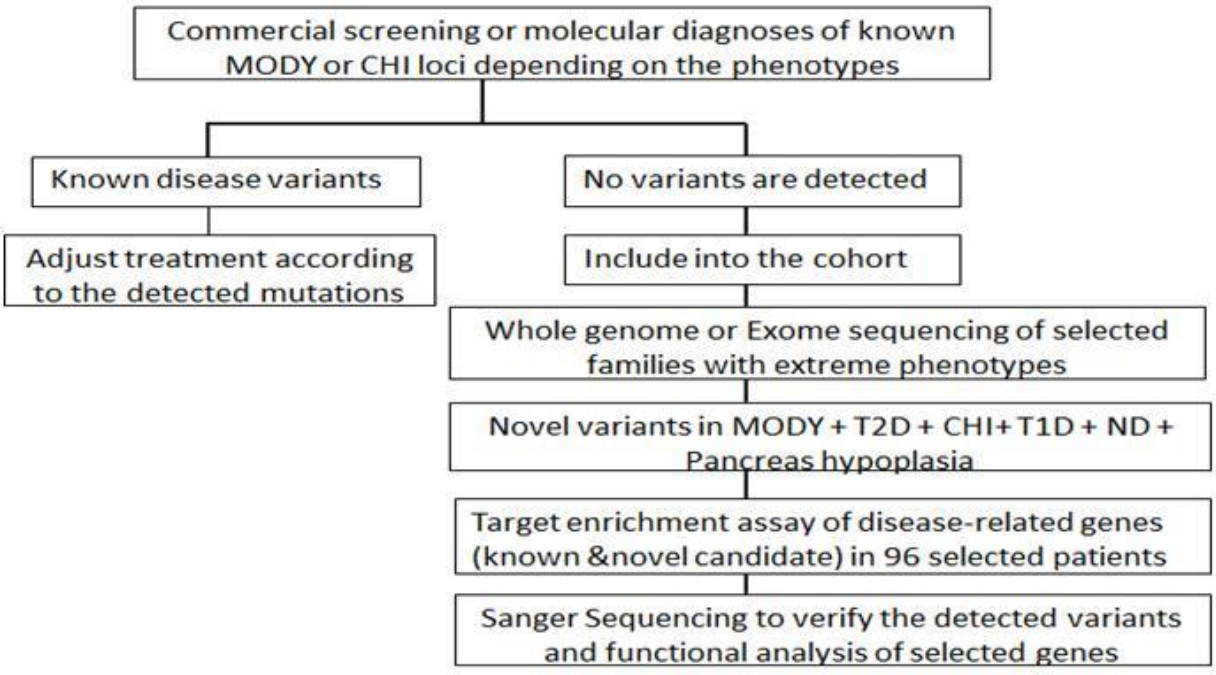

Figure 11: Molecular screening procedure of the recruited patients

Families with still unknown pathogenesis were then selected for next generation sequencing (i) exome sequencing or whole genome sequencing in families with extreme phenotype. As candidate gene numbers started to expand rapidly, we then switched to start directly with targeted enrichment of all available genes (known or experimental) with implications to monogenic diabetes and subjected these to next-generation-sequencing. In extension, we selected a most recent cohort of 96 patients who had suggestive phenotypes for monogenic diabetes (Figure 11). They were investigated by target enrichment next-generation sequencing, the detected genes were then confirmed by Sanger sequencing in the index-subjects and their family members. The rare variants were also excluded from our 200 pediatric normal controls.

2.2.3 Blood DNA extraction

Twelve-hour fasting blood of all patients was collected in Ethylenediamine-N,N,N',N –tetraacetate (EDTA) and citrate treated tubes on ice, and then the blood was stored at –20^{o}C within 2 hours. DNA was extracted according to the manufacture's manual (QIAmap DNA blood Mini kit, QIAGEN Germany); 1 set (18) of 15 ml tubes, 2 sets of Sarstedt tubes (2.0 ml), 1 set of Eppendorf tubes (2.0 ml) and 1 set of QIAamp columns were labeled according to the sample identity number (ID). First step, 90 μl of proteinase K (20 mg/ml) was added to the 15 ml tubes. Then, 1000 μl of each blood and 1000 μl of AL-Buffer (preheated to 70^{o}C in water bath) were added into the same 15 ml tubes, which were later covered and mixed for 10 seconds followed by 40 minutes incubation at 70 ^{o}C (swing down the blood 2-3 times in between). After the incubation process, 1100 μl of isopropanol was added into each tube and vortex for 10 seconds. The lysate was then applied to the corresponding labeled QIAamp-spin columns with extension tubes on the vacuum box, in which the vacuum was created with 4-10 liter per minute (LPM). The washing procedure was carried out with 1x 900 μl of AW-I Buffer, 3x 900 μl of AW-II Buffer. Further, the residual of the washing buffer was removed intensively by centrifuging at 10000 rpm, 1 minute after washing. DNA was extracted with 400 μl of ddH_2O preheated at 85^{o}C into the Sarstedt tubes two times with spinning at 12000 rpm, 2 minutes. DNA was therefore eluted into 800 μl of ddH_2O. DNA concentration and purity were measured using spectrophotometer with optical density (OD) at 260 nm (OD_{260}) and 280 nm (OD_{280}) in the ultraviolet spectrum (dilution factor 1:20). The DNA concentration was calculated as the following:

Double-stranded DNA concentration in μg/ml: $Measured\ OD_{260} \times \frac{50\mu g/ml}{1-OD_{260}} \times 20$

DNA from every sample has a ratio of $OD_{260}/\ OD_{280}$ approximately 1.8, which indicated the DNA samples are of relatively high purity.

2.2.4 Sanger sequencing

The DNA Sequencing was carried out using the dideoxy nucleotide chain-termination method [100] with dye terminator labeling on purified PCR products (Table 1). The primers were designed using the program Oligo 6.1(Molecular Biology Insights, Inc., USA). The PCR products were purified using two methods: *QIAGEN gel extraction kit* or *Nuclear Spin kit*, depending on the specificity of the PCR. If the PCR bands were not very distinct, purification was done using *QIAGEN gel extraction kit* following the manual. Otherwise, the PCR products were purified using the *Nuclei Spin kit* following the product instruction. The purified PCR products were buffered in sterile water and the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit was used to set up the sequencing reactions. Every PCR fragment was sequenced with both forward and reverse primers, of which 2.5 pmol of each was used separately. About 20 - 40 ng of PCR fragments were used for one reaction (5 ng per 100 bps). Sequences were read on *ABI 3730 DNA Analyzer* and automated base calling was performed using the Sequence Analysis software and multiple sequence alignment was performed with the program Seqman in DNAstar (DNASTAR.Inc).

2.2.5 Total RNA extraction, RT-PCR, and quantitative PCR

Total RNA was extracted from the patients (family 62) and normal controls PBMC with the use of an RNeasy Plus Mini Kit (QIAGEN, Germany) according to the manufacture's instructions. In brief; sample volume was adjusted to 100 µl with nuclease-free H_2O. Then, 350 µl of RLT buffer was added and mixed well. Subsequently, 250 µl of 100% ethanol were added, mixed, samples were transferred to spin columns and centrifuged for 15 seconds at 10,000 rpm in a tabletop centrifuge (Eppendorf). Membranes with bound RNA were washed twice by adding 500 µl of RPE buffer and centrifuging for 15 seconds at 10,000 rpm. Residual ethanol was removed by centrifuging for 1 minute at full speed. To elute RNA, 30-50 µl of nuclease-free H_2O were added to the membrane, incubated for 2 minutes and centrifuged for 1 minute at 10,000 rpm. The extracted total RNA was stored at -80°C.

Two micrograms of total RNA in every sample were diluted in RNase-free distilled water to 22.5 µl and placed into a PCR reaction tube. After adding 1µl of random primers, the RNA samples were incubated in the master cycler for 10min at 70°C to linearize RNA strands and cooled down at 4°C to prevent recreation. Following, 11.5µl of a reaction mixture (Table 1) was added and samples were incubated at 37°C for 30min.

Table 1: Reaction mix of DNase I treatment

Components	Volume [µl]	final concentration
M-MLV-RT 5x reaction buffer	8	1x
dNTP mix (each 10mM)	1	0,3mM
DNase (2U/µl)	2	4U

The reaction was finally heated for 5min at 75°C in order to stop the reaction. Reverse transcription of mRNA was then initiated with 1µl MMLV- RT and 0.5µl RNasin® and the cDNA was synthesized at 42°C for 1h. The polymerase was inactivated by heating at 95°C for 5min. The cDNA samples were stored at -20°C.

The PCR was carried out in the master cycler with the 25µl batches under following conditions (Table 2). The quality of PCR products was verified by capillary electrophoresis in a 2% Agarose gel with 0.00003% (v/v) EtBr. 12µl of cDNA sample and 7µl of DNA-ladder were loaded on the agarose gel. Electrophoretic separation was carried out at 80V in 1x TAE buffer and cDNA bands were visualized and photographed by the UV recorder UV solo and Video Copy Processor. The quality of cDNA was verified by comparing the molecular size of the cDNA product with the DNA-ladder. Bands of the pure cDNA or contamination with genomic DNA were visible.

Table 2: PCR Reaction process of control-PCR with β-actin

Reaction	temperature [°C]	time [min]	
Initial denaturation	94	2	
Subsequent denaturation	94	0.5	
Annealing	58	0.5	30 cycles
Elongation	72	0.5	
Final elongation	72	10	

SYBR® Green chemistry (Applied Biosystems) was used for quantitative PCR. The composition of SYBR® Green RT-qPCR mixes is outlined in Table 3, whereas the reaction conditions are present in Table 4. Primers used for quantification of gene expression in both SJ-beta cells and pancreatic islet are shown in Chapter 2.1.4. The same biological replicate was loaded into four different wells (4 technical replicates) and the reactions were carried out in StepOnePlus™ Real-Time PCR System. Data was analyzed by delta-delta-Ct (ddCt) method. Briefly, the average threshold cycle (Ct) value for each gene was calculated. The expression of a gene of interest (gi) was normalized to that of a house-keeping (hk) gene (*ActB* and *Ppib).* Finally, the expression of the treated sample was normalized to the expression of the untreated sample and the fold change was calculated according to the formula

Fold change = $2^{-((Ct_{\text{treated gi}}-Ct_{\text{treated hk}})-(Ct_{\text{untreated gi}}-Ct_{\text{untreated hk}}))}$.

Statistical significance was determined by Mann-Whitney rank sum test with p values of <0.05 considered to be statistically significant. GraphPad Software was used for statistical analysis and preparation of charts.

Table 3: SYBR Green RT-qPCR mix

Reagent	Stock concentration	Volume, µl	Final concentration
Nuclease-free H_2O	-	5	-
Power SYBR® Green PCR Master Mix	2x	7.5	1x
Forward primer	10 µM	0.25	167 nM
Reverse primer	10 µM	0.25	167 nM
cDNA	5-20 ng/µl	2	2.5-10 ng/µl

Table 4: The SYBR RT-PCR program

Reaction		temperature [°C]	time [min]	
UNG hydrolysis		50	2	
Initial denaturation		95	10	
Denaturation		95	0.25	40 cycles
Annealing/Extension		60	1	
melting curve	Denaturation	95	0.25	
	Annealing	60	1	
	Denaturation	95	0.25	

2.2.6 *Kcnq1* knock out mice

Kcnq1 KO C57BL/6 mice 12-14 weeks of age were kindly provided by the research group of Prof. Dr. Jakob Voelkl (Institute of Physiology, Medical faculty, University of Tubingen, Germany). All mice were fed with normal laboratory chow. The pancreata of *Kcnq1* KO and wild type mice were kindly given by the research group of Prof. Dr. Maik Gollasch, ECRC, Charité after the operation.

Kcnq1 KO mice were described previously [80, 101]. *Kcnq1* was inactivated by the insertion of a neomycin resistance gene cassette into exon 2, which is the first exon common to all known isoforms of KCNQ1. The genotyping of these mice was performed by PCR with genomic DNA, a forward primer (5′-CCAGGAGTGGGTGGTTCTAC-3′), a reverse primer (5′-GCCAGCACTAAAGATCTTGC-3′), and a neo-forward primer (5′-CGCTTCCTCGTGCTTTACG-3′) [80].

2.2.7 Pancreas preparation from the *Kcnq1* knock out and wild type mice

2.2.7.1 Pancreas preparation for immunohistochemistry

Mice were sacrificed by cervical dislocation following Max-Delbrück-Centrum practice guidelines for mouse experiments.

Pancreata were removed from freshly-sacrificed mice, placed into fixation medium with 4%PFA overnight at 4^o C, and then deep-frozen (-80°C) by Tissue Freezing medium and

mounted on a Microm HM560 microtome. Sections (12 µm) were cut with the machine (Microtome HM560, Thermo Scientific, Walldorf, Germany) and stored at -80^{o} C, average 150-180 slices for every mouse pancreas.

2.2.7.2 Isolation of pancreatic islets

Abdominal cavity of mouse was opened completely. The bile duct was identified as being attached to the duodenum at the papilla, and collagenase (1mg/ml)/HBSS mixture was injected into the bile duct. The pancreas was removed as a whole and digested at 37^{o}C for 15 minutes. After digestion, islets were further purified by filtration under centrifugation at 1500 rpm for 1 minute. The pellet was then suspended in Histopaque gradient solution (3.5ml of Histopaque 1119, 3ml of Histopaque 1110, 2.5ml of Histopaque 1080, 2ml of Histopaque 1060). Finally, islets were hand-picked with pipette under an OLYMPUS CKX31 microscope and snap-frozen in liquid nitrogen before being stored at -80^{o} C.

2.2.8 Immunohistochemistry of the pancreatic islets

First day, pancreatic sections were blocked at room temperature (RT) for at least 1 hour in specific blocking solution (Table 5). Primary insulin and glucagon antibodies were diluted using appropriate blocking solutions. After blocking, the pancreas sections were incubated with the primary antibody, anti-insulin guinea pig and anti-glucagon rabbit in 5% donkey blocking solution, respectively, at 4°C overnight. Second day, the specimen were washed with PBST at RT for 3 times, and 10 minutes/ time. Secondary fluorescein-conjugated donkey anti-guinea pig IgG and fluorescein-conjugated donkey anti-rabbit was applied with the respective blocking solution of the first antibody and incubated at room temperature in the dark for 1hour. Finally, the sections were mounted with Vectashield HardSet Mounting Medium with DAPI.

Table 5: Dilution, blocking and carrier solutions for specific antibodies

Antibody(dilution)	blocking solution	secondary antibody(dilution)
anti-insulin guinea pig 1:500	5% donkey blocking solution	FITC-conjugated donkey anti-guinea pig IgG 1:500
anti-glucagon rabbit 1:500	5% donkey blocking solution	FITC-conjugated donkey anti-rabbit IgG 1:500

2.2.9 Analysis of β cell mass

In order to calculate β cell mass, every 10th section of pancreas was selected and therefore, approximately 15-18 sections represent one individual mouse pancreas. After immunohistochemistry with insulin and glucagon antibodies, the slices were automatically scanned by fluorescent microscope BZ-8100E, then the areas of β cells in relation to the whole pancreas section area was calculated by Image J software (National Institutes of Health, USA).

2.2.10 Construction of plasmids

2.2.10.1 Mutagenesis of the *KCNQ1* constructs and transformation into E.coli

The human h*KCNQ1* cDNA was kindly provided by Prof. G. Abbott (University of California, California, USA) and subcloned into the pIRES2-AcGFP1 vector. We performed mutagenesis with the primers (Chapter 2.1.3) for all three mutations (R397W, G292D, E13del) according to the manufacture's instructions (QuikChange II Site-Directed Mutagenesis Kit, Agilent Technologies, USA). The reaction and the PCR program were performed as shown below (Table 6). After the PCR reaction, load the products on 1.0% agarose gel for control, afterwards, add 1 µl of DpnI enzyme in 41µl of the PCR product and incubate at 37 ^{o}C for 1 hour. Transform the mutagenesis products into E.coli according to the standard protocol for plasmid mini preparation.

Table 6: The PCR reaction and program for the mutagenesis of the h*KCNQ1* constructs

Reagent		**Volume, µl**
10Xbuffer		5
Mutagenesis Primer forward		1
Mutagenesis Primer reverse		1
dNTP-Mix		1
Quick Solution		3
Pfu-Turbo-Polymerase		1
DNA (10ng/µl)		1.7
Necleasfree H_2O		37.3
Total		50
Reaction	**temperature [°C]**	**time**
Initial denaturation	95	1 minute
subsequent denaturation	95	50 second
annealing	60	50 second
elongation	68	5 minutes
Final elongation	68	7 minutes

12 cycles

2.2.10.2 Extraction of plasmid DNA

Plasmid DNA was extracted using commercially available Qiagen kits: QIAprep® Spin Miniprep Kit (minipreps) or QIAfilter™ Plasmid Maxi Kit (maxipreps) according to the manufacturer's instruction. DNA concentration was determined using NanoDrop ND-1000 spectrophotometer.

2.2.10.3 Extraction of minipreps

The transformed Ecoli bacteria were cultured at 37°C overnight with shaking at 220 rpm. They were centrifuged at 8000g for 5 minutes, resuspended in Resuspension buffer, and then

transferred into microcentrifuge tube. The bacteria were lysed by 250µl of lysis buffer and 350 µl of Neutralization buffer was added into microcentrifuge tube and then centrifuged again at 13000 rpm for 10 minutes. The supernatant was transferred into QIAprep 2.0 spin column and centrifuged at 13000 rpm for 1 minute. The QIAprep 2.0 spin column was washed with 500 µl of PB buffer and 750 µl of PE buffer. To eluted DNA, 50 µl of Elution buffer was added into QIAprep 2.0 spin column, allowed to stand for 1 minute, and centrifuged for 1 minute. The eluted DNAs were then Sanger sequenced for selecting the positive constructs (Kcnq1 mutated & wild type).

2.2.10.4 Extraction of maxipreps

The starter culture containing 100 µl/ml of ampicillin was incubated for 6-8 hours at 37°C with shaking at 220 rpm according to the following instructions: dilute the starter culture 1/1000 into selective LB medium and grow at 37°C for 12-16 hours with shaking at 220 rpm. Harvest the cells by centrifugation at 3500 rpm for 10 minutes at 4°C, resuspend the pellets in 10 ml Buffer P1 and add 10 ml Buffer P2, then incubate them at room temperature for 5 minutes. 10 ml of chilled Buffer P3 were added. Then the lysate was poured into the barrel of the QIAfilter Cartridge, incubated at room temperature for 10 minutes. Then QIAGEN-tip 500 was equilibrated by applying 10 ml Buffer QBT. The tip was washed twice with 30 ml of Buffer QC and DNA was eluted using 15 ml of Buffer QF. DNA was precipitated by adding 10.5 ml isopropanol followed by centrifugation at 4°C for 30 minutes at 15,000g. The DNA pellets were washed with 5 ml 70% ethanol and centrifuged at 15000 rpm for 10 minutes. The pellets were air-dried for 5 minutes and the DNA in was redissolved 200-300 µl of nuclease-free water.

2.2.11 Cell culture and transfection

SJ pancreatic β cells were kindly provided by Prof. Carmen Birchmeier's lab at the MDC in Berlin. The cells were used between passages 13 and 25 and grown in Dulbecco's modified Eagle's medium (DMEM) containing 4.5g/l glucose + GlutaMAX + pyruvate, 15% (vol/vol) heat inactivated fetal bovine serum (FBS), 70µmol/l β-mercaptoethanol, 100U/ml penicillin, and 100U/ml streptomycin in a humidified atmosphere at 37°C with 5% CO_2. When cell confluence reached 70-75%, cells were transiently transfected with 2µg of hKCNQ1 constructs of wild type and mutations pIRES-AcGFP1 vector), respectively using Amaxa^{TM} Cell Line Nucleofector^{TM} Kit V and Amaxa^{TM} Nucleofector^{TM} 2b device.

Cells were first trypsinized, centrifuged, resuspended in 20 ml of DMEM medium and counted. 4x10^6 cells were transferred into a 1.5 ml tube and centrifuged at 90g for 10 minutes. The medium was removed. Cells were resuspended in 100 µl of Cell Line Nucleofector^{TM} Solution V and mixed with *KCNQ1* constructs. Cells were transferred to the cuvette and electroporated. The solution was quickly transferred into a 1.5 ml tube again and the cuvette

was washed with 500µl of the RPMI 1640 medium. The transfected cells were subsequently incubated at 37°C for 10 minutes to recover and seeded in culture plates with DMEM medium.

2.2.12 Insulin secretion in highly glucose-sensitive mouse β-cells

The insulin secretion from SJ β-cells was measured after incubation with the various secretagogues [102]. SJ β-cells were seeded in 4-6 wells of a 12-well plate (6 X 10^5 - 1 X 10^6 cells/well) after transfection (Chapter 2.2.11). After 72 hours, culture medium was aspirated and cells were washed with 1 ml of the Secretion buffer. 1ml of the Secretion buffer containing 5.5 mM of glucose was added and cells were incubated at 37°C for 30 minutes. Then, the buffer was aspirated and cells were washed with 1 ml of Secretion buffer again. One millimeter of secretion buffer with either 3.3mM or 16.7mM of glucose, or 30mM KCL was added separately, and the cells were incubated at 37°C for another 30 minutes.

In order to measure the amount of the secreted insulin, supernatant was aspirated from the cells and transferred into a 1.5ml tube, then centrifuged at 200g for 5 minutes. Subsequently, the upper phase of the supernatant was transferred into another tube.

In order to measure the cell insulin content and the amounts of the total cell protein, the supernatant was removed and cells were lysed with protease inhibitor cocktail in 50µl of RIPA buffer on ice for 10 minutes. 2µl of cell lysate was diluted to 1:2000 with Secretion buffer, it was used to determine cell insulin content. Total cell protein amount was determined using BCA™ Protein Assay Kit as outlined in Chapter 2.2.13.1.

Insulin secreted and cell insulin content was measured using Mouse High Range Insulin ELISA kit according to manufacturer's protocol. All controls and samples were run in triplicates. 5 µl of each standard, control and sample were pipetted into the wells of an ELISA plate followed by 75 µl of a Working Strength Conjugate. The plate was sealed and incubated for 2 hours while shaking at 700-900 rpm on a High Speed Microplate Shaker. Then the solution was transferred and the plate was washed 6 times with 350 µl of Working Strength Wash buffer per well. After that, 100µl of TMB Substrate was pipetted into each well and incubated for 15 minutes at RT with shaking at 700-900 rpm on a High Speed Microplate Shaker. Finally, 100 µl of Stop Solution was pipetted into each well, and the plate was gently shaken to mix the contents and the absorbance was read at 450 nm and 630 nm in the SpectraMax M2 Multi-Mode Microplate Readers. The reference absorbance at 630 nm was subtracted from the absorbance at 450 nm.

The secreted insulin was normalized to total protein concentration or to total produced insulin, where the produced insulin was the sum of secreted insulin and cell insulin content. Subsequently, the insulin secretion of the cells, stimulated with 16.7 mM glucose, was normalized to the secretion of cells, stimulated with 3.3 mM glucose and then all the values

were normalized to *KCNQ1* control transfected samples. The calculations and statistical analysis were done using GraphPad Software.

2.2.13 Protein biochemical analysis

2.2.13.1 Protein isolation and quantification

The transfected cells were collected into respective tubes after washing twice with PBST, and then add 100 µl RIPA buffer containing protease inhibitor then incubated for 20min at 4°C and centrifuged at 13,000 g for 10 min at 4°C. The supernatant containing the cytosolic proteins was collected to new tubes and stored at -80°C immediately. The cytosolic protein concentration was determined by the BCA Protein Assay reagent kit. The absorbance was measured photometrically in the Microplate Reader equipped with a 590 nm filter. The cytosolic protein concentration of transfected cell lysate was determined based on the standard curve.

2.2.13.2 SDS-PAGE

SDS-PAGE was used to separate proteins according to their molecular size. 20 µg of protein was diluted in Aqua dest, and 2 x Laemmli loading buffer were added. The protein was heat-denatured at 95°C for 5min and cooled down at 4°C. Protein marker and protein samples were loaded on 4-20% Tris-glycine gels vertically aligned to the electrophoresis chamber and filled with 1 x electrophoresis buffer.

2.2.13.3 Western blot

The proteins were then electrotransferred to PVDF membranes under semidry conditions. After membranes were blocked with 5% dry milk in PBST for 1 hour, primary antibodies were added at the required dilution and membranes were sealed into plastic boxes and stored overnight at 4°C. The second day was followed by three washing steps for 10 min each in PBST, then incubation with a corresponding secondary antibody in 5% dry milk in PBST for 1 hour at RT. After repeating the washing steps with PBST, detected agents was added on the membrane, and positive signals were acquired by a ChemiDoc™ MP imaging system. To save time and material, FVDF membranes were recycled. The membranes were stripped by striping buffer 2 times for 15 minutes/time. Then the membranes were blocked with 5% dry milk in PBST for 1 hour and the above steps, repeated above steps. The protein's molecular size was quantified by using Image J software.

2.2.14 Analysis and statistics

All clinical data were analyzed by SPSS17.0 software (SPSS Inc., Illinois, USA). The primers were designed using program *Oligo 6.1*(Molecular Biology Insights, Inc., Cascade, USA). The results of Sanger sequencing were analyzed by ABI 3730 Sequence analysis

software (Applied Biosystems, Wrington, UK) and DNASTAR (DNASTAR.Inc). The statistical analysis of immunohistochemistry and western blot results, Image J software (National Institutes of Health, USA) and the t test ware used. All values were given as mean and standard error of the mean (SEM). All graphic and statistical analyses were performed using the Graph Pad Prism 5.0 software (GraphPad Software, Inc.CA, USA). A *p*-value less than was 0.05 considered to be significant.

3. Results

3.1 Analysis of the clinical data

3.1.1 Clinical characteristics of the cohort with β cell dysfunction

The large cohort of individuals and families with extremely rare types of pancreatic β cell disorders are all of unknown pathogenesis. The patients were first screened with known candidate genes. Phenotypes include neonatal diabetes, MODY-like diabetes, paediatric-onset type-2 diabetes, diabetes syndromes with associated disease of e.g. exocrine pancreas, kidney, central nervous system (CNS) or heart, pancreatic hypoplasia, and congenital hyperinsulinism. All these conditions are very rare (1:100.000 – 1:1.000.000) and represent unique phenotypes, which are very valuable for molecular genetic investigation. Familial β cell disorders are inherited either in a dominant or a recessive mode of inheritance; the patients without family history are considered to be sporadic cases. In our investigation, we have collected DNA samples of 520 individuals (including family members and 279 individuals with diabetes or CHI). According to their inheritance and diabetes type (ADA-classification), all of these diabetes patients were categorized in accordance with the American Diabetes Association guideline [34]. All had been autoantibody-negative, but remarkably differed in terms of needs for insulin treatment, inheritance of diabetes and consanguinity, as well as onset of diabetes or obesity grade (Table 7). Our typing was done as follows: (i) sporadic, insulin-dependent but non-immune diabetes (type 1B diabetes, n=87), (ii) obesity-associated diabetes (type 2 diabetes) (iii) autosomal dominant, type 3 diabetes, AD-MODY, assumed recessive diabetes (consanguinity, recessive inheritance assumed AR-MODY), pancreas aplasia (PA), syndromic diabetes, or neonatal diabetes (transient or persistent, onset before 6 months of age). Along with our intention to study naturally occurring, human loss-of-function or gain-of-function mechanisms in single genes, we studied rare diabetes conditions with diabetes onset before age 18 years and autoantibody-negative. Diabetes onset ranges from neonatal diabetes until late adolescent age under 18 years, which is considered to be as an inclusion criterion of the disease-onset age.

Table 7: Overview of the beta cell dysfunction cohort

	Classification of human beta cell dysfunctions						
Diabetes type prevalence (< 18 years)	T1B	T2D	AD-MODY	AR-MODY	PA/ND	Syndromal	CHI
	1:10.000	1:5.000	1:10.000	1:40.000	1:100.000	1:100.000	1:20.000
	87	47	7	3	12	8	37

AD: Autosomal dominant, AR: Autosomal recessive, PA: Pancreas aplasia / hypoplasia, ND: neonatal diabetes, syndromic: diabetes syndromes, CHI: congenital or childhood-onset hyperinsulinism.

The ethnic origins of the families are central Europe (n=154), Arabic countries (n=50), Turkey (n=66), and Asia (n=9). 279 patients took part in this investigation, the gender ratio was 1.2:1 (152 male: 127 female) and age of onset was from birth to 18 years old (mean age was 6.7±5.95 years old). Our molecular screening procedure of this cohort can be seen in figure 11 in chapter **2.2.2**

3.1.2 Genetic investigation of the cohort with β cells dysfunction

To date, we have sequenced 10 complete genomes, 45 exomes sequencing in this cohort. We have identified *PCBD1* [103] and other 58 novel candidate genes. All these new findings were included in the following target enrichment NGS in 96 samples with extreme phenotypes. Of the 96 patients who are performed by target enrichment NGS, 8 cases (8%) were diagnosed CHI, 6 cases (6%) were diagnosed as ND, 8 cases (8%) were diagnosed as MODY, and 8 cases (8%) were diagnosed as pancreatic hypoplasia (PH), the remaining were diagnosed as T1D or T2D. Our custom Exon-capture assay (Nextera Illumina, figure 5) includes (i) all known monogenic diabetes genes (38), (ii) type 2 diabetes susceptibility genes (18), (iii) additional genes causing congenital hyperinsulinism (10), (iv) loci which are important in the pancreas and islet development from mice (37), and (v).new candidate genes from our next-generation sequencing project (exome and complete sequencing) with high suspect in human diabetes (58).

Table 8: The overview of the selected target genes

Resources	Known MODY loci	T2DB loci	Mouse loci	PH	CHI loci	ECRC exome results
Loci Nr	38	18	37	3	10	58

PH: Pancreas hypoplasia

From the previous mentioned exome, whole genome sequencing and target enrichment NGS experiments, we filtered and analysed the sequences obtained in each family following different strategies according to their individual inheritance of phenotypes.

In total, combining all our strategies, except for the discovered 58 novel candidate genes, we also detected around 40 cases with known candidate gene mutations, which may be overlooked in the conventional screening strategy with Sanger sequencing.

3.1.2.1 Detected mutations in known candidate genes in the patients with beta cells dysfunction

As mentioned before, we have detected around 40 cases harbouring known candidate gene mutations (Table 9). Of the 11 known candidate genes, *KCNJ11* genes are the most frequent gene with detected mutations in 7 patients (table 9). They were diagnosed as PND, TND, CHI and T1DB, respectively. The patients (ID: 26_119, 26_129, 26_131and ID: 56_383, 56_384) come from 2 different families, respectively (table 9). *ABCC8* genes mutations were detected in 6 patients. In these patients with ABCC8 mutations, 4 patients diagnosed as CHI come from 4 different families, respectively (table 9). The other 2 patients were diagnosed as T2D. For the mutations detected in the *GCK*, five patients were diagnosed as MODY2. Four patients of pancreas hypoplasia were detected harboring *PTF1A* enhancer mutations and the two PH patients harbored *GATA6* mutations. In Glis3, one patient was diagnosed as T1D, and the other one was diagnosed as TND. Two patients with *HNF1A* and two patients with *HNAF4A* were diagnosed as MODY3 and MODY1, respectively. One patient with INS mutation was diagnosed as T1D, an INS mutation was also detected in her mother. The patient harboring INSR mutation was diagnosed as T1D, INSR mutations were detected in her parents. One patient with *PCBD1* mutation was diagnosed as T1BD and mild hyperphenylalaninemia (Table 9). We found a novel candidate gene, *KCNQ1*, mutations in this gene result in ND and CHI in our cohort (see Chapter 3.2).

Table 9: List of mutations identified in the known candidate genes of beta cell dysfunction.

Known Gene	Age of onset	gender	Model of inheritance	c.Pos	p.Pos	phenotype	Known variants (MAF)	Treatment
ABCC8								
16_87	2 months	Female	Recessive	c. 4661G>T	G1554V	CHI	1/121112	NA
30_154	23 days	Male	Dominant	c.4260C>T	Ser1419Leu,	CHI	Novel	diazoxide
1B_193	14 years	Male	Sporadic	c.1836G>A	Glu612Asp	T2D	Novel	Insulin, then metformin
59_196	13 years	Male	Recessive	c.1616A>C	Tyr539Cys	T2D	rs193922397 (NA)	metformin
35_214	1 month	Female	Dominant	c.3926G>A	Gly1309Glu	CHI	Novel	10% glucose, glucagon
38_227	1 month	Female	Recessive	c.4159_4161del	1387-1388 FS/S	CHI	rs72559718(1/38092)	Pancreatectomy (97%)
GCK								
1B_140	12 years	Female	Sporadic	c.943C>T	Leu315Phe	MODY2	rs193922338 (NA)	metformin
1B_141	4 years	Male	Sporadic	c.148C>G	His50Asp	MODY2	Novel	metformin
43_268	14 years	Male	Recessive	c.C626T	Thr209Met	MODY2	Novel	NA
1B_157	3 years	Male	Sporadic	c.537A>C	Asn179Thrfs*25	MODY2	Novel	NA
39_230	13 years	Female	Recessive	c.G1318T	Glu440stop	MODY2	Novel	NA
GATA6								
4_15	2 years	Male	*De novo*	c.754_905del	Ala252Hisfs*160	PH	Novel	insulin
1B_74	1 day	Male	*De novo*	c.951_954dupGCCG	Leu319Alafs*145,	PH	Novel	insulin
GLIS3								
3_40	9 years	Male	Recessive	c. 734A>G	His245Arg	T1D	rs376031632(6/10606)	insulin
27_145	20 days	Male	Recessive	c. 256C>T	Pro86Ser	TND	rs149916569 (0.001)	insulin
HNF1A								
50_367	15 years	Male	Sporadic	c. 347C>T	Ala116Val	MODY3	Novel	lifestyle
1B_391	16 years	Male	Sporadic	c. 824A>C	Glu275Ala	MODY3	rs199890776(4/109258)	metformin
HNF4A								
1B_17	16 years	Female	Sporadic	c. 790G>A	Val264Met	MODY1	rs139779712(2/12136)	Insulin

46_355		Male	Recessive	c. 266G>A	Arg89Gln	MODY1	Novel	NA
INS								
19_31	14 years	Female	Dominant	c.138G>A	Arg46Gln	T1BD	rs121908260(NA)	insulin
19_99	27 years	Female	Recessive	c.138G>A	Arg46Gln	GDM	rs121908260(NA)	Metformin, insulin
INSR								
15_84	14 years	Female	Recessive compound het	c.2715C>G & c.3090G>C	Phe905Leu & Gly1030Arg	T2D	Novel	metformin
KCNJ11								
21_105	2 month	Male	De novo	c.175G>A	Val59Met	PND	rs80356616 (NA)	insulin
23_112	8 month	Male	Dominant	c.36C>A	Tyr12Stop	CHI	rs104894236 (NA)	NA
26_119	1 month	Male	Dominant	c. 149G>A	Arg50Gln	TND	rs80356611 (NA)	sulfonylurea
26_129	30 years	Male	Dominant	c. 149G>A	Arg50Gln	T2D	rs80356611 (NA)	NA
26_131	42 years	Male	Dominant	c. 149G>A	Arg50Gln	T2D	rs80356611 (NA)	NA
56_383	16 years	Female	Sporadic	c. 481G>A	Ala161Thr	T1BD	Novel	insulin
56_384	16 years	Female	Sporadic	c. 481G>A	Ala161Thr	T1BD	Novel	insulin
PTF1a **enhancer**								
1B_94	3 days	Female	Recessive	10:23508437 A>G		PH	[104]	insulin
1B_95	3 days	Female	Recessive	10:23508437 A>G		PH	[104]	insulin
Lond1_451	1 day	Male	Recessive	10:23508437 A>G		PH	[104]	NA
Lond2_457	1 day	Female	Recessive	10:23508437 A>G		PH	[104]	NA
PCBD1								
1_7	14 years	Female	Recessive	c.46del	Leu16cysfs*5	T1BD &HPA	Novel	insulin then meglitinide

MOI: modes of inheritance: CHI: congenital hyperinsulinism; T2D: type 2 diabetes; T1BD: type 1B diabetes; GDM: gestational diabetes mellitus; PH: pancreas hypoplasia; TND: transient neonatal diabetes; PND: permanent neonatal diabetes; MODY: maturity onset of diabetes in the young. HPA: hyperphenylalaninemia; MAF: minor allele frequency from the ExAC database; NA: not available. Transcripts: ABCC8 (NM_000352), *GCK* (NM_000162), *GATA6* (NM_005257.4), *GLIS3* (NM_152629, NM_001042413), HNF1A (NM_000545), *HNF4A* (NM_000457), *INS, INSR, KCNJ11* (NM_000525), *PCBD1* (NM_000281.2).

3.1.2.2. Target enrichment next-generation sequencing of beta cell dysfunction disorders.

Large scale, complete exome sequencing is limited by huge amounts of amplified variants and a coverage that is still low in some critical regions. As a consequence, pathogenic mutations might be missed. Therefore, we performed our first round of target enrichment screening in 96 samples with extreme phenotypes. Through this strategy, isolating high-priority segments of genomes greatly enhances the efficiency of next-generation sequencing (NGS) by allowing researchers to focus on their regions of interest, reduces the cost per sample and opens up the possibility analysing multiple bar-coded samples simultaneously in one sequencing run.

In our experiments, we have detected 26 cases with known candidate gene mutations and 33 cases harbouring mutations of our previously discovered novel candidate genes (Figure 12). The discovery ratio has thus reached 62%, which confirms its high efficiency in identifying molecular causes of human disorders

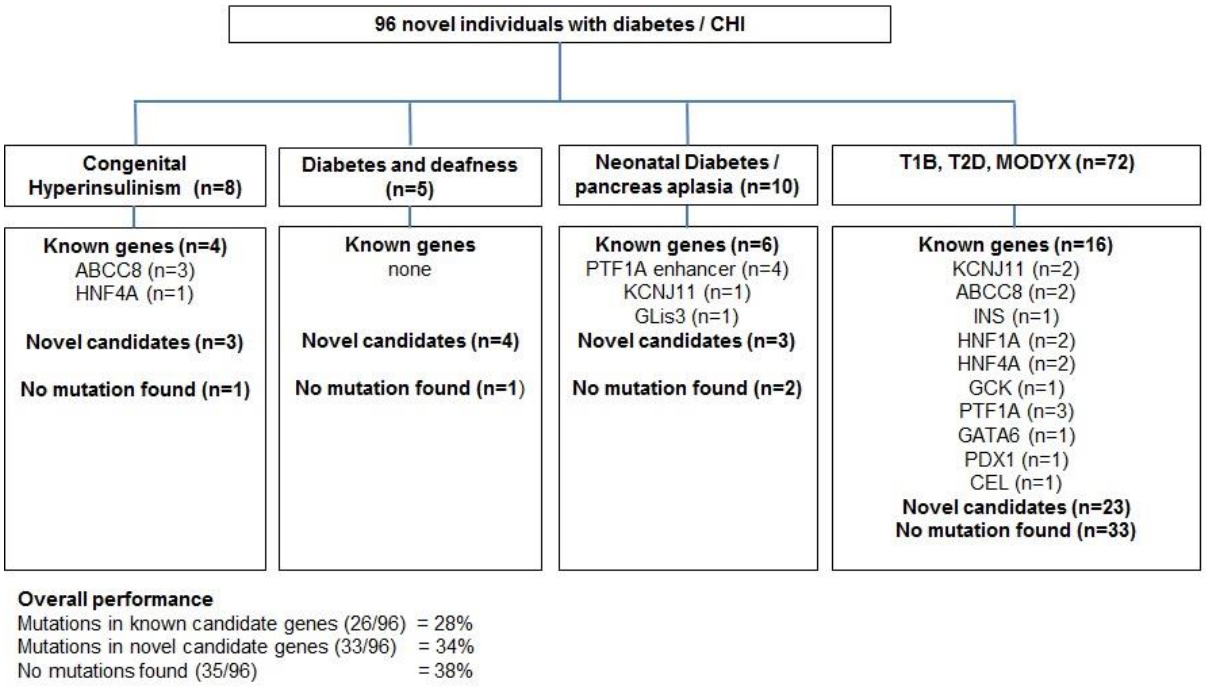

Figure 12: Detection rate of our custom-designed targeted enrichment/ NGS assay in patients with different insulin secretion defects of unknown origin. Highest rate of mutations had been identified in patients with ND (2/6), CHI (4/8) and PH (7/8), while T1D or T2D displayed a lower detected rate. Novel gene mutations had a higher detected rate in ND (3/5).

3.2 *KCNQ1* mutation causes insulin secretion defect in the patients

Of our 58 identified novel candidate genes in disorders of beta cells dysfunction, *KCNQ1* mutations were detected in three patients in this cohort. These three patients were diagnosed as PND, CHI in combination with LQTS, and CHI respectively (Table 10). For the PND patient, one homozygous *KCNQ1* mutation (R397W) was detected, and two heterozygous *KCNQ1* variants (E13del & G292D) were detected in the two CHI patients (Table 10 and figure 13)

Table 10: Clinical characteristics of patients with *KCNQ1* mutations

Patient ID	**28_148**	**62_405**	**48_241**
Age of onset	1 day	6 months	10 months
Gender	male	male	male
Ethnicity	Turkish	German	German
Consanguinity	Yes	Yes	No
Family history	Both parents: healthy	Father: CHI &LQTS, patient's brother: LQTs, both harbor the same KCNQ1 mutation; mother: healthy.	Both parents: healthy
Phenotype	PND	CHI	CHI
Glucose(mg/dl)	293	26	NA
Symptoms	intrauterine growth retardation	LQTS	No
Detected variant	11:2608860	11:2797188	11:2594170
g.Pos (Hg19)	C>T	A>G	G>A
c.pos*	1189	Exon 13 acceptor site	875
AA change	Arg397Trp	del531-560 fs619	Gly292Asp
Zygosity	homozygous	heterozygous	heterozygous
Known variant (MAF)	rs199472776 (0.000016)	Novel	rs199472736 (NA)
Function Change	Arg397Trp	Splice acceptor lost	Gly292Asp
Prediction **	Disease causing	Disease causing	Disease causing
Treatment	Insulin	Frequent feeds	Frequent feeds
Reference	[105]	Unpublished	[106]

AA: Amino acid: MAF: minor allele frequency from ExAc database, NA: not available
* ENST 00000155840
** Base on prediction programs of Polyphen, SIFT, and Mutation Taster

The PND patient (ID: 28_148) comes from a consanguineous family. He was found to have hyperglycemia one hour after birth (glucose: 293mg/dl) without c-peptide. The patient was born at 37 gestational weeks with intrauterine growth retardation, and his

birth weight was 1628 grams. Clinical investigation showed no other clinical manifestation of Jerwell and Lange-Nielsen syndrome, which is known to be caused by biallelic *KCNQ1* mutations, including normal ECG normal hearing. His parents are cousins, the parents and the siblings are until now healthy (Figure 13 A). The homozygous variant (hg19:11:2608860 C/T, R397W) from the patient was first found in our exome sequencing and confirmed by Sanger-sequencing (Table 10). Even the heterozygous variant detected in the parents is extremely rare with a minor allele frequency of 0.000016 (1.6×10^{-6}) from the ExAC database with 6700 cases.

The first CHI patient (ID: 62_405) (Figure 13 C) was diagnosed as CHI and LQTS at 6 months after birth, no other clinical manifestation; his father (ID: 62_406) with LQT-syndrome knew about his risk for hyperinsulinemic hypoglycemia and this was confirmed recently in an oGTT with a late drop of glucose below 50 mg/dl after 120 min post glucose; his younger brother (ID: 62_407) was diagnosed with LQTS but had a normal oGTT in terms of diabetes and hypoglycemia currently. Through molecular screening, a splicing acceptor lost (AG>GG) was found which resulting in deletion of exon13 (Figure 13, D) in all three affected family members in this family. This is a novel detected variant, which may result in a new isoform of KCNQ1.

The second CHI patient (ID: 1B_241) (Figure 13 E, Table 10) is one sporadic case, except for the hyperinsulinemic hypoglycemia, no other clinical features were found in him. One de novo heterozygous was detected in the patient with a missense variant (G292D, Figure 13 F and Table 10). His parents are both healthy and don't harbor this variant. The detected variant, G292D, is a quite rare variant as that of the PND variant, there is no MAF information available of this variant from the known databases. This variant locates at the helix-S5-S6 of the channel, which is important for normal KCNQ1 channel deactivation [107]. The other two detected variants (E13 deletion & R397W) locate in the intracellular C-terminal (CT) domain, which is responsible for channel traumatization, proper channel trafficking and biophysical properties, this domain plays a critical role in gate regulation as well [108]. Therefore, mutations in this domain could cause severer phenotypes compared to the mutation locating at helix-S5-S6. The molecular findings match the phenotype features of all the three patients.

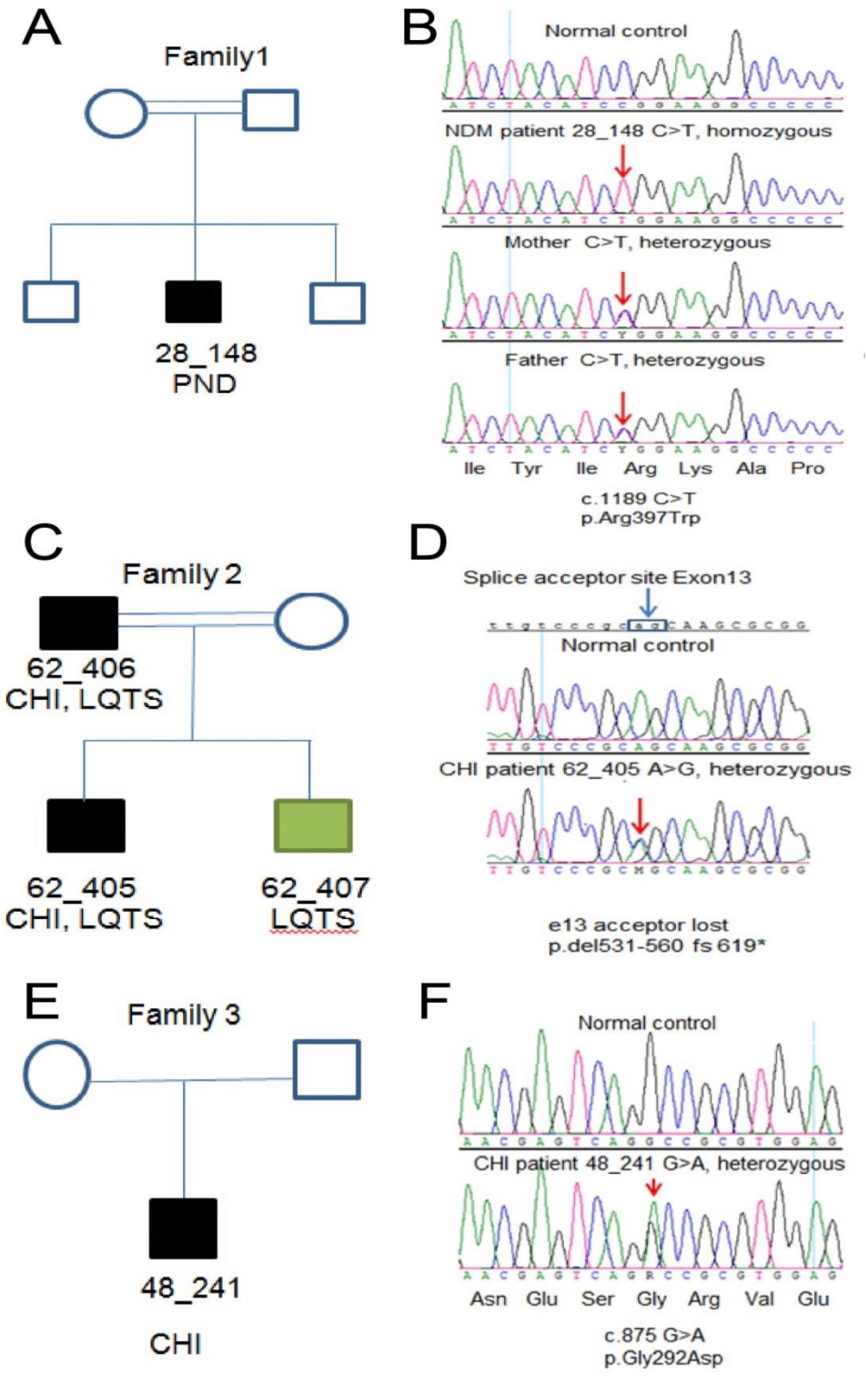

Figure 13: Mutations in *KCNQ1* cause PND and CHI respectively. A: Pedigree of family 1 with PND patient due to homozygous mutation R397W. **B**: Sanger sequencing shows the homozygous and heterozygous variants from the index patient and his parents, respectively. **C**: Pedigree of family 2 shows that patient and his father were both diagnosed as CHI and LQTS, and patient's brother was diagnosed as only LQTS. **D**: Sanger sequencing showed the patient and his father harboring one splicing variant (AG>GG). **E**: The second CHI patient has one heterozygous DE novo *KCNQ1* variant: G292D. **F:** Sanger sequencing of the de variant.

3.2.1 Verification of the detected splicing mutation in *KCNQ1*

In order to verify the splicing variants, RNAs was extracted from the index patient (ID: 62_405), his father (62_406), his brother (62_407), and normal controls from peripheral blood mononuclear cells (PBMC). Four pairs of primers were designed, first pair is from exon 11 to exon 13 and the reverse primer locates only inside exon13, the second pair spans from junction exon11 & exon12 to exon14, the third spans junction of exon12 & exon13 (4 bps of exon13 in the forward primer) to exon14, and the last one spans between exon11 and exon 14 in the wild type *KCNQ1* of coding DNA sequence. As expected, the RT-PCR experiments with four previously mentioned primer pairs confirmed the deletion of exon13 in one allele of the affected patients in this family. For the reverse primer locates only inside exon13, we could only detect one band in this family (the wild type), however, with the primers which span exon 13, we could detect two bands from the PBMC cells of the three patients and one band from PBMC cells of the normal control sample. This exon 13 deletion may cause a new *KCNQ1* isoform (Figure 14).

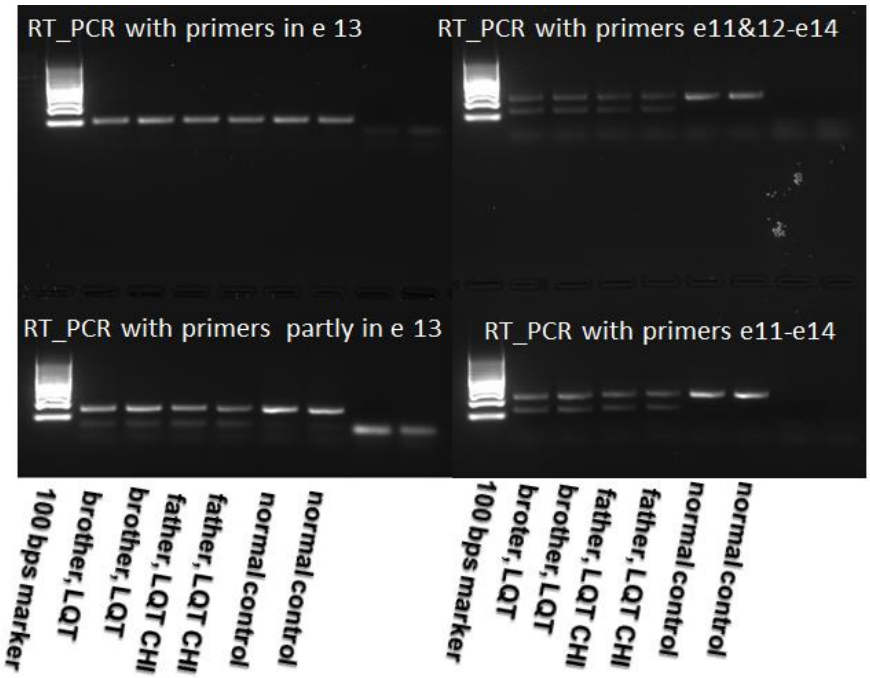

Figure 14: RT-PCR with LQTS patient ficoll cells confirmed *KCNQ1* e13 is spliced out. Two bands of the PCR products are shown for the three pairs of primers spanning exon 13, and the size of the shorter bands was shown in exon13 that was spliced out.

We further sequenced all the above mentioned bands with Sanger sequencing, and as expected, for the short bands, after the sequence of exon12, the sequence of exon14 was shown continuously from the sequence, which confirmed the deletion of exon13 from one DNA allele of the patient's blood cells (Figure 15).

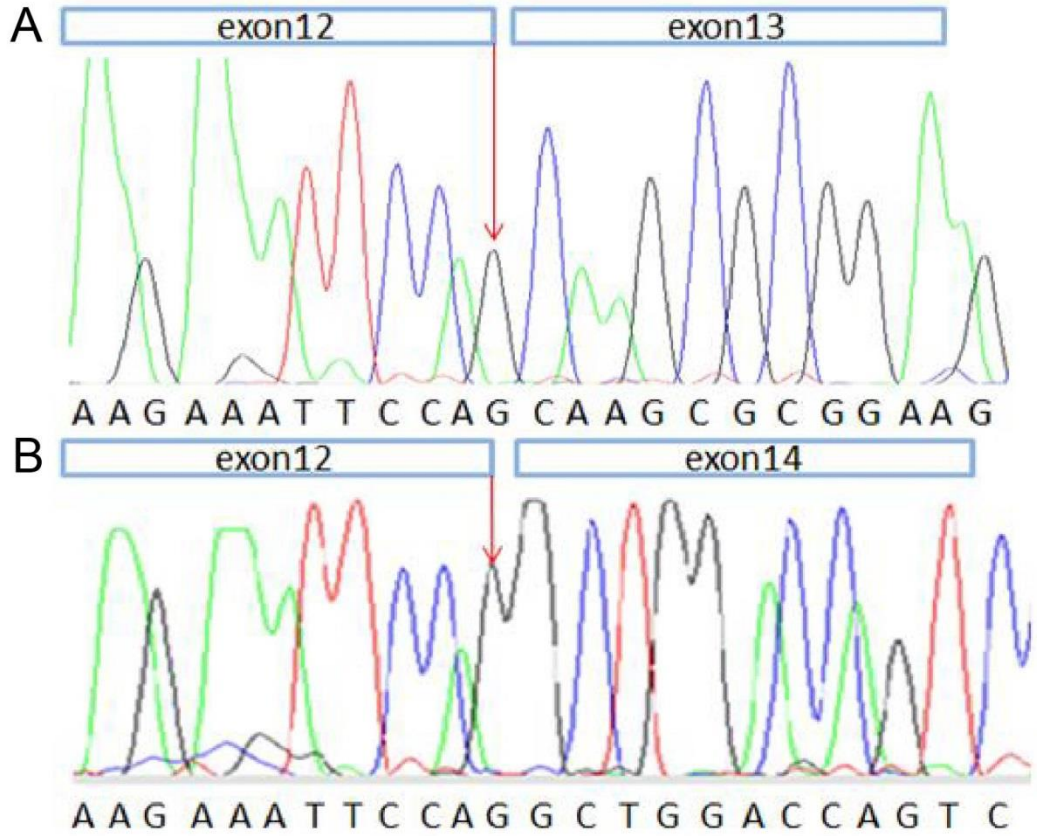

Figure 15: The confirmation of the exon 13 deletion from the CHI & LQTs patients. A. The PBMCs cDNA sequencing results from a normal control. It is the same as the patients' long band sequencing. B: the shorter bands from PBMC cells cDNA sequencing of the patients.

3.2.2 *KCNQ1* mutations cause bipolar insulin secretion defects

To verify that the three variants cause abnormal insulin secretion in pancreatic beta cells, we transfected the *KCNQ1* constructs with the two CHI mutations (G292D and e13 deletion), diabetes mutation, together with wild type into Mouse SJ β cells with four replicates for each. After 72 hours' transfection, the insulin secretion of all transfected and non-transfected mouse SJ β cells was measured. We found that the Insulin secretion in both CHI *KCNQ1* transfected mouse β cell variants significantly increased compared with that in the wild type *KCNQ1* transfected mouse β cells regardless whether the cells were treated with different concentration of glucose (3.3mM, 16.7mM) 30mM of KCL (Figure 16A), in contrast, significantly reduced insulin secretion was measured in the diabetic variant (R397W) transfected mouse SJ β cells compared with that of in the wild type *KCNQ1* transfected cells by the above mentioned glucose or KCL regulation(Figure 16A).

However, the insulin production in all three *KCNQ1* variants (CHI & diabetes), transfected cells decreased significantly compared with that in the *KCNQ1* wild type

transfected mouse β cells (Figure 16B). Therefore, we found that only insulin secretion, but not insulin production is affected by this membrane protein abnormality. We repeated this experiment four times with the similar results.

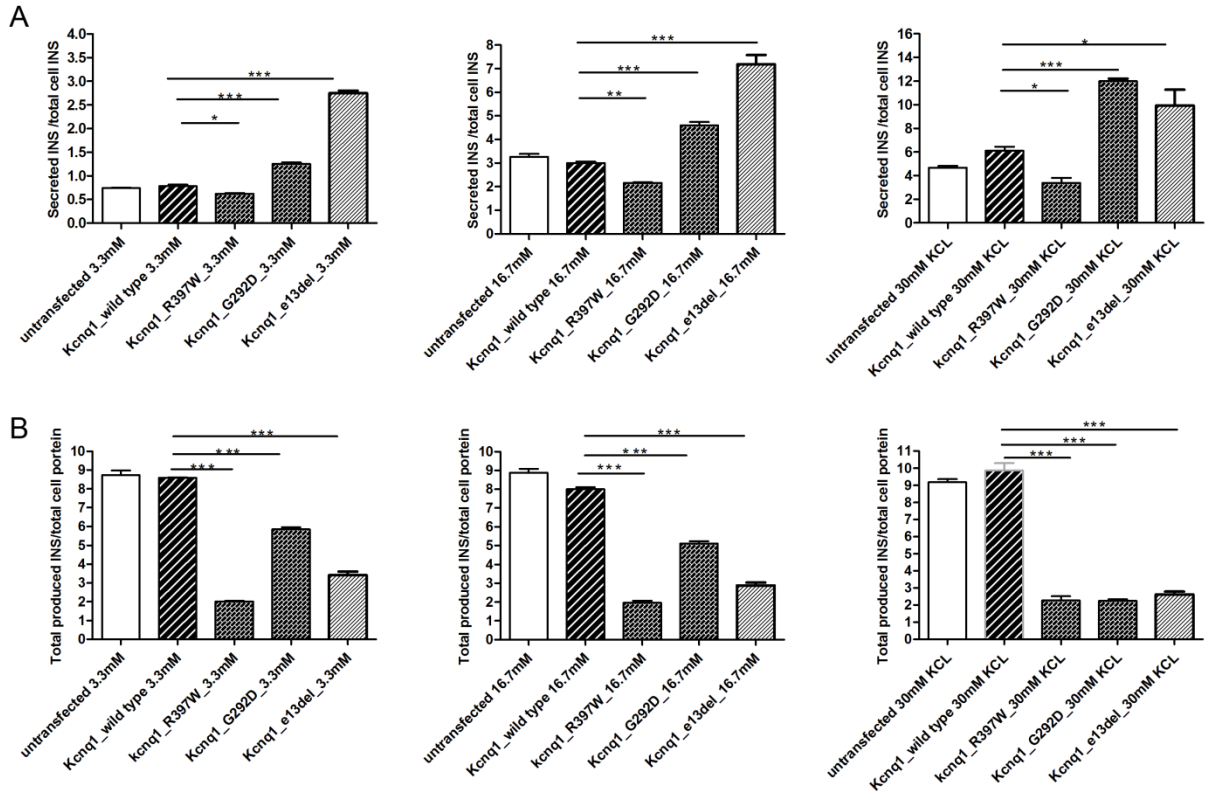

Figure 16: The insulin secretion assay in the *KCNQ1* diabetic variant, CHI variants and wild type *KCNQ1* transfected mouse β cells. **A**: The insulin secretion in both CHI *KCNQ1* variants, transfected mouse β cells was significantly increased compared with the wild type *KCNQ1* or non-transfected mouse β cells, whereas the insulin secretion in diabetic *KCNQ1* variant reduced than that of wild type *KCNQ1* transfected mouse β cells . **B.** The total cell insulin production was significantly decreased in both the *KCNQ1* diabetic variant and CHI variants transfected mouse β cells compared with that of the *KCNQ1* wild type transfected cells. *: P<0.05, **: P<0.01, ***: P<0.001.

3.2.3 The expression abnormality caused by *Kcnq1* mutations in mouse β cells

In order to understand the molecular mechanism of the abnormality in insulin secretion in *KCNQ1* mutations, we performed quantitative real-time PCR (qPCR) with RNAs isolated from the mouse SJ β cells transfected with all three variants (G292D, E13 Del & R397W) and wild type construct. We took the non-transfected cells with internal controls. We checked the expression levels of the transcriptional factors involved in the regulation of insulin secretion in β cells (*Pdx1, Neurod1, Foxa2, Gck, Hnf1α, Hnf1β, Hnf4α, Ucn3, Nkx6.1, MafA, Foxo1, Scl2a2, Abcc8, Kcnj11, Ins1,* & Ins2). We also checked of the transcription factors for maintaining the pancreatic islet α cells

(*Gcg, MafB, Irx1, Arx, Nkx2.2*), PP cells (*Pp*), or δ (*Sst*) cells to explore the conversion possibility from the of β cell to other cell types.

Not surprisingly, we detected decreased insulin transcriptional levels of both mouse *Ins1* and *Ins2* in SJ β cells transfected with all the detected *Kcnq1* mutations compared with those transfected with *Kcnq1* wild type construct and non-transfected mouse β cells, which is inconsistent with the reduced insulin production of insulin assay (figure 17).

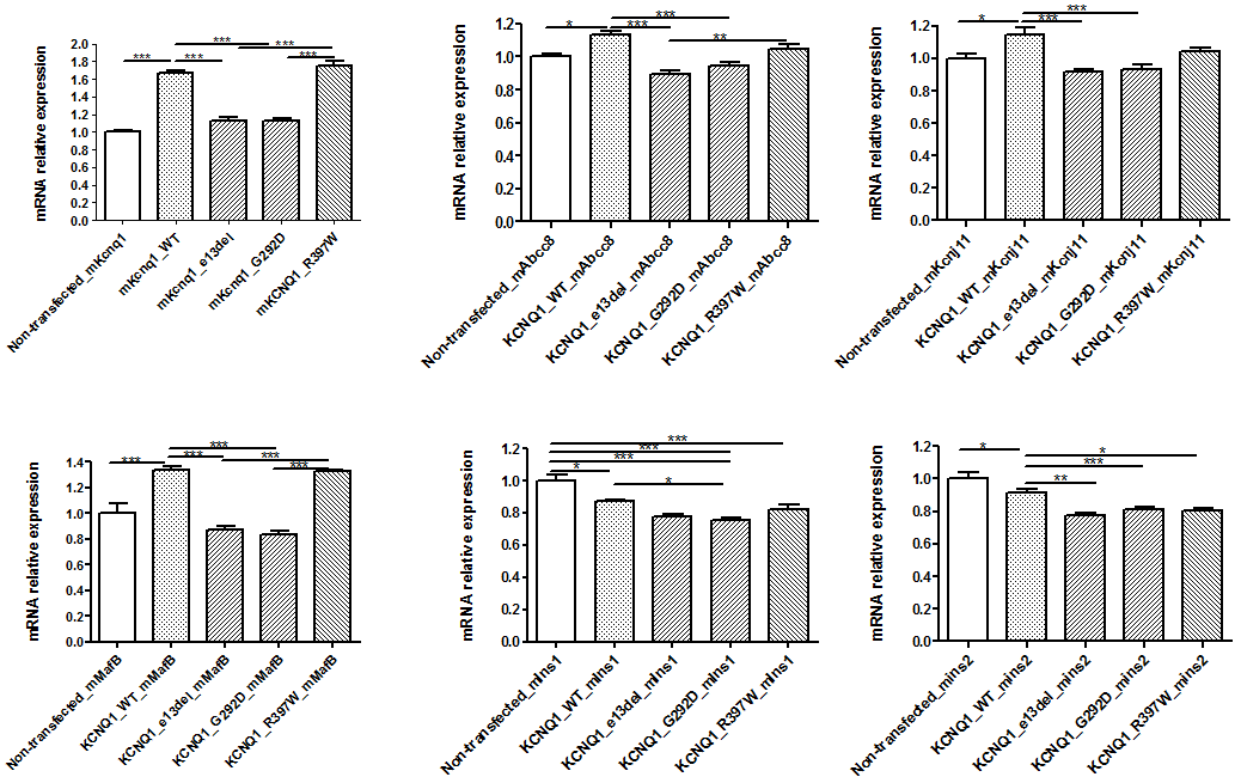

Figure 17: The *Kcnq1* mutations affected the expression levels of *Ins, Abcc8, kcnj11*, and *MafB*. CHI variants: G292D & e13del; Diabetic variant: R397W. *: P<0.05, **: P<0.01, ***: P<0.001.

On the transcription levels, transfected *KCNQ1* itself generated from the wild type construct and the diabetes associated mutation R397W was significantly higher than in non-transfected β cells or cells transfected with the two CHI mutations (G292D & e13 del). Furthermore, the expression of *MafB*, which is exclusively expressed in mature pancreatic α-cells, was used as marker for possible transdifferentiation, as it binds and activates the α-cell–specific promoter control element (termed G1) in the endogenous glucagon gene [31]. We detected a similar expression pattern of *MafB* as with the *Kcnq1* expression, which indicates that the CHI variants strengthen the β cell identity in contrast to that of the diabetes mutation R397W.

We showed previously that the insulin secretion is increased by CHI mutations and decreased by diabetes mutation in mouse β cells despite the decreased insulin production caused by all the mutations. In addition, *Kcnq1* channel function might interact with the K_{ATP} channel expression (*Abcc8* & *Kcnj11*) and Kv7.1 (*Kcnq1* & *Kcne2*) itself. Finally, the two *Kcnq1* CHI mutations (G292D & e13 Del) downregulate the expression levels of both *Abcc8* and *Kcnj11*, while the *Kcnq1* variant R397W has no significant effect on the expression of K_{ATP} channel transcription (figure 12). Therefore, the observed down-regulation of *Abcc8* and *Kcnj11*, coding for the K_{ATP} channel by itself might be one mechanism by which insulin secretion is increased by the CHI mutations. For the diabetogenic mutation, the disrupted insulin secretion might be caused by the transdifferentiation from β cells into α cells.

For any of other transcriptional factors, we could not detect significantly different expressions among the *Kcnq1* CHI &diabetic mutations transfected β cells compared to the *Kcnq1* transfected wild type β cells or non-transfected β cells. We repeated this experiment four times with similar results in both 24 hrs and 48 hrs transfection.

3.2.4 The *KCNQ1* mutations regulate protein expression of *Pdx1*, *Foxa2* and *Pax6* in mouse β cells

To understand at the protein level if the critical transcription factors involved in the insulin secretion are affected by the *Kcnq1* mutations, we performed Western blot with protein-lysates from transfected mouse β cells (transfection time: 24hrs and 48hrs) with constructs of *Kcnq1* wild type; the three variants (R397W, G292D, and E13Del), always compared to non-transfected cells. Briefly introduced, *Pdx1*, *Foxa2*, and *Neurod1* are three crucial transcription factors for β cell identity and function, while *Pax6* is expressed in both pancreatic β and α cells. *Pax6* plays an important role of β cell differentiation, and is also critical for α-cell function by coordinating glucagon gene expression as well as glucagon biosynthesis and secretion. We therefore tested if these important transcription factors would be affected by individual *KCNQ1* mutations. We incubated the electro-transferred PVDF membranes with primary antibodies of *Pdx1, Foxa2, Neurod1, Pax6*, and *Kcnq1*, and selected anti-Vinculin, anti- β actin, and anti-α tubulin as housekeepers (Figure 18A). We quantified the Western blot bands with software Image J. We repeated the experiments (24 hrs & 48 hrs transfection) four times separately.

We kept on detecting the significant up-regulation of the two *Kcnq1* CHI variants (G292D &E13del) to the expression of *Pdx1* compared to that of the *Kcnq1* wild type in

mouse SJ β cells (Figure 18B), even though the *Kcnq1* RNA expression was not affected by the two mutations. However, the expression of *Pdx1* was similar in SJ β cells transfected with the diabetic mutation (R397W) to that of those transfected with the *Kcnq1* wild type constructs (Figure 18B).

For *Foxa2*, differently, we detected significant down-regulation of *Foxa2* expression in β cells transfected with the diabetes-associated mutation. Furthermore, we found no different expression of *Foxa2* among the two CHI variants (E13 Del & G292D) and wild type constructs transfected β cells (Figure 18).

The expression of *Pax6*, however, is significantly up-regulated in the diabetic *Kcnq1* mutation (R397W) transfected cells, down regulated in the *Kcnq1* E13del transfected cells compared to those of transfected with *Kcnq1* wild type constructs. In the *Kcnq1* G292D transfected β cells, the expression of *Pax6* is also lower than those transfected with *Kcnq1* wild type construct, but the reduction is not significant (Figure 18). We could hypothesize a different molecular mechanism of the insulin secretion defect caused from the two CHI mutations and the diabetic mutation.

As for the expression of *Kcnq1* itself, we detected higher expression in *Kcnq1* diabetic mutation transfected β cells, lower or no expression in the CHI mutation transfected β cells than those transfected with *Kcnq1* wild type construct. This result is consistent with previous studies that over expression of *Kcnq1* in MIN6 cells impaired the insulin secretion [79], in contrast, inhibiting the *Kcnq1* channel enhances glucose-stimulated insulin secretion in mice [66]. We hence hypothesize that the abnormal channel function affected the protein expression of the curial transcriptional factors like *Pdx1, Foxa2,* or *Pax6*, but not the RNA expression levels.

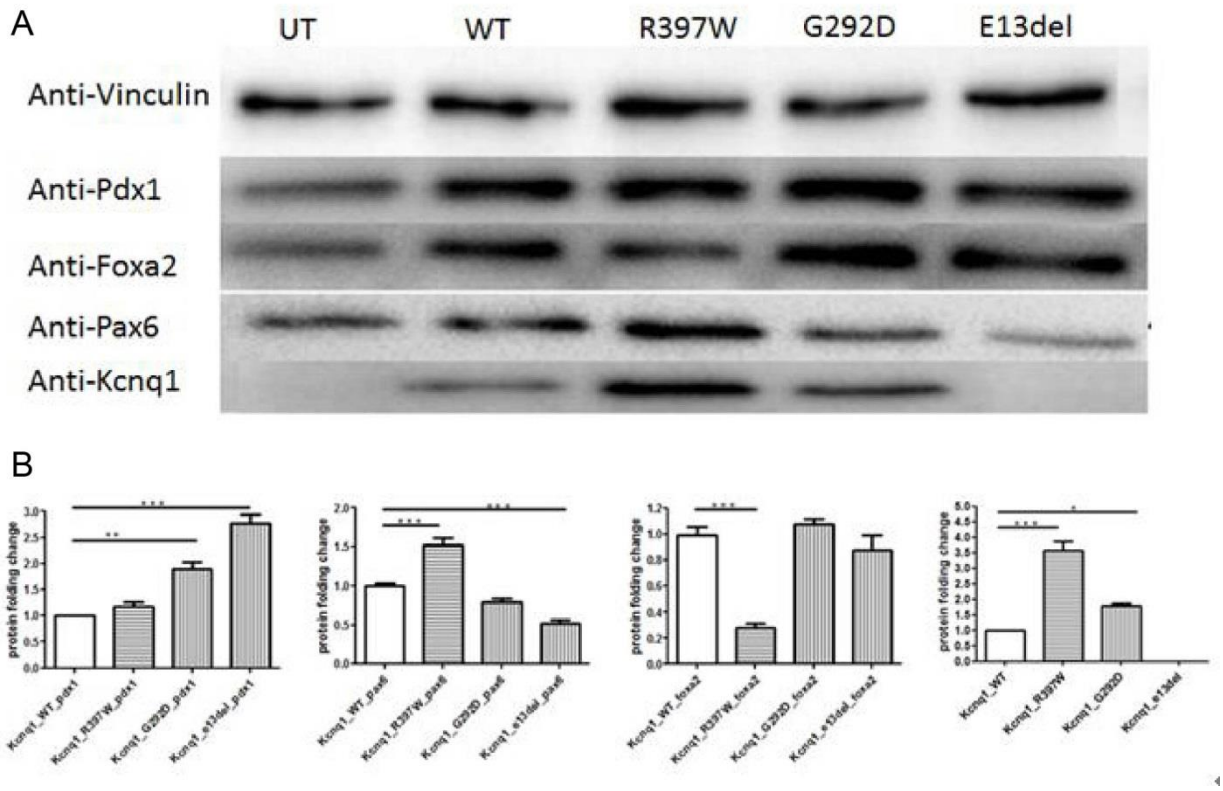

Figure 18: *Kcnq1* mutations cause abnormal expression of *Pdx1, Pax6* and *Foxa2* in mouse β cells. A. The western blot (WB) in mouse β cells. UT: Un-transfected β cells; WT: wild type; R397W: diabetic mutation in Arg397Trp; G292D: CHI mutation in Gly292Asp, Del 13: CHI mutations in exon 13; Pmax: positive control. The results of western blot with *Pax6* were at 48h after transfection, the others were at 24h after transfection. **B.** Quantification of the protein expression. *: P<0.05, **: P<0.01, ***: P<0.001.

3.2.5 *Kcnq1* mutation cause reduced pancreatic β cell mass

As shown before, the *KCNQ1* mutations caused an insulin secretion defect. In order to further understand the pathogenesis process, we checked the pancreatic β cell mass from the *Kcnq1* knock out (KO) and wild type (WT) mice. These mice were kindly donated by Prof. Voelkl (Institute of Physiology, Medical faculty, University of Tubingen) as a collaboration project. Own expression studies indicated that in this mouse strain, pancreas-specific reduction of *Kcnq1* expression was only by approx. 60%, so we handled with a knock-out model, at least concerning endocrine pancreas. Before starting the experiments, we first measured the weight of all the mice, there is no significant difference between the KO and wild type mice. Secondly, we measured the weight of the pancreas of all the mice, there is no significant difference between the KO and wild type mice either. Thirdly, we sliced the pancreas of each mouse into approx. 160 slices, one slice was selected from every 10 slices (around 16 slices for each animal) that they were immunostained with DAPI, Insulin, and Glucagon antibodies

respectively. After scanning the slices by fluorescent microscope BZ-8100E, we counted the areas of insulin immunostaining (β cell mass) and the whole pancreas of all the selected slices with software Image J. The relative β cell mass was calculated by dividing of both (insulin stained area/ whole pancreas area) areas.

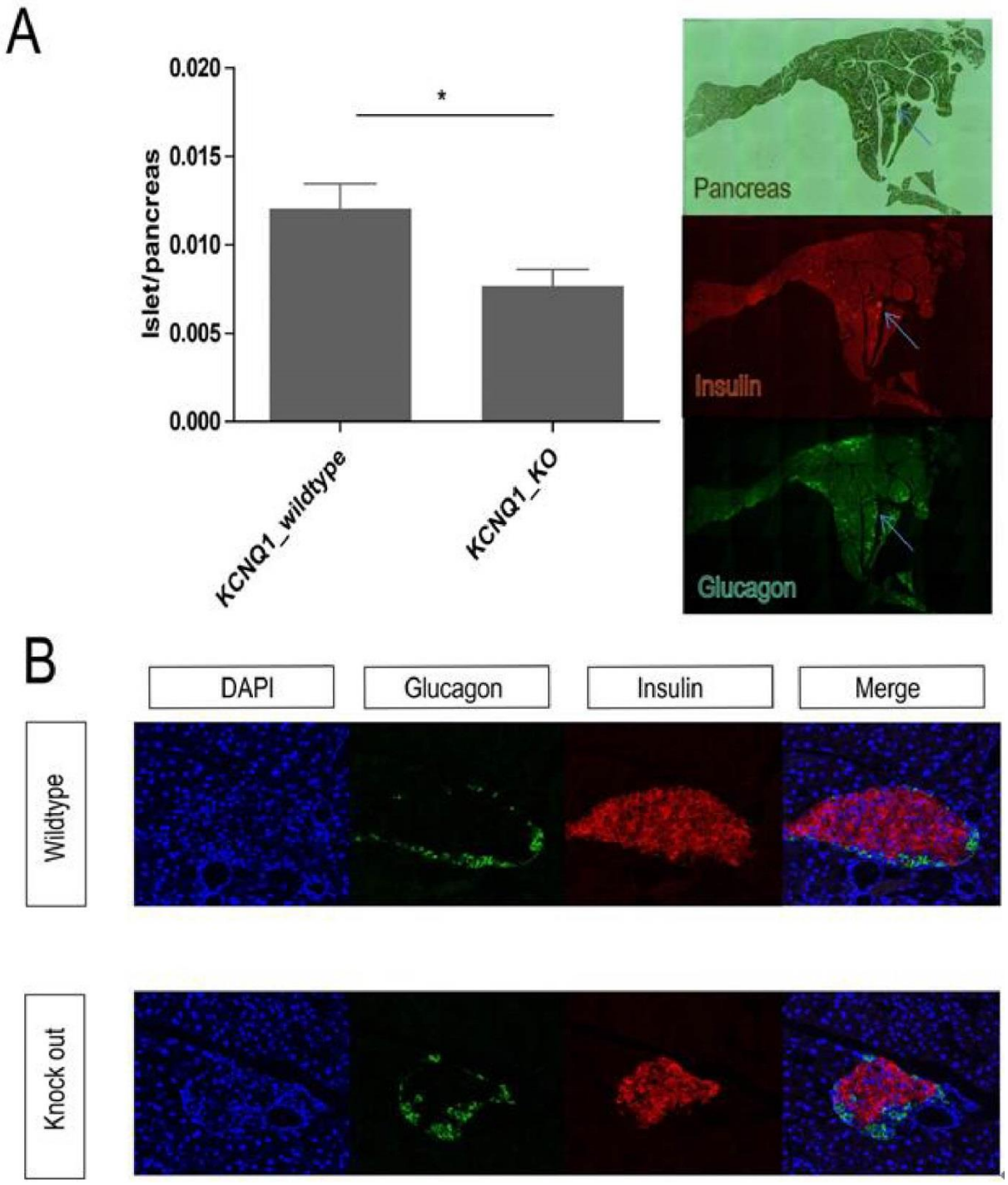

Figure 19: Reduced pancreatic β cell mice in *Kcnq1* KO mice. (A, left) β cell mass for *Kcnq1* KO and WT. *: P<0.05, **(A, right)** indicate a cross section area of pancreas (red point shows islet (blue arrow). The β cell mass was determined by the ratio of the area positive for insulin immunostaining (red) to total pancreatic area. **(B)** Indicate islet of pancreas between wild type and KO in *Kcnq1* mice under fluorescence confocal microscopy.

We found a relevant reduction of β cell mass (insulin-positive cells) in *Kcnq1* KO compared to that in WT mice (P<0.05), with 41% less for the KO mice than that in the wild type mice (Figure 19 A), indicating the important role of *Kcnq1* in β cell or islet development.

3.2.6 The expression of the transcription factors in *Kcnq1* knock out mice pancreas islets

As shown before, the β cell mass of the *Kcnq1*-deficient mice is significantly reduced compared to that of the *Kcnq1* wild type mice. To understand the molecular mechanism involved in this abnormality, we checked the gene expression levels of the β cell specific markers, like *Pdx1, Foxa2, NeuroD1, Ins1,* and *Ins2*, together with the α cell specific markers *Arx, Irx1* and *Mafb* in RNAs extracted form islets of *Kcnq1* KO and WT mice. We found significantly reduced expression of *Kcnq1* isoform1 and isoform 7 in KO compared to WT mice (P<0.05 and P<0.01), respectively (Figure 20). The expression levels of two β cell specific markers: *Pdx1* & *Foxa2*, were both detected to be significantly decreased in the *Kcnq1* KO mice compared to the wild type (P<0.05) (Figure 20). For the *Ins1*, *Ins2* and *Neurod1*, however, their expression levels in the islets were detected to be similar between *Kcnq1* and wild type mice (data not shown). The expression levels of α cell markers: *Arx1* & *Irx1* were also detected to be significantly lower in *Kcnq1* KO mice compared to the wild type, P<0.01 (Figure 20).

In summary, reduced expression levels of both α & β cell markers indicate that normal *Kcnq1* expression has a critical role in both dominant endocrine cell lines producing the vital islet hormones insulin and glucagon.

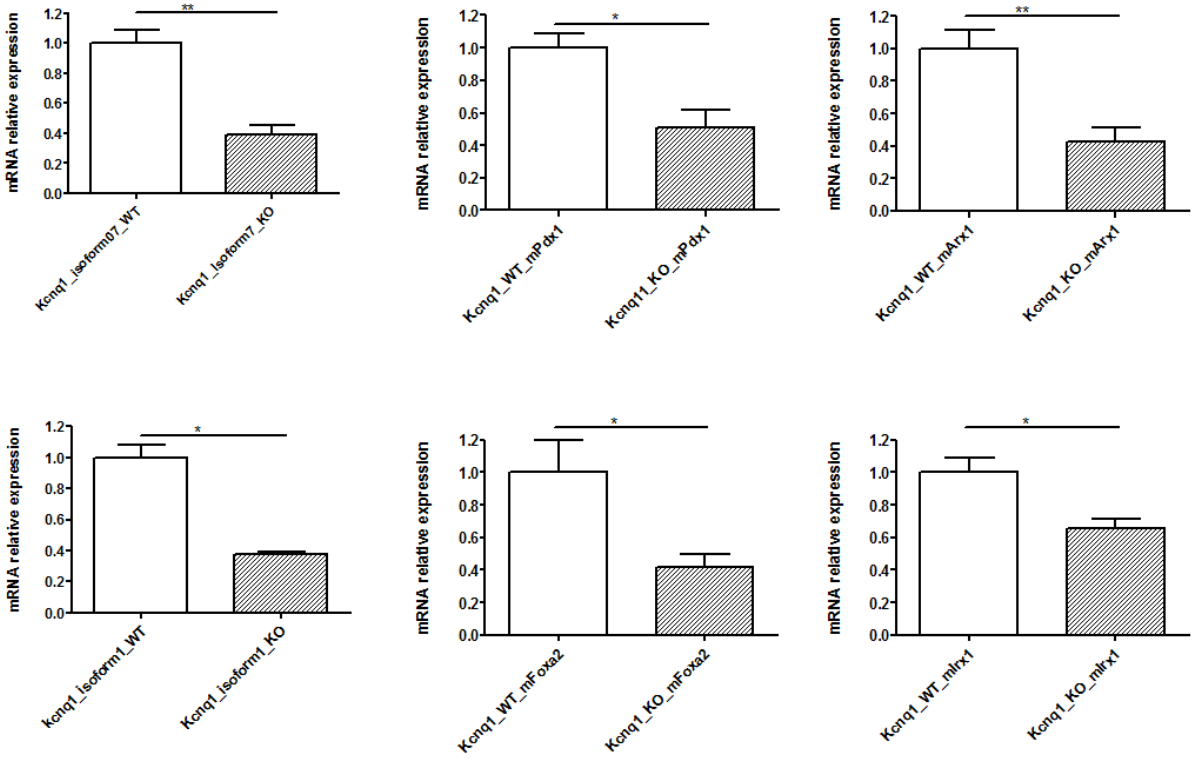

Figure 20: Decreased mRNA expression levels *Kcnq1*-isoform1, *Kcnq1*-isoform7, *Pdx1*, *Foxa2*, *Arx1*, and *Irx1* in *Kcnq1* KO mice islets. *: P<0.05, **: P<0.01.

4. Discussion

Diabetes mellitus (DM) is a public health challenge affecting hundreds of millions of people all over the word. The pathophysiological process of DM is complex. Except for the environmental factors, the genetic mutations resulting in diabetes have attracted the attention of researchers and publishers. It was reported that a single gene mutation resulting in β cell dysfunction accounts for approximately 1-2% of all diabetes cases [109].

In our research, we focus on studying rare disorders with pancreatic β cell dysfunction, especially monogenetic diabetes, congenital hyperinsulinism, and pancreas hypoplasia-caused diabetes. In order to better investigate the pancreatic β cell dysfunction disorders, we established our international cohort with neonatal diabetes, MODY, non-autoimmune T1D, paediatric-onset type-2 diabetes, pancreas hypoplasia, and CHI, separately. We screened this cohort with different studying strategies like exome sequencing, complete genome sequencing and target enrichment /next generation sequencing. The ECRC research group could detect variants with known or assumed pathogenic link to diabetes or hyperinsulinism in nearly half of the patients. Of the detected mutations, we found three *KCNQ1* mutations in one patient

with permanent neonatal diabetes and two CHI patients, separately. We further verified all the variants with conventional Sanger sequencing. We validated the functional abnormality of the three *KCNQ1* mutations with different studying strategies. In addition, we also studied if *KCNQ1* abnormality results in abnormal pancreas development in mice and in the hope of finding an effective therapeutic tool for patients with *KCNQ1* mutations in the future.

4.1 The clinical importance of molecular screening

The exact diagnosis of diabetes mellitus has become difficult due to the overlapping clinical features between the complex diabetes (type 1 & type 2 diabetes) and monogenetic diabetes. Molecular screening, therefore, becomes one very important tool for distinguishing both complex and phenotype and monogenetic diabetes. Molecular screening has been proved to be very necessary for diagnosing patients with pancreatic β cell dysfunction, including MODY, ND, CHI, and pancreas hypoplasia. Molecular screening is also needed for patients diagnosed as T1D or T2D, especially the patients with early onset and/or family diabetic history. Some patients were defined as T1D or T2D previously and were finally diagnosed as monogenic diabetes using molecular screening strategies [47].

MODY is the most common type of monogenic diabetes. In the past, MODY was applied to any child or young adult who had persistent, asymptomatic hyperglycemia without progression to diabetic ketosis or ketoacidosis However, recently, the concept of MODY was redefined at the molecular genetics level by molecular genetic testing [71]. Since patients with MODY are mainly young and non-obese, they are often misdiagnosed as T1D or T2D [71]. In these cases, molecular genetic testing plays a key role for avoiding misdiagnosis and further guide treatment. For early-onset diabetic patients, it is important to apply molecular screening for precise diagnosis and better treatment.

In the present study, we first performed exome sequencing to discover novel candidate genes. We then applied these candidate genes together with the known monogenetic candidate genes, the loci associated with T2D and the crucial genes involved in the pancreas development for targeted enrichment next-generation sequencing. The advantage of this strategy is simultaneous analysis of hundreds of genes of interest within 96 samples. It is much more efficient compared to the conventional sequencing method, which could only manage to analyze one or a few genes at a time. However, the detected genetic mutations were confirmed by

conventional Sanger sequencing. It is a common sense that molecular diagnosis could help to avoid misdiagnosis and finally would improve further therapy and prognosis. For example, neonatal diabetes cases with k_{ATP} channel mutations often respond to sulfonylureas therapy, Which has successfully replaced insulin injection using oral sulfonylureas. Therefore, clinical outcome has been improved by the obvious improvement of glycemic control [110]. With the successful molecular diagnosis, we could elucidate the etiology of the disorders, and explain the other associated clinical features. Molecular diagnosis is not only quite important for optimal treatment but also could improve patients' life quality. Moreover, molecular diagnosis can offer an appropriate genetic counseling to the relative of patients.

4.2 Molecular screening of the cohort with pancreatic β cell dysfunction

4.2.1 Genetic identification in patients with congenital hyperinsulinism

Congenital hyperinsulinism (CHI) is defined as the unregulated secretion of insulin from pancreatic β-cells in relation to the blood glucose concentration, it is the commonest cause of persistent hypoglycemia in neonatal and infancy periods [61]. CHI can be classified as transient, persistent, mild or severe. Severe forms of CHI can cause obvious problems in the first hour of life, such as the patient with ID 62_405, the patient was diagnosed with CHI at the first hour of life. Patients with milder forms of CHI could only be detected in later years or occasionally through physical examination, such as the patient's father with ID 62_406 (Fig 13C). In the neonatal or infancy period, persistent or reduplicated hypoglycemia usually accompanies hypoglycemia brain injury.

It was reported that several genes' mutations responsible for CHI: *ABCC8, KCNJ11, GCK, GLUD1, HADH, HNF4A, HNF1A, SLC16A1, UCP2*, respectively [111]. Mutations in *ABCC8* and *KCNJ11* were the most common cause of CHI [73]. These two genes affect mainly K_{ATP} channel in the cell membrane. The inactivity mutation in *ABCC8* or *KCNJ11* decreases or loses activity of the K_{ATP} channel which leads to an uncontrollable insulin secretion and finally resulting in hypoglycemia. Recessive inactivity mutation in *ABCC8* and *KCNJ11* genes is the most common cause of CHI [62].

In our cohort, a total of 37 patients were diagnosed as CHI, 8 patients (22%) were chosen for molecular screening according to their extreme phenotype and *ABCC8* gene mutations of six patients (75%) were detected in these 8 patients. Recessive

mutations account for the majority parts in *ABCC8* gene in our cohort, this result is consistent with the previous studies [62]. Diazoxide-responsiveness is the most significant difference between recessive and dominant mutations [62]. For example, one patient with *ABCC8* dominant mutation was able to maintain normal glucose level with diazoxide and dietary control, but the three patients harboring *ABCC8* recessive mutations had difficulties to maintain normal glucose level with diazoxide alone. Further, one of the patients with *ABCC8* recessive mutations underwent pancreatectomy, 97% of the total pancreas was excised at 3 months old. The patients with *ABCC8* dominant mutations are normally better treated with diazoxide than the patients with *ABCC8* recessive mutations. Very likely after treatment with the diazoxide, the dominant mutation can display normal channel assembly and trafficking to the plasma membrane, the glucose level can then be controlled at a normal range to avoid pancreatectomy [62]. Meanwhile, we detected *KCNQ1* mutations in two patients with CHI. This is the first study showing *KCNQ1* mutations in CHI. Chapter 4.2.5 will discuss how *KCNQ1* mutations cause CHI

4.2.2 Gene mutations detected in neonatal diabetes of this cohort

Neonatal Diabetes (ND) is a specific form of monogenetic diabetes [34]. It has been defined as diabetes with age of onset less than 6 months and negative autoantibody for T1D [48]. Until now, 22 genetic mutations have been identified as the cause of ND, which includes permanent and transient diabetes. *ABCC8* and *KCNJ11* mutations are found to be the most common causes of ND [112]. Although our ND sample size is small (6 patients), we detected gene mutations in four ND patients. One of the patients (ID: 26_119) with *KCNJ11* mutation was treated with sulfonylurea. Potassium channel gene mutations in ND are sensitive to sulfonylurea treatment. The other two ND cases with Glis3 and *KCNJ11* mutations were both treated with insulin. In one PND case, a *KCNQ1* mutation was detected which will be discussed also in the chapter 4.2.5.

4.2.3 The detected known candidate gene mutations in MODY

MODY is the most common type of monogenetic diabetes and accounts for 2-5% of all patients with diabetes [43]. Eleven gene mutations were recently identified to cause MODY. Of these, *HNF1A, GCK*, and *HNF4A* gene mutations account approximately for 70% of patients with MODYs [113]. It is commonly known that patients with MODY are usually young and non-obese. Therefore, they are frequently misdiagnosed as T1D or T2D, and molecular diagnosis plays an important role to avoid this kind of misdiagnosis.

In our cohort, we identified gene mutations in 10 MODY patients: 6 patients have *GCK* mutations, 2 have *HNF1A* mutations, and 2 with *HNF4A* mutations. These 3 mutated genes (*GCK, HNF1A* and *HNF4A*) play a major role in the cause of MODY, which is consistent with the previous studies [42].

4.2.4 Mutated genes causing pancreatic hypoplasia

Pancreatic hypoplasia (PH) was defined as poorly developed pancreatic size but still showing ventral and dorsal pancreas. Pancreatic agenesis was a completely missing pancreas or at least parts arising from dorsal (corpus and tail) or ventral (head) pancreatic bud. Only a few genes have been related with pancreatic hypoplasia in humans, with mutation *GATA6, PDX1* and *FTF1A* [114]. The majority of PH patients display symptoms like abdominal pain or diabetes. PH cause rarely diabetes. In our cohort, 7 diabetic patients (7/96) were diagnosed with PH by examining the pancreas image. Seven of the eight patients showed gene mutations. We found 2 diabetic patients with *GATA6* heterozygous mutations and 4 diabetic patients with homozygous *PTF1A* enhancer mutation. *GATA6* gene mutations cause not only PH but also other congenital abnormality like congenital cardiac defects, congenital biliary tract anomalies, neurocognitive abnormalities and so on [114]. In our cohort, 2 patients with *GATA6* gene mutations showed congenital abnormality of both pancreas and heart. This is in agreement with previous studies [114]. The first patient had tetralogy of Fallot and suffered from abdominal pain and the second patient had intrauterine growth retardation. Earlier research showed that *GATA6* is expressed in embryonic pancreatic multipotent progenitors and plays a critical role in mouse pancreatic development [114]. Functional research indicates that heterozygous *GATA6* gene mutations result in a loss of function and cause pancreatic hypoplasia [114]. Similarly, *PTF1a* gene is also an important factor in sustaining pancreatic development. *PTF1a* gene mutations are the most common cause of pancreatic hypoplasia [104]. Pancreatic hypoplasia with *PTF1a* mutation usually accompanies extra-pancreatic abnormalities. It was reported that patients with pancreatic hypoplasia have several neurological features by recessive PTF1a gene mutation [115].

In this study, we found 4 PH patients with *PTF1a* enhancer mutations and all of them were from different consanguineous families. All 4 patients had intrauterine growth retardation and other cognitive disorders like exocrine insufficiency after birth.

4.3 *KCNQ1* mutations cause abnormal pancreatic islet development and β cell dysfunction

4.3.1 *KCNQ1* is associated with T2D and causes congenital hyperinsulinemia in LQTs patients

KCNQ1 is a potassium channel protein and is encoded by *KCNQ1* gene. Further *KCNQ1* is widely expressed in cardiomyocytes, stomach, kidney, inner ear, intestine and pancreas [86]. It must be noted that *KCNQ1* was reported to be expressed mainly in cardiomyocytes and pancreas. *KCNQ1* gene mutations are associated with LQTS, short QT syndrome [116], Ventricular tachycardia [117] and familial atrial fibrillation [118] in the cardiomyocytes. In pancreas, *KCNQ1* gene mutations are found to be associated with T2D [75], GDM and hyperinsulinemia [119].

Recently, patients with *KCNQ1* LQTS were found to have hyperinsulinemic hypoglycemia[119]. Hypoglycemia can cause sympathetic activation and this is one of the causes of arrhythmias and sudden death [119]. Some of the patients with hypoglycemia may have hypokalemia. This is presumably due to insulin trigger Na-K pump, move K^+ from extracellular to intracellular [120]. Hypokalemia can also cause arrhythmias and sudden death. Hypoglycemia can result in brain injury and cause epilepsy or cerebral palsy in infants and children.

T2D is a kind of polygene disease and has a complicated pathogeny. It was closely related with environment and inheritance. Until now, about 70 candidate genes have been reported to be associated with T2D, including *FCF7L2, ADRA2A, KCNJ11, RREB1, HHEX, CDKAL1, KCNQ1*, and many others. SNP (single nucleotide polymorphism) refer to single nucleotide variants caused DNA sequence variants at genome level. Yasuda et al. found that *KCNQ1* gene variant (rs2237895) is significantly associated with T2D in a multistage genome-wide association study in 100000 SNPs [75]. Yasuda et al. further analyzed the other SNPs of *KCNQ1* from NCBI, rs2237892 was detected to show the more significant association with T2D compared to rs2237895 [75]. At the same time, Hiroyuki et al. also found that SNPs of *KCNQ1*, rs2283228, rs2237895 and rs2237897, were strongly associated with T2D in cohorts from Japan, Singapore and Denmark [121].

The research of SNPs in *KCNQ1* gene is closely associated with T2D, and the risk alleles of *KCNQ1* was found to cause impaired insulin secretion [79]. However, the process of diabetes with *KCNQ1* gene mutation is not fully understood. Yamagata et al. found that the density of current of *KCNQ1* and density of total K^+ current were

significantly increased when *KCNQ1* was transfected into the MIN6 mouse β cells [79]. In addition, insulin secretion was significantly impaired in overexpress in *Kcnq1* in MIN6 β cells [79], whereas inhibition of *Kcnq*1 in β cells increases insulin secretion [119].

So far, there is no report that *KCNQ1* mutations directly cause pancreatic β cells disorders. We are the first group to have detected *KCNQ1* mutations in both disorders of CHI and diabetes.

4.3.2 *KCNQ1* mutations cause permanent neonatal diabetes and congenital hyperinsulinism

In this study, we detected two heterozygous *KCNQ1* mutations in two CHI patients separately, and one *KCNQ1* homozygous mutation in a PND patient (Table.10) through our molecular screening strategies (Chapter 2.2.2). All three mutations are really rare or novel and predicted to be very deleterious with different functional prediction programs (Polyphen, SIFT, Mutationtaster, CADD etc.).

The first CHI patient (48_241) had only a mild phenotype of CHI and a missense *KCNQ1* mutation: G292D. This is a de novo mutation in this family. His other family members don't harbor this mutation. The second patient had a stronger CHI phenotype together with LQT syndrome. He was detected a heterozygous splicing accepter lost mutation, which results in the deletion of exon 13. His father (CHI & LQTS) and one brother (LQTS) were both detected harboring the same mutation. The third patient had PND and harbored one homozygous missense mutation: R397W, the parents of the patient harbor the heterozygous variant and both were healthy.

All the three detected mutations locate at two different domains in *KCNQ1*. The first CHI mutation locates at the helix-S5-S6 (G292D, Figure 9, Figure 21) of the channel, which is important for normal *KCNQ1* channel deactivation [107]. Mutation studies of the same domain showed altering channel gating associated with inactivation and deactivation [107]. The second CHI and diabetic mutations locate at intracellular C-terminal (CT) domain (E13 deletion & R397W, figure 21), which is responsible for channel traumatization, proper channel trafficking and biophysical properties, it plays a critical role in gate regulation as well [108]. In addition, the C terminus provides an interface for other interacting proteins [122]. In the proximal CT domain, the diabetic mutation locate at the helix A, and the e13 deletion between the helix B and helix C, both interact with calmodulin (CaM) C lobe and N lobe respectively. The two mutations may perturb the interactions with CaM, change the protein expression and current density [108].

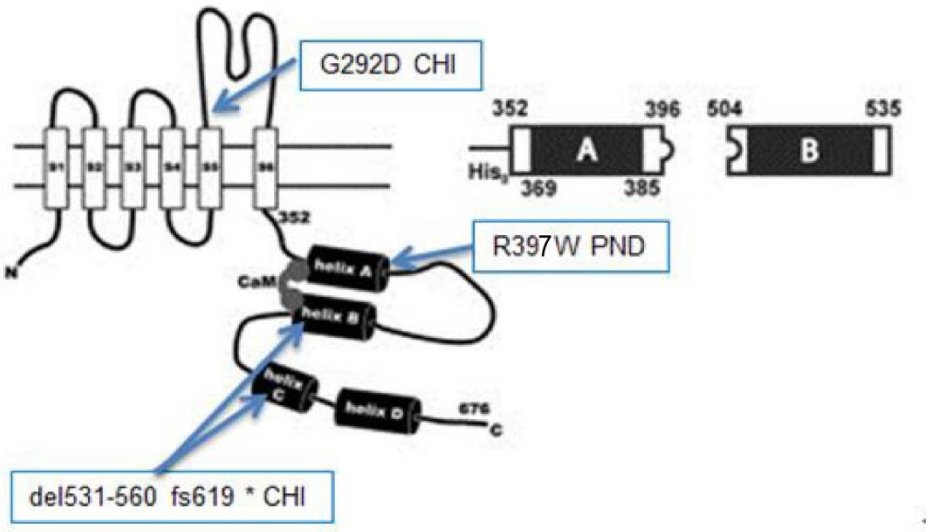

Figure 21: A schematic representation of the *Kv7.1* α subunit, with the mutation location. The proteins in helices A and B bound to CaM. Adapted from [108].

Through functional analysis in mouse pancreatic β cells and *Kcnq1* knock out mice. We, therefore, have further investigated how the *KCNQ1* mutations leading to diabetes and congenital hyperinsulinism.

4.3.3 *KCNQ1* mutations cause abnormal insulin secretion in pancreatic β cells

As shown in the chapter 1.4, in pancreatic β cells, the insulin secretion was controlled by a series of regulation in nucleus by increase of ADP/ATP, closures of K^+ channel, membrane depolarization and Ca^{2+} influx [70]. In this study, we identified mutations in the other potassium channel protein (*KCNQ1*): a voltage-gated potassium channel (Kv7.1), this channel, which is present in the cell membranes of cardiac tissue and pancreas, contributes to the repolarization of the cell.

In order to verify if the *KCNQ1* variants cause insulin defect resulting in ND and CHI, we transfected the constructs with the three detected *KCNQ1* variants and wild type separately together one empty vector into mouse pancreatic β cells. After 72 hours transfection, we measured the insulin secretion of the β cells by treating the cells with 3.3 mM of glucose, 16.7 mM of glucose and 30 mM of KCL separately, which represents three conditions: the basic insulin secretion, glucose activated insulin secretion, and induced depolarization insulin secretion. In this study, we found that transfected β cells the insulin secretion was significantly reduced in all three conditions in the diabetic mutation (3.3mM glucose, 16.7mM glucose and 30mM KCL) compared to that in the *Kcnq1* wild type transfected cells and untransfected cells. Whereas, in the two CHI mutations transfected β cells, we detected significantly increased insulin secretion than that in the wild type and untransfected cells in all three conditions. These

results were in accordance with the patients' phenotypes caused by different *KCNQ1* mutations. However, insulin production is all significantly reduced in all three mutation transfected β cells compared to those transfected with wild type and untransfected cells, indicating the mutations cause the insulin secretion defect. However, the reduced insulin production in all the variants, might be affected by the protein-protein interaction, and in the end, it affected the insulin transcriptional levels. This hypothesis was confirmed by the qPCR expression experiments showing that both the RNA expression levels of *Ins1* and *Ins2* were significantly decreased in all the three *Kcnq1* mutation transfected β cells compared to those in the *Kcnq1* wild type transfected and non-transfected β cells.

It was reported that insulin secretion was obviously decreased by induced glucose, pyruvate, or tolbutamide in *Kcnq1*-MIN6 cells [79]. The overexpression of *Kcnq1* did not only reduce insulin secretion at low glucose concentration, but also reduced insulin secretion at high glucose concentration [79], and the T2D disease alleles of *KCNQ1* gene are associated with impaired insulin secretion [75]. The impaired insulin secretion is multifactorial and may be involved in many factors like β cell mass reduction, impaired β cell glucose sensing, impaired transcription factors for maintain β cell maturation and so on. It was also reported that *KCNQ1* mutations affect insulin secretion in T2D by the reduction of depolarization-evoked and the reduction of granule docking [78]. Whereas premature repolarization of action potential could impair insulin secretion during increased *KCNQ1* function, the inhibition of *KCNQ1* channel by chromanol 293B shows that insulin was obviously increased. The loss of function in *KCNQ1* also results in a rise in insulin secretion. In our research, *KCNQ1* diabetic mutation model results in insulin secretion reduction, and CHI *KCNQ1* mutations result in an insulin secretion increase. These results were consistent with other studies about insulin secretion. It was confirmed that blocking *KCNQ1* increases insulin secretion in vivo, perhaps the period of depolarization of β cell was extended further caused Ca^{2+} influx and promote insulin exocytosis [119]. In this way, blocking *KCNQ1* can be applied for the treatment of diabetes patient(s) with *KCNQ1* mutation(s). It was reported that T2D patients with *KCNQ1* mutations were treated with repaglinide [123]. However, the pathogenesis of ND is not completely understood, therefore, we treated our ND patient with *KCNQ1* mutation with insulin substitution.

4.3.4 Different transcription factors are involved in the pathogenesis of CHI and diabetes separately

4.3.4.1. CHI mutations up-regulate *Pdx1* and down-regulate K_{ATP} channel-proteins in the pancreatic β cells

As mentioned before, pancreas development is regulated by many critical transcription factors, some of the transcription factors keep on playing their roles in the mature pancreatic β cells for differentiation, and maintenance. Therefore, we examined the protein level of some transcription factors such as *Pdx1*, *Neurod1*, *Foxa2*, *Pax6*, and *Kcnq1* in beta cells transfected with WT *Kcnq1* and our human *KCNQ1* mutations. All these factors are important in pancreas development and β cell differentiation.

Among these factors, *Pdx1* plays a crucial role in the early stage of pancreas development, β cell differentiation and in maintaining mature β cell function [22]. In our research, the *Pdx1* protein expression level was reduced in diabetic cell model of *Kcnq1* mutation (24h and 48h after transfection) even though it was not significant. In contrast, *Pdx1* expression level was increased significantly in CHI models of *Kcnq1* mutations (24h and 48h after transfection). In CHI cell model of *Kcnq1* mutations, the higher *Pdx1* expression level increases β cell differentiation and β cell function, causing more insulin secretion and further increased hyperinsulinemia. However, in the diabetic cell model, the expression of *Pdx1* did not show a significant decline. We hypothesized that other transcription factors might play a role in the insulin secretion defect caused by the diabetes mutation. The pathogenesis of CHI and diabetes from *KCNQ1* mutations might be different from each other.

PDX1 is mainly expressed in the gut region at an early stage of embryonic development [16]. *PDX1* expression maintains cell differentiation during pancreas development, however it restricts mature β cell differentiation by inducing expression of insulin and glucose transporter 2 [21]. The loss of *PDX1* function can cause pancreatic hypoplasia in humans and mice [22]. It was reported that *Pdx1* heterozygous mice have glucose intolerance, and there was an increase of β cells apoptosis and decreased β cell mass. Therefore, *Pdx1* plays an important role in maintaining the glucose level in normal range [124]. *PDX1* heterozygous mutations in humans were also detected in patients with MODY4. *PDX1* has been a major switch in order to control the activation of other downstream of transcription factors in β cells [125]. The extent of *PDX1* up-regulation by the mutation G292D is slightly weaker than that by the E13 del mutation, which consists of the relatively milder phenotype caused by the

G292D than that by the E13 del mutation, it moreover emphasizes the important role of *PDX1* in controlling insulin secretion in β cells.

Previous studies showed that loss-of-function of K_{ATP} channel mutations cause hyperinsulinism and gain-of-function of K_{ATP} channel mutations cause neonatal diabetes [72]. Indeed, we detected reduced expression of the two genes encoding the K_{ATP} channel (*ABCC8* & *KCNJ11*) selectively in *KCNQ1*-hyperinsulinism mutations. For the diabetes mutation, the expression of the K_{ATP} channel per se is not affected. We therefore conclude that the *KCNQ1* CHI mutations suppress the K_{ATP} channel opening and, therefore, decrease membrane potential, which in turn increases the rate of Ca^{2+}-dependent insulin secretion.

4.3.4.2. The *Kcnq1* diabetes mutation down regulates the expression level of *Foxa2* in mouse β cell

Due to the reduction of β cell mass in patients with diabetes mellitus and in *Kcnq1* KO mice, we hypothesized that transcription factors maintaining β cell function and identity might be regulated by the *Kcnq1* mutation R397W. Forkhead box a2 (*Foxa2*) is a member of the mammalian forkhead transcription factor gene family and plays significant roles in pancreas development and in keeping pancreatic β cells differentiated [126]. *Foxa2* controls vesicle docking and insulin secretion in mature β cells [126]. In early stage of pancreatic development, the alternative inactive *Foxa2* gene causes failure of differentiation of α cells [127], nevertheless, ablation of *Foxa2* in β cells affects insulin secretion [126]. Recently, it was reported that *Foxa2* combines with *Neurod1* and *Insm1* to maintain mature pancreatic β cell function [102].

In our research, we examined the expression levels of *Foxa2* (24h and 48h after transfection) in all three *Kcnq1* mutations and wild type transfected β cells, and found that the expression of *Foxa2* was downregulated by diabetic mutation in β cells after 24 and 48 hours transfection, respectively. However, the expression level is similar to that of *Kcnq1* wild type and CHI variants. The expression level of *Foxa2* was not affected by the CHI mutations. Even though, *Foxa2* controls *Pdx1* gene expression in pancreatic transfected β cells [128], the expression level of *Pdx1* in the diabetic cell models was somewhat reduced but no significantly different compared to that of the wild type cells. The insulin secretion defect caused by the diabetic mutation is not likely due to the down-regulation of *Pdx1*. As *Foxa2* regulates multiple pathways of insulin secretion, β cells lacking *Foxa2* are essentially deficient for both subunits KIr6.2 and SUR1 of the K_{ATP} channel [129]. Some other transfection factors might also be regulated, thus the

defective maintenance of pancreatic β cells by the reduction of *Foxa2* might be the pathogenesis of the diabetes mellitus. As shown before, FoxA2 is an upstream transcription factor, and plays a role at an early stage of the pancreas development, the expression level of *Foxa2* in our study was also down-regulated at an the early stage (24hrs transfection), after 48hrs transfection, its expression level was similar to that in the wild type and CHI cell models.

Therefore, the molecular mechanism of the CHI and diabetic mutations are from different regulation pathways.

4.3.4.3. The conversion from pancreatic β cell to α cells by diabetes mutation

Both α-cells and β-cells are pancreatic endocrine cells residing in the islets of Langerhans and sensitive to blood glucose changes. They share a long developmental pathway before the two cell types finally diverge, which is mainly controlled by differential expression of transcription factors [130]. In addition, it has been commonly observed that in diabetic conditions, there is not only β-cell/insulin deficit but also α-cell/glucagon excess [131]. In our diabetic case, we hypothesized that the *Kcnq1* mutation R397W might cause this conversion as well. Therefore, we examined the expression level of *Pax6* in all the *Kcnq1* mutations and wild type transfected cells. *Pax6* is an important transcription factor in endocrine cell development and is subsequently required for differentiation of α cells [132]. Previous studies in mice showed that homozygous *Pax6* deficiency results in a lack of α cells [133], suggesting that *Pax6* is required primarily for determining α cell fate.

In our research, the expression of *Pax6* shows a significant increase in diabetic *Kcnq1* mutation cellular model, and a significant decrease in the CHI *Kcnq1* e13del cellular model after 48hr transfection compared to that in the KCNQ1 wild type cellular models, whereas the expression of *Pax6* in the CHI G292D cellular model is only slightly decreased compared to that in the wild type cellular model, which is also consistent with the milder phenotype. The expression levels of *Pax6* showed no difference in any of the three mutations and wild type cellular models after 24hrs transfection. *Pax6* is a transcription factor for determining α cell fate, therefore, higher regulation of *Pax6* in the diabetic cellular model indicates the conversion from β cells to α cells, which finally results in the disruption of β cells maintenance and weakens the β cells function.

This phenomenon is further confirmed by the RNA expression experiments. We performed real-time PCR (qPCR) with RNA_S isolated from the mouse β cells

transfected with the two CHI and one diabetic mutations (G292D, E13 Del & R397W) and wild type constructs, together with the un-transfected cells. We detected a significant up-regulation of *MafB* in both the *Kcnq1* wild type and diabetic cellular models compared to that in the non-transfected cells and CHI mutation cellular models, which indicates that over expression of *Kcnq1* enhances the conversion from β cells to α cells. Even the down-regulation of *MafB* in the CHI mutation cellular model is not significantly different compared to that in the untransfected cells, but is significantly decreased compared to that in the *Kcnq1* wild type cellular model. As mentioned before, *MafB* is only expressed in pancreatic α cells. Combining the *Pax6* protein expression results (Western blot) with *MafB* RNA expression levels, the diabetic *Kcnq1* mutation might cause the dedifferentiation of pancreatic β cells and convert into the glucagon producing α cells.

The expression pattern of *Mafb* is very similar to that of *Kcnq1*.The expression level of *Kcnq1* itself in the diabetic cellular model is much higher than that in the non-transfected cells or CHI mutation cell models. These results are consistent with previous studies, which showed that loss-of-function or gain-of-function of this channel protein would cause either higher or lower insulin secretion [78, 79, 119]. We hypothesize that the gain-of-function *Kcnq1* mutation(s) contribute to the cell conversion from β to α, leading to a corresponding insulin deficiency in the patients.

4.3.5 Insulin secretion and Islet β and α-cell mass in *Kcnq1* KO mice

Except for the pancreatic β cell models, we would like to know how *KCNQ1* plays a role in pancreas development. In collaboration with the Voekl group in Tubingen, we obtained *Kcnq1* knock out (KO) mice and age- & gender-matched wild type mice for investigation. It was reported that insulin sensitivity was increased in this kind of *Kcnq1* KO mice [134], and that the insulin secretion from the islets isolated from these *Kcnq1* KO mice shows no difference compared to wild type mice [80]. We measured the insulin secretion from the islets isolated from the mice as the mentioned study, and obtained similar results as those reported in the literature, which indicates that the reduced *Kcnq1* channel activity might be compensated by other ion channel activity of the islets, like $K_{V2.1}$ channel or K_{ATP} channel.

It has been proven that insulin is an important hormone to maintain homeostasis of glucose and more insulin is only produced by pancreatic β cells. When loss of β cells causes T1D, β cell failure to provide enough insulin will result in T2D. Therefore, β cell function and β cell mass play key roles in adjusting the glucose level to the normal

range in the body. Experiments with *Kcnq1* variants mice models showed that *Kcnq1* variants have a close implication with diabetes mellitus [80].

In our research, in order to know if the β cell mass is affected by the *Kcnq1* knock out, we examined the β cells mass from the isolated pancreas in *Kcnq1* KO and control mice. β cell mass is significantly reduced in *Kcnq1* KO mice compared to that in the wild type mice, this result is consistent with recent findings [80]. In an ordinary adult pancreas, β cell mass accounts for approximately 20% of pancreas weight, pancreas weigh ranges from 60g to 100g, therefore, the weight of β cell mass is approx.1-2g [135]. About 1000 β cells together with others cells form an islet, and about 1 million islets exist in the pancreas [135]. On one hand β cell mass were measured accurately that could increase our understanding of pathogenesis of T1D, T2D and monogenic diabetes in living individuals. On the other hand, β cell mass were measured that can assess the effect of medication and glycemic control, further guide treatments for next step. However, these islets are scattered throughout the whole pancreas. It is impossible to directly measure the β cell mass in a living human individual.

The loss of β cell mass is caused by the loss of function of pancreas. This is confirmed by histological evidence that shows that islet number and insulin-positive area are reduced in autopsy samples from patients with T1D [136]. T1D shows that loss of β cell mass happens at an early stage of the disease or before its development [137]. Sherr et al. showed that there is a significant reduction of β cell mass in patients with T1D at an early stage compared to those with late stage [138]. Further, β cells destruction is an inevitable process of the development of T1D, but more or less β cells can survive in patients with T1D. Compared to the limited amount of β cell functional research in human, the animal model has been widely used for research in β cells function. In order to confirm β cell mass and function are gradually and progressively reduced in patients with T1D over time. Sreenan et al. [139] measured β cell mass and insulin secretion in mice 8, 9, 13, 18 weeks of age and found that β cell mass was significantly reduced in 18 week old diabetic mice. Accordingly, insulin secretion reduction accompanies reduction of β cell mass.

Due to the patients with T2D have insulin resistance or relative insulin secretion insufficiency, thus, theoretically, β cell mass in patients with T2D should increase for response the requirement of insulin in body or at least should not decrease. However, in fact, β cell mass in patients with T2D are decreased [140]. The balance of β cell mass was adjusted by β cells loss and newly formed β cells. The balance was

destroyed by reduction of newly formed β cells or increase of β cells loss that can results in DM. It was reported that increased β cell apoptosis leads to reduced β cell mass in patients with T2D, β cell replication and neogenesis did not take part in the reduction of β cell mass [141].

In recent years, It was found that transdifferentiation of β cell to α cell was a reason which deal to β cell loss in diabetic rodent models [142]. The loss of β cells leads to reduction of β cell mass, which causes reduction of insulin secretion and rise of glucose, and further results in DM. It has been reported that β cell mass was reduced by 20-40% in patients with impaired glucose tolerance and impaired fasting glycaemia [143].

The decreased expression of *Kcnq1* (37% of the wild type) and the two key transcription factors: *Pdx1* & *Foxa2* in *Kcnq1* KO mice indicates a total islet development defect including both β cell mass and α cell mass. *Pdx1* does not only affect the development of the whole pancreas but also affects the differentiation of β cells [20]. Disruption of *Foxa2* prevents full differentiation of fetal α- and β-cells [144]. This hypothesis is further confirmed by RNA expression experiments with the α cell markers: *Arx* & *Irx1*. The expression levels of both α cells markers in the islet isolated from the *Kcnq1* knock out mice are significantly decreased compared to those from the wild type mice. *Arx* is one of the known transcriptional factors to marker α cell and is required for α cell development [11] and *Irx1* also plays a role in the regulation of alpha-cell-specific gene expression [145].

In our cell model study, we found that the *Kcnq1* expression in the CHI mutations cellular models is much lower than those in the *Kcnq1* wild type and diabetic mutation cellular models. From that experiment, the beta cell maintenance and function seems to be enhanced by the CHI mutations through up regulation of *Pdx1*, the insulin secretion is activated over by down-regulation of the K_{ATP} channel. The decreased insulin production is very likely due to the regulation of the transcription factors. For the diabetic mutation cellular model, it is *Foxa2* which down-regulated the expression of *Pdx1*. However, is not affected by the diabetic mutation. Additionally, the diabetic mutation induces the β cells' conversion to α cells, which finally decrease both the insulin secretion out of the β cells and production inside the β cells.

Even though the *Kcnq1* expression is also reduced in the CHI cell models, the impact of insulin regulation is obviously different from that of the *Kcnq1* KO mice. In *Kcnq1* KO mice, the differentiation of both β cells and α cells was disrupted. The insulin secretion

assay only measured the insulin production and secretion from some definite amount of islet cells, which cannot represent the failure of the development.

In conclusion, we report for the first time single-gene mutations in *KCNQ1*, leading to pathological insulin secretion in humans and as a consequence to neonatal diabetes or hyperinsulinemic hyperinsulinism. The in-vitro functional analysis verified that the insulin secretion pathways are different from the mutations locating in different protein domains, and studying islet-specific RNA-expression and simply size of insulin-positive cells demonstrated that *Kcnq1* primarily regulates beta cell mass rather than insulin secretion. Thus, genetic and experimental evidence further underlines the critical role of the voltage-gated potassium channel, Kv7.1, in insulin secretion. Why our diabetes mutation has such a pancreas-specific phenotype is an interesting question that might point to novel drugs interacting with *KCNQ1* but in a pancreas-selective fashion, diminishing cardiac side effects. Screening band developing such compounds may finally lead to a different approach for the treatment of common type 2 diabetes.

5. References

1. Desgraz R, Bonal C,Herrera PL, beta-cell regeneration: the pancreatic intrinsic faculty. Trends Endocrinol Metab, 2011.22:34-43.
2. Prado CL, Pugh-Bernard AE, Elghazi L, Sosa-Pineda B,Sussel L, Ghrelin cells replace insulin-producing beta cells in two mouse models of pancreas development. Proc Natl Acad Sci U S A, 2004.101:2924-9.
3. Lian G, Yue X, Xianxiang Z, Yong L, Weijuan L,Bing C, Insulinization: A promising strategy for the treatment of type 2 diabetes mellitus. Exp Ther Med, 2013.6:1300-1306.
4. Granata R, Baragli A, Settanni F, Scarlatti F,Ghigo E, Unraveling the role of the ghrelin gene peptides in the endocrine pancreas. J Mol Endocrinol, 2010.45:107-18.
5. Ye L, Robertson MA, Hesselson D, Stainier DY,Anderson RM, Glucagon is essential for alpha cell transdifferentiation and beta cell neogenesis. Development, 2015.142:1407-17.
6. Moon JS,Won KC, Pancreatic alpha-Cell Dysfunction in Type 2 Diabetes: Old Kids on the Block. Diabetes Metab J, 2015.39:1-9.
7. Bernard-Kargar C,Ktorza A, Endocrine pancreas plasticity under physiological and pathological conditions. Diabetes, 2001.50 Suppl 1:S30-5.

8. Teta M, Long SY, Wartschow LM, Rankin MM,Kushner JA, Very slow turnover of beta-cells in aged adult mice. Diabetes, 2005.54:2557-67.
9. Georgia S,Bhushan A, p27 Regulates the transition of beta-cells from quiescence to proliferation. Diabetes, 2006.55:2950-6.
10. Maulis M,Gianani R, Beta-cell regeneration in human pancreas: the lessons of pancreatic pathology. Adv Exp Med Biol, 2012.771:310-8.
11. Bramswig NC,Kaestner KH, Transcriptional regulation of alpha-cell differentiation. Diabetes Obes Metab, 2011.13 Suppl 1:13-20.
12. Murtaugh LC, Pancreas and beta-cell development: from the actual to the possible. Development, 2007.134:427-38.
13. Bouwens L,Rooman I, Regulation of pancreatic beta-cell mass. Physiol Rev, 2005.85:1255-70.
14. Kim SK,MacDonald RJ, Signaling and transcriptional control of pancreatic organogenesis. Curr Opin Genet Dev, 2002.12:540-7.
15. Scharfmann R, Control of early development of the pancreas in rodents and humans: implications of signals from the mesenchyme. Diabetologia, 2000.43:1083-92.
16. Ackermann AM,Gannon M, Molecular regulation of pancreatic beta-cell mass development, maintenance, and expansion. J Mol Endocrinol, 2007.38:193-206.
17. Jennings RE, Berry AA, Strutt JP, Gerrard DT,Hanley NA, Human pancreas development. Development, 2015.142:3126-37.
18. Jensen J, Gene regulatory factors in pancreatic development. Dev Dyn, 2004.229:176-200.
19. Miller CP, McGehee RE, Jr.,Habener JF, IDX-1: a new homeodomain transcription factor expressed in rat pancreatic islets and duodenum that transactivates the somatostatin gene. EMBO J, 1994.13:1145-56.
20. Melloul D, Transcription factors in islet development and physiology: role of PDX-1 in beta-cell function. Ann N Y Acad Sci, 2004.1014:28-37.
21. Holland AM, Gonez LJ, Naselli G, Macdonald RJ,Harrison LC, Conditional expression demonstrates the role of the homeodomain transcription factor Pdx1 in maintenance and regeneration of beta-cells in the adult pancreas. Diabetes, 2005.54:2586-95.
22. Kaneto H,Matsuoka TA, Role of pancreatic transcription factors in maintenance of mature beta-cell function. Int J Mol Sci, 2015.16:6281-97.

23. Kristinsson SY, Thorolfsdottir ET, Talseth B, Steingrimsson E, Thorsson AV, Helgason T, Hreidarsson AB,Arngrimsson R, MODY in Iceland is associated with mutations in HNF-1alpha and a novel mutation in NeuroD1. Diabetologia, 2001.44:2098-103.
24. Wang H, Gauthier BR, Hagenfeldt-Johansson KA, Iezzi M,Wollheim CB, Foxa2 (HNF3beta) controls multiple genes implicated in metabolism-secretion coupling of glucose-induced insulin release. J Biol Chem, 2002.277:17564-70.
25. Tabassum R, Chavali S, Dwivedi OP, Tandon N,Bharadwaj D, Genetic variants of FOXA2: risk of type 2 diabetes and effect on metabolic traits in North Indians. J Hum Genet, 2008.53:957-65.
26. Zhang C, Moriguchi T, Kajihara M, Esaki R, Harada A, Shimohata H, Oishi H, Hamada M, Morito N, Hasegawa K, Kudo T, Engel JD, Yamamoto M,Takahashi S, MafA is a key regulator of glucose-stimulated insulin secretion. Mol Cell Biol, 2005.25:4969-76.
27. Wang H, Brun T, Kataoka K, Sharma AJ,Wollheim CB, MAFA controls genes implicated in insulin biosynthesis and secretion. Diabetologia, 2007.50:348-58.
28. Heller RS, Jenny M, Collombat P, Mansouri A, Tomasetto C, Madsen OD, Mellitzer G, Gradwohl G,Serup P, Genetic determinants of pancreatic epsilon-cell development. Dev Biol, 2005.286:217-24.
29. Gosmain Y, Marthinet E, Cheyssac C, Guerardel A, Mamin A, Katz LS, Bouzakri K,Philippe J, Pax6 controls the expression of critical genes involved in pancreatic {alpha} cell differentiation and function. J Biol Chem, 2010.285:33381-93.
30. Artner I, Hang Y, Mazur M, Yamamoto T, Guo M, Lindner J, Magnuson MA,Stein R, MafA and MafB regulate genes critical to beta-cells in a unique temporal manner. Diabetes, 2010.59:2530-9.
31. Artner I, Le Lay J, Hang Y, Elghazi L, Schisler JC, Henderson E, Sosa-Pineda B,Stein R, MafB: an activator of the glucagon gene expressed in developing islet alpha- and beta-cells. Diabetes, 2006.55:297-304.
32. Whiting DR, Guariguata L, Weil C,Shaw J, IDF diabetes atlas: global estimates of the prevalence of diabetes for 2011 and 2030. Diabetes Res Clin Pract, 2011.94:311-21.
33. Forouhi NG,Wareham NJ, Epidemiology of diabetes. Medicine (Abingdon), 2014.42:698-702.

34. American Diabetes A, Diagnosis and classification of diabetes mellitus. Diabetes Care, 2014.37 Suppl 1:S81-90.
35. Cooke DW,Plotnick L, Type 1 diabetes mellitus in pediatrics. Pediatr Rev, 2008.29:374-84; quiz 385.
36. Kitabchi AE, Umpierrez GE, Miles JM,Fisher JN, Hyperglycemic crises in adult patients with diabetes. Diabetes Care, 2009.32:1335-43.
37. Emerging Risk Factors C, Seshasai SR, Kaptoge S, Thompson A, Di Angelantonio E, Gao P, Sarwar N, Whincup PH, Mukamal KJ, Gillum RF, Holme I, Njolstad I, Fletcher A, Nilsson P, Lewington S, Collins R, Gudnason V, Thompson SG, Sattar N, Selvin E, Hu FB,Danesh J, Diabetes mellitus, fasting glucose, and risk of cause-specific death. N Engl J Med, 2011.364:829-41.
38. Rother KI, Diabetes treatment--bridging the divide. N Engl J Med, 2007.356:1499-501.
39. American Diabetes A, (2) Classification and diagnosis of diabetes. Diabetes Care, 2015.38 Suppl:S8-S16.
40. Rubio-Cabezas O, Hattersley AT, Njolstad PR, Mlynarski W, Ellard S, White N, Chi DV, Craig ME, International Society for P,Adolescent D, ISPAD Clinical Practice Consensus Guidelines 2014. The diagnosis and management of monogenic diabetes in children and adolescents. Pediatr Diabetes, 2014.15 Suppl 20:47-64.
41. Irgens HU, Molnes J, Johansson BB, Ringdal M, Skrivarhaug T, Undlien DE, Sovik O, Joner G, Molven A,Njolstad PR, Prevalence of monogenic diabetes in the population-based Norwegian Childhood Diabetes Registry. Diabetologia, 2013.56:1512-9.
42. Pihoker C, Gilliam LK, Ellard S, Dabelea D, Davis C, Dolan LM, Greenbaum CJ, Imperatore G, Lawrence JM, Marcovina SM, Mayer-Davis E, Rodriguez BL, Steck AK, Williams DE, Hattersley AT,Group SfDiYS, Prevalence, characteristics and clinical diagnosis of maturity onset diabetes of the young due to mutations in HNF1A, HNF4A, and glucokinase: results from the SEARCH for Diabetes in Youth. J Clin Endocrinol Metab, 2013.98:4055-62.
43. Gat-Yablonski G, Shalitin S,Phillip M, Maturity onset diabetes of the young--review. Pediatr Endocrinol Rev, 2006.3 Suppl 3:514-20.

44. Ellard S, Bellanne-Chantelot C, Hattersley AT,European Molecular Genetics Quality Network Mg, Best practice guidelines for the molecular genetic diagnosis of maturity-onset diabetes of the young. Diabetologia, 2008.51:546-53.
45. Fajans SS,Bell GI, MODY: history, genetics, pathophysiology, and clinical decision making. Diabetes Care, 2011.34:1878-84.
46. Shields BM, Hicks S, Shepherd MH, Colclough K, Hattersley AT,Ellard S, Maturity-onset diabetes of the young (MODY): how many cases are we missing? Diabetologia, 2010.53:2504-8.
47. Murphy R, Ellard S,Hattersley AT, Clinical implications of a molecular genetic classification of monogenic beta-cell diabetes. Nat Clin Pract Endocrinol Metab, 2008.4:200-13.
48. Greeley SA, Tucker SE, Worrell HI, Skowron KB, Bell GI,Philipson LH, Update in neonatal diabetes. Curr Opin Endocrinol Diabetes Obes, 2010.17:13-9.
49. Rubio-Cabezas O, Klupa T, Malecki MT,Consortium C, Permanent neonatal diabetes mellitus--the importance of diabetes differential diagnosis in neonates and infants. Eur J Clin Invest, 2011.41:323-33.
50. Flanagan SE, Edghill EL, Gloyn AL, Ellard S,Hattersley AT, Mutations in KCNJ11, which encodes Kir6.2, are a common cause of diabetes diagnosed in the first 6 months of life, with the phenotype determined by genotype. Diabetologia, 2006.49:1190-7.
51. Hattersley AT,Ashcroft FM, Activating mutations in Kir6.2 and neonatal diabetes: new clinical syndromes, new scientific insights, and new therapy. Diabetes, 2005.54:2503-13.
52. Owen KR,McCarthy MI, Genetics of type 2 diabetes. Curr Opin Genet Dev, 2007.17:239-44.
53. Imamura M,Maeda S, Genetics of type 2 diabetes: the GWAS era and future perspectives [Review]. Endocr J, 2011.58:723-39.
54. Farrar D, Duley L, Medley N,Lawlor DA, Different strategies for diagnosing gestational diabetes to improve maternal and infant health. Cochrane Database Syst Rev, 2015.1:CD007122.
55. Feig DS, Zinman B, Wang X,Hux JE, Risk of development of diabetes mellitus after diagnosis of gestational diabetes. CMAJ, 2008.179:229-34.
56. Van Ryswyk E, Middleton P, Hague W,Crowther C, Clinician views and knowledge regarding healthcare provision in the postpartum period for women

with recent gestational diabetes: a systematic review of qualitative/survey studies. Diabetes Res Clin Pract, 2014.106:401-11.

57. Lee AJ, Hiscock RJ, Wein P, Walker SP,Permezel M, Gestational diabetes mellitus: clinical predictors and long-term risk of developing type 2 diabetes: a retrospective cohort study using survival analysis. Diabetes Care, 2007.30:878-83.
58. Kim C, Newton KM,Knopp RH, Gestational diabetes and the incidence of type 2 diabetes: a systematic review. Diabetes Care, 2002.25:1862-8.
59. Sacks DA, Hadden DR, Maresh M, Deerochanawong C, Dyer AR, Metzger BE, Lowe LP, Coustan DR, Hod M, Oats JJ, Persson B, Trimble ER,Group HSCR, Frequency of gestational diabetes mellitus at collaborating centers based on IADPSG consensus panel-recommended criteria: the Hyperglycemia and Adverse Pregnancy Outcome (HAPO) Study. Diabetes Care, 2012.35:526-8.
60. Tieu J, Crowther CA,Middleton P, Dietary advice in pregnancy for preventing gestational diabetes mellitus. Cochrane Database Syst Rev, 2008:CD006674.
61. Hussain K, Diagnosis and management of hyperinsulinaemic hypoglycaemia of infancy. Horm Res, 2008.69:2-13.
62. Rahman SA, Nessa A,Hussain K, Molecular mechanisms of congenital hyperinsulinism. J Mol Endocrinol, 2015.54:R119-29.
63. Senniappan S, Alexandrescu S, Tatevian N, Shah P, Arya V, Flanagan S, Ellard S, Rampling D, Ashworth M, Brown RE,Hussain K, Sirolimus therapy in infants with severe hyperinsulinemic hypoglycemia. N Engl J Med, 2014.370:1131-7.
64. Yorifuji T, Congenital hyperinsulinism: current status and future perspectives. Ann Pediatr Endocrinol Metab, 2014.19:57-68.
65. Lebreton F, Pirog A, Belouah I, Bosco D, Berney T, Meda P, Bornat Y, Catargi B, Renaud S, Raoux M,Lang J, Slow potentials encode intercellular coupling and insulin demand in pancreatic beta cells. Diabetologia, 2015.58:1291-9.
66. Liu L, Wang F, Lu H, Ren X,Zou J, Chromanol 293B, an inhibitor of KCNQ1 channels, enhances glucose-stimulated insulin secretion and increases glucagon-like peptide-1 level in mice. Islets, 2014.6:e962386.
67. Martin GM, Chen PC, Devaraneni P,Shyng SL, Pharmacological rescue of trafficking-impaired ATP-sensitive potassium channels. Front Physiol, 2013.4:386.

68. Bennett K, James C,Hussain K, Pancreatic beta-cell KATP channels: Hypoglycaemia and hyperglycaemia. Rev Endocr Metab Disord, 2010.11:157-63.
69. McTaggart JS, Clark RH,Ashcroft FM, The role of the KATP channel in glucose homeostasis in health and disease: more than meets the islet. J Physiol, 2010.588:3201-9.
70. Bonfanti DH, Alcazar LP, Arakaki PA, Martins LT, Agustini BC, de Moraes Rego FG,Frigeri HR, ATP-dependent potassium channels and type 2 diabetes mellitus. Clin Biochem, 2015.48:476-82.
71. Bennett JT, Vasta V, Zhang M, Narayanan J, Gerrits P,Hahn SH, Molecular genetic testing of patients with monogenic diabetes and hyperinsulinism. Mol Genet Metab, 2015.114:451-8.
72. Ortiz D,Bryan J, Neonatal Diabetes and Congenital Hyperinsulinism Caused by Mutations in ABCC8/SUR1 are Associated with Altered and Opposite Affinities for ATP and ADP. Front Endocrinol (Lausanne), 2015.6:48.
73. Kapoor RR, Flanagan SE, Arya VB, Shield JP, Ellard S,Hussain K, Clinical and molecular characterisation of 300 patients with congenital hyperinsulinism. Eur J Endocrinol, 2013.168:557-64.
74. Gloyn AL, Siddiqui J,Ellard S, Mutations in the genes encoding the pancreatic beta-cell KATP channel subunits Kir6.2 (KCNJ11) and SUR1 (ABCC8) in diabetes mellitus and hyperinsulinism. Hum Mutat, 2006.27:220-31.
75. Yasuda K, Miyake K, Horikawa Y, Hara K, Osawa H, Furuta H, Hirota Y, Mori H, Jonsson A, Sato Y, Yamagata K, Hinokio Y, Wang HY, Tanahashi T, Nakamura N, Oka Y, Iwasaki N, Iwamoto Y, Yamada Y, Seino Y, Maegawa H, Kashiwagi A, Takeda J, Maeda E, Shin HD, Cho YM, Park KS, Lee HK, Ng MC, Ma RC, So WY, Chan JC, Lyssenko V, Tuomi T, Nilsson P, Groop L, Kamatani N, Sekine A, Nakamura Y, Yamamoto K, Yoshida T, Tokunaga K, Itakura M, Makino H, Nanjo K, Kadowaki T,Kasuga M, Variants in KCNQ1 are associated with susceptibility to type 2 diabetes mellitus. Nat Genet, 2008.40:1092-7.
76. Jonsson A, Isomaa B, Tuomi T, Taneera J, Salehi A, Nilsson P, Groop L,Lyssenko V, A variant in the KCNQ1 gene predicts future type 2 diabetes and mediates impaired insulin secretion. Diabetes, 2009.58:2409-13.
77. Mussig K, Staiger H, Machicao F, Kirchhoff K, Guthoff M, Schafer SA, Kantartzis K, Silbernagel G, Stefan N, Holst JJ, Gallwitz B, Haring HU,Fritsche A,

Association of type 2 diabetes candidate polymorphisms in KCNQ1 with incretin and insulin secretion. Diabetes, 2009.58:1715-20.

78. Rosengren AH, Braun M, Mahdi T, Andersson SA, Travers ME, Shigeto M, Zhang E, Almgren P, Ladenvall C, Axelsson AS, Edlund A, Pedersen MG, Jonsson A, Ramracheya R, Tang Y, Walker JN, Barrett A, Johnson PR, Lyssenko V, McCarthy MI, Groop L, Salehi A, Gloyn AL, Renstrom E, Rorsman P,Eliasson L, Reduced insulin exocytosis in human pancreatic beta-cells with gene variants linked to type 2 diabetes. Diabetes, 2012.61:1726-33.
79. Yamagata K, Senokuchi T, Lu M, Takemoto M, Fazlul Karim M, Go C, Sato Y, Hatta M, Yoshizawa T, Araki E, Miyazaki J,Song WJ, Voltage-gated K+ channel KCNQ1 regulates insulin secretion in MIN6 beta-cell line. Biochem Biophys Res Commun, 2011.407:620-5.
80. Asahara S, Etoh H, Inoue H, Teruyama K, Shibutani Y, Ihara Y, Kawada Y, Bartolome A, Hashimoto N, Matsuda T, Koyanagi-Kimura M, Kanno A, Hirota Y, Hosooka T, Nagashima K, Nishimura W, Inoue H, Matsumoto M, Higgins MJ, Yasuda K, Inagaki N, Seino S, Kasuga M,Kido Y, Paternal allelic mutation at the Kcnq1 locus reduces pancreatic beta-cell mass by epigenetic modification of Cdkn1c. Proc Natl Acad Sci U S A, 2015.112:8332-7.
81. Wrobel E, Tapken D,Seebohm G, The KCNE Tango - How KCNE1 Interacts with Kv7.1. Front Pharmacol, 2012.3:142.
82. Tombola F, Pathak MM,Isacoff EY, How does voltage open an ion channel? Annu Rev Cell Dev Biol, 2006.22:23-52.
83. Schmitt N, Schwarz M, Peretz A, Abitbol I, Attali B,Pongs O, A recessive C-terminal Jervell and Lange-Nielsen mutation of the KCNQ1 channel impairs subunit assembly. EMBO J, 2000.19:332-40.
84. Dahimene S, Alcolea S, Naud P, Jourdon P, Escande D, Brasseur R, Thomas A, Baro I,Merot J, The N-terminal juxtamembranous domain of KCNQ1 is critical for channel surface expression: implications in the Romano-Ward LQT1 syndrome. Circ Res, 2006.99:1076-83.
85. Labro AJ, Boulet IR, Choveau FS, Mayeur E, Bruyns T, Loussouarn G, Raes AL,Snyders DJ, The S4-S5 linker of KCNQ1 channels forms a structural scaffold with the S6 segment controlling gate closure. J Biol Chem, 2011.286:717-25.
86. Peroz D, Rodriguez N, Choveau F, Baro I, Merot J,Loussouarn G, Kv7.1 (KCNQ1) properties and channelopathies. J Physiol, 2008.586:1785-9.

87. Barhanin J, Lesage F, Guillemare E, Fink M, Lazdunski M,Romey G, K(V)LQT1 and lsK (minK) proteins associate to form the I(Ks) cardiac potassium current. Nature, 1996.384:78-80.
88. Wang Q, Curran ME, Splawski I, Burn TC, Millholland JM, VanRaay TJ, Shen J, Timothy KW, Vincent GM, de Jager T, Schwartz PJ, Toubin JA, Moss AJ, Atkinson DL, Landes GM, Connors TD,Keating MT, Positional cloning of a novel potassium channel gene: KVLQT1 mutations cause cardiac arrhythmias. Nat Genet, 1996.12:17-23.
89. Hedley PL, Jorgensen P, Schlamowitz S, Wangari R, Moolman-Smook J, Brink PA, Kanters JK, Corfield VA,Christiansen M, The genetic basis of long QT and short QT syndromes: a mutation update. Hum Mutat, 2009.30:1486-511.
90. Hibino H, Nin F, Tsuzuki C,Kurachi Y, How is the highly positive endocochlear potential formed? The specific architecture of the stria vascularis and the roles of the ion-transport apparatus. Pflugers Arch, 2010.459:521-33.
91. Wangemann P, Liu J,Marcus DC, Ion transport mechanisms responsible for K+ secretion and the transepithelial voltage across marginal cells of stria vascularis in vitro. Hear Res, 1995.84:19-29.
92. Jain RN, Brunkan CS, Chew CS,Samuelson LC, Gene expression profiling of gastrin target genes in parietal cells. Physiol Genomics, 2006.24:124-32.
93. Elso CM, Lu X, Culiat CT, Rutledge JC, Cacheiro NL, Generoso WM,Stubbs LJ, Heightened susceptibility to chronic gastritis, hyperplasia and metaplasia in Kcnq1 mutant mice. Hum Mol Genet, 2004.13:2813-21.
94. Asano S, Morii M,Takeguchi N, Molecular and cellular regulation of the gastric proton pump. Biol Pharm Bull, 2004.27:1-12.
95. Nguyen N, Kozer-Gorevich N, Gliddon BL, Smolka AJ, Clayton AH, Gleeson PA,van Driel IR, Independent trafficking of the KCNQ1 K+ channel and H+-K+-ATPase in gastric parietal cells from mice. Am J Physiol Gastrointest Liver Physiol, 2013.304:G157-66.
96. Jespersen T, Grunnet M,Olesen SP, The KCNQ1 potassium channel: from gene to physiological function. Physiology (Bethesda), 2005.20:408-16.
97. Cirovic S, Markovic-Lipkovski J, Todorovic J, Nesovic-Ostojic J, Jovic M, Ilic S, Tatic S,Cemerikic D, Differential expression of KCNQ1 K+ channel in tubular cells of frog kidney. Eur J Histochem, 2010.54:e7.

98. Vallon V, Grahammer F, Volkl H, Sandu CD, Richter K, Rexhepaj R, Gerlach U, Rong Q, Pfeifer K,Lang F, KCNQ1-dependent transport in renal and gastrointestinal epithelia. Proc Natl Acad Sci U S A, 2005.102:17864-9.
99. Arnoux JB, Verkarre V, Saint-Martin C, Montravers F, Brassier A, Valayannopoulos V, Brunelle F, Fournet JC, Robert JJ, Aigrain Y, Bellanne-Chantelot C,de Lonlay P, Congenital hyperinsulinism: current trends in diagnosis and therapy. Orphanet J Rare Dis, 2011.6:63.
100. Sanger F, Nicklen S,Coulson AR, DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci U S A, 1977.74:5463-7.
101. Casimiro MC, Knollmann BC, Ebert SN, Vary JC, Jr., Greene AE, Franz MR, Grinberg A, Huang SP,Pfeifer K, Targeted disruption of the Kcnq1 gene produces a mouse model of Jervell and Lange-Nielsen Syndrome. Proc Natl Acad Sci U S A, 2001.98:2526-31.
102. Jia S, Ivanov A, Blasevic D, Muller T, Purfurst B, Sun W, Chen W, Poy MN, Rajewsky N,Birchmeier C, Insm1 cooperates with Neurod1 and Foxa2 to maintain mature pancreatic beta-cell function. EMBO J, 2015.34:1417-33.
103. Simaite D, Kofent J, Gong M, Ruschendorf F, Jia S, Arn P, Bentler K, Ellaway C, Kuhnen P, Hoffmann GF, Blau N, Spagnoli FM, Hubner N,Raile K, Recessive mutations in PCBD1 cause a new type of early-onset diabetes. Diabetes, 2014.63:3557-64.
104. Weedon MN, Cebola I, Patch AM, Flanagan SE, De Franco E, Caswell R, Rodriguez-Segui SA, Shaw-Smith C, Cho CH, Lango Allen H, Houghton JA, Roth CL, Chen R, Hussain K, Marsh P, Vallier L, Murray A, International Pancreatic Agenesis C, Ellard S, Ferrer J,Hattersley AT, Recessive mutations in a distal PTF1A enhancer cause isolated pancreatic agenesis. Nat Genet, 2014.46:61-4.
105. Crotti L, Tester DJ, White WM, Bartos DC, Insolia R, Besana A, Kunic JD, Will ML, Velasco EJ, Bair JJ, Ghidoni A, Cetin I, Van Dyke DL, Wick MJ, Brost B, Delisle BP, Facchinetti F, George AL, Schwartz PJ,Ackerman MJ, Long QT syndrome-associated mutations in intrauterine fetal death. JAMA, 2013.309:1473-82.
106. Van Langen IM, Birnie E, Alders M, Jongbloed RJ, Le Marec H,Wilde AA, The use of genotype-phenotype correlations in mutation analysis for the long QT syndrome. J Med Genet, 2003.40:141-5.

107. Seebohm G, Westenskow P, Lang F,Sanguinetti MC, Mutation of colocalized residues of the pore helix and transmembrane segments S5 and S6 disrupt deactivation and modify inactivation of KCNQ1 K+ channels. J Physiol, 2005.563:359-68.
108. Sachyani D, Dvir M, Strulovich R, Tria G, Tobelaim W, Peretz A, Pongs O, Svergun D, Attali B,Hirsch JA, Structural basis of a Kv7.1 potassium channel gating module: studies of the intracellular c-terminal domain in complex with calmodulin. Structure, 2014.22:1582-94.
109. Alkorta-Aranburu G, Carmody D, Cheng YW, Nelakuditi V, Ma L, Dickens JT, Das S, Greeley SA,del Gaudio D, Phenotypic heterogeneity in monogenic diabetes: the clinical and diagnostic utility of a gene panel-based next-generation sequencing approach. Mol Genet Metab, 2014.113:315-20.
110. Rafiq M, Flanagan SE, Patch AM, Shields BM, Ellard S, Hattersley AT,Neonatal Diabetes International Collaborative G, Effective treatment with oral sulfonylureas in patients with diabetes due to sulfonylurea receptor 1 (SUR1) mutations. Diabetes Care, 2008.31:204-9.
111. Arya VB, Mohammed Z, Blankenstein O, De Lonlay P,Hussain K, Hyperinsulinaemic hypoglycaemia. Horm Metab Res, 2014.46:157-70.
112. De Franco E, Flanagan SE, Houghton JA, Lango Allen H, Mackay DJ, Temple IK, Ellard S,Hattersley AT, The effect of early, comprehensive genomic testing on clinical care in neonatal diabetes: an international cohort study. Lancet, 2015.386:957-63.
113. Steck AK,Winter WE, Review on monogenic diabetes. Curr Opin Endocrinol Diabetes Obes, 2011.18:252-8.
114. Lango Allen H, Flanagan SE, Shaw-Smith C, De Franco E, Akerman I, Caswell R, International Pancreatic Agenesis C, Ferrer J, Hattersley AT,Ellard S, GATA6 haploinsufficiency causes pancreatic agenesis in humans. Nat Genet, 2012.44:20-2.
115. Al-Shammari M, Al-Husain M, Al-Kharfy T,Alkuraya FS, A novel PTF1A mutation in a patient with severe pancreatic and cerebellar involvement. Clin Genet, 2011.80:196-8.
116. Moreno C, Oliveras A, de la Cruz A, Bartolucci C, Munoz C, Salar E, Gimeno JR, Severi S, Comes N, Felipe A, Gonzalez T, Lambiase P,Valenzuela C, A new

KCNQ1 mutation at the S5 segment that impairs its association with KCNE1 is responsible for short QT syndrome. Cardiovasc Res, 2015.107:613-23.

117. Xiong Q, Cao Q, Zhou Q, Xie J, Shen Y, Wan R, Yu J, Yan S, Marian AJ,Hong K, Arrhythmogenic cardiomyopathy in a patient with a rare loss-of-function KCNQ1 mutation. J Am Heart Assoc, 2015.4:e001526.
118. Chen YH, Xu SJ, Bendahhou S, Wang XL, Wang Y, Xu WY, Jin HW, Sun H, Su XY, Zhuang QN, Yang YQ, Li YB, Liu Y, Xu HJ, Li XF, Ma N, Mou CP, Chen Z, Barhanin J,Huang W, KCNQ1 gain-of-function mutation in familial atrial fibrillation. Science, 2003.299:251-4.
119. Torekov SS, Iepsen E, Christiansen M, Linneberg A, Pedersen O, Holst JJ, Kanters JK,Hansen T, KCNQ1 long QT syndrome patients have hyperinsulinemia and symptomatic hypoglycemia. Diabetes, 2014.63:1315-25.
120. Clausen T, Hormonal and pharmacological modification of plasma potassium homeostasis. Fundam Clin Pharmacol, 2010.24:595-605.
121. Unoki H, Takahashi A, Kawaguchi T, Hara K, Horikoshi M, Andersen G, Ng DP, Holmkvist J, Borch-Johnsen K, Jorgensen T, Sandbaek A, Lauritzen T, Hansen T, Nurbaya S, Tsunoda T, Kubo M, Babazono T, Hirose H, Hayashi M, Iwamoto Y, Kashiwagi A, Kaku K, Kawamori R, Tai ES, Pedersen O, Kamatani N, Kadowaki T, Kikkawa R, Nakamura Y,Maeda S, SNPs in KCNQ1 are associated with susceptibility to type 2 diabetes in East Asian and European populations. Nat Genet, 2008.40:1098-102.
122. Haitin Y,Attali B, The C-terminus of Kv7 channels: a multifunctional module. J Physiol, 2008.586:1803-10.
123. Dai XP, Huang Q, Yin JY, Guo Y, Gong ZC, Lei MX, Jiang TJ, Zhou HH,Liu ZQ, KCNQ1 gene polymorphisms are associated with the therapeutic efficacy of repaglinide in Chinese type 2 diabetic patients. Clin Exp Pharmacol Physiol, 2012.39:462-8.
124. Brissova M, Shiota M, Nicholson WE, Gannon M, Knobel SM, Piston DW, Wright CV,Powers AC, Reduction in pancreatic transcription factor PDX-1 impairs glucose-stimulated insulin secretion. J Biol Chem, 2002.277:11225-32.
125. Vishwakarma SK, Rahamathulla S, Bardia A, Tiwari SK, Srinivas G, Raj A, Tripura C, Sandhya A, Habeeb MA, Khan AA, Pande G, Reddy KP,Reddy PY, In vitro quantitative and relative gene expression analysis of pancreatic

transcription factors Pdx-1, Ngn-3, Isl-1, Pax-4, Pax-6 and Nkx-6.1 in trans-differentiated human hepatic progenitors. J Diabetes Investig, 2014.5:492-500.

126. Gao N, White P, Doliba N, Golson ML, Matschinsky FM,Kaestner KH, Foxa2 controls vesicle docking and insulin secretion in mature Beta cells. Cell Metab, 2007.6:267-79.
127. Lee CS, Sund NJ, Behr R, Herrera PL,Kaestner KH, Foxa2 is required for the differentiation of pancreatic alpha-cells. Dev Biol, 2005.278:484-95.
128. Lee CS, Sund NJ, Vatamaniuk MZ, Matschinsky FM, Stoffers DA,Kaestner KH, Foxa2 controls Pdx1 gene expression in pancreatic beta-cells in vivo. Diabetes, 2002.51:2546-51.
129. Lantz KA, Vatamaniuk MZ, Brestelli JE, Friedman JR, Matschinsky FM,Kaestner KH, Foxa2 regulates multiple pathways of insulin secretion. J Clin Invest, 2004.114:512-20.
130. Zhang Y, Fava GE, Wang H, Mauvais-Jarvis F, Fonseca VA,Wu H, PAX4 Gene Transfer Induces alpha-to-beta Cell Phenotypic Conversion and Confers Therapeutic Benefits for Diabetes Treatment. Mol Ther, 2015.
131. Liu Z, Kim W, Chen Z, Shin YK, Carlson OD, Fiori JL, Xin L, Napora JK, Short R, Odetunde JO, Lao Q,Egan JM, Insulin and glucagon regulate pancreatic alpha-cell proliferation. PLoS One, 2011.6:e16096.
132. Hart AW, Mella S, Mendrychowski J, van Heyningen V,Kleinjan DA, The developmental regulator Pax6 is essential for maintenance of islet cell function in the adult mouse pancreas. PLoS One, 2013.8:e54173.
133. Gerace D, Martiniello-Wilks R, O'Brien BA,Simpson AM, The use of beta-cell transcription factors in engineering artificial beta cells from non-pancreatic tissue. Gene Ther, 2015.22:1-8.
134. Boini KM, Graf D, Hennige AM, Koka S, Kempe DS, Wang K, Ackermann TF, Foller M, Vallon V, Pfeifer K, Schleicher E, Ullrich S, Haring HU, Haussinger D,Lang F, Enhanced insulin sensitivity of gene-targeted mice lacking functional KCNQ1. Am J Physiol Regul Integr Comp Physiol, 2009.296:R1695-701.
135. Weir GC,Bonner-Weir S, Islet beta cell mass in diabetes and how it relates to function, birth, and death. Ann N Y Acad Sci, 2013.1281:92-105.
136. Zheng S,Mathews CE, Metabolic abnormalities in the pathogenesis of type 1 diabetes. Curr Diab Rep, 2014.14:519.

137. Pinto J, Sudeep K,Venkatesha BM, A rare case of type 1 diabetes mellitus with pituitary hypoplasia. Diabetes Metab Syndr, 2014.8:184-6.
138. Sherr JL, Ghazi T, Wurtz A, Rink L,Herold KC, Characterization of residual beta cell function in long-standing type 1 diabetes. Diabetes Metab Res Rev, 2014.30:154-62.
139. Sreenan S, Pick AJ, Levisetti M, Baldwin AC, Pugh W,Polonsky KS, Increased beta-cell proliferation and reduced mass before diabetes onset in the nonobese diabetic mouse. Diabetes, 1999.48:989-96.
140. Saisho Y, beta-cell dysfunction: Its critical role in prevention and management of type 2 diabetes. World J Diabetes, 2015.6:109-24.
141. Butler AE, Janson J, Bonner-Weir S, Ritzel R, Rizza RA,Butler PC, Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes. Diabetes, 2003.52:102-10.
142. Talchai C, Xuan S, Lin HV, Sussel L,Accili D, Pancreatic beta cell dedifferentiation as a mechanism of diabetic beta cell failure. Cell, 2012.150:1223-34.
143. Meier JJ, Breuer TG, Bonadonna RC, Tannapfel A, Uhl W, Schmidt WE, Schrader H,Menge BA, Pancreatic diabetes manifests when beta cell area declines by approximately 65% in humans. Diabetologia, 2012.55:1346-54.
144. Ejarque M, Cervantes S, Pujadas G, Tutusaus A, Sanchez L,Gasa R, Neurogenin3 cooperates with Foxa2 to autoactivate its own expression. J Biol Chem, 2013.288:11705-17.
145. Petri A, Ahnfelt-Ronne J, Frederiksen KS, Edwards DG, Madsen D, Serup P, Fleckner J,Heller RS, The effect of neurogenin3 deficiency on pancreatic gene expression in embryonic mice. J Mol Endocrinol, 2006.37:301-16.

6. Affidavit

“I, Lei Liang, certify under penalty of perjury by my own signature that I have submitted the thesis on the topic “Human *KCNQ1* mutations cause neonatal diabetes and hyperinsulinemic hypoglycemia: Clinical and functional characterization”. I wrote this thesis independently and without assistance from any third parties, I used no other aids than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see "uniform requirements for manuscripts (URM)" the ICMJE www.icmje.org) indicated. The sections on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) correspond to the URM (s.o) and are answered by me. My interest in any publications to this dissertation corresponds to those that are specified in the following joint declaration with the responsible person and supervisor. All publications resulting from this thesis and which I am author correspond to the URM (see above) and I am solely responsible.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

Date Signature

7. Curriculum Vitae

My curriculum vitae does not appear in the electronic version of my paper for reasons of data protection.

8. Publication

At present, there is no publication for this study.

9. Acknowledgements

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10. Supplementary material

10.1 Equipments

Applied Biosystems® GeneAmp® PCR System 9700	Thermo Scientific, Darmstadt, Germany
Megafuge 1.0 R	Thermo Scientific, Bonn, Germany
Microtome HM560	Thermo Scientific, Walldorf, Germany
ChemiDoc™ MP imaging system	Bio-Rad, Munich, Germany
NanoDrop® Spektrophotometer, ND-1000	NanoDrop Technologies, Wilmington, USA
OLYMPUS CKX31 microscope	OLYMPUS, Tokyo, Japan
Fluorescent microscope BZ-8100E	KEYENCE, Tokyo, Japan
Real-time PCR System StepOnePlus	Applied Biosystems,Darmstadt
Thermomixer Comfort	Eppendorf, Hamburg,Germany
Trans-Blot® SD Cell	Bio-Rad, Munich, Germany
V-1 plus vertex mixer	Biosan, Riga,Latvia
Mini Centrifuge/Vortex FVL-2400N	Biosan, Riga,Latvia
Amaxa™ nucleofector™ Apparate Electro Transfection system	Lonza, Germany
Olympus IX71 inverted micoscope	OLYMPUS, Tokyo, Japan
ABI 3730 DNA analysis system (Sequencer)	Applied Biosystems, Wrington, UK
Multiclamp 700B amplifier	Molecular Devices, Sunnyvale, USA
Digidata 1440a data acquisition system	Molecular Devices, Sunnyvale, USA
High Speed Microplate Shaker	Illumina, San Diego, California, USA

10.2 Chemicals

4-20% mini precast Tris-glycine gels	Bio-Rad, Munich, Germany
10x DreamTaq™ green buffer	Fermentas, St. Leon-Rot, Germany
50x TAE Electrophoresis buffer	Fermentas, St. Leon-Rot, Germany
Agarose	Sigma-Aldrich, Steinheim,

	Germany
Aqua ad iniectabilia	B. Braun, Melsungen, Germany
BSA	Sigma-Aldrich, Steinheim, Germany
Complete Protease Inhibitor Cocktail Tablets	Roche Diagnostics, Mannheim, Germany
DNase I	Qiagen, Hilden, Germany
dNTP Mix (10mM)	Promega, Mannheim, Germany
dNTP Mix (2 mM)	Fermentas, St. Leon-Rot, Germany
Normal Donkey serum	Jackson ImmunoResearch, Suffolk, UK
DreamTaq DNA Polymerase	Fermentas, St. Leon-Rot, Germany
Ethanol	Merck Millipore, Darmstadt, Germany
Ethidium bromide (10 mg/ml)	Roth, Karlsruhe, Germany
Glucose	Roth, Karlsruhe, Germany
Glycerin	Roth, Karlsruhe, Germany
Glycine	Merck Millipore, Darmstadt, Germany
HCl	Sigma-Aldrich, Munich,Germany
Isopropanol	Merck Millipore ,Darmstadt, Germany
Ketamin 10%	Bela Pharm, Vechta, Germany
β-Mercaptoethanol	Roth, Karlsruhe, Germany
Methanol	J.T. Baker, Griesheim, Germany
Powdered milk	Roth, Karlsruhe, Germany
M-MLV Reverse Transcriptase	Promega, Mannheim, Mannheim
M-MLV RT 5x Reaction buffer	Promega, Mannheim, Mannheim
Paraformaldehyde	Merck Millipore ,Darmstadt, Germany
10x PBS	Bio-Rad, Munich , Germany
peqGOLD RNApure	PEQLAB Biotechnologie, Erlangen, Germany
Ponceau S Solution	Sigma-Aldrich, Steinheim, Germany

Protease inhibitor cocktail tablets	Roche Diagnostics, Mannheim, Germany
Proteinase K	Roth, Karlsruhe, Germany
Random Primer (500µg/ml)	Promega, Mannheim, Germany
RIPA buffer (pH 7.4)	Sigma-Aldrich, Steinheim, Germany
RNase A	Sigma-Aldrich, Steinheim, Germany
RNase-free distilled water	Qiagen, Hilden, Germany
RNasin® Ribonuclease Inhibitor (40U/µl)	Promega, Mannheim, Germany
Rox high (50x)	PEQLAB Biotechnologie, Erlangen, Germany
Kapa Probe Fast qPCR MM (2x)	PEQLAB Biotechnologie, Erlangen, Germany
Sodium *citrate*	Sigma-Aldrich, Steinheim, Germany
Sodium dodecyl sulfate	Sigma-Aldrich, Steinheim, Germany
collagenase	Sigma-Aldrich, Steinheim, Germany
Tissue Freezing Medium	Triangle Biomedical Sciences, Durham, USA
Tris base	Roth, Karlsruhe, Germany
Triton X-100	Sigma-Aldrich, Steinheim, Germany
Tween® 20	Roth, Karlsruhe, Germany
Vectashield HardSet Mounting Medium(DAPI)	Vector Laboratories, Burlingame, CA, USA
10x Tris Glycin SDS	Bio-Rad, Munich, Germany
Laemmli	Bio-Rad, Munich, Germany
Power SYBR® Green PCR Master Mix	Applied Biosystems, Foster City, CA, USA

10.3 Solutions and buffer

Agarose gel (1%/ 2%)	1%/2% Agarose (w/v) in 1x TAE buffer
5% donkey blocking solution	1% BSA (w/v)+0,3 % Triton X-100 (v/v)+5% normal donkey serum (v/v) in 1XPBS+ 0,3% Triton X-100

10x electrophoresis buffer	1,92 M Glycine+250 mM Tris-HCl (pH8,1)+1% SDS (w/v)
2x Laemmli buffer(pH 6,8)	0,1% Bromophenol blue +50% Glycerine +5% β-Mercaptoethanol+2% SDS (w/v)+ 0,125 M Tris-HCl
Paraformaldehyde (4%)	4% (w/v) Paraformaldehyde in PBS (pH 7,4)
Proteinase K solution	0,0005% Proteinase K (v/w) in PBS (pH 7,5)
RNase A solution	0,01% RNase A (v/w)+ 150 mM NaCl+ 15mM sodium citrate
Milk (5%)	5% Powered Milk (w/v) in TBST
10x TBS buffer (pH 7,4)	0,2 M Tris-HCl (pH 7,4)+ 1,5 M NaCl
TBST	10 M Tris-HCl (pH 7,4)+ 150 mM NaCl +0,1% Tween® 20 (w/v)
Transfer buffer (pH 9,2)	39 mM Glycine+48 mM Tris+20% Methanol (v/v)
Strip buffer	0,15M Glycine+ 0,4% SDS adjusted to PH 2,5 by HCl
PBST	0,3% Triton X-100 (v/v) in PBS
Hams F-12 Medium	Merck Millipore ,Darmstadt, Germany
Dulbecco's modified Eagle's medium (DMEM)	Life Technologies**,** Darmstadt, Germany
10x HBSS	Life Technologies**,** Darmstadt, Germany
PIPA	Beyotime Bio, Shanghai, China
RPMI 1640 medium	Life Technologies, Carlsbad, CA, USA
Immobilon™ Western Chemiluminescent HRP Substrate	Merck Millipore ,Darmstadt, Germany
Histopaque®-1119	Sigma-Aldrich, Steinheim, Germany
FBS	Sigma-Aldrich, Steinheim, Germany
Extracellular bath solution	135mM NaCl, 5mM KCl, 1,3 mM $MgCl_2$, 5mM HEPES, 2,5mM $CaCl_2$, 10mM d-glucose, adjusted to pH 7,4 using NaOH
Intracellular solution	10mM NaCl, 120mM KCl, 2mM $MgCl_2$, 11mM HEPES, 1mM $CaCl_2$, 11mM EGTA, adjusted to pH 7,2 using KOH
Secretion buffer	137 mM NaCl, 0.9 mM CaCl2, 2.7 mM KCl, 1.5 mM KH2PO4, 0.5 mM MgCl2, 8.1 mM Na2HPO4, 20 mM HEPES pH 7.4, 0.2% BSA

DEPC-ddH_2O	0.1% DEPC in ddH_2O
1x FA gel running buffer	1x FA gel buffer, 0.74% formaldehyde
10x FA gel buffer pH 7.0	200 mM MOPS, 50 mM NaOAc, 10 mM EDTA
5x RNA loading buffer	4x FA gel buffer, 4 mM EDTA, 2.7% formaldehyde, 20% glycerol, 3% formamide, 0.00064 % bromphenol blue