UNIVERSITAT DE BARCELONA

Beta cell dedifferentiation in the treatment of type 2 diabetes

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BETA CELL DEDIFFERENTIATION IN THE TREATMENT OF TYPE 2 DIABETES

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BETA CELL DEDIFFERENTIATION IN THE TREATMENT OF TYPE 2 DIABETES

Thesis presented by Claudia Fernández Alegre to qualify for the PhD degree by the Universitat de Barcelona.

The work presented in this dissertation has been carried out by Claudia Fernández Alegre under the guidance of Dr. Eduard Montanya Mias and Dr. Noèlia Téllez Besolí in the Departament de Ciències Clíniques of the Universitat de Barcelona at Institut d'Investigació Biomèdica de Bellvitge (IDIBELL).

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III. ABBREVIATIONS

ABCC8: ATP Binding Cassette subfamily C member 8
ADA: American Diabetes Association
ADP: Adenosine Diphosphate
AIDS: Acquired Immune Deficiency Syndrome
ALDH1A3: Aldehyde Dehydrogenase 1 Family Member A3
AMPK: 5' AMP-activated Protein Kinase
ANOVA: One-way Analysis of Variance
ARX: Aristaless-Related Homebox, X-linked
ATF4: Activating Transcription Factor 4
ATF6: Activating Transcription Factor 6
ATP: Adenosine Triphosphate
BIP: Binding Immunoglobulin Protein
BMI: Body Mass Index
BSA: Bovine Serum Albumin
CaM: Calmodulin
cAMP: Cyclic Adenosine Monophosphate
CAT: Catalase
cDNA: Complementary DNA
ChgA: Chromogranin A
CHOP: C/EBP Homologous Protein
CMRL: Connaught Medical Research Laboratories
CN: Calcineurin
CV: Cardiovascular
DAG: Diacylglycerol
DAPI: 4'6-diamidino-2-phenylindole dihydrochloride
DDIT3: DNA Damage Inducible Transcript 3
DNA: Deoxyribonucleic Acid
DNMT3A: DNA Methyltransferase 3 Alpha
DPC: Days Post Conception
DPP-4: Dipeptidyl Peptidase-4
DTZ: Dithizone
EDTA: Ethylenediaminetetraacetic Acid
EIF2AK3: Eukaryotic Translation Initiation Factor 2-Alpha Kinase 3
ELISA: Enzyme-Linked ImmunoSorbent Assay
EPAC2: Exchange Protein directly Activated by cAMP 2
ER: Endoplasmic Reticulum
ERAD: ER-Associated Degradation
FACS: Fluorescence-Activated Cell Sorting
FBS: Fetal Bovine Serum
FGF: Fibroblast Growth Factor
FLTP: Flattop
FOXO1: Forkhead Box Protein O1
G6PC2: Glucose-6-Phosphatase Catalytic subunit 2 protein
GAST: Gastrin

GATA6: GATA Binding Protein 6
GCG: Glucagon
GCK: Glucokinase
GFP: Green Fluorescent Protein
GIP: Glucose-dependent Insulinotropic Polypeptide
GLP-1: Glucagon-like Peptide-1
GLP1R: Glucagon-like Peptide-1 Receptor
GLP-1RAs: GLP-1 Receptor Agonists
GLUT: Glucose Transporter
GPCR: G-Protein-Coupled Receptors
GRP78: Glucose-Regulating Protein 78
GSIS: Glucose-Stimulated Insulin Secretion
GWAS: Genome-Wide Association Studies
HBA1C: Glycated hemoglobin
HEK: Human Embryonic Kidney
HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
HES1: Hairy and Enhancer of Split 1
HH: Hedgehog
HIV: Human Immunodeficiency Virus
HK1: Hexokinase 1
HNF4: Hepatocyte Nuclear Factor-4 Alpha
HRP: Horseradish Peroxidase
HSPA5: Heat Shock Protein Family A (Hsp70) Member 5
IAPP: Islet Amyloid Polypeptide
IC50: Half-maximal Inhibitory Concentration
IDF: International Diabetes Federation
IDIBELL: Institut d'Investigació Biomèdica de Bellvitge
IEQ: Islet Equivalent
IFU: Infectivity Units
IgG: Immunoglobulin G
IL-1: Interleukin-1
INS: Insulin
INSM1: Insulinoma-associated antigen 1
IP3: Inositol triphosphate
IR: Insulin Receptor
IRE1: Inositol-Requiring Protein 1
ISL-1: Islet-1
JNK: c-Jun N-terminal Kinase
KATP channels: ATP-sensitive Potassium Channels
KCL: Potassium Chloride
KCNJ11: Potassium Inwardly Rectifying Channel Subfamily J Member 11
KRBH: Krebs-Ringer Bicarbonate Hepes Buffer
LDB1: LIM Domain Binding
LDHA: Lactate Dehydrogenase A

MAFA: V-maf musculoaponeurotic fibrosarcoma oncogene homolog A
MAFB: V-maf musculoaponeurotic fibrosarcoma oncogene homolog B
MAMs: Mitochondria-Associated Membranes
MAPK: Mitogen-Activated Protein Kinase
MCT1: Monocarboxylate Transporter 1
MIN6: Mouse Insulinoma 6
miRNA: Micro RNA
MNX: Motor Neuron and Pancreas Homeobox
MODY: Maturity-Onset Diabetes of the Young
MPC: Multipotent Pancreatic Progenitor
mRNA: Messenger RNA
mtDNA: Mitocondrial DNA
mTOR: Mammalian Target of Rapamycin
MYC: Myelocytomatosis proto-oncogene
NAD: Nicotinamide Adenine Dinucleotide
NAFLD: Non-Alcoholic Fatty Liver Disease
ND: Not Determined
NEUROD1: Neurogenic Differentiation Factor 1
NFAT: Nuclear Factor of Activated T-cells
NF-κB: Nuclear Factor κB
NGN3: Neurogenin 3
NGT: Normal Glucose Tolerance
NKX2.2: NK2 homeobox 2
NKX6.1: NK6 homeobox 1
NLRP3: NLR Family Pyrin Domain Containing 3
NO: Nitric Oxide
NOS: Nitric Oxide Synthase
NPY: Neuropeptide Y
PAX4: Paired Box 4
PAX6: Paired Box 6
PBA: 4-Phenylbutyrate
PBS: Phosphate Buffered Saline
PC: Prohormone Convertase
PCP: Planar Cell Polarity
PCSK1: Proprotein Convertase Subtilisin/Kexin Type 1
PDI: protein disulfide isomerases
PDX1: Pancreatic Duodenal Homebox 1
PERK: Protein Kinase R-like ER Kinase
PFA: Paraformaldehyde
PKA: Protein Kinase A
PP: Pancreatic Polypeptide
PVDF: Polyvinylidene Fluoride
PYY: Peptide YY
qPCR: Quantitative Polymerase Chain Reaction

RAP1: Ras-Associated Protein 1
RAS: Renin-angiotensin system
RER: Rough Endoplasmic Reticulum
REST: Repressor Element 1 Silencing Transcription Factor
RFX6: Regulatory Factor X6
RIN: RNA Integrity Number
RNA: Ribonucleic Acid
ROI: Region of Interest
ROS: Reactive Oxygen Species
RP: Reserve Pool
RPLP0: Ribosomal Protein Large P0
RQ: Relative Quantity
RRP: Readily Releasable Pool
RT-qPCR: Reverse Transcription Quantitative Polymerase Chain Reaction
RyR: Ryanodine Receptor
SAH: Subarachnoid Hemorrhage
scRNA-seq: Single-cell RNA sequencing
SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEM: Standard Error Mean
SER: Smooth Endoplasmic Reticulum
SERCA: Sarco/Endoplasmic Reticulum Ca2+-ATPase
SI: Stimulation Index
SLC16A1: Solute Carrier Family 16 Member 1
SNARE: Soluble N-ethyl-maleimide-sensitive Attachment Protein Receptor
SOD: Superoxide Dismutase
SOX9: SRY-Box 9
SST: Somatostatin
SUR: Sulfonylurea Receptor
SUs: Sulfonylureas
SYT: Synaptotagmin
T1DM: Type 1 Diabetes Mellitus
T2DM: Type 2 Diabetes Mellitus
TBI: Traumatic Brain Injury
TBP: TATA-box Binding Protein
TBS-T: Tris-buffered saline + Tween-20
TFs: Transcription Factors
TGF-β: Transforming Growth Factor Beta
TRAF2: Tumor Necrosis Factor 2
TRB3: Tribbles-Related Protein 3
TUDCA: Tauroursodeoxycholic Acid
TUNEL: Terminal Deoxynucleotidyl Transferase Biotin-dUTP Nick-End Labelling
TUs: Transducing Units
UCN3: Urocortin3
UPR: Unfolded Protein Response

USA: United States of America
UW: University Wisconsin Solution
VDCC: Voltage-dependent Ca2+ channels
WFS1: Wolfram syndrome 1
WHO: World Health Organization
WNT: Wingless/Integrated
WT: Wild-Type
XBP1: X-box binding protein 1
ZNT8: Zinc Transporter 8

IV. SUMMARY

Type 2 Diabetes Mellitus (T2DM) is the most common type of diabetes (90-95 %) traditionally associated with peripheral insulin resistance that entails compensatory β-cell mass expansion and hyperinsulinemia during the initial stages of the disease. Despite this early adaptation, later on, blood glucose levels cannot be maintained, and it leads to progressive β-cell failure that results from reduced β-cell function and β-cell mass. Therefore, pancreatic β-cell failure, due to the interplay of genetic and acquired factors, is key to the onset and progression of T2DM. Originally reduced functional β-cell mass was attributed to β-cell apoptosis but novel mechanisms such as β-cell dedifferentiation have shed more light on the matter. β-cell dedifferentiation or the loss of β-cell identity and maturity has been evidenced as a mechanism of β-cell failure during stress-induced glucotoxicity and its downstream pathways: oxidative stress, ER stress, the hexosamine pathway, inflammation and hypoxia. Interestingly, β-cell dedifferentiation could be a reversible process, these dedifferentiated cells could potentially be rescued and induced to redifferentiate into mature β-cells, opening new possibilities for the prevention and treatment of T2DM.

Sulfonylureas (SUs) are oral hypoglycemic agents widely used for its lower cost. These drugs induce glucose independent insulin secretion by inhibiting potassium flux through the ATP-dependent potassium channels (K_{ATP}) in β-cells. It has been observed that after a long-term treatment, patients start to show a failure to respond to the drug (2ry failure). However, the cellular mechanism underlying such failure is still not fully understood. Indeed, there are some studies conducted in mice suggesting β-cell exhaustion after chronic hyperexcitability and hypersecretion that could be induced by SUs. However, no conclusive data in human islets has been reported to understand these sulfonylurea-induced effects.

A novel T2DM therapy is the use of GLP-1R agonists. Glucagon-like peptide-1 (GLP-1) is an incretin hormone mainly secreted by intestinal enteroendocrine L-cells, as the active form GLP-1 (7-36)NH_2, that contributes to glucose homeostasis, primarily by potentiating glucose-induced insulin secretion in β-cells. GLP-1 binds to a specific receptor, GLP-1R, activating several pathways within the β-cell resulting in increased

insulin biosynthesis, improved β-cell function, and increased β-cell mass. As well as, preventing β-cell apoptosis.

The hypothesis of this work was that chronic exposure to glibenclamide, a second-generation SU, could induce β-cell dedifferentiation resulting in a reduction of the functional β-cell mass, which could contribute to the progression of secondary failure to sulfonylureas. Considering the protective effects of GLP-1R agonists on β-cell viability and function, it was hypothesized that this process could be attenuated by GLP-1R agonists, resulting in the preservation of mature β-cell function. The main aim of the present work has been to investigate the effect of T2DM treatments and the diabetogenic environment on human β-cell phenotype, which included specific aims such as analysis of chronic glibenclamide exposure on β-cell function, β-cell apoptosis, and β-cell identity; elucidate the underlying mechanisms contributing to the glibenclamide-induced effects on β-cell function and identity; analyze the impact of the GLP-1R agonist exendin-4 (Ex-4) on glibenclamide-induced effects. Lastly, to analyze the impact of high glucose on β-cell function, viability and identity.

Pancreata from deceased organ donors were processed for islet isolation. Islet preparations above or equal to 80% purity were used for all experiments. Human islets were cultured for 4 to 7 days with glibenclamide, chemical chaperone PBA, Ex-4 or at high glucose. At the end of the culture in the different conditions, islets were collected to determine β-cell function (glucose-stimulated insulin secretion), β-cell apoptosis (TUNEL), ER stress and oxidative stress (gene expression analysis by RT-qPCR and Western Blot), and β-cell dedifferentiation (gene expression and immunohistochemistry).

Human islets cultured with glibenclamide showed a lower stimulation index than control islets due to a higher basal insulin secretion. Islet insulin content was significantly reduced in the glibenclamide group after one week. B-cell apoptosis was increased in the glibenclamide group. Gene expression analyses provided for the first time results showing glibenclamide-induced reduction of insulin and key β-cell transcription factors. Protein expression analyses confirmed the reduction on insulin and some TFs, however

lineage tracing could not corroborate the increase of β-cell dedifferentiated cells in the glibenclamide group. Thus, glibenclamide seems to generally induce loss of β-cell identity without signs of disallowed or progenitor endocrine markers expression. Glibenclamide provoked ER stress observed through the induction of UPR markers. Interestingly, once ER stress was prevented by the chemical chaperone PBA, glibenclamide-induced loss of β-cell identity was avoided. Glibenclamide effects on β-cell function were not prevented by PBA due to the fact that PBA alone exhibited a reduction on the stimulation index.

The addition of Ex-4 concomitantly to the culture medium could not prevent the glibenclamide-induced deleterious effects as hypothesized, although Ex-4 alone did not show any detrimental effects on β-cell function or viability. Moreover, induction of ER stress and loss of β-cell identity was also not prevented. The consideration of another β-cell dedifferentiation inducer such as hyperglycemia was tested, and results demonstrated that high glucose does not recapitulate the gene expression effects observed with glibenclamide. Despite both stressors glibenclamide and high glucose resulted in β-cell dysfunction, the mechanisms underlying such failure are very distinct.

In conclusion, our results provide a demonstration that loss of human β-cell identity and maturity is driven by glibenclamide-induced ER stress. Thus, we present a mechanism of adaptation of primary human β-cells to ER stress through the loss of β-cell identity and maturity. This mechanism may contribute to the decline in functional β-cell mass that is linked to the secondary failure upon sulfonylurea treatment in T2DM, opening up novel therapeutic possibilities. Therefore, reducing ER stress may aid in the preservation of β-cell identity and β-cell function.

V. RESUM

La diabetis mellitus tipus 2 (DM2) és el tipus de diabetis més freqüent (90-95 %) associada tradicionalment a la resistència perifèrica a la insulina que comporta una expansió massiva compensatòria de les cèl·lules β pancreàtiques i hiperinsulinemia durant les etapes inicials de la malaltia. Malgrat aquesta adaptació primerenca, més endavant, els nivells de glucosa en sang no es poden mantenir i condueixen a una fallada progressiva de les cèl·lules β que resulta de la reducció de la funció i de la massa cel·lular β. Per tant, la fallada de cèl·lules β, a causa de la interacció de factors genètics i adquirits, és clau en l'aparició i progressió del la DM2. Originalment, la reducció de la massa funcional de cèl·lules β es va atribuir a l'apoptosi d'aquestes, però nous mecanismes com la desdiferenciació d'aquestes cèl·lules han aportat més llum sobre la matèria. S'ha evidenciat la desdiferenciació de cèl·lules β o la pèrdua d'identitat i maduresa d'aquestes cèl·lules com un mecanisme de fracàs de cèl·lules β durant la glucotoxicitat induïda per l'estrès i les seves vies aigües avall: estrès oxidatiu o de reticle endoplasmàtic (RE), via hexosamina, inflamació i hipòxia. Curiosament, la desdiferenciació de cèl·lules β podria ser un procés reversible, aquestes cèl·lules desdiferenciades podrien potencialment ser rescatades i induïdes a redifferenciar-se en cèl·lules β madures, obrint noves possibilitats per a la prevenció i el tractament de la DM2.

Les sulfonilurees (SUs) són agents hipoglucemiants orals molt utilitzats pel seu menor cost. Aquests fàrmacs indueixen la secreció d'insulina independent de glucosa inhibint el flux de potassi a través dels canals de potassi dependents de l'ATP (K_{ATP}). S'ha observat que després d'un tractament a llarg termini, els pacients comencen a mostrar una falta de resposta al fàrmac (fallada secundaria). No obstant això, el mecanisme cel·lular subjacent a aquest fracàs encara no s'entén del tot. De fet, hi ha alguns estudis en ratolins que suggereixen l'esgotament de les cèl·lules β després de la hiperexcitabilitat crònica i la hipersecreció d'insulina que podria ser induïda per les SUs. No obstant això, no s'ha descrit cap dada concloent en illots humans per entendre aquests efectes de les SUs.

Una nova teràpia DM2 és l'ús d'agonistes del receptor de GLP-1 (GLP-1R). El GLP-1 és una hormona incretina secretada principalment per cèl·lules L enteroendocrines intestinals que contribueix a l'homeòstasi de la glucosa, principalment potenciant la

secreció d'insulina induïda per glucosa en cèl·lules β. El GLP-1 s'uneix al seu receptor específic, GLP-1R, activant diverses vies dins de la cèl·lula β donant lloc a un augment de la biosíntesi de la insulina, una millora de la funció de les cèl·lules β i un augment de la massa cel·lular β. Així com, prevenir l'apoptosi de les cèl·lules β.

La hipòtesi d'aquest treball era que la glibenclamida, una sulfonilurea de segona generació, podria induir la desdiferenciació de cèl·lules β donant lloc a una reducció de la massa funcional de cèl·lules β, la qual cosa podria contribuir a la progressió de la fallada secundària a les sulfonilurees. Tenint en compte els efectes protectors dels agonistes de GLP-1R sobre la viabilitat i la funció de les cèl·lules β, es va plantejar la hipòtesi que aquest procés podria ser atenuat pels agonistes de GLP-1R, donant lloc a la preservació de la funció de cèl·lules β madures. L'objectiu principal del present treball ha estat investigar l'efecte del tractaments de la DM2 i l'entorn diabetogènic sobre el fenotip de cèl·lules β humanes, incloent objectius específics com l'anàlisi de l'exposició crònica a glibenclamida sobre la funció cel·lular β, l'apoptosi de cèl·lules β, i la identitat cel·lular β; dilucidar els mecanismes subjacents que contribueixen als efectes induïts per glibenclamida sobre la funció, viabilitat i identitat de les cèl·lules β; estudiar l'impacte de l'agonista GLP-1R exendina-4 (Ex-4) sobre els efectes induïts per glibenclamida. Finalment, analitzar els efectes de l'exposició a alta glucosa sobre la funció, viabilitat i identitat de les cèl·lules β.

Es van processar pàncrees de donants d'òrgans morts per a l'aïllament dels illots. Es van utilitzar preparats d'illots superiors o iguals al 80% de puresa per a tots els experiments. Els illots humans van ser cultivats de 4 a 7 dies amb glibenclamida, la xaperona química PBA, Ex-4 o amb alta glucosa. Al final del cultiu en les diferents condicions, es van recollir illots per determinar la funció de les cèl·lules β (secreció d'insulina estimulada per glucosa), l'apoptosi de cèl·lules β (TUNEL), l'estrès de RE i l'estrès oxidatiu (anàlisi de l'expressió gènica per RT-qPCR i Western Blot), i la desdiferenciació de cèl·lules β (expressió gènica i immunohistoquímica).

Els illots humans cultivats amb glibenclamida van mostrar un índex d'estimulació més baix que els illots control a causa d'una major secreció basal d'insulina. El contingut

d'insulina dels illots va mostrar una reducció significativa en el grup de glibenclamida. L'apoptosi de cèl·lules β es va incrementar en el grup glibenclamida. Les anàlisis d'expressió gènica van proporcionar per primera vegada resultats que mostraven una reducció induïda per glibenclamida del gen de la insulina i factors clau de transcripció (TF) de cèl·lules β. Les anàlisis d'expressió de proteïnes van confirmar la reducció de la insulina i alguns TF, però el rastreig de llinatges no va poder corroborar l'augment de cèl·lules β desdiferenciades en el grup glibenclamida. Així, la glibenclamida sembla induir una pèrdua general d'identitat β sense signes d'expressió de marcadors endocrins no permesos o progenitors. La glibenclamida va provocar estrès de RE observat mitjançant la inducció de marcadors UPR. Curiosament, quan l'estrès de RE va ser evitat pel PBA, es va evitar la pèrdua d'identitat de les cèl·lules β induïda per la glibenclamida. Els efectes de la glibenclamida sobre la funció de cèl·lules β no van ser impedits pel PBA ja que el PBA sol ja va mostrar una reducció en l'índex d'estimulació.

L'addició d'Ex-4 de manera concomitant al medi de cultiu no va poder evitar els efectes deleteris induïts per la glibenclamida com havíem hipotetitzat, tot i que l'Ex-4 per si sol no va mostrar cap efecte perjudicial sobre la funció o viabilitat de les cèl·lules β. A més, tampoc es va evitar la inducció de l'estrès de RE i la pèrdua d'identitat de les cèl·lules β. Es va considerar un altre inductor de la desdiferenciació de cèl·lules β com és la hiperglucèmia, i els resultats van demostrar que l'alta glucosa no recapitula els efectes d'expressió gènica observats amb glibenclamida. Tot i que tant la glibenclamida com l'alta glucosa van donar lloc a una disfunció de cèl·lules β, els mecanismes subjacents a aquest fracàs són molt diferents.

En conclusió, els nostres resultats proporcionen una demostració de que la pèrdua d'identitat i maduresa de cèl·lules β humanes està impulsada per l'estrès de RE induït per la glibenclamida. Així, presentem un mecanisme d'adaptació de les cèl·lules β humanes a l'estrès de RE a través de la pèrdua d'identitat i maduresa de cèl·lules β. Aquest mecanisme pot contribuir a la disminució de la massa funcional de cèl·lules β que està vinculada a la fallada secundaria al tractament de sulfonilurees en la DM2, obrint noves possibilitats terapèutiques. Per tant, la reducció de l'estrès de RE pot ajudar a la preservació de la identitat cel·lular β i, en conseqüència, a la seva funció.

VI. INTRODUCTION

1. Human pancreas development and histology

The human pancreas is a retroperitoneal organ located in the upper left abdomen behind the stomach attached to the duodenum (**Figure 1**). The pancreas is essential for nutrient metabolism and has both exocrine and endocrine functions. The exocrine pancreas consists of acinar and ductal cells, whose prime role is nutrient digestion. The acinar cells secrete pancreatic juice containing enzymes that catalyze the breakdown of proteins, carbohydrates, and lipids. Functioning largely independently from the exocrine cells, the endocrine pancreas is responsible for regulating glucose homeostasis by releasing hormones into the blood stream. The endocrine pancreas consists of small clusters, called islets of Langerhans, containing five endocrine cell types, namely glucagon-producing α-cells, insulin-producing β-cells, somatostatin-producing δ-cells, pancreatic polypeptide-producing PP cells, and ghrelin-producing ε-cells.

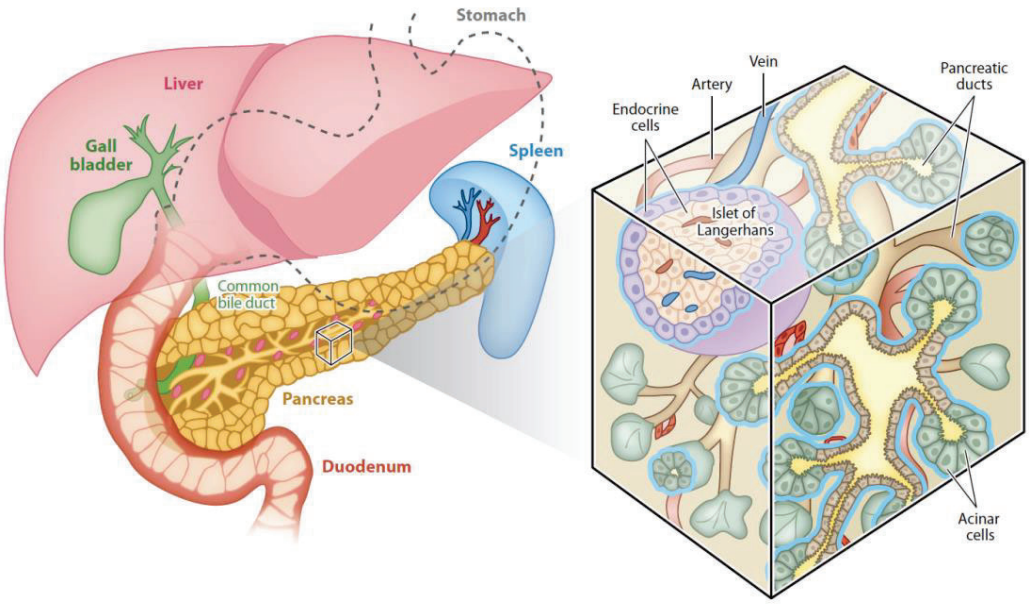


Figure 1. Overview of adult pancreas anatomy. The pancreas lies behind the stomach (outlined in dashed line the picture) in the abdomen and attaches to the duodenum. The inset shows exocrine acinar cells that secrete the pancreatic juice which is transported through a ductal network that released the juice into the duodenum. And the endocrine pancreas which consists of small clusters called islets of Langerhans, containing five endocrine cell types. Pancreatic islets are surrounded by a dense capillary network through which hormones are released into the blood stream. From (Shih et al., 2013).

Pancreas development begins with the outgrowth of the foregut endoderm into a dorsal and ventral pancreatic bud at E9.0-E9.5 (embryonic day 9-9.5) in mice and 26 dpc

(days post-conception) in humans (Gittes, 2009; Pan & Wright, 2011). As a result of gut rotation, the two buds (ventral and dorsal) come into contact with each other, and they fuse. This fusion process occurs at E12-E13 in mice and 37-42 dpc in humans (Gittes, 2009; Jørgensen et al., 2007) to form the main pancreatic duct. Following fusion, the biliary secretions, and pancreatic secretions from the whole pancreas gain access to the duodenum by the ventral pancreatic duct. The specification of different pancreatic cell types culminates in the formation of tip and trunk domains from 12.5 in mice (47 dpc in humans) until birth; at this stage, cells occupying the trunk domain are either duct or endocrine progenitor cells, while acinar progenitors are at the tip (Pan & Wright, 2011; Shih et al., 2013). Acinar cells arise from the extending tip epithelium and continue to undergo active proliferation to increase the number of acinar tips by duplication processes.

After the tip and trunk domains have separated, cells in the trunk undergo extensive morphogenetic changes to form a 3-D network of tubules lined by a single layer of polarized epithelial cells. This network of tubules is referred to as the primitive ducts or progenitor cords and is the epithelium that gives rise to pancreatic endocrine cells. A subset of cells in the progenitor cords initiate expression of the transcription factor (TF) Neurogenin3 (Ngn3), which marks the onset of endocrine cell differentiation. Trunk epithelial cells that do not activate Ngn3 eventually contribute to the ductal tree. During late gestation and in the first weeks of postnatal life, endocrine cells start to coalesce and round up into mature islets (**Figure 2**).

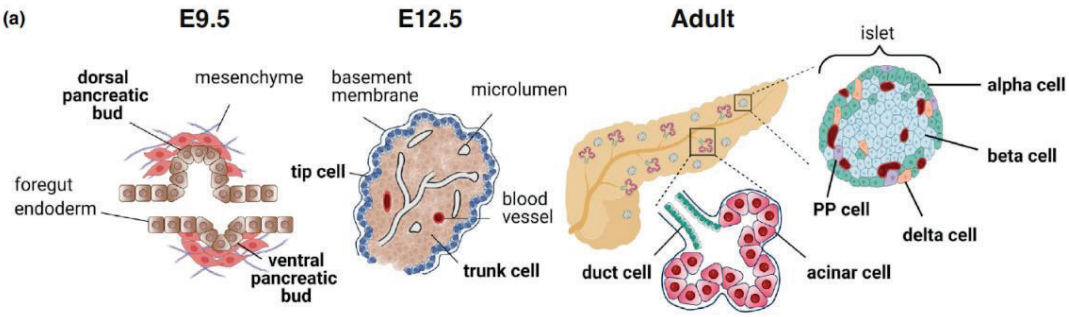


Figure 2. Schematic representation of the developing pancreas. Developmental stages in the mouse embryo and after birth. PP, pancreatic polypeptide. From (Isaacson & Spagnoli, 2021).

Endocrine differentiation and maturation

Many transcription factors (TFs) have been identified to function in endocrine subtype lineage allocation, differentiation, and maturation (Pan & Wright, 2011). These TFs include *Pdx1, Mnx1, Nkx2.2, Nkx6.1, Pax4, Pax6, Arx, Foxa1, Foxa2, HNF4, Islet1, Insm1, Rfx6, MafA and MafB*. They can be divided into 3 classes: (1) general endocrine differentiation (*NeuroD1, Islet1, Insm-1, Rfx6, Nkx6.1*), (2) lineage-allocation (*Pdx1, Pax4, Arx, Nkx2.2*), and (3) maturation factors (*MafA, MafB, Foxa1, Foxa2, NeuroD1*).

(1) General endocrine factors facilitate the formation of proper endocrine cell numbers by maintaining survival and/or stimulating proliferation of endocrine progenitors/intermediates. Several TFs that fall into this category (*e.g.*, *NeuroD1, Insm-1, Rfx6, and Islet1*) are downstream, primarily direct targets, of Ngn3. Nkx6 family TF genes (*Nkx6.1* and *Nkx6.2*) could be the only non-Ngn3 direct target that are included in this class. Nkx6 might act upstream of *Ngn3* since *Nkx6.1* or *Nkx6.1/Nkx6.2* null mice have dramatically reduced number of $Ngn3^{+}$ endocrine progenitors and islet precursors (Henseleit et al., 2005).

(2) Lineage-specific factors control the flux of endocrine progenitors towards a specific endocrine type. As a consequence of removing such factors, endocrine cell numbers are largely unaffected, but fractional islet allocations are often severely altered. *Nkx2.2, Pax4, Arx* and *Pdx1* mutants highlight the extreme lineage-switching caused by such single factor removals. Pax4 also directly inhibits expression of *Arx*, which encodes a homeodomain TF that is key α-cell specification factor (Collombat et al., 2003). Pax4 and Arx are required for β-cell and α-cell allocation, respectively. Pdx1, apart from its early roles in multipotent pancreatic progenitor cells (MPC) outgrowth and expansion, is also required for β-cell proliferation and survival. It has been also proposed that one of the β-cell functions is to inhibit proliferation of adjacent islet cell types, and that endocrine sub-type intercommunication is required to generate the appropriate proportions of the various endocrine subtypes (Gannon et al., 2008).

(3) Maturation factors seem to control later aspects of the final move towards physiological readiness, although in some cases there may be a degree of overlap

with a harder to decipher role in the preceding phases of lineage allocation and commitment. Loss of these factors causes aberrant function postnatally or in adulthood. *MafA/B* are expressed relatively later in development and are essential to acquire and maintain the mature state of hormone-expressing cells by activating genes important for β-cell function (*insulin, Pdx1, GLUT2, Nkx6.1, G6pc2*). Expression of *Foxa2* begins very early in the definitive endoderm, and persists throughout the early pancreatic epithelium. *Foxa1*/*Foxa2* maintain the normal physiological status of the mature pancreas controlling multiple aspects of insulin secretion by regulating many genes involved in glucose sensing, metabolism, and granule exocytosis machinery (N. Gao et al., 2007, 2010). NeuroD1 is required for the complete transition to β-cell maturity, and maintenance of full glucose-responsiveness (Gu et al., 2010).

Differences between rodent and human

Human pancreas formation largely mimics the process in the mouse (Pan & Wright, 2011). Dorsal and ventral pancreas budding is first evident at 26-35 dpc, and fusion of the anlagen occurs at 6 weeks of gestation (G6w, gestation 6 weeks). The dorsal bud produces most of the head, body, and tail of the mature pancreas, whereas the ventral bud contributes to the inferior part of the head of the organ, the uncinate process (Piper et al., 2004). Although only limited studies have been performed, PDX1, NGN3 and Islet1 (ISL-1) have been immunolocalized at a few stages in human fetal pancreas (Hanley et al., 2010; Piper et al., 2004). The spatiotemporal patterns of these TFs suggest that similar transcriptional regulatory mechanisms operate in both human and mice (**Figure 3**). Pdx1 is found in pancreatic progenitors broadly through the epithelium from G7w onwards, and later in insulin-producing cells. $Ngn3^{+}$ cells are found scattered in the epithelium at G8w, concomitant with the emergence of β-cells at G8w, which is followed by α-cells, and δ-cells at G9w (Jeon et al., 2009). A rare population (<5%) of insulin+/glucagon+ cells was also reported in the early human fetal pancreas.

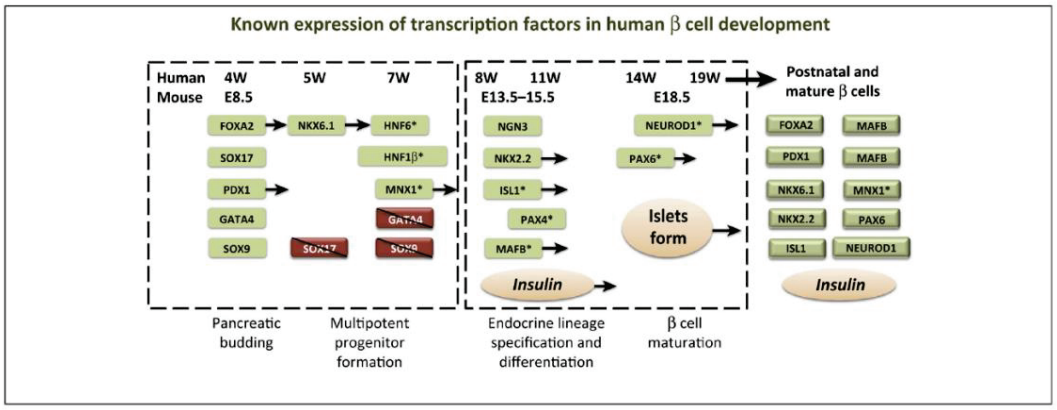


Figure 3. Approximate timeline of transcription factor expression during human β-cell development. E: embryonic day; W: weeks gestation. From (Conrad et al., 2014).

Humans and mice are different in their endocrine specification. In humans, the mono-hormonal insulin-secreting cells appear first (Jennings et al., 2013), while poly-hormonal cells appear between 9 and 16 weeks of gestation after development of mono-hormonal cells; these two stages are reversed in mice, with the poly-hormonal cells appearing first.

Among the different TFs directly implicated in endocrine differentiation and moreover, playing critical roles in both formation and maintenance of function of the endocrine compartment during adulthood we would like to highlight:

Sox factors

Sox17 and Sox9 are involved in pancreatic development, with *Sox17* expressed at an early stage of pancreatic development, subsequently disappearing from pancreatic cells (Jennings et al., 2013). Sox9 is involved in proliferation, survival, and maintenance of pancreatic progenitors. The first-wave of Sox9-positive progenitors produce cells of all three pancreatic lineages. *Sox9* expression then becomes limited to the trunk ductal/endocrine progenitor domain during the secondary transition, while at later stages of pancreas development Sox9 expression is restricted to ductal cells. Mature endocrine cells do not express *Sox9*, but this factor activates the pro-endocrine gene *NGN3* (Lynn et al., 2007).

Forkhead box factors

Forkhead box A1, A2, and O1 (FOXA1, FOXA2, and FOXO1) are involved in pancreatic development and their expression starts in the early stages of pancreatic development. Foxa1 and Foxa2 are co-localized in the foregut endoderm, which differentiates into the pancreatic buds. The highest expression levels of both genes are found in pancreatic islets (N. Gao et al., 2008). Both Foxa1 and Foxa2 are important for the development and functionality of pancreatic β cells. Different functions for FoxO1 in β-cells have been reported, particularly in maintaining pancreatic β-cell identity (Benner et al., 2014; Kitamura et al., 2005). In β-cells, FOXO1 is normally found in the cytoplasm, but mild hyperglycemia causes nuclear translocation of FOXO1 (Talchai et al., 2012), possibly contributing to the maintenance of MAFA and NEUROD1 expression and preservation of β-cell function (Kitamura et al., 2005).

Pancreatic and dudodenal homeobox 1 (PDX1)

PDX1 is expressed in all pancreatic precursor cells and appears to be crucial for early pancreatic development. During early endocrine specification, *PDX1* expression disappears, while at later stages of β-cell development its expression is upregulated. During embryonic development, expression of *Pdx1* in the duodenum is considered to be the first evidence of pancreatic morphogenesis, with continued expression in the bud area throughout pancreatic development, later becoming localized to β and δ-cells. In humans, *PDX1* expression begins at 4 weeks and later becomes restricted to β-cells (Jennings et al., 2013). In adult pancreas, *PDX1* expression is not restricted to islet cells, as it is detected in the exocrine pancreas in mice and humans (Pan & Wright, 2011). PDX1 also regulates the expression of some genes including insulin, glucose transporter 2 (*GLUT2*), glucokinase (*GCK*), and islet amyloid polypeptide (*IAPP*), that are crucial for β-cell function. Pdx1 has been previously reported to be indispensable for the maintenance of β-cell identity, mainly by repressing the α-cell program (Chera et al., 2014; T. Gao et al., 2014; L. Guo et al., 2013).

Homeobox protein NKX family

Three homeobox protein NK-homolog genes (Nkx2.2, Nkx6.1, and Nkx6.2) are expressed during pancreatic development. Nkx6.1 plays an essential role in the development of pancreatic β-cells as well as in the insulin secretion process. The pattern of Nkx2.2 expression shows differences between mice and humans. In mice, Nkx2.2 is expressed in MPCs before development of endocrine progenitors (Sussel et al., 1998). However, in humans, NKX2.2 is not detected in MPCs of the fetal pancreas, but appears after endocrine specification (Jennings et al., 2013). Nkx2.2 is important in maintaining pancreatic β-cell identity (Papizan et al., 2011) and lies upstream of Nkx6.1 (Sander et al., 2000). Nkx2.2 stimulates *Neurod1* expression in pancreatic progenitors, which is crucial for β-cell development (Mastracci et al., 2013). Nkx6.1 and Nkx6.2 are important for islet endocrine cell development, however, the loss of Nkx6.2 alone is compensated by Nkx6.1 (Henseleit et al., 2005).

Neurogenin 3 (NGN3)

NGN3 marks islet cell precursors and can drive the early differentiation of islet cells into endocrine progenitors, which later can further differentiate into different cell types; NGN3 thus can be considered a marker for the pancreatic endocrine progenitors. Induction of Ngn3 during pancreatic differentiation causes the activation of several TFs, including *Pax4*, *Arx*, *Nkx2.2*, *Nkx6.1*, *Isl-1*, and *Neurod1*. These TFs are responsible for differentiating the endocrine precursors into mono-hormonal islet cells (Gouzi et al., 2011). Ngn3 has been generally considered a marker for putative endocrine progenitors because is transiently expressed in endocrine progenitors before disappearing in mature endocrine cells. However, reports in mice and humans pancreas have demonstrated that Ngn3 is also expressed in differentiated adult mice islets (S. Wang et al., 2009) and human exocrine pancreas (D. L. Gomez et al., 2015).

Neurogenic differentiation factor D1 (NEUROD1)

In humans, expression of the neurogenic differentiation factor 1 (NEUROD1), a downstream target of NGN3, starts at gestational week 15 and continues to be

expressed in the endocrine cells of mature islets (Jennings et al., 2013; Jeon et al., 2009). In mouse, Neurod1 has been suggested to play a role in assembling the insulin exocytotic machinery, and being essential for downregulation of genes that impair insulin secretion in β-cells (Gu et al., 2010). Additionally, a regulatory role for Neurod1 in endocrine-cell type specification in mice has been proposed, where its repression by Nkx2.2 in Pdx1-positive progenitors generates α-cells, whereas its activation generates β-cells (Mastracci et al., 2013). Interestingly, NEUROD1 interacts physically with other key TFs such as INSM1 and FOXA2, and co-occupies β-cell-specific regulatory elements, wherein mutations were shown to result in β-cell dysfunction in humans (Roscioni et al., 2016).

Paired box-encoding gene (PAX) family (PAX4 and PAX6)

Pax4 expression begins during pancreatic development and at a later stage, its expression becomes restricted to β-cells. Pax4 is downstream of Ngn3 and is activated after the induction of Nkx6.1 and Nkx2.2 (Gradwohl et al., 2000). Pax4 and Arx exhibit transcriptionally antagonistic actions to each other; they are involved in islet cell lineage bifurcation, contributing to specification towards α/PP and β/δ cell fate, respectively (Collombat et al., 2005). Pax4/Arx activation may also serve as an on/off switch between α/β cells, as illustrated by induced neogenesis and a consequent shift into cells exhibiting a β-cell phenotype on forced expression of Pax4 in embryonic glucagon^{+} cells (Collombat et al., 2009). Pax6, is required for pancreatic endocrine cell development. In the human pancreas, the expression of PAX6 starts at 14-16 weeks and continues in all mature islet cells (Lyttle et al., 2008).

V-musculoaponeurotic fibrosarcoma oncogene homolog (MAF)

During the later stages of β-cell differentiation, the MAFB and MAFA TFs play a prominent role. MAFB expression in β-cells precedes an increase in PDX1 expression that marks the start of insulin transcription. MAFA expression follows shortly after insulin expression (Artner et al., 2006) and persists in adult β-cells. MAFA is essential for the development of pancreatic β-cells, but MAFB is essential for the development of both α and β-cells (Artner et al., 2010). Involvement of MafA in regulation of insulin production has been reported by multiple studies (H. Wang et al., 2007; C. Zhang et al.,

2005). Interestingly, in mice, MafB disappears from the pancreatic β-cells after birth (Artner et al., 2010) but its expression continues in human β-cells (Dai et al., 2012).

GATA factors

The GATA binding protein 4 (GATA4) is expressed during early human pancreatic budding (between 4 and 5 weeks of age) and at the same time as PDX1, implying a role of GATA4 in human pancreas development (Jennings et al., 2013). GATA4 expression then becomes significantly reduced in pancreatic progenitors, persisting only in mature acinar cells. Gata6 expression has been localized to endocrine pancreas and islets and Gata6, but not Gata4, interacts physically with Nkx2.2, thus Gata6 plays an important role in the pancreas specification as well as in the development of endocrine cell types (Decker et al., 2006). GATA6 has also been shown to be more important during human pancreatic development than in mice (De Franco et al., 2013).

1.1. Endocrine pancreas. Islets of Langerhans

The endocrine fraction of the pancreas represents around 1.5% of the pancreatic volume, embedded within the acinar tissue. It is primarily formed by the islets of Langerhans, first described by the German pathological anatomist Paul Langerhans in 1869, which are intermingled with blood vessels, neurons, and a mesodermally-derived stromal component. The intimate interaction between endocrine and vascular cells regulates hormone release, establishing a fine-tuned glucose homeostasis in the body.

The pancreas of a healthy adult contains on average 1.65 million islets. These islets range widely in size, after isolation, their sizes are frequently estimated by normalizing to spherical structures with a 150-µm diameter (islet equivalent or IEQ). Such a theoretical islet equivalent contains about 12-14 ng of insulin, for a total of ~10.5 mg of insulin in the entire pancreas (Henquin, 2019), consisting of 1500-2000 cells. Islets typically consist of four different secretory cell types; glucagon-producing α-cells, insulin-producing β-cells, somatostatin-producing δ-cells and PP producing cells. In recent years a fifth mammalian islet cell type, the ghrelin-producing ε-cell has also been described (Wierup et al., 2002).

There has been prolonged controversy over important functional differences between the human “mixed-islet” architecture and the canonical core (β-cell) and mantle (other endocrine) structure that is found in mice (**Figure 4**).

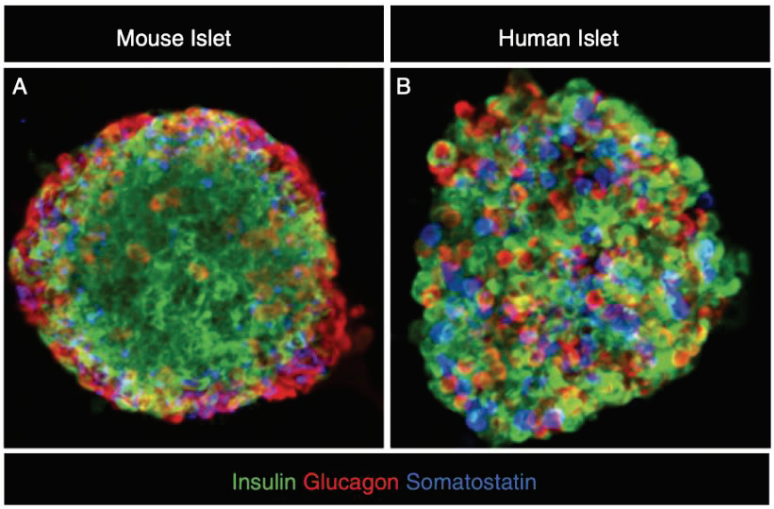


Figure 4. Islet morphology differences between mice and humans. (A) Mouse and (B) human islets labelled for insulin (green), glucagon (red) and somatostatin (blue). Mouse islets have been considered to have the majority of β-cells located in the islet core that contacts other β-cells. By contrast, human islets have a greater or similar proportion of α-cells to β-cells, and β-cells more frequently have direct contact with α-cells. From Aamodt and Powers (Aamodt & Powers, 2017).

Islet-like clusters appear from approximately G11w in human pancreas. Unlike the mixed-islet architecture observed in human adult pancreas, the aggregated insulin and glucagon-expressing cells in the human fetal islets (around G14-16w) seem to be arranged similarly to the mouse adult islet (Jeon et al., 2009) with a β-cell core and peripheral mantle of α and δ-cells. It has been proposed that at G19w there is a transient separation of peripheral α-cells and δ-cells, away from the β-cell core, to form juxtaposed homogeneously mono-hormone-producing clusters. Presumably, the mono-hormone islets reintegrate amongst each other after G22w, and there is significant intermixing to generate the adult islet architecture.

The rodent and human pancreas show significant differences in the proportion of individual hormone-producing cell types, although as in mice any specific islet cell has

its origin in multiple progenitors (Scharfmann et al., 2008). Greater numbers of PP cells are located in the human head region, with more α-cells and β-cells in the neck, body and tail regions. Mouse islets comprise ~ 75% β-cells, ~ 20% α-cells, and ~ 5% other endocrine cells; while human islets contain ~ 50% β-cells, ~ 40% α-cells, ~ 10% δ-cells, and a few PP cells (Brissova et al., 2005). Mouse islets are dependent on homotypic cellular communication between β-cells, resulting in the formation of a functioning syncytium with coordinated islet activity, whereas human islets are rich in heterotypic islet cell contacts (Bosco et al., 2010; Stožer et al., 2013). The importance of these differences and any relevance to endocrine cell function and glucose/energy metabolism will be reviewed in the next section.

1.1.1. B-cells

B-cells reside within the islets of Langerhans scattered throughout the exocrine pancreas and are the most abundant cell type in islets. B-cells play a crucial role in controlling normoglycaemia, thanks to its ability to synthesize and secrete the polypeptide hormone insulin. Insulin synthesis starts with the precursor preproinsulin, which is cleaved into proinsulin in the rough endoplasmic reticulum (ER) of the β-cell. Proinsulin, a more stable peptide, is packaged into immature granules within the Golgi network and is matured by the action of the enzyme prohormone convertase 1/3 (PC1/3) into active insulin (59). This maturation process creates two different peptides: insulin and C-peptide, with 6Kb and 3Kb, respectively (**Figure 5**). Both insulin and C-peptide are stored in secretory granules until released on metabolic demand. In β-cells, processed insulin is stored in secretory granules that have an electron-dense core in ultrastructural analysis (T. Kim et al., 2006). These intracellular dense core granules harbor several protein components, including proinsulin, insulin, islet amyloid polypeptide (IAPP), granins encoded by *chromogranin A* (*CHGA*) and *chromogranin B* (*CHGB*), and transmembrane proteins like IA2 (also called ICA152) (T. Kim et al., 2006; Suckale & Solimena, 2010).

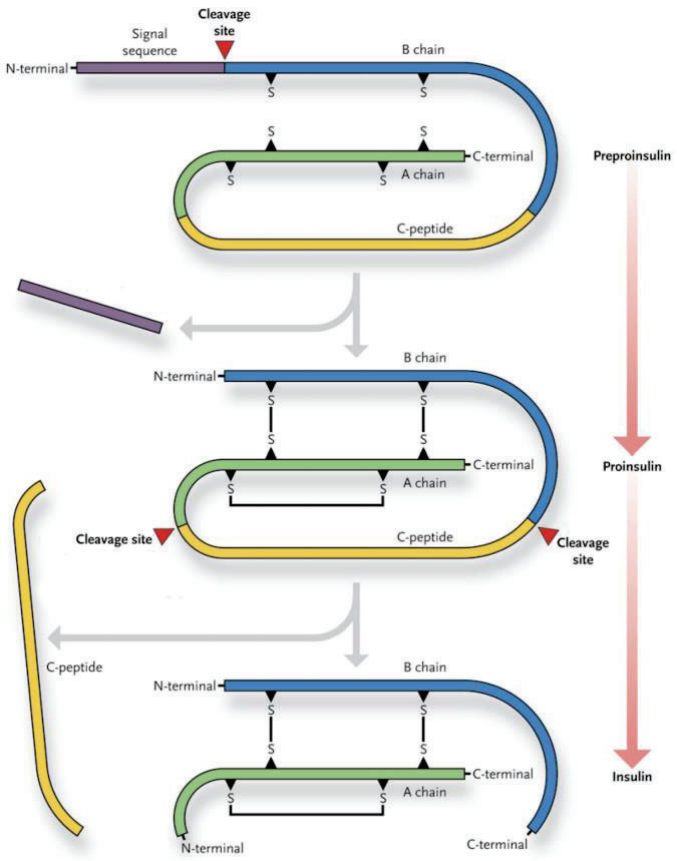


Figure 5. Schematic representation of insulin biosynthesis (adapted from (Kaufman, 2011)**.**

Most of the current understanding of β-cell maturation is derived from rodent studies. In mouse, embryonic β-cells are described as immature, highly proliferative and unipotent though still plastic. At this stage β-cells present insulin granules and display high basal insulin secretion. Right after birth, β-cells appear to be abundant and organized in proto-islets (Bastidas-Ponce et al., 2017). However, at this stage these cells have not yet acquired a mature phenotype to properly secrete insulin in response to glucose levels (Martens et al., 2013). The events that conclude maturation occur in postnatal stages, following a biphasic pattern of maturation (Dore et al., 1981) that is adapted to the neonatal type of diet. The first wave of maturation takes place during the first two weeks after birth where there is an increase of the endocrine mass, β-cells are still proliferative, characteristic that is progressively lost (Bonner-Weir et al., 2016) and acquire all the TFs and machinery necessary for the establishment of adult β-cell identity. The second wave coincides with the diet change from milk-based diet to solid-

adult diet that occurs with the weaning period (third week of life) where β-cells define their mature functional landscape by differentially regulating molecular and metabolic changes. This process can be described by three aspects that contribute to the complex identity of mature β-cells: markers, functionality and signaling pathways.

Markers of β-cell maturation

At birth, β-cells possess signature genes that are important for the establishment and maintenance of identity: *Nkx2.2*, *Pdx1*, *Nkx6.1* and *Neurod1* for example. The acquisition of urocortin3 (Ucn3) marks the early phase of postnatal maturation. The role of Ucn3 is still not completely known. UCN3 is expressed, in humans, in both α and β-cells (Van der Meulen et al., 2012). Similarly, a member of Synaptotagmin (Syt) family, Syt4, is upregulated during postnatal stages. The role of this protein is fundamental for the reduction of calcium sensitivity, high in immature β-cells, that directly regulates the exocytosis of insulin granules (Chen Huang et al., 2018). Contrary to Ucn3 and Syt4, Neuropeptide Y (NPY) marks immature β-cells in both mouse and human. Its role seems to contribute to the maintenance of the immature phenotype, promoting proliferation and high basal insulin secretion, referred as secreting high insulin levels in response to low glucose stimuli (Rodnoi et al., 2017).

Among TFs, the Maf family is important for the establishment of β-cell identity and functionality. In rodents *MafB* is highly expressed in embryonic β-cells and is rapidly downregulated after birth, and is progressively substituted by *MafA* marking the late phase of postnatal maturation. Different from rodents, in humans, *MAFB* remains highly expressed in adult β-cells (Conrad et al., 2015; Dai et al., 2012). However, a recent report has demonstrated a role of MafB related to the maintenance of β-cell identity found in rodent islets (Z. Deng et al., 2022).

Another marker recently characterized is Flattop (Fltp), a Wnt/Planar Cell Polarity (PCP) effector protein. This marker has been described to distinguish an immature pool ($Fltp^-$) and terminally mature ($Fltp^+$) β-cells. $Fltp^-$ β-cells are characterized by a higher proliferation rate and differential mRNA expression of G-protein-coupled receptors (*GPCR*), Wnt and MAPK signaling components. The $Fltp^+$ cells feature higher expression

of functional genes (i.e., *Slc2a2, Nkx6.1, Ucn3, MafA*), increased number of secretory granules, enhanced mitochondrial physiology and higher glucose-stimulated insulin secretion (GSIS) (Bader et al., 2016). Similarly, in humans, the surface marker ST8SIA1 and CD9 distinguish four antigenic subgroups of β-cells, β1 through β4, in adult islets. Interestingly, each subpopulation had distinct gene expression profiles and insulin secretion capabilities; the most abundant β1 population had the lowest basal insulin secretion, whereas the β4 population displayed the highest insulin secretion capabilities (Dorrell et al., 2016).

Functional characteristics of mature β-cells

The presence or absence of certain markers is associated with functional characteristics that distinguish immature from mature β-cells. However, not all β-cells develop at the same time and some maintain distinct immature characteristics during adult life. This heterogeneity mostly appears during the perinatal age and it is maintained in adulthood. Several studies have demonstrated the existence of different types of β-cells within the islets (Bader et al., 2016; Dorrell et al., 2016; Van der Meulen et al., 2017). The ratio of the different subpopulations often shifts under metabolic stress or diabetes progression.

The proliferative capacity of β-cells, both in mouse and humans, is strictly confined to the early stages of life or to high demanding physiological conditions such as pregnancy, demonstrated in rodents (Baeyens et al., 2016). In healthy adult conditions, only a small pool of β-cells retain proliferative capacity, which is linked to an immature functional phenotype (Bader et al., 2016; Marta Szabat et al., 2009).

A mature β-cell is exquisitely sensitive to nutrient environment and can respond to extremely small changes in blood glucose concentrations (between 4.5 and 8 mM in humans), triggering significant changes in insulin secretion within minutes through the complex process of stimulus-secretion coupling (Heimberg et al., 1993). The biological processes beneath the GSIS are activated only during the postnatal development. In fact, the neonatal islets are considered as “leaky”, showing high basal insulin secretion (Blum et al., 2012; Henquin & Nenquin, 2018). Once the key components are acquired,

such as Syt4, the exocytosis of insulin granules is less sensitive to low calcium levels, therefore β-cells secrete low insulin concentrations at low glucose levels and high insulin concentrations at high glucose concentrations, in a biphasic fashion (Z. Wang & Thurmond, 2009). It has been known for nearly 40 years that insulin secretion is biphasic (Curry et al., 1968), and that insulin concentrations in plasma increase rapidly to a peak at 2–4 min, decrease to a nadir at 10–15 min, and then gradually increase progressively to a pseudo-steady state at 2–3 h. The initial spike response is generally referred to as first-phase insulin release, and the subsequent increase in insulin secretion is considered to represent the second-phase insulin release.

Thus, the GSIS is the key functional characteristic of mature β-cells. The current consensus attributes glucose-induced insulin secretion to activation of two main pathways: the triggering pathway (K_{ATP} channel-dependent pathway) and amplifying pathway (K_{ATP} channel-independent pathway) (Henquin et al., 2009). First, β-cells sense glucose, which enters via the specific glucose transporter (Glut2 for rodents, GLUT1 for humans (De Vos et al., 1995; McCulloch et al., 2011)). The expression of this transporter is one of the main functional characteristics that distinguish a mature from an immature or diseased β-cell. After entering to the β-cells, glucose is quickly converted into glucose-6-phosphate (G6P) by GCK, a low-affinity type IV hexokinase. GCK regulates the rate of glucose utilization and is therefore considered as the glucose sensor in β-cells (Matschinsky, 2009). G6P is redirected in the two main metabolic processes: glycolysis and oxidative phosphorylation. The events result in production of ATP and other molecules that also serve as coupling factors. The increase of the cytoplasmic ATP/ADP ratio leads to the closure of the ATP-sensitive potassium (K_{ATP}) channels that generates depolarization of the plasma membrane. This triggers the membrane action potential that opens the voltage-gated Ca^{2+} channels. The influx of this ion results in increased intracellular calcium concentration, $[Ca^{2+}]_i$, which in turn, triggers exocytosis of the insulin vesicles by fusion of the insulin granules to the plasma membrane in a soluble *N*-ethyl-maleimide-sensitive factor attachment protein receptor (SNARE)-dependent process (**Figure 6**). Following its release into the bloodstream, secreted insulin acts on the target organs (chiefly the liver) where it promotes glucose storage until normal

blood glucose is restored. When this has occurred, insulin secretion stops by the reversal of the process described. Thus, insulin secretion is under feedback control via changes in plasma glucose.

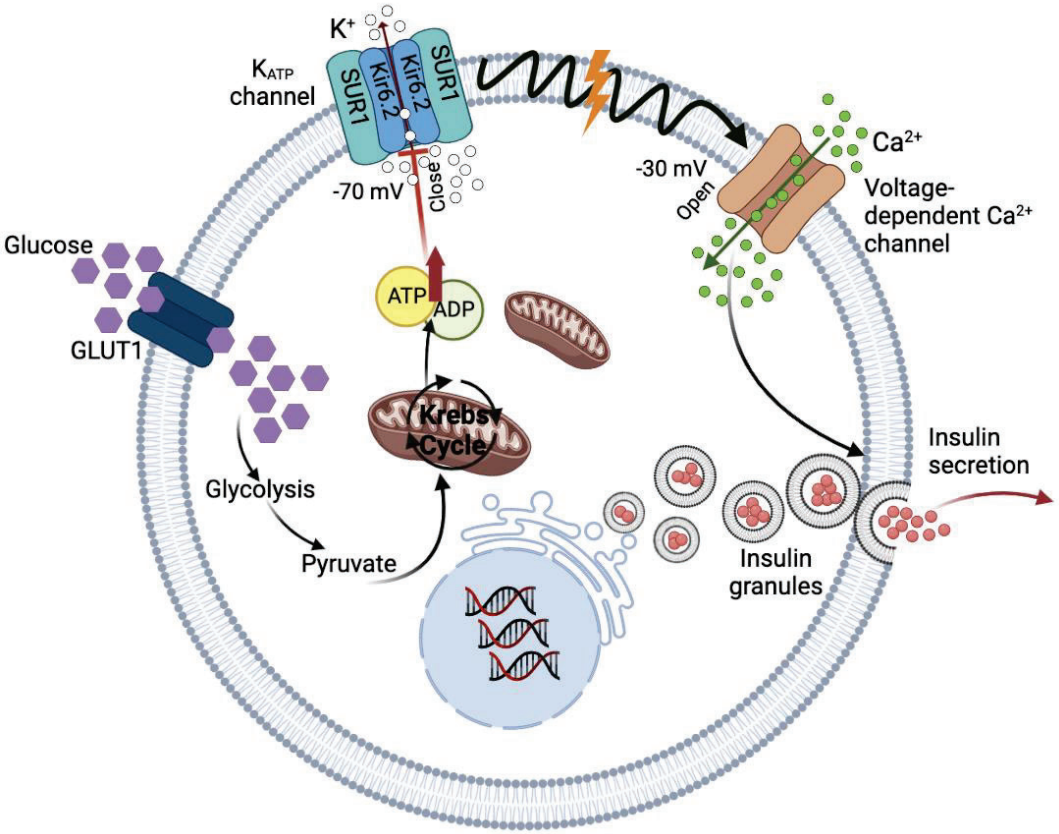


Figure 6. Glucose stimulated insulin secretion pathway in pancreatic β-cells. Glucose enters the β-cell via GLUT1 (in humans) where through glycolysis and oxidative phosphorylation an ATP/ADP ratio shift occurs, inhibiting the K_{ATP} channels, resulting in membrane depolarization and insulin release. Created in *BioRender.com.*

β-cell electrical activity is tightly correlated with insulin secretion (Rorsman & Ashcroft, 2018). At low (<3 mM) glucose (when insulin is suppressed), ATP/ADP ratio is quite low and the K_{ATP} current is large enough to keep the plasma membrane hyperpolarized (at ~ -70 mV) close to the equilibrium potential K^+, which maintains β-cells electrically silent. Increasing glucose concentrations ≥6 mM (i.e. close to the normal plasma glucose levels) induces membrane depolarization. When the membrane potential reaches a threshold of ~ -50 mV, the β-cells start generating oscillatory

electrical activity consisting of bursts of action potentials that originate from depolarized plateau separated by electrically silent repolarized intervals. The burst of action potentials represent intermittent opening of voltage-dependent Ca^{2+} channels, leading to increases in cytosolic [Ca^{2+}] that trigger exocytosis of the insulin-containing secretory granules by mechanisms similar to those involved in neurotransmitter release (Rorsman & Ashcroft, 2018). Hence, bursts of action potential induce oscillations of $[Ca^{2+}]_c$, whereas continuous electrical activity induces a sustained $[Ca^{2+}]_c$ increase (Gilon et al., 2014).

In β-cells, the glucose-induced membrane depolarization and associated initiation of action potential firing are mediated by changes in K_{ATP} channel activity. Under hypoglycemic conditions, K_{ATP} channel activity is high because of a low cytoplasmic ATP/ADP ratio and the combination of a negative membrane potential and low membrane resistance prevents electrical activity (Q. Zhang et al., 2020). When plasma glucose rises, accelerated glucose uptake into the β-cells and the associated stimulation of metabolism increase the cytoplasmic ATP/ADP ratio and thereby causes K_{ATP} channel closure.

Of note, many other factors can influence insulin secretion, as the amino acids arginine and leucine, or the hormone GLP-1. Lipid metabolism is also important for regulating as well as modulating insulin secretion (Nolan et al., 2006).

In contrast to this established triggering pathway, the metabolic amplifying pathway is more complex, and its mechanism is less well studied. It has been suggested that metabolic signals generated by glucose, such as ATP (Detimary et al., 1994; Eliasson et al., 1997; N. Takahashi et al., 1999), as well as, intracellular signals such as cAMP, DAG and IP_3, evoked by hormonal and neuronal inputs (Henquin, 2009), are important for normal regulation of insulin secretion but this pathway does not function if the triggering pathway is not operational. It requires a high $[Ca^{2+}]_c$ and it involves an amplification by glucose of the efficacy of cytosolic Ca^{2+} on granule translocation and/or exocytosis (Henquin, 2000; Ravier et al., 2010). The amplifying action of glucose thus augments insulin secretion which seems to account for up to 40-50% of the global

response. Early human studies of insulin secretion distinguished initiating and potentiating actions of glucose (Cerasi, 1975; Pfeifer et al., 1981). The greater response to non-glucose stimuli at elevated blood glucose levels has been attributed to a potentiating action of glucose, which becomes defective in subjects with type 2 diabetes (Ward et al., 1984).

Regarding the dynamics of insulin granule exocytosis is thought to involve several steps, including recruitment, docking, priming and fusion (Eliasson et al., 2008). It has been suggested that secretory vesicles in pancreatic β-cells exist in functionally distinct pools and that the sequential release of these pools underlies the separable components in the dynamics of exocytosis (Rorsman & Renström, 2003). Pancreatic β-cells contain at least two pools of insulin secretory granules that differ in release competence: a reserve pool (RP) accounting for the vast majority of granules, and a readily releasable pool (RRP) accounting for the remaining <5% (Straub et al., 2004).

Recent studies that addressed the topic of the functional β-cell heterogeneity in healthy adult islets have shown that putative pacemaker β-cells, called “hub” or leader cells, are heavily connected with neighbor cells and through rhythmic calcium oscillations coordinate the electrophysiological response to glucose stimuli (Johnston et al., 2016; Salem et al., 2019). Additional subpopulations of β-cells, including “extreme” β-cells located in the center of the islet, that have a distinct polarization pattern and higher proinsulin and ribosomal RNA content (Farack et al., 2019); and “virgin” β-cells, which are postulated to represent a population of immature β-cells that form a neogenic niche at the periphery of the islet, have also been identified (Van der Meulen et al., 2017).

Signaling pathways activated in mature β-cells

To understand the β-cell maturation process it is important to include what is known regarding the signaling pathways that are differentially regulated. The mammalian target of rapamycin (mTOR) pathway orchestrates different cellular activities including proliferation and growth (Saxton & Sabatini, 2017) in response to nutrients and survival signals. This pathway is also the predominant pathway activated in early postnatal β-cell

development. The beginning of the oral feeding after birth, the transition from blood supplied nutrition type to an enteral one, induces the upregulation of the mTOR signaling pathway. The mTORC1 complex contributes to the progression of functional maturation, while the mTORC2 branch regulates islet architecture and mass (Sinagoga et al., 2017).

Another pathway that has been described in the context of β-cell maturation is 5' AMP-activated protein kinase (AMPK) signaling. It is a cellular energy and stress sensor, responding to the available intracellular levels of ATP (Lin & Hardie, 2018). This pathway is already target of diabetes treatments and it is known to be involved in the regulation of β-cell mass and insulin secretion, among others (A. Fu et al., 2013). The weaning process, switching from high-fat milk to high-carbohydrate food, induces activation of AMPK signaling, which antagonizes and inhibits mTORC1 complex. Thus, AMPK signaling is at the center of the second phase of maturation, inhibiting the mTORC1 pathway and enhancing mitochondrial biogenesis and oxidative metabolism (Jaafar et al., 2019).

The Wnt signaling pathway is known to be fundamental for the endocrinogenesis process during the embryonic development (Scheibner et al., 2019; Sharon et al., 2019). For instance, the Wnt/PCP effector protein, Fltp, has been used to demonstrate the association between non-canonical Wnt signaling and the β-cell maturation phenotype. However, the molecular mechanisms through which Wnt/PCP regulates the maturation process have not been deciphered yet.

Differences between rodent and human

Besides differences in islet cytoarchitecture, rodent and human islets differ substantially in cell proliferation (Spears et al., 2021), being the proliferation rate of human β-cells much lower than in rodents; susceptibility to destruction (Eizirik et al., 1994), being human islets more resistant than rodent islets and β-cell function regarding the glucose threshold for the secretion of insulin (Harrison et al., 1985; Henquin et al., 2006) and the ionic channels expressed (Drews et al., 2010; Rorsman & Ashcroft, 2018; Rorsman & Braun, 2013). Two genes encode insulin in rodents, *Ins1* and *Ins2*, whereas only one gene, *INS*, is present in humans (Melloul et al., 2002). Strikingly, human islets

secrete more insulin at baseline glucose levels, but less in response to a stimulatory glucose challenge compared to rodent islets (Dai et al., 2012). Furthermore, human β-cells differ from rodent β-cells in glucose transporter gene expression (predominantly GLUT1 instead of GLUT2) (Ferrer et al., 1995). The 100-fold lower GLUT2 abundance in human versus rat β-cells is associated with a 10-fold slower uptake of alloxan, explaining their resistance to this rodent diabetogenic agent. Human and rat beta-cells exhibit comparable GCK expression with similar flux-generating influence on total glucose utilization (De Vos et al., 1995).

Additionally, regarding the electrical activity of β-cells within an islet, in the mouse appears to be synchronized because of electrical coupling through gap junctions (Ravier et al., 2005). Therefore, $[Ca^{2+}]_c$ oscillations are synchronized between β-cells and induce oscillations of insulin secretion that can be detected at the level of single islets (Bergsten et al., 1994; Gilon et al., 1993). The role of connexin 36 is essential in this coupling, as seen in mice islets (Gilon et al., 1993). In human islets, although coupling that involves connexin 36 is also apparent (Serre-Beinier et al., 2009), "connectivity" is less than in mice islets, perhaps because of the more heterogeneous disposition of the various cell types where β-cells are separated from each other by non-β-cells (Cabrera et al., 2006; Hodson et al., 2013; Quesada et al., 2006; Serre-Beinier et al., 2009).

2. Type 2 Diabetes Mellitus

Diabetes Mellitus comprises a group of heterogeneous disorders characterized by hyperglycemia that results from defects in insulin secretion, insulin resistance or both. Uncontrolled hyperglycemia is associated with long-term complications such as retinopathy with potential loss of vision, nephropathy leading to renal failure, peripheral neuropathy with risk of foot ulcers and amputation or cardiovascular disease, which is the main cause of death in patients with diabetes (Einarson et al., 2018).

In 2021, diabetes mellitus affected approximately 537 million people of all ages worldwide (10.5 % of world's population) and it is estimated that by 2030 will affect 643 million people (11.3 % of the population) rising to 783 million by 2045 (12.2%) (International Diabetes Federation, 2021).

Diabetes mellitus is divided into multiple categories based on pathogenesis, including type 1 diabetes mellitus (T1DM), type 2 diabetes mellitus (T2DM), that accounts for 90% to 95% of all cases of diabetes, and monogenic diabetes. The American Diabetes association (ADA) defines T1DM as autoimmune β-cell destruction, usually leading to absolute insulin deficiency, and type T2DM as progressive loss of β-cell insulin secretion frequently occurring on the background of insulin resistance.

Only 1–2% of T2DM results from a single gene mutation. These monogenic forms of diabetes can be subdivided into neonatal diabetes (appearing during the first year of life) and young-onset diabetes (appearing before 25- year-old). The identified genes can be classified as genes involved in glucose sensing (*GLUT2* (Sansbury et al., 2012; Van De Bunt & Gloyn, 2012)*, GCK* (Shammas et al., 2013) and K_{ATP} channels (Ashcroft, 2010; Ashcroft & Rorsman, 2012) the insulin gene itself (Støy et al., 2010) and transcription factors mostly involved in β-cell development. Indeed, mutations in genes encoding Kir6.2 (*KCNJ11*) and SUR1 (*ABCC8*), the two subunits of the K_{ATP} channels (**Figure 6**), account for 50% of the permanent and 30% of the transient form of neonatal diabetes. Because these gain-of-function mutations impair the ability of glucose to close K_{ATP} channels, the sugar cannot depolarize the plasma membrane, drive Ca^{2+} influx and trigger insulin secretion (Ashcroft & Rorsman, 2012; Benninger et al., 2011). This is opposite to the loss-of-function mutations of K_{ATP} channels that induce a permanent depolarization of the plasma membrane leading to congenital hyperinsulinism associated with persistent hypoglycemia.

2.1. Epidemiology and pathophysiology of T2DM

Globally, an estimated 10.5% of the world's population (536.6 million individuals) are affected by T2DM (International Diabetes Federation, 2021) and it is ranked the ninth leading cause of mortality according to the World Health Organization (WHO). The prevalence of T2DM is high and rising across all regions. The rise is driven by population aging, economic development and increasing urbanization leading to more sedentary lifestyles and greater consumption of unhealthy foods linked with obesity (Basu et al., 2013).

The pathophysiology of T2DM is characterized by peripheral insulin resistance, occurring in the three major target tissues (liver, muscle, and adipose tissue) (Yki-Järvinen, 1995), impaired regulation of hepatic glucose production (Radziuk & Pye, 2002), and declining β-cell function (Levy et al., 1998), eventually leading to β-cell failure.

Insulin resistance was first described to be inherited by a set of genes (*PPARG, KCNJ11, TCF7L2, CDKAL1, IGF2BP-2, CDKN2A/2B, H4EX, SLC30A, FTO, WFS1, JAZF1*) (DeFronzo & Tripathy, 2009; Eriksson et al., 1989; Groop & Lyssenko, 2008; Kashyap et al., 2003; Morino et al., 2005; Pendergrass et al., 2007; Rothman et al., 1995) and although the origins can be traced to the genetic background (Groop & Lyssenko, 2008), the epidemic of T2DM has been long considered a disease of the affluent “western" countries related to the epidemic of obesity and physical inactivity (James, 2008). However, recent evidence shows that early loss of β-cell function plays an important role in the pathogenesis of T2DM, especially in nonobese individuals such as South Asians (Unnikrishnan et al., 2017).

In 2009, Prof Ralph DeFronzo defined the “Ominous Octet” as the eight factors that contributed to the pathophysiology of T2DM (Defronzo, 2009) (**Figure 7**):

- Muscle insulin resistance → reduced glucose uptake
- Hepatic insulin resistance → excessive glucose production
- Adipocyte insulin resistance → accelerated lipolysis and elevated circulating levels of FFA and insulin-resistance provoking adipocytokines
- Progressive β-cell failure and apoptosis→ decreased insulin secretion
- Increased alpha cell secretion of glucagon and increased hepatic sensitivity to glucagon
- Reduced incretin effect due to beta cell resistance to GLP-1 and GIP
- Increased renal glucose production
- Elevated renal tubular glucose reabsorption
- Brain insulin resistance and altered neurotransmitter dysfunction leading to impaired appetite suppression and weight gain

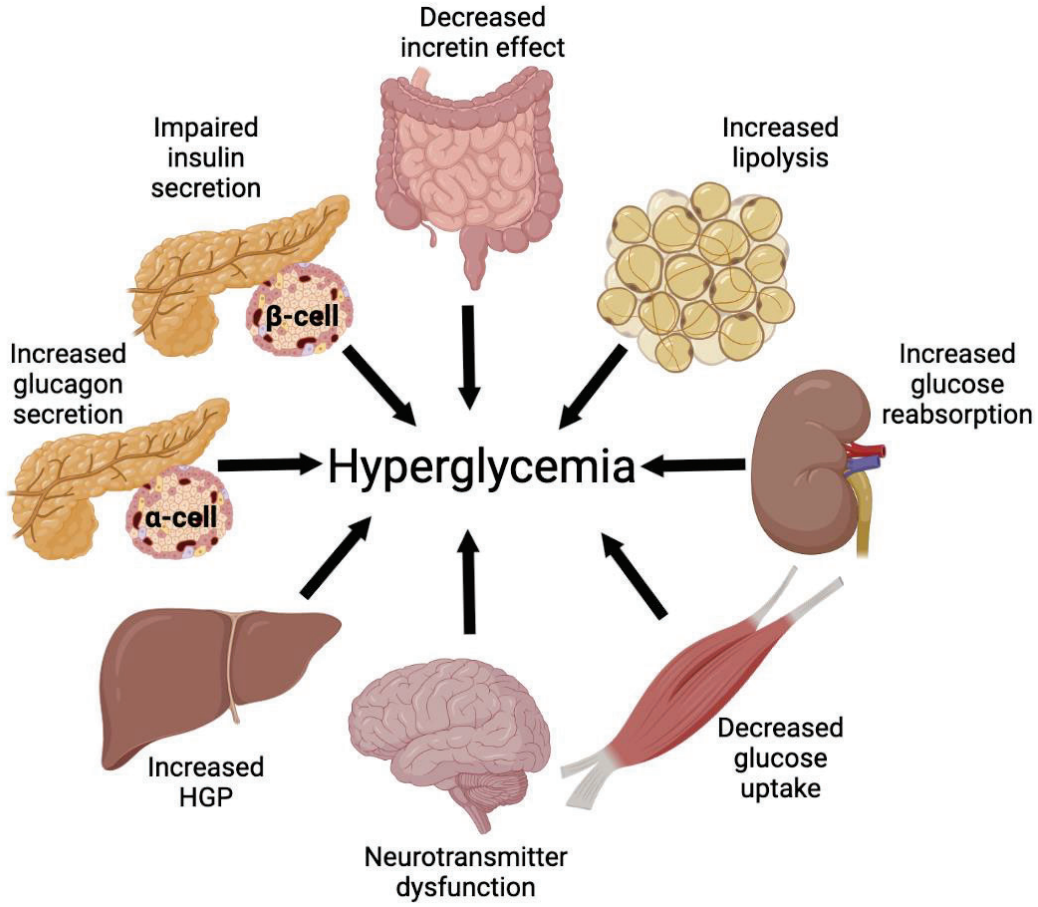


Figure 7. Type 2 diabetes is characterized by multiple pathophysiologic abnormalities which collectively have been referred to as the Ominous Octet. HGP: Hepatic glucose production. Adapted from (Defronzo, 2009). Created in *BioRender.com.*

The prevailing view is that insulin resistance causes elevation of plasma glucose levels, which promotes increased demand on pancreatic β-cells to produce and secrete more insulin (Prentki & Nolan, 2006). This compensatory hyperinsulinemic response by the β-cells is multifactorial and initially restores euglycemia in the prediabetic state; however, chronic exposure to excess glucose and lipids eventually leads to β-cell dysfunction and/or cell death to cause overt diabetes. During the past years, however, the idea that insulin resistance precedes β-cell dysfunction has been challenged, and there is a growing appreciation that, at least in a subset of patients, the contribution of islet β-cell hyperresponsiveness is a primary event in the development of glucose intolerance (Corkey, 2012; Nolan & Prentki, 2019). Ultimately, however, regardless of

whether the initial trigger is nutrient excess, insulin resistance, or both, the transition from adaptive β-cell response to a pathological β-cell response represents a critical step in the progression to T2DM.

The latest advances in genome-wide association studies (GWAS) have allowed the identification of over 400 genetic variants (Mahajan et al., 2018; Vujkovic et al., 2020) each of them having only a moderate or small effect on the risk of T2DM. These genetic variants influence multiple processes in tissues and cells, including β-cells (islet development, islet senescence, islet function), adipocytes, skeletal muscle, liver, and other tissues. Importantly, as mentioned previously, lifestyle and environmental factors modify the natural course of T2DM. Combination of genetic variants and physiologically characterized pathways has allowed the division of individuals with T2DM into subgroups characterized by impaired β-cell function and insulin resistance, providing a better understanding of the linking of T2DM to genetic susceptibility (Dimas et al., 2014; Udler et al., 2018).

2.2. B-cell failure

The origin of β-cell failure in T2DM is considered to involve both a partial reduction in β-cell mass (number) and a progressive deterioration of β-cell function (the ability of an individual β-cell to enhance its insulin secretion in response to glucose).

2.2.1. Loss of β-cell mass

In an insulin resistance state, blood glucose levels remain in the normal range due to precisely regulated increases of insulin secretion by β-cell compensatory responses that include increased insulin synthesis and secretion, and increased β-cell mass via both proliferation and hypertrophy (Ahrén & Pacini, 2002; Okamoto et al., 2006; H. Zhang et al., 2010). The capacity of the β-cell to increase is crucial because it usually prevents most obese and insulin resistant subjects from developing T2DM (Kahn et al., 1993; Meigs et al., 2006), yet this β-cell compensation often fails and becomes the culprit of the onset of T2DM.

It is a long-held notion that β-cell mass (defined by insulin immunolabeling) is decreased in patients with T2DM compared with non-diabetic (ND) donors (Butler et al., 2003). In autopsy series, a reduction in β-cell relative volume (Butler et al., 2003; Meier

et al., 2009) or mass (Rahier et al., 2008; Saisho et al., 2013) has been reported in T2DM; other morphological and histochemical evidence documents structural disarray and amyloid infiltration in human diabetic islets (Hull et al., 2004). In addition, islets from T2DM donors are smaller and on average have fewer than half the islet equivalent (IEQs) of ND donors (S. Deng et al., 2004). The decrease of β-cell mass has historically been explained by β-cell death through apoptosis due to gluco/lipotoxicity (Butler et al., 2003; Fontés et al., 2010; Halban et al., 2014). However, apoptosis may not be the only explanation for a decrease insulin-positive cells in the setting of T2DM. More recently, some studies have addressed β-cell loss by explaining dedifferentiation (Nordmann et al., 2017; Talchai et al., 2012; Weir et al., 2013) or transdifferentiation of β-cells into non-β-cells (Brereton et al., 2014; Spijker et al., 2013) (**Figure 8**). Given the current inability to longitudinally monitor an individual's β-cell mass using the latest imaging techniques, it is unclear whether β-cell mass declines in those who progress to T2DM or whether those individuals had a lower β-cell mass at baseline, making them more susceptible to T2DM when metabolically challenged (Elsakr & Gannon, 2017; Linnemann et al., 2014).

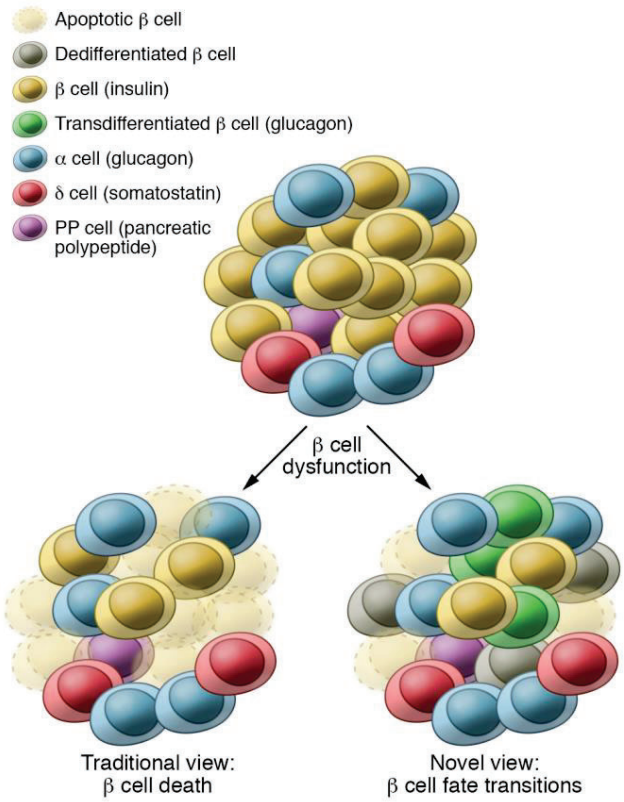


Figure 8. Fates of overexerted β-cells. Upon overexertion, in many animal models and humans, β-cells initially undergo functional compensation, which can be followed by a pathogenic response. In the past, overexerted β-cells were thought to predominantly undergo cell death. More recently, there has been evidence from animal models and human pancreatic tissue that β-cells can respond by undergoing dedifferentiation, transdifferentiation, or β-cell subtype transitions. From (Hudish et al., 2019).

2.2.2. Deterioration of β-cell function: overexerted β-cells

Besides the reported reduction in β-cell mass, a substantial deficit in β-cell function is evident in T2DM patients (Ferrannini et al., 2005; Gastaldelli et al., 2004; Jensen et al., 2002). Despite evidence that β-cells can mount compensatory response to insulin resistance and overnutrition, in individuals who develop T2DM the ability to compensate is transient. Over time, production of large amounts of insulin by compensating β-cells exerts continuous demand on the ER for proper protein synthesis, folding, trafficking, and secretion. Ultimately, β-cells are unable to sustain the increased workload and the initial adaptive responses become progressively maladaptive.

In normal pancreatic β-cell, glucose metabolism leads to insulin secretion via coupling to electrical excitation, as previously mentioned. In diabetes, this link will normally be maintained, such that "hyperstimulation" by exposure to elevated glucose, will be coupled to electrical "hyperexcitation" with membrane depolarization and chronically elevated $[Ca^{2+}]_c$. In the diabetic state, the notion of β-cell "hyperstimulation", as a result of the ambient hyperglycemia, which is suggested to be linked to "hyperxcitability", chronically elevated $[Ca^{2+}]_c$, and chronic hypersecretion, results in "β-cell exhaustion" (Remedi & Nichols, 2008) (**Figure 9**).

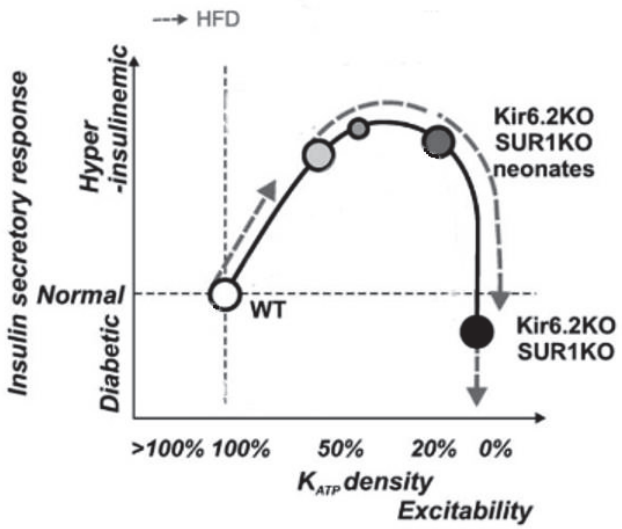


Figure 9. Proposed "inverse U" model response for enhanced β-cell excitability. Normal islets (white circle) secrete normally, but following a high-fat diet (HFD; grey dashed arrow) progress to insulin hypersecretion. HFD causes further enhancement of excitability, beyond the threshold driving those islets ''over the top'' (dashed) to an undersecretory phenotype. Conversely, Kir6.2- and SUR1-KO islets (zero K_{ATP} channel activity), which have maximally enhanced excitability, hypersecrete as neonates (grey circle on solid line), but rapidly progress to an undersecretory phenotype (black circle on solid line), and are positioned on the ''descending limb'' (Miki et al., 1998; Seghers et al., 2000; Shiota et al., 2002). Adapted from (Remedi & Nichols, 2008).

The concept "β-cell exhaustion" as a consequence of chronic stimulation of glucose metabolism, excitation and insulin secretion cannot be sustained by the β-cell and there is evidence that insulin depletion is an important part of the reduced or absent insulin response to glucose seen after chronic hyperglycemia *in vivo* and elevated glucose *in*

vitro. “β-cell exhaustion” could also induce ER stress, and some markers of ER stress are induced by elevated glucose (Elouil et al., 2007).

However, Nichols et al. (Nichols & Remedi, 2012) explained that the diabetic “glucotoxic” effect is the result of hyperstimulation of metabolism, not of excitation and secretion. They proved through animal experiments, where they broke the link between [glucose]-K_{ATP} channel activity either pharmacologically or genetically, that hyperexcitability *per se* does not lead to β-cell death *in vivo.* Conversely, even in chronically underexcited, non-secreting, islets, systemic diabetes leads to dramatic loss of β-cell insulin content, which may be reversible following lowering of blood glucose.

Another indication of β-cell dysfunction in T2DM is the observed change in the dynamics of insulin release, which includes loss of the first-phase response (Brunzell et al., 1976; Gerich, 2002; Mezza et al., 2021) and disruption of the regular oscillatory release patterns (Lang et al., 1981; Polonsky et al., 1988). These are more likely to be the result of functional alterations than changes in cell mass.

3. B-cell dedifferentiation

3.1. Concept of β-cell dedifferentiation

Before diving into the concept of β-cell dedifferentiation, a point to take into consideration is the difference between β-cell “differentiation” and “maturation” which has been recently defined by Barsby & Otonkoski (Barsby & Otonkoski, 2022). They refer to β-cell “differentiation” as the acquisition of a terminally differentiated insulin-positive cell identity throughout in utero development or *in vitro* stem-cell-based protocols, conversely to the “maturation” term defined as a measurement of the phenotypic properties of β-cells and their ability to respond to, and control, blood glucose levels through GSIS, which occurs postnatally in rodents (Blum et al., 2012) and humans (Otonkoski et al., 1988) and beyond the attainment of fetal β-cell identity. The current understanding of β-cell maturation is that such a process is a spectrum rather than a binary state (Velazco-Cruz et al., 2020; Veres et al., 2019; Zeng et al., 2017) and takes several years to be completed in humans (Avrahami et al., 2020). Functional maturation can be a reversible process, as β-cell dedifferentiation and senescence, with resultant

functional deterioration, are known to be associated with the onset of diabetes (Salinno et al., 2019; Thompson et al., 2019; Weir & Bonner-weir, 2004).

B-cell differentiation is usually defined by the upregulation of a set of β-cell marker genes (including *INS, PDX1, NKX6.1, NEUROD1, MAFA* and *UCN3*). However, the presence of these genes is not necessarily an indication of mature β-cell functionality (Wortham & Sander, 2021; Zeng et al., 2017). Indeed, upregulation of *UCN3* occurs during the postnatal maturation of β-cells (Blum et al., 2012; Zeng et al., 2017) but UCN3 itself appears to be functionally redundant in driving this maturation process (J. L. Huang et al., 2020). It is therefore important to understand the difference between genes that are critical for maintaining β-cell identity and those that further determine the functional properties of β-cells, and the overlap between these two groups. For instance, MAFA may help repress 'disallowed' metabolic genes while maintaining expression of specific glucose transporter genes (GLUTs), GCK and PGC1α (coding for a regulator of mitochondrial biogenesis and circadian oscillation).

Overall, β-cell identity is defined as having monohormonal insulin expression, production and retention, expression of β-cell-enriched transcription factors (e.g. *PDX1, NKX6.1, NKX2.2, NEUROD1)* and expression of voltage-dependent ion channels and membrane polarisation machinery associated with triggering of insulin release. Whereas β-cell maturity refers to having the following features: glucose-sensitive insulin release (or GSIS) at physiologically relevant glucose concentrations; insensitivity to pyruvate/lactate-stimulated insulin release; absence of 'disallowed' genes (e.g. *SLC16A1, LDHA, HK1*); glucose-responsive mitochondrial oxidative metabolism; K_{ATP} channel-independent metabolic amplification pathways for GSIS modulation; appropriate balance between energy-sensing mTOR/AMPK pathways in basal and stimulatory conditions; ability to respond to and maintain circadian entrainment; expression of transcription factors/transcriptional regulators associated with advanced maturation (e.g. MAFA, SIX2, SIX3, BMAL1/ARNTL, NR1D1); and epigenetic and microRNA signatures associated with mature functionality and 'disallowed' gene repression (Barsby & Otonkoski, 2022).

The “β-cell dedifferentiation” term was originally proposed more than two decades ago to interpret the loss of mature β-cell phenotype, characterized by a regression to a less differentiated or a progenitor-like state (Jonas et al., 1999; Weir & Bonner-weir, 2004). It has been classically defined as reduced expression of β-cell-enriched genes, including key transcription factors (*Nkx6.1, Nkx2.2, Pdx1, MafA, MafB, Neurod1, Pax6, Foxo1*), insulin, glucose sensing and metabolism genes (*Glut2, Gck, Gdp2*), protein processing (*Pcsk2, Pcsk1/3*), stimulus-secretion coupling genes (*Kcnj11, Abcc8*), amplification of insulin secretion genes such as *Glp1r*; the concomitant upregulation of genes suppressed or expressed at very low levels in normal β-cells (*Ldha, Hk1, Slc16a1*), called disallowed or forbidden genes (Lemaire et al., 2016; Rutter et al., 2015), in addition to the re-expression of β-cell progenitor markers, such as *Ngn3, Sox9, Myc,* and in some cases even *Nanog* and *Oct4* (Diedisheim et al., 2018; Oshima et al., 2018; Talchai et al., 2012; Z. Wang et al., 2014). These alterations lead to metabolic reprogramming and structural reconfiguration of β-cells and ultimately defective insulin secretion. However, increasing evidence suggests that the adequacy of dedifferentiation term remains debated (Nimkulrat et al., 2021). In contrast, some researchers hold the view that β-cell dedifferentiation is the consequence of the disruption of specific genes related to glucose metabolism, the secretory pathway, and protein processing and is accompanied by the upregulation of β-cell forbidden genes (Lemaire et al., 2016, 2017; Rutter et al., 2015) without a regression to β-cell precursor-like stage, thus no re-expression of progenitor transcription factors (Blum et al., 2014; S. Guo et al., 2013; Neelankal John et al., 2018) or found that dedifferentiated β-cells resemble immature (neonatal) β-cells to some extent but are not Ngn3-expressing progenitors (Dahan et al., 2017; Sachs et al., 2020).

Christensen and Gannon (Christensen & Gannon, 2019) stated that the term dedifferentiation may best be reserved for situations where β-cells revert to a more immature or progenitor state similar to an earlier stage in their normal developmental program, rather than a novel cell state not normally found in development where it may be more appropriate to use the term “loss of β-cell identity”. In the same line of thought, Blum’s group (Nimkulrat et al., 2021) have described an alternative model to

demonstrate that β-cell dedifferentiation is dependent on the degree of the mature identity disruption, resulting in their own, but not reverse-ordered, developmental ontogeny. This model, which they call the Anna Karenina model (based on the opening sentence in Tolstoy's novel by the same name, "All happy families resemble one another, each unhappy family is unhappy in its own way") suggests that β-cell dedifferentiation is a stress type–specific process caused by disruption of specific gene regulatory networks by the diabetogenic environment, thus resulting in a stress type–specific loss of functional maturity, without assuming a "true" cell progenitor identity (S. Guo et al., 2013) (**Figure 10**).

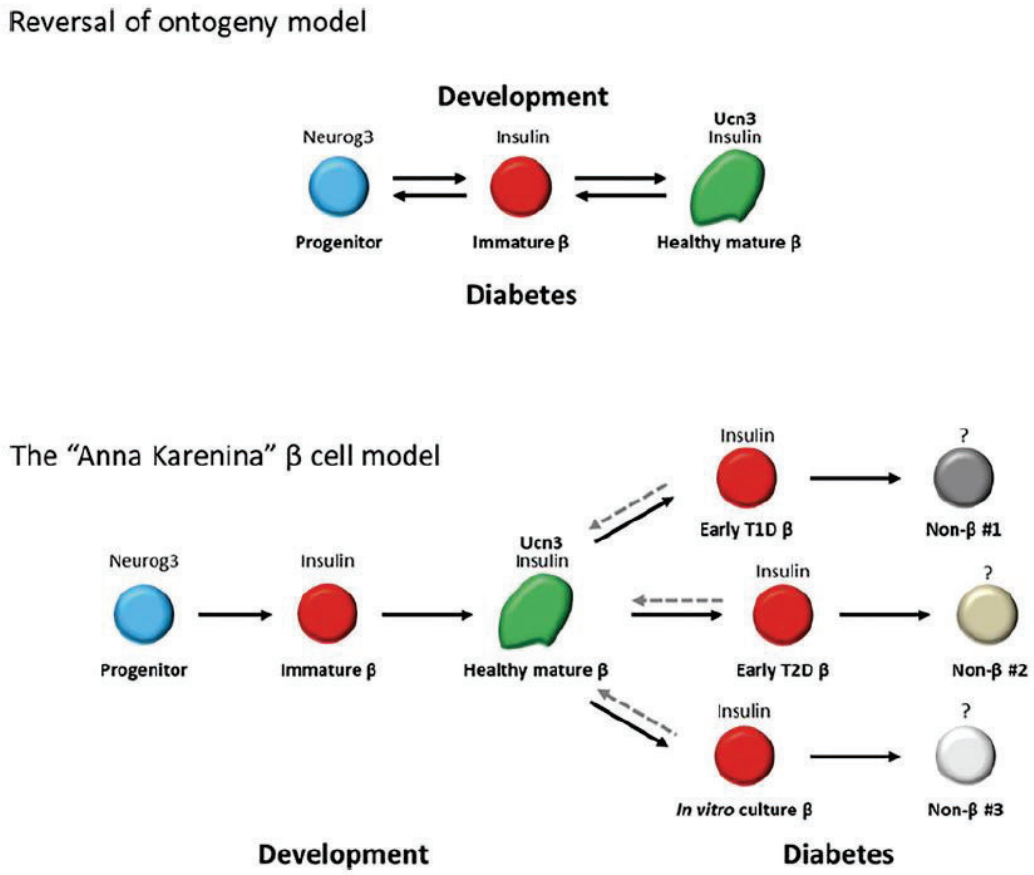


Figure 10. The Anna Karenina β-cell model to demonstrate an alternative mechanism of β-cell dedifferentiation. The reversal ontogeny model predicts that β-cells dedifferentiate in diabetes in a reverse order of their normal ontogeny during development (top). The Anna Karenina β-cell model predicts that, in each type of diabetes, β-cells will lose their mature phenotype in a unique manner, depending on how their genetic network is perturbed by a particular diabetogenic environment (bottom). Adapted from (Nimkulrat et al., 2021).

The concept of β-cell maturity comprises not only the genes that β-cell should express, but also the set of genes that must be actively repressed in order to function properly, the so called “disallowed genes” or tissue-specifically repressed genes which are upregulated under conditions of metabolic stress, such as T2DM, while markers of β-cell identity are downregulated. Many of the disallowed genes are in fact expressed in immature β-cells (Dhawan et al., 2015; Thorrez et al., 2011). The repression of these ubiquitous proteins prevents the inappropriate β-cell function such as elevated basal insulin secretion or insulin release triggered by exercise. Two of the identified disallowed genes encode ubiquitously expressed proteins for anaerobic glycolysis and lactate shuttles (Ishihara et al., 1999; Sekine et al., 1994; Thorrez et al., 2011; Zhao et al., 2001): lactate dehydrogenase A (LDHA) and lactate/pyruvate transporter [monocarboxylate transporter 1 (MCT1) which is encoded by *SLC16A1* gene]. Repression of both genes explains the pyruvate paradox of β-cells: the incapacity of extracellular pyruvate to stimulate insulin release, whereas intracellular pyruvate is a crucial metabolite in the stimulus-secretion coupling of glucose-induced insulin release. Another example of disallowed gene is *HK1* encoding for the high-affinity hexokinase 1. In most other cell types, HK1–HK3 are responsible for the first step of glycolysis. For pancreatic β-cells (Matschinsky, 2002) and hepatocytes (Agius, 2008), HK1–HK3 are unfit to sustain normal glucose homeostasis because are saturated by micromolar glucose concentrations, leading to support glycolysis even when circulating glucose is low and cause hepatic glucose uptake and basal insulin secretion between meals. Another example of a disallowed gene is the transcriptional repressor REST (repressor element 1 silencing transcription factor), when not repressed, β-cells show insufficient functional β-cells mass through a combination of compromised β-cells development and underperformance of regulated exocytosis of insulin secretory granules (Martin et al., 2008). Repression of disallowed genes is controlled by both histone modification and DNA methylation, via DNMT3A, which connect epigenetic regulation to metabolism (Gut & Verdin, 2013; Lempradl et al., 2015), and it has been shown in islets (Dhawan et al., 2015; Thorrez et al., 2011), together with the upregulation of tissue-specific miRNAs which provide an extra level for the required repression (Martinez-Sanchez et al., 2015).

Importantly, the loss of β-cell identity and maturity is not unidirectional. Successful redifferentiation of dedifferentiated β-cells was reported in both mouse and human islets (Gershengorn et al., 2004; Ouziel-Yahalom et al., 2006; Z. Wang et al., 2014). Through targeted pharmacological therapy, β-cell stress can be alleviated, potentially restoring β-cell function and glucose homeostasis (Sachs et al., 2020; Taylor et al., 2018). There is even demonstrations in rodent models that β-cell dedifferentiation can be reversed following a pair-feeding (Ishida et al., 2017) or long-term caloric restriction protocol in *db/db* mice (Sheng et al., 2016); or after gastric bypass surgery in GK rats (Qian et al., 2014), a non-obese spontaneous T2DM model, or with chronic insulin therapy (Z. Wang et al., 2014).

3.2. Conditions that promote β-cell dedifferentiation

Earlier reports have established the major role of glucotoxicity in β-cell dedifferentiation (Bensellam, Laybutt, et al., 2012), including its downstream pathways: oxidative stress (S. Guo et al., 2013; R. P. Robertson & Harmon, 2006), ER stress (Lipson et al., 2008; Lombardi et al., 2012; Pirot et al., 2007; M. Szabat et al., 2011), the hexosamine pathway (Kaneto et al., 2001; Yoshikawa et al., 2002), inflammation (Nordmann et al., 2017; Sun et al., 2019) and hypoxia (N. Liu et al., 2020; Puri et al., 2013; Sato et al., 2014, 2017). However, the precise molecular mechanisms involved are still under investigation.

One of the first studies to show β-cell dedifferentiation was the Weir's group demonstration that chronic mild to severe hyperglycemia in pancreatectomized rats triggered loss of β-cell differentiation (Jonas et al., 1999). This observation was reinforced in a genetic model when Talchai et al. (Talchai et al., 2012) generated a β-cell-specific deletion of the transcription factor FoxO1 (FoxO1 βCKO mice), a major target of insulin signaling and regulator of metabolic homeostasis in many tissues. These mice subjected to pathophysiologic models of β-cell stress, such as aging and multiple pregnancies, and analyzed through lineage tracing, demonstrated that the mutant β-cells were not lost through apoptosis, but became dedifferentiated to revert to a more progenitor-like state. This observation has been confirmed by many other studies where

the adoption of a dedifferentiated phenotype is shown as a protective mechanism to support β-cell survival under conditions of stress (Bensellam et al., 2018).

Inducible ablation of the transcription factor LIM Domain Binding 1(LDB1) in mature β-cells also resulted in impaired insulin secretion and glucose homeostasis due to a reduction in β-cell identity genes and induction of the endocrine progenitor marker Ngn3 (Ediger et al., 2017). Using the well-established T2DM mouse model *db/db*, which has a mutation on the leptin receptor, Neelankal et al. used transcriptome analysis of 12-week-old islets from WT, *db* heterozygotes, and *db/db* mutants to demonstrate that dedifferentiation was also occurring in a model of insulin resistance (Neelankal John et al., 2018).

Evidence of dedifferentiated β-cells, defined by the presence of hormone-negative endocrine cells, was also reported in a study of human T2DM patients with adequate glucose control (Sun et al., 2019). A recent Japanese study showed that the proportion of dedifferentiated cells that retain the pan-endocrine marker chromogranin A without expressing the four major islet hormones was increased in islets from patients with diabetes compared to that from healthy individuals, and chromogranin A expression levels increased significantly during long-standing diabetes (Amo-Shiinoki et al., 2021). However, without lineage tracing analyses and more extensive progenitor marker analyses, dedifferentiation is more difficult to prove in humans. To overcome this, Diedisheim et al. (Diedisheim et al., 2018) used the functional human β-cell line EndoC-βH1 to show that treatment with the growth factors FGF1 and FGF2 induces β-cell dedifferentiation and that this process was reversible under specific conditions. They also identified novel human β-cell dedifferentiation markers SOX9, HES1, MYC, PYY, GAST, and NGN3 in addition to the previously reported ALDH1A3 marker described by Cinti et al. (Cinti et al., 2016).

Besides altered expression and subcellular localization of transcription factors, downregulation of β-cell-enriched genes also involves gene repression by epigenetic mechanisms and miRNAs. Thus, in the islets of T2DM patients, increased DNA methylation in the promoter and enhancer regions of *Pdx1* has been proposed to be involved in its downregulation under hyperglycemia (Yang et al., 2012), as well as

increased methylation in the *Glp1r* promoter region (Hall et al., 2013) and there are reports implicating several miRNAs in the inhibition of insulin gene expression and upstream transcription factors (Fred et al., 2010; J. W. Kim et al., 2013; Sebastiani et al., 2015; Xu et al., 2013).

Butler et al. have argued that β-cell dedifferentiation or degranulation is minor stating that the hormone-negative chromogranin+ cells observed in T2DM pancreata are not dedifferentiated β-cells found in mouse models, but rather immature β-cells arising during regeneration (Butler et al., 2016). Dedifferentiation is defined by the coordinated downregulation of cell maturity and identity genes, and cell degranulation is determined by assessing the number of secretory granules per cell (Jain et al., 2022). Therefore, the analytic approach is important, and to resolve differences between these investigations, a coordinated and systematic study at the single-cell level is necessary.

In a recent study by Sachs et al. in 2020, new markers and pathways associated with β-cell dedifferentiation were identified in an scRNA-seq comparison of mouse islets from wild-type and streptozotocin-induced severely diabetic mice (Sachs et al., 2020). Interestingly, dedifferentiated β-cells could be protected from ER stress-mediated cell death by targeted delivery of a GLP-1–oestrogen conjugate, and it was further shown that insulin treatment triggered IR pathway activation in β-cells and restored β-cell identity and function for diabetes remission in mice.

As already mentioned, the presence of progenitor cell markers (Sox9, Aldh1a3, Ngn3) has been observed in the dedifferentiated islets of diabetic rodents (Kim-Muller et al., 2016; Talchai et al., 2012; Z. Wang et al., 2014) and in some studies using human islets (Cinti et al., 2016; Diedisheim et al., 2018; Sun et al., 2019). However, as demonstrated with the “Anna Karenina model” (Nimkulrat et al., 2021) found in rodents, other groups have confirmed the absence of upregulated expression of β-cell progenitor markers in rodents islets (Z. Deng et al., 2022) and human islets (Avrahami et al., 2020; S. Guo et al., 2013).

Even in T1DM patients, where diabetes is thought to result from an autoimmune attack on insulin-producing β-cells involving islets and cells of the immune system (Atkinson et al., 2014; Boldison & Wong, 2016), it has been observed that following the

disease progression at the islet level, alteration of the β-cell phenotype (reduced expression of β-cell markers) precedes β-cell destruction (Damond et al., 2019; Lam et al., 2019; Seiron et al., 2019). It has been discussed that this altered phenotype potentially may allow escape from detection by the immune system (Rui et al., 2017) which was further confirmed afterwards in a non-obese diabetic mice model study by Engin's group (H. Lee et al., 2020).

Other pathways that have been suggested to be implicated in β-cell dedifferentiation include activation of Hedgehog (Hh) signaling in β-cell which reduced expression of both genes critical for β-cell function and TFs associated with β-cell mature phenotype, synchronizing with increased expression of the pioneer cell markers Hes Family BHLH Transcription Factor 1 (Hes1) and Sox9 (Landsman et al., 2011). Renin-angiotensin system (RAS) activation induced β-cell dedifferentiation and impaired insulin secretion via NF-κB signaling, ultimately leading to β-cell failure (H. Chen et al., 2018). There is some evidence suggesting that the Notch signaling pathway may play a role in β-cell dedifferentiation. Notch signaling is reactivated during dedifferentiation in human β-cells (Bar et al., 2008). As well as TGF-β signaling, where activin B, a TGF-β ligand, is upregulated during tumorigenesis and drives the loss of β-cell maturity in a mouse insulinoma model (Ripoche et al., 2016). Another molecule, adenosine, a key extracellular signaling mediator that regulates several aspects of metabolism by activating 4 G-protein-coupled receptors, including A2A adenosine receptor, have been implicated in loss of β-cell identity and increased expression of dedifferentiation markers SRY-Box 2 (Sox2) and Hes1 (Csóka et al., 2017). The discovery that numerous signaling pathways may be involved in β-cell dedifferentiation raises the possibility that these pathways could be used as potential targets for the treatment of diabetes.

4. Endoplasmic reticulum stress

4.1. Concept of endoplasmic reticulum stress

Over the last decades, the ER stress pathway has emerged as an important mechanism implicated in the pathogenesis of several diseases including diabetes (Delépine et al., 2000; Ladiges et al., 2005; Laybutt et al., 2007; Senee et al., 2004; Thameem et al., 2006) and its following complications including diabetic retinopathy

and nephropathy (J. Li et al., 2009; Lindenmeyer et al., 2008; G. Liu et al., 2008; Morse et al., 2010).

The ER, a membranous intracellular organelle, is the first compartment of the secretory pathway. It has been is classically divided into the rough ER (RER) and smooth ER (SER), depending on the presence or absence of ribosomes on the cytosolic face of the membrane respectively (Almanza et al., 2019). More recently, a novel classification was proposed based on membrane structure rather than appearance. According to this classification, the ER comprises the nuclear envelope, sheet-like cisternae and a polygonal array of tubules connected by three-way junctions (Shibata et al., 2006). The ER occupies an extensive cell-type specific footprint within the cell and is in contact with many other intracellular organelles. It forms physical contact sites with mitochondria named mitochondria-associated membranes (MAMs), which play a crucial role in Ca^{2+} homeostasis (Hayashi et al., 2009).

The ER main function is protein synthesis, contributes to the storage and regulation of calcium, to the synthesis and storage of lipids, and to glucose metabolism. These diverse functions indicate a pivotal role for the ER as a dynamic 'nutrient sensing' organelle that coordinates energetic fluctuations with metabolic reprogramming responses, regulating metabolism and cell fate decisions. The ER is involved in secretory and transmembrane protein synthesis, folding, maturation, quality control and degradation, and ensures that only properly folded proteins are delivered to their site of action (Stefan et al., 2011). About 30% of all proteins are cotranslationally targeted to the ER (Braakman & Bulleid, 2011). Protein processing within the ER by chaperones and foldases, includes signal sequence cleavage, N-linked glycosylation, formation, isomerization or reduction of disulfide bonds (by protein disulfide isomerases (PDIs), oxidoreductases), isomerization of proline or lipid conjugation, all of which ultimately result in a properly folded conformation (Aebi et al., 2010; Hebert & Molinari, 2007; Wallis & Freedman, 2013). Misfolded proteins are potentially detrimental to cell function and are therefore tightly controlled. Although protein misfolding takes place continually, it can be exacerbated during adverse intrinsic and environmental conditions. The ER has developed quality control systems to ensure that there are

additional opportunities to correct misfolded proteins or, if terminally misfolded, to be disposed of by the cell. Terminally misfolded secretory proteins are eliminated by a process called ER-associated degradation (ERAD) (Meusser et al., 2005).

The ER, as the main cellular compartment for Ca^{2+} storage, plays a pivotal role in the regulation of Ca^{2+} levels and reciprocally many ER functions are controlled in a Ca^{2+}-dependent way, thereby regulating the calcium homeostasis of the whole cell (Meldolesi & Pozzan, 1998). Cytosolic Ca^{2+} is taken up by the ER mainly by sarco-endoplasmic reticulum Ca^{2+}-ATPases (SERCAs). B-cells possess a well-developed ER which takes up Ca^{2+} via SERCA2b (ATP2A2), a ubiquitously expressed isoform, and SERCA3 (ATP2A3), an isoform that is expressed only in β-cells within islets (Arredouani et al., 2002; Váradi et al., 1996). The release of Ca^{2+} from the sarco-endoplasmic reticulum, as in various cell types, are triggered by inositol 1,4,5-trisphosphate (IP_3R) and ryanodine (RyR) receptors.

The maintenance of the ER is essential for preserving cellular function and viability. Disruption in ER homeostasis caused by, for example, the depletion of ER calcium, perturbations in the ER redox state, and/or the accumulation of the misfolded proteins within the ER results in what is commonly referred to as “ER stress”. This stress is sensed by ER transmembrane proteins that activate the unfolded protein response (UPR), an adaptive response whose function is to restore ER homeostasis and thus alleviate ER stress (**Figure 11**) (Back & Kaufman, 2012; Schröder & Kaufman, 2005; Szegezdi et al., 2006). This is achieved by: decreasing the ER synthetic load through inhibiting protein synthesis; clearing the ER of misfolded proteins by increasing the expression of components of ER-associated degradation (ERAD), which translocate misfolded proteins out of the ER for subsequent proteasomal degradation; and enhancing the synthesis and folding capacity of the ER by stimulating an increase in both ER mass and function.

The ER luminal domains of all three ER stress sensors are normally bound by the ER resident chaperone, heat shock protein A5 [heat shock protein family A (Hsp70) member 5, also known as glucose-regulated protein 78 (GRP78) and binding immunoglobulin protein (gene *GRP78*) (BiP)], keeping them in an inactive state (Bertolotti et al., 2000; J.

Shen et al., 2002). Accumulating misfolded proteins in the ER lumen engage BiP thus releasing the three sensors inducing its activation.

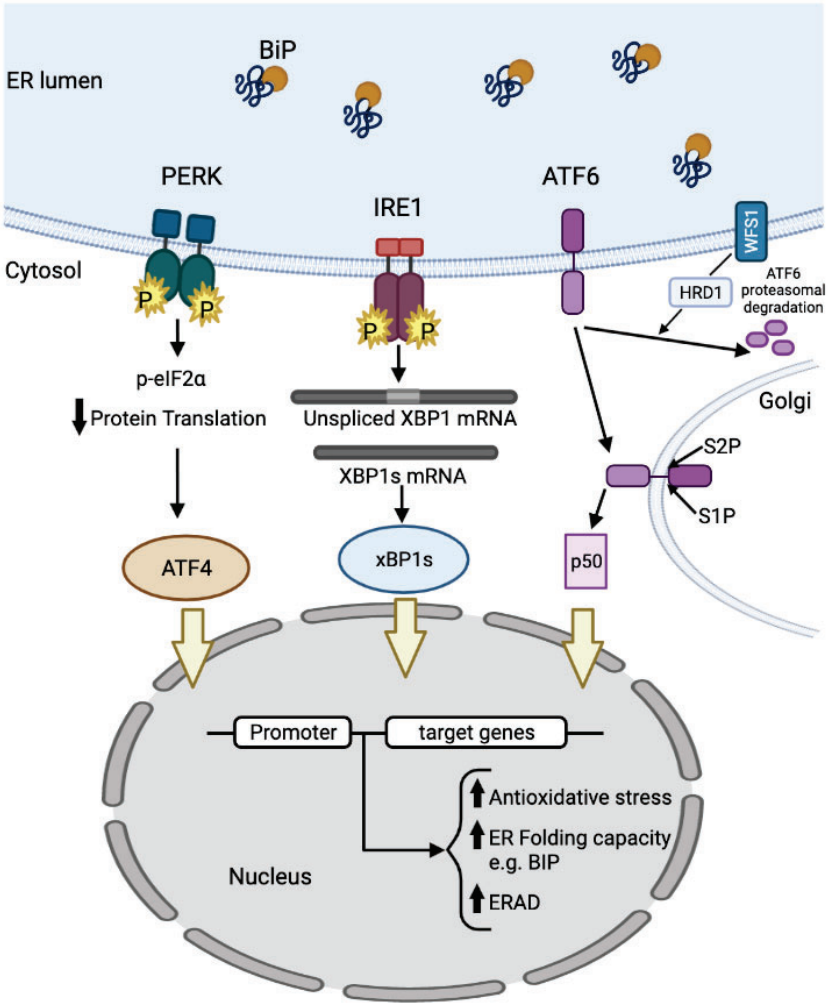


Figure 11. A simplified overview of the adaptive UPR. PERK, IRE1 and ATF6 signal to the nucleus through the action of the transcription factors ATF4, XBP1s, and p50, which bind to response elements within promoters to induce transcription of mRNAs whose products are important in increasing ER folding capacity, increasing ERAD, and reducing oxidative stress. WFS1 inhibits ATF6 through ubiquitination and proteasomal degradation, by targeting HRD1 (E3 ubiquitin ligase) to ATF6. Adapted from (Cnop et al., 2017; Herbert & Laybutt, 2016), created in BioRender.com.

Several small molecules that induce ER stress through a variety of mechanisms have been identified (Jin et al., 2014; Stechmann et al., 2010). Stressors such as tunicamycin target the N-linked glycosylation of proteins (Olden et al., 1979; Schultz & Oroszlan,

1979) or thapsigargin that targets SERCA which induces ER stress by reducing ER Ca^{2+} concentration and impairing protein folding capacity (Thastrup et al., 1990).

4.2. Unfolded protein response in β-cells

B-cells are highly specialized secretory cells that synthesize large amounts of proinsulin in response to glucose stimulation (Ling & Pipeleers, 1996). To cope with this heavy ER load, β-cells are endowed with a fundamental adaptive response to prevent accumulation of misfolded or unfolded proteins in the ER when protein load overwhelms the ER folding capacity. Unfolded protein response (UPR) activity is orchestrated by three sensors located in the membrane of the ER, the pancreatic ER kinase (PERK also known as EIF2AK3), the endoribonuclease/kinase inositol requiring 1 (IRE also known as ERN1) and the activating transcription factor 6 (ATF6) (**Figure 11**).

Active PERK phosphorylates the α-subunit of the eukaryotic translation initiation factor 2 (eIF2α) (Shi et al., 1998) thereby leading to a transient and global attenuation of protein translation to decrease ER load in parallel with a paradoxical increase in the translation of special transcripts such as activating transcription factor 4 (ATF4) (Harding et al., 2000). This transcription factor upregulates the expression of chaperones and antioxidant genes as well as proapoptotic genes like DNA damage-inducible transcript (*DDIT3)*, also known as C/EBP homologous protein (CHOP), activating transcription factor 3 (ATF3*)* and tribbles-related protein 3 (TRB3) (also known as TRIB3) that contribute to β-cell death under prolonged or unresolved ER stress. Activation of IRE1 catalyzes the removal of a 26-bp sequence from the mRNA encoding the bZIP transcription factor XBP1 (X-box binding protein) resulting in a frame shift and the production of a transcriptionally active "spliced" form of XBP1 (XBP1s) (Y. Chen & Brandizzi, 2013; Yoshida et al., 2001). This key transcription factor, together with active ATF6, stimulates the expression of *XBP1*, chaperones, such as BiP and GRP94, foldases and genes of the ER-associated degradation (ERAD) pathway thereby leading to improved ER folding capacity and enhanced clearance of unfolded proteins from the ER (Schröder & Kaufman, 2005). However, XBP1s can also participate in the regulation of numerous metabolic pathways such as lipid biosynthesis (So et al., 2012; Sriburi et al., 2004), glucose metabolism (Y. Deng et al., 2013; J. Lee et al., 2011; Junli Liu et al., 2016; S. W. Park et al., 2014; Zhou et al., 2011), insulin signaling (Akiyama et al., 2013; Ozcan

et al., 2004), redox metabolism (Y. Liu et al., 2009), DNA repair (Tao et al., 2011) and it influences cell fate including cell survival (Wu & Kaufman, 2006), cell differentiation (Blais et al., 2005; Reimold et al., 2001; Sha et al., 2009) and development (Masaki et al., 1999; Reimold et al., 2000; Sone et al., 2013).

ATF6 activation is initiated by the unmasking of a Golgi localization signal by dissociation of BiP (J. Shen et al., 2002). This allows ATF6 to translocate to the Golgi where it is cleaved by site-1 protease (S1P) and site-2 protease (S2P) resulting in the release of 50-kD N-terminal fragment (p50) encoding a bZIP transcription factor (Ye et al., 2000). P50 translocates to the nucleus where it activates genes involved in protein folding, processing and degradation (Oslowski & Urano, 2011), having overlapping functions with and compensatory functions to those of XBP1s (Yamamoto et al., 2004).

WFS1, known as wolframin, is a protein localized to the ER membrane shown to be a UPR component with a protective role against ER stress and, conversely, that chronic ER stress is caused by loss of function of WFS1 (Fonseca et al., 2005). WFS1 suppresses ER stress signaling acting as a negative regulator of ATF6, under non-stressed conditions, WFS1 inhibits ATF6α through ubiquitination and proteasomal degradation, by stabilizing HRD1 (E3 ubiquitin ligase), bringing ATF6α to the proteosome, enhancing its ubiquitination and proteosome-mediated degradation (Fonseca et al., 2010). In stress conditions, ATF6 dissociates from WFS1 and is released from the ER membrane leading to activation of ATF6 signaling pathway. WFS1 also plays a critical role in maintaining ER and cytosolic Ca^{2+} homeostasis (Hara et al., 2014; Zatyka et al., 2015), regulating sterile inflammation (Morikawa et al., 2022), and insulin granule acidification in β-cells (Hatanaka et al., 2011). A recent work by Groen et al. (Groen et al., 2021) showed that during the conversion of β-cell to α-cells there is an intermediate state where WFS1 is downregulated (**Figure 12**). So, β-cells have an increased expression of WFS1 but when β-cells lose their identity, WFS1 is downregulated.

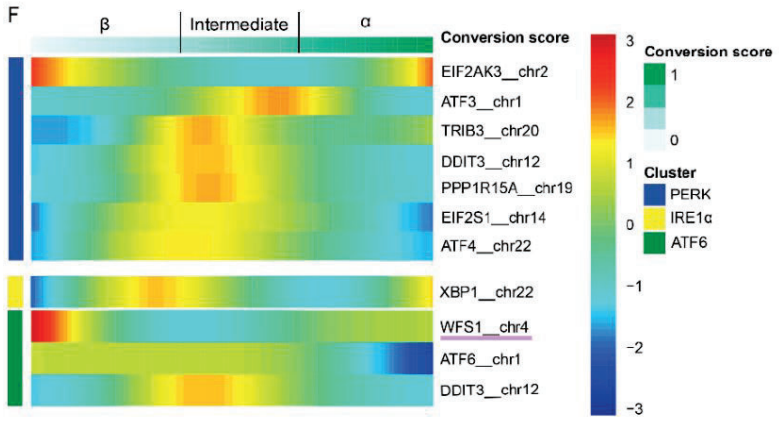


Figure 12. Scaled expression of Unfolded Protein Response (UPR) genes across cell identity score from β- to α-cells. From scRNAseq on primary human islets, Groen et al. have associated loss of identity with upregulation of stress markers. The figure displays cell identity score of canonical β-cells centered around zero and of canonical α-cells centered around one, 'intermediate cells' show a score in-between both populations. The gene-cluster (rows) represent the three UPR pathways: PERK (blue), IRE1α (yellow) and ATF6 (green). Genes involved in all three arms are mostly affected around the transition between β-cells and intermediate cells. IRE1α gene (XBP1) is affected earlier, followed by PERK genes and ATF6 expression is lost when α-cell identity is acquired. From (Groen et al., 2021).

If UPR action fails to restore ER homeostasis under prolonged or intense ER stress, an apoptotic program is triggered in human and rodents (Oslowski & Urano, 2010; Scheuner & Kaufman, 2008). Two apoptotic pathways are described: the intrinsic (mitochondrial) or extrinsic (death receptor) pathways of apoptosis. Both pathways trigger activation of caspase proteases that dismantle the cell, and all three branches of the UPR are involved in apoptosis but it is mediated primarily by the chronic activation of IRE1 and/or PERK (Y. Chen & Brandizzi, 2013; Szegezdi et al., 2006). Chronic PERK activation causes the ATF4-dependent increase in the expression of the proapoptotic protein C/EBP homologous protein (CHOP), otherwise known as DDIT3 (Oyadomari & Mori, 2004; B. Song et al., 2008). Chronic IRE1 activation leads to the recruitment of tumor necrosis factor 2 (TRAF2) (Urano et al., 2000) and the activation of c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinases (MAPK), resulting in an increased expression of proapoptotic proteins. This program involves the complex action of several effectors including TRB3 (Bromati et al., 2011; Liew et al., 2010), ATF3

(D. Li et al., 2008), and likely perturbations of Ca^{2+} homeostasis (Scorrano et al., 2003; Tsujimoto & Shimizu, 2007).

ER stress has also been connected to autophagic cell death. For instance, activated PERK can induce autophagy through ATF4 (Sano & Reed, 2013). Autophagy not only promotes cell survival, but can also mediate nonapoptotic cell death under experimental conditions when apoptosis is blocked, or in response to treatments that specifically trigger caspase-independent autophagic cell death (Høyer-Hansen & Jäättelä, 2007).

Glucose stimulation exerts opposite effects on the PERK-eIF2α-ATF4 and IRE1-XBP1s/ATF6 arms of the UPR. Thus, PERK-eIF2α-ATF4 arm is maximally activated at low glucose concentrations (PERK and eIF2α phosphorylation, upregulation of ATF4 and downstream target genes such as *Ddit3*), where protein synthesis, ATP levels and $[Ca^{2+}]_{ER}$ are low, while IRE1-XBP1 arm is maintained at basal level. Glucose stimulation rapidly triggers *Xbp1* pre-mRNA splicing leading subsequently to upregulation of XBP1s and a wide array of downstream target genes to face the ER synthetic load (Bensellam et al., 2009; Elouil et al., 2007; E. Gomez et al., 2008; Jonas et al., 2009; Moore et al., 2011).

Furthermore, an interesting study by Sharma et al. (Sharma et al., 2015) suggested that induction of UPR functioned as a sensor of insulin demand and induced β-cell proliferation through the activation of ATF6 in mouse and human islets. Studies in islets from human T2DM patients also show a doubling of the ER size compared with controls, indicative of the presence of ER stress responses (Marchetti et al., 2017). These studies demonstrate the remarkable ability of β-cells to adapt to increased metabolic demand and provides evidence that UPR plays a positive role in these important compensatory adaptations protecting β-cells from the detrimental effects of ER stress.

Until recently, the accepted concept was that excessive stimulation of the UPR under chronic/unresolved ER stress played an important role in β-cell decompensation and failure. Several reports supported that statement demonstrating increased activation of ER stress sensors and downstream effectors and target genes in different *in vitro* and *in vivo* models exposed to elevated glucose levels (Bensellam et al., 2009; Elouil et al., 2007; Jonas et al., 2009; Lipson et al., 2008; Seo et al., 2008) in addition to human T2DM

(Hartman et al., 2004; Laybutt et al., 2007). These findings have been reinforced by genetic manipulations of the UPR components in β-cell lines and mouse models. Thus, high glucose-induced downregulation of insulin gene expression in INS-1 cells has been shown to be significantly prevented by Atf6 silencing. In contrast, active Atf6 overexpression in INS-1 cells inhibited insulin secretion and gene expression in parallel to a marked reduction in Pdx1 and MafA mRNA and protein levels (Seo et al., 2008). Similarly, strong overexpression of Xbp1 in rat islet cells reduced the degree of β-cell differentiation, inhibited GSIS, and increased β-cell apoptosis (Allagnat et al., 2010).

This concept was further explored and questioned by Laybutt's group (Chan et al., 2013) by comparing time-dependent mRNA expression changes in islets of prediabetic and diabetic *db/db* mice and diabetes-resistant *ob/ob* mice. They demonstrated that increased UPR genes paralleled downregulation of some key β-cell transcription factor genes and other β-cell-enriched genes (in 6-week-old *ob/ob* and *db/db* mice). Interestingly, the β-cell phenotype recovered in 16-week-old *ob/ob* mice. However, they also observed that 16-week-old diabetic mice showed decreased UPR gene expression with decreased expression of β-cell-enriched genes, introducing the hypothesis that that β-cell dysfunction in T2DM may result from a failure of the UPR to adequately adapt rather than from a maladaptive UPR (Herbert & Laybutt, 2016).

In humans, the expression of BiP, XBP1s, and CHOP was lower in cultured islets isolated from subjects with T2DM compared with those isolated from subjects without diabetes (Marchetti et al., 2007). A recent study has confirmed and extended this finding by showing that numerous UPR, ER-Golgi (retrograde)transport, ER quality control, ERAD and (retro)translocon-related genes were significantly downregulated in islets of T2DM donors vs BMI-matched non-diabetic subjects (Bugliani et al., 2013). Collectively, these observations support the notion that adaptive UPR inactivation occurs during the progression towards diabetes in rodents and humans and may play an important role in β-cell dedifferentiation.

Regarding the mechanisms behind the inactivation of the UPR, hyperglycemia and the ensuing glucotoxicity are primary candidates (Walters et al., 2013). Reports suggest a role of hypoxia and inflammatory signaling in the downregulation of the adaptive UPR

in T2DM. It has been shown that hypoxia-response genes were upregulated in the islets of diabetic, but not prediabetic *db/db* mice in an inverse relationship with UPR gene expression and that the inhibition of the adaptive UPR by hypoxia was associated with impaired ER-to-Golgi protein trafficking and was implicated in increased apoptosis under hypoxic stress. These effects were mediated by the activation of JNK and DDIT3 (Bensellam, Laybutt, et al., 2012). Furthermore, it is hypothesized that the inactivation of the UPR in T2DM leads to progressive accumulation of unfolded proteins in the ER; this potentiates hyperglycemia-induced reactive oxygen species (ROS) production and oxidative stress, which leads to a gradual loss of β-cell identity (Bensellam et al., 2018).

4.3. Alleviation of ER stress to reestablish homeostasis

Several small molecules have been reported to inhibit one or more arms of the UPR including IRE1 inhibitors, PERK inhibitors and ATF6 modulators with promising beneficial effects (Almanza et al., 2019). Besides these small molecules targeting a specific arm of the UPR, treatments to alleviate or prevent ER stress by increasing ER folding capacity using pharmacological chaperones such as tauroursodeoxycholic acid (TUDCA), an endogenous bile acid able to resolve ER stress in islet cells by inhibiting phosphorylation of eIF2α (Y. Y. Lee et al., 2010), or 4-phenylbutyrate (PBA), which reduces the accumulation of misfolded proteins in the ER by interacting with hydrophobic domains (Malo et al., 2013), are being explored, even in clinical trials to treat T2DM, T1DM, obesity, NAFLD (Almanza et al., 2019).

Reports show that the maintenance of an intact adaptive UPR is essential for prevention of β-cell failure (Back & Kaufman, 2012; Eizirik et al., 2008; Scheuner & Kaufman, 2008). To target this issue, several groups have used TUDCA, PBA and the more recently discovered azoramide to alleviate or prevent ER stress demonstrating that can restore rodent islet function both *in vitro* and *in vivo* (Cadavez et al., 2014; Chan et al., 2013; S. Fu et al., 2015; Y. Y. Lee et al., 2010; Sharma et al., 2015; Tang et al., 2012). In humans, PBA has also been shown to partially alleviate lipid-induced β-cell dysfunction (Xiao et al., 2011). Notably, the administration of antioxidants also reduces ER stress and preserve β-cell function (B. Song et al., 2008; Tang et al., 2012). Interestingly, downregulation of β-cell-enriched genes in *db/db* mouse islets was

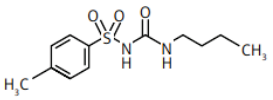
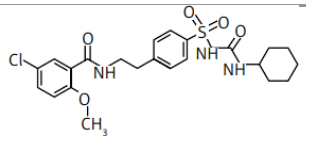
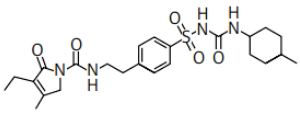
partially restored with the chemical chaperone PBA suggesting that the inactivation of the adaptive UPR plays a role in β-cell dedifferentiation (Chan et al., 2013).

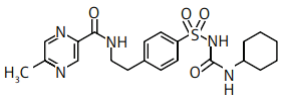
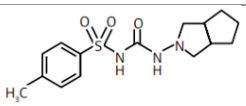
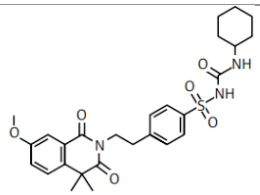
5. Sulfonylureas

5.1. Mechanism of action

Sulfonylureas (SUs) are the oldest oral antidiabetic agents currently available, and with metformin, are the most commonly prescribed drugs for treatment of T2DM in the world (Amod, 2020; Scheen, 2021). The capacity of synthetic sulfur containing compounds to lower blood sugar was reported in 1941 by Auguste Loubatières in France (Jackson & Bressler, 1981). In 1956, the first sulfonylurea used to purposefully lower blood glucose, tolbutamide, was introduced commercially in Germany followed by chlorpropamide, acetohexamide, and tolazamide, the first-generation sulfonylureas (Seltzer, 1980). Investigators at the Carlo Erba Research Institute in Milan, Italy, developed the "second generation" compounds, glyburide (glibenclamide) and glipizide, by expanding the radical group on urea opposite the benzene ring found in acetohexamide (Tommasini, 1975) (**Table 1**). Both glyburide and glipizide were approved for use in the US in 1984, following their introduction in Europe by several years (Gerich, 1989; Tommasini, 1975).

Table1. Examples of different generations of sulfonylureas. From (Sola et al., 2015).

Molecules	Gen.	Dose [mg]	Duration of action* T1/2	Activity of metabolites T1/2	Elimination	Structure
Tolbutamide	I	500-2000	Short 4.5 to 6.5 h	Inactive	Urine 100%	
Glibenclamide	II	2.5-15	Intermediate to long 5 to 7 h	Active 10h	Bile 50%	
Glimepiride	II	1-6	Intermediate 5 to 8 h	Active 3 to 6 h	Urine 80%	

Glipizide	II	2.5-20	Short to intermediate 2 to 4 h	Inactive	Urine 70%	
Gliclazide	II	40-320	Intermediate 10h	Inactive	Urine 65%	
Gliquidone	II	15-180	Short to intermediate 3 to 4 h	Inactive	Bile 95%	

*short duration activity means <12h; intermediate 12-24 h; long over 24h.

The clinically most significant effect of sulfonylureas is to induce glucose independent insulin release from β-cells by inhibiting potassium flux through K_{ATP} channels (Seino et al., 2012). K_{ATP} channels are widely distributed throughout the body, but are heterogeneous with respect to protein composition. All consist of hetero-octamers composed of 4 potassium pore units (KIR), and 4 sulfonylurea receptors (SUR) that serve as regulatory subunits (Burke et al., 2008; Seino et al., 2010). The 2 KIR isoforms (KIR6.1 and KIR6.2) and the 3 SUR isoforms (SUR1, SUR2A, and SUR2B) are expressed across a range of tissues (Burke et al., 2008; Seino et al., 2010, 2012). However, pancreatic β-cells predominantly express SUR1, while cardiomyocytes and skeletal muscle express SUR2A, and vascular smooth muscle cells largely express SUR2B (Burke et al., 2008; Seino et al., 2010, 2012). Consequently, the combination of SUR1/KIR6.2 is predominant in pancreatic β-cells, while the combinations of SUR2A/KIR6.2 and SUR2B/KIR6.2 or SUR2B/KIR6.1 are predominant in cardiomyocytes and vascular smooth muscle, respectively (Burke et al., 2008; Seino et al., 2010). In β-cells, the K_{ATP} channels are typically open, allowing K^+ efflux, and polarization of β-cell membranes (Ashcroft, 2005; Burke et al., 2008). Increased ATP/ADP ratios associated with the glucose metabolism that follows a blood sugar rise, increases binding of ATP to cytoplasmic portions of both KIR6.2 and SUR1 (Ashcroft, 2005). This closes β-cell K_{ATP} channels, depolarizing the β-cell membrane, opening voltage regulated Ca^{2+} channels, and stimulating the fusion of insulin vesicles with the cell membrane (Ashcroft, 2005).

Nucleotide regulation of K_{ATP} channels is unique among K^+ channels: the channel is rapidly and reversibly inhibited by binding of cytoplasmic adenosine nucleotides and exhibits complex activation by nucleotide tri- and diphosphates (Nichols, 2006). ATP inhibits the K_{ATP} channel by binding to the KIR6.2 subunit, whereas Mg ADP activates it by binding to the SUR subunit (Nichols et al., 1996), which is also the site of action of pharmacological channel openers and blockers. By directly binding to the SUR subunit, SUs inhibit K_{ATP} channel activity, causing membrane depolarization and activation of insulin secretion. Tolbutamide, an example of the first generation SUs, blocks the K_{ATP} channels with an IC_{50} of 7 μM; whereas second generation SUs (like glibenclamide) inhibit channel activity at nanomolar concentrations (Q. Zhang et al., 2020). As a result, these drugs can decouple metabolism from electrical activity, stimulating insulin secretion regardless of the metabolic state of the β-cell.

In addition to SUR1 binding, sulfonylureas likely exert a portion of their effects through binding with exchange protein directly activated by cAMP (Epac2). Epac2 is a guanine nucleotide exchange factor that interacts with the Rap1 protein to increase the availability of insulin vesicles to fuse with the β-cell plasmalemma (Seino et al., 2010). Epac2 typically responds to cAMP generated by alternative signaling pathways. However, recent evidence indicates that multiple sulfonylureas, with the exception of gliclazide, bind to both SUR1 and Epac2 (Herbst et al., 2011; Seino et al., 2012), and the activation of Epac2/Rap1 is required for sulfonylurea to exert their full effects on stimulation of insulin secretion (**Figure 13**).

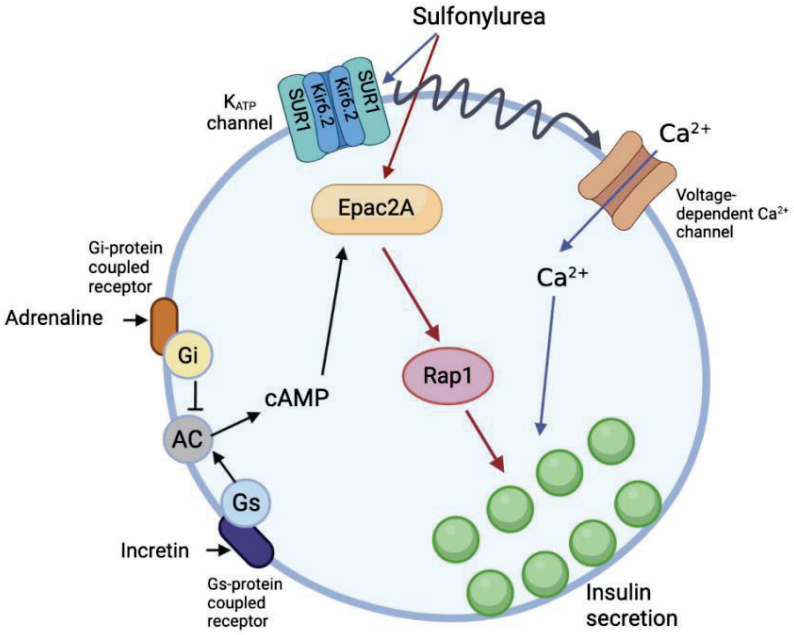


Figure 13. Sulfonylurea mechanism of action in pancreatic β-cells. Epac2A is a target of both sulfonylurea and cAMP. Closure of K_{ATP} channels is essential for sulfonylurea to stimulate insulin secretion by inducing Ca2+ influx. Activation of Epac2A/Rap1 signaling is required for SU to exert the full effects on insulin secretion. AC, adenylate cyclase; Gs, Gs proteins; Gi, Gi protein. Created in BioRender.com

SUs have also been described to have extra-pancreatic effects such as attenuation of ischemically induced changes in cardiac electrical properties and prevention of malignant arrythmias and reduced insulin clearance. Accumulating evidence suggests that the activation of mitochondrial K_{ATP} channels plays a crucial role in the mechanical protection that results from ischaemic preconditioning (Billman, 2008). SUs, and glipizide in particular, reduce hepatic uptake and the metabolic clearance rate of insulin in humans; an effect not observed with glibenclamide (Barzilai et al., 1995). Other but more controversial extra-pancreatic effects include inhibition of glucagon secretion from pancreatic α-cells (Cryer, 2012), and enhanced insulin sensitivity (Overkamp et al., 2002; Pernet et al., 1985; Tracewell et al., 1998).

The most common side effect shared by all SUs is hypoglycemia, in particular with the use of glyburide (glibenclamide) and first-generation formulations. Therefore, these drugs should be avoided in patients at highest risk for hypoglycemia, such as elderly, those with poor nutrition or that have concomitant renal, hepatic, or cardiovascular (CV)

disease. Another side effect that has been extensively debated is its CV safety, this topic was called into question with a first-generation SU (tolbutamide), but recent clinical studies (ADVANCE and CAROLINA) have shown neutral results regarding the CV safety profile of second-generation SU (Scheen, 2021).

Some studies have demonstrated that second generation SU glibenclamide accumulates progressively in the β-cell (Ling et al., 2006). Moreover, autoradiography studies have shown that SUs are internalized by the β-cell and bind to intracellular sites such as secretory granules (Carpentier et al., 1986; Geng et al., 2003). In 2003, Kamp et al. (Kamp et al., 2003) were the first to show that SUs cross phospholipid bilayer membranes rapidly by free-diffusion mechanism.

5.2. Effects of glibenclamide in pancreatic β-cells

Glibenclamide is a K_{ATP} channel blocker and broad-spectrum ATP-binding cassette transporter (ABC) inhibitor included in the second-generation sulfonylurea group, as explained in previous section (see **Table 1**).

In 1981, Hakan Borg and Andersson first described the long-term effects of sulfonylureas using isolated mouse islets cultured for one week with glibenclamide (Håkan Borg & Andersson, 1981). They proved that glibenclamide stimulated insulin secretion the initial 3 days of culture, but no enhancement of insulin secretion was shown during the final days. At the end of the culture period, they tested the islets to observe the capacity for insulin release in response to an acute glucose challenge, and demonstrated that insulin release was abolished by culture in the presence of glibenclamide.

In 1992, Davalli et al. (Davalli et al., 1992) using human islets, treatments for 48h with tolbutamide, a first generation SU, proved that human islets become selectively desensitized to the same stimuli given acutely after a chronic treatment, showing a reduced insulin response with preserved insulin content. In 1999, (Kawaki et al., 1999), using MIN6 cells with chronic SUs treatment, proved that desensitization of β-cells was the result of a reduced number of functional K_{ATP} channels on the plasma membrane. They also found that following this chronic treatment, β-cells showed significantly increased basal insulin secretion, reduced insulin content and significantly increased basal intracellular [Ca^{+2}] when compared to control cells. The desensitization result after

treatment with glibenclamide occurs not only following the same stimuli (glibenclamide or other SU) but also following stimulation with glucose, L-alanine, L-arginine, and KCL (Ball et al., 2000).

Glibenclamide shows a prolonged binding in comparison to other SUs like glicazide or glimepiride (Mriller et al., 1994) which its binding is very reversible, thus could explain the negative effects associated to *in vitro* treatment with glibenclamide shown in the literature. Sawada et al. (Sawada et al., 2008) *in vitro* experiments in rodent β-cell have demonstrated that glibenclamide, out of 3 different SUs analyzed, showed the most prominent negative effects: stimulating apoptosis (after 48h) and increasing ROS production (after 24h). Indeed, there are some proapoptotic effects *in vitro* with rodent β-cells (Efanova et al., 1998; Iwakura et al., 2000) and with human islets cultured *ex vivo* (Del Guerra, Marselli, et al., 2005; Maedler et al., 2005). The apoptotic effect of glibenclamide is thought to be derived by increased intracellular [Ca^{2+}] (Iwakura et al., 2000) and may involve an increase of intracellular ROS (Kimoto et al., 2003) or increased ER stress (J. Y. Kim et al., 2012). In contrast, chronic treatment of gliclazide protects pancreatic β-cells from apoptosis (Del Guerra et al., 2007; Sawada et al., 2008), protects against oxidative stress by upregulating the antioxidant defense status of β-cells, increasing SOD and CAT activity (Gier et al., 2009; O'Brien et al., 2000).

Regarding several other effects of glibenclamide on β-cells, it is observed that with glibenclamide treatment there is a decrease on intracellular pH and with other SU such as tolbutamide the decrease is much smaller (Kamp et al., 2003); it has also been observed that glibenclamide, by binding to ABCC8 which encodes for SUR1, decreases insulin gene expression (Zertal-Zidani et al., 2013) and induces decrease on insulin content resulting in degranulation of β-cells (Ling et al., 2006). It has been observed in rodent islets that β-cell insulin stores depletion is enhanced by the lack of increased (pro)insulin biosynthesis (Alarcon et al., 2006; Boland et al., 2017). This activation of insulin secretion is prolonged even in the absence of glibenclamide or glucose. This altered functional state is calcium dependent and involves protein translational activity (Ling et al., 2006). As mentioned in the previous section, glibenclamide is accumulated intracellularly in the membranes of secretory vesicles and mitochondria and it is

attributed to a protein kinase C activation following glibenclamide stimulation of diacylglycerol synthesis. Glibenclamide activates protein translation at the levels of initiation and elongation due to the sustained elevation in intracellular calcium, activating mTOR signaling via PKA/PKB-dependent and independent pathways, leading to phosphorylation of 4E-BP1 (promotes cap-dependent translation initiation) and rpS6 (increases ribosomal biosynthesis) (Q. Wang et al., 2008). Finally, glibenclamide treatment may help protecting β-cells against autoimmune attack, which triggers the development of type 1 diabetes (Lamprianou et al., 2016). Regarding some off-target effects of glibenclamide, it is observed that glibenclamide inhibits activation of inflammasome (NLRP3) (Lamkanfi et al., 2009).

Glibenclamide can counteract the metformin suppression of protein translation that results from inhibition of mTOR pathway, by activating both mTOR and PKA (Q. Wang et al., 2011). In contrast to glibenclamide, high glucose could not antagonize metformin inhibition of translation, although it activated mTOR and translation in the absence of metformin. These observations indicate that glibenclamide and glucose regulate different pathways upstream of mTOR. Glibenclamide-induced signals can activate mTOR when AMPK is active, suggesting that glibenclamide acts downstream of AMPK. In contrast, the glucose-induced mTOR activation is blocked by AMPK activation, suggesting that glucose acts upstream of AMPK.

Regarding the *in vivo* effects of glibenclamide, Remedi and Nichols (Remedi & Nichols, 2008) elegantly demonstrated the reversible effects of SUs while treating wild-type mice with glibenclamide pellets. Pellet-implanted mice became progressively and consistently glucose-intolerant, with greatly reduced insulin secretion in response to glucose, and freshly isolated islets from these animals showed a reduction of secretory capacity. Strikingly, however, secretory capacity was fully restored in these islets within hours of drug washout *ex vivo*. These results show that chronic glibenclamide treatment causes loss of insulin secretory capacity due to β-cell hyperexcitability, but further reveal the rapid reversibility of this secretory failure.

5.3. Primary and secondary sulfonylurea failure

Cardiovascular safety, glucose-lowering potency, hypoglycemia risk, effect on body weight, and cost are important considerations to take into account while selecting drugs to manage T2DM. Most guidelines state that metformin should be first-line therapy followed by various options for second-line treatment if sufficient glycemic control is not achieved after metformin monotherapy (World Health Organization, 2018). Sulfonylureas and dipeptidyl peptidase-4 (DPP-4) inhibitors are the most commonly used second-line glucose-lowering treatments in many countries (Montvida et al., 2018). Sulfonylureas are used mainly based on their low cost, well-established glucose-lowering action, and a longstanding experience in clinical practice. However, not all patients with T2DM respond to the antidiabetic action of sulfonylureas (primary failure), and most patients who respond well initially may experience a loss of effective antidiabetic response after several years of treatment (secondary failure).

It was suggested back in the 80s that sulfonylureas present acute β-cell unresponsiveness, meaning that T2DM patients chronically treated with SUs often progress to a failure of β-cells to secrete insulin despite intensive therapy (Dunbar & Foà, 1974; Karam et al., 1986). This secondary failure has been later studied and confirmed in clinical trials such as the UKPDS (Matthews et al., 1998) as well as the ADOPT study (Kahn et al., 2006). De Fronzo, when describing the ominous octet, highlighted that sulfonylureas only improve insulin secretion, which is transient, and that these agents do not preserve β-cell function (Defronzo, 2009; DeFronzo et al., 2013; Ryder & Defronzo, 2015).

The reasons underlying this secondary failure have long been debated. One hypothesis that has attracted considerable interest is the notion that sulfonylurea-mediated hyperexcitation of β-cells may trigger excitotoxic reactions leading to increased rates of β-cell apoptosis (Maedler et al., 2005). As a result, β-cell mass decreases, and this is seen as the major cause of the developing insulin deficiency (Lupi & Del Prato, 2008). An alternative scenario is suggested by findings in mouse models. Mice with reduced K_{ATP} channel activity show the expected increased β-cell electrical activity and, consequently, hypersecretion of insulin (Remedi et al., 2006). Mouse models with complete lack of functional K_{ATP} channels have been generated by ablation

of either the K^+ channel pore KIR6.2 or the regulatory sulfonylurea receptor 1. Surprisingly, both models exhibit a paradoxical undersecretory phenotype, in spite of continuous β-cell electrical activity and an apparently normal pancreatic β-cell mass (Miki et al., 1998; Seghers et al., 2000; Shiota et al., 2002). These findings suggest that a bell-shaped relationship exists between electrical activity and insulin secretion, and that hyperexcitation leads to β-cell failure without β-cell death (**Figure 9**).

SUs are also prescribed as treatment of maturity-onset diabetes of the young (MODY), which is an unusual form of diabetes caused by a defect on a single gene. Mutations in three genes (HNF1A, HNF4A, GCK) account for about 95% of all MODY cases and oral hypoglycemic agents (generally sulfonylureas) are recommended for patients with mutations in the HNF4A and HNF1A genes, as well as ABCC8 and KCNJ11 genes. When used from diagnosis, SUs are known to remain effective in patients with HNF1A diabetes for many years at small doses (Pearson et al., 2000). The patients needing to return to insulin tended to have had a longer duration of diabetes. Progressive loss of pancreatic β-cell function is seen in HNF1A diabetes resulting in increasing glycemia and increasing treatment requirements (Pearson et al., 2001). In this type of diabetes primary failure of sulfonylurea medications is almost rare and has actually been detected in only a few patients carrying the c.618G->A mutation (Demol et al., 2014). The development of absolute insulinopenia, or insulin deficiency, due to secondary failure has been reported after 3–25 years of SU treatment (Shepherd et al., 2009), with a decrease in insulin secretion of about 1% per year of treatment. These data suggest that response to sulfonylurea also reduces with duration of diabetes.

6. Incretin treatments: GLP-1 receptor agonists

Incretins such as glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), which are secreted from the intestine upon meal ingestion, amplify insulin secretion from pancreatic β-cells in a glucose concentration–dependent manner (Daniel J. Drucker, 2006; Gheni et al., 2014). This glucose dependency of incretin action provided the basis for recently developed incretin-based therapies, which have less risk for hypoglycemia. GLP-1 and GIP bind to their specific receptors on pancreatic β-cells, G-protein coupled receptors, increasing the intracellular

cAMP level through the activation of adenylate cyclase, thereby leading to the potentiation of insulin secretion. This potentiation is mediated by both protein kinase A (PKA)-dependent and PKA-independent pathways, the latter involving Epac2, a protein possessing guanine nucleotide exchange activity toward the small GTPase Rap (Kashima et al., 2001; Ozaki et al., 2000; Renström et al., 1997) .

Experiments in rodent cell lines and islets first demonstrated that activation of GLP-1 receptor signaling leads to enhanced expression of mRNA transcripts for *Gck*, *Glut2*, *Pdx1*, and insulin (D. J. Drucker et al., 1987; Perfetti et al., 2000; Stoffers et al., 2000; X. Wang et al., 1999; Y. Wang et al., 1995, 1997). The studies also showed the positive impact of GLP-1 by improving β-cell function and increase β-cell mass (D. J. Drucker et al., 1987; Y. Wang et al., 1995) through enhanced proliferation of existing β-cells in rodents (Edvell & Lindström, 1999). Additionally, GLP-1 has shown an effect inhibiting H_2O_2-induced β-cell apoptosis (Hui et al., 2003) by acting through GLP-1 receptor signaling which directly modifies the susceptibility to apoptotic injury (Y. Li et al., 2003).

GLP-1 has a short half-life (1-2 min) as it is rapidly degraded in the circulation by a serine exopeptidase dipeptidyl peptidase 4 (DPP-4) (Nauck & Meier, 2018). Consequently, GLP-1 receptor agonists (GLP-1RAs) therapies have been developed generating stable derivatives of GLP-1 with prolonged action resistant to DPP-4 (Nauck et al., 2021). GLP-1RAs, such as lixisenatide, liraglutide, exenatide, dulaglutide, albiglutide and semaglutide, are a group of glucose-controlling drugs that are widely used in the treatment of T2DM due to their ability to promote insulin secretion, lower glucagon secretion and promote gastric emptying and weight loss. Their glucose-lowering properties are comparable with those of insulin therapy (Abd El Aziz et al., 2017). Exenatide (synthetic Exendin-4), was the first GLP-1RA introduced in 2005, GLP-1RAs are delivered by subcutaneous injection. However, in 2020, the first orally administered GLP-1RA was approved for the treatment of T2DM (Chaplin, 2020). Another GLP-1RA, PF-06882961, was optimized to promote endogenous GLP-1R signaling at nanomolar concentrations. PF-06882961 was shown to increase insulin levels in primates and produce a dose-dependent decline in serum glucose levels in healthy humans (Griffith et al., 2022).

Coadministration of incretin and SU has been found to enhance insulin secretion in humans (Aaboe et al., 2009; Gutniak et al., 1996). Also observed in C57BL/6 mice models where islets cultured *ex vivo* for 2 days with glibenclamide and GLP-1 exhibited insulin secretion higher in combination showing an additive effect. The authors found that Epac2A is a target of both incretin and sulfonylurea. This suggests the possibility of interplay between incretin and sulfonylurea through Epac2A/Rap1 signaling in insulin secretion (H. Takahashi et al., 2015). Epac2A rather than PKA plays a major role in the augmentation of SU-induced insulin secretion by cAMP signaling. The combination of GLP-1 with glibenclamide or glimepiride, but not gliclazide, enhances Epac2A/Rap1 signaling and that PKA activation is not involved in Epac2A/Rap1 activation.

GLP-1 and Ex-4 have been reported to also activate β-cell replication in mice and young human islet grafts (Tian et al., 2011) and prevent β-cell exhaustion and ER stress-induced apoptosis in an *in vivo* mice model and rodent β-cells (Tsunekawa et al., 2007; Yusta et al., 2006). There is another study suggesting the protective effect of Ex-4 against glibenclamide-induced β-cell apoptosis in rodent cells through decreased expression of SERCA and ER Ca^{2+} depletion (J. Y. Kim et al., 2012).

VII. HYPOTHESIS AND AIMS

Progressive pancreatic β-cell failure that results from reduced β-cell function and β-cell mass is central in the development of T2DM. The loss of functional β-cell mass was originally attributed to β-cell apoptosis, but a novel mechanism referred to as β-cell dedifferentiation has emerged as a possible contributor during stress-induced glucotoxicity and its downstream pathways: oxidative stress, endoplasmic reticulum (ER) stress, the hexosamine pathway, inflammation and hypoxia. Importantly, precedent studies show that β-cell dedifferentiation can be a reversible process.

Chronic exposure to sulfonylureas, K_{ATP} channel blockers used as oral hypoglycemic agents in the treatment of T2DM, results in impaired β-cell function and loss of β-cell mass that could account for the secondary failure to sulfonylurea treatment described in T2DM. Indeed, there are some studies conducted in mice suggesting β-cell exhaustion after chronic membrane hyperexcitability and insulin hypersecretion due to SUs. However, no conclusive data in human islets has been reported to understand these sulfonylurea-induced effects.

GLP-1 based therapies, a T2DM treatment, that potentiate glucose-induced insulin secretion in β-cells, have been shown to increase insulin biosynthesis, improve β-cell function, increase β-cell mass, and prevent β-cell apoptosis.

HYPOTHESIS

The hypothesis of this thesis was that chronic exposure to glibenclamide could induce β-cell dedifferentiation resulting in a reduction of the functional β-cell mass, which could contribute to the progression of secondary failure to sulfonylureas. Considering the protective effects of GLP-1R agonists on β-cell viability and function, it was hypothesized that this process could be attenuated by GLP-1R agonists, resulting in the preservation of mature β-cell function.

The aim of the study was to investigate the effect of T2DM treatments and the diabetogenic environment on human β-cell phenotype. The specific aims of the study were:

- To determine the effects of chronic glibenclamide exposure on β-cell function, β-cell apoptosis and β-cell identity.

- To elucidate the underlying mechanisms contributing to the glibenclamide-induced effects on β-cell function and identity.
- To analyze the impact of the GLP-1R agonist Exendin-4 on glibenclamide-induced effects.
- To analyze the impact of high glucose on β-cell function, β-cell apoptosis and β-cell identity.

VIII. MATERIALS AND METHODS

1. Tissue procurement

1.1. Human islet isolation

Human islet isolation is a time sensitive and intensive procedure that requires a teamwork approach. The islet isolation was performed in the clean room facilities at the Unit of Pancreatic Islet Transplantation of the Institute d'Investigació Biomèdica de Bellvitge (IDIBELL, Barcelona, Spain). Use of human islets was approved by the local Ethics Committee of Hospital Universitari de Bellvitge (identification number: PR037/17).

1.1.1. Pancreas processing

Human pancreatic islets were isolated from adult deceased pancreas donors, for which signed consent was obtained from donor relatives. Inclusion and exclusion criteria were verified before proceeding to islet isolation (**Table 2**).

The pancreas was removed from the donor as part of multiorgan procurement by the Unit of Hepatic Transplantation of the General and Digestive Surgery Service. The pancreas was placed in University Wisconsin preservation solution (UW solution; DuPont Pharma, Mississauga, ON, Canada) at 4°C. Ideally, islet isolation should be performed as soon as possible once the pancreas is harvested in order to minimize cold ischemia time. The quality of the pancreas is critically important, damage of the pancreatic tissue can cause exocrine tissue degranulation and non-specific activation of endogenous proteolytic enzymes. The body and tail region were used for islet isolation. On arrival at our laboratory, the pancreas was decontaminated by placing it in Ringer's acetate (B. Borun, Meelsungen, Germany) containing 0.05% povidone iodine (Meda manufacturing, Bordeux, France).

Table 2 Pancreas donor qualifications.

Inclusion criteria (the donor or pancreas must meet these criteria)
1. Donors accepted by the Transplant Coordination Service of Hospital Universitari de Bellvitge 2. Donor age: Over 15 years old 3. *In situ* hypothermic perfusion
Exclusion criteria
1. History or biochemical evidence of type 1 or 2 diabetes 2. Cold ischemia time greater than 20 hours 3. Hot ischemia time greater than 10 minutes 4. Prolonged periods of hypotension which cause biochemical abnormalities such as increase of transaminase serum levels 5. Evidence of clinical or active viral hepatitis (B, C) 6. AIDS or HIV seropositivity (HIV-I or HIV-II) 7. Inadequate organ preservation (temperature above 4°C, without a preservation solution) 8. Elevated serum levels of alpha-amylase

1.1.2. Pancreas perfusion

The main pancreatic duct was cannulated and a solution with methylene blue (Farmacia Dr. Carreras, Barcelona, Spain) was injected with a 5 ml syringe (BD Bioscience, Franklin Lakes, NJ, USA) to ensure that pancreatic capsule was intact or to identify leakage sites. An important consideration of pancreas procurement for islet isolation is to maintain the integrity of the pancreas capsule to prevent leaking of the enzyme during perfusion. The pancreas was perfused with 100 ml of a cold three-enzyme mix solution containing collagenase and proteases (Collagenase HA with thermolysin and clostripain, VitaCyte, Indianapolis, IN, USA) for 30 minutes. The enzyme concentrations were 7.5 Wunsch Units (collagenase)/ 0.03 mg (thermolysin)/ 0.04 mg (clostripain)/ml. During this process the temperature and the pressure of the pancreas

was controlled in order to achieve a correct distension and perfusion of the organ. In addition, the peripancreatic fat was removed maintaining the capsular integrity during the last 10 minutes. Finally, the perfused pancreas was cut into 4-6 pieces and placed in a Ricordi digestion chamber (**Figure 14 A**).

1.1.3. Digestion

The digestion step was performed using the Ricordi method (Ricordi et al., 1988) with some modifications (Goto et al., 2004) using the Ricordi chamber and a continuous digestion. The pancreas was digested through a mechanical agitation and continuous flow of the enzyme mixture (**Figure 14 B**). The temperature of the digestion chamber was controlled at 37±1°C. Samples were taken every 2 minutes from the Ricordi chamber to assess the size of the dissociated tissue and the presence of free islets by DTZ (Sigma-Aldrich, St. Louis, MO, USA) staining. A few drops of a DTZ solution [prepared as follows: 10 mg of DTZ dissolved in 300 µl of 70% ethanol, NaOH 1M, diluted with 20 ml of Ringer's acetate] were added to a petri dish along with the sample and visualized under a microscope. The solution must be freshly prepared and filtered (Millex-HA Filter Unit, 0.45 µm, Merk Millipore, Billerica, MA, USA) before use.

When most of the islets were separated from exocrine tissue in the assessed samples, the digestion was stopped by diluting the tissue solution with the diluting buffer at 37°C [Ringer's acetate solution supplemented with 25 mM glucose (Sigma-Aldrich), 10 mM nicotinamide (Sigma-Aldrich), 5 mM sodium pyruvate (Sigma-Aldrich), 0.025% sodium bicarbonate (Gibco, Gaithersburg, MD, USA), 15 mM HEPES (Biological Industries, Kibbutz BeitHaemek, Israel), 0.20 µg/ml DNase (Pulmozyme, Roche Diagnostics, Mannheim, Germany)]. In order to neutralize the enzymes, the diluting solution was collected in 250 ml-collection tubes that contained 2% ABO compatible human serum (Blood and Tissue Bank, BST, Barcelona, Spain), and they were placed in an ice water bath. The digested pancreas was incubated with UW solution supplemented with 10 nM nicotinamide and 5 mM sodium pyruvate for 90 minutes.

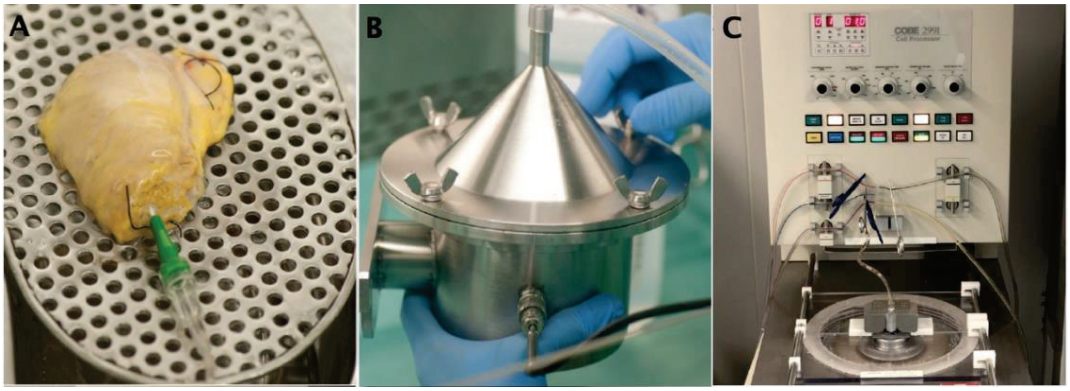


Figure 14. Human islet isolation procedure. (A) Pancreas perfusion with enzyme mix solution containing collagenase and proteases. (B) Pancreas digestion in the Ricordi chamber. (C) Purification in the COBE 2991 cell processor.

1.1.4. Islet purification

Digested tissue was purified on a continuous density gradient and centrifuged in a refrigerated COBE 2991 cell processor (COBE BCT, Lakewood, CO, USA) at 4-8°C (**Figure 14 C**). Seven 25 ml-fractions were collected in 250 ml conical tubes (Corning, Corning, NY, USA) prefilled with 225 ml of Ringer's acetate supplemented with 100 U/ml Penicillin/Streptomycin (Laboratorios Normon, Madrid, Spain), 25 mM glucose, 10 mM nicotinamide, 5 mM sodium pyruvate, 0.025% sodium bicarbonate, 15 mM HEPES and 2.4% ABO compatible human serum. Islet purity of the fractions was evaluated by DTZ staining and examined by light microscopy. Each fraction was pooled with fractions of equivalent purity (**Figure 15**).

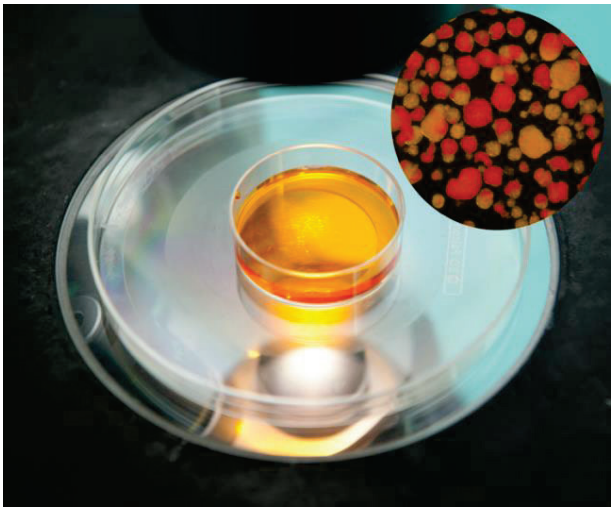


Figure 15. Human islet fraction after isolation procedure. Islet purity assessed by DTZ staining.

1.1.5. Islet culture

Immediately after purification, islets were cultured overnight in ultra-low attachment cell culture plates (Corning, NY, USA) with CMRL-1066 medium (5.6 mM glucose) (Connaght Medical Research Laboratories; Mediatech, Manassas, VA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (v/v), 2 mM L-glutamine (Life Technologies, Grand Island, NY, USA), 10 mM nicotinamide (Sigma-Aldrich, St. Louis, MO, USA), 10 mM HEPES (Biological Industries, Kibbutz BeitHaemek, Israel), 250 ng/ml amphotericin B (Fungizone, Life Technologies), 40 µg/ml gentamicin (Laboratorios Normon, Madrid, Spain) and 20 µg/ml ciprofloxacin (Fresenius-Kabi, Barcelona, Spain) at 37°C and 5% CO_2. Islet purity was determined by dithizone staining (Sigma-Aldrich) and only ≥80% purity preparations were used for all experiments. After overnight culture, medium was changed to complete CMRL-1066 at 5.6 mM and islets were cultured for one week in the different experimental conditions.

2. Experimental design

Human islets were cultured for 4 or 7 days in complete CMRL-1066 at 5.6 mM glucose (control condition), and Glibenclamide (Glib) (Sigma, G0639) at 1 µM, or Glib (1 µM) and the chemical chaperone 4-phenylbutyrate (PBA) (Sigma, SML0309) at 2,5 mM, or Glib (1 µM) and the GLP-1 receptor agonist exendin-4 (Ex-4) (Sigma, E7144) at 10 nM, or at 22.2 mM glucose (high glucose). Culture medium was changed every 48h. At the end of the culture in the different conditions, islets were collected to determine β-cell function (glucose-stimulated insulin secretion) (GSIS), β-cell apoptosis (immunohistochemistry), ER stress and oxidative stress (gene expression analysis by quantitative PCR and Western Blot), β-cell dedifferentiation (gene expression and immunohistochemistry).

Human β-cell line

EndoC-βH3 cells (Univercell-Biosolutions, Toulouse, France), a conditionally immortalized human pancreatic β-cell line, was cultured according to the manufacturer's instructions. Briefly, cells were cultured in coated (βcoat®; Univercell-Biosolutions) 100 mm TPP plates at a density of $5.4x10^6$ cells/plate in culture medium

(Optiβ1®; Univercell-Biosolutions) supplemented with 10 µg/ml puromycin (Invivogen) and passaged every 7 days. The immortalizing transgenes were removed by a 21-day treatment with 1µM 4-hydroxy tamoxifen (Sigma-Aldrich). Excised cells were cultured in 12-well TPP plates (Sigma-Aldrich) at a density of $4x10^5$ cells/well with Optiβ1 culture medium with 5.6 or 22.2 mM glucose for one week. At the end of the culture, cells were collected for gene expression analysis.

Mouse islet isolation and culture

Mouse islets were isolated from C57BL/6J male mice (Janvier laboratory), 9-22 weeks of age, by collagenase digestion and Histopaque gradient as described. After isolation, islets were hand-picked under the stereomicroscope to obtain a pure islet population and cultured overnight in non-tissue culture treated plastic ware at 37°C and 5% CO_2 in RPMI-1640 (Sigma-Aldrich) supplemented with 10% FBS (v/v), 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin and 11.1 mM glucose. After the overnight culture, medium was changed to complete RPMI medium with 11.1 or 22.2 mM glucose and islets were cultured for one week to determine GSIS. Animal experimental procedures were reviewed and approved by the Ethical Committee for Animal Experimentation of the University of Barcelona (CEEA 480/16).

Rat β-cell line

Rat insulinoma INS-1 cells, a generous gift from Prof. Claes B. Wollheim, were cultured between passages 74 and 86 in TC Dish 100 Cell+ (Sarstedt AG & Co. KG) in a complete medium composed of RPMI 1640 (Sigma-Aldrich), supplemented with 10% FBS (v/v), 100 U/ml penicillin, 100 U/ml streptomycin, 1 mM sodium pyruvate, 50 µM 2-mercaptoethanol, 10 mM HEPES, 2.4 mM $NaHCO_3$, and 11.1 mM glucose at 37°C and 5% CO_2. Cells were split weekly at 10^3 cells/mm^2 and the medium was changed every 2 to 3 days. INS-1 cells were seeded at a density of $1.15x10^5$ cells/ml in TC Plate 24-well Cell+ (Sarstedt AG & Co. KG) with complete RPMI medium and after 65-72 hours, the medium was then replaced by new complete RPMI medium with 11.1 or 22.2 mM glucose. Medium was changed after 48 hours of culture. After 96 hours, gene expression analyses were performed.

3. Techniques

3.1. B-cell function

3.1.1. Glucose-stimulated insulin secretion

After one week culture in experimental conditions, groups of 20 islets handpicked using a low dithizone (Sigma-Aldrich) concentration (0.023 mM) that does not impair β-cell function (Latif et al., 1988), were washed three times with Krebs-Ringer Bicarbonate Hepes (KRBH) buffer [115 mM NaCl (Sigma-Aldrich); 24 mM $NaHCO_3$ (Sigma-Aldrich); 5 mM KCl (Sigma-Aldrich); 1 mM $MgCl_2$ (Sigma-Aldrich); 2.5 mM $CaCl_2 \cdot 2H_2O$ (Sigma-Aldrich); 10 mM HEPES; and 0.5% BSA, pH 7.4] with 2.8 mM glucose. They were pre-incubated in 1 ml fresh KRBH buffer containing 2.8 mM glucose for 1 h in a shaking water bath at 37°C. Then, they were sequentially incubated with 1 ml of KRBH buffer containing 2.8 mM (basal) glucose for 1h and an additional hour with 1 ml of KRBH buffer containing 20 mM (stimulated) glucose with continuous shaking. Supernatants were collected and stored at -80°C until assayed for insulin content (Mercodia human insulin ELISA; Mercodia AB, Uppsala, Sweden) according to the manufacturer's instructions. Absorbance was measured at 450 nm in ELx808^{TM} Absorbance Microplate Reader (BioTek, Winooski, VT, USA). Insulin secretion was expressed as percentage of insulin content. The stimulation index (SI) was calculated as the secreted insulin ratio between stimulated (20 mM) and basal (2.8 mM) glucose concentrations.

3.1.2. Insulin and DNA content

After the GSIS assay, islets were rinsed three times with PBS to remove the BSA and were sonicated in 1 ml ddH_2O. For insulin content, an aliquot of the homogenate was extracted with acid-ethanol solution and stored at -80°C until insulin quantification by ELISA as described above. The remaining homogenate was stored at -80°C, and DNA content was measured by fluorimetry with Hoechst 33258 (Sigma-Aldrich) (3.33 µg/ml) on a fluorescence spectrophotometer (FLUOstar Omega, BMG Labtech, Ortenberg, Germany) (excitation wavelength 360 nm, and emission wavelength 460 nm).

3.2. Immunostaining

3.2.1. B-cell apoptosis

Islets were pelleted, fixed overnight in 4% paraformaldehyde-PBS (PFA; Merck KgaA, Darmstadt, Germany) and processed for paraffin embedding. Sections were double

stained by immunofluorescence for apoptotic nuclei with the terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) technique (ApopTag Plus Fluorescein *In Situ* Apoptosis Detection Kit; Merk Millipore), and insulin using a rabbit anti-human insulin antibody (Santa Cruz Biotechnology Inc., Dallas, TX, USA) (final dilution 1:100). Preparations were visualized by immunofluorescence with donkey anti-rabbit IgG conjugated with Alexa Fluor 555 (Life Technologies) (final dilution 1:400). Nuclei were stained with 300 nM DAPI (Life Technologies). TUNEL-positive β-cells were assessed using a BX51 fluorescence microscope (Olympus). Cell imaging was done through DP Controller software (Olympus) and the different labeled pictures for each pellet of islets were overlapped for quantification using GIMP2 software. The number of $TUNEL^{+}insulin^{+}$ cell was divided by total number of $insulin^{+}$ cells and the β-cell apoptosis rate was calculated and presented as percentage of β-cell apoptosis. A minimum of 1,000 cells per sample were counted.

3.2.2. Fluorescence intensity quantification

Islets were pelleted, fixed overnight in 4% paraformaldehyde-PBS (PFA; Merck KgaA, Darmstadt, Germany) and processed for paraffin embedding. Sections were double stained for insulin using a guinea pig anti-Insulin antibody (Abcam) (final dilution 1:500), and NKX6.1 using a mouse anti-NKX6.1 (DSHB) (final dilution 1:100), NKX2.2 using a mouse anti-NKX2.2 (DSHB) (final dilution 1:100), or MAFA using a rabbit anti-MafA (Bethyl laboratories) (final dilution 1:250). Preparations were visualized by immunofluorescence with goat anti-guinea pig IgG conjugated with Alexa Fluor 647 (Life Technologies) (final dilution 1:400) and goat anti-mouse IgG conjugated with Alexa Fluor 555 (Life Technologies) (final dilution 1:400) or donkey anti-rabbit IgG conjugated with Alexa Fluor 555 (Life Technologies) (final dilution 1:400). Images of double-labelled sections were acquired using a Carl Zeiss LSM 880 spectral confocal laser scanning microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) equipped with multiline argon laser (458nm, 488 and 514nm), 405nm and 561nm diode lasers and 633nm He/Ne laser (*Centres Científics i Tecnològics, Universitat de Barcelona*, Bellvitge Campus, Barcelona, Spain) using a 25x oil immersion objective (0.6 numerical aperture) operated using Zen Black Edition (Zeiss, version 3.2). ImageJ software (version 2.1.0) was used to determine the number of cells that were positive for insulin and NKX6.1, or insulin and

NKX2.2, or insulin and MAFA, and were manually counted. Quantification of insulin fluorescence intensity was performed using ImageJ software (version 2.1.0). Briefly, images were subjected to an auto-threshold before measuring the mean gray value (or average of intensity units in selection). Quantification of nuclear NKX6.1, NKX2.2 and MAFA fluorescence intensity of INS^+ cells was performed using ImageJ. First, images were subjected to an auto-threshold before selecting for INS^+ region of interest (ROI), which was then used to identify the $DAPI^+$ ROI. Then, only $DAPI^+$ ROI was used to measure the mean gray value of the transcription factor staining. Results were expressed as arbitrary units.

3.3. Gene expression analysis

3.3.1. RNA isolation

RNA was isolated from human islet preparations after 4 or 7 days of culture, and from INS-1 cells after 96h. Total RNA was extracted with RNeasy Mini or Micro Kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. The principle for the extraction is based on the stringency of guanidine-isothiocyanate lysis with the speed and purity of silica-membrane purification (RNeasy Plus Mini Handbook 12/2014, Qiagen). Cultured islets were immersed in lysis buffer containing 350 µl guanidine isothiocyanate-containing buffer plus 3.5 µl ß-mercaptoethanol (BioRad, Hercules, CA, USA). Samples were homogenized and lysed by mechanical disruption with repeated pipetting. The lysate was then passed through a gDNA eliminator spin column. This column, in combination with the optimized high-salt buffer, allows efficient removal of genomic DNA. Ethanol was added to the flow-through to provide appropriate binding conditions for RNA, and the sample was then applied to an RNeasy spin column, were total RNA binds to the membrane. RNA was then eluted in water.

RNA quantity and quality was assessed with the Bioanalyzer 2100 (Agilent Technologies, Inc., Palo Alto, CA, USA) or using the NanoDropTM ND-2000c Spectrophotometer (Thermo Fisher Scientific). The RNA Integrity Number (RIN) estimates the integrity of total RNA samples. The RIN values range from 10 (intact) to 1 (totally degraded). The RIN score of our samples ranged from 8 to 10.

3.3.2. Reverse transcriptase PCR

cDNA (complementary DNA) synthesis was performed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster, CA, USA) according to the manufacturer's instructions. Random primers were used to ensure that the first strand synthesis occurs efficiently with all RNA molecules. To remove RNA complementary to the cDNA, samples were incubated with RNase H at 37°C for 20 min. The reactions were performed in a 2720 Thermal Cycler (Applied Biosystems).

3.3.3. Real-time qPCR

qPCR was run in a 7900HT fast Real-Time PCR system (Applied Biosystems) with 384-well optical plates. Reactions were performed using TaqMan Gene Expression Assays and TaqMan Gene Expression Master Mix (Applied Biosystems) following the manufacturer protocol in a final volume of 20 µl with 10 ng of cDNA in each reaction. A full listing of assays (Applied Biosystems), gene names, and assay identification number is given in **table 3**. Relative quantities (RQ) were calculated using the software Gene Expression Suite v1.0.3 (Applied Biosystems) using the $2^{-\Delta\Delta CT}$ analysis method with 60S acidic ribosomal protein P0 (RPLP0) and TATA-box binding protein (TBP) as endogenous controls.

Table 3 Gene expression assays used for real-time qPCR.

Gene name	Gene symbol	Assay ID
HUMAN		
ATP binding cassette subfamily C member 8	ABCC8	Hs01093752_m1
Aldehyde dehydrogenase 1 family member A3	ALDH1A3	Hs00167476_m1
Aristaless related homeobox	ARX	Hs00292465_m1
Activating transcription factor 6	ATF6	Hs00232586_m1
Catalase	CAT	Hs00156308_m1
DNA damage inducible transcript 3	DDIT3	Hs00358796_g1
Eukaryotic translation initiation factor 2 alpha kinase 3	EIF2AK3	Hs00984003_m1

Forkhead box O1	FOXO1	Hs00231106_m1
Glucagon	GCG	Hs01031536_m1
Glucagon like peptide 1 receptor	GLP1R	Hs00157705_m1
Hexokinase 1	HK1	Hs00175976_m1
Hexokinase 3	HK3	Hs01092850_m1
Insulin	INS	Hs00355773_m1
Potassium voltage-gated channel subfamily J member 11	KCNJ11	Hs00265026_s1
Lactate dehydrogenase A	LDHA	Hs01378790_g1
MAF bZIP transcription factor A	MAFA	Hs01651425_s1
MAF bZIP transcription factor B	MAFB	Hs00534343_s1
Neurogenin 3	NGN3	Hs01875204_s1
NK2 homeobox 2	NKX2.2	Hs00159616_m1
NK6 homeobox 1	NKX6.1	Hs00232355_m1
Paired box 4	PAX4	Hs00173014_m1
Paired box 6	PAX6	Hs01088114_m1
Proprotein convertase subtilisin/kexin type 1	PCSK1	Hs01026107_m1
Proprotein convertase subtilisin/kexin type 2	PCSK2	Hs00159922_m1
Pancreatic and duodenal homeobox 1	PDX1	Hs00236830_m1
Ribosomal protein lateral stalk subunit P0	RPLP0	Hs99999902_m1
Solute carrier family 2 member 1	SLC2A1	Hs00892681_m1
Solute carrier family 2 member 2	SLC2A2	Hs01096908_m1
Superoxide dismutase 2, mitochondrial	SOD2	Hs00167309_m1
SRY-box 9	SOX9	Hs00165814_m1

TATA-box binding protein	TBP	Hs99999910_m1
Wolframin ER transmembrane glycoprotein	WFS1	Hs00903605_m1
X-box binding protein 1 (spliced)	XBP1s	Hs03929085_g1
	RAT	
Forkhead box O1	FoxO1	Rn01494868_m1
Insulin 2	Ins2	Rn01774648_g1
MAF bZIP transcription factor A	MafA	Rn00845206_s1
NK6 homeobox 1	Nkx6.1	Rn01450076_m1
Catalase	Pdx1	Rn00755591_m1
TATA-box binding protein	Tbp	Rn01455646_m1

3.4. Western Blot

3.4.1. Protein extraction

Total proteins from 3000 human IEQs were extracted as followed: centrifuged, washed three times in PBS at 4°C, and the pellet resuspended in ice-cold RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, pH 7.4) containing protease and phosphatase inhibitors (Sigma-Aldrich). Islets were disaggregated by gentle continuous pipetting every 10 min for a total incubation of 30 min on ice. After centrifugation at 14,000 rpm for 10 min at 4°C, supernatants were collected and stored at -80°C.

3.4.2. Protein quantification

Proteins were further quantified using a colorimetric assay for measuring total protein concentration, the Pierce BCA protein assay kit (Thermo Fisher Scientific). Assays were conducted according to the manufacturer's protocol.

3.4.3. Western blotting

Equal amount of proteins (30 µg) were subjected to SDS-PAGE and then transferred onto PVDF membranes. Blots were blocked for 1h with TBS-T (Tris-buffered saline + 0.05% Tween-20) + 5% non-fat dry milk and then incubated overnight at 4°C with specific primary antibodies (**Table 4**) diluted in TBS-T + 1% non-fat dry milk. HRP-conjugated goat

anti-mouse/rabbit secondary antibodies (**Table 4**) were used for developing blots using chemiluminescent detection. Images were acquired using the Amersham Imager 680 system. Target protein levels (normalized to ACTIN) were quantified by densitometric analysis using ImageJ software.

Table 4 Antibodies for Western Blot.

Antibody	Brand	Reference	MW (kDa)	Dilution
ACTIN	EMD Millipore	MAB1501R	43	1:10000
CHOP	Cell Signaling	2895S	27	1:500
XBP1s	Cell Signaling	12782	60	1:500
Anti-Mouse HRP	Abcam	ab205719	-	1:5000
Anti-rabbit HRP	Abcam	ab205718	-	1:10000

3.5. B-cell tracing

3.5.1. Lentiviral vector production

Transfection of human embryonic kidney 293T (HEK 293T) cells for lentiviral vector production was obtained using the packaging vector psPAX2 and the envelope vector pMD2.G, and plasmids: pTrip-RIP400-nlscre-DeltaU3, pTrip-CMV-Lox-PURO-Lox-eGFP (eGFP), and pLVX-IRES-tdTOMATO (tdTomato). Concentration of lentiviral vectors was performed based on ultracentrifugation (90.000 g) with a 30% sucrose gradient. The pellets were resuspended in complete medium and kept a -80 °C until used.

3.5.2. Lentiviral vector titration

To titrate the lentiviral particles, first p24 ELISA which determines the amount of virus p24 proteins present in vector cores, was used to determine a relative lentiviral particle titers. Then, functional titration assays based on vector-encoded reporter gene expression (eGFP and tdTomato) in rat INS-1 cells were analyzed using fluorescence-activated cell sorting (FACS) analysis to obtain the IFU (infectivity units) per ng p24, calculated using the formula (infectivity units (IFU)$\cdot \mu l^{-1}$= P x N /100x V, where P= % of fluorescent cells, N= number of cells/well at the time of infection, and V= volume (µl)

virus/well) (Tiscornia et al., 2006). After 4 different functional titration assays with its linear regression equation and solving it for each lentiviral vector, the linear regression equation of the functional titration assay that had a closer approximation to all lentiviral particles tested was selected (**Figure 16**).

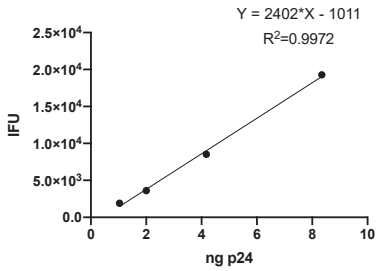


Figure 16. Linear regression equation selected from functional titration assay using LV-tdTomato in INS-1 cells and analyzed by FACS 4 days after infection. Graph represents infective units (IFU) as a function of the amount (ng) of vector (p24) added.

3.5.3. Efficiency of lentiviral infection

The linear equation from previous graph (**Figure 16**) was solved to calculate the IFUs needed to infect whole islets in suspension from ng of p24 of our lentiviral vectors. Infection of whole human islets in suspension was performed using 3.6x10^6 total IFUs equivalent to 1.5x10^3 ng p24. The concentration (ng p24/µl) of each lentiviral vector obtained by p24 ELISA was used to calculate the volume of lentiviral vector needed to infect with 1.5x10^3 ng p24. After lentiviral infection, the efficiency of infection was analyzed 4 days (experimental time-point 0) and 11 days post-infection (experimental time-point 7) using Fluorescence-activated Cell Sorting (FACS). Briefly, islets were washed with PBS+1% BSA prior to trypsinization. Islets were disaggregated by gentle continuous pipetting in trypsin–EDTA 0.05% (Sigma) with DNase (1 mg/ml) (RQ1 RNases-Free DNase, Promega, Madison, WI, USA) for 6 min. in a water bath at 37 °C (Téllez et al., 2005). Dispersed islet cells were washed once with PBS and incubated with LIVE/DEAD dye (Live/Dead® Fixable Dead Cell Stain Kits, Live Technologies) at RT for 30 min, protected from light. Following one 1% BSA-PBS wash, cells were fixed in 4% paraformaldehyde–PBS at room temperature (RT) for 15 min. Prior to flow cytometry, labelled cells were washed twice with FACS buffer (PBS, 2 mM EDTA, 0.5%BSA) and transferred into 35 µm nylon mesh cell strainer caped-tubes (BD biosciences, Erembodegem, Belgium). Sorting was done using a Beckman-Coulter MoFlo Astrios cell

sorter equipped with a 100-µm flow tip and operated at a sheath pressure of 25 psi (Carballar et al., 2017). The laser illumination was set for eGFP excitation at 488 nm, for LIFE/DEAD blue day at UV, and for tdTomato excitation at 554 nm.

3.5.4. Lentiviral vector infection system

For β-cell lineage tracing, 2000 human IEQs were transduced with two lentiviral vectors: pTrip-RIP400-nlscre-DeltaU3 and pTrip-CMV-loxP-PURO-loxP-eGFP using 3.6x10^6 total IFUs. Human IEQs were transduced overnight in complete CMRL medium containing 8 µg/ml polybrene. After overnight incubation, the medium was refreshed. Four days post-transduction, labelled islets were exposed to 1 µM Glibenclamide for 7 days islets and then were fixed overnight in 4% paraformaldehyde-PBS (PFA; Merck KgaA, Darmstadt, Germany) and processed for paraffin embedding. Sections were double stained for GFP using a chicken anti-GFP antibody (Abcam) (final dilution 1:250) and for insulin using a rabbit anti-human insulin antibody (Santa Cruz Biotechnology Inc., Dallas, TX, USA) (final dilution 1:50). Preparations were visualized by immunofluorescence with goat anti-chicken IgG conjugated with Alexa Fluor 488 (Life Technologies) (final dilution 1:400) and donkey anti-rabbit IgG conjugated with Alexa Fluor 555 (Life Technologies) (final dilution 1:400). Nuclei were stained with 300 nM DAPI (Life Technologies). B-cell dedifferentiation was expressed as percentage of GFP^+ $insulin^+$ cells. A minimum of 1,000 cells per sample was counted.

4. Statistical analysis

Data were expressed as mean ± SEM. Statistical analysis were performed using GraphPad Prism 9 software. Normality for all variables was tested using Kolmogorov-Smirnov test. Statistical significance was determined with the Unpaired Student t-test or Multiple t-test with Bonferroni-Dunn correction as appropriate. A p value <0.05 was considered significant.

IX.RESULTS

1. Procurement of human islets

Human pancreatic islets were isolated from 27 adult deceased multi-organ donors, 40.7% female, 57.3±1.78 years (range: 33-73), BMI 26.24±0.86 (Kg/m^2) (range: 19.53-42.58). All donors were Caucasian and the most common causes of death of donors were stroke (n=12) and anoxia after cardiac arrest (n=6). Donors with known history of diabetes mellitus were excluded. In addition, glycated hemoglobin (HbA1c) and/or blood glucose was used to confirm that the donors did not have diabetes (**Table 5**).

Table 5. Islet donor characteristics.

Donor number	Sex	Age	BMI	Hb1aC (%)/ Blood glucose (mM)	Cause of death	Cold ischemia time (min)
1	F	52	ND	4.6 mM	SAH	590
2	F	66	22.2	5.6 mM	SAH	785
3	F	53	22.5	ND	Ischemic stroke	853
4	M	51	29.4	6.1 mM	Cerebral hypoxia	976
5	F	50	22	5.3 %	SAH	792
6	M	52	31.2	7.4-8.9 mM	Cerebral hypoxia	825
7	M	59	24.1	4.4 %	Hemorrhagic stroke	419
8	M	73	26.1	5.6 %	CVA	457
9	M	69	26.9	5.3 mM	SAH	930
10	M	67	25.3	5.5 %	CVA	946
11	M	48	29.3	4.8 %	CVA	699
12	M	53	27.8	5.6 %	CVA	722
13	M	53	26.1	5.5 %	Anoxia after cardiac arrest	909
14	F	58	29.7	6.1 %	Anoxia after cardiac arrest	722

15	M	63	27.1	5.6 %	Anoxia after cardiac arrest	692
16	F	33	22.9	5.2 %	CVA	923
17	M	72	27.5	5.7 %	CVA	741
18	F	61	23.0	5.5 %	Stroke	640
19	F	56	19.5	5.6 %	Cranioencephalic trauma	913
20	F	62	23.4	5.2 %	Stroke	939
21	F	68	21.9	5.4 %	Cranioencephalic trauma	720
22	M	68	25.3	5.8 %	Anoxia after cardiac arrest	864
23	M	45	27.7	5.1 %	Stroke	890
24	M	48	24.8	5.7 %	Anoxia after cardiac arrest	1227
25	M	59	27.6	5.4 %	Stroke	706
26	F	51	42.6	5.0 %	Cranioencephalic trauma	838
27	M	58	26.2	5.3 %	Anoxia after cardiac arrest	913

BMI, body mass index; Hb1ac, glycated hemoglobin; F, female; M, male; ND, not determined; SAH, subarachnoid hemorrhage; CVA, cerebrovascular accident.

The purest islet fractions (≥80% purity), usually the first fractions obtained from the cell processor were used for this project. The mean islet purity of the islet fractions, determined by DTZ staining, was 84.4±1.38% (range: 75-95%).

PART 1.

2. Effects of glibenclamide exposure on human pancreatic β-cells

2.1. Glibenclamide-induced effects on β-cell function

The GSIS assay was used to determine the β-cell function of cultured islets. Islets exposed to 1 µM glibenclamide for one week showed an impaired GSIS as indicated by a lower SI (control: 6.39±1.31; glibenclamide: 1.80±0.22, p=0.004; n=8) (**Fig. 17A**). The lower SI in glibenclamide group was accounted by combination of a significant increase in basal insulin secretion at 2.8 mM glucose (control: 1.13±0.21%; glibenclamide:

2.71±0.66%, p=0.04; n=8) and a non-significant reduction in stimulated insulin secretion at 20 mM glucose (control: 8.34±2.86%; glibenclamide: 5.19±1.8%, p=0.35; n=8) (**Fig. 17B**). Islet insulin content was reduced by 31.7% (p<0.001) (**Fig. 17C**) in glibenclamide exposed islets, raw values were the following (control: 258.2±63.6; glibenclamide: 160.7±39.7 ng Ins/µg DNA, n=8). DNA quantification did not differ between groups (control: 0.61±0.1 µg DNA; glibenclamide: 0.54±0.09 µg DNA, p=0.64; n=8).

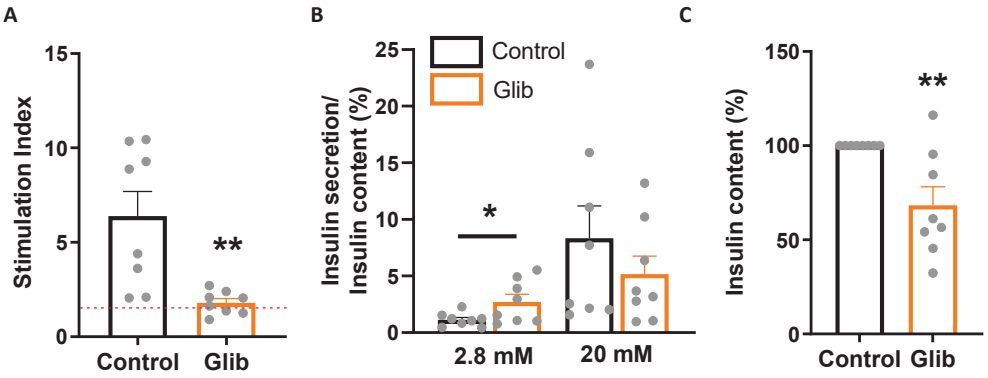


Figure 17. B-cell function assessed after chronic exposure to glibenclamide. Cultured islets from human donors were cultured at 5.6 mM glucose (control) or 1 µM glibenclamide (Glib) for one week. (A) Stimulation index is expressed as ratio between stimulated (20 mM glucose) and basal (2.8 mM glucose) insulin secretion. (B) Insulin secretion of control and glibenclamide-exposed islets at 2.8 mM glucose and 20 mM glucose normalized by insulin content. (C) Islet insulin content after GSIS. All bars show mean ± SEM (n=8). Each dot represents one experiment performed with islets from a different islet donor. *p value<0.05; **p value<0.01; ***p value<0.001 with Student t-test.

2.2. Glibenclamide-induced effects on β-cell apoptosis

In order to study if glibenclamide had a detrimental effect on β-cell viability, β-cell apoptosis was determined by TUNEL assay. After one week of culture, β-cell apoptosis was significantly increased in islets exposed to glibenclamide (control: 0.75±0.05%, glibenclamide: 1.49±0.24%; p=0.02; n=4) (**Fig. 18 A-B**).

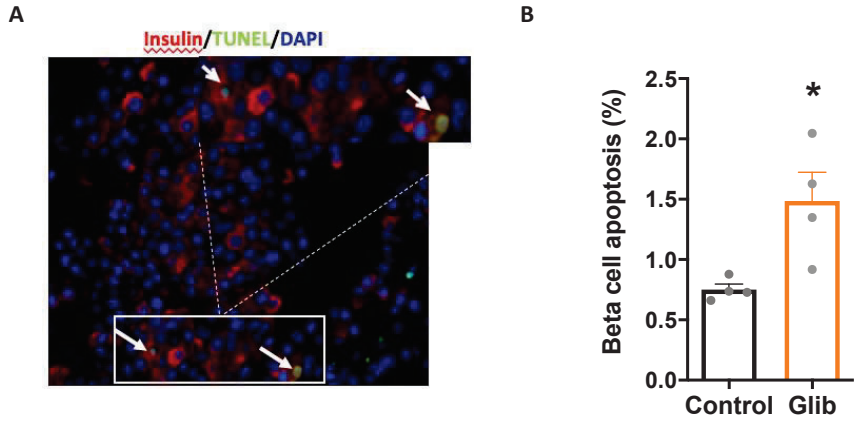


Figure 18. B-cell apoptosis assessed after chronic exposure to glibenclamide. (A) Representative immunofluorescence image showing double staining for TUNEL (green) and insulin (red). Nuclei are stained in blue with DAPI. (B) Quantification of β-cell apoptosis after one week of culture. All bars show mean ± SEM (n=4). Each dot represents one experiment performed with islets from a different islet donor. *p value<0.05 with Student t-test.

2.3. Glibenclamide-induced effects on β-cell identity

The expression of β-cell specific transcription factors *MAFA*, *MAFB, PDX1, NKX6.1, NKX2.2*, *PAX6* as well as insulin gene and transcription factor *FOXO1* were significantly reduced in glibenclamide-treated islets (**Fig. 19**). Prohormone convertase 2 (PC2; gene *PCSK2*) involved in the processing of mature insulin, the β-cell marker *GLP1R* and *KCNJ11*, encoding the Kir6.2 protein of K_{ATP} channel were also significantly reduced in glibenclamide-treated islets (**Fig. 19**). Gene expression of prohormone convertase 1/3 (PC1/3; gene *PCSK1*) also involved in the processing of mature insulin was significantly increased.

As mentioned in the introduction when describing the β-cell dedifferentiation concept, depending on the model used, some but not all, describe a metabolic reprogramming with an increase of β-cell disallowed genes concomitant to a possible increase of endocrine progenitor-related genes. In our study, when analyzing the glibenclamide-treated human islets, no differences were observed in the expression of disallowed genes (*HK1, HK3, LDHA*) or endocrine progenitor-related genes (*ALDH1A3, NGN3, SOX9*), with *NGN3* not being detected at all (**Fig. 19**).

We sought to analyze the α-cell-specific markers expression. Gene expression of the α-cell-specific marker *ARX* was reduced, and glucagon showed a tendency to reduced expression in the glibenclamide group.

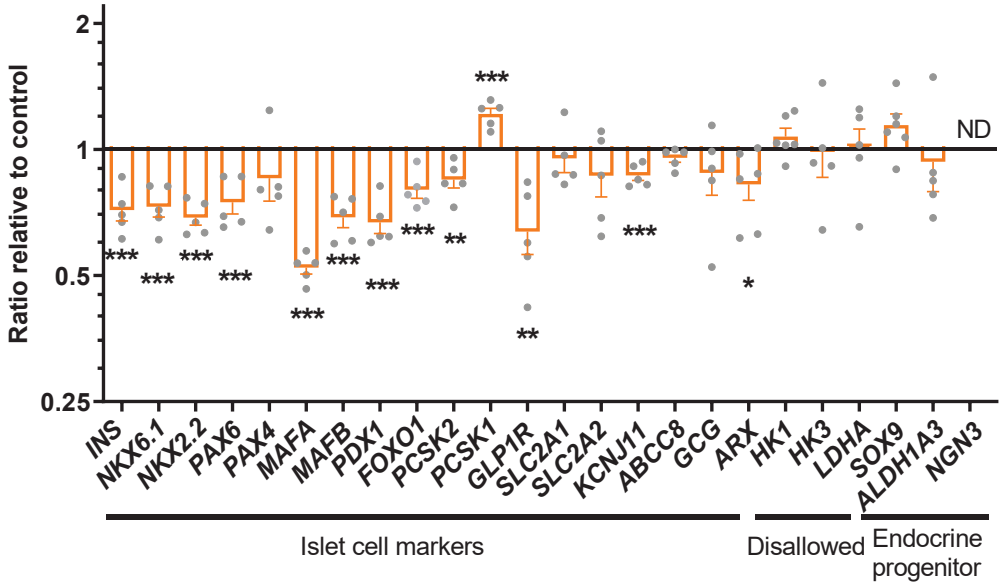


Figure 19. Changes in β-cell gene expression of glibenclamide-exposed islets. mRNA expression of β-cell identity, disallowed and endocrine progenitor markers. Expression is shown as a ratio between control islets and islets cultured with 1 µM glibenclamide for one week in a scale Log2. Each dot represents one experiment performed with islets from a different islet donor. ND: non detectable. Bars show mean ± SEM (n≥5), *p value<0.05; **p value<0.01; ***p<0.001 with Student t-test.

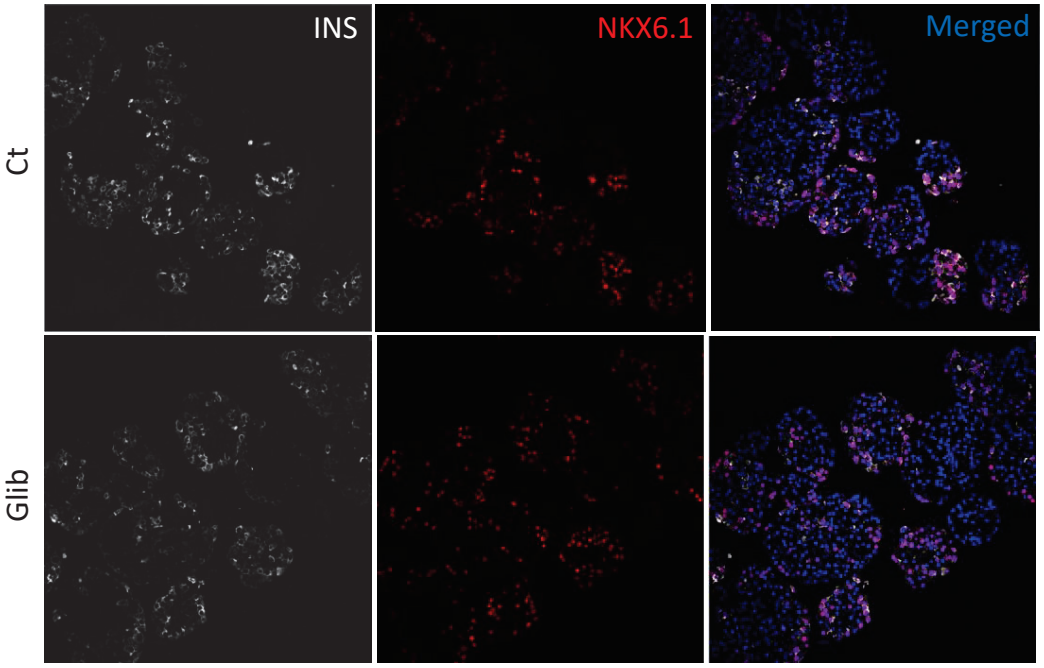
The quantification at the protein level of insulin and key β-cell transcription factors that were downregulated at the gene expression level such as *NKX6.1*, *NKX2.2* and *MAFA*, showed no differences between control and glibenclamide groups (**Table 6**). We did not observe differences in the percentage of INS^+ cells between control and glibenclamide group (p=0.64; n=5). Nor were any significant differences found in the percentage of $NKX6.1^+$ (p=0.71; n=5), $NKX2.2^+$ (p=0.55; n=5) or $MAFA^+$ cells (p=0.23; n=5).

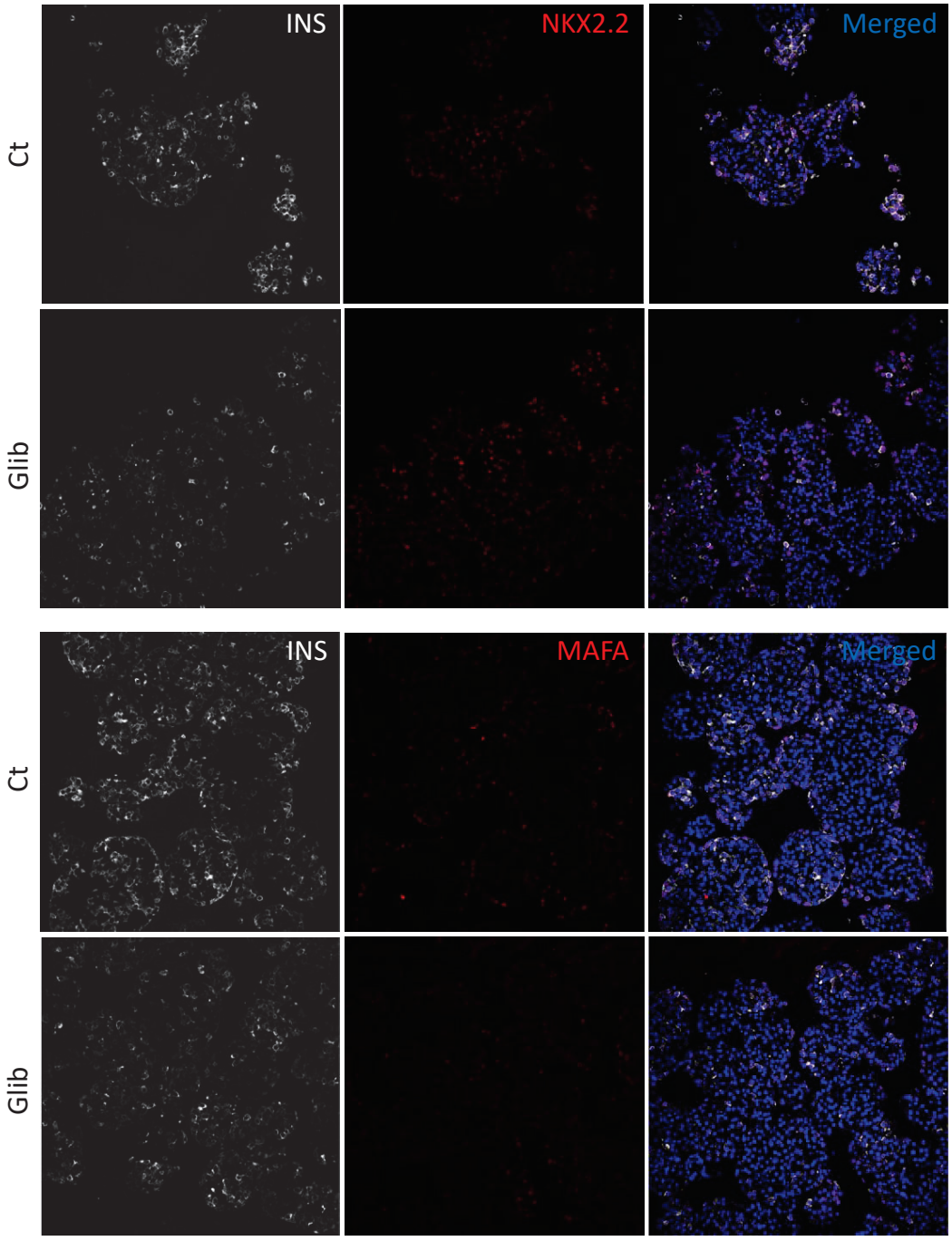
Table 6. Percentage of insulin-positive cells and NKX6.1, NKX2.2 or MAFA-positive cells.

	INS$^+$/DAPI (%)	NKX6.1$^+$/DAPI (%)	NKX2.2$^+$/DAPI (%)	MAFA$^+$/DAPI (%)
Control	40.7±5.76	24.3±6.78	32.0±7.12	17.9±4.42
Glibenclamide	37.1±4.78	20.5±7.03	39.7±10.35	10.5±3.64
	(p=0.64)	(p=0.71)	(p=0.55)	(p=0.23)

However, insulin fluorescence intensity, nuclear NKX6.1 and nuclear MAFA fluorescence intensity in insulin-positive β-cells were significantly decreased in glibenclamide-treated islets (**Fig.20**). Nuclear NKX2.2 fluorescence intensity in β-cells was also analyzed but no differences were observed between groups (**Fig. 20**).

A




INS
NKX2.2
Merged
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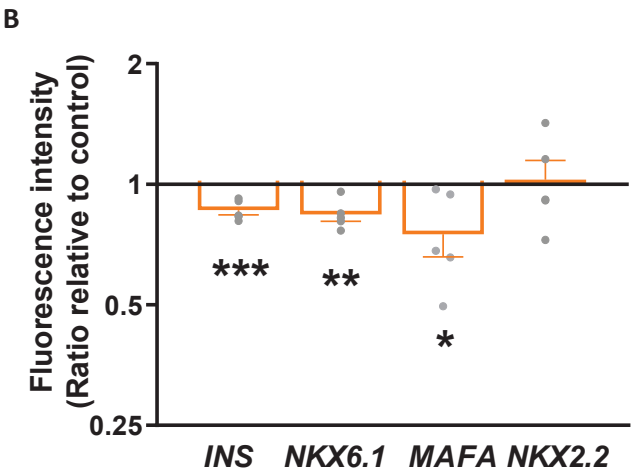

Figure 20. Insulin, NKX6.1, NKX2.2 and MAFA protein expression changes of glibenclamide-exposed islets. (A) Representative immunofluorescence images showing double staining for insulin (white) and transcription factor NKX6.1, NKX2.2 or MAFA (red). Nuclei are stained in blue with DAPI. (B) Quantification of insulin fluorescence intensity, nuclear NKX6.1 fluorescence intensity in β-cells, nuclear MAFA fluorescence intensity in β-cells and nuclear NKX2.2 fluorescence intensity in β-cells. Each dot represents one experiment performed with islets from a different islet donor. Bars show mean ± SEM (n=5). * p value<0.05; **p value<0.01; ***p<0.001 with Student t-test.

2.4. Glibenclamide-induced effects on β-cell dedifferentiation

Set up lentiviral infection method with human islets for β-cell tracing

Lentiviral vector purification, titration and the infection system were set up with human islets as stated in the Materials and Methods section. The efficiency of lentiviral infection in human islets was evaluated at two time-points (4 days and 11 days post-infection) comparing the efficiency of lentiviral infection between islets infected on isolation day (day 0) versus day 1 post-isolation (day 1). Lentiviral vectors used on day 1 post-isolation showed greater efficiency of infection at 4 days (day 0: 11.80±2.87%; day 1: 34.79±8.8%; p=0.03) and at 11 days post-infection (day 0: 22.36±4.36%; day 1: 48.38±11.89%; p=0.08) (**Fig. 21**). There is only one lentiviral vector that was tested on the day of isolation and also on day 1 post-isolation. There was slightly greater efficiency of infection at day 1 post-isolation (day 0: 17.75%; day 1: 26%). Thus, seems that infecting on day 1 post-isolation has greater efficiency of infection than infecting on isolation day.

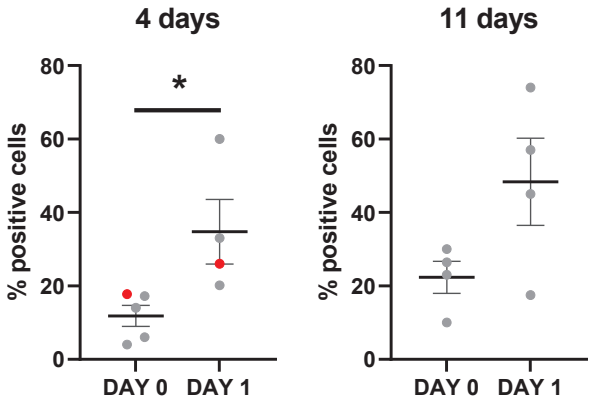

Figure 21. Comparison of lentiviral efficiency of infection between isolation day or day 1 post-isolation at 4 days and 11 days after infection. Red-colored dots represent the only data coming from the same lentiviral vector. Data are mean ± SEM (n=4-5), *p<0.05 Student's t-test.

The efficiency of infection depending on the amount of time the lentiviral vectors used had been stored at -80 °C was analyzed. The results showed that longer storage time did not mean worst efficiency of infection (**Fig. 22**). We concluded that the amount of time stored at -80°C does not affect the efficiency of infection.

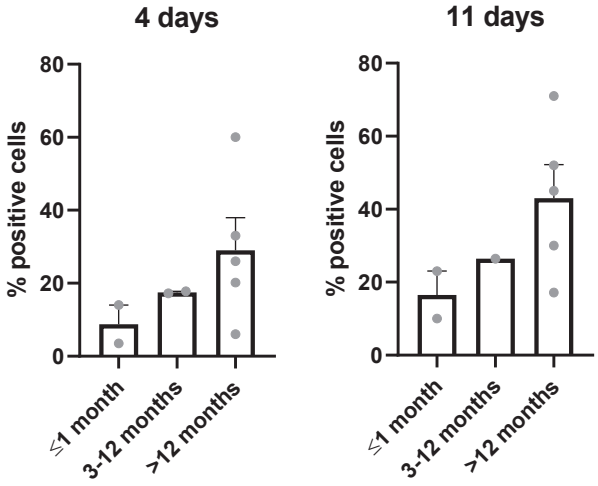

Figure 22. Efficiency of infection comparing the time of storage of lentiviral vectors at -80 °C. Data are mean ± SEM (n=2-5).

From the FACS analysis we have detected that the percentage of reporter-positive cells increased from day 4 to day 11 after lentiviral infection by 2-fold (ratio of increase: 2.05±0.47; n=8). The increase is due to two factors: the detection power by FACS and the difference of cell death between traced and non-traced cells.

Traced human islets with lentiviral vectors pTrip-RIP 400-nlsCre and pTrip-CMV-lox-PURO-lox-eGFP were analyzed by FACS using LIVE/DEAD dye to detect cell death. From the FACS plots (LIVE/DEAD vs GFP) it was observed that the intensity of fluorescent reporter protein (eGFP) was increased at 11 days post-infection (**Fig. 23A**). The results demonstrated that at 11 days post-infection fluorescent reporter proteins showed more intensity due to increased amount of protein accumulated (4 days: 5375±905.1; 11 days: 35833±8002; p=0.01; n=6-8), consequently there was an increased percentage of reporter-positive cells because the sorter was able to detect those traced cells that were not detected at day 4 post-infection (**Fig. 23B**). To sum up, FACS is not able to detect all traced cells (GFP+) at day 4 post-infection because the intensity of the fluorescent reporter protein (GFP) is not enough to be detected as positive. Thus, traced cells are underestimated by FACS at 4 days post-infection.

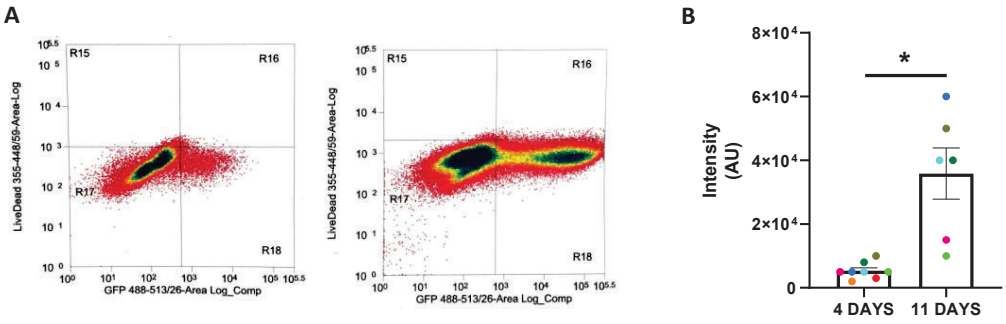

Figure 23. Efficiency of infection by FACS analysis between 4 days post-infection and 11 days post-infection. (A) Representation of FACS analysis plots of dispersed human islets traced with GFP using LIVE/DEAD dye. Left plot: analysis 4 days post-infection, Right plot: analysis 11 days post-infection. (B) Increase of fluorescent reporter protein intensity from 4 days to 11 days after lentiviral infection. AU: Arbitrary Units. Each colored dot represents the same experiment. Data are mean ± SEM (n>6), *p<0.05 Student's t-test.

An increment in cell death amongst the non-traced population (GFP-) in comparison to traced cells (GFP+) (4 days: 33-fold increase; 11 days: 20-fold increase) was observed

(**table 7**). Thus, there is an enrichment of traced cells. As expected, from day 4 to day 11 post-infection, there is around 3-fold increase of dead cells from both populations (3.4-fold GFP+ cells and 2.3-fold GFP- cells).

Table 7. Percentage of dead cells after FACS analysis of traced cells (GFP+) and non-traced cells (GFP-) using LIVE/DEAD dye.

Dead cells (%)	MEAN±SEM (n=4)	
	4 DAYS	**11 DAYS**
GFP+	0.38±0.13	1.30±0.36
GFP-	12.3±5.36	28.9±13.7
fold	33.4	20.8

Evaluation of glibenclamide-induced effects by β-cell tracing

To directly evaluate β-cell dedifferentiation, GFP-traced β-cells were analyzed by double immunofluorescence with insulin and GFP antibodies (**Fig. 24A**). First, the efficiency of the lineage tracing system in human islets was similar in control (39±5.93% of INS^+ traced cells) and glibenclamide group (32.7±6.39% of INS^+ traced cells) (**Fig. 24B**). To detect β-cell dedifferentiation, we looked for cells that had lost the insulin staining maintaining the GFP labelling. No differences were found and after glibenclamide exposure the percentage of INS^-GFP^+/GFP^+ cells was similar in both groups (control: 21.1±4.11%, glibenclamide: 15.3±1.15%; p=0.2; n=5) (**Fig. 24C**).

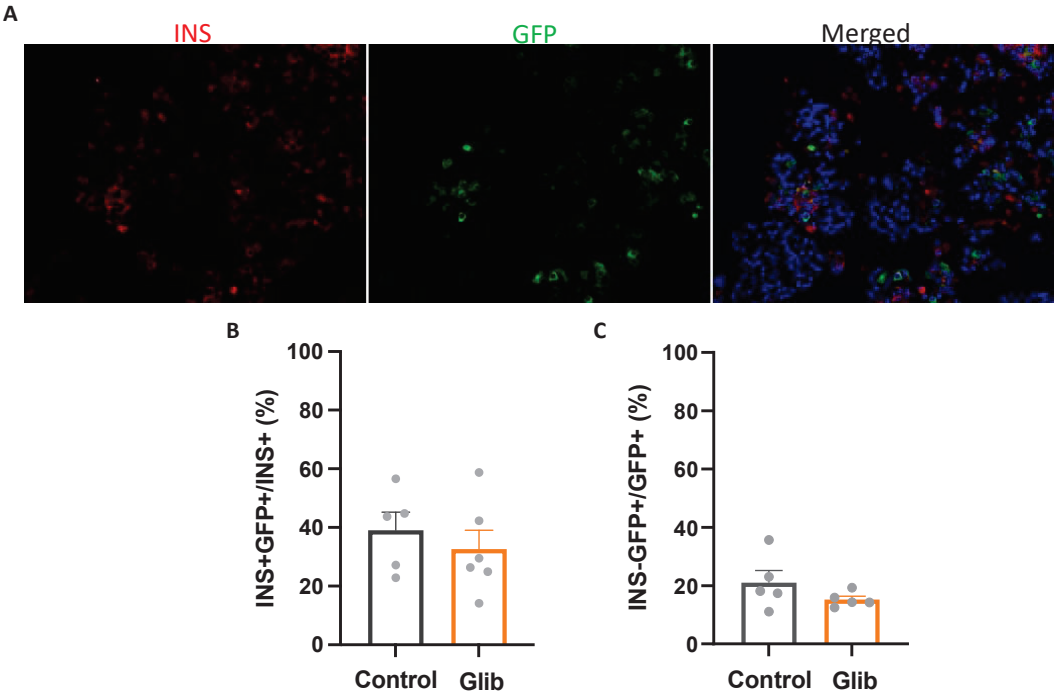

Figure 24. Genetic cell tracing as a direct analysis of β-cell dedifferentiation. (A) Representative immunofluorescence image of traced β-cells showing double staining for insulin (red) and GFP (green). Nuclei are stained in blue with DAPI. (B) Percentage of insulin cells that express GFP showing the efficiency of the lineage tracing. (C) Quantification of traced (GFP^+) β-cells that had lost insulin expression. Each dot represents one experiment performed with islets from a different islet donor. Bars show mean ± SEM (n≥5).

To complement the genetic β-cell tracing analysis, we evaluated the percentage of insulin-positive cells that had lost expression of β-cell TFs NKX6.1, NKX2.2 and MAFA as well as the percentage of TFs-positive cells that had lost insulin expression, from the double-immunofluorescence staining presented in **Fig. 20**. **Table 8** shows the results obtained of INS^+ cells that were $NKX6.1^-$ which showed no differences between control: 48.1±7.61% and glibenclamide: 56.5±9.77% groups (p=0.5, n=5). The percentage of INS^+ cells that were $NKX2.2^-$ showed no differences between control: 43.1±5.23% and glibenclamide: 41.3±7.35% groups (p=0.8, n=5). As well as the percentage of INS^+ cells that were $MAFA^-$ showed no differences between control: 61.1±6.26% and glibenclamide: 72.2±5.88% groups (p=0.2, n=5). However, the number of cells that maintained staining for the transcription factors ($NKX6.1^+$, $NKX2.2^+$ or $MAFA^+$) but were

INS$^-$ showed a tendency to increase in the glibenclamide group (**Table 9**). A 75% increase observed of INS$^-$NKX6.1$^+$/NKX6.1$^+$ cells (control: 7.81±0.95%; glibenclamide: 13.6±3.07%, p=0.1, n=5), a 43% increase of INS$^-$NKX2.2$^+$/NKX2.2$^+$ cells (control: 36.7±6.25%; glibenclamide: 52.8±6.16%, p=0.1, n=5) and 92% increase of INS$^-$ MAFA$^+$/MAFA$^+$ cells (control: 13.5±5.99%; glibenclamide: 25.4±6.29%, p=0.2, n=5).

Table 8. Percentage of insulin-positive cells that were negative for NKX6.1, NKX2.2 or MAFA.

	INS$^+$NKX6.1$^-$ /INS$^+$ (%)	INS$^+$NKX2.2$^-$ /INS$^+$ (%)	INS$^+$MAFA$^-$ /INS$^+$ (%)
Control	48.1±7.61	43.1±5.23	61.1±6.26
Glibenclamide	56.5±9.77	41.3±7.35	72.2±5.88
	(p=0.5)	(p=0.8)	(p=0.2)

Table 9. Percentage of TFs-positive cells that were negative for insulin.

	INS$^-$ NKX6.1$^+$/ NKX6.1$^+$ (%)	INS$^-$ NKX2.2$^+$/ NKX2.2$^+$ (%)	INS$^-$ MAFA$^+$/MAFA$^+$ (%)
Control	7.81±0.95	36.7±6.25	13.5±5.98
Glibenclamide	13.6±3.07	52.8±6.16	25.4±6.29
	(p=0.1)	(p=0.1)	(p=0.2)

2.5. Glibenclamide-induced effects on ER stress and prevention by PBA

To study the mechanisms involved in the effect of glibenclamide exposure upon human pancreatic islets, gene expression of ER stress and oxidative stress markers was evaluated by RT-qPCR. As mentioned previously, pancreatic β-cells exposed to sulfonylureas are subjected to chronic stimulation to secrete insulin which could result in ER stress.

Islets exposed to glibenclamide for 4 days of culture showed increased levels of the ER stress markers spliced XBP-1 *(XBP1s)* (p=0.02), an indicator of the unfolded protein

sensor IRE1 activation, and *DDIT3* (p=0.04), a downstream gene known to be induced by UPR (**Fig.25**). At 7 days of culture, glibenclamide-cultured islets maintained increased levels of spliced XBP-1 *(XBP1s)* (p=0.02), and *DDIT3* (p=0.02), and the expression of *WFS1*, whose absence leads to upregulated ER stress, was significantly decreased (p<0.001) (**Fig. 25**). Other markers of ER stress (or activated UPR) were assessed but no differences were found in *ATF6* or *EIF2AK3* mRNA levels (**Fig. 26**). The expression of two well-established markers of oxidative stress catalase *(CAT)* and superoxide dismutase 2 *(SOD2)* was not modified by glibenclamide neither at 4 or 7 days of culture (**Fig. 26**).

The addition of the chemical chaperone PBA to the culture medium prevented the activation of ER stress markers *XBP1s, DDIT3,* and *WFS1* in glibenclamide-cultured islets (**Fig. 25**). PBA was able to prevent the mRNA changes of all three genes.

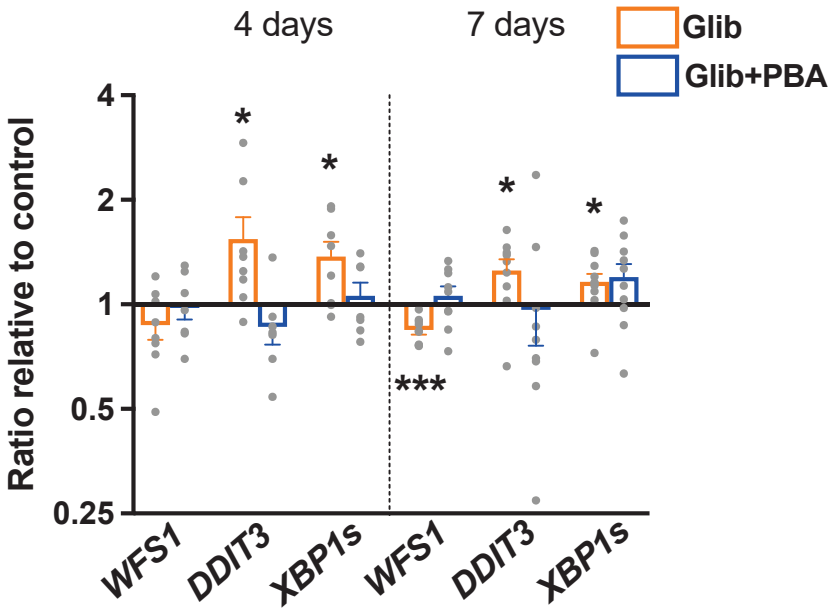

Figure 25. mRNA changes on ER stress markers of glibenclamide-exposed islets with chaperone PBA. Gene expression of ER stress markers at 4 and 7 days of culture. Expression is shown as a ratio between control islets and islets cultured with 1 µM glibenclamide and 2,5 mM PBA in a scale Log2. Each dot represents one experiment performed with islets from a different islet donor. Bars show mean ± SEM (n≥7). *p value<0.05; ***p<0.001 vs control group; Multiple t-test with Bonferroni correction.

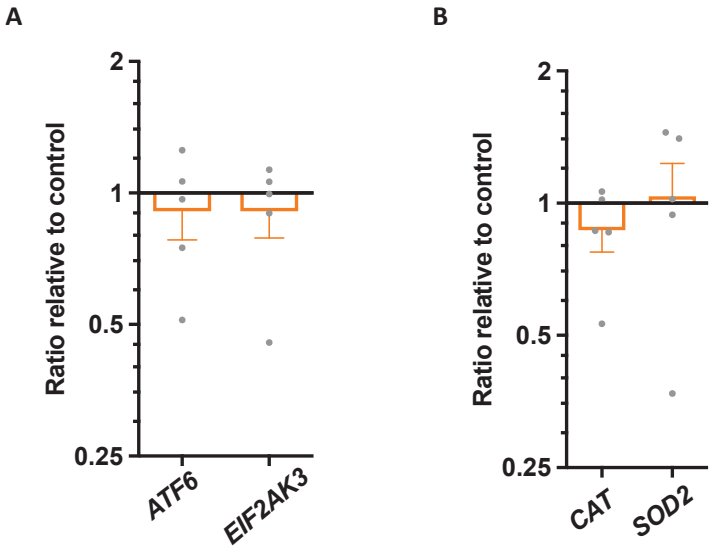

Figure 26. mRNA changes on additional ER stress markers and oxidative stress markers of glibenclamide-exposed islets. (A) mRNA expression of ER stress markers *ATF6*, *EIF2AK3*; (B) oxidative stress markers *CAT* and *SOD2*. Expression is shown as a ratio between control islets and islets cultured with 1 µM glibenclamide for one week in a scale Log2. Each dot represents one experiment performed with islets from a different islet donor. Bars show mean ± SEM (n≥4), *p value<0.05 with Student t-test.

Glibenclamide increased the expression of XBP1s and CHOP proteins after 4 days of culture and PBA was able to prevent it (**Fig. 27 A-C**).

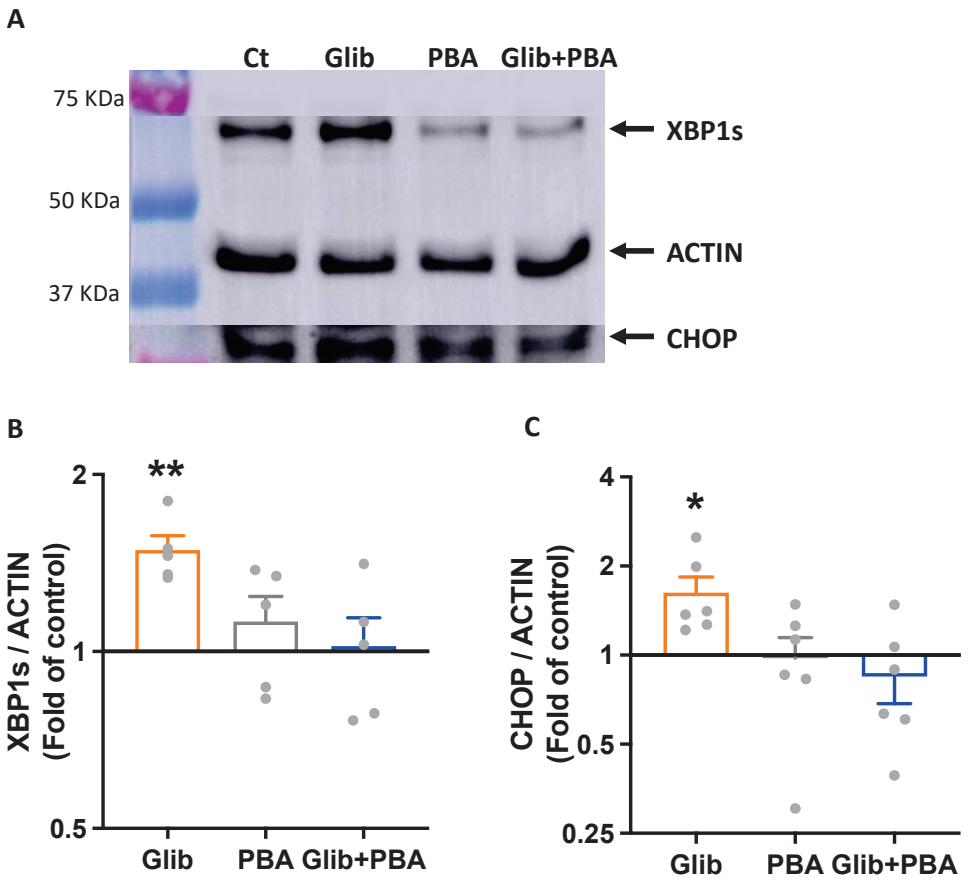

Figure 27. Protein expression changes on ER stress markers of glibenclamide-exposed islets with chaperone PBA. (A) Western blot representative image of ER stress markers at 4 days of culture. (B,C) Quantification of XBP1s and CHOP protein expression after Western blot assay after 4 days of culture. Each dot represents one experiment performed with islets from a different islet donor. Bars show mean ± SEM (n=6). *p value<0.05; **p<0.001 vs control group; Multiple t-test with Bonferroni correction.

2.6. Effects of ER stress alleviation on β-cell identity

To decipher if the increased ER stress observed after glibenclamide exposure could be contributing to the glibenclamide-induced loss of β-cell identity, PBA was added to the medium.

Islets exposed to glibenclamide for 4 days of culture showed decreased expression of *INS* gene and *MAFA*, *PDX1*, *FOXO1*, *GLP1R* and *KCNJ11* mRNAs (**Fig.28**), and PBA was able to prevent the downregulation of all mRNAs except for *INS*, *MAFA* and *GLP1R*.

At 7 days of culture, the addition of PBA to the culture medium prevented the downregulation of the β-cell identity markers *NKX6.1, NKX2.2, PAX6*, *MAFA, FOXO1* and *KCNJ11* (**Fig. 28**). PBA exposure still displayed a decreased expression of *INS*, *PDX1* and *GLP1R* mRNAs in glibenclamide-treated islets, but it was less pronounced. Glibenclamide seems to have a time-dependent effect where the longer the exposure to the drug, more profound is the loss of β-cell identity observed (**Fig. 28**).

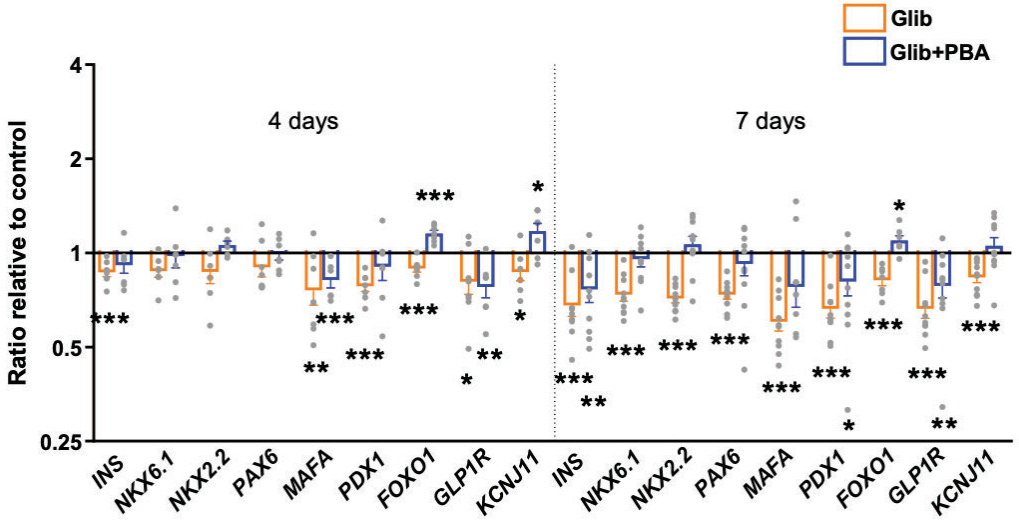

Figure 28. mRNA changes on β-cell identity markers of glibenclamide-exposed islets with chaperone PBA. Gene expression of β-cell identity markers. Expression is shown as a ratio between control islets and islets cultured with 1 µM glibenclamide and 2,5 mM PBA in a scale Log2. Each dot represents one experiment performed with islets from a different islet donor. Bars show mean ± SEM (n≥6). *p value<0.05; **p value<0.01; ***p<0.001 vs control group; Multiple t-test with Bonferroni correction.

Next, we evaluated if the gene expression changes observed after glibenclamide and PBA exposure were confirmed at the protein level. In line with the gene expression results, decrease of nuclear NKX6.1 and nuclear MAFA fluorescence intensity in β-cells was prevented by PBA (**Fig. 29**). However, decrease of insulin fluorescence intensity was not prevented by the addition of PBA in the culture medium, following the same pattern observed at the mRNA level (**Fig. 29**).

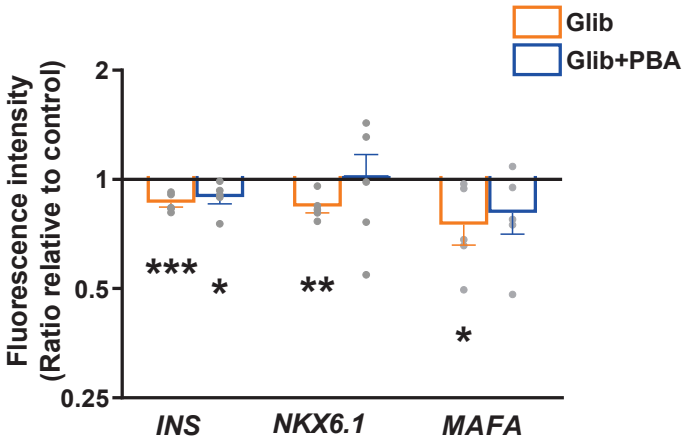

Figure 29. Insulin, NKX6.1 and MAFA protein expression changes of glibenclamide-exposed islets with chaperone PBA. Quantification of insulin fluorescence intensity, nuclear NKX6.1 and MAFA fluorescence intensity in β-cells, after double insulin-NKX6.1 or insulin-MAFA immunofluorescence. Expression is shown as a ratio between control islets and islets cultured with 1 µM glibenclamide and 2,5 mM PBA for one week in a scale Log2. Each dot represents one experiment performed with islets from a different islet donor. Bars show mean ± SEM (n=5). *p value<0.05; **p value<0.01; ***p<0.001 vs control group; Multiple t-test with Bonferroni correction.

Following the observation that PBA prevented glibenclamide-induced loss of β-cell identity, we evaluated if PBA could also prevent glibenclamide-induced β-cell dysfunction by GSIS assay. However, since exposure to PBA alone altered β-cell function, PBA was not a valid tool to determine the effect of ER prevention on glibenclamide-induced β-cell dysfunction (**Fig. 30**).

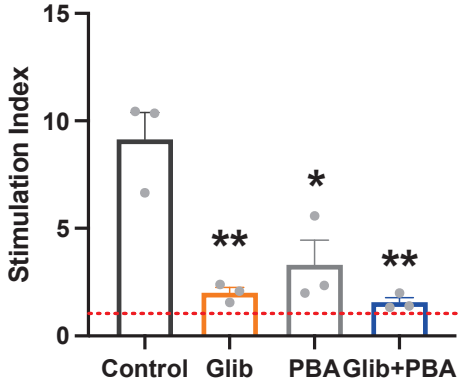

Figure 30. B-cell function of glibenclamide-exposed islets with chaperone PBA. Stimulation index is expressed as ratio between stimulated (20 mM glucose) and basal (2.8 mM glucose) insulin secretion. Each dot represents one experiment performed with islets from a different islet donor. Bars show mean ± SEM (n=3). *p value<0.05; **p value<0.01 vs control group; Multiple t-test with Bonferroni correction.

3. Effects of GLP1 receptor agonist, exendin-4, on glibenclamide-treated islets

3.1. B-cell function and β-cell apoptosis

We tested whether the addition of exendin-4, a GLP-1R agonist, to the culture medium could prevent the detrimental effect of glibenclamide on β-cell function and β-cell apoptosis. Considering the protective effects of GLP-1R agonists on β-cell viability and function, we hypothesized that the deleterious effects of glibenclamide could be prevented by a GLP-1R agonist. Exendin-4 (Ex4) alone did not show any detrimental effect on β-cell function whereas glibenclamide deleterious effects were confirmed with a decreased SI (**Fig. 31A**). Ex-4 could not prevent the glibenclamide-induced β-cell dysfunction (**Fig. 31A**) due to the increased basal insulin secretion observed in the Glib+Ex4 group (p=0.06) (**Fig. 31B**). No differences between groups were found regarding the total insulin content of the islets (**Fig. 31C**).

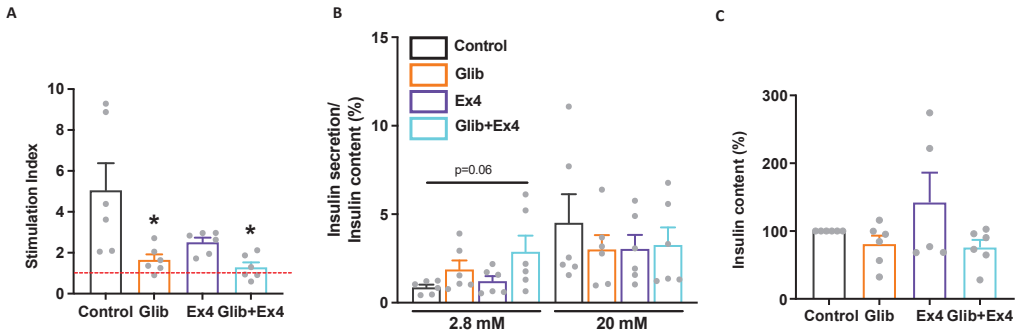

Figure 31. B-cell function of glibenclamide-exposed islets with Exendin-4. Human islets were cultured at 5.6 mM glucose (control), with 1 µM glibenclamide (Glib), 10 nM exendin-4 (Ex4) or both for one week. (A) Stimulation index is expressed as ratio between stimulated (20 mM glucose) and basal (2.8 mM glucose) insulin secretion. (B) Insulin secretion of islets at 2.8 mM glucose and 20 mM glucose normalized by insulin content. (C) Islet insulin content after GSIS. All bars show mean ± SEM (n=6). Each dot represents one experiment performed with islets from a different islet donor. *p value<0.05 vs control group; Multiple t-test with Bonferroni correction.

The addition of Ex4 to the culture medium did not prevent β-cell apoptosis induced by glibenclamide (**Fig. 32**).

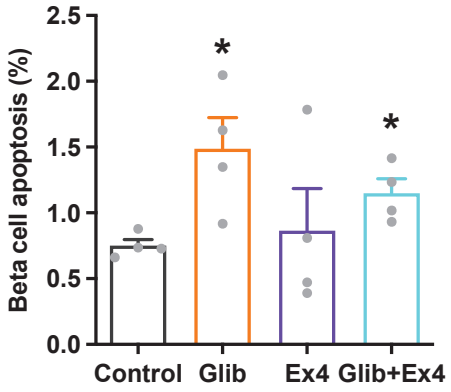

Figure 32. B-cell apoptosis of glibenclamide-exposed islets with Exendin-4. Cultured islets from human donors were cultured at 5.6 mM glucose (control), 1 µM glibenclamide (Glib), 10 nM exendin-4 (Ex4) or both for one week. Quantification of β-cell apoptosis after one week of culture. All bars show mean ± SEM (n=4). Each dot represents one experiment performed with islets from a different islet donor. *p value<0.05 vs control group; Multiple t-test with Bonferroni correction.

3.2. ER stress and β-cell identity

Addition of Ex4 was tested to determine if it could prevent the increased ER stress observed after glibenclamide exposure *in vitro*. mRNA changes observed with glibenclamide (decreased *WFS1* and increased *DDIT3* and *XBP1s)* were not prevented by Ex4 (**Fig. 33**).

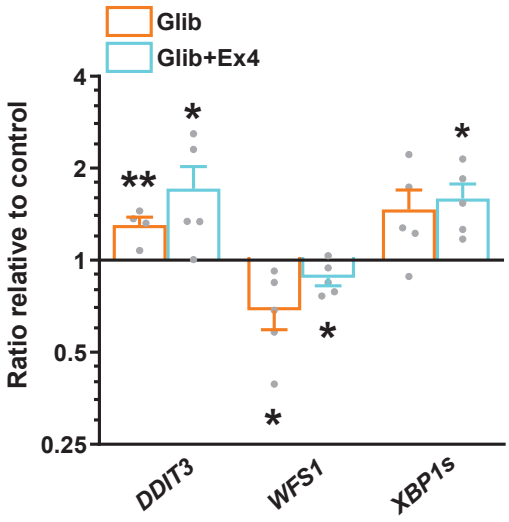

Figure 33. mRNA changes on ER stress markers of glibenclamide-exposed islets with Exendin-4. mRNA expression of ER stress markers. Expression is shown as a ratio between control islets and islets cultured

with 1 µM glibenclamide and 10 nM Ex4 for one week in a scale Log2. Each dot represents one experiment performed with islets from a different islet donor. ND: non detectable. Bars show mean ± SEM (n=5), *p value<0.05; **p value<0.01 vs control group; Multiple t-test with Bonferroni correction.

B-cell identity changes observed after glibenclamide exposure with decreased *INS*, *NKX6.1*, *NKX2.2*, *PAX6*, *MAFA*, *PDX1*, *GLP1R* and *PCSK2* mRNAs were also not prevented by Ex4 (**Fig. 34**).

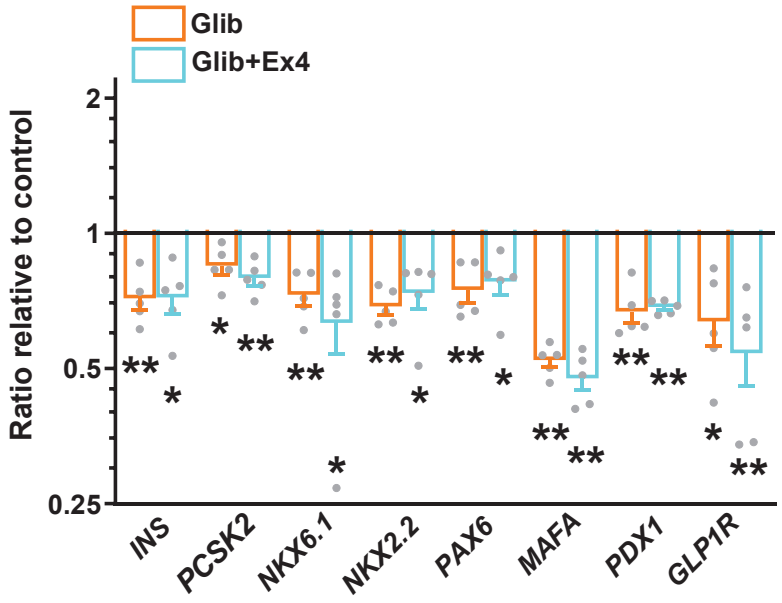

Figure 34. mRNA changes on β-cell identity markers of glibenclamide-exposed islets with Exendin-4. mRNA expression of β-cell identity markers. Expression is shown as a ratio between control islets and islets cultured with 1 µM glibenclamide and 10 nM Ex4 for one week in a scale Log2. Each dot represents one experiment performed with islets from a different islet donor. Bars show mean ± SEM (n=5), *p value<0.01; **p<0.001 vs control group; Multiple t-test with Bonferroni correction.

PART 2.

4. Effects of high glucose exposure on human islets

To discern and better understand the underlying molecular pathways affected by glibenclamide that resulted in changes in β-cell identity and maturity, we explored another β-cell dedifferentiation inducer such as the exposure to high glucose concentrations. Both stressors, glibenclamide and high glucose culture induce sustained

stimulation of insulin secretion, and chronically used result in β-cell dysfunction. Thus, hyperexcitability and hyperstimulation were studied with human islets *in vitro*.

4.1. Effects of high glucose on β-cell function

Human islets cultured for one week at 22.2 mM glucose showed an impaired β-cell function indicated by a lower SI compared to islets cultured at 5.6mM glucose (5.6 mM: 5.05±1.33; 22.2 mM: 1.56±0.13, p=0.041, n=5-6). The lower SI in islets cultured at high glucose resulted mainly from an increased basal insulin secretion (5.6 mM: 0.87±0.15%; 22.2 mM: 4.42±1.8%, p=0.058, n=5-6) (**Fig. 35**, **A** and **B**). Islet insulin content was reduced by 47% (p<0.001) in islets cultured at high glucose (**Fig. 35C**), raw values were the following (5.6 mM: 294.1±80.5; 22.2 mM: 158.8±48.2 ng Ins/µg DNA, n=6).

In contrast, experiments with C57BL/6J mouse islets, showed a similar SI after one week culture at 11.1 mM and 22.2 mM glucose (11.1mM: 3.32±0.61; 22.2 mM: 5.01±0.79, p=0.14, n=5-7). Both basal and stimulated insulin secretion were significantly increased in islets cultured at high glucose, resulting in an overall preserved stimulation index (**Fig. 35, D** and **E**). Despite no differences found on SI, islet insulin content was reduced by 59% (p<0.001) in islets cultured at high glucose (**Fig. 35F**), raw values were the following (11.1 mM: 4283±1223; 22.2 mM: 1810±749.5 ng Ins/µg DNA, n=5).

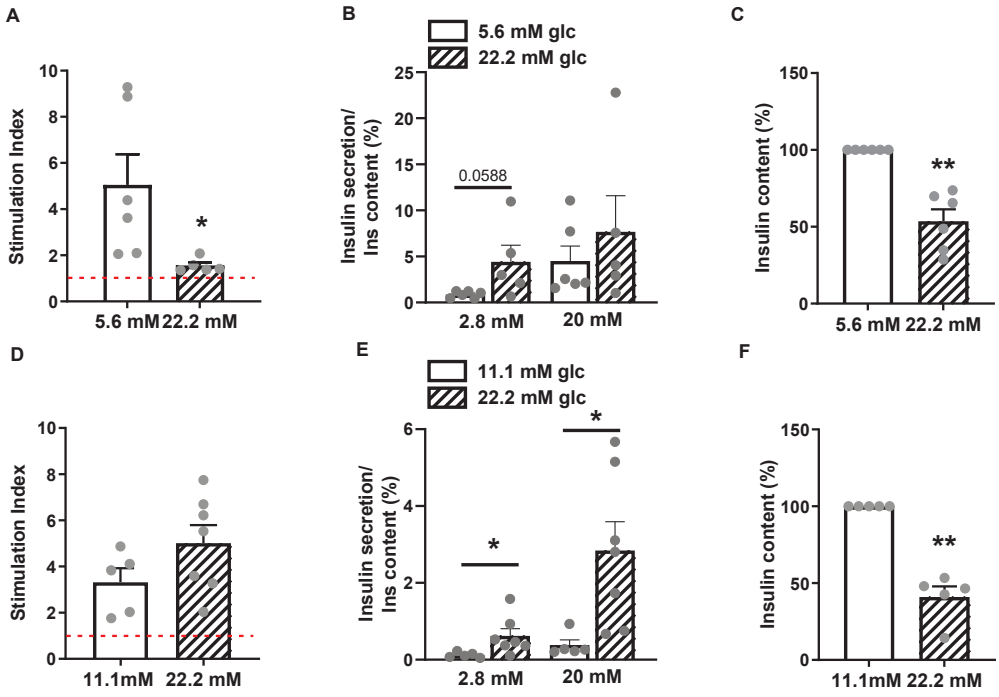

Figure 35. B-cell function determined after high glucose exposure on human and C57BL/6J mice islets. Stimulation index of human (A) or C57BL/6J (D) islets after one week of culture at 5.6 mM or 11.1 mM, respectively, and 22.2 mM glucose. Basal (2.8 mM glucose) and stimulated (20 mM glucose) insulin secretion expressed as percentage of insulin content (B, E). Islet insulin content of human (C) and C57BL/6J (F) islets. Each dot represents one experiment performed with islets from a different islet donor or sample. All bars show mean ± SEM (n=5-7). *p value<0.05; **p value<0.001 with Student t-test.

B-cell apoptosis was similar in human islets after one week of culture at low and high glucose (5.6 mM: 0.75±0.04%; 22.2 mM: 0.50±0.13%, p=0.1, n=3-4).

4.2. Effects of high glucose on β-cell identity

Gene expression analyses on β-cell identity markers were performed to evaluate if β-cell dysfunction observed with high glucose could be associated to the loss of β-cell identity. Human islets cultured at high glucose showed increased expression of insulin and *NKX2.2* genes (p<0.05), and no significant changes in *NKX6.1, PAX6, PDX1, MAFA* and *GLP1R* gene expression (**Fig. 36A**). The expression of *PSCK2* was significantly reduced (p<0.05). *SOX9* and *LDHA* showed a tendency of increased expression. The human β-cell line ENDOC-βH3 cultured at high glucose showed no significant changes in gene expression of β-cell identity markers (**Fig. 36B**). On the contrary, the murine cell

line INS-1 cultured at 22.2 mM glucose showed a significantly reduced expression of the key β-cell transcription factors *Pdx1, Nkx6.1, FoxO1, MafA* as well as insulin gene ($p<0.05$) (**Fig. 36C**).

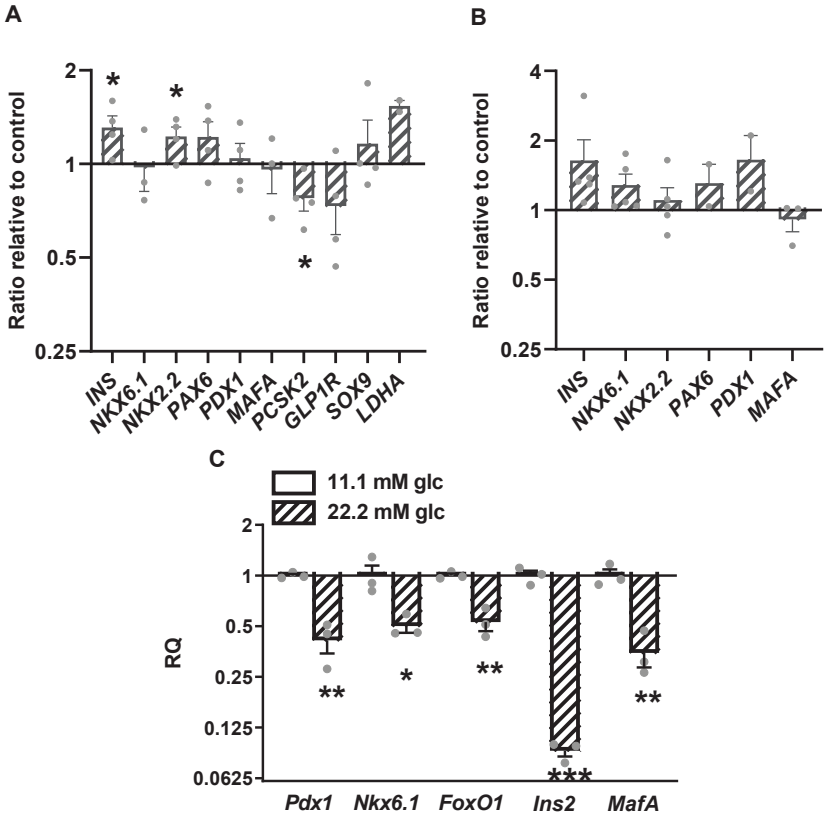

Figure 36. mRNA changes on β-cell identity markers of human islets, EndoC-βH3 and INS-1 cells. Gene expression of β-cell identity markers in human islets (A), EndoC-βH3 cells (B) after one week culture at 5.6 mM vs 22.2 mM glucose and INS-1 cells cultured at 11.1 mM and 22.2 mM glucose for 96 hours (C). Each dot represents one experiment performed with islets from a different islet donor or cell sample and bars show mean ± SEM (n=3-5), *p value<0.05; **p value<0.01; ***p value<0.001 vs control group with Student t-test.

4.3. Effects of high glucose on ER stress

Human islets cultured at high glucose showed a similar expression of ER stress markers (*ATF6, EIF2AK3, DDIT3, WFS1*) than islets cultured at 5.6 mM glucose (**Fig. 37A**), except for *XBP1s* mRNA that showed a tendency of increased expression. Concordantly, no differences were detected in the expression of ER stress markers in the ENDOC-βH3 cells cultured at 5.6 mM and 22.2 mM glucose (**Fig. 37B**).

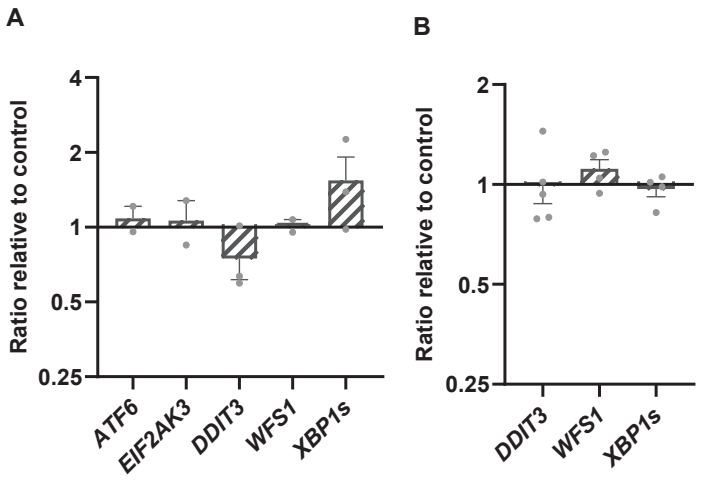

Figure 37. mRNA changes on ER stress markers of human islets and EndoC-βH3 cells. Gene expression of ER stress markers in human islets (A) and EndoC-βH3 cells (B) after one week of culture at 5.6 mM and 22.2 mM glucose respectively. Each dot represents one experiment performed with islets from a different islet donor or cell sample. Bars show mean ± SEM (n=2-5).

X. DISCUSSION

T2DM is a complex metabolic disease characterized by chronic hyperglycemia secondary to the decline of functional β-cells in the context of peripheral insulin resistance which develops as a result of the interplay of lifestyle (diet and exercise) with a myriad of genetic variants (Vujkovic et al., 2020), some of these are in important proximity of UPR genes such as the well-established *WFS1* risk variants (Sandhu et al., 2007) that are associated with reduced insulin secretion (Wood et al., 2017), although the role of the genetic predisposition remains debated.

Studies on the timing and relationship between changes in β-cell molecular architecture, insulin secretion, insulin sensitivity, and β-cell functional defects have identified the latter as the primary requirement for hyperglycemia development (Costes et al., 2013; Weir & Bonner-weir, 2004). As a result, it has been proposed that chronic metabolic stress causes dysfunctional mature β-cells to phenotypically dedifferentiate or transdifferentiate into other islet endocrine cell types over time, eventually leading to "β-cell exhaustion"(Mezza et al., 2019). Stress-induced dedifferentiation is well described in plants and mammalian somatic cells, such as Schwann cells, cardiac myocytes, germ cells, and β-cells (Bersell et al., 2009; Z.-L. Chen et al., 2007; Talchai et al., 2012). Cells can use dedifferentiation as an adaptive mechanism to minimize damage (Puri et al., 2015; Shoshani & Zipori, 2011).

To date, numerous drugs for the treatment of T2DM are available, e.g. insulin secretagogues (including sulfonylureas, glinides, GLP-1 receptor agonists and DPP-4 inhibitors) have been developed targeting insulin secretion to achieve the reduction of high blood glucose levels (I. Song et al., 2015). B-cell restoration or regeneration, as well as the preservation of functional islet integrity should be cautiously considered for T2DM treatment and possibly cure.

Our results demonstrated that human islets chronically exposed to glibenclamide display a significant decrease on GSIS, in line with previous studies reporting β-cell dysfunction in β-cell lines (Ball et al., 2000; Kawaki et al., 1999) rodent (Anello et al., 1999; Gullo et al., 1991; Rabuazzo, 1992) and human islets cultured at 10 μM glibenclamide for 24h (Del Guerra, Marselli, et al., 2005) and at 0.1 or 10 μM for 4 days

(Maedler et al., 2005). The reduced stimulation index was due to significant increased basal insulin secretion (at 2.8 mM glucose), such feature has already been reported with glibenclamide in the literature using MIN6 cells (Kawaki et al., 1999). The increase on basal insulin secretion has been associated with immature β-cells with downregulated β-cell TFs, that are not able to correctly sense glucose and inaccurately secrete insulin at non-stimulatory glucose concentrations (Arnes et al., 2016; Blum et al., 2012). Several mechanisms have been proposed to explain this feature such as insensitivity of the ATP-regulated K^+ channel (Richardson et al., 2007); reduced expression of glucose transporters (Richardson et al., 2007); low activity of glucokinase (Taniguchi et al., 2000); low levels of the β-cell selective gap junction protein connexin36 (Carvalho et al., 2010). Importantly, reports show that after 48h wash-out of 24h-glibenclamide pretreated human islets, this impairment in β-cell function disappeared (Del Guerra, Marselli, et al., 2005).

Our results on islet insulin content showed a significant reduction in the glibenclamide group. Similarly, islet insulin content results of previous *in vitro* studies showed that glibenclamide exposure resulted in a decreased insulin content (Ball et al., 2000; Kawaki et al., 1999; A. Takahashi et al., 2007), comparable to the results of *ex vivo* human islets cultured for 24h which presented a decrease on insulin content (Del Guerra, Marselli, et al., 2005). However, other *in vivo* studies with chronic glibenclamide treatment demonstrated no changes on insulin content (Remedi & Nichols, 2008).

Previous data on β-cell apoptosis with glibenclamide was confirmed through our study which showed that islets chronically exposed to glibenclamide had detrimental effects on β-cell viability. Other studies have shown significant increase in β-cell apoptosis in cultured human islets for 4 days (Maedler et al., 2005). However, when the culture time was shorter (24 hours) there were no changes on β-cell apoptosis (Del Guerra, Marselli, et al., 2005). Using β-cell lines, other groups have been able to prove that the glibenclamide-induced β-cell apoptosis is due to enhancement of Ca^{2+} influx caused by glibenclamide exposure (Efanova et al., 1998; Iwakura et al., 2000).

Our study demonstrated that glibenclamide long term exposure induced loss of β-cell identity in human islets with downregulation of *insulin* mRNA and key transcription factors that maintain β-cell identity and function like *PDX1*, *NKX6.1*, *NKX2.2*, *PAX6*, *MAFA*, *MAFB*, *FOXO1,* as well as *PCSK2*, *KCNJ11* and *GLP1R*. However, there were no significant changes on the expression of disallowed genes or the β-cell dedifferentiation markers analyzed. Gene expression results were confirmed at the protein level after quantification of nuclear β-cell staining, decreased expression was detected in insulin, MAFA and NKX6.1 proteins. However, we did not detect differences in NKX2.2 protein expression. Previous reports from (S. Guo et al., 2013) are in accordance with our results, they demonstrated that T2DM human islets showed profound loss of MAFA, MAFB, PDX1, and NKX6.1 protein levels without changes in NKX2.2.

Several evidences have described a role for NKX6.1 in T2DM: first, genome-wide association studies suggested that variants of NKX6.1 associate with T2DM (Yokoi et al., 2006). Second, decreased NKX6.1 expression has been shown to accompany the development of T2DM in rodents and humans (S. Guo et al., 2013; Talchai et al., 2012). Third, *in vitro* studies in β-cell lines and isolated islets suggest a possible role for Nkx6.1 in the regulation of GSIS as well as β-cell proliferation (Schisler et al., 2005, 2008). Additionally, Nkx6.1 expression was shown to be necessary and sufficient to confer β-cell identity to differentiating endocrine precursors in the embryo (Schaffer et al., 2013), raising the possibility that Nkx6.1 could also contribute to maintain the differentiated state of adult beta cells. MAFA can further enhance GSIS to maintain glucose homeostasis (C. Zhang et al., 2005). For instance, PDX1 controls insulin gene transcription by forming a transcriptional activation complex with Neurod1 and by upregulation of MafA and Ngn3 expression, which are needed for insulin transcription (Y. Zhu et al., 2017). *PDX1* also has a role regulating β-cell survival under ER stress by interacting with *ATF4* (Juliana et al., 2018). PDX1 has been described as a protein that plays many distinct roles in the life of a β-cell, including involvement in β-cell differentiation, β-cell function, β-cell survival and β-cell proliferation (Fujimoto & Polonsky, 2009; Hayes et al., 2013; Spaeth et al., 2017; Yao et al., 2020; X. Zhu et al., 2021; Y. Zhu et al., 2017). To do so, PDX1 controls the expression of other genes that

play important roles in the β-cell fate. These genes include *INS, NGN3, SOX9, HNF6, HNF1b, FOXA2, MAFA, NKX2.2, NEUROD, SLC2A2, GCK, IAPP, NKX6.1, CCND1, CCND2, TRPC3* (Hayes et al., 2013; Jiangman Liu et al., 2021; Spaeth et al., 2017; Y. Zhu et al., 2017). Such findings strongly suggest that the decrease in GSIS properties in our islets may result from reduced levels of these key transcriptional regulators.

Glibenclamide does not lead to beta-to-alpha cell reprogramming, implied by the decrease expression of *ARX* mRNA and the undetected changes on *GCG* mRNA. Regarding β-cell disallowed genes analyzed (*LDHA, HK1, HK3*), no changes were detected in glibenclamide-treated islets. The hypothesis that this could be due to results being extracted from whole islets RNA samples rather than single β-cells has been discarded after Lemaire et al. (Lemaire et al., 2017) published its work confirming that expression of disallowed genes in FACS purified β-cells was found to be equally to the expression in whole islets. Another hypothesis to explain our results could be based on the differences in gene expression between rodents and human β-cells, but it has been discarded due to the fact that induction of disallowed genes has also been observed in human samples perturbed with cell stress (Darden et al., 2022; N. Liu et al., 2020). Therefore, as previously stated by Nimkulrat et al., depending on the model and the stressor implicated, the dedifferentiation process has its own characteristics. In agreement, previous models have shown loss of β-cell TFs without concomitant upregulation of disallowed genes (e.g. *Ldha*) such as *ob/ob mice,* NOD mice (Nimkulrat et al., 2021) and INS-1E$^{Nkx6.1-/-}$ cells (Pavluch et al., 2023).

The expression of endocrine progenitor markers analyzed *(NGN3, ALDH1A3* and *SOX9),* was not detected nor increased. *NGN3* has been shown to also be undetected in human islets exposed to metabolic and inflammatory stress (Darden et al., 2022). These human islets also exhibited unchanged expression of pancreatic hormone genes (*INS, GCG, SST*). Their results indicate that islets undergo global suppression of genes that maintain islet cell identity and function in response to high glucose and IL-1β, but remain resistant to complete reversion to an endocrine progenitor state during acute stress, resembling the results we have obtained after glibenclamide-induced chronic ER stress conditions. We have not detected increased *ALDH1A3* mRNA. The upregulation of

Aldh1a3 may indicate the dysfunction of oxidative phosphorylation and mitochondrial function (Kim-Muller et al., 2016). Perhaps glibenclamide-induced β-cell loss of identity is not related to mitochondrial metabolism dysfunction, although we cannot discard this hypothesis since mitochondrial function analyses were not performed.

In line with our results, Guo et al. (S. Guo et al., 2013) found changes in β-cell identity supported by the loss of many cell-enriched transcription factors in human T2DM islets including *MAFA, MAFB, NKX6.1* and *PDX1*, but there was no evidence for increased *NGN3*, *NANOG*, and *MYCL1* expression. In the same study, many physiological important genes which are activated by the β-cell-enriched transcription factors already mentioned were diminished in T2DM islets, including insulin, glucose transporter type 1 (SLC2A1), and GLP1R, in line with our results. Aging also leads to changes in the β-cell transcriptional identity and compromises β-cell structure function (S. Shrestha et al., 2022), human pancreases showed significant decreases in the nuclear protein levels of PDX1, NKX6.1 and NKX2.2 in old β-cells, as we have seen in our experiments. Decreased expression of key β-cell TFs, upregulated ER stress, and impaired autophagy are found in T1DM and T2DM β-cells, which are functionally impaired and/or transcriptionally immature (Avrahami et al., 2020; S. Guo et al., 2013; Laybutt et al., 2007; Muralidharan et al., 2021).

Accordingly to the literature, alterations in β-cell gene expression, including transcriptional noise (Enge et al., 2017), senescence (Aguayo-Mazzucato et al., 2017, 2019) and compromised identity, are linked to impaired β-cell function during aging and/or T2DM pathophysiology (Camunas-Soler et al., 2020; S. Guo et al., 2013; S. Shrestha et al., 2021). Collectively, gene expression and protein analyses of human islets indicated that glibenclamide exposure induces loss of β-cell identity that correlates with loss of β-cell function. B-cell dedifferentiation was further analyzed with genetic β-cell tracing using lentiviral vectors.

Our lineage tracing experiments to evaluate β-cell dedifferentiation were based on the assumption that dedifferentiated β-cells would have lost insulin expression while maintaining GFP expression. As reported in the results section, we were able to reach

around 34% of β-cell tracing, similar to the 40% found in dispersed human islets used by De Koning's group (Spijker et al., 2013). However, glibenclamide-exposed islets did not differ from the control group in the number of dedifferentiated β-cells. In our study, human islets were transduced using $3.6x10^6$ total IFUs, which were approximately the same IFUs ($4x10^6$ TUs) used to transduce human islets as previously reported by Ravassard's group (Scharfmann et al., 2008). Previous studies have reported that the percentage of dedifferentiated endocrine cells, defined as hormone-negative/chromogranin A-positive endocrine cells, is around 12% in T2DM subjects and around 4% in controls (Cinti et al., 2016; J. Song et al., 2022; Sun et al., 2019; Y. Wang et al., 2020). Although their method of detection was not based on genetically traced islets, considering the highest % of dedifferentiation seen in the literature, we calculated what we could expect in our control and glibenclamide islets, based on extrapolation from numbers of T2DM samples. If we assume 50% of all endocrine cells are β-cells, we would expect that a maximum of 2% of total islet cells could be dedifferentiated β-cells in controls vs 6% in T2DM subjects. Having reached around 34% of β-cell tracing, and assuming stochastic cell infection and tracing, we would have only been able to detect a range from 0.7% (control) to 2% (stressed-cells) of dedifferentiated β-cells, which are relatively low numbers. Therefore, it is possible that the lack of differences perceived between control and glibenclamide groups lies in the fact that we were just not capable of detecting them with the method used. We cannot exclude that improvements on the β-cell tracing method used for the experiments plus adding additional single cell transcriptomic analysis could potentially help to detect differences between groups. Furthermore, another point to consider regarding the β-cell dedifferentiation analysis through genetic β-cell tracing is based on the observation that the loss of insulin observed in our experiments is a general loss of expression and not due to a subpopulation of cells that have completely lost insulin expression. This result is supported by the reduction on insulin fluorescence intensity measured on β-cells and the lack of differences found on the total number of insulin-positive cells. Despite the general loss of insulin and TFs expression upon glibenclamide exposure, it was observed that a subpopulation of cells had lost insulin protein expression while maintaining TF

protein expression in the glibenclamide group, although the increase on the percentage of these cells has not significantly changed the overall result. Thus, β-cell tracing results, may suggest that the differences in gene and protein expression observed with the glibenclamide-treated islets were not due to an increase of dedifferentiated β-cells, or β-cells that have completely lost insulin expression, rather it seems a result of a general downregulation or loss of TFs and insulin.

The pancreatic β-cells have highly developed ER structures and ER stress-transducer proteins because the processing of insulin requires actively regulated ER function (Harding & Ron, 2002; Scheuner & Kaufman, 2008). The β-cells of the pancreas rely heavily on a highly efficient and functional ER to meet the metabolic demand of insulin production. A disarrangement of ER homeostasis may result in β-cell dysfunction. The ER stress response is an adaptive cellular process triggered during times of increased demands for protein folding, and that involves the activation of three interconnected signaling branches, namely, the PERK-eiF2a-ATF4, ATF6, and IRE1 pathways. Together, these pathways enable stressed cells to maintain homeostasis by increasing the expression of chaperone proteins, lipid synthesis, protein degradation pathways and diminishing mRNA expression (Walter & Ron, 2011). *In vitro* studies on rodent and human islets have identified ER stress as a mechanism leading to β-cell failure, increased proinsulin misfolding and decreased insulin production in T2DM (Brusco et al., 2023; N. Shrestha et al., 2021).

SUs are antidiabetic drugs that act on the β-cell by inhibiting the K_{ATP} channels, causing membrane depolarization, activation of voltage-dependent Ca^{2+} channels and subsequent increase on cytosolic Ca^{2+} that triggers insulin granule exocytosis. Previous studies on rodent β-cell lines have demonstrated that sustained exposure to SUs leads to deleterious effects related to the increased basal intracellular [Ca^{+2}] (Iwakura et al., 2000; Kawaki et al., 1999). Furthermore, dysregulation of cytosolic [Ca^{2+}] has been linked to ER stress in older and stressed β-cells (Arda et al., 2016; Enge et al., 2017; Tong et al., 2020; Y. J. Wang et al., 2016; Westacott et al., 2017). For instance, depletion of [Ca^{2+}] in the ER is thought to cause misfolding of proteins (Cnop et al., 2010), which leads to an overload of the ER, causing the accumulation of misfolded proteins and ER stress. In

order to cope with the stress, the UPR is activated to restore ER homeostasis. However, when the UPR is not able to restore the ER homeostasis and there is a chronic increase of insulin demand, this will result in ER-stress-induced β-cell death. As the results of our study demonstrated, sustained glibenclamide exposure resulted in activation of the UPR involving the PERK pathway, seen with an increase of the proapoptotic TF *DDIT3* mRNA and CHOP protein levels, fitting with the increased β-cell apoptosis results observed. The IRE1 pathway was also activated, seen with an increase of XBP1 spliced form. Regarding the 3rd UPR sensor, ATF6 was also disrupted, seen by a downregulation of its negative regulator, *WFS1* mRNA. *DDIT3* can also be a target gene in the ATF6 arm.

In old β-cells (S. Shrestha et al., 2022), upregulation of the ER stress response (all three branches including XBP1, ATF6 and loss of ATF4 plus downstream targets such as DDIT3), and impaired autophagy are due to higher protein synthesis loads, which are expected to arise because of increased transcriptional output and higher mRNA processing in the nucleolus, inducing increases in nucleolar size. Even though we have shown increase of ER stress in our glibenclamide-perturbed β-cells, this characteristic feature was not analyzed in our β-cells. Moreover, this increased ER stress response has been associated with compromised expression of key β-cell markers (INS, PDX1, NKX6.1, NKX2.2, MAFA) and secretory function (S. Shrestha et al., 2022; J. Song et al., 2022). Remarkably, Song et al. showed decreased expression of UPR markers, XBP1s and chaperone GRP94, during aging which paralleled the β-cell identity loss and acquisition of progenitor-like properties.

There is a controversy among ER stress studies, already stated in the introduction, with studies showing increased UPR markers expression and others showing a lower expression of UPR markers, also referred to impaired adaptive or maladaptive UPR. These differences probably reflect the time point of analysis (early vs late), or the set-up of the experiment itself (short vs long exposure). ER stress has a time-dependency, and when samples are analyzed at the beginning of the insult, the results will show increased expression of UPR markers, whereas if the experiment is set to be analyzed after a long period of exposure to the insult, such as diabetes itself, results will show a decreased expression of the UPR markers. This concept has been demonstrated by

Gromada's group (Xin et al., 2018) in single cell RNA sequencing in human β-cells, that identified three dynamic states that include β-cells with 1) low UPR and low insulin expression (transition state), 2) low UPR and high insulin expression (insulin production state), or 3) high UPR and low insulin expression (reflecting a stress-coping mechanism of UPR activation) leading to downregulation of *INS* gene expression also observed in rodent β-cells (Lipson et al., 2006). Thus, previous data suggest that β-cells undergo cycles of insulin biosynthesis and stress recovery. Off note, markers of β-cell dedifferentiation (*NGN3, OCT4, NANOG)* were not detected in these human nondiabetic samples.

Reactive oxygen species (ROS) are defined as atoms or molecules that possess one or more unpaired electrons, making them highly reactive (C. Zhang et al., 2021). High levels of ROS, which are mainly produced by the mitochondria, are extremely toxic to the cell, leading to DNA breaks, lipid peroxidation, and protein aggregation, denaturation and fragmentation (Len et al., 2019). Most cells are equipped with a battery of antioxidant genes with the role of neutralizing it, and maintaining an equilibrium between ROS production and the antioxidant defense, but when ROS levels exceed the ability of the cell to detoxify it and the equilibrium is lost, the cells enter into what is referred to as oxidative stress (Pizzino et al., 2017). B-cells are highly vulnerable to oxidative stress due to the fact that they express low levels of several antioxidant genes (Baumel-Alterzon et al., 2021; Del Guerra, Lupi, et al., 2005). Reports of low expression in islets of the antioxidant gene *CAT* have been linked before to the vulnerability of β-cells to oxidative stress (Gurgul et al., 2004). Our stressor glibenclamide did not induced changes in expression of *CAT* or *SOD2*, two surrogate markers of oxidative stress, suggesting that glibenclamide may not induce oxidative stress or not sufficient to be able to detect a counterregulatory response.

Our data demonstrated that the chemical chaperone PBA, used to prevent ER stress, was able to attenuate the glibenclamide-induced reduction in key β-cell TFs, indicating that ER-stress contributes to the glibenclamide-induced loss of β-cell identity. Although, the exact molecular pathways implicated in the ER stress-induced effects on β-cell

identity have not been elucidated in the duration of this project, we believe it will be of interest to analyze Ca^{2+} dysregulation in glibenclamide-exposed islets.

Previous studies have made an association between increased ER stress and loss of β-cell identity, examples are found using *in vivo* T2DM mice models (Walters et al., 2013), in a mice model of neonatal diabetes (Riahi, Israeli, Yeroslaviz, et al., 2018) and in old β-cells. It has been already shown that genes linked to all three ER stress branches are up-regulated in human old β-cells, including their downstream targets such as HSPA5, and the ER stress TF XBP1 (S. Shrestha et al., 2022). Moreover, murine studies have demonstrated that age is positively associated with increased percentage of dedifferentiated β-cells (Aguayo-Mazzucato et al., 2019; S. Shrestha et al., 2022; J. Song et al., 2022; Téllez et al., 2016). Regarding human studies, Song et al. were able to provide evidence for age-related increase in dedifferentiated cells in nondiabetic humans, seen as hormone-negative/chromogranin A-positive endocrine cells, FoxO1 cytoplasm localization due to its translocation from the nucleus, and *UCN3* reduced levels. Furthermore, these previous studies have suggested that defects in the UPR might be responsible of β-cell dedifferentiation, genes involved in the ER stress response (*XBP1*, *DDIT3*, *ATF6 or HSPA5*) are activated but they show that gene regulatory networks required for proper response to stress are impaired in aging β-cells (S. Shrestha et al., 2022; J. Song et al., 2022).

Recently it has been shown that XBP1 is key for maintenance of mature β-cell identity, repression beta-to-alpha cell transdifferentiation and it is required for β-cell protection against diabetes in insulin resistance states (K. Lee et al., 2022). In fact in a T1DM model, it was shown that deletion of IRE1alpha (upstream of XBP1) in β-cell results in transient β-cell dedifferentiation (H. Lee et al., 2020). Furthermore, knockdown of chaperone GRP94 or XBP1 in Min6 cells confirmed the link between UPR and β-cell identity by demonstrating a decrease in mRNA of *Pdx1, Unc3, NeuroD1*, and *MafA* (J. Song et al., 2022), also showing an upregulation of *Oct4* and *Ngn3*.

Our results suggest that the stressor, in this case glibenclamide, potentially drives loss of β-cell identity and maturity resulting in impaired glucose-stimulated insulin

secretion, which will aggravate T2DM, thus loss of β-cell identity and maturity precedes diabetes. In this line, other stress-related drivers such as cancer-related signals or ER stress in neonatal diabetes have been shown to induce β-cell dedifferentiation as an initial trigger for β-cell failure, independent of the metabolic state (Riahi, Israeli, Yeroslaviz, et al., 2018; J. Song et al., 2022) and suggested to be secondary to ER stress *per se*, in contrast with the common view that β-cell dedifferentiation in diabetes is secondary to chronic hyperglycemia (Z. Wang et al., 2014).

There are two recent studies from the Carlotti's group that have linked loss of human pancreatic β-cell identity and function to ER stress (Groen et al., 2021) and oxidative stress (Leenders et al., 2021). The first one showed alteration on β-cell identity upon disruption of islet-integrity, and exposure to ER stressor thapsigargin (TG). Interestingly, among the two stressors used in the study, TG was able to induce gene expression of endocrine progenitor markers SOX9, HES1 and c-MYC (no differences in NGN3); in contrast, islet integrity disruption treatment did not lead to increased expression of progenitor markers. Confirming that each β-cell stressor leads to a stress type-specific disruption of gene regulatory networks, without always assuming a progenitor cell identity (Nimkulrat et al., 2021). Their data indicated that the activation of the three arms of the UPR is associated with the transition from mature β-cells to cells displaying severe alterations in identity. The second study demonstrated that oxidative-stress is linked to loss β-cell identity and function in primary human islets (Leenders et al., 2021) and was able to show prevention of loss of β-cell identity using TUDCA, which has been shown to have anti-oxidant capacities in neurological disorders (Oveson et al., 2011; Rosa et al., 2017).

The most recent study in human samples (Brusco et al., 2023) has been able to link changes in expression of ER stress markers, insulin synthesis and β-cell identity to *in vivo* specific metabolic features of insulin secretion and sensitivity in the same individual in a cohort of different conditions (normal glucose tolerance (NGT), impaired glucose tolerance (IGT) and overt T2DM). This study has confirmed that ER stress markers are significantly increased with T2DM progression and correlate with altered proinsulin/insulin expression and insulin processing, and negatively correlate with

specific β-cell markers expression. They have been able to solve the difficulties encountered with the high degree of heterogeneity among islets and classify islet profiles into three different stages from normal to fully dysfunctional correlating them to the different conditions (NGT, IGT, TD2M). Importantly, it is the first study able to reconstruct, at the islet level, the progressive damage in islets from individuals stratified depending on the glucose tolerance. Altered proinsulin/insulin expression and insulin processing can result in increased proinsulin secretion, despite not being able to confirm such effect with glibenclamide during our study, it has been demonstrated that elevated proinsulin circulating levels (Then et al., 2020; Yoshioka et al., 1988) associated with its increased biosynthesis represent the primary driver of ER protein load in β-cells, causing accumulation of misfolded proinsulin (Arunagiri et al., 2019; M. Liu et al., 2005; Scheuner & Kaufman, 2008) which leads to adaptive UPR and, if unresolved, to terminal UPR and ER stress (Riahi, Israeli, Cerasi, et al., 2018; Zito et al., 2010).

The studies highlighted here confirm that among all the mechanisms involved in β-cell dysfunction (Ghosh et al., 2019), ER stress represents a determinant link between disturbed insulin processing and loss of β-cell identity and dysfunction in T2DM, which could be a plausible hypothesis to explain the results from our study.

Based on our results of the gene expression differences between 4-days vs 7-days of culture, and with the reinforcement of previous publications, we have hypothesized that our stress effector glibenclamide has a progressive “cascade effect” in human islets which starts affecting some TFs (FOXO1, MAFA, PDX1) and as time progresses and stress accumulates, other set of TFs are affected (such as NKX6.1, NKX2.2 and PAX6). This is in line with a report on *db/db* mice (S. Guo et al., 2013), where they show that upon oxidative stress there is an initial translocation of FOXO1 from cytoplasm to nucleus at 6 weeks, followed by changes in MAFA (concomitant to GLUT2) at 8 weeks and finally changes in NKX6.1 at 10 weeks.

In summary, we present a mechanism of adaptation of primary human β-cells to ER stress through the loss of β-cell identity and maturity. This mechanism may contribute to the decline in functional β-cell mass that is linked to the onset and progression of

diabetes, but importantly, this mechanism of adaptation offers the possibility to potentially rescue those β-cells. Furthermore, reducing ER stress may aid in the preservation of β-cell identity and consequently function, opening up the possibilities to potentially redifferentiate β-cells.

Due to the positive effects of GLP-1 based therapies observed in β-cells, we thought about the addition of Ex-4 to the culture media as a strategy to prevent the glibenclamide-induced negative effects on β-cells. Our results of GSIS determined that Ex-4 was not able to prevent impaired β-cell function induced by glibenclamide. Although previous studies have used both drugs in rodent islets or cell lines and observed an increase on β-cell function, the differences with our study are considerable due to the fact that they culture islets just for 2 days (H. Takahashi et al., 2015), probably not being enough to capture the whole effect of glibenclamide, and they add Ex-4 1h prior to the glibenclamide treatment (J. Y. Kim et al., 2012). Thus, adding Ex-4 prior to the sustained exposure to glibenclamide could be considered as an option to prevent the negative effects on β-cell function.

Preventing or reversing β-cell dedifferentiation is a promising approach to restore glycemic control in people with diabetes, in particular T2DM. Thus, drugs that work by directly reversing or preventing β-cell dedifferentiation could be very valuable (Sachs et al., 2020; Tahrani et al., 2016). Sachs et al. (Sachs et al., 2020) presented a model of rodent β-cell dedifferentiation counteracted by GLP1-oestrogen therapy which prevented ER stress-induced apoptosis by increasing the ERAD pathway. Ex-4 is a molecule with a promising effect on the replication of human β-cells (Tian et al., 2011). Thus, we wondered if Ex-4 effect could counteract with the loss of identity induced by glibenclamide. Although Ex-4 has been linked to increased expression of TFs key for the maintenance of β-cell identify such as PDX1 (M. Gao et al., 2018; Pinney et al., 2011), Ex-4 was not able to counteract the effects of glibenclamide on the expression of TFs. Importantly, glibenclamide-induced loss of β-cell identify and in particular, on *GLP1R* expression, is thought to be key in order to explain the lack of Ex-4 effects.

Studies in rodent and human islets have identified that GLP-1RA alleviates β-cell apoptosis (Buteau et al., 2004; Farilla et al., 2003; Chenghu Huang et al., 2015; Y. Li et al., 2003; Moffett et al., 2015) induced by several stressors such as glucolipotoxicity (Cunha et al., 2009; Ding et al., 2019; S.-H. Park et al., 2015), which may trigger oxidative (X. Shen et al., 2020) and ER stress (Cunha et al., 2009). However, we have not been able to confirm the preventive effect exerted by Ex-4 on glibenclamide-exposed islets. Nonetheless, there is a report on a hamster derived insulin secreting HIT-T15 cells and dispersed rat islets that showed prevention of glibenclamide-induced β-cell apoptosis and increased ER stress by pretreating the cells with Ex-4 for 1h (J. Y. Kim et al., 2012). The authors hypothesized that Ex-4 action on preventing glibenclamide effects is through a PKA-dependent pathway because when using ERK inhibitor or PI3K inhibitor, Ex-4 effect on glibenclamide was not modified.

Moreover, our gene expression analyses have not been able to confirm the preventive effect of Ex-4 on glibenclamide-induced ER stress and, in consequence, glibenclamide-induced loss of β-cell identity. Therefore, if glibenclamide-induced ER effects are not counteracted, as observed with Ex-4, the loss of β-cell identity cannot be prevented. A demonstration of this hypothesis is clearly validated with our PBA experiments. Nonetheless, we can hypothesize that if the Ex-4 exposure was previous to the glibenclamide treatment, the ER stress could have been prevented. Thus, pretreatment with GLP1R agonists may be considered as a therapeutic strategy. Collectively, our results revealed that GLP-1 analog (Ex-4) did not have any effect on human islets treated with glibenclamide and this could be due to the contribution of reduced *GLP1R* gene expression induced by glibenclamide.

As previous studies have proven (Nimkulrat et al., 2021), β-cell dedifferentiation is a stress type–specific process resulting in a stress type–specific loss of functional maturity, cells will lose their mature phenotype in a unique manner, depending on how their genetic network is perturbed by a particular diabetogenic environment. Thus, in order to understand the underlying mechanisms contributing to the glibenclamide-induced loss β-cell identity and function, we thought to analyzed the effects of sustained stimulation of insulin secretion driven by hyperglycemia, or so called

"hyperstimulation". No conclusive data in human islets has been reported to understand if the hyperexcitability and hypersecretion produced by chronic sulfonylurea induced K_{ATP} channel closure is more damaging for β-cells than the metabolic hyperstimulation of persistent hyperglycemia (Nichols & Remedi, 2012).

Rodent studies have proven that the islet transcriptome is deeply modulated by glucose (Bensellam et al., 2009; Moreno-Asso et al., 2013). Glucose serves as a main survival factor for pancreatic β-cells (Hoorens et al., 1996) and β-cell function is correlated with glucose regulation of many β-cell genes (Martens & Pipeleers, 2009; Schuit et al., 2002). It is in this regard that glucose activates and represses the expression of genes involved in a large variety of biological functions (Moreno-Asso et al., 2013). Previous reports in murine models confirmed that changes in glucose metabolism can influence gene expression (Porat et al., 2011; Terauchi et al., 2007), which supports the concept that changes in glucose flux, with or without hyperglycemia, can lead to the changes in β-cell phenotype that are responsible for the marked functional deterioration. They also support the conclusion that glucose exerts toxic effects at least in part through changes in gene expression, further justifying use of the term glucotoxicity (Weir, 2020). However, reports on human islets may differ from the well-studied effects of hyperglycemia in rodent β-cells. For instance, there are studies demonstrating that although human islets cultured at high glucose display β-cell dysfunction, decrease expression of β-cell TFs is not observed (Brun et al., 2015; Marselli et al., 2020).

In our experiments, human islets cultured with high glucose for one week did not exactly recapitulate the glibenclamide observed effects. B-cell function was impaired but no other glibenclamide effects could be found with high glucose in human islets. Indeed, high glucose diminished β-cell function, with an increased basal insulin secretion, and insulin content was decreased by 50 %, as previously reported in human islets cultured with elevated glucose for one week (Eizirik et al., 1992; Federici et al., 2001; Ling & Pipeleers, 1996; Maedler et al., 2002; Marshak et al., 1999). However, results on C57BL6/J islets cultured with high glucose did not show detrimental effect on β-cell function, only reduced insulin content, emphasizing the differences among

species in terms of vulnerability to glucose. Previous reports had emphasized the different effects of hyperglycemic-like conditions depending on the species of origin. For instance, non-obese diabetic mice cultured at 28mM glucose for 7 days showed increased insulin release with decreased insulin content (Eizirik et al., 1991).

Although previous studies in human islets have detected increased β-cell apoptosis following high glucose culture conditions at 16.7 mM glucose (Federici et al., 2001), 25 mM glucose (Brun et al., 2015) or at 33.3 mM glucose (Maedler et al., 2002), we were not able to detect differences in β-cell apoptosis analyzed by TUNEL assay. Furthermore, differences in organ donor characteristics and isolation procedures, as well as methods used to measure insulin secretion and apoptosis, are thought to be responsible for the wide functional heterogeneity between islet preparations and high variability among studies, making results comparison difficult (Hart & Powers, 2019; Poitout et al., 2019).

Our study demonstrated that β-cell identity markers were not diminished in human islets nor in human β-cell line EndoC-βH3 cultured at high glucose concentration. For instance, *INS* and *NKX2.2* gene expression was increased and *PSCK2* gene expression was decreased, whereas all other TFs expression was not altered. Previous data has demonstrated that human islets culture at high glucose concentrations (22 mM for 3 to 4 days) do not exhibit decreased expression of β-cell identity markers such as *MAFA*, *MAFB, INSM1* and *PAX6* (Marselli et al., 2020), while exhibiting β-cell dysfunction (Brun et al., 2015). Marselli et al. demonstrated that high glucose effects differ from lipotoxic conditions were decreased on β-cell TFs expression was detected (Marselli et al., 2020). In contrast, data published using human islets at 33 mM for 4 and 9 days analyzed by Northern blot showed reduced *INS* and *PDX1* mRNA (Marshak et al., 1999; Sacco et al., 2019), which could be due to differences on the analysis methods or glucose concentrations used. However, shorter culture-time periods did not display a reduction on *INS* nor *PDX1* mRNA (Dai et al., 2012; Fred et al., 2010; Shalev et al., 2002).

B-cell identity gene expression results differ from the majority of the work published in T2DM rodent islets or rodent cell lines. For instance, we were able to detect downregulation of TFs such as *Pdx1, Nkx6.1, Foxo1, MafA* by high glucose

concentrations in rat INS-1 cells, possibly due to increased oxidative stress, which is in accordance with previous reports of murine cell line (Neelankal John et al., 2017; Olson et al., 1993; R. Robertson et al., 2007), *db/db* mice (Kjørholt et al., 2005; Sacco et al., 2019), mice fed with HFD (Bosma et al., 2021; Glavas et al., 2019; Reimer & Ahrén, 2002) or rodent islets after pancreatectomy (Ebrahimi et al., 2020). Concordantly to the *in vivo* rodent studies, islets from T2DM subjects showed reduced GSIS paralleled to decreased *INS, GLUT1, GLUT2* and *GCK* mRNAs but increased *FOXO1* and *PDX1* mRNAs (Del Guerra, Lupi, et al., 2005), emphasizing the *in vivo* hyperglycemia, or perhaps lipotoxic effects.

Our results demonstrated that ER stress markers were not altered in human islets nor human β-cell line EndoC-βH3 cultured with high glucose. In agreement with our results showing absence of β-cell apoptosis upon high glucose exposure. In T2DM human islets, it was initially reported no increase in expression of ER stress genes (Marchetti et al., 2007). In contrast, analyses of T2DM pancreatic sections from two other groups were able to detect increased expression of various UPR markers (DDIT3 and chaperones) (Chang-jiang Huang et al., 2007; Laybutt et al., 2007). Some rat islet and cell line studies have also shown increases in ER stress markers (Elouil et al., 2007; H. Wang et al., 2005). However, it seems that *in vitro* glucotoxic alterations of β-cell function and survival are better correlated with oxidative stress than ER stress (Jonas et al., 2009; A. P. Robertson, 2004). Studies comparing gene expression in rat vs C57BL6 mouse islets showed that low vs intermediate glucose concentration induced apoptosis and oxidative stress markers in both rat and mouse islets, whereas, in contrast with rat islets, culture in high vs intermediate glucose did not induce apoptosis and oxidative stress markers such as *Mt1a, c-Myc, Hmox1* and *Atf3* in mouse islets despite similar increases in Xbp1 pre-mRNA splicing (Jonas et al., 2009). As mentioned, high glucose induces *c-Myc* upregulation, which can be activated by oxidative stress and has been shown to play an important role in β-cell dysfunction and apoptosis (Elouil et al., 2005; Kaneto et al., 2002; Pascal et al., 2008; Pelengaris et al., 2002), upregulation of *c-Myc* is known to suppress insulin gene expression (Kaneto et al., 2002; Pascal et al., 2008), and this decrease of insulin gene expression is prevented by antioxidants, which points towards the role of oxidative stress (R. P. Robertson & Harmon, 2006; Tanaka et al.,

1999). Furthermore, high glucose also altered mitochondrial metabolism by reducing the expression of 11 mitochondrial DNA (mtDNA)-encoded respiratory chain subunits in human islets (Brun et al., 2015). Thus, it is tempting to hypothesized that lack of upregulation of β-cell disallowed genes with glibenclamide treatment could be due to the absence of glucose metabolism and oxidative stress involvement.

As proved by a previous study, and despite the differential effects observed by high glucose and glibenclamide, both stressors coincide to stimulate islet nitric oxide synthase (NOS)-NO system (Lundquist et al., 2016) which contributes to β-cell dysfunction (Mosén et al., 2008; Muhammed et al., 2012; Salehi et al., 2008), as explained by their work, imposed and persisting overstimulation of this system by glibenclamide and/or high glucose will result in an excessive production of NO with the capability of inhibiting crucial steps along the insulin secretory signaling pathways.

Interestingly, we detected a trend for an increase in *LDHA* mRNA, a disallowed marker, not observed in glibenclamide-treated islets. In line with other reports, β-cells from hyperglycemic animals also display increased mRNA levels of genes expressed at low or very low levels in normal β-cells, including the glycolytic enzymes Hk1 (Ghanaat-Pour et al., 2007; Jonas et al., 1999; Kjørholt et al., 2005; Laybutt et al., 2003) and LdhA (Bensellam et al., 2009; Jonas et al., 1999; Marselli et al., 2010), Hmox1, Gpx1, Txnip (J. Chen et al., 2008; Minn et al., 2005; Shalev et al., 2002) and several TFs such as c-Myc (Kaneto et al., 2002), Atf3 (Hartman et al., 2004), Ddit3 (B. Song et al., 2008). Most of these genes are typically induced by various types of stress, including oxidative stress (A. P. Robertson, 2004), ER stress (Pirot et al., 2007), cytokine treatment (Cardozo et al., 2001) and hypoxia (N. Liu et al., 2020). In this case, the *in vivo* hyperglycemia effects have also been observed in islets cultured in high glucose for a few days, suggesting that they constitute early sensitive markers of β-cells glucotoxicity (Bensellam et al., 2009). For instance, the mRNA levels of many hypoxia-inducible factor (HIF)- target genes increased exponentially between 5 and 30 mM glucose, suggesting a role for hypoxia, at least *in vitro*, in β-cell glucotoxicity (Bensellam, Duvillié, et al., 2012). In line with this results, Liu et al. (N. Liu et al., 2020) revealed that hypoxia-inducible factor HIF-1α, which

is activated by hyperglycemia, inflammation and IAPP, induces its downstream targets NGN3 and ALDH1A3. Additionally, two studies have reported increased mRNA levels of the pre-endocrine gene *SOX9* (Bugliani et al., 2013; Marselli et al., 2010). Evidence suggests that *SOX9* upregulation in T2DM may result from hypoxia and activation of HIF1 and could play a role in the downregulation of β-cell identity genes (Puri et al., 2013). As seen in our experiments, glibenclamide was not able to induce their expression suggesting that although hyperglycemia and glibenclamide can induce cell stress and β-cell dysfunction, the underlying mechanisms leading to such result may differ, therefore each stressor activates different set of genes resulting in a stress-type specific loss of β-cell identity or dedifferentiation. For instance, as mentioned in the introduction, glibenclamide and glucose present several differences in terms of pathway activation at the level of protein translation (Q. Wang et al., 2011).

In summary, high glucose exposure results suggested that human islet β-cell dysfunction is not due to increase ER stress and subsequent loss of β-cell identity. In contrast, rodent β-cells display loss of β-cell identity, probably due to IRE1α activation and increased ER stress as seen in previous rodent studies (Lipson, 2006), but it does not lead to β-cell dysfunction. Importantly, our high glucose results on human islets emphasize the link between ER stress upregulation and loss of β-cell identity. In this case, the absence of ER stress correlates with unmodified β-cell identity markers expression.

Collectively our results provide a demonstration that loss of human β-cell identity and maturity is driven by glibenclamide-induced ER stress. Glibenclamide treatment provokes increase of insulin demand, leading to activation of the UPR and ER stress genes, resulting in loss of β-cell identity and alterations in β-cell function. This loss of β-cell phenotype, which is suggested to be a protective mechanism against stress overload, contributes to the progression of the secondary failure to sulfonylurea treatment described in T2DM. Importantly, the process could be prevented by amelioration of ER stress, leading to preserved β-cell identity and β-cell function. Overall, the results support that strategies to decrease β-cell workload will help prevent and/or delay β-cell failure in T2DM.

XI.CONCLUSIONS

1. Human islets exposed to glibenclamide showed increased basal insulin secretion, which resulted in lower stimulation index, indicating that *in vitro* exposure of glibenclamide impaired β-cell function.

2. Human islets exposed to glibenclamide had increased β-cell apoptosis compared to control islets, indicating that glibenclamide contributes to β-cell death.

3. The expression of insulin and β-cell identity markers was lower in human islets exposed to glibenclamide, whereas no differences in expression of disallowed genes or progenitor-related markers were observed. Thus, glibenclamide seems to induce loss of β-cell identity without evidences on metabolic reprogramming or conversion to a progenitor-cell.

4. The expression of ER stress markers was higher in human islets exposed to glibenclamide. Alleviation of ER stress by the chemical chaperone PBA prevented glibenclamide-induced loss of β-cell identity. Thus, the results suggest that increased ER stress mediates the loss of β-cell identity in human islets exposed to glibenclamide.

5. Addition of exendin-4, a GLP-1 receptor agonist, to the culture medium of human islets exposed to glibenclamide did not prevent the deleterious effects induced by glibenclamide on β-cell function, survival, ER stress and identity.

6. High glucose exposure had a detrimental effect on human β-cell function but did not induce ER stress nor loss of β-cell identity.

XII. REFERENCES

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XIII. SCIENTIFIC CONTRIBUTIONS

RESEARCH ARTICLES

Carballar, R., Canyelles, ML., **Fernández, C.,** Martí, Y., Bonnin, S., Castaño, E., Montanya, E., Téllez, N. (2017) Purification of replicating pancreatic β-cells for gene expression studies. Sci Rep. 2017; 7: 17515.

COMMUNICATIONS

Fernández C., Téllez N., Rivera K., Nacher M., Montanya E. (2022) Loss of β-cell identity and endoplasmic reticulum stress in human islets treated with glibenclamide. Oral presentation delivered at 13th CIBERDEM Annual Meeting, Mataró, November 2022.

Fernández C., Téllez N., Gutiérrez V., Rivera K., Nacher M., Montanya E. (2020) Endoplasmic reticulum stress contributes to the loss of β-cell identity in human pancreatic islets treated with glibenclamide. Oral presentation (online) delivered at IDIBELL PhD Day, November 2020.

Fernández C., Téllez N., Gutiérrez V., Rivera K., Nacher M., Montanya E. (2020) Endoplasmic reticulum stress contributes to the loss of β-cell identity in human pancreatic islets treated with glibenclamide. Oral presentation (online) delivered at 11th CIBERDEM Annual Meeting, November 2020.

Fernández C., Téllez N., Gutiérrez V., Rivera K., Nacher M., Montanya E. (2020) Endoplasmic reticulum stress contributes to the loss of beta cell identity in human pancreatic islets treated with glibenclamide. Oral presentation (online) delivered at 56th Annual Meeting of the European Association for the Study of Diabetes, September 2020.

Fernández, C., Téllez, N., Nacher, M., Montanya, E. (2019) Effects of glibenclamide on β-cell dedifferentiation in human islets. Poster presentation delivered at 10th CIBERDEM Annual Meeting, 2019.

Fernández, C., Carballar, R., Canyelles, ML., Martí, Y., Bonnin, S., Castaño, E., Montanya, E., Téllez, N. (2018) Purification of replicating pancreatic β-cells for gene expression studies. Poster presentation delivered at 6th DZD Diabetes Research School of the German Center of Diabetes Research (DZD), Berlin, October 2018.