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PhD THESIS

ROLE OF PAX4 AND PAX8 IN PANCREATIC ISLETS PHYSIOLOGY AND PATOPHYSIOLOGY

presented by

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A tí abue,

Siempre

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53BP1: Tumor suppressor p53-binding protein 1
5-HT: 5-Hydroxytryptophan
A1C: Glycated hemoglobin
ADP: Adenosine diphosphate
AI-2: Insulinoma-associated protein 2
Arx: Aristaless related homeobox
ATF6: Activating Transcription Factor 6
ATP: Adenosine triphosphate
AVV: Adeno-Associated Virus (AAV)
Bcl2: B-cell lymphoma 2
Bcl-xL: B-cell lymphoma-extra large
Bip: Binding immunoglobulin protein
BMI: Body Mass Index
BrdU: 5-bromo-2'-deoxyuridine
Brn4: Brain-specific POU-box gene
BSA: Bovine Serum Albumin
Ca^{2+}: Calcium
Calr: Calreticulin
CCNA2: cyclin A2
Ccnb2: Cyclin B2
Cdc16: Cell division cycle protein 16 homolog
CDC6: cell division cycle 6
Cdk4/6: Cyclin-dependent kinase 4/6
Cdkn1a/p21: Cyclin-dependent kinase inhibitor 1A
Cdkn2a: Cyclin-dependent kinase inhibitor 2A
CDS: coding DNA sequence
CITR: Collaborative Islets Transplant Registry
CNS: Central Nervous System
COX2: Ciclooxigenase 2
C-peptide: Connecting peptide
DAG: Diacylglycerol
DAPI: 4',6-diamidino-2-phenylindole
DDIT3/CHOP: DNA-damage-inducible transcript 3
Derl3: Derlin-3
DKA: Diabetic Ketoacidosis
DM: Diabetes Mellitus
Dnaja2: Hsp40
DOX: Doxycycline
DT: Diphteria Toxin
DTT: Dithiothreitol
E: Embryonic days
E2F1: E2F transcription factor 1
EAD: Experimental Autoimmmune Diabetes
ELISA: Enzyme-Linked ImmunoSorbent Assay
EMSA: Electrophoretic Mobility Shift Assay
ER: Endoplasmic Reticulum
ERAD: Endoplasmic-Reticulum-Associated Protein Degradation
ESCs: Pluripotent Embryonic Stem Cells
EtBr: Ethidiumbromide
$FADH_2$: flavin adenine dinucleotide
FBS: Fetal Bovine Serum
FFA: Fatty Acids
FoXM1: Forkhead box protein M1
FoxO1: Forkhead box protein O1

FPG: Fasting Plasma Glucose
FSC: Forward Scatter
FT_4: Free Tetraiodothyronine
GCK: Glucokinase
GD65: Glutamic Acid Decarboxylase
GDM: Gestational Diabetes Mellitus
GFP: Green fluorescent protein marker
GIP: Glucose-dependent Insulinotropic Polypeptide
GLIS3: GLIS family zinc finger protein 3
GLP-1/2: Glucagon-Like Peptide 1/2
GLUT1/2/4: Glucose Transporter 1/2/4
GSIS: Glucose-Stimulated Insulin Secretion
GTD: Gestational Thyroid Dysfunction
GWAS: Genome-Wide Association Studies
HD: Homeodomain
Hes1: Hairy and enhancer of split-1
Hex: Haematopoietically expressed homeobox
HHS: Nonketotic Hyperosmolar Syndrome
Hlxb9: Homeobox HB9
Hnf1α/4α: hepatocyte nuclear factor 1/4 homeobox A
Hnf6: Hepatocyte nuclear factor 6
HRP: Horse Peroxidase Reaction
Hspa5: Heat Shock 70kDa Protein 5
Htr2b: Gα(q)-linked serotonin receptor 5-hydroxytryptamine receptor-2b
IA1: Insulinoma-associated cDNA-1
IAPP: Islet Associated Polypeptide
Id2: DNA-binding protein inhibitor ID-2
IF: Immunofluorescence
Ifit1/3/44: Interferon-induced protein with tetratricopeptide repeats 1/3/44
IGF-1: Insulin-like Growth Factor 1
IKK: Iκβ kinase
IL-1Ra: Interleukin-1 receptor antagonist
IL-1β: Interleukin-1β
INS-1E: Rat insulinoma cell line
IP_3: Inositol 1,4,5-trisphosphate
IPA: Ingenuity Pathways
iPSCs: Induced Pluripotent Stem Cells
IQGAP1: GTPase-Activating Protein 1
IRE1: Inositol Requiring Enzyme 1
IRS-1: Insulin-receptor substrate 1
Isl1: Insulin gene enhancer protein Isl1
KEGG: Kyoto Encyclopedia of Genes and Genomes
KLF4: Kruppel-like factor 4
KRBH: Krebs-Ringer bicarbonate-HEPES buffer
LADA: Latent Autoimmune Diabetes in Adults
Lgals9: galectin-9
Lman1/2: lectin, mannose-binding 2
MafA: v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog A
MafB: v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog B
Mapk10/Jnk3: Mitogen-activated protein kinase 10
MCF7: Michigan Cancer Foundation-7 (breast Cancer Cell line)
MCP: Multipotent Progenitor Cells
MCP1: Monocyte Chemoattractant Protein 1
MDI: Multiple Daily Injection
Men1: Multiple endocrine neoplasia type I
MIDD: Mitochondrial Diabetes and Deafness
MIN6: Mouse Insulinoma cell line

MIP-LUC: Mouse Insulin Promoter-Luciferase
MODY: Maturity Onset Diabetes of the Young
MOI: Multiplicity Of Infection
mPL-I: murine Placental Lactogen
MSCs: Mesenchymal Stem Cells
MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADH: Nicotinamide adenine dinucleotide
NeuroD/β2: Neurogenic differentiation 1
Ngn3: Neurogenin 3
NIS: Sodium/Iodine Symporter
Nkx2.2: NK2 homeobox 2
Nkx6.1/6.2: NK6 homeobox 1/2
NOS2: Nitric oxide synthase 2
OC: Octapeptide
OCT4: Octamer-binding transcription factor 4
OGTT: Oral Glucose Tolerance Test
Orc4: Origin recognition complex subunit 4
p27: Cyclin-dependent kinase inhibitor 1B
Pax: Paired box
Pax1-9: Paired box 1-9
PAX4v: PAX4 splicing variant
PBS: Phosphate Buffered Saline
PC1/2: Prohormone convertases 1/2
PCR: Polymerase Chain Reaction
PD: Paired Domain
Pdia4: Protein Disulfide Isomerase family A, member 4
PDL: Pancreatic Duct Ligation
Pdx1: Pancreatic and duodenal homeobox 1
PERK: PKR-like ER kinase
PI3K: Phosphatidylinositol 3-kinase
PISCES: Pancreatic Islet Cell Enhancer Sequence
PL: Placental Lactogen
Plaa: Phospholipase A2-activating protein
PLC: Phospholipase C
PNET: Pancreatic Neuroendocrine Tumors
PP1: Protein Phosphatase 1
PPARγ: peroxisome proliferator-activated receptor γ
PPY: Pancreatic Injury
Prl: Prolactin
Prlr: Prolactin-receptor
PSCs: Pancreatic Stem Cells
Ptf1a: Pancreas transcription factor 1 subunit α
PVDF: Polyvinylidene difluoride
RB: Retinoblastoma
rhPRL: Recombinant human prolactin
RIN: RNA integrity Number
RIP: Rat insulin II promoter
ROS: Reactive Oxygen Species
Sec23b: Sec23 Homolog B
SERCA: ER Ca^{2+}-ATPases
SFFV: Spleen Focus-Forming Virus
SOX2/9: sex determining region Y box 2/9
SSC: Sideward Scatter
Ssr3: Signal Sequence Receptor Subunit γ
STZ: Streptozotocin
Syvn1: synovial apoptosis inhibitor 1
T1DM: Diabetes mellitus Type 1

T2DM: Diabetes mellitus Type 2
T_4: thyroxine
TBE: Tris/Borate/EDTA
TCF7L2: Transcription factor 7-like 2 (T-cell specific, HMG-box)
TFAM: Mitochondrial Transcription Factor A
Tg: Thyroglobulin
TNF: Tumor Necrosis Factor
TP53: Tumor suppressor protein 53
Tph1/2: Tryptophan hydroxylase 1/2
TPO: Thyroperoxidase
TRAF2: TNFα-receptor-associated factor 2
TSH: Thyroid Stimulating Hormone
TTF-1/2: Thyroid Transcription Factor 1/2
UBIQ: Ubiquitin
Ufd1l: Ubiquitin fusion degradation protein 1 homolog
UPR: Unfolded Protein Response
WB: Western blot
WDPNETs: Well-Differentiated Pancreatic Neuroendocrine Tumors
WG: Weeks of Gestation
WPRE: Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element
XBP1: Transcription factor X-box protein binding 1
ZnT8: Zinc transporter 8

INTRODUCTION

CHAPTER 1_INTRODUCTION

1. DIABETES: A WORLDWIDE DISEASE

1.1. Definition of Diabetes Mellitus

Diabetes Mellitus (DM) is a metabolic disorder of multiple etiology characterized by chronic hyperglycemia with disturbances in carbohydrate, fat and protein metabolism resulting from defects in insulin secretion by the endocrine pancreas and/or insulin responsiveness on the target tissues such as skeletal muscle, adipose tissue, brain and liver *(WHO, 1999)*. Insulin is a hormone produced by the β-cells of the endocrine pancreas whose main action is to lower plasma glucose levels through suppression of hepatic glucose production and stimulation of glucose utilization in skeletal muscle and adipose tissue (Figure 1) *(DeFronzo, 2004)*.

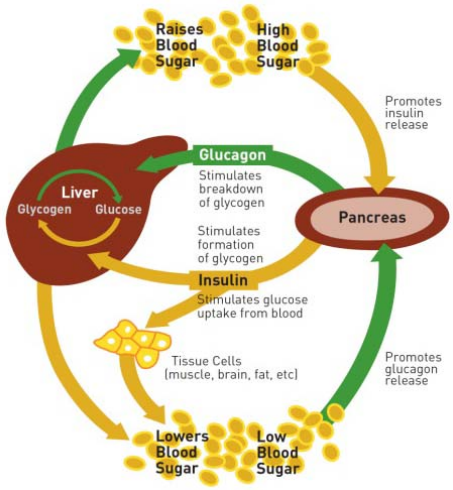

Figure 1: Regulation of glucose metabolism by insulin action.

1.2. Epidemiology of a Worldwide Disease

According to the International Diabetes Federation (IDF), 386.7 million people were affected by DM in 2014 (8.3% worldwide prevalence). The prevalence of the disease is expected to rise to 592 million by 2035 (10.1% prevalence) reaching worldwide pandemic magnitudes. This presumes an increase of 55% of cases in two decades. By 2014, diabetes has caused 4.9 million deaths (every seven seconds a person dies from diabetes) (Figure 2). It is important to highlight that there are still 179.2 million people with DM undiagnosed, which represents 50% of people suffering DM (Figure 2). Consistent with the World Health Organization (WHO) data, this alarming increase in the incidence of DM is mainly due to the augmentation of T2DM cases in most of the countries. Interestingly, the age of onset of this disease shows an increasing trend towards younger people developing DM (the highest percentage of people with DM is in the age group between 40 and 59 years old). In addition, more

than 79,000 children developed T1DM in 2013. Furthermore, 21 million pregnancies were affected by gestational diabetes in 2013 *(IDF, 2014)*.

Figure 2: Worldwide prevalence of diabetes mellitus in 2014. *Figure adapted from International Diabetes Federation (IDF) Atlas. 6th Edition (revision 2014) (IDF, 2014).*

In Europe, 52 million people (1 in 13 adults) suffer DM, which accounts for 7.9% of European population between 20-79 years old in 2014. These values will rise up to 68.9 million of people (prevalence of 10.3%) by 2035. Alarmingly, 33.1% of the cases are undiagnosed. Frighteningly, Europe has the highest prevalence of children with T1DM (112,000 cases in 2010 under age 14 years). Moreover, in our continent approximately 537,000 deaths are caused from DM in 2014, 23.1% of them under the age of 60.

Among the top countries in Europe for the number of people with DM (20-79 years age range) in 2013, Spain is in the forth place with 3.8 million people affected that indicate a prevalence of 8.15% of the population. Diabetes is the eighth cause of death in our country with 25,000 deaths in 2013 (8% mortality) *(Soriguer et al., 2012), di@bet.es study)*.

The total global budget for DM is estimated in 612.2 USD billion in healthcare expenses (11% of total spending on adults) in 2014. Worryingly, Europe assumes 24% of worldwide expenditure reaching 116 EUR billion in 2014. Of note, Spanish total direct annual cost of DM was 5,809 EUR million, representing 8.2% of the total country health expenditure (overall healthcare cost per person with DM is approximately 3,000 EUR per year) *(Carlos Crespo and Noemí López-Martínez, 2013; IDF, 2011, 2014;*

L'Heveder and Nolan, 2013; SED, 2014). These data makes DM a major social and economical global problem.

1.3. Diabetes Mellitus Complications and Risk Factors:

DM is a chronic disease that requires continuous medical care and patient self-management education and support to prevent acute complications and to reduce the risk of long-term problems. To prevent these complications is required a glycemic control. Current WHO diagnostic criteria *(World Health Organization, 2006)* for DM are fasting plasma glucose (FPG) values ≥126 mg/dl; plasma glucose levels after oral glucose tolerance test (OGTT) ≥200 mg/dl 2 hours after 75 grams of glucose intake and A1C cut point ≥6.5%.

People with DM have an increased risk of developing a number of serious health problems. Consistently, chronic hyperglycemia, or high blood glucose levels, is associated with polyuria (increased urinary frequency), polydipsia (chronic excessive thirst), weight loss, sometimes with polyphagia (great hunger), blurred vision and tingling, pain, or numbness in the hands/feet (Figure 3). Frequently, in T2DM patients these symptoms are mild to absent complicating the diagnosis of DM.

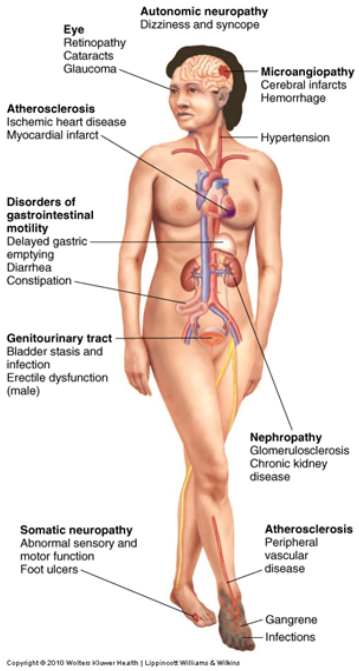

Figure 3: Main complications of diabetes mellitus.

Although long-term complications of DM develop gradually, they can eventually be disabling or even life-threatening. Some of the potential complications of persistent hyperglycemia include:

- **Retinopathy**, an important cause of blindness resulting of long-term accumulated damage in the small blood vessels of the retina. One percent of global

blindness can be attributed to DM *(World Health Organization, 2012)*. Approximately 84-90% of diabetic patients develop retinopathy after 10 to 15 years.

- **Nephropathy** causing renal failure. About 50% of T1DM patients and 35% of T2DM patients develop evidence of diabetic kidney disease over the course of their lifetime *(De Boer, 2014)*.
- **Peripheral neuropathy** (nerve damage) with risk of foot ulcers, amputations and Charcot joints (progressive degeneration of a weight bearing joint, a process marked by bone destruction) and **autonomic neuropathy** leading to gastrointestinal, genitourinary and cardiovascular symptoms and sexual dysfunction. Prevalence of neuropathy is 7% at 1 year, increasing to 50% after 25 years of disease.
- Frighteningly, patients with DM have an increased incidence of artherosclerotic cardiovascular, peripheral arterial and cerebrovascular disease. Hypertension and abnormalities in lipoprotein metabolism are often found in diabetic people. Altogether, increases the risk of **heart disease and stroke**. It has been estimated that 37-42% of all ischemic strokes in both African Americans and caucasians are attributable to the effects of DM alone or in combination with hypertension. The prevalence of heart disease or stroke in patients with DM is approximately 33.7% and it is responsible of 50% deaths of diabetic patients *(Morrish et al., 2001)*. The peripheral vascular disease incidence is about 26% in patients with DM aged ≥ 30 years *(http://www.clevelandclinicmeded.com)*.
- In the most severe cases, life-threatening consequences are hyperglycemia with **ketoacidosis** (DKA, high concentrations of ketone bodies) or **nonketotic hyperosmolar syndrome** (HHS), which leads to coma and, presumably, death without an effective treatment (Figure 3). Prevalence of DKA in T1DM youth was 31.1% during the years 2008-2010 and, this percentage was notably higher in those diagnosed at a younger age (~39% in those between the ages of 0 and 4 and ~23% in those between the ages of 15 and 19 years old) *(Dabelea et al., 2014; Elding Larsson et al., 2011).* HHS is usually presented in older patients with T2DM and carries a higher mortality than DKA, estimated at approximately 10-20%. The overall incidence of HHS is less than 1 case per 1000 person-years, making it significantly less common than DKA. Nevertheless, as the prevalence of T2DM increases, the incidence of HHS will likely increase as well *(Nugent, 2005)*.

1.3.1. Cancer, an Important Complication of Diabetes Mellitus

Over the last eight decades, several epidemiological studies have revealed a **connection between T2DM and several types of cancer** *(Joost, 2014; Joslin et al., 1959; Kessler, 1970; Vigneri et al., 2009)*. T2DM is associated with increased risk by 20-50% for different types of cancers such as **cancer of pancreas** *(Burney et al., 2014; Everhart and Wright, 1995; Huxley et al., 2005)*, colorectum *(Larsson et al., 2005; Limburg et al., 2006)*, liver *(El-Serag et al., 2006)*, kidney *(Lindblad et al., 1999)*, breast *(Larsson et al., 2007)*, bladder *(Larsson et al., 2006)*, endometrium *(Friberg et al., 2007)*

and non-Hodgkin lymphoma *(Mitri et al., 2008)*. On the other hand, DM has been also linked with reduced risk of prostate cancer *(Rodriguez et al., 2005)*.

Of particular interest are the Pancreatic Neuroendocrine Tumors (PNETs), a group of endocrine tumors arising in the pancreas that account for 3% to 5% of all pancreatic malignancies. PNETs incidence is lower than 1 per 100,000 persons per year, with slightly more males being affected than females. However, this incidence is higher in autopsy studies, ranging from 0.8% to 10% suggesting that these tumors frequently go unnoticed and people harbor asymptomatic PNETs. Moreover, PNETs incidence increases with advancing age, with a peak reported in the 6th and 7th decades of life. The average age at diagnosis was 58.5 years. Most PNETs are apparently indolent obstructing its diagnostic with better prognosis than the more common pancreatic exocrine tumors. Nevertheless, overall 5-year survival rate is only about 42% *(Garcia-Carbonero et al., 2010; Halfdanarson et al., 2008; SEER Cancer Statistics Review, 2014)*. Nonetheless, PNETs are the most common neuroendocrine tumors *(Yao et al., 2008)*. PNETs are frequently classified into 1) functioning tumors including insulinomas, gastrinomas, VIPomas, somastitonomas, glucagonomas and other syndromes as multiple endocrine neoplasia syndrome type 1 (MEN1) whose main characteristic is the hormonal overproduction; and 2) non-functioning tumors, that represents 15% of PNETs and comprise a large group of neoplasms that do not produce syndromes of hormonal excess *(Ro et al., 2013)*. The behavior of these types of cancers is highly variable and range from benign to extremely aggressive; therefore, it is very important to increase the understanding and the recognition of efficient treatment strategies. In this context, up to 45% of the pancreatic cancer cases can present as new-onset diabetes *(Jemal et al., 2006; Niederhuber et al., 1995)*.

A temporal or a causal relationship between diabetes and cancer has been difficult to established *(Chari et al., 2008; Li et al., 2012)* given the late appearance of symptoms of pancreatic cancer (approximately 10 years are needed to diagnose symptomatic pancreatic cancer) *(Pezzilli et al., 2009; Pezzilli and Pagano, 2013)*. Most of the epidemiological data supporting the association between diabetes and pancreatic cancer have been generated in cohort and case-control studies and meta-analyses *(Everhart and Wright, 1995; Huang et al., 2014; Huxley et al., 2005)*. These epidemiological evidences point towards a reciprocal link between these two pathologies, but the temporal association and the mechanism remain yet to be determined (diabetes could be a secondary manifestation of pancreatic cancer) as well as the pathophysiological mechanisms behind it *(Burney et al., 2014; Gullo, 1999)*. The importance to establish the association between T2DM and cancer is due to two main causes: 1) the possible use of recent onset diabetes as a marker of the disease, in particular, as a specific marker of pancreatic cancer, and 2) the selection of a population at risk for development of pancreatic cancer.

The association between T2DM and pancreatic cancer may partly be due to shared potential risk factors between the two diseases, such as: 1) non-modifiable risk

factors such as advanced age (individuals aged 55 years and older), sex (men have slightly higher risk than women), ethnicity (African Americans are more vulnerable to develop and die from cancer and diabetes than other race- *((CDC), 2014)*-and family history; and 2) modifiable risk factors that include lower socioeconomic status, obesity, diet, physical activity, tobacco smoking and alcohol consumption *(Giovannucci et al., 2010)*.

Besides the common potential risk factors, pathological alterations derived from T2DM can also increase the incidence of cancer. The possible mechanisms underlying a direct link between T2DM and pancreatic cancer involve inflammatory cytokines, chronic oxidative stress, accumulation of advanced glycation end-products, increased production of ROS and, subsequent oxidative damage to DNA and other biomolecules *(Giovannucci et al., 2010; Huang et al., 2014; Vigneri et al., 2009)*. Furthermore, insulin resistance with compensatory hyperinsulinemia and increased levels of insulin-like growth factor 1 (IGF-1) may promote the proliferation of cancer cells *(Gapstur et al., 2000)*. Recently, it has been proposed two powerful mechanisms to explain the relationship between these important diseases: 1) chronic activation of IGF-1 and 2 signaling which is known to promote tumor formation *(Handelsman et al., 2013)* and/or 2) dysfunction of the GTPase-activating protein 1 (IQGAP1), a growth factor and nutrient-sensor that couples cell growth and division, and regulates glucose-stimulated insulin secretion (GSIS) in β-cells. Moreover, IQGAP1 has been associated with several carcinomas and T2DM in humans *(Osman et al., 2013)*. Additional observational data reveals an emerging role of certain anti-diabetic medications like metformin, which seems to reduce the risk of cancer by 30% in individuals with T2DM *(Giovannucci et al., 2010; Leone et al., 2014)*. These evidences suggest that treatment of DM may influence in an early diagnosis and treatment of important diseases such as cancer.

1.4. Classification of Diabetes Mellitus:

According to World Health Organization (WHO), DM can be classified into 6 major types:

A. Type I Diabetes Mellitus (T1DM) results from an autoimmune destruction of the β-cells of the pancreas causing absolute deficiency in insulin secretion. This type of DM affects 20 million people worldwide *(IDF, 2014)* representing 5-10% of DM cases (Figure 4). This form of DM has also been known as insulin-dependent diabetes or juvenile-onset diabetes. The rate of the autoimmune attack is highly variable being rapid mainly during childhood, while in adults is frequently found with a slow progression. Children and adolescents particularly may manifest ketoacidosis as initial sign of the disease. Other patients exhibit a modest fasting hyperglycemia that can rapidly become severe hyperglycemia and/or ketoacidosis in the presence of infection or other stress. Particularly, adult patients may retain residual β-cell function sufficient to prevent ketoacidosis for many years. Nevertheless, they become insulin-dependent

for survival and are at risk for ketoacidosis. At latter stage of the disease, there is little or no insulin secretion as manifested by low or undetectable levels of plasma C-peptide *(Hother-Nielsen et al., 1988)*. A number of pancreatic autoantigens play an important role as markers of the autoimmune injury in the islets such as autoantibodies to glutamic acid decarboxylase (GD65), insulin, insulinoma-associated protein 2 (AI-2), and the zinc transporter ZnT8. One or more of them are present in 85-90% of individuals when fasting hyperglycemia is initially detected. Interestingly, these markers are probably not involved in the initiation of the injury being released later in the autoinmune process *(Pescovitz et al., 2009; Wong et al., 2004)*. Specific T1DM diagnosis criteria include the presence of autoantibodies (e.g values considered as positive when plasma levels of GD65 and AI-2 autoantibodies are greater than 5.0 U/mL and 7.5 U/mL, respectively *(Huici-Moreno, 2011)* and ketones levels measured in urine or blood samples higher than 240 mg/dl. Risk factors that contribute to the increased incidence of T1DM, in addition to genetic predisposition, are environmental factors which include viral infections *(Filippi and von Herrath, 2008)*, immunizations *(Hviid et al., 2004)*, diet i.e. exposition to cow´s milk at early age *(Vaarala, 2005)*, vitamin D deficiency *(Hypponen, 2010)*; and perinatal factors such as maternal age, history of peeclampsia and neonatal jaundice. Moreover, high weight at birth and early weight gain could influence in the development of T1DM *(Harder et al., 2009)*. Patients affected with this disease are not usually obese, but obesity is not incompatible with the etiology. Currently, the most widely used treatment requires insulin injections throughout the patient´s lifespan.

B. Type II Diabetes Mellitus (T2DM) is a more progressive disease caused by a combination of resistance to insulin action and inadequate compensatory insulin secretory response. Insulin resistance supposes a reduction in peripheral insulin sensitivity causing a decrease in glucose uptake by target tissues (skeletal muscle, adipose tissue, brain and liver) and an increment in hepatic glucose release. As a result, β-cells are subsequently forced to increase insulin production, which produces an early phase of hyperinsulinemia to compensate circulating glucose levels. In long-term, β cells are overwhelmed by insulin over-production, resulting in cell death. T2DM represents 90-95% of DM cases (Figure 4). It is known as non-insulin-dependent diabetes or adult-onset diabetes, although the number of children diagnosed with T2DM is increasing due to a growing incidence of overweight in the young population *(Dubinina et al., 2014; Macaulay et al., 2014; Rosenbloom et al., 1999; Zeitler et al., 2014)*. At early stage of the disease, hyperglycemia is compensated by a hyperinsulinemia and β-cell proliferation, but in latter stages insulin secretion is impaired when inflammation and a progressive loss of β-cell mass occurs *(Butler et al., 2003; Rahier et al., 2008)*. Overweight (Body Mass Index-BMI- ≥25-30 kg/m^2) or obese (BMI ≥30 kg/m^2) individuals have a higher risk for developing T2DM compared with individuals whose BMI is considered within the normal range (BMI 18.5-25 kg/m^2). Indeed, obesity is a state that causes itself insulin resistance. Obesity is believed to

account for 80-85% of the risk of developing T2DM, and recent research suggests that obese people are up to 80 times more likely to develop this disease than those with a BMI≤22kg/m^2 *(Sattar and Gill, 2014)*. In T2DM patients, ketoacidosis rarely appears and, when seen, is due to infection or stress. T2DM is often associated with a strong genetic predisposition. That is supported by data such as: 1) the lifetime risk of developing this disease is 40% for individuals who have one parent which suffer T2DM and almost 70% if both parents are affected *(Köbberling J, 1982)* and, 2) the differences in prevalence between ethnic groups. Other important risk factors are age, lack of physical activity and other factors such as the intrauterine environment *(Groop et al., 1996)* and low birth weight *(Hales et al., 1991)*. In addition, T2DM is frequent in individuals with hypertension and dyslipidemia.

T2DM is a progressive disease that involves a state named **prediabetes**, which usually is not diagnosed because coursing asymptomatically. Prediabetes means that the blood glucose is higher than normal, but it is not yet high enough to be classified as T2DM being described as a condition of high risk of developing DM. In general, prediabetes is defined as impaired FPG in the 100-125 mg/dl range and impaired glucose tolerance at two-hours of OGTT with levels between 140-199 mg/dl. The A1C values are between 5.7-6.4%. Progression from prediabetes to T2DM is preventable with healthy lifestyle changes, but without them 15-30% prediabetic patients will develop T2DM within five years. The main therapy for prediabetes and T2DM is a combination of weight reduction, diet, increased physical activity and/or pharmalogical treatment of hyperglycemia with antidiabetic drugs *(Knowler et al., 2002; Tuomilehto et al., 2001)*. However, during the progression of the disease, it might require insulin injections as part of the therapy.

C. Gestational Diabetes (GDM) has been defined as any degree of glucose intolerance with onset or first recognition during pregnancy. Although most cases resolve with delivery, the definition applied whether or not the condition persisted after pregnancy and did not exclude the possibility that unrecognized glucose intolerance may have antedated or begun concomitantly with the pregnancy. Approximately, GDM affects up to 15% of pregnant women aged 20-49 worldwide (0.6% of global DM cases) ranging from 1-25% depending on the population studied and the diagnostic test employed (more than 200,000 cases annually worldwide) (Figure 4). GDM is most often diagnosed through a prenatal screening at 24-28 weeks of gestation by a standard OGTT test after an overnight fast, rather than by reported symptoms which are similar to T2DM, if present. GDM is diagnosed if the OGTT levels are ≥ 140 mg/dL 2 hours after 75 g glucose bolus. Individuals at high risk for GDM are 1) older women (≥30 years old); 2) those with BMI ≥ 26; those with previous history of glucose intolerance, GDM in previous pregnancies and/or DM antecedents in first degree family; and 3) those with a history of large babies (macrosomia), spontaneous abortion, too much amniotic fluid or high blood pressure. Untreated GDM can have severe consequences including fetal complications like neonatal macrosomia,

hypoglucemia, hypocalcemia and hyperbilirubinemia and mother complications like caesarean section, obstructed labour, and pre-eclampsia. Importantly, GDM also has long-term health impact, with 10% of mothers with GDM developing T2DM immediately following the pregnancy and more than 50% of them will develop T2DM within 5-10 years after the delivery. Moreover, infants of women with GDM have a higher prevalence of overweight and obesity, and higher risk of developing T2DM later in life. Current treatment for GDM consists in the monitoring of blood glucose and a simple lifestyle therapy with special diet and physical activity (80-90% women). In the cases that insulin resistance persists, insulin supplementation will be necessary.

D.Genetic defects of β-cell function:

D.1. Maturity Onset Diabetes of the Young (MODY) is associated with monogenic defects in β-cell function and mass which accounts for up to 2% of all young patients with diabetes (0.1% of the total DM cases) (Figure 4). These forms of DM are frequently characterized by onset of a mild hyperglycemia at an early stage, generally before 25 years. The most common MODY forms are involved in mutations in genes participating in glucose sensing and β-cell transcription factors (Table 1) *(Fajans and Bell, 2011; Froguel and Velho, 1999; Mastracci and Sussel, 2012)* which leads to an impaired insulin secretion with minimal or not defects in insulin action in the absence of ketoacidosis. They are inherited in an autosomal dominant pattern, in contrast to the polygenic recessive origin of T1DM and T2DM. Requirements for MODY diagnosis are a family history of multigenerational diabetes and, usually, absence of obesity (to differentiate it from T2DM) and negativity for islets autoantibodies and measurable C-peptide levels (to differentiate it from T1DM).

Tabla 1: Genes implicated in Maturity Onset Diabetes of the Young (MODY). *Modified from (Kovler Diabetes Center, 2014; Mastracci and Sussel, 2012):*

MODY	GENE	GENE NAME	RESULTING DEFECT	CLINICAL FEATURES	REFERENCE
MODY 1	*HNF4α*	hepatocyte nuclear factor-4α	insulin secretion and β-cell mass	Familial, early-onset DM. Insulin therapy is not immediately acquired. Often responds well to sulfonylurea pills.	Bell GI et al., PNAS 1991; Stoffel M et al., PNAS 1996
MODY 2	*GCK*	glucokinase	glucose sensing by β-cells; hepatic glucose storage	Second most common type of MODY. Stable, mildly elevated blood glucose.	Froguel P et al., Nature 1992
MODY 3	*HNF1α*	hepatocyte nuclear factor-1α	insulin secretion and β-cell mass	Most common type of MODY. Familial, early-onset DM. Insulin therapy is not immediately required. Often responds well to sulfonylurea pills.	Vaxillaire M et al Nat Gen 1995; Menzel S. et al., Diabetes 1995
MODY 4	*PDX1*	pancreas/duodenum homeobox protein-1	transcriptional regulation of β-cell development and function	Very rare. Can cause exocrine pancreatic insufficiency, meaning digestive function of pancreas is impaired	Stoffer DA et al Nat Gen 1997
MODY 5	*HNF1β*	hepatic nuclear factor 1β	insulin secretion and β cell mass; 'renal cysts and diabetes syndrome'	Can cause either diabetes or developmental problems in kidneys. May also be abnormalities of the reproductive organs.	Horikawa Y et al., Nat Gen 1997
MODY 6	*NEUROD1*	neurogenic differentiation 1	transcription/regulation of β-cell development and function	Very rare	Malecki M T et al., Nat Gen 1999
MODY 7	*KLF11*	kruppeHike factor-11	impaired insulin promoter activation leading to decreased insulin expression	Very rare	Neve B et al., PNAS 2005
MODY 8	*CEL*	carboxyl-ester lipase	lipase function in pancreatic acinar cells; 'exocrine pancreatic dysfunction	Can cause exocrine pancreatic insufficiency, meaning digestive function of pancreas is impaired. Pancreas may be small.	Raeder H et al., Nat Gen 2006
MODY 9	*PAX4*	paired box gene 4	repressed activity of the insulin and glucagon promoters	Very rare	Plengvidhya N et al., J Clin Endocr Metab. 2007
MODY 10	*INS*	insulin	insulin gene processing	Can cause neonatal diabetes, antibody negative T1DM and MODY	Edghill EL et al., Diabetes 2008; Molven A et al., Diabetes 2008
MODY 11	*BLK*	tyrosine kinase, B-lymphocyte specific	β-cell function (insulin synthesis and secretion)	Very rare	Kim SH et al., Diabetes 2004; Borowiec M et al., PNAS 2009

D.2. Neonatal diabetes is diagnosed in the first six months of life and it does not show the typical pattern of T1DM. Neonatal diabetes can either be transient or permanent. **Transient neonatal diabetes** is caused by a genetic defect on ZAC/HYAMI imprinting, whereas **permanent neonatal diabetes** is most commonly due to a defect in the gene encoding the Kir6.2 subunit of the β-cell K_{ATP} channel. Children suffering the last one can be easily treated with sulfonylurea.

D.3. Mitochondrial diabetes and deafness (MIDD) is characterized for point mutations in mitochondrial DNA. The most common mutations occur in tRNA of leucine genes. MIDD represents only 1% of all DM cases *(Maassen et al., 2001)*. This disease is maternally inherited and genetic studies reveled that it might be sporadic *(Rotig et al., 1996)*.

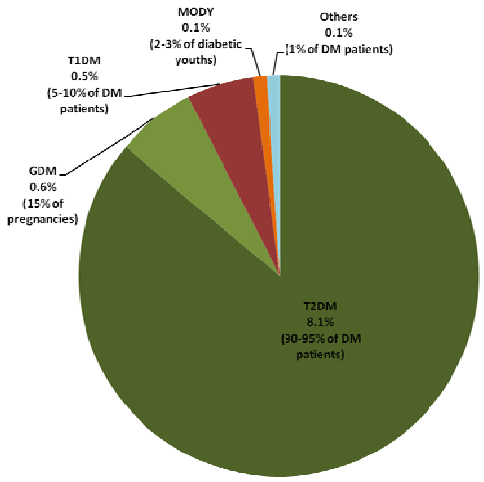

Figure 4: Worldwide prevalence of different types of Diabetes Mellitus (DM).

1.4.1. Potential Relationship Between the Pathophysiology of T1DM and T2DM

Despite being classified as two different types of DM, T1DM and T2DM may share common pathogenic mechanisms such as inflammation that is present in both forms of DM. Diabetes Mellitus (DM) is characterized by a deterioration and progressive failure of the pancreatic β-cells leading to compromised insulin secretion and subsequent hyperglycemia *(Wentworth et al., 2009)*. These β-cell cytotoxicity conveys not only to impaired β-cell function, but also a gradual loss of β-cell mass from 0 to 65% in T2DM, and approximately 70-100% in T1DM *(Cnop et al., 2005; Donath, 2004; Kloppel et al., 1985; Matveyenko and Butler, 2008; Pipeleers et al., 2008)*. Loss of β-cell mass is induced through β-cell apoptosis. β-cell death in T1DM is due predominantly to autoimmunity. In T2DM, β-cell death occurs as the combined consequence of increased circulating glucose (hyperglycemia) and saturated fatty acids together with adipocyte secreted factors (dyslipidemia) and chronic activation of the innate immune system *(Donath et al., 2003)*. Additionally, obesity and insulin resistance are associated with a chronic but subclinical inflammatory process that impairs insulin action in most tissues and could also hamper pancreatic β-cell function. Accordingly, a mutual thread might be the activation of innate intra-islet inflammatory mediators/autoinmmunity pathways which leads, in summary, to β-cell dysfunction in T2DM, insulin resistance in both T2DM and T1DM, and enhanced adaptive immunity that destroy β-cells in T1DM *(Wentworth et al., 2009)* triggering a final common pathway that lead to β-cell apoptosis *(Cnop et al., 2005; Donath, 2004; Donath et al., 2003; Itariu and Stulnig, 2014; Kloppel et al., 1985; Wentworth et al., 2009)*. Therefore, analysis of the evidence for putative final common pathways of β-cell death in both DM types is under study.

Multiples mechanisms are implicated in the inflammatory response. Elevated glucose concentrations (glucotoxicity) increased the metabolic activity of islets cells, leading to formation of ROS and increased oxidative stress. ROS promote the

activation of the inflammasome (multiprotein oligomer component of innate immune system responsible for activation of the immflamatory processes), thus enabling the production of mature interleukin-1β (IL-1β) *(Maedler et al., 2002; Zhou et al., 2010)*, the main cytokine involved in the pathogenesis of T2DM, with an action similar to the IL-1β presents in T1DM *(Alexandraki et al., 2006)*. IL-1β induces various cytokines and chemokines, including IL-6, IL-8, tumor necrosis factor (TNF) and monocyte chemoattractant protein-1 (MCP1) that lead to the recruitment of immune cells which induce islets dysfunction *(Alexandraki et al., 2006; Butcher et al., 2014; Donath, 2014; Maedler et al., 2002)*. In the same context, insulin overproduction induces **Endoplasmic Reticulum (ER) Stress** which also 1) activates the crosstalk with the inflammasome triggering apoptosis in the case of prolonged pathophysiological circumstances (Figure 5) *(Donath, 2014; Mollereau et al., 2014; Oslowski et al., 2012; Zhang et al., 2009)* and 2) additionally, has been involved in β-cell mass decrease. It has been proposed that the increase in proinsulin biosynthesis causes disruption in ER homeostasis and accumulation of unfolded and misfolded proinsulin protein in the ER lumen which leads to ER stress *(Scheuner et al., 2005)*. Failure of the ER adaptative capacity activates the **Unfolded Protein Response (UPR)**, a signaling network composed by three canonical branches mediated by three ER-membrane associated proteins. In the first branch, the **PKR-like ER kinase (PERK)** which induces translation attenuation by phosphorylation of eIF2α resulting in release of NF-κβ from its inhibitor Iκβ. NF-κβ translocates to the nucleus and induces the expression of the cytokines IL-1β and TNFα among others. In the second branch, **Inositol Requiring Enzyme 1 (IRE1)** undergoes autophosphorylation leading the splicing of a different isoform of the transcription factor X-box protein binding 1 (XBP1) mRNA, which regulates chaperone and endoplasmic-reticulum-associated protein degradation (ERAD) gene expression. Moreover, IRE1 recruits TNFα-receptor-associated factor 2 (TRAF2) which interacts with cellular stress pathways, JNK and Iκβ kinase (IKK) and activates them. These inflammatory kinases then phosphorylate and activate downstream mediators of inflammation. The third branch of the UPR, the **Activating Transcription Factor 6 (ATF6)** is also able to activate NF-κβ (Figure 5). Furthermore, there is a crosstalk between the three branches inducing the production of the inflammatory cytokines IL-8, IL-6, and MCP1 *(Fonseca et al., 2011; Hotamisligil, 2010; Szegezdi et al., 2006)*. In this line, the proapoptotic transcription factor DNA-damage-inducible transcript 3 (DDIT3/CHOP) is one of the major mediators of ER stress downstream PERK and ATF6 branches *(Eizirik et al., 2008; Song et al., 2008)*, leading to the induction of inflammatory mediators which, finally, activate both apoptotic pathways, death-receptor- and mitochondrial-mediated apoptotic pathways and their specific subsets of caspases associated. Caspases 12, 3, 6, 7, 8 and 9 has been observed in different studies of ER stress, although the cohort of caspases linked to ER stress has not yet been conclusively established *(Szegezdi et al., 2003)*.

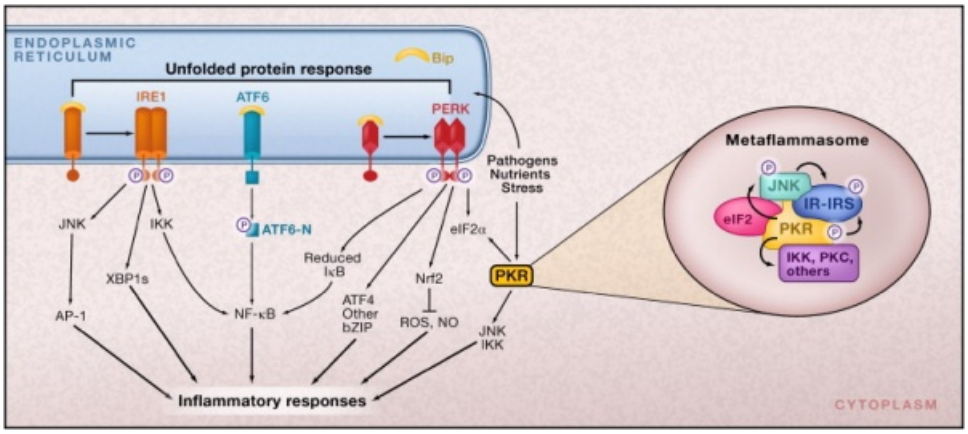

Figure 5: ER stress is linked to inflammatory signaling. *Figure from (Hotamisligil, 2010).*

Another additional feature that connets both diabetes types is that there are multiple DM variations between T1DM and T2DM as diabetes is a chronic, variable progressing and systemic disease. Singularly, T2DM patients often develops a T1DM-like phenotype after 20 years of disease by islets autoantibodies detection and insulin dependence, whereas it is well-known that T1DM patients show features of the metabolic syndrome associated with T2DM after long duration of disease *(Thorn et al., 2005)*. **Latent autoimmune diabetes in adults (LADA)** is one form of diabetes which appears to straddle the two major types, T1DM and T2DM. About 20% of the patients diagnosed with T2DM may have LADA *(Landin-Olsson, 2002)*. LADA is found in about 10% of initially non-insulin-requiring DM patients, and it is therefore probably more prevalent than T1DM. The usual features of LADA patients reported are onset of diabetes greater than 30 years of age *(Groop and Pociot, 2014; Groop et al., 2006; Schloot, 2014)*, clinical presentation masquerading as non-obese T2DM, unlikely to have a family history of T2DM, initial control of hyperglycemia with diet and oral antidiabetic agents requirement for 6 months after diagnosis, high risk of progression to insulin dependency within months, and some features of T1DM such as low fasting C-peptide and positive for islet directed antibodies, most often GADA, but they may also have other antibodies such as IA2A, ICA and IAA *(Brahmkshatriya et al., 2012; Falorni and Calcinaro, 2002; Itariu and Stulnig, 2014; Pozzilli and Di Mario, 2001; Zimmet et al., 1999)*.

Considering that the genetic predisposition is an important feature in T1DM and T2DM, genome-wide association studies (GWAS) have been carried out in the last years attempting to find the link between both diseases. Although very little genetic overlap has been discovered, some genes such as GLIS3 *(Barrett et al., 2009; Cooper et al., 2009; Dupuis et al., 2010)*, TCF7L2 *(Cervin et al., 2008)* and PPARγ *(Raj et al., 2009)* showed evidence of association between T1DM and T2DM *(Groop and Pociot, 2014)*.

2. PANCREAS: KEY ORGAN IN DIABETES

2.1. Definition, Function and Anatomical Organization of the Pancreas

Adult pancreas is a dual endocrine and exocrine glandular organ that forms part of the digestive system in vertebrate animals. It is located behind the stomach in the upper, back part of the abdomen and is surrounded by other organs including small intestine, liver, kidneys and spleen. Macroscopically, the pancreas has an elongated shape and a yellowish-pink aspect with a lumpy consistency depending on the level of fibrosis and fat accumulation in the organ. Pancreas size depends on species, increasing with the body size (for example, the volume of a human pancreas is several thousand times bigger than the rodent one). Human pancreas has a length of 18 to 25cm and an average weight of 68 g *(Ogilvie, 1937)*.

Anatomically, this organ is subdivided into three main regions: the widest part of the gland, called the head of the pancreas, is found in the right part of abdomen and closely nestled in the distal two-thirds of the duodenum. The common bile duct enters this area of the pancreas. The head is attached to the main part of the pancreas, the body, which is overlaid by the posterior wall of the stomach. The superior mesenteric blood vessels run behind this part of the gland. The thin end of the gland is called the tail. This region is located in the left part of abdomen in close proximity with the spleen (Figure 6).

The pancreas has two main functions: an **exocrine** function that helps in digestion and an **endocrine** function that regulates glucose homeostasis.

The **exocrine pancreas** represents 95-99% of the total pancreatic mass, and its main function is to secrete digestive enzymes to the duodenum. It is organized into acini, clusters composed by acinar cells which secrete enzymes into a complex network of ducts (Figure 6). Among the enzymes produced by the acinar cells, the vast majority are proteases like chymotrypsinogen, trypsinogen, procarboxypeptidases and proelastase which are released as proenzymes to avoid auto-digestion of the tissue. However, other enzymes such as pancreatic lipase, pancreatic α-amylase, ribonucleases (RNases) and desoxyribonucleases (DNases) are secreted as active enzymes. Moreover, an additional cell type, the centro-acinar cells (situated closely to the ducts) actively secretes the pancreatic juice rich in bicarbonate and mucins. These secretions are collected into the pancreatic intercalated ducts that are fused into the major pancreatic duct or **duct of Wirsung.** The duct of Wirsung is connected with the common bile duct at the level of the hepatopancreatic ampulla or **ampulla of Vater**. Together, they are opened into the duodenum through the papilla major or **papilla of Vater** through which the digestive enzymes are emptied into the duodenum to contribute in food processing *(Slack, 1995)*.

The **endocrine pancreas** accounts for approximately 1-5% of the pancreas. It is composed by round-shaped micro-organs scattered throughout the exocrine tissue named **pancreatic islets** or **islets of Langerhans** (Figure 6). The islets of Langerhans are

composed for five different hormone-secreting cell subtypes: α-, β-, δ-, ε-, and PP-cells secreting glucagon, insulin, somatostatin, ghrelin, and PP (pancreatic polypeptide), respectively. Pancreatic islets play a crucial role in glucose homeostasis regulating the levels of glucose on the blood circulation through a coordinate action of these hormones *(Andralojc et al., 2009; Cabrera et al., 2006; Konstantinova et al., 2007; Orci, 1976; Prado et al., 2004; Slack, 1995; Wierup et al., 2004)*.

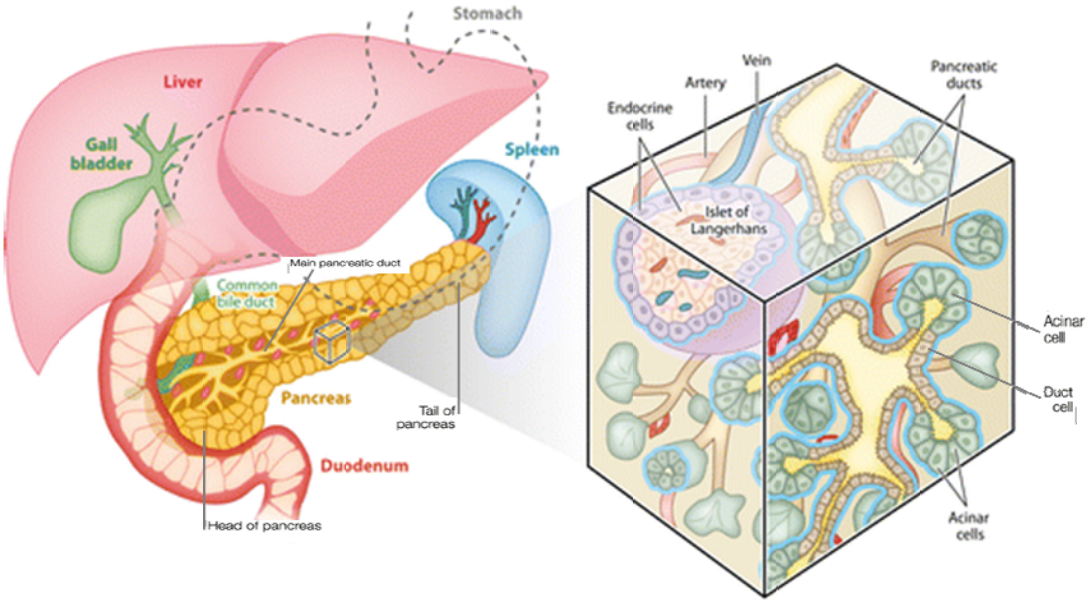

Figure 6: Schematic representation of the endocrine pancreas anatomy. *Figure modified from (Shih et al., 2013).*

3. PANCREATIC ISLETS

3.1. Definition, Cell Types and Functions of Pancreatic Islets

Islets of Langerhans are complex micro-organs surrounded by a thin collagen capsule and a glial sheet *(Hughes et al., 2006; Smith, 1975)* that defines and separates them from the exocrine tissue. Islets are highly vascularized to establish an intimate interaction with capillaries with the aim to regulate the hormone release to the bloodstream *(Andralojc et al., 2009; Cabrera et al., 2006; Konstantinova et al., 2007; Orci, 1976; Prado et al., 2004; Slack, 1995; Wierup et al., 2004)*.

Mammalian pancreatic islets, even though account for more than a million in adult human pancreas, 600 in rats and 200 in mice, represent only 1-5% of the organ. Total number of islets increases with the size of the species but islet size remains constant between 100 and 200 µm *(Hellman and Taljedal, 1969; Kim et al., 2009)*. Additionally, islets vary from small clusters of only a few cells to large aggregates of thousands of cells (1000-2000 cells) composed by five different cell types. Roughly, the classification of the pancreatic islets cells is *(Andralojc et al., 2009; Cabrera et al., 2006; Orci, 1976; Wierup and Sundler, 2004)*:

A. α-cells: cell type that secretes **glucagon**, a 29 amino acid peptide hormone derived from proglucagon (180 amino acids) through proteolityc cleavage, in addition to other products such as glucagon-like peptide-1 (GLP-1), glucagon-like peptide-2 (GLP-2) and glicentin. Facing a hyploglycemia situation, α-cells secrete glucagon that produce the stimulation of glucose release (Figure 7) and, consequently, raising its concentration in the bloodstream through 2 processes: 1) glycogenolysis, process in which glycogen is converted into glucose when glucagon binds to the glucagon receptors in the hepatocytes and/or 2) gluconeogenesis if glycogen store is not enough to compensate the glucose requirements. Glucagon is stored in secretory granules with an electrondense core and a grayish peripheral mantle *(Deconinck et al., 1971)*.

B. β-cells secrete the hormone **insulin**, a 51 amino acid peptide, which has the opposite effect of glucagon having a strong hypoglycemic action (Figure 7). It induces glucose uptake by somatic cells to reduce circulating glucose levels. Insulin is first synthesized as preproinsulin, an oligopeptide which contains a 24 amino acid signal peptide. After removal of this signal that directs the translocation into the ER, the incoming proteolityc precursor is the proinsulin. This biologically inactive proinsulin is cleaved into three parts: 1) a carboxy-terminal A-chain (21 amino acids) and 2) the amino-terminal B-chain (30 amino acids), which remain connected by two disulfide bonds. These two chains forming the biologically active insulin molecule through the action of the cellular endopeptidases, the prohormone convertases (PC1 and PC2) and the exoprotease carboxypeptidase E and 3) the central fragment of proinsulin called C chain or C-peptide (Connecting peptide), which is released together with insulin in a 1:1 molar ratio *(Orci, 1986)*. C-peptide measurement is an indicator of endogenous insulin production (to differentiate it from the insulin injected). β-cells also co-secrete Islet Associated Polypeptide (IAPP, also called amylin), a 37-amino acids peptide. During maturation process, insulin is stored into cytoplasmic secretory vesicles with an electrondense core and a clear peripheral mantle where this hormone crystallized with zinc forms this dense core. The Zn presence is still unclear, but results very helpful in islet isolation procedures, where zinc-chelating dyes like dithizone can be used in determining islet yield and purity *(Hou et al., 2009; Maske, 1957)*.

C. δ-cells release **somatostatin** which has two active forms produced by alternative cleavage of a single preproprotein, the prosomatostatin: one of 14 aminoacid (SS-14) and other of 28 amino acids (SS-28). This hormone inhibits the secretion of both insulin and glucagon in a paracrine manner (Figure 7) but also suppresses the pancreatic exocrine enzyme secretion *(Adrian, 1978; Corleto, 2010; Csaba and Dournaud, 2001; Roncoroni et al., 1983)*.

D. ε-cells secrete the 28 amino acid hormone **ghrelin**, a paracrine regulator of β-cells whose action is the suppression of glucose-stimulated insulin secretion, without significantly influencing insulin secretion at basal and low glucose concentrations *(Bosco et al., 2010)*. Furthermore, ghrelin is known as an orexigenic factor (stimulate appetite) (Figure 7) *(Kojima et al., 1999)*.

E. PP cells deliver the hormone **pancreatic polypeptide** (36 amino acids) that regulates the endocrine and exocrine secretions of the pancreas as well as gastrointestinal secretions and the hepatic glycogen levels. In contrast with ghrelin, this hormone has anorexigenic effect *(Batterham et al., 2003)*. Somatostatin has an inhibitory effect on PP levels, meanwhile acute hypoglycemia, fasting, exercise and protein-rich meal have a stimulatory effect *(Adrian, 1978; Adrian et al., 1978)*.

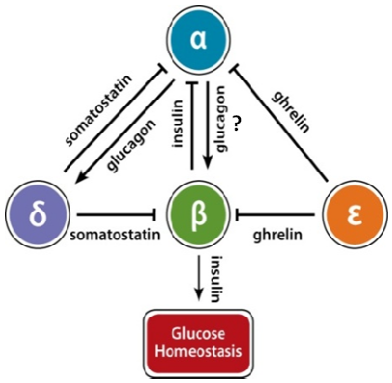

Figure 7: Cross-regulation between the different islet cell types. *Figure modified from (Jiang and Morahan, 2012).*

3.2. Differences between Human and Mouse Pancreatic Islets

Several studies elucidate important differences between the architecture and composition of the islets of Langerhans in humans and rodents that may explain the divergences in their physiological and functional behavior in responding to the insulin demand under normal and pathophysiological conditions. Because there are important differences both intra- and interspecies, comparative studies between human and murine islets are needed to increase the understanding of the complexity and the plasticity of islet *(Bosco et al., 2010; Brissova et al., 2005; Cabrera et al., 2006; Grippo et al., 2011; Kim et al., 2009; Steiner et al., 2010)*.

A. Cell types distribution:

Murine islets have a highly ordered and well-defined structure with a central core composed by β-cells, representing 60-80% of the cells of the islet and a mantle surrounding the core formed by the other endocrine cell types including α-cells (15–20% of the cells of the islet), δ-cells (<10% of islet cells) and PP-cells (<1% of cells). Notwithstanding, ghrelin-expressing ε-cells within the mouse pancreas do not represent a terminally differentiated endocrine population. Ghrelin cells give rise to significant numbers of α- and PP-cells and rare β-cells in adult islet *(Arnes et al., 2012; Wierup et al., 2014)*. There may also be regional differences in islet distribution in the rodent pancreas. Some studies suggest that there is a higher number of islets in the tail of the pancreas compared to the head *(Elayat et al., 1995)*. However, other authors postulate a homogenous distribution in the different regions of the adult mouse pancreas *(Kim et al., 2009)*. In detail, PP-cells are more common in the islets of the

head region of the pancreas, while α-cells are more frequent in the islets of the tail *(Ku and Lee, 2006; Park and Bendayan, 1992)*.

Human islets are more heterogeneous in cellular composition and, moreover, they tend to contain fewer β-cells and more α-cells compared to rodent islets *(Brissova et al., 2005; Cabrera et al., 2006; Grube and Bohn, 1983; Kharouta et al., 2009; Kim et al., 2009; Morrish et al., 2001)*. In human islets, α-, β- and δ-cells appear to be randomly distributed throughout the islet, although ε-cells are located at the periphery *(Cabrera et al., 2006; Kharouta et al., 2009; Kim et al., 2009)*. Adult human islet is composed by approximately 50% β-cells, 40% α-cells, 10% δ-cells, ≤1% PP-cells and 1% ε-cells *(Brissova et al., 2005; Cabrera et al., 2006; Wierup et al., 2002)*. Because of this distribution, more than 70% of human β-cells have direct physical contact with other cell types *(Steiner et al., 2010)*. Nevertheless, small human islets appear to be composed mainly of β-cells with a similar distribution as mouse islets *(Bosco et al., 2010; Kim et al., 2009)*. Interestingly, human fetal architecture resembles that found in adult mice islets suggesting that these islets undergo a reorganization process to adopt their classical morphology *(Jeon et al., 2009)*. It is worthy to mention that the number of islets in men increase towards the tail of the pancreas *(Saito et al., 1978; Wittingen and Frey, 1974)*, although these differences in distribution between the regions seems to be minimal. It is reported a greater number of PP-cells in the head, of α-cells in the neck and β-cells and ε-cells were evenly distributed between the neck, body and tail *(Brissova et al., 2005; Cabrera et al., 2006; Stefan et al., 1982; Wierup et al., 2014)*.

B. Anatomical relationship with vasculature:

Islets are highly vascularized due to their endocrine function. Islets receive 7–10% of total pancreatic blood flow *(Jansson and Carlsson, 2002)*. For that reason, several studies are trying to demonstrate the relationship between blood vessels and the islets cells. For this purpose, some authors like Cabrera, Kim and Bosco showed that all islets cell types are in close contact with the blood vessels in human islets in contrast with murine islets where β-cells are centered in the core of the islet. This determines a different microcirculatory pattern for both, human and mouse islets being predominantly a core to mantle circulation model in the islets of the rodents but, in the case of human islets nowadays still results controversial and it is needed more investigation *(Kim et al., 2009; Nyman et al., 2008)*.

3.3. Pancreatic Development and Key Transcription Factors

Understanding of molecular mechanism of pancreas development and maintenance of mature endocrine fate of this organ is an important subject to generate promising and innovative diabetes therapies *(Ben-Othman et al., 2013; Cano et al., 2014; Conrad et al., 2014; Jennings et al., 2013; Mastracci and Sussel, 2012; Rieck et al., 2012; Shih et al., 2013; Sussel, 2012)*.

Mouse animal models have been a useful tool to elucidate the intricate cascade of transcriptional factors in pancreas morphogenesis. In this context, human and mouse pancreas organogenesis is largely conserved sharing common features, however some crucial differences between these two species during this process have been discovered thanks to studies in human fetal pancreatic tissue (limited by availability and ethical restrictions), human β-cell lines or genome-wide analyses of various forms of diabetes *(Conrad et al., 2014; Jennings et al., 2013).*

Pancreas formation initiates between embryonic days (E) 8.5-9 in mouse and 4 weeks of gestation (WG) in humans, when the endoderm layer of the developing embryo becomes specified towards a pancreatic fate forming the primitive gut tube *(Pan and Wright, 2011)*. At this stage, the miss-expression of sonic hedgehog (Shh) in transgenic mice revealed that this transcription factor is necessary for the induction of prepancreatic markers (*Apelqvist et al., 1997)*.

Subsequent this endoderm patterning, pancreas morphogenesis is subdivided into two main waves of development in mouse, the primary and secondary transitions. By contrast, human development lacks the first wave of endocrine cell formation (Figure 8A and B) *(Cano et al., 2014; Jennings et al., 2013)*.

A. The **primary transition** occurs between E9.0-11.5 in mouse and about the 4-7 WG. This period is marked for the emergence of two independent primordia from the foregut epithelium whose development is temporally distinct *(Slack, 1995)* and received different signals from their adjacent mesoderm-derived tissues. These pancreatic buds, the dorsal and ventral primordia, are composed of highly proliferative undifferentiated multipotent progenitor cells (MPC) *(Gu et al., 2004)* which will give raise the different cell types of the mature pancreas. In this first wave of endocrine cells formation, main cell subtypes detected are insulin$^+$ and insulin$^+$/glucagon$^+$ cells *(Herrera, 2000)*.

During the primary transition, several transcription factors mediate the expansion of pancreatic progenitor cells and maintain the pancreatic fate. Lacking of this factors provoke pancreas agenesis or hypoplasia, but the pancreatic budding still remains what shown a cross regulation between this factors *(reviewed in (Cano et al., 2014; Pan and Wright, 2011; Sussel, 2012))*. Nevertheless, due to its importance during embryonic development of the pancreas, it is worth mentioning in more detail the role of Pdx1. This factor is widely expressed at different stages of pancreas organogenesis and in the adult organ. Pdx1 is first detected at E8.5 and, as development progresses, becomes down regulated in the endocrine cells and, finally, restricted to β-cells just before birth *(Offield et al., 1996)*. Pdx1 expression, in combination with Ptf1a, determines the commitment towards a pancreatic fate. In this context, Pdx1 and Ptf1a null mice show pancreas agenesis *(Ahlgren et al., 1996; Kawaguchi et al., 2002; Offield et al., 1996)*. In humans, PDX1 expression appears slightly later in development than in mice *((Jennings et al., 2013); reviewed in (Conrad et al., 2014))*. Mutation in this gene leads to Maturity Onset Diabetes of the Young 4 (MODY 4) *(Oliver-Krasinski and*

Stoffers, 2008; Stoffers et al., 1997a). In adult stages, Pdx1 plays an important role in β-cell function since this transcription factor is involved in normal glucose regulation of insulin secretion leading to impaired insulin secretion, hyperglycemia and late onset β-cell apoptosis in heterozygous Pdx1 mice *(Ahlgren et al., 1998; Brissova et al., 2002; Johnson et al., 2003)* due to defective respiratory chain activity by TFAM down-regulation *(Gauthier et al., 2004; Gauthier et al., 2009)*.

At E11.5, the gut tube suffers a first rotation which brings the dorsal and the ventral buds into close proximity for their fusion in a single organ latter at E18.5 on it development process.

B. At the onset of the **secondary transition** (E12.5), the segregation of the pancreatic epithelium into "tip" or "trunk" domains occurs. Based on lineage tracing, this second wave of endocrine cells does not seem to arise from the early primitive endocrine cells detected at E9.5 *(Herrera, 2000)*. This stage is marked by a high proliferation in this epithelium forming multiple protrusions. The tip domain represents the outer cells, meanwhile the trunk domain results from the inner cells. Notch signaling plays an essential role in this step promoting the trunk while repressing tip identity *(Afelik and Jensen, 2013; Afelik et al., 2012)*. Cross-repression between the master regulators Ptf1a and Nkx6.1/6.2 (NK6 homeobox 1/2) mediates tip/trunk compartmentalization, being Ptf1a a promoter for tip domain and Nkx6.16.2 for trunk domain *(Schaffer et al., 2010)*. During this secondary transition (E13.5-e16.5), the epithelium undergoes a massive differentiation into the three main pancreatic lineages (acinar, ductal and endocrine): 1) Tip cells appear to contain MPC which adopt the acinar phenotype *(Pan et al., 2013)*; and 2) Trunk cells which are bipotential for the ductal and endocrine cell fate *(Kopinke et al., 2012; Kopinke et al., 2011; Kopp et al., 2011; Solar et al., 2009)*. The ductal versus endocrine fate decision is controlled by Notch activity, but is the pro-endocrine basic helix-loop-helix (bHLH) transcription factor Ngn3 (neurogenin 3) expression that balance the endocrine /ductal phenotype during the endocrine cell differentiation *(Gradwohl et al., 2000; Johansson et al., 2007)*. High Notch activity regulates the expression of Hes1 and Sox9 resulting in the repression of Ngn3 that promote the ductal fate *(Greenwood et al., 2007)*. Conversely, low Notch signaling promotes the expression of Sox9 alone activating Ngn3 which leads to endocrine differentiation (Figure 8A and B) *(Delous et al., 2012; Shih et al., 2013)*.

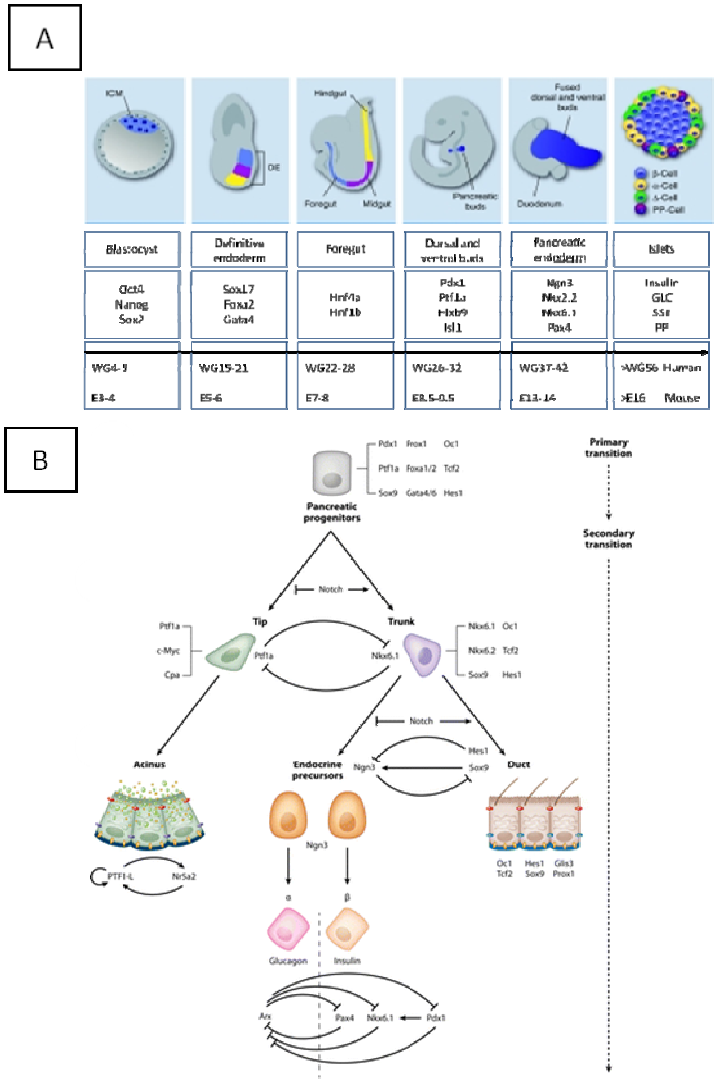

Figure 8: A) Illustration of pancreas development in human and mouse and B) Key transcription factors governing lineage decisions during pancreas development. *Figure modified from (Naujok et al., 2011) and (Shih et al., 2013).*

3.3.1. A Focus in Pancreatic Islets Cells Development

Expression of Ngn3 is the starting point for the activation of additional transcription factors *(Gouzi et al., 2011; Johansson et al., 2007)* which allow the trunk precursor cells to generate the five different endocrine cell types (Figure 9). Between E13.5-15.5, fully differentiated insulin-expressing β-cells and glucagon-expressing α-cells emerge. First somatostatin-expressing δ-cells appear at E16.5 and ghrelin-expressing ε-cells, at E8.5 *(Wierup et al., 2014)*. Over the next few days (E14.5-e18.5), endocrine cells coalesce into small aggregates *(Pictet et al., 1972)*. Shortly before birth, at E18.5, PP-expressing cells differentiate and, finally, endocrine cells begin to form well-organized islets of Langerhans. It should be mentioned that timing of expression of Ngn3 seems to determine the final fate of the endocrine precursors. In this context, induction of Ngn3 expression at E11.5 favored differentiation towards β and PP-cells, whereas at E14.5 prompted the differentiation towards δ-cells *(Johansson et al., 2007)*.

Briefly, a combination of several transcription factors such as **Arx**, **Brn4**, **MafB** and **Pax6** define the α-cell lineage, while β-cell commitment requires combined expression of **Pax4**, **IA1**, **MafA**, **Nkx2.2**, **Nkx6.1**, **Isl1, Pax6** and **Pdx1** (in a second wave of expression) (Figure 9) *(Collombat et al., 2005; Collombat et al., 2003)*. In the case of the other endocrine cell types, little is known *(Bernardo et al., 2008)*.

Between the key transcriptional factors that could modulate the lineage identity of the endocrine precursors are **Pax4** (Paired-box 4) and **Arx** (Aristaless related homeobox) whose expression pattern becomes mutually antagonistic for proper islet cell specification. Pax4 is restricted to β- and δ-cells, whereas Arx is expressed in α-, ε- and PP-cells *(Collombat et al., 2005; Collombat et al., 2003; Sosa-Pineda, 2004; Sosa-Pineda et al., 1997)*. Collombat *et al.* demonstrated that Arx global mutants exhibit loss of α-cells and increased β- and δ-cells. Futhermore, misexpression of Pax4 or Arx in mature endocrine cell types induces α-to-β or β-to-α conversion *(Collombat et al., 2007; Collombat and Mansouri, 2009; Courtney et al., 2013)*. The analysis of the pancreas of Pax4-deficient animals demonstrated that this factor is an important player in processes underlying β-cell genesis. Interestingly, co-ablation of Arx and Pax4 causes an early-onset loss of mature β- and α-cells, concomitantly with a massive increase in δ-cells *(Collombat et al., 2005)*.

In humans, endocrine cells formation is slightly delayed in comparison to rodents (approximately two weeks, probably due the loss of the first wave of differentiation) *(Jennings et al., 2013)*. Nevertheless, islet-like structures emerges earlier in human embryo development being detectable at the end of the first trimester, meanwhile islets appears just at birth in mice (E18.5) (Figure 8A) *(Cano et al., 2014; Herrera et al., 1991; Jeon et al., 2009; Piper et al., 2004; Sarkar et al., 2008)*.

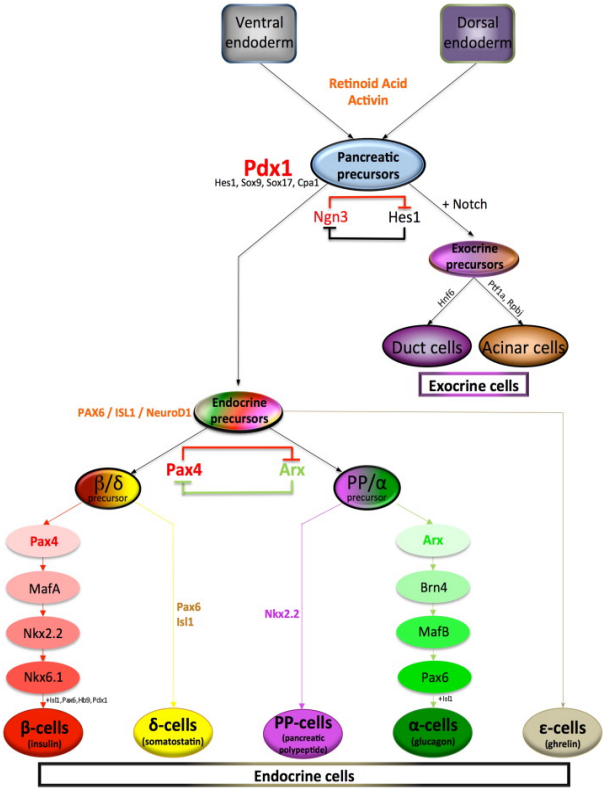

Figure 9: Schematic representatic of key transcription factors involved in pancreatic islets development. *Figure from (Ben-Othman et al., 2013).*

3.3.2. Maintenance of β-cell Identity

Once islets cells are differentiated into a specific endocrine lineage, they must maintain their identity. Several transcription factors are involved in the maturation and maintenance of the different cell types into mono-hormone cells. Focusing in β-cells, whose main function is insulin release, some of the crucial genes necessary for the activation of the *Insulin promoter* are **Pax6**, **MafA/MafB**, **NeuroD1/β2**, **Nkx2.2**, **Pdx1** and **Pax4**.

Pax6 expression is restricted to islets at birth. Inactivation of this transcription factor during development in mouse embryos leads to a defective islet morphology and a reduction in the entire islets cells with a concomitant decrease in hormone production *(Ashery-Padan et al., 2004; Sander et al., 1997; St-Onge et al., 1997)*.

The expression of the basic leucine-zipper transcription factors **MafA** and **MafB** is critical for α- and β-cell maturation. MafB is needed for α- and β-cell differentiation, becoming restricted to α-cells later in the development process. Nevertheless, MafA is β-cell specific *(Artner et al., 2010; Nishimura et al., 2006; Zhang et al., 2005)*. MafB-deficient mice exhibit a reduced number of α- and β-cells *(Artner et al., 2010; Artner et al., 2006)*. On the contrary, MafA mutant mice display normal β-cells development, but newborn mice develop glucose intolerance *(Zhang et al., 2005)*. This phenotype suggests that MafA is not necessary for embryonic development, while it is important for the maintenance of β-cells mass and function. In relation with this idea, MafA is crucial for glucose-responsive β-cells through interaction with key transcription

factors, like Pdx1 and NeuroD1, in the induction of insulin and Glut2 transcription *(Aramata et al., 2005; Hang and Stein, 2011)*. Interestingly, MAFB expression is observed in human β-cells *(Dai et al., 2012; Dorrell et al., 2011)*.

The bHLH transcription factor **NeuroD1/β2** is required for β-cell maturity and glucose responsiveness *(Gu et al., 2010)*. After birth, its expression is restricted to insulin-producing cells. Knock-out NeuroD1/β2 mice die of severe diabetes shortly after birth; their β-cells are poorly differentiated, impaired islets formation, and the number of β-cells is decreased *(Naya et al., 1997)*. Furthermore, mutations in human NEUROD1/β2 have been reported to cause MODY6 *(Malecki et al., 1999)*. This factor also activates the transcription of insulin by forming heterodimers with the bHLH protein E47 in the insulin promoter *(Naya et al., 1995)*.

Nkx2.2 is confined to β-cells and its expression is observed in mice from early stages of development, while in human is delayed being a significant difference between both species. Only later in this process, its expression overlaps *(Jennings et al., 2013; Sussel, 2012; Sussel et al., 1998)*. Inactivation of Nkx2.2 in mice results in not fully differentiated β-cells *(Prado et al., 2004; Sussel et al., 1998)*. Furthermore, this factor binds to the insulin gene promoter affecting insulin gene expression *(Cissell et al., 2003)*.

Pdx1 is restricted to β-cells at later stages of pancreatic development in a second wave of expression *(Bonal and Herrera, 2008)*. Inactivation of Pdx1 in transgenic mice results in pancreas agenesis. In this context, mutations in human PDX1 have been associated to MODY4 and adult onset of T2DM *(Stoffers et al., 1997a; Stoffers et al., 1997c)*. Furthermore, Pdx1 not only acts synergistically in the regulation of insulin gene with other genes such as NeuroD1/β2, Ngn3 and MafA, but also activate many β-cells genes like Glut2 or GCK (glucokinase) *(Kaneto et al., 2005)*.

Pax4 transcripts are already detected at E9.5; it is not until E13.5-E15.5 that reaches its maximal expression. The timing of Pax4 expression coincides with the secondary transition phase, suggesting an important role for this gene in the expansion and/or survival of the β-cell lineage *(Pictet et al., 1972; Sosa-Pineda et al., 1997)*. Pax4 expression ensures normal development of β-cells, since ablation of Pax4 gene results in the absence of mature β-cells and δ-cells, with a concomitant enrichment for α-cells and ε-cells *(Pictet et al., 1972; Sosa-Pineda et al., 1997)*. Remarkably, Pax4 has been linked to MODY9, disease associated with repressed activity of human insulin and glucagon gene promoters *(Plengvidhya et al., 2007)*. Pax4 also plays a major role in the adaptive response of β-cell mass in the adult pancreas, and the evidence will be discussed in detail in next epigraphs.

3.4. Regulation of the Principal Function of Pancreatic β-cells: Glucose Uptake and Insulin Action

The primary function of β-cells is to store and release insulin in order to regulate glucose levels in the bloodstream. Glucose uptake and metabolism are two

essential steps in the "glucose-stimulated insulin secretion" (GSIS) pathway which leads to an increase in insulin secretion in response to augmented extracellular and intracellular glucose levels.

Extracellular glucose enters β-cells by facilitated diffusion through specific glucose transporters (GLUT-1 in humans; GLUT-2 in rodents) and it is retained in cytoplasm through its phosphorylation by the enzyme glucokinase (GCK), which serves as a glucose sensor that determines the rate of glucose utilization by β-cells over a range of physiological glucose levels. Glucose phosphorylation determines the metabolic flux through glycolysis. The main end product of glycolysis in β-cells is pyruvate, which is efficiently transferred to the mitochondria activating the Krebs cycle. As a result of this metabolic pathway, more than 90% of glucose-derived carbons are converted into CO_2 (3-fold higher than in other cell types) *(Ishihara et al., 1999; Schuit et al., 1997)* and in reducing equivalents NADH and $FADH_2$ which act as a source of electrons for the mitochondrial respiratory chain. Electrons form an electrochemical gradient across the inner mitochondrial membrane that is used to produce ATP via oxidative phosphorylation. ATP is transported from the mitochondria to the cytosol in exchange for cytosolic ADP. This incremented ATP:ADP ratio leads to the closure of ATP-sensitive K^+(K_{ATP}) channels and, therefore, plasma membrane depolarization triggering the opening of voltage-dependent Ca^{2+} channels thus allowing the extracellular Ca^{2+} influx (in humans also participate voltage-dependent Na^+ flux). This increase in cytosolic Ca^{2+} leads to activation of phospholipase C (PLC) promoting the generation of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). These metabolic products bind to protein receptors on the membrane of endoplasmic reticulum (ER) stimulating the release of ER-Ca^{2+} via IP_3 gated channels further increasing the intracellular Ca^{2+} concentration. This increase in intracellular Ca^{2+} is the signal that triggers insulin secretion by exocytosis of existing insulin granules after their fusion with the plasma membrane of β-cells (Figure 10) *(Newsholme et al., 2010; Quesada et al., 2008; Wiederkehr and Wollheim, 2006)*.

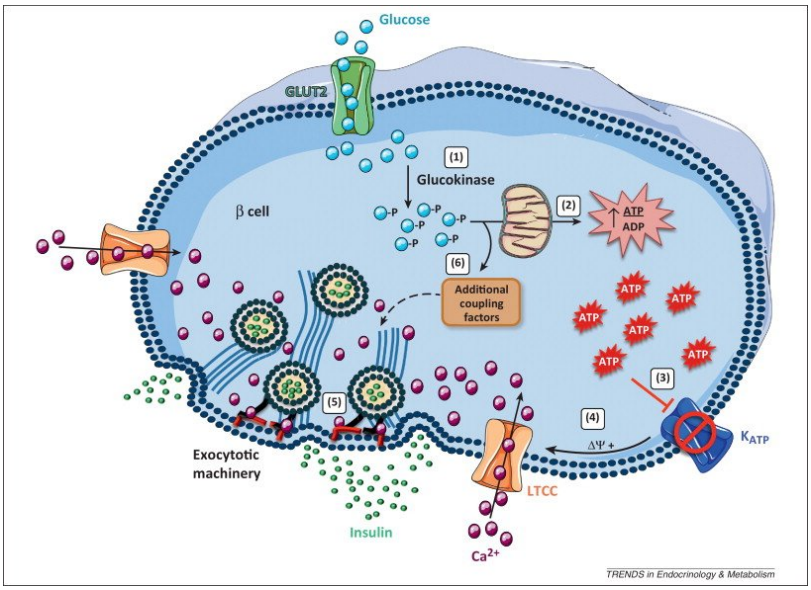

Figure 10: Glucose-dependent insulin release in pancreatic β-cells. *Figure from (Mancini and Poitout, 2013).*

Insulin secretion in adult islets follows a **biphasic kinetics** dependent of Ca^{2+} influx (Figure 11) *(Henquin, 2009)*. These phases affect different pools of insulin-secretory granules. The first phase of insulin release, called K_{ATP} channel-dependent pathway, occurs very rapidly in response to increased glucose levels in the blood. This phase has been explained immediately above. The second wave, named K_{ATP} channel-independent pathway, is produced by a sustained and slow release of the newly formed vesicles and requires trafficking to the plasma membrane *(Straub and Sharp, 2002)*.

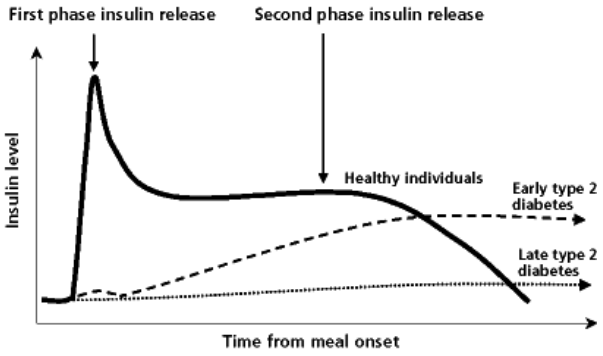

Figure 11: Biphasic kinetics of insulin secretion in healthy pancreatic islets and in T2DM patients' islets. *Figure from MannKind Corporation.*

GSIS is regulated and affected at three complementary levels: 1) **nutrients** such as amino acids (leucine, arginine and glycine) which act similarly to glucose by altering the membrane potential of the β-cell, and fatty acids (FFAs) that cause peripheral insulin resistance by inhibiting insulin-stimulated glucose uptake and glycogen synthesis. FFAs support between 30 and 50 % of basal insulin secretion and potentiate glucose-stimulated insulin secretion *(Boden, 2003; Torres et al., 2009; Yang et al.,*

2010); 2) **nervous system** via parasympathetic nervous system through the acetylcholine that triggers insulin secretion *(Lechin and van der Dijs, 2006)* and 3) **hormones** through the inhibitory effect of somastotatin *(Corleto, 2010)*, ghrelin, epinephrine and norepinephrine *(Hoffman, 2007)* and, on the other side, the stimulatory effect of glucagon and the two incretin hormones, glucagon-like peptide (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) *(Green and Flatt, 2007)*.

Insulin action starts with the binding of the hormone to it specific receptor present on the surface of all insulin target tissues *(Whitehead et al., 2000)*. The insulin receptor is composed by two extracellular α-subunits and two transmembrane β-subunits. Insulin, binding to the extracellular α-subunits, provokes the phosphorylation of the two transmembrane β-subunits of the receptor activating their tyroxine kinase domain. Following its activation, insulin receptor tyroxine kinase phosphorylates specific intracellular proteins. On insulin target tissues, insulin-receptor substrate 1 (IRS-1) is the major protein that interacts with this receptor and undergoes tyroxine phosphorylation mediating the insulin effect on hepatic glucose production, gluconeogenesis and glycogen formation *(Kerouz et al., 1997)*. Phosphorylated IRS-1 actives phosphatidylinositol 3-kinase (PI3K) leading to the synthesis of glycogen by activation of glycogen synthase kinase-3 and the protein phosphatase 1 (PP1), the main regulators of glycogen metabolism. This process generates different biological responses on insulin target tissues:

1) Facilitates glucose uptake in skeletal muscle, adipose tissue and other tissues. Insulin-activated cascade induces the fusion of vesicles containing GLUT4 transporter with the plasma membrane, thereby giving the cell an ability to efficiently take up glucose;

2) In liver, insulin has two main functions: on one hand, it stimulates the storage of glucose in the form of glycogen by the hepatocytes. In this organ, insulin activates the enzyme hexokinase which catalyzes the phosphorylation of glucose to yield glucose-6-phosphate. Moreover, the hormone not only inhibits the role of the glucose-6-phosphate, but also activates the functions of the enzymes phosphofructokinase and glycogen synthase which are involved in glycogen synthesis by glycogenesis. On the other hand, insulin promotes the synthesis of free fatty acids when glycogen is accumulated in the hepatocytes in high levels (roughly 5% of liver mass). These fatty acids are exported from liver in lipoproteins providing free fatty acids for use in other tissues, including adipocytes.

3) In the adipose tissue, insulin inhibits breakdown of fat by inhibiting the intracellular lipase that hydrolyzes triglycerides to release free fatty acids (Figure 12).

4) In brain, insulin has a significant effect on glucose metabolism mainly in the cerebral cortex. This may be either a direct effect of insulin stimulating glucose uptake and metabolism or an indirect effect achieved via insulin-stimulated neuronal activation with secondary increment in cell glucose metabolism. In the case of a direct effect of

the insulin, the increased uptake is brought about by recruitment of glucose transporter GLUT1, the major transporter of glial cells, to the plasma membrane of cells within the brain. Glucose may then be converted into lactate for neuronal use or into glycogen *(Bingham et al., 2002)*.

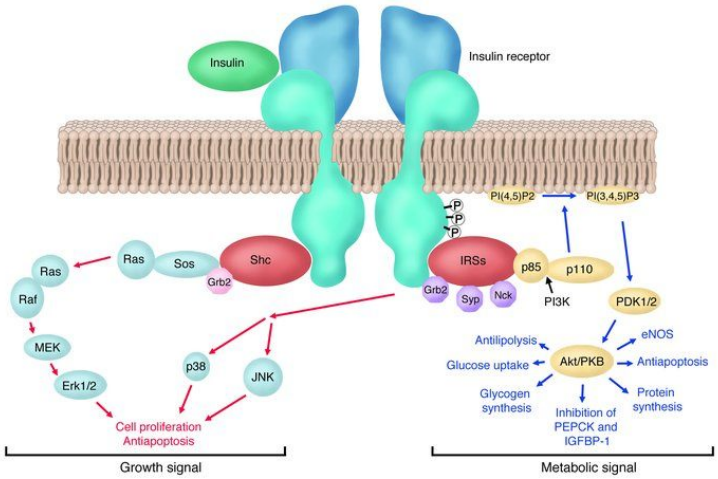

Figure 12: Schematic representation of the insulin action pathway. *Figure from (Van den Berghe, 2004).*

4. DIABETES THERAPY

Modern medical care uses a vast array of lifestyle and pharmaceutical interventions aimed at preventing and controlling hyperglycemia as well as to decrease the likelihood that target tissues of the body are harmed by hyperglycemia. However, due to important inconvenients of current treatments, novel therapies of treating DM are urgently needed to find new, better and more sustainable treatments for diabetic patients.

4.1. Current Therapies for Diabetes Mellitus

4.1.1. Insulin Therapy:

Patients with T1DM require daily insulin therapy for life. T2DM patients can initially be treated with lifestyle changes and healthy diet without the requirement for insulin supplementation. However, with the progression of the disease, in many T2DM patients (more than 50% after 5 years) these interventions are unable to maintain the normoglycemia becoming dependent on insulin therapy as a consequence of the natural progression of T2DM *(UKPDS, 2008)*.

Insulin is the oldest therapy available for DM since it was discovered in 1921, although clinical testing in humans started in 1922. Insulin is derived from various sources such as animal (cows and pigs) as well as synthetic origin such as the use of recombinant DNA. Nowadays, approximately 70% of worldwide insulin for clinical use in humans is produced by genetic engineering techniques in which human insulin DNA is inserted into the host cells *Escherichia coli*. This biosynthetic insulin is considered

just as safe as animal insulin, but it is cheaper to produce so it can be generated in large quantities and it is absorbed more rapidly than animal derived insulin. Moreover, it is indistinguishable from human insulin produced in the pancreas and therefore, is less likely to cause allergic reactions in diabetic patients *(IDF, 2014; Keen et al., 1980)*.

The goal of best insulin therapy is to mimic physiological insulin replacement as closely as possible. For that reason, synthetic insulin analogues (or insulin receptor ligands) have been developed. The commonly used types of insulin are:

A. Fast-acting types, which begin to work within 5 to 15 minutes and are active for 3 to 4 hours (these insulin analogs do not form hexamers being their action very fast).
B. Short-acting analogues, which begin working within 30 minutes and are active for 5-8 hours.
C. Intermediate-acting analogs start to work in 1-3 hours and continue to be active for about 16-24 hours.
D. Long-acting types begin to work in 4-6 hours and are active for longer than 24 hours.
E. Combination of different insulin analogues which include a mixture of either fast-acting or short-acting insulin with longer-acting insulin.

Crucial problems of insulin clinical treatment are the mode of administration and the selection of the correct dose and timing. Nevertheless, like nearly all other proteins, insulin cannot be administered by oral via into the gastrointestinal tract due to its rapid breakdown into single amino acids, so subcutaneous injections are required for insulin therapy using single-use syringes with needles, repeated-use insulin pens with needles or insulin pumps. For insulin administration, there are two main therapeutic approaches: multiple daily injection therapy (MDI) or insulin pump therapy. MDI is the accepted standard care to maintain near-normal blood glucose to reduce the risk of complications. Insulin pumps have the advantage of maintaining a better control over basal insulin dosage, but this therapy has some limitations such as the cost, the potential hypoglycemic and hyperglycemic episodes and infection and/or ulcerations due to catheter problems. However, to solve these issues, a recent technology has been prompted through an experimental integration of a continuous glucose monitoring and an insulin pump called bioartificial pancreas to improve the insulin treatment in T1DM, although this technology is still at an early stage of development *(Yoshida et al., 2014)*.

4.1.2. Anti-diabetic Drugs:

Several anti-diabetic drugs are administered to diabetic patients, mainly T2DM patients, to control blood glucose levels into a normal range and minimize chronic diabetic complications. Most of them are classified into: 1) **insulin sensitizers** which increase the sensitivity of target tissues to insulin; 2) **insulin secretagogues**, medicaments that increase the amount of insulin secreted by pancreas; 3) **incretin-**

based therapies are based in gut-derived peptide hormones that augments glucose-induced insulin secretion; and 4) other types of diabetogenic drugs (Table 2) (Figure 13) *(Ching Sun et al., 2014; Wajchenberg, 2010)*.

Table 2: Current anti-diabetic drugs for diabetic patients.

MEDICATION	ACTION	CANCER RISK	ADVANTAGES	DISADVANTAGES
Insulin sensitizers (increase sensitivity of target organs to insulin)				
Biguanides (Metformin)	Increase glucose uptake by inhibition of hepatic gluconeogenesis and improvement of insulin sensitivity by skeletal muscle and adipose tissue. Acitvation of AMPK Favourable effects on insulin receptor expression, tyrosine kinase activity and incretins pathway (increase GLP-1) Oral administration	Lower or not effect	Do not cause hypoglucemia Available in combination with other oral diabetic drugs First line agent Inexpensive Familiar Weight loss	No use in cardiovascular, pulmonary or hepatic insufficiency (risk of lactic acidosis)
Thiazolidinediones	Binds to PPARγ regulating transcription of insulin-sensitive genes Reduce insulin resistance and endogenous insulin levels in muscle, adipose tissue and mildly, in liver Oral administration	High risk of bladder cancer Low risk of breast and lung cancer	Do not cause hypoglucemia	Increase risk of heart failure Weight gain Fluid retention (edema) Increase risk f fractures
Insulin secretagogues (increase amount of insulin secreted by pancreas)				
Sulfonylureas (Glibenclamide)	Direct binding to sulfonylurea receptor SUR1 that inhibits K_{atp} channels. This causes Ca^{2+} influx leading to insulin release Binds strongly to plasma proteins Oral administration	Higher (increased risk of cancer-related death compared with insulin sensitizers)	Low cost (second line agent after metformin) Reduction of microvascular complications	Hypoglucemia (in particular, patients with renal failure and elderly) Only for T2DM patients Impair cardiac ischemia preconditions Implicated in cardiovascular risk Weight gain No use in pregnancy
Meglitinide	Same action that sulfonylureas (bind to K_{ATP} channels, but in a different site) Called "short-acting secretagogues" Oral administration	Higher		Hypoglucemia (lower risk than sulfonylureas) Weight gain
Incretin-based therapies				
Glucagon-like peptide-1 receptor agonist (GLP-1)	Stimulation of insulin synthesis and secretion Reduction of glucagon release Subcoutaneous administration	High risk of thyroid and pancreatic cancer	Expansion of β-cell mass by stimulation of β-cell proliferation and neogenesis and inhibition of β-cell apoptosis Weight loss (increase saciety, gastroparesis and o inhibition of digestive secretions)	Rapidly inactivated by DPP-4 enzyme (short half-life) Increased risk of acute pancreatitis Nausea (decreased gastric motility)
Dipeptidyl peptidase-IV inhibitors (DPP-IV)	Reduction of degradation of circulating GLP-1 Oral administration	High risk of pancreatic cancer	Expansion of β-cell mass by stimulation of β-cell proliferation and neogenesis and inhibition of β-cell apoptosis No hypoglucemia Weight neutral	Increased risk of acute pancreatitis Hypoglycemia Increased risk of infections (nasopharyngitis) Headache
Other diabetes therapies				
Alpha glucosidase inhibitors	No direct effect on insulin secretion or sensitivity Alpha-glucosidase enzyme inhibition in small intestine preventing the conversion of polysaccharides to monosaccharides Triggers slow release of carbohydrates Oral administration	Neutral	Do not cause hypoglucemia Available in combination with other oral diabetic drugs	Only effective on the earliest stage
Sodium-glucose-linked cotransporter-2 inhibitors (SGLT2)	Promote glycosuria acting in the reabsorption of the filtered glucose in the kidney's proximal tubuler from the urine back into the bloodstream Oral administration	Neutral	Improves blood pressure Weight loss	No for T1DM patients Reproductive and urinary infections Polyuria
Amylin analogs	Slow gastric emptying Supress glucagon Subcoutaneous administration	Neutral	Treatment of T1DM and T2DM patients	Nausea

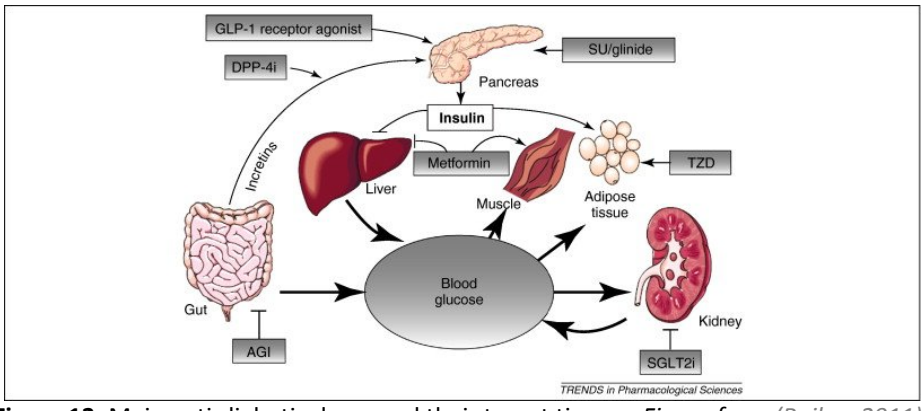

Figure 13: Main anti-diabetic drugs and their target tissues. *Figure from (Bailey, 2011).*

4.2. Experimental Therapies for Diabetes Mellitus

4.2.1. Pancreatic Islets Transplantation:

An alternative therapy to achieve glycemic control in DM patients is β-cell replacement by transplantation. This therapy can be accomplished by 1) whole pancreas transplantation or 2) pancreatic islets allo-transplantation or auto-transplantation, although these two therapies are complementary *(Fiorina et al., 2008; Johnson and Jones, 2012)*.

A. Whole pancreas transplantation was first performed in 1966. This approach usually combines simultaneous pancreas and kidney transplant. This process results in insulin-independence rates of 85% 1 year after transplantation *(Demartines et al., 2005; Mora et al., 2010; Ojo et al., 2001)* and also can revert some secondary complications of T1DM *(Jahansouz et al., 2011)*. Nevertheless, the main disadvantage is that transplant is a major surgery that involves a greater risk of complications related to the transplanted exocrine tissue rather than the endocrine (graft thrombosis, graft pancreatitis and pancreatic fistulae) and even mortality rates up to 4%.

B. Pancreatic islets allo-transplantation is a procedure which consists in the transplantation of purified islets from the pancreas of deceased donors into a T1DM patient. In the case of T2DM patients (but impossible in T1DM patients), pancreatic **islets auto-transplantation** could be performed. This process consists in a total pancreatectomy, following purification of islets and, then, transplantation of their own healthy islets. Both methods were based in a collagenase digestion of pancreas first developed in 1967 by Lacy´s group. In 2000, Shapiro et al. improves this islets allo-transplantation protocol, known as Edmonton protocol, where they introduced a new combination of immunosuppressive medications *(Shapiro and Lakey, 2000; Shapiro et al., 2000)*. Pancreatic allo-transplantation is performed in patients with uncontrolled diabetes for a minimum of 5 years and the lower age limit is 18 years of age. The transplant process is currently performed as a percutaneous transhepatic cannulation of the portal vein into the liver. Islets are usually received by two infusions with an average of 500,000 islets per infusion (the second graft is usually given between 3-6 months after the initial one) to get enough functioning islets to eliminate or reduce the insulin injections. According to the 2010 annual report of the Collaborative Islets

Transplant Registry (CITR), about 60% of transplant recipients achieved insulin independence during the year following transplantation, 50% by the end of the second year and only 24% after five years *(Appel, 2009; Appel et al., 2004; Ryan et al., 2005)*. Unfortunately, this therapy has major disadvantages which are not only the side effects of the immunosuppressive treatment for preventing islets rejections (not in the case of pancreatic islets auto-transplantation), but also and much more important, it is the shortage of islets availability since most islets-transplanted patients requires islets from two or more donors to achieve euglycemia. Moreover, pancreas digestion is a very aggressive method which leads to an enormous islets mortality pre- and post-transplantation. To circumvent the problem of pancreatic islets allo-transplantation, main sources are single-donor islets transplants *(Shapiro, 2011)*, xenotransplants of porcine islets *(Abouaish et al., 2011; Cooper and Casu, 2009)*, encapsulated islets to avoid the antigen recognition and protect islets from immune system *(Calafiore et al., 2006a; Calafiore et al., 2006b)* and new immunological strategies to preserve islets from toxic immunosuppression such as Efalizumab (a blocking monoclonal antibody directed to the most important integrins on lymphocytes LFA1-*(Berney et al., 2003)*-); $CD34^+$ cells; anti-CD3-specific antibodies (it shows slow the progression to permanent diabetes in humans with recent-onset diabetes-*(Keymeulen et al., 2005)*-); antithymoglobulin *(Lopez et al., 2006)* and the regulatory and effector T-cells *(Zheng et al., 2003)*. Despite progress in immune system knowledge, no effective immunotherapy has been identified at the moment.

C. Gene therapy for islets transplantation is a novel and powerful strategy to modify islets for a favorable outcome of the islets survival post-transplantation. Gene therapy employ viral vectors capable of transferring useful genes, modulate gene expression and inhibition of endogenous genes for the improvement of islet survival, islet function, block apoptosis and confer local immunoregulation post-transplantation. This approach consists in the *ex vivo* transduction of isolated islets with gene transfer vectors prior to transplantation *(Hughes et al., 2010; Levine, 1997) (Wong et al., 2010)*. Gene therapy includes two different methods for gene transfer to pancreatic islets: 1) nonviral-mediated gene transfer and 2) viral-mediated gene transfer methods.

C.1. Non viral-mediated gene transfer methods such as calcium phosphatase, co-precipitation, lipofection, direct-microinjection, electroporation, biolistics and protein transduction *(Bartlett et al., 1998; Bilbao et al., 2002; Kenmochi et al., 1998; Lakey et al., 2001; Mahato et al., 2003; Narang et al., 2005; Noguchi and Matsumoto, 2006; Rodriguez Rilo et al., 1998; Saldeen et al., 1996)*. Non-viral vectors offer high clinical safety, no immunogenicity and easy production. Nevertheless, several disadvantages are demonstrated including low efficiency in islets transduction and transient gene expression *(Benhamou et al., 1997; Lakey et al., 2001; Saldeen et al., 1996)*.

C.2. Viral-mediated gene transfer methods include four major types of viral vectors: 1) adenovirus (adV); 2) adeno-associated virus (AAV); 3) Herpex simplex virus and 4) lentivirus whose main features are summarized in Table 3.

Table 3: Viral vectors commonly used for islet gene therapy. *Table adapted from (Hughes et al., 2010).*

Viral vector type	Adenovirus (AdV)	Adeno-Associated Virus (AAV)	Herpes Simplex Virus	Lentivirus
Duration of Expression	Transient	Stable, Long-term	Stable, Long-term	Stable, Long-term
Immunogenecity	High	Low	Low	Low
Repeated dosing	Not possible	Possible	Possible	Possible
Clinical Trials	Yes	Yes	Yes	Yes
Advantages	Infection of both dividing and non-dividing cells Transient expression (high short time of expression) High titer and viral stocks	Infection of both dividing and non-dividing cells Integration into host genome Long-term expression *in vivo* Minimal immune response High viral titers	Large genome Non-pathogenic Unable to reactivate broad host range Long-term expression	Infection of both dividing and non-dividing cells Integration into host genome Long-term expression Reduced immunity
Disadvantages	Immunogenic Cause mild respiratory disease in humans	Requires helper virus Slow expression onset Inefficient large-scale virus production Small genome limiting the virus packaging capacity	Potentially provoks antiviral responses	Safety concerns Production inefficient

Transduction efficiencies of viral vectors are quite low, approximately 7-30% of intact islets cells are transduced in adenoviral infections *(Barbu et al., 2006; Kvell et al., 2005; Takahashi et al., 2007)* and 3-50% in lentiviral transductions *(Chou and Sytwu, 2009; Fenjves et al., 2008; Gallichan et al., 1998; Giannoukakis et al., 1999; Kobinger et al., 2004; Leibowitz et al., 1999)*. Recent studies reported increased infection rates of up to 30-90% throughout the whole islet *(Barbu et al., 2006; Kvell et al., 2005; Takahashi et al., 2007)*. Nevertheless, these transduction protocols used an enormous virus dosage leading to cytotoxic side effects on islets and are hardly reproducible *(Barbu et al., 2005; Rajalingam et al., 2001)*. Unfortunately, in most protocols, successful transduction of cells within islets only occur at the periphery of the islets, meanwhile cells residing in the core of islets are difficult to transduce. For optimal gene modulation by gene therapy prior to intact islets transplantation is necessary the future development of 1) efficient and reliable easy-to-use transduction protocol; 2) transduction of high percentage of whole islet cells, including cells residing in the islet core; 3) long-term expression of the inserted genes and 4) absence of immunogenicity post-transplantation.

4.2.2. Stem cell-derived Insulin-producing Cells:

The lack of donors and/or the necessity of new sources of islets have led to the development of strategies for the generation of an unlimited supply of new human β-cells for therapeutic replacement. This therapy has gained significant excitement in the

last two decades (Figure 15). Several sources have been considered for the *in vitro* generation of bioengineered insulin-producing cells including pancreatic stem cells (PSCs) *(Jiang and Morahan, 2012; Ramiya et al., 2000)*, bone marrow mesenchymal stem cells (MSCs) *(Aguayo-Mazzucato and Bonner-Weir, 2010; Ianus et al., 2003)*, pluripotent embryonic stem cells (ESCs) *(Thompson, 2001)* and induced pluripotent stem cells (iPSCs) *(Lowry et al., 2008; Nakagawa et al., 2008; Yu et al., 2007)*. Remarkable features of ESC and iPSC are that they possess the capacity to differentiate into the three embryonic layers (endoderm, mesoderm and ectoderm) and, finally, into all functional cell types of the body (including β-cells) and, in addition, they proliferate indefinitely and at high speed. The differentiation protocols to produce insulin-producing cells are based in the sequenced recapitulation of the developmental events driving cells from foregut endoderm into fully mature β-cells *in vitro*. Several differentiation protocols have been developed with high efficiencies guiding hESCs though the key pancreatic developmental steps from pluripotent cells into definitive endoderm, foregut, pancreatic endoderm, endocrine progenitor and finally into pancreatic endocrine cells by treatment with a sequential cocktail of growth factors (Activin A-*(D'Amour et al., 2006)*-, betacellulin and nicotinamide-*(Cho et al., 2008)* -,retinoic acid-*(Shim et al., 2007)*-sodium butirate-*(Jiang et al., 2007a)*-,bFGF-*(Jiang et al., 2007b)*-), and bioactive small molecules (IDE1/2-*(Borowiak et al., 2009)*-, stauprimide-*(Zhu et al., 2009)*-, WS6-*(Shen et al., 2013)*-, BRD7552-*(Yuan et al., 2013)*-*(Hou et al., 2013)*) resulting cells with a sustained expression of the important transcription factors Pdx1, NeuroD1, Ngn3, MafA or Isl1, and positives for insulin and C-peptide detection *(D'Amour et al., 2005; D'Amour et al., 2006; Jiang et al., 2007b; Kroon et al., 2008; Rezania et al., 2012; Shim et al., 2007)*. These cells can differentiate into mature and functional β-cells within 3-4 months once transplanted into the renal capsule of rodents *(Kroon et al., 2008; Rezania et al., 2012)*. Unfortunately, stem-cells entail considerable problems: 1) cells fails to release insulin appropriately in response to various concentrations of glucose *(D'Amour et al., 2006)*; 2) cells presents very low insulin content; 3) for still unknown reasons, these cells fail to express as time passes some key β-cell-specific genes, including Pdx1, Nkx6.1,MafA, Pax4, Glut2 and GCK *(Xie et al., 2013b)*; and 4) cells were a heterogeneous polyhormonal population of mixed phenotypes including cells with double-positive for insulin and glucagon or insulin and somastotatin suggesting an improper endocrine specification *(D'Amour et al., 2006)* and undifferentiated cells which could give rise to teratomas after implantation (Figure 14) *(Fujikawa et al., 2005)*. Importantly, Melton and colleagues have recently developed a promising protocol to generate the called stem-cell-derived β-cells (SC-β) which express mature β-cells factors, flux Ca^{2+} in response to glucose and enough quantity of insulin that respond in a glucose-regulated manner in transplanted mice where ameliorates hyperglycemia *(Pagliuca et al., 2014)*.

For future *in vitro* experimentation to improve the generation of insulin-producing cells, alternative approach must be validated. One strategy would be to

discover the complete and proper signals to specify β-cells fate taking in account that human pancreas development displays unique features that differ to the mouse embryonic process *(Richardson et al., 1997)*. A second strategy would be to identify a "three-dimensional environment" where novel culture conditions are necessary to maintain the nature islets niche composed by the extracellular matrix and the adjacent endothelial and mesenchymal cells. Moreover, mature β-cells express non-coding RNAs (microRNAs and long non-coding RNAs), which modulate their functionality *(Moran et al., 2012)*. In that direction, some methodologies wager for the creation of physiological competent β-cells from ESC/iPS-derived pancreatic progenitor via viral-, plasmid- or RNA-based expression of missing genes *(Okita et al., 2010; Warren et al., 2010; Xie et al., 2013a)*. Other recent alternative procedures are the artificial induction of pluripotency in human somatic cells though enforced viral expression of OCT4, SOX2, c-Myc and KLF4 transcription factors *(Pandian and Sugiyama, 2012)* or the innovative therapy know as patient-derived iPS cells (DiPS) which consist in iPS cells derived from the skin fibroblast of the patient with T1DM with the advantages that can be reprogrammed to secrete insulin in a dose-dependent manner and overcoming the immune rejection *(Dominguez-Bendala et al., 2012; Dominguez-Bendala and Ricordi, 2012; Maehr et al., 2009; Minami and Seino, 2013; Naujok et al., 2011; Pagliuca and Melton, 2013; Pagliuca et al., 2014; Pandian et al., 2014; Rezania et al., 2014)*. Interestingly, last year, a first patient was successfully implanted with the embryonic stem cell-derived islet replacement product, VC-01™. ViaCyte's VC-01 delivers pancreatic precursor cells (called PEC-01™ cells) that are designed to further differentiate and mature after surgical implantation into fully functioning insulin-producing β-cells and in other pancreatic endocrine cell types *(www.viacyte.com)*.

Transplanted cell type	Jiang et.al. Non-enriched INSULIN+	Phillips et.al. Non-enriched INSULIN+	Shim et.al. Non-enriched PDX1+	Eshpeter et.al. Non-enriched INSULIN+	Kroon et.al. Non-enriched PDX1+	Mao et.al. Non-enriched INSULIN+	Kelly et.al. Enriched progenitor	Kelly et.al. Enriched endocrine	Rezania et.al. Non-enriched INSULIN+	Basford et.al. Enriched INSULIN+	Rezania et.al. Non-enriched progenitor	Schulz et.al. Non-enriched PDX1+
Polyhormonal cells *in vitro*	NA	NA	✓	✓	✓	✓	✓	✓	✓	✓	✓	NA
In vitro GSIS	NA	NA	NA	NA	NA	✗	NA	NA	✗	✗	NA	NA
In vivo GSIS	NA	✗	NA	NA	✓	NA	NA	NA	NA	NA	✓	✓
Amelioration of diabetes	✓ 30%	✗	✓	✗	✓	✓ If fasted	✓	✗	✗	✗	✓	✓

Figure 14: Characteristics of hESC-derived pancreatic cells that have been differentiated toward insulin-secreting cells. *Figure from (Schiesser and Wells, 2014).*

4.2.3. Pancreatic β-cells Regeneration:

Due to the obstacles of the therapies previously exposed, recent strategies have focused on developing innovative approaches based on islet-cells plasticity. Currently, potential sources of new β-cells include: A) β-cell **neogenesis** from exocrine progenitors (facultative stem cells located among the ductal, acinar o centroacinar cells); B) **Transdifferentiation** from other mature cells (α-cells and acinar cells) and C) β-cell regeneration by **replication** of pre-existing β-cells. These processes involve the

coordinated expression of key transcription factors which operate at various developmental stages (Figure 15).

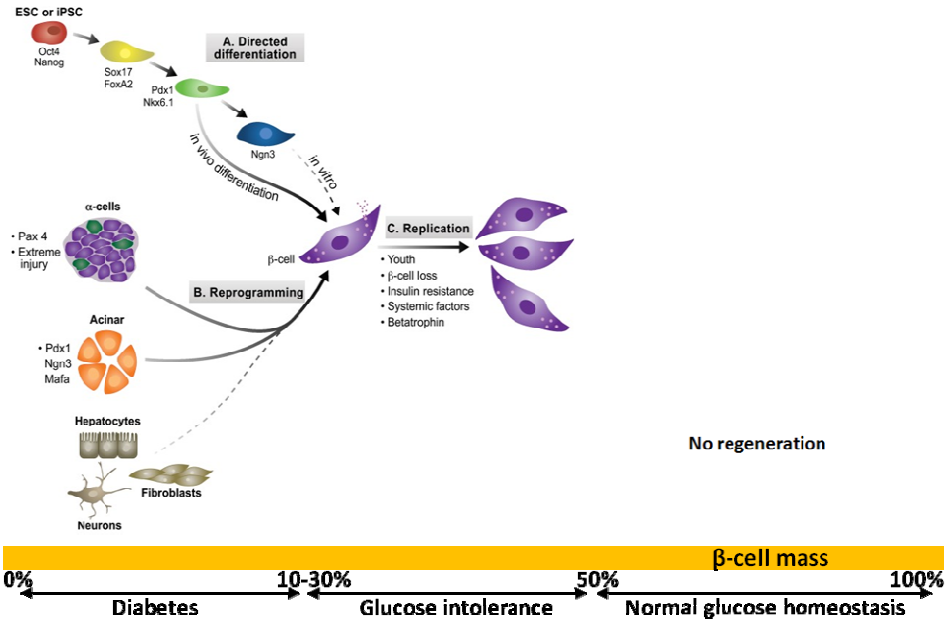

Figure 15: Strategies to develop new sources for β-cell regeneration. *Figure modified from (Pagliuca and Melton, 2013).*

A. Neogenesis consists in the generation of β-cells through a process that recapitulates development. It is reported the putative existence of undifferentiated endocrine progenitor located within the adult pancreas, but their presence is still controversial *(Bonner-Weir, 2000b; Xiao et al., 2013b; Ziv et al., 2013)*. Reinforcing this hypothesis, a source of new insulin-positive cells could be precursor cells located in the proximity of pancreatic ductal epithelium after pancreatic injury (PPY) and pancreatic duct ligation (PDL) *(Bonner-Weir et al., 2010)*. This cells are thought to be able to reactive the endocrine program. In addition, an increase in ductal cell proliferation and duct-associated insulin positive cells are observed in obese and diabetic humans *(Butler et al., 2010b)*. It is supported by the presence of mature islets located close to the ducts in adult pancreas *(Bouwens and Pipeleers, 1998)*. The mechanism involved in neogenesis remains unresolved highlighting the importance of discovering the function of genes that define the pancreatic progenitors. One of the most important genes in this process for human and rodents is Ngn3 *(Cano et al., 2014; McKinnon and Docherty, 2001; Stoffers et al., 1997b)*. Using a PDL protocol, a population of cells that form islets-like structures presents a huge induction of Nng3 and a variety of precursors markers such as Pdx1, Ptf1a, Sox9 and Hnf6 *(Xu et al., 2008)*. Furthermore, a recent Ptf1a lineage tracing study remark the existence of Ptf1a-positive acinar cells that transdifferentiate into Ngn3-positive cells after PDL and, finally, differentiate into new endocrine cells *(Pan et al., 2013)*. However, other investigators fail to identify new

β-cells from ductal cells following PDL method *(Rankin et al., 2013; Solar et al., 2009; Xiao et al., 2013a; Xiao et al., 2013c)*.

B. Transdifferentiation is the conversion of existing mature pancreatic cells into β-cells. This process could be: 1) acinar to β-cell reprogramming, 2) α-cell to β-cell reprogramming or/and 3) δ-cell to β-cell reprogramming.

B.1. Acinar to β-cell reprogramming: direct conversion of mouse acinar cells to β-cells *in vivo* has been done (Figure 15) through viral expression of three transcription factors, Ngn3, Pdx1 and MafA with an efficiency of 25% of infected cells. These insulin-positive cells also co-express key markers of β-cells such as Nkx6.1, Glut2 and GCK and, indeed, improve glycemic control of diabetic mice *(Zhou et al., 2008)*. Nevertheless, only these factors are not enough to reprogram other cells types (fibroblast, skeletal muscle, liver). Recently, a successful progression in this approach has been made through the addition of Pax4 in combination with the other genes previously described and the supplementation of the culture medium with compounds including betacellulin, exedin-4 and nicotinamide *(Lima et al., 2013)*. For the potential therapeuthical application, this reprogramming approach has to be safe and reproducible in humans.

B.2. α-cell to β-cell reprogramming: endocrine lineages of pancreas are closely related. The functional similarity, the shared ancestry and gene expression programs of the different endocrine cell types and, specially, among α- and β-cells, might make the interconversion between them a powerful source for generating new β-cells. In the murine α- to β-cell trasdifferentiation process (Figure 15), the transcription factor Pax4 acts as a master regulator playing a crucial role. Ectopic expression of Pax4 in α-cells drives their conversion into β-cells *in vivo* leading to an amelioration of hyperglycemia *(Collombat and Mansouri, 2009; Collombat et al., 2009)*. It is interesting to note that a recent study linked Pax4 in the conversion of α- to β-cells by pancreatic duct-lining precursor cells that re-express the developmental gene Ngn3 adopting a β-like cell identity. This process results in a 10 times increase of islets size after 20 months *(Al-Hasani et al., 2013)*. Conversely, loss of Pax4 in favor of the expression of α-cell-specific transcription factor Arx in β-cells leads to a concomitant increase in the number of α- or PP-cell population *(Collombat et al., 2007; Sosa-Pineda et al., 1997)* highlighting the possibility that forced expression of Pax4 and/or ablation of Arx could forced the reprogramming program through transdifferentiation to β-cells. Using a transgenic mouse model of α-lineage tracing system, after a 99% of β-cells ablation using the diphtheria toxic receptor, resulted in a regeneration of insulin-producing cells from mature α-cells and, these cells expressed the β-cells markers Pdx1, Nkx6.1 and, importantly, insulin *(Nir et al., 2007; Thorel et al., 2010)*. Worth to mention that recently the transcription factor Nkx6.1 has been established as β-cell reprogramming factor since it has been able to define α- to β-cell fate *(Schaffer et al., 2013)*. In this regard, next generation sequencing and RNA profiling studies of the epigenetic landscape of human α- and β-cells identified that α-

cells harbor bivalent chromatin signatures in genes crucial for β-cells, such as Pdx1 and MafA suggesting that this cell type might possess a plastic capacity to be reprogrammed into a β-cells *(Bramswig et al., 2013)*. As a conclusion, understanding of molecular mechanism as well as genes involved in these processes could help to induce the transdifferentiation therapy as potential DM treatment.

B.3. δ-cell to β-cell reprogramming: recently, it has been described a new mechanism for diabetes recovery by the restoration of insulin-producing cells due to the spontaneous en masse reprogramming of somatostatin-producing δ-cells after injury during early postnatal life. This transdifferentiation event is produced by the the combined action of the transcription factor FoxO1 and downstream effectors *(Chera et al., 2014)*.

C. Replication of pre-existing β-cells: in 2004, Dor et al. published study using mice engineered to express a tamoxifen-dependent Cre recombinase specifically within β-cells**.** They reported that proliferation of pre-existing β-cells, localized by alkaline phosphatase staining, is the major mechanism regulating β-cell regeneration in adult rodents *(Dor et al., 2004)*. The β-cell self-replication has been confirmed by a transgenic model for β-cell ablation combined with a double *in vivo* thymidine analogue-labelling strategy *(Nir et al., 2007; Xiao et al., 2013a)* and by an innovative DNA double-labeling approach to trace the new insulin-producing cells origin *(Teta et al., 2007)*.

Evidences that support the endogenous generation of new and more β-cells are that T1DM patients (approximately 16% of them) islets still retain some remnant β-cells *(Scholin et al., 2004)*. Furthermore, several studies have suggested that islet-cells maintain an intrinsic replication capacity, although it decline over human and mice aging since proliferation rates vary from 2.5-3% in fetal β-cells to less than 0.2-0.5% in old stages *(Cano et al., 2008; Kushner, 2013; Perl et al., 2010; Stolovich-Rain et al., 2012; Teta et al., 2005)*.

Dynamic changes in β-cell mass are also observed in challenging metabolic demands such as pregnancy and insulin resistance associated to obesity indicating that β-cells retain a certain compensatory capacity (Figure 15).

An adaptative increase in β-cell mass has been linked to obesity in both human and rodents *(Bonner-Weir, 2000a; Kloppel et al., 1985; Rahier et al., 2008)*. In humans, an increment of 30-60% in β-cell mass of nondiabetic obese individuals has been reported *(Meier et al., 2008; Saisho et al., 2013)* and a β-cell mass greater than double of normal in mice after 20 weeks feed on a high-fat diet *(Terauchi et al., 2007)*. In addition, glucose itself induces β-cells replication in mice and humans via metabolism by glucokinase *(Bonner-Weir et al., 1989; Porat et al., 2011)*. Mice haploinsufficient for glucokinase, which main function is serve as glucose sensor, show a decreased in β-cells number *(Terauchi et al., 2007)*. By contrast, the glucokinase mutation V91L in humans that leads to increase the affinity of GCK to glucose, results in large islets *(Kassem et al., 2010)*. Changes in β-cell mass in response to insulin resistance have

been explained thanks to the generation of liver insulin receptor knockout (LIRKO) mice used to investigate the role of insulin signaling in hepatocytes. Lack of insulin receptor of hepatocytes resulted not only in an increase of insulin resistance, but also in an augmentation of six-fold of β-cells mass *(Michael et al., 2000; Okada et al., 2007)*. Moreover, *in vitro* experiments the use of LIRKO-derived serum in cultures of human islets was sufficient to regulate β-cell proliferation upon insulin resistance conditions *(El Ouaamari et al., 2013)*. Subsequently, betatrophin has been identified as a β-cell-specific growth factor that improves hyperglycemia due to insulin resistance *(Yi et al., 2013)*.

During pregnancy, mothers maintain a nutrient flow to the developing fetus creating a physiological state of insulin resistance. To prevent maternal hyperglycemia, it is trigger a β-cell mass expansion by increasing proliferation regulated by the action of prolactin (Prl) and placental lactogen (PL) hormones as key promoters of these adaptative changes in β-cell growth in mice *(Sorenson and Brelje, 1997)* and humans *(Butler et al., 2010a; Parsons et al., 1992; Van Assche et al., 1978)*. Interestingly, β-cell replication, and subsequently, β-cell mass increase during the first two-thirds of gestation in mice reaching a peak around day 14 and day 18, respectively (Figure 16) *(Rieck et al., 2009)*. Furthermore, transgenic mice in which murine placental lactogen (mPL-I) is targeted to β-cell using the rat insulin II promoter (RIP-mPL-I) display a marked increase in islet mass, even though these changes are associated with hypoglycemia *(Vasavada et al., 2000)*. Prolactin and placental lactogen may act via Prolactin-receptor (Prlr) through the Jak2/Stat5 pathway *(Rieck and Kaestner, 2010)*. Indeed, Prlr-deficient mice present a 30% reduction of β-cell mass and islets density since the neonatal phase revealing a key function of Prl signaling during embryogenesis and postnatal period *(Auffret et al., 2013; Freemark et al., 2002)*. Other studies have suggested that lactogens act via the action of FoXM1 which repress the transcriptional regulator Menin and, consequently, the cyclin-dependent kinase inhibitors p18 and p27 and, finally, leading to cell cycle progression *(Karnik et al., 2007; Zhang et al., 2010)*. However, the exact molecular mechanisms by which lactogenic hormones are involved in this process are poorly understood. In this context, a recent study has demonstrated that the neurotransmitter serotonin acts downstream of lactogen signaling pathway to stimulate β-cell proliferation *(Kim et al., 2010b)*. Lactogenic signaling increased the transcription of serotonin synthetic enzyme tryptophan hydroxylase-1 (Tph1) which leads the conversion of tryptophan to serotonin (5-HT) through the tryptophan hydroxylase activity and it is secreted from the β-cell along with insulin. Once released, serotonin then activates the Gα(q)-linked serotonin receptor 5-hydroxytryptamine receptor-2b (Htr2b) on β-cells surface. Htr2b expression is increased in maternal islets during pregnancy and normalized just before parturition correlating with the period of increased β-cell replication and expansion of β-cell mass *(Kim et al., 2010b)*. Corroborating this hypothesis, transcriptome analysis performed at different mice gestation days throughout pregnancy highlight the up-regulation of the

key factors that correlates with the peak of β-cell proliferation and/or β-cell mass expansion including Tph1/2, Prlr and Pax8, and potential cell cycle regulators such as Fbwx15, Fbxl17, Fbxl21 and Fbxo27 *(Rieck et al., 2009)*. Elegantly, Georgia and colleagues propose that the activation of Prlr increases the expression of serotonergic genes (Tph1 and Htr2b) and FoxM1 and decreases Men1 levels leading to β-cells mass expansion *(Georgia and Bhushan, 2010).*

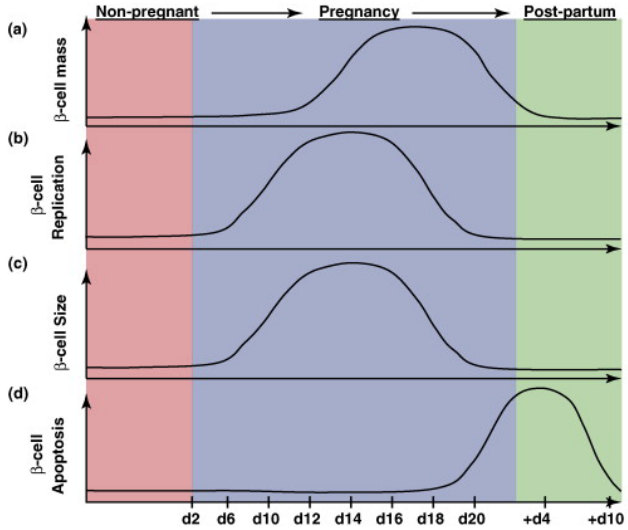

Figure 16: Dynamic changes in β-cell mass during pregnancy in mouse. *Figure from (Rieck et al., 2009).*

It is interesting to note that screening for small-molecule effectors that modulate replication could be an interesting strategy to enhance β-cell replication. Treatment with exedin-4 *(Xu et al., 1999a)*, glucagon-like peptide 1 (Glp1), gastric inhibitor polypeptide (GIP) *(Meloni et al., 2013; Stoffers et al., 2000)* and WS6 *(Shen et al., 2013)* in mice improve β-cell survival and β-cell mass expansion.

The major goal to develop efficient and powerful anti-diabetic therapies is the identification of targets that allows the therapeutical β-cell stimulation by not only enhancing β-cell mass, but also protecting them from death.

5. PAX GENES FAMILY: KEY GENES FOR β-CELL REGENERATION

Paired box (**Pax**) genes encode a family of transcription factors that contain a highly conserved DNA-binding domain called the Paired Domain (PD), whereby this family is named. Nine Pax genes (Pax1-Pax9) have been identified in mammals which have been evolutionary conserved among orthologs present in worms, flies, frogs, fish and birds sharing a common ancestral *(Vorobyov and Horst, 2006)*. Pax gene family members have been subclassified into four groups (I-IV) based on their genomic structure according to the presence or absence of an octapeptide region and the presence, absence or truncation of a homeodomain (Table 4) *(Blake et al., 2008; Blake*

and Ziman, 2014; Chi and Epstein, 2002; Lang et al., 2007; Li and Eccles, 2012; Mahajan et al., 2014; Robson et al., 2006).

Table 4: Pax gene family: description of structure, expression during embryogenesis and in adult tissue and their association with cancers *(modified from (Li and Eccles, 2012) and (Robson et al., 2006))*.

The defining feature of all Pax proteins is the presence of the **paired domain** (PD), a 128 amino acids in length DNA-binding motif with two structural subdomains, PAI and RED (PAI+RED= PAIRED) *(Jun and Desplan, 1996)*. The PAI domain (N-terminal) interacts directly with the DNA, meanwhile the RED domain does not have a direct interaction to DNA, although it contributes to the overall binding interactions of the PD. Seven of the Pax genes contain a second highly conserved 60-amino acid DNA-binding motif found in all Hox gene products named **homeodomain** (HD). Members of subgroup III (Pax3 and Pax7) and IV (Pax4 and Pax6) possesses a complete homeodomain, meanwhile the subgroup II (Pax2, Pax5 and Pax8) only carry a truncated homeodomain. An additional conserved domain found in all Pax proteins (except Pax4 and Pax6) is the 8-amino acid **octapeptide** (OC) motif which is located between the PD and the HD and serves as a binding motif of protein co-factors for inhibition of downstream gene transcription *(Eberhard et al., 2000)*. Finally, all Pax members include a transactivation domain located in the C-terminal amino region (Figure 17) *(Underhill, 2012)* that differs between the different family members. It is

important to highlight that all Pax genes (except Pax4, Pax5 and Pax9) can produce alternative splice variants that differ in their structure and, subsequently, in the binding activity of the PD and HD motifs modifying the transcriptional activity of downstream genes *(Blake and Ziman, 2014; Lang et al., 2007; Underhill, 2012)*. Moreover, evidences suggest both DNA-binding motifs could also function as protein-protein interaction domains *(Underhill and Gros, 1997)*.

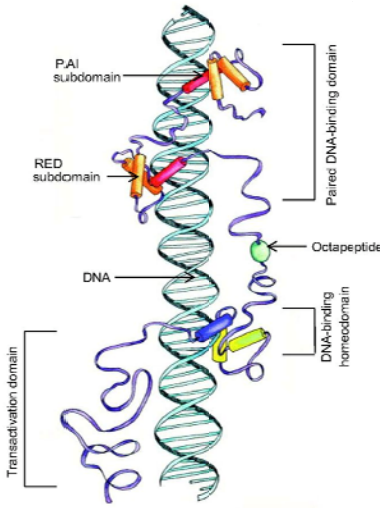

Figure 17: DNA structure of Pax family genes. *Figure from (Blake and Ziman, 2014).*

Pax genes play important functions as master regulators of multiple tissue/organ development and cellular differentiation in embryos promoting cell proliferation, cell-lineage specification, migration and survival *(Robson et al., 2006)*. Expression and role of Pax genes during development has been studied by Pax loss-of-function mutations causing potent morphogenetic phenotypes in animal models and genetic diseases in humans that, generally, results in prenatal death or soon after birth *(Chi and Epstein, 2002; Wang et al., 2008b)*. However, after embryogenesis, expression of most Pax genes attenuates *(Dahl et al., 1997)*. Interestingly, Pax genes expression either continues or their re-expression is possible in some adult tissues. Pax expression in adult tissues is often required for adult stem cell maintenance and/or tissue regeneration under patophysiological circumstances to maintain the tissue homeostasis. Furthermore, Pax genes expression is involved with a wide variety of cancer types that originate from tissues that require the expression of these genes during development or in homeostasis (Table 3) *(Li and Eccles, 2012; Robson et al., 2006)*. It is interesting to note that subgroups I (Pax1 and Pax9) and IV (Pax4 and Pax6) are less often linked with cancer. Indeed, Pax6 and Pax9 are related with a favorable outcome, and the first one is associated with a tumor-suppression function. This may be explained by their structural organization, since members of subgroups II and III which contains OC and HD are related with more aggressive cancers and, moreover, some of them are associated with chromosomal translocations in specific tumors (Pax3/Pax7-FKHR; Pax8-PPARγ and Pax5-IGH). By contrast, members of subgroups I and IV only possess one of these domains and, thereby, linked to a better cancer

prognosis. Notwithstanding, Pax4 expression is associated with tumorigenesis and development of human insulinomas *(Brun et al., 2007; Miyamoto et al., 2001)* and in primary lymphomas and hematologic malignancies *(Li et al., 2006)*. Pax6 is presented in pancreatic adenocarcinomas participating actively in cancer progression *(Lang et al., 2007; Mascarenhas et al., 2009)*. Due to the characteristics explained above, Pax genes could be useful tumor markers *(Robson et al., 2006)*.

5.1. TRANSCRIPTION FACTOR PAX4: A STAR IN β-CELL PLASTICITY DURING DEVELOPMENT AND ADULTHOOD

The transcription factor Pax4 plays a crucial function during embryonic development underlying the specification towards the endocrine commitment and, specifically, to β-cell fate. Moreover, in recent times, the contribution of Pax4 during adulthood in human, mouse and rat islets has reached a great importance pinpointing as an excellent candidate for β-cell regeneration by protecting this cell type from apoptosis and promoting proliferation *(Brun et al., 2004; Brun and Gauthier, 2008)* with the aim to be an advancement of current treatments for DM patients. Accordingly, and notwithstanding its low endogenous levels *(Bonnavion et al., 2013; Dorrell et al., 2011; Smith et al., 1999; Smith et al., 2000; Sosa-Pineda et al., 1997)*, it might be hypothesize that under physiological or patophysiological conditions in which β-cell mass needs to be compensated due an increasing insulin demand such as pregnancy and obesity, Pax4 could acts as an adaptative factor that permits β-cell regeneration; and, in contrast, mutations and polymorphisms that weaken Pax4 activity would contribute to reduction of β-cell survival and/or proliferation leading to apoptosis and the concomitant loss of insulin-producing cells causing insulin deficiency, hyperglycemia and, finally, diabetes.

5.1.1. PAX4: Master Regulator of Pancreatic Islets Development

During development of mouse embryos pancreas, Pax4 expression is initially detected at E9.5 of development in a few cells of the dorsal pancreatic bud. Around E11.0, Pax4-expressing cells were also detected in the ventral pancreatic bud *(Sosa-Pineda et al., 1997)*. The population of Pax4-positive cells increased during the following days of embryogenesis becoming maximal at E13.5-15.5. This peak of Pax4 expression coincides with a period of massive proliferation and endocrine differentiation including the confinement of this Pax4 expression exclusively in the endocrine cell of islets, specifically, in β-cells indicating that Pax4 is critical for sustaining the phenotype as well as the proliferation and/or survival of the insulin-producing cells *(Greenwood et al., 2007; Pictet et al., 1972; Sosa-Pineda et al., 1997)*. At these stages, Pax4 is colocalized with other endocrine markers such as Ngn3, Isl1, Nkx2.2, Pax6, insulin and glucagon suggesting that the onset of Pax4 expression occur concomitant with, or shortly after endocrine specification *(Smith et al., 2003; Smith et al., 2000; Sosa-Pineda et al., 1997; Wang et al., 2004)*. Thereafter, Pax4 levels are

declining exponentially towards the end of embryonic development, being restricted to β-cells. In adult pancreatic human and rodent islets, very low expression levels of Pax4 have been detected *(Brun et al., 2004; Brun and Gauthier, 2008; Dohrmann et al., 2000; Kojima et al., 2003; Smith et al., 1999; Theis et al., 2004; Wang et al., 2004; Zalzman et al., 2003)*.

In a first study, Pax4 was inactivated by homologous recombination in mice. Pax4-nullizygous newborn died 3-5 days after birth as a result of a completely deficiency of pancreatic β- and δ-cells, duodenal endocrine cells and serotonin cells of the stomach *(Larsson, 1998; Sosa-Pineda et al., 1997)* and the missexpression of early developmental markers of insulin-producing β-cells such as Pdx1, Hlxb9 and MafA *(Sosa-Pineda, 2004; Wang et al., 2004)*. Interestingly, Pax4-deficient mice showed a significantly expansion of glucagon- and ghrelin-producing cells suggesting that the lack of Pax4 expression forced endocrine progenitor to adopt an α-cells fate rather than a β- and δ-cell program *(Dohrmann et al., 2000; Prado et al., 2004; Sosa-Pineda et al., 1997; Wang et al., 2008a)*. In addition, by irreversibly tagging the progeny of cells taking advantage of Cre/LoxP technology, transgenic mice conditionally and ectopically expressed Pax4 in glucagon-producing cells being detected by β-galactosidase staining that allows lineage-tracing experiments *(Herrera, 2000)*.

5.1.1.1. Regulation of PAX4 Expression

5.1.1.1.1. PAX4 Transcriptional Regulation:

Human and murine Pax4 exhibit similar transcription initiation sites for Pax4 RNA *(Smith et al., 2000)*. Moreover, a fragment containing approximately 2 kb of the upstream region of mouse Pax4 contains all necessary elements and it is sufficient to drive its pancreas-specific expression. This region displays an equivalency by 88% in comparison with the human Pax4 sequence *(Brink et al., 2001; Brink and Gruss, 2003; Smith et al., 2000)*. Analysis of this region reveals binding sites for master transcription factor during pancreas development such as Hnf4α, Hnf1α, Pdx1 and NeuroD1 RNA. Interestingly, mutations in these genes result in MODY disease *(Brink et al., 2001; Smith et al., 2000)*. Similarly, Pax4 gene promoter region (4 kb upstream of the transcription initiation site of Pax4) contains several binding sites for Pax4 itself suggesting a strong negative autorregulatory effect *(Smith et al., 2000)*. Additionally, regulation of Pax4 expression appears to require the synergic interaction between Ngn3 and Hnf1α and requires the activation domains of both factors *(Courtney et al., 2013; Smith et al., 2003)*.

5.1.1.1.2. PAX4 Transcriptional Targets:

Pax4 and Pax6, subgroup IV members, could bind to a pancreatic islet cell enhancer sequence (PISCES) which is present in the glucagon, insulin, somastotatin and ghrelin promoters *(Wang et al., 2008b)* and this interaction is necessary for the regulation of these genes. However, Pax4 binding has lower affinity than Pax6 *(Collombat et al., 2005; Fujitani et al., 1999; Petersen et al., 2000; Sander et al., 1997;*

Smith et al., 1999; Wang et al., 2008b). This could be explained by possible post-translational modifications (phosphorylation, sumoylation and ubiquitination among others) or the interaction with other proteins that could modify its DNA-binding properties *(Epstein et al., 1994)*. In order to elucidate these interactions, in a study using a rat glucagon-producing cell line, exogenous overexpression of Pax4 reduced the endogenous expression of glucagon gene and hampered the activity of a glucagon-promoter-controlled reported gene *(Petersen et al., 2000)*. Moreover, Pax4 also inhibit an insulin-promoter-controlled reported gene in the absence of Pax6, but not in the cotransfection with both factors *(Campbell et al., 1999; Petersen et al., 2000)*. Another study support the fact that Pax4 binds with high affinity to Pax6 target sites of glucagon promoter suggesting an inhibitory competition between them *(Ritz-Laser et al., 2002)*. This is sustained by the circumstance that a strong repressor domain in the carboxy-terminal region of Pax4 has been described *(Fujitani et al., 1999; Smith et al., 1999)*. Taken together these data, one hypothesis could be that Pax4 might inhibit the glucagon transcription by competing with Pax6 for DNA binding sites in the glucagon promoter. In turn, this allows Pax6 to bind to the insulin promoter achieving the insulin expression in β-cell precursors *(Fujitani et al., 1999; Sander et al., 1997; Smith et al., 1999)*.

5.1.2. PAX4 as Master Regulator of Mature Pancreatic Islets: Coordinator of β-cell Plasticity

5.1.2.1. Genetic Studies Linking PAX4 with the Development of Diabetes:

A distinctive attribute of Pax4 is that several genetic studies have highlighted mutations and polymorphisms in this gene associated with both T1DM and T2DM in several ethnic populations that underline the importance of Pax4 on islet physiology.

Mutations in PAX4 gene have been identified in the codon located in the position C397T (**R133W**) just after the paired domain and in the codon C109T (**R37W**) in the amino-terminal end of the paired domain. These mutations predispose West African population to a ketosis-prone diabetes (rare form of T2DM) being the second mutation, R37W, more severe than the first one, R133W. Patients harboring these mutations present altered insulin secretion during insulin tolerance test and *in vitro* studies using a luciferase assay show a reduced repression of target genes *(Mauvais-Jarvis et al., 2004)*. In addition, two haplotypes at the Pax4 locus have been associated with T1DM in Scandinavian families *(Holm et al., 2004)*. Consistent with the latter, an independent study has identified a single nucleotide polymorphism in exon 9 of PAX4, **A1168C**, linked to T1DM in German and Swiss population. Interestingly, isoform PAX4-C was frequent in T1DM children (73%) and rare in the control group (32%) *(Biason-Lauber et al., 2005)*. However, this polymorphism does not present correlation with T1DM in Finnish, Hungarian and UK population *(Hermann et al., 2005; Martin et al., 2006)*. Discrepancies between these studies most likely arise from genetic heterogeneity and gene-environment interactions. Later, two more mutations in Pax4,

at codon 164 (**C492T/R164W**) placed in the homeodomain and in the nucleotide 1 at the intron 7 G/A (**IVS7-1G>A**), have been linked to MODY in the Thai population *(Plengvidhya et al., 2007)*. More recently, a novel heterozygous 39-base deletion in exon 3 that results in a frameshift in PAX4 gene (**c.374-412 del39**) has been described in a Japanese family with MODY. In this mutant Pax4, a part of the homeodomain critical for binding to the target genes is lacked, losing the transcriptional repressor function *(Jo et al., 2011)*. Moreover, the PAX4 **R192H** polymorphism generates a protein with defect in transcriptional repressor activities on its targets genes which lead to β-cell dysfunction related to impaired glucose-induced insulin secretion in MODY and in early onset-age T2DM patients *(Kooptiwut et al., 2012; Plengvidhya et al., 2007)*. This mutation has been considered to be associated with MODY9 subtype *(OMIM, 2014; Plengvidhya et al., 2007).* In the same context, a meta-analysis of GWAS for early onset-age T2DM in Southern Han Chinese and European populations has characterized a novel diabetes-associated locus, **rs10229583**, located downstream of PAX4 *(Ma et al., 2013)*.

Of particular interest is the homozygous missense mutation **R121W** found in a T2DM Japanese population *(Shimajiri et al., 2001)*. This mutation is located in the arginine codon 121 (R129 in mouse Pax4) of exon 3 of the carboxy-terminal end of the paired domain. This region is highly conserved among Pax family genes, since its contact directly with the major groove formed by the DNA consensus sequence *(Xu et al., 1999b)*. Consequently, R121W mutation may affect severely the PAX4 transcription activity by inhibiting its DNA binding capacity. The mutant allele frequency is 2% among T2DM Japanese population. However, an independent study in Okinawan population increased this value to 6.2% *(Shimajiri et al., 2003)* suggesting that this mutation is commonly distributed in T2DM Japanese patients. Homozygous R121W patients suffer an early onset of diabetes and severe defects in first-phase insulin secretion requiring insulin dependency, without autoimmune-mediated process *(Shimajiri et al., 2001; Shimajiri et al., 2003; Tokuyama et al., 2006)*. Furthermore, R121W mutant Pax4 lacked the inhibitory effect on Pax6 transcriptional activity on the glucagon-promoter-controlled reported gene *(Shimajiri et al., 2001)*. In order to estimate the DNA-binding activity of the mutation, EMSA analysis was performed using the R129W mouse mutant Pax4, equivalent to R121W human mutant PAX4, due to the low binding of the human protein to the probe. This experiment confirmed a decreased DNA binding property of Pax4R129W to the binding sites of the rat glucagon promoter G3 element *(Shimajiri et al., 2001)*. In conclusion, the mutation PAX4R121W is a fascinating objective to further analysis in order to determine the role of Pax4 in expansion and survival of β-cell mass. An extensive work is required to elucidate the impact of human PAX4R121W/mouse Pax4R129W mutation in adult β-cell plasticity.

5.1.2.2. Studies Highlight the Role of PAX4 in Adult β-cell Plasticity:

5.1.2.2.1. *In vitro* Studies of Pax4 Role in Mature Pancreatic Islets:

In vitro evidences for the implication of Pax4 in β-cell plasticity in response to physiological situations has been provided in studies performed with the proinflammatory cytokine interleukin 1β (IL-1β). It has been shown a dose-dependent relationship between IL-1β and Pax4 expression. On one hand, low concentrations of IL-1β induced both endogenous Pax4 expression and β-cell proliferation being beneficial on islets mass in human islets. On the other hand, high levels of this cytokine suppressed Pax4 expression and induced apoptosis becoming detrimental *(Maedler et al., 2006)*. This phenomenon is mimicking in T1DM and T2DM in which a gradual loss of β-cell mass happens by autoinmmunity *(Donath, 2004)*. According with the previous data, Pax4 seems to be an integrator of the Fas/Flip signal transduction pathway, in which cell proliferation or death are determined by the expression levels of IL-1β *(Donath, 2004)*. Contributing to the idea of Pax4 as an important factor for β-cell plasticity in adulthood and a mediator of the cytokines signaling pathway, the cytokine ciliary neurotrophic factor also significantly increased Pax4 mRNA levels promoting the neonatal rat islets survival through the anti-apoptotic gene Bcl2 *(Rezende et al., 2007)*. This sustains the premise that Pax4 is a key mediator of cytokine-signaling pathway and for β-cell plasticity in adult islets.

Demonstration of Pax4 as a key regulator of β-cell mass adaptation has been provided by *in vitro* experiments in which the Pax4 transcription levels were induced by the mitogens activin A and betacellulin in adult rat islets with a concomitant three-fold increase in β-cell replication *(Brun et al., 2004; Ueda, 2000)*. Wortmanin (a PI_3 kinase inhibitor) suppressed betacellulin-induced Pax4 expression, implicating the phosphatidylinositol 3-kinase (PI_3) signaling pathway. Furthermore, overexpression of murine Pax4 by a doxycycline-inducible adenoviral system in mature rat and human islets conferred protection against cytokine-mediated apoptosis and detonate β-cell replication. Pax4 overexpression in rat islets results in more than 3.5-fold increase in β-cell proliferation by induction of the c-myc/Id2 proliferative pathway and the anti-apoptotic gene Bcl-xL *(Brun et al., 2004; Cheung et al., 2004; Lu et al., 2007; Pelengaris et al., 2002)*. Nevertheless, the diabetes-linked R129W (human R121W) mutant variant was found to be less efficient showing an attenuate protection against cytokine-induced cell death in rat and human islets. Therefore, this study proposed that by modulation of apoptosis through Bcl-xL expression and proliferation via c-myc levels, Pax4 may regulate the total population of β-cells and, ultimately, islet mass *(Brun et al., 2004)*.

Once the potential beneficial effect of Pax4 on β-cell replication and survival has been established in rodent islets, a similar positive outcome was also demonstrated in human islets *(Brun et al., 2008)*. Pancreatic islets derived from T2DM donors with a BMI between 22 and 26 presented elevated levels of PAX4 transcripts in comparison with control non-diabetic donors. Although islets of T2DM donors with

BMI greater than 26 exhibited PAX4 levels indistinguishable to those of non-diabetic islets. These results demonstrate that hyperglycemia is an important inducer of PAX4 expression and potentially of cell replication in diabetic islets *(Brun et al., 2008; Gunton et al., 2005)*. However, prolonged lipotoxicity may repress hyperglycemia-induced PAX4 expression and, finally, attenuation of β-cell proliferation by inducing apoptosis *(Brun et al., 2008; Lupi et al., 2002; Maedler et al., 2001)*. To study in more detail this premise, adenoviral overexpression of mouse Pax4, but not human PAX4, in mature human islets in culture was capable of inducing cell replication and conferred protection against cytokine-mediated apoptosis. The failure of human PAX4 could be explained by EMSA studies that suggest a weaker interaction of human protein with its DNA binging motif when compared with the mouse protein. Moreover, alignment of mouse, rat and human Pax4 protein revealed a 50% divergence in the carboxy-terminal end of the human sequence where a repressor domain is located being less efficient in its trans-activation function *(Brun et al., 2008; Fujitani et al., 1999; Smith et al., 1999)*. Interestingly, a novel Pax4 variant lacking the carboxy-terminal end of the protein is presented at high levels in human insulinomas *(Miyamoto et al., 2001)*. Additionally, overexpression of human PAX4 resulted in a slight increase in Bcl-xL levels while Id2 expression and cell replication was unchanged. Similar to murine islets, activin A, betacellulin and the incretin GLP-1 (which effect seems to be glucose dependent) augment PAX4 mRNA levels in human islets, although this stimulation is lower than in rat islets suggesting that human islets are more resistant to growth factors. Astonishingly, addition of this mitogens or GLP-1 failed to induce replication in human islets. This study highlights fundamental differences between human and rodent Pax4 *(Brun et al., 2008)*.

Protective and proliferative effect of Pax4 must be demonstrated in an *in vivo* model mimicking the physiological conditions. Moreover, an *in vivo* physiological model would permit to evaluate the role of Pax4 in β-cell plasticity in a wide range of harmful situations such as chemical stroptozotocin- and/or experimental autoimmune-induced β-cell death models. In addition, an *in vivo* model of T2DM linked-R121W mutation would provide further information regarding the contribution of Pax4 in the diabetes etiology. New therapeutic strategies using Pax4 and identifying its downstream targets genes are necessary to improve islet cell survival and β-cell expansion in diabetic patients.

5.1.2.2.2. *In vivo* Studies of Pax4 Role in Mature Pancreatic Islets:

In regard to the *in vivo* function of Pax4 in mature pancreatic islets, this transcription factor displays a recently uncovered capacity to reprogram α-cells to β-cells. Forced Pax4 expression in adult α-cells one week following birth induces a continuous conversion into a mature β-cell phenotype with a concomitant reduction by 77% of glucagon-producing cells. Besides, it has been shown a dramatic increase in islet size in pancreas ectopically-expressing Pax4. Interestingly, 41% of these younger animal treated with streptozotocin (STZ) to induce diabetes survived after two months

post-STZ injection due to a progressive normalization of glycemia thanks to the functionality of new insulin-producing cells derived from the transdifferentiation processes *(Collombat and Mansouri, 2009; Collombat et al., 2009)*. Additionally, misexpression of Pax4 in glucagon(+) cells age-independently induces their conversion into β-like cells resulting in islet hypertrophy and islet neogenesis due to the continuous mobilization of pancreatic duct-lining precursor cells that re-express the developmental gene Ngn3. Importantly, these processes can repeatedly regenerate the whole β cell mass and, thereby, reverse several rounds of toxin-induced diabetes *(Courtney et al., 2013)*. Concerning with the endocrine cell subtype specification process, the transcription factors Pax4 and Arx antagonize each other´s expression during the course of pancreas morphogenesis and endocrine cell fate allocation to establish β- and α-phenotype, respectively. To gain insight into the potential mechanism by which Pax4 achieves reprogramming of α- to β-cells, several independent studies have been performed in mice:

1) Arx loss-of-function mutant mice by homologous recombination in embryonic stem cells using Cre/LoxP system *(Collombat et al., 2003)*;

2) Transgenic mouse models allowing either the constitutive inactivation of *Arx* in all glucagon-producing cells as soon as they initiate hormone expression or the inducible loss of *Arx* in α-cells at different ages *(Courtney et al., 2013)*;

3) Inducible, tamoxifen-dependent Cre/LoxP-based lineage tracing system where the diphtheria toxin receptor was specifically expressed in β-cells (RIP-DT) allowing the controlled ablation of approximately 99% of β-cells *(Thorel et al., 2010)*;

4) Combination of pancreatic duct ligation (PDL) coupled with elimination of pre-existing β-cells with alloxan treatment *(Chung et al., 2010; Chung and Levine, 2010)*.

These studies highlight that the neogenic β-cells arise primarily from mature α-cells. Additionally, the generation and analysis of Arx and Pax4 double-mutant mice has provided evidences that Pax4 is indispensable for β-cell regeneration, indicating that this gene represents the main trigger of α-cell mediated β-like cell neogenesis and it is sufficient to restore a functional β-cell mass in abscense of Arx *(Collombat et al., 2007; Courtney et al., 2013)*. Moreover, this approach suggests that Arx and Pax4 inhibit transcription of one another by direct interaction with their respective promoter regions leading to the proper reorganization of endocrine progenitor cells *(Collombat et al., 2007)*. In summary, the phenotypic alterations observed in pancreas of mice deficient in Pax4/Arx indicate that the activity of Pax4 is essential for the formation of mature β-cells. Nevertheless, we are focus on elucidating the intricate regulatory network and the key factors/signal driving the specific role played by Pax4 in pancreatic islets as target candidate for DM therapies.

5.1.3. PAX4 as Oncogene

Pax gene family members have been involved in cancer development. Nevertheless, subgroup IV (Pax4 and Pax6) is linked with cancer to a reduced degree *(Robson et al., 2006)*. Notwithstanding, human insulinomas show upregulation of a novel PAX4 splicing variant (PAX4v) generated by lack of exon 7 resulting in the replacements of the entire carboxy-terminal region with a 35 novel amino acids sequence, but does not affect the amino-terminal region of protein that contains the paired and homeodomain responsible of DNA binding *(Miyamoto et al., 2001)*. Furthermore, elevated expression of Pax4 levels has been found in rat insulinoma cell lines *(Brun et al., 2007)*. To address the potential oncogenic function of Pax4, this transcription factor has been repressed by RNA interference in the insulinoma INS-1E cell line that normally express high levels of this gene *(Brun et al., 2007; Li et al., 2006)*. Inhibition of Pax4 provoked apoptosis and further sensitized cells to cytokine-induced cell death through approximately 50% reduction of the anti-apoptotic gen Bcl-xL expression without altering cell proliferation suggesting that Pax4 acts a survival factor in this cell line *(Brun et al., 2007)*. Aberrant expression of Pax4 was also discovered in primary lymphoma and hematologic malignances caused by epigenetic modifications *(Li et al., 2006)* similar that those imposed on Pax4 gene in mature pancreatic islets *(Brun et al., 2007)*. In contrast, ectopic Pax4 expression acts as a tumor suppressor in melanomas decreasing cell growth of malignant cells *(Hata et al., 2008)*. As a conclusion, pending of the Pax4 regulation and the metabolic circumstances, this transcription factor may thus qualify as a potential oncogene joining other Pax family members involved in cancer *(Brun and Gauthier, 2008)*.

5.2. TRANSCRIPTION FACTOR PAX8: A RISING STAR IN β-CELL PLASTICITY

The transcription factor **Pax8** belongs to subgroup II of paired box (Pax) gene family together with two more members, Pax2 and Pax5. The defining feature is the presence of a truncated homeodomain, in addition to the paired domain and the octapeptide in its structure. A comparison of the mouse and human Pax8 coding sequences reveals two small deletions in the human PAX8 gene that do not change the reading frame. Indeed, the coding regions from both species show a 91.3% homology *(Plachov et al., 1990)*.

Pax8 gene is a master regulator of embryonic development of several organs, although it is also expressed during adulthood in specific glands. During adulthood, Pax8 contribution is related with normal mature-state maintenance and plays a principal role in cell survival and apoptosis, including tumorogenesis, in these organs where it is normally expressed, postulating that this transcription factor could possesses capacity to be used as diagnostic and, potentially, important prognostic marker of cancer.

5.2.1. PAX8 as Master Regulator in Development and Adulthood: Contribution to Disease

During fetal development, Pax8 is expressed in thyroid gland *(De Felice and Di Lauro, 2011; Tong et al., 2009)*; in the midbrain–hindbrain boundary in early central nervous system development *(Stoykova and Gruss, 1994)*; in the inner ear development; in epithelial cells of female and male genital tracts *(Mittag et al., 2007)* and the cells of the pronephric, mesonephroic and metanephric lineages of kidney formation *(Bouchard et al., 2002; Li and Eccles, 2012; Pfeffer et al., 1998; Plachov et al., 1990; Tong et al., 2009)*.

The first event in thyroid gland development is the induction of a specific gene expression pattern. The development of thyroid is orchestrated by Pax8 and four more genes, thyroid transcription factor-1 (TTF-1), thyroid transcription factor-2 (TTF-2), Nkx2.2 and Hex *(De Felice and Di Lauro, 2011; Lang et al., 2007)*. The earliest evidence in embryonic development of Pax8 expression is about E9-9.5 in mice and 22WG in humans *(De Felice and Di Lauro, 2004; Zannini et al., 1997)*. Indeed, Pax8 expression is maintained in follicular cells of adult thyroid gland where this transcription factor perform one of its more significant functions maintaining the biosynthesis of specific thyroid hormones, including the thyroglobulin (Tg), thyroperoxidase (TPO) and the sodium/iodine symporter (NIS), all of which are essential for thyroid hormone synthesis *(De Felice and Di Lauro, 2011; Plachov et al., 1990; Poleev et al., 1992; Tong et al., 2009; Zannini et al., 1997)*. This is sustained by studies with Pax8-knockout mice whose do not survive to weaning unless they were treated with the thyroid hormone T_4 (thyroxine) that allows the survival of these mice up to six months *(Christ et al., 2004; De Felice and Di Lauro, 2004; Friedrichsen et al., 2003; Friedrichsen et al., 2004; Mittag et al., 2009; Mittag et al., 2007; Santisteban and Bernal, 2005)*. In this context, Pax8 homozygous null mutant mice display normal kidney development, but they present congenitally smaller thyroids and absence of follicles in comparison with heterozygous mutant or wild-type littermates, and suffer from hypothyroidism and growth retardation *(Mansouri et al., 1998)*. In humans, mutations of PAX8 are associated with thyroid disorders such as congenital thyroid hypoplasia which is characterized by inadequate production of thyroid hormones *(Macchia et al., 1998; Missero et al., 1998; Vilain et al., 2001)* or absence of thyroid glands *(Mittag et al., 2005)*. Furthermore, PAX8 is detected in follicular cell-derived thyroid neoplasm *(Nonaka D, 2008)*. Most hypothyroidism cases are caused by mutated segments in the paired domain of Pax8 presented by a hypoplasia etiology easily treated with external sources of thyroid hormones *(Congdon et al., 2001; Missero et al., 1998)*.

Pax8 is also required for specification of nephric lineage specification and/or survival. In addition, Pax8 and Pax2 share an overlapping pattern of expression during murine embryogenesis *(Plachov et al., 1990)* such as demonstrated in Pax8-knockout mice with a background of heterozygous null Pax2 mutation in which a major loss, or

complete depletion, respectively, of the nephric cell lineage results through increased apoptotic cell death *(Bouchard et al., 2002; Narlis et al., 2007)*.

Pax8 is involved in the development of genital tracts. Mutations on this transcription factor cause infertility in male and female mice because of the malformation and obstruction of these reproductive organs *(Mittag et al., 2007; Wistuba et al., 2007)*.

In central nervous system (CNS) development, Pax8 displays an additional important role. Untreated Pax8-deficient animals exhibit growth and mental retardation as well as many neurological deficits such as ataxia and completely distorted cellular composition of anterior pituitary *(Bernal, 2005; Friedrichsen et al., 2004; Mittag et al., 2009; Mittag et al., 2007)*. Furthermore, a study suggests that the redundant function between Pax2 and Pax8 is essential for specifying GABAergic and glycinergic neuronal fates *(Batista and Lewis, 2008)*. Recently, positive nuclear immunoreactivity for Pax8 has been detected in renal extraneural hemangioblastoma. Hemangioblastoma is a benign, morphologically distinctive neoplasm of disputed histogenesis that typically occurs in the CNS. In the kidney, renal hemangioblastomas have demonstrated similar immunophentypic profiles to those of the CNS *(Zhao et al., 2013)*.

5.2.1.1. Regulation of PAX8 Expression

Pax8 is related to retinoblastoma (RB)-E2F1 pathway, which has a central role in regulating cell cycle. Silencing of PAX8 by RNA interference causes a reduction in E2F1 mRNA levels and in its target genes (including CCNA2 and CDC6) in cancer cell lines, as well as in the proteasome-dependent destabilization of RB protein levels which provokes the induction of G_1/S cell-cycle arrest and onset of cellular senescence *(Li et al., 2011)*. PAX8 transcriptionally regulates the E2F1 promoter directly recruiting RB for PAX8-binding site, thereby forming a negative feedback loop that, finally, upregulate the RB-E2F1 pathway and, therefore, growth of cancer cells *(Li et al., 2011; Park et al., 2006)*. Furthermore, other studies reported that PAX8 activate transcriptionally BCL2 and repress TP53 expression which contributes to tumor cell survival *(Hewitt et al., 1997; Stuart et al., 1995)*. PAX8 also regulate telomerase in colorectal and glioma cell lines proposing that this gene could be critical for maintenance of telomeres facilitating immortalization and survival of cancer cells *(Chen et al., 2008)*.

5.2.2. Evidences of PAX8 as a Novel Target Gene for Regulation of β-cell Plasticity in Diabetes

Surprisingly, Pax8 has recently emerged as a new member of Pax family involved in pancreatic islets physiology. Although previous reports have not detected Pax8 expression neither in developing pancreas or in mature pancreatic islets *(unpublished data from our group; (Goode and Elgar, 2009))*, new studies claimed strong PAX8 expression in normal human islets, as well as in pancreatic neuroendocrine tumors (PNETs) *(Haynes et al., 2011; Laury et al., 2011; Long et al.,*

2010; Ozcan et al., 2011; Sangoi et al., 2011). In the pancreas, the reported PAX8 expression is restricted to the endocrine pancreas *(Ozcan et al., 2011; Sangoi et al., 2011)*. Additionally, a genome-wide linkage and admixture mapping study has linked PAX8 as a T2DM potential candidate gene in African Americans families *(Elbein et al., 2009)*. Accordingly, transcriptome profile performed on isolated islets from pregnant mice revealed a robust induction of Pax8 at day 14.5 of gestation correlating with the peak of β-cell proliferation (Figure 16) *(Rieck and Kaestner, 2010; Rieck et al., 2009)*. In this sense, prolactin is one of the hormones that plays a crucial role regulating β-cell mass during pregnancy. Prolactin-receptor (Plrl) heterozygous mice exhibit a failure on β-cell adaptation caused by a lower number of β-cells, lower serum insulin and higher blood glucose levels *(Huang, 2013)*. These data corroborate the importance of intact prolactin receptor (Prlr) signaling for β-cell proliferation under these circumstances. Following this argument, important genes in Prlr signaling implicated in β-cell mass modulation by their role in regulation of β-cell proliferation such as Tph1/2 and Prlr appears up-regulated in transcriptome analysis at day 14.5 of gestation coinciding with the peak of β-cell replication *(Huang, 2013; Kim et al., 2010a; Kim et al., 2010b; Rieck et al., 2009; Schraenen et al., 2010a; Schraenen et al., 2010b)* (see epigraph *4.1.3.3.C. "Pancreatic β-cells regeneration: replication of pre-existing β-cells"*). Of note, prolactin possesses cytoprotective properties improving in 26% β-cell viability and survival in human islets and achieving normoglycemia in transplanted mice *(Yamamoto et al., 2008)*. Other interesting *in vitro* data reveals that recombinant human prolactin (rhPRL) has been shown to improve glucose-stimulated insulin secretion in cultured human pancreatic islet cells *(Labriola et al., 2007)* and may protect against apoptosis in these isolated human islets *(Terra et al., 2011)*. In this line, genetic variation in the PRLR gene could increase the risk of development of GDM in a Chilean population *(Le et al., 2013)*. Interestingly, GDM is associated with gestational thyroid dysfunction (GTD). GTD is defined as elevated thyroid stimulating hormone (TSH), decreased free tetraiodothyronine (FT_4) or both *(Karakosta et al., 2012; Tirosh et al., 2013)*, hormone synthesis where Pax8 plays a crucial role. Interestingly, tyroxine has been shown to improve T1DM complications *(Leong et al., 1999)* and enhances glucose-responsive insulin secretion through MafA as well as promote β-cell development and differentiation in rat pancreatic islets *(Aguayo-Mazzucato et al., 2013)*. Recently, a heterozygous novel PAX8 mutation was identified in a Portuguese family of Azores *(Carvalho et al., 2013)*. This polymorphism consists in the substitution of proline to arginine at codon 25 (P25R) located in the highly conserved paired domain. P25R-PAX8 mutation is responsible for a severe form of dominantly inherited congenital hypothyroidism. Interestingly, the only pregnant female member of this family harboring this Pax8 mutation developed GDM *(Dr. Anselmo, personal communication)*. Identification of this subject in combination with the previous studies indicating that Pax8 is induced during pregnancy strongly advocates for an important role of Pax8 in islets physiology.

This robust induction of Pax8 during pregnancy suggests a potential role of this gene in the adaptation to situations of higher metabolic demands characteristic of this metabolic state suggesting that this transcription factor could be involved in GDM etiology and/or development. Consequently, further investigations are required for the identification of Pax8 contribution to maintain β-cell mass and to determine its role in islets plasticity. Furthermore, intrinsic gene networks controlled by this gene in islets could illuminate potential novel strategies for development of therapies to treat GDM.

5.3.3. Pax8 as a Potential Oncogenic Marker

PAX8 has emerged as a potential diagnostic marker for most adult and paedriatic thyroid cancers (Wilms tumors) *(Lui et al., 2005; Mahajan et al., 2014; Scouten et al., 2004)*; ovarian carcinoma *(Bowen et al., 2007; Cheung et al., 2011; Schaner et al., 2003)*; renal cell carcinomas *(Li et al., 2011)*; breast carcinomas *(Nonaka et al., 2008)*; bladder cancer *(Laury et al., 2011; Pellizzari et al., 2006; Robson et al., 2006)* and pancreatic neuroendocrine tumors (PNETs) *(Haynes et al., 2011; Koo et al., 2012; Laury et al., 2011; Long et al., 2010; Ordonez, 2012; Ozcan et al., 2011; Sangoi et al., 2011; Xiang and Kong, 2013)*. Furthermore, PAX8 has been also detected in placental cancer *(Ferretti et al., 2005)*, colorectal cancer and glioma cell lines *(Chen et al., 2008)*. Nevertheless, little is known about the biological significance of PAX8 expression in cancer and how PAX8 could directly or indirectly influence in cell growth and survival.

Additionally, Pax8 is involved in a translocation mutation t(2;3)(q13;p25)that results in a fusion protein with peroxisome proliferator-activated receptor γ(PPARγ) to form the rearrangement PAX8-PPARγ oncogene. This fusion protein is expressed in both malignant and benign thyroid tumors, but it is appearance is significantly greater in follicular thyroid carcinoma seeming to confer resistance to apoptosis to tumoral cell and increase cell cycle transit *(Powell et al., 2003)*. Indeed, PAX8-PPARγ is presented in 20-53% of follicular thyroid carcinomas and 55% of follicular thyroid adenomas *(Castro et al., 2006; Cheung et al., 2003; Kroll et al., 2000; Lacroix et al., 2004; Lui et al., 2005; Marques et al., 2002)*.

In recent years, several clinical studies have recognized PAX8 expression in pancreatic neuroendocrine tumors (PNETs) with a diagnostic utility *(Haynes et al., 2011; Koo et al., 2012; Laury et al., 2011; Long et al., 2010; Ordonez, 2012; Ozcan et al., 2011; Sangoi et al., 2011; Xiang and Kong, 2013)*. One of the most challenging areas in PNETs biology is the identification of specific biomarkers that can morphologically and histochemically distinghish well-differentiated pancreatic PNETs (WDPNETs) from other types of well-differentiated neuroendocrine tumors (WDNETs) leading to support a better prognosis and treatment, as well as to determine the origin of secondary metastatic neoplasms. It is highly relevant to determine the origin of WDNETs due to the effectiveness of antineoplasmic agents *(Garcia-Carbonero et al.,*

2010; Garcia-Carbonero et al., 2014; Garcia-Carbonero et al., 2011). PAX8 expression was studied in WDNETs resulting in PAX8-positive nuclear detection in 35-67% of primary and metastatic PNETs and 50% in liver metastases from primary PNETs, whereas no cases of ileal, pulmonary, duodenal and rectal neuroendocrine tumor metastases where Pax8-positive *(Haynes et al., 2011; Laury et al., 2011; Long et al., 2010; Ordonez, 2012; Ozcan et al., 2011; Sangoi et al., 2011)*. Of note, PAX8 expression can distinguish PNETs from acinar cell carcinoma, but exists a weak overlap with solid-pseudopapillary neoplasm *(Sangoi et al., 2011)* and with poorly differentiated neuroendocrine carcinoma *(Haynes et al., 2011)*. Moreover, they established a correlation between PAX8 expression and WHO category. Benign behavior tumors (WHO 1.1) showed PAX8 detection and, PAX8-positive tumors were significantly smaller than PAX8-negative tumors *(Long et al., 2010)*.However, later studies suggested that PAX8 expression could not be used as biomarker of aggressiveness in PNETs *(Sangoi et al., 2011)*. Furthermore, clinical investigators have proposed a flowchart in which Pax8 was included in a panel of inmunohistochemical staining profile to determine the origin of WDNETs *(Sangoi et al., 2011)*. These investigators concluded that selective expression of Pax8 could be a useful marker to determine the primary origin site of metastatic well-differentiated PNETs from others anatomic sites *(Haynes et al., 2011; Laury et al., 2011; Long et al., 2010; Ordonez, 2012; Ozcan et al., 2011; Sangoi et al., 2011)*.Interestingly, it is noteworthy highlight that all previous clinical studies have been used the same polyclonal antibody to demonstrate PAX8 staining in PNETs.

For future work, it is essential the validation of the specificity of antibodies before embarking into large-scale inmunohistochemistry studies. In this regard, further investigations are necessary to determine the specificity and sensitivity of Pax8 for diagnostic application as a reliable biomarker for primary and metastatic PNETs.

CHAPTER II_OBJECTIVES

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Diabetes Mellitus causes destruction of β-cells, which decreases the potential of insulin secretion and, as a consequence, the normal control of glycemia. In view of the pandemic magnitude that this illness has reached, it is of great need to develop innovative therapeutic strategies to preserve or restore the functional β-cell mass in diabetic patients. This concept is known as *in vivo* regeneration. Therefore, the definitive therapeutic approach for the treatment of DM should aim to: 1) block the destruction of β-cells, 2) replace or regenerate lost β-cells and 3) preserve the mass and functionality of the β-cells. We propose to investigate the physiological function and the underlying molecular mechanisms regulated by the transcription factors Pax4 and Pax8 as potential key players for innovative regenerative therapies that lead to the development of effective treatments to DM.

Pax4) To investigate the **role of Pax4 in adult β-cell plasticity *in vivo***, our group generated two transgenic animal models that conditionally expressed either the mouse Pax4 or the diabetes-linked variant Pax4R129W specifically in β-cells. These animals were:

- challenged with STZ, a chemical tool to induce β-cell destruction.
- cross with a mouse model of T1DM, the RIPB7.1 mouse model of experimental autoimmune diabetes (EAD).

These conditionally expressing Pax4 animal models under the previous experimental conditions will allow us to:

1. Unravel Pax4 and Pax4R129W function in β-cell expansion, protection and/or survival in adult murine islets.
2. Determine the contribution of Pax4R129W variant in the development of DM in humans.
3. Decipher the molecular networks by which Pax4 and/or Pax4R129W might contribute in β-cell plasticity.

Pax8) In order to elucidate the **functional role of Pax8 in pancreatic islets physiology** and characterize a potential novel regulator of islet plasticity, we propose to study the Pax8 expression pattern in islets during development and post-natal maturation:

1. Establish Pax8 expression pattern during development and its mature function in mouse pancreatic islets.
2. Determine Pax8 expression profile during pregnancy in mouse and human islets and its correlation with gestational β-cell mass expansion.
3. Evaluate Pax8 detection in well-defined pancreatic neuroendocrine tumors and its application as a clinical biomarker.

4. Decipher the genes and molecular networks enriched by Pax8 overexpression. In order to achieve this aim, we perform:

    4.1. Generation of a lentiviral vector that allow Pax8 overexpression in mouse and human islets.

    4.2. Development of a reliable transduction protocol to infect intact mouse and human pancreatic islet.

CHAPTER III_MATERIALS AND METHODS

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1. HUMAN ISLETS:

Human islets were kindly provided by Dr.D. Bosco (The Cell Isolation and Transplantation Centre, Department of Surgery, Geneva, Switzerland) and by Prof. F. Pattou (Cell Therapy for Diabetes-CHRU de Lille, Lille, France) under JDRF grant.

2. ANIMAL STUDIES:

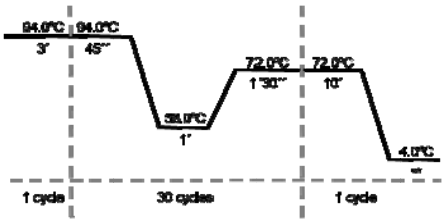
C57BL/6J genetic background was employed for the generation of the inducible Pax4 mice or the mutant variant Pax4R129W, we used the pIRES2-DsRed-Express (Clontech; #632540) backbone vector. The final construct contained the tetracycline responsive promoter, the rabbit β-globinintron followed by the Pax4 or Pax4R129W coding sequence. Myc-epitope and polyhistidine tags were added for detection purposes. DsRedexpress was included in the constructs in order to follow Pax4 induction using noninvasive in vivo imaging. Pax4 transgenic animals were crossed to RIPrtTA mice to generate double transgenic descendants with conditional expression of Pax4 or Pax4R129W specifically in β-cells *(Hu He et al., 2011b)*.

In order to create the quadruple transgenic BPTL mice in the Dr. Gauthier group, Pax4-rtTA and PAx4R129W-rtTA were crossed with MIP-LUC-VU animals (available in our laboratory) to generate PTL triple transgenic mice. The RIP-B7.1 animal model of experimental autoimmmune diabetes (EAD) *(Karges et al., 2002)* was then mated to PTL mice to derive BPTL quadruple transgenic mice used in our studies.

Induction of Pax4 transgene expression or the diabetes-linked variant Pax4R129W was achieved by providing 1g/L of doxycycline (Sigma-Aldrich, #D9891) in the drinking water. Bottles were protected from light exposure to prevent inactivation of the doxycycline. Drinking water was changed three times per week. Doxycycline was administrated after weaning for 1 month and continued for the duration of the experiment. Only females were used for our experiments. Animals were housed in a pathogen-free facility and kept under controlled environmental conditions (12 h-light–dark cycle, 23 ± 1 °C with 30–50% relative humidity). Food and water was ad libitum. Animal experiments were performed in accordance with CABIMER animal committee approved protocol.

Mouse genotyping was performed by PCR in a 0.4mm tail sample obtained at day 10 after birth. For extraction and amplification of genomic DNA from mouse tailsthe REDExtract-N-AmpTM Tissue PCR Kit (Sigma-Aldrich; #031M6112) was used. PCR was performed with the specific primers outlined in Table5:

Table 5: Primer sequences used for mice genotyping.

Genotype	Primers	PCR conditions	Product size
Pax4	Fw: 5'-ATAAATTCTGGCTGGCGTGG-3' Rv: 3'-CACAGCTGGTGATCTGAGTT-5'		600bp
RIP-rtTA	Fw: 5'-AGAGCTGCTTAATGAGGTCG-3' Rv: 5'-GCGACTTGATGCTCTTGATC-3'		400bp
B.7	Fw: 5'-CAAACAACAGCCTTACCTT-3' Rv: 5'-GCCTCCAAAACCTACACATCCT-3'		300bp
MIP-LUC	Fw: 5'-GGATCTCTGGCATGCGAG-3' Rv: 5'-TTGTTTCCAAAAAGGGGTTG-3'		300bp

For pregnancy studies, three to five-months-old female C57BL/6J mice were purchased from Janvier Laboratories (Saint Berthevin, Cedex, France). Mating of animals was conducted overnight in a ratio of 1 mal e: 3 females. Successful mating was confirmed by the presence of a vaginal mucous plug the following morning. Once females were pregnant, males were removed. Pax8 analysis was performed in either non-pregnant females as control or pregnant female mice at d10.5, d14.5, d16.5 and d18.5 of gestation. Glucose levels and weight were measured weekly.

2.1. Islets source:

Mice were sacrificed by cervical dislocation and pancreatic islets were isolated by 0.9 mg/ml collagenase V (Roche, #C9263) digestion and handpicked as previously described *(Brun T, 2004).*

2.2. Islets culture:

Mouse islets were cultured in RPMI 1640 at 11.1 mM glucose (Sigma-Aldrich; #R0883) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich; #F7524), 2 mM L-glutamine (Sigma-Aldrich; #G7513), 10 mM HEPES (Gibco; #15630-056), 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma-Aldrich; #P4333), 1 mM sodium pyruvate (Sigma-Aldrich; #S8636) and 50 µM β-mercaptoethanol (Gibco; #31350-10). Prior to culturing, mouse islets were washed with 1X phosphate buffered saline (PBS) (Sigma-Aldrich; #P3813) containing 100 U/ml penicillin and 100 µg/ml streptomycin to avoid and minimize post-isolation contaminations.

Human islets preparations were maintained in CMRL-1066 (Cellgro, #99-663-CV) supplemented with 10% FBS or 10% Charcoal stripped FBS (nearly free of endogenous steroids to avoid skewing of results) (PAA, #A11-119), 100 U/ml penicillin, 100 µg/ml streptomycin and 100 µg/ml gentamycin (Sigma-Aldrich; #G1397).

Pregnancy was reproduced in an *in vitro* model by incubating human islets with recombinant human prolactin (Sigma-Aldrich, #L4021), the main hormone produced during this metabolic period. Human islet equivalents (IEQs) were cultured in CMRL-1066 medium for 48 hours at 37ºC for removing traces of hormones. After medium was renewed, prolactin treatment was performed adding 200ng/ml prolactin daily up to 96 hours.

2.2.1. Thapsigargin treatment of mouse islets:

For thapsigargin-induced ER-stress and cell death determinations, Pax4-rtTA, Pax4R129W and control islets were initially treated or not with 1µg/L Dox for 96 h to induce Pax4 overexpression. Then, islets were incubated or not with 1µM thapsigargin for 48 hours. Apoptosis was then assessed using Cell Death Detection Elisa Plus Kit (Roche; #11544675001) (see section 3.9). Fluorescence correlating with Pax4 expression was monitored in groups of 10 islets plated on µ-Plate 96 welllibiTreat (IBIDI, Martinsried, DE) in a final volume of 150 µl complete RPMI medium. Images were captured using an ImageXpress microsystem (Molecular devices, Spain).

2.3. Streptozotocin (STZ) treatment:

Pax4-rtTA and control animals were challenged with a single or double high dose of STZ to destroy β-cells. STZ was prepared in 0.1 mM sodium citrate at pH 4.5 and administered by intraperitoneal injection (200 mg/kg body wt). Blood glucose was determined before STZ injection and then three times per week.

3. CELL CULTURE:

The mouse insulinoma β-cell line, MIN-6 *(Ishihara, 1993)* was cultured in Dulbecco's modified eagle medium (DMEM) at 25 mM glucose (Sigma-Aldrich; #5796) supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mм L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES and 50 µM β-mercaptoethanol, and was maintained at 37°C in a 5% CO_2 environment in Sarsted culture flask with filter caps (Sarsted; #831813002). Media was changed every 3 days and passages were performed twice per week (passage 20-70). To harvest cells for sample preparation or culture passage, cell monolayers at 85-90% confluence were detached by exposure to 1X trypsin (Gibco; #15400054) for 5 min at 37ºC.

The rat INS-1E insulinoma cell line (kindly provided by Claes Wollheim, (University of Geneva, Switzerland) wascultured in RPMI-1640 medium at 11.1 mM glucose supplemented with 10% (vol./vol.) heat-inactivated FBS, 10 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 1 mM sodium pyruvate and 50 µM β-mercaptoethanol. Cells were incubated at a humified (95%) atmosphere (5% CO_2) at 37°C in TPP tissue culture flasks with filter caps (TPP, #90076). When confluence was approximately 80-90%, culture was washed with PBS, trypsinized and incubated for 5min. Trypsinization was stopped by adding complete RPMI 1640 media. Passages were performed weekly and media were changed every 3 days. Passages 20-80 were used for our experiments.

Human Embryonic Kidney 293T cells and the cell line MCF7 were cultured in Dulbecco's modified eagle medium (DMEM) (25 mM glucose) supplemented with 10% heat-inactivated FBS, 100 units/ml penicillin, 100 µg/ml streptomycin and 2 mм L-glutamine. Cells were maintained at 37°C in a 5% CO_2 environment. Media was changed every 3 days and passages were performed three times per week using exclusively early passages (passages 8-20). To harvest cells for sample preparation or

culture passage, cell monolayers at 90-95% confluency were digested by exposure to 1X trypsin for 5 min at 37ºC.

4. BIOCHEMICHAL - MOLECULAR BIOLOGY ASSAYS:

4.1. RNA isolation:

Total RNA from at least 200 islets was extracted using the RNeasy Micro Kit (Qiagen; #74004) according to the manufacturer's instructions.

RNA from various cells lines (MIN6, INS-1E, 293T, MCF7) was extracted using the commercially available RNeasy mini kit (Qiagen) according to the manufacturer's instructions. An intermediate treatment with Turbo DNAse (Ambion, #AM2238) for 30 minutes at 37ºC was included to remove traces of DNA contamination.

RNA concentration was measured using a spectrophotometer (NanoDrop1000; Thermo Scientific). An Agilent 2100 Bioanalyser was used to determine the RNA integrity number (RIN). The degradation level was estimatedby visualization of the 18S and 28S ribosomal bands. RNA samples with 260/280 ratio in the range of 2.0 to 2.2 and RIN 7-10 were selected for further analysis.

4.2. Complementary DNA synthesis:

One µg total RNA was converted into cDNA using radom hexamers (Roche, #11277081001) and Superscript II Reverse transcriptase (Invitrogen; #18064-014) *(Gauthier BR, 1999)*. Then, the mixture was heat at 65 ºC for 5 min, 25 ºC for 10 min, and 42 º C for 50 min and, finally, 70 º C for 15 min.

4.3. QT-PCR:

QT-PCRs were performed as described previously *(Gauthier et al., 2004)*. The PCR reaction within the exponential phase of the amplification curve was performed for 50 cycles. Primers were designed using the Primer 3 software (Centro of Excellence in Genomics, University of Tartu, Estonia). Several primers have been designed and the best one for each gene has been selected after validation. In addition, several primer concentrations have been evaluated. Primer sequences synthesized by Sigma-Aldrich are listed in Table 6. Real time PCR was performed using an ABI 7000 Sequence Detection System (Applera Europe) and PCR products were quantified fluorometrically using the FastStart Universal SYBR Green Master (Roche Diagnostics, # 04913850001). Amplifications were performed in duplicate for each sample, and normalized to the reference mRNAs of cyclophylin and β-actin.

Table 6: Primer sequences used for QT-PCR.

Gene	Sequences
mouse Pax8	Fw: 5´-GTTTGAGCGGCAGCATTAC-3´ Rv: 5´-GTAAGGGCAGTGGGTACAGC-3´
human PAX8	Fw: 5´-CCCCCTACTCCTCCTACAGC-3´ Rv: 5´-GGCCTTGATGTGGAACTGTAA-3´
mouse Tph1	Fw: 5´-TGTTGACTGCGACATCAGCCGA -3´ Rv: 5´-GGAAACCAAGGGACAGTCTCCA-3´
human TPH1	Fw: 5´-TTCTGACCTGGACCATTGTGCC-3´ Rv: 5´- ACGGTAGACATTGTCTTTGAAGCC-3´
mouse Pdx1	Fw: 5´- CGCGTCCAGCTCCCTTT-3´ Rv: 5´- CCTGCCCACTGGCCTTT-3´
human PDX1	Fw: 5´- TCTTGTCCTCCTCCTTTTTCCA-3´ Rv: 5´-GCTGGCTGTCATGTTGAACTT -3´
mouse Pax4	Fw: 5´-GAAGACACGGTGAGGGTTTG -3´ Rv: 5´- TGCTTCCCATTTCAGCTTCT-3´
mouse Pax6	Fw: 5´- CAACCTGGCTAGCGAAAAGC-3´ Rv: 5´-TCTGCCCGTTCAACATCCTT -3´
mouse Cdkn1a (p21Cip)	Fw: 5'-AATCCTGGTGATGTCCGACC-3' Rv: 5'-TTGCAGAAGACCAATCTGCG-3'
mouse Cdk4	Fw: 5'-TATGAACCCGTGGCTGAAAT-3' Rv: 5'-CCTTGATGTCCCGATCAGTT-3'
mouse NOS2	Fw: 5´-GGGCTGTCACGGAGATCA-3´ Rv: 5´-CCATGATGGTCACATTCTGC-3´
mouse Cox2	Fw: 5´-CCAGCACTTCACCCATCAGTT-3´ Rv: 5´-ACCCAGGTCCTCGCTTATGA-3´
mouse Lgals9	Fw: 5'-TCAGTGCCCAGTCTCCATACA-3´ Rv: 5´- CTCCTTGGATTGGTCCAGTAAAG-3´
Mouse Calr	Fw: 5´-CCTGCCATCTATTTCAAAGAGCA-3´ Rv: 5´-GCATCTTGGCTTGTCTGCAA-3´
mouse c-myc	Fw: 5´-GCTGGAGATGATGACCGAGT-3´ Rv: 5´-AACCGCTCCACATACAGTCC-3´
mouse RPS29	Fw: 5´- CACGGTCTGATCCGCAAAT-3´ Rv: 5´- GCGTACTGCCGGAAGCA-3´
mouse β-actin	Fw: 5´-TCCTGTGGCATCCACGAAACTACA -3´ Rv: 5´- ACCAGACAGCACTGTGTTGGCATA-3´
mouseCyclophylin A	Fw: 5´-ATGGCAAATGCTGGACCAA -3´ Rv: 5´-GCCATCCAGCCATTCAGTCT -3´
human CYCLOPHYLIN A	Fw: 5´- TACGGGTCCTGGCATCTTGT-3´ Rv: 5´- CCATTTGTGTTGGGTCCAGC-3´

4.4. Transfection:

To introduce the specific DNA of different Pax genes (Pax4, Pax6 and Pax8) into various cells lines and control their expression, cells were plated at 3×10^5 cells/well into 6-well plates in a final volume of 2 ml (6×10^5 cells/well for MIN6) or 1×10^4 cells/well (2×10^4 cells/well for MIN6) into 24-well plates in a 0.5 ml total volume with complete medium under standard cell culture conditions (5% CO_2, 37°C) and were allowed to adhere for 24 hours. Cells were transfected at approximately 70-80% confluency. Culture medium was changed 2 hours after transfection for medium without antibiotics.

For transfection, Lipofectamine 2000 Reagent (Invitrogen) was diluted 4 times in serum-free medium Opti-MEM and incubated for 5 min at room temperature. In parallel, 4 µg of plasmid DNA was resuspended in OptiMeM according to the manufacturer´s protocol. Each transfection consisted of either the empty control plasmid pcDNA3.1 (from Dr. Benoit Gauthier group) or the expression plasmids for hPax4, hPax6 and h/mPax8-pCMV6-XL5 (TrueClone, Origene). Plasmid DNA-lipid complexes were prepared by mixing both solutions. After 25 min incubation at RT, DNA-lipid complexes were added directly to the cells cultured in absence of antibiotics. Six hours post transfection, medium was replaced with complete medium. Finally, cells

were incubated for 24 hours (48 hours in the case of MIN6 and INS-1E cell lines) at 37°C.

4.5. Protein isolation and Western Blot:

For protein isolation, cell lines at 90% confluence were washed and scraped in ice-cold PBS. After centrifugation at 1500 rpm for 5 min, cell pellets (from $3x10^5$ cells seeded) were lysated in Whole Cell Buffer, WCB (20mM Hepes pH 7.7, 300 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X100, 1X Protease Inhibitor Cocktail Tablets SigmaFast (Sigma-Aldrich, #S8830), 0.5mM DTT). Following, cells were incubated for 1 hour at 4°C with rotation and then centrifuged at maximum velocity for 5 min at 4°C. Protein content of the supernatant was determined using the Quick Start Bradford 1X Protein Assay Kit (Bio-Rad, #500-0205) at 562 nm according to the manufacturer's instructions.

For islets protein extraction, approximately 200 islets were harvested in WCB and proteins were isolated and quantified following the same procedure explained for cell lines.

Twenty to thirty micrograms of total protein was mixed with 20mM dithiothreitol (DTT) (Sigma-Aldrich, #43816) and 5X loading buffer (250 mM TrisHCl pH=6.8, 0.5% bromophenol blue, 5% SDS and 50% glycerol) to a final volume of 15ul, heated to 95°C for 3 min, chill on ice for 2 min and separated by 10% SDS-PAGE (4X Separating buffer (1.5M TrisHCl pH=8.8, 0,4% SDS), 30% (w/v) acrylamide/bis-acrylamide solution (Sigma-Aldrich, #A3574), 10% ammonium persulphate (APS) and N,N, N', N'-tetramethylethylenediamine (TEMED)) for 1 hour at 110 V. Afterwards, proteins were transferred by humid transference to a PVDF membrane with a pore size of 0.45 mm (GE healthcare) for 1 hour at 100 V using Towbin system buffer (25 mM Tris-HCl pH 8.3, 192 mM glycine, 20% (vol:vol) methanol).

After transfer, the membrane was blocked with 5% dried milk in TBST (NaCl 0.19 M, TRIS-HCl pH 7.5 0.05 M, 0.1% Tween 20) for 1 hour at room temperature and subsequently, incubated with corresponding primary antibodies listed in Table 7 diluted in 3% bovine serum albumin (BSA) in TBST overnight at 4ºC. After washing with TBST, the membranes were incubated with the corresponding fluorescence-HRP secondary antibody (Table 8) for 1 hour at room temperature. After washing, immunodetection was performed using the ECL Western Blotting detection reagent (GE Healthcare, # RPN2106) on KODAK BioMax light films (Sigma-Aldrich, # Z370398). β-Actin or α-Tubullin was used as a loading control.

4.6. Immunofluorescence techniques:

4.6.1. Immunofluorescence in culture cells and disaggregated islets cells: For immunofluorescence studies, approximately 50 islets per condition from either human or mouse origin were dissociated with trypsin for 5 min at 37ºC to obtain single-cell suspensions. Dissagregated islets or different cell lines preparations were seeded in 12mm diameter glass coverslips (Menzel-Glaser, #J1800AMNZ) treated with

poly-L-lysine (Sigma-Aldrich, #P4707) for 20 min at room temperature, at a density of $2x10^4$ cells per well in 24-mutiwell plates and cultured for 24 hours in complete medium to allow the adherence. Samples were then fixed in 4% paraformaldehyde (Sigma-Aldrich, #P6148) for 10 min at room temperature. Permeabilization was achieved in ice-cold methanol 100% for 20 min at -20ºC; after three washes with PBS/0.1% Tween-20, cells were exposed to specific primary antibodies (listed in Table 7) diluted in PBS containing 3% BSA (Sigma-Aldrich, #A3294) and 0.1% Tween-20 (Sigma-Aldrich, #P2287) for overnight (~20 hours) at 4 °C. Controls without primary antibodies were used for each experiment. Afterwards, to remove excess antibody, coverslips were then rinsed three times in PBS/0.1% Tween-20 for 5 min and incubated with the appropriate secondary antibodies conjugated with fluorophores (in PBS/ 0.1% Tween-20) (Table 8) for 60 min at room temperature in dark. Samples were then washed three times with PBS/0.1% Tween-20 for 5 min each. Visualization of nuclei was performed by staining with 1 µg/ml DAPI (Sigma-Aldrich, #D9542) for 5 min at room temperature. Coverslips were mounted using DAKO fluorescent mounting medium (Dako Diagnostics; #S3023) and left to dry overnight at 4°C.

Images were acquired at 40X on a Leica TCS SP5 (AOBS) confocal microscope (Leica Microsystems, Heerbrugg, Switzerland).

4.6.2. Immunohistochemistry in paraffin sections: mouse pancreas was dissected from adult mice and fixed in 4% paraformaldehyde overnight at 4°C. Dehydratation, embedding and sectioning were performed at the Hystology platform at CABIMER. Paraffin embedded tissue were sectioned into slices of 5 µm using a microtome Leica RM 2255(Leica Microsystems, Heerbrugg, Switzerland). Sections were mounted on SuperFrost Plus slides (Menzel-Glaser). Every 10 sections, one slide was stained with hematoxylin-eosin.

Sections were deparaffinized in xylene and rehydrated by immersion in decreasing concentrations of ethanol from 100% to 70%, followed by several washes in water (Xylene 5min/2x; Ethanol 100% 1min/2x; Ethanol 90% 1 min; Ethanol 80% 1 min; Ethanol 70% 1 min; water). After deparaffinization, sections were subjected to heat-induced antigen retrieval using 10 mM sodium citrate buffer (pH 6.0) and heating in the microwave on high power for 3x3 min or in autoclave (Selecta, 4001745) at 121ºC for 40 min depending on the antibody. Samples were cold down for 20 min at room temperature.

After washing twice with PBS and once with PBS + 0.5% Triton X-100 (Sigma-Aldrich, #T8787), blocking was performed with PBS + 0.1% Triton X-100 containing 3% BSA and 3% donkey serum (Sigma-Aldrich, #D9663) for 1 hour at room temperature. Primary antibodies (Table 7) were diluted in PBS + 0.1% Triton X-100 containing 3% BSA + 3% donkey serum and incubated overnight at 4ºC in a humid chamber. Subsequently, sections were washed twice with PBS and once with PBS + 0.2% Triton X-100 and incubated with corresponding secondary antibodies (Table 8) diluted in PBS + 0.1% Triton-100 for 1 hour at room temperature in dark. Nuclei counterstaining was

performed by staining with 1µg/ml DAPI for 5 min at room temperature. After wash three times with PBS for 5 min each, sections were mounted using DAKO fluorescent mounting medium and left to dry overnight at 4°C.

Pictures were acquired at 40X using briefly camera DFC 500 or DFC 390 FX for fluorescence on a confocal microscope (Leica Microsystems, Heerbrugg, Switzerland).

4.6.3. HRP (Horse Peroxidase Reaction) in paraffin sections: mouse and human tissues were processed as previously described for immunohistochemistry in paraffin sections. Paraffin embedded sections of pancreatic neuroendocrine tumors, as well as, human kidney and liver as controls from anonymous patients were provided for Drs. RocíoCarbonero and Lourdes Gómez from the Department of Oncology and Pathology, respectively, at Virgen del Rocío Hospital (Seville, Spain).

The endogenous peroxidase was blocked by use of 3% hydrogen peroxide in 100% methanol for 15 min. Sections were washed twice with PBS for 5 min and blocked in PBS supplemented with 3% BSA for 1 hour at room temperature. Thereafter, samples were incubated with the corresponding primary antibody (Table 7) diluted in the blocking solution overnight at 4ºC in a humid chamber. To remove excess antibody, sections were rinsed three times in PBS for 5 min and incubated with proper biotinylated IgG (Table 8) for 1 hour at room temperature. Sections were then washed twice with PBS for 5 min each. Visualization of the immunoreaction was performed by using Vectastain Elite ABC (Vector Laboratories, #PK-6100) y 3,3-diaminobenzidine, DAB Substrate (Vector Laboratories, #SK-4100) according with the manufacturer protocol. The color reaction was stopped by immersing the sections in PBS before they were rinsed twice in water for 5 min each. Dehydration of the tissues was subsequently performed by placing the slides in an ascending ethanol series (70%, 80%, 90%, 96%, 2×100% ethanol for 1 min each one) and through two changes of xylene for 5 min each. Section were counterstained with hematoxylin (Merk, #1.05174.0500) for 10 seconds and washed with water several times until obtained the desired colour. Sections were mounted with DPX (Sigma-Aldrich, #44581) and air-dried.

4.6.4. Islets embedding on paraffin: approximately 50 human or mouse islets were fixed in 10% Formalin (Panreac Appli. Chem.; #252931) for 48 hours at room temperature. Subsequently, islets were mixed with approximately 100µl of 150-300 µm diameter Affi-Gel blue beads (Bio-Rad, #153-7301) and included in warm (70°C) HistoGel (Thermo Scientific; #R904012). After cooling, histogel containing the islet-bead mixture was embedded in paraffin following the standard procedures of CABIMER Histology Core facility and sectioned in 5 µm thicknes sections. Sections were then mounted on SuperFrost Plus slides. Every 10 sections, one slide was stained with hematoxylin-eosin. Subsequently, immunochemistry studies were performed following the protocol described above.

Table 7: Primary antibodies used for Immunofluorescence and Western blot (IF: Immunofluorescence; HRP: Horse Peroxidase Reaction; WB: Western blot).

Primary antibodies	Dilution	Source	Technique IF/HRP/WB
Rabbit polyclonal anti-Pax8	1:500/1:500/1:1000	ProteinTech, #19120-1-AP	IF/HRP/WB
Mouse monoclonal anti-Pax8 (PAX8R1)	1:100/1:100/1:500	Abcam, #ab53490	IF/HRP/WB
Mouse monoclonal anti-Pax8 (BC12)	1:100/1:500	Abcam, #ab124445	IF/WB
Mouse monoclonal anti-Pax6	1:200/1:200/1:1000	DSHB	IF/HRP/WB
Rabbit polyclonal anti-myc Tag	1:100/1:1000	Cell Signaling, #2272	IF/WB
Mouse monoclonal anti-Pdx1	1:200	DSHB	IF
Mouse monoclonal anti-Ki67 (SP6)	1:200	Thermo-Scientific, #RM9106	IF
Rabbit polyclonal anti-MafA	1:200	Bethyl, #A300-611A	IF
Rabbit polyclonal anti-Glut2	1:200	Provided by C.Wright, Vanderbilt University	IF
Rabbit polyclonal anti-Ki67	1:200	Thermo-Scientific, #MA14520	IF
Rat monoclonal anti-BrdU [BU1/75 (ICR1)]	1:500	Abcam, #6326	IF
Mouse monoclonal anti-BrdU(Clone BU-33)	1:200	Sigma-Aldrich, #B8434	IF
Mouse monoclonal anti-Insulin	1:500	Sigma-Aldrich, #I2018	IF
Rabbit polyclonal anti-Insulin	1:200	Santa Cruz Biotechnology, #sc-9168	IF
Guinea pig polyclonal anti-Insulin	1:250	Abcam, #ab7842	IF
Mouse monoclonal anti-Glucagon	1:200	Sigma-Aldrich, #G2654	IF
Rabbit polyclonal anti-Glucagon	1:200	Cell Signaling, #2760	IF
Goat polyclonal anti-Somatostatin	1:200	Santa Cruz Biotechnology, #sc-7819	IF
Goat polyclonal anti-GFP	1:200/1:1000	Abcam, #ab6673	IF/WB
Rabbit polyclonal anti-GFP	1:200/1:1000	Sigma-Aldrich, #G1544	IF/WB
Rabbit polyclonal anti-cleaved Caspase3 (D175)	1:200	Cell Signaling, #9661S	IF
Mouse monoclonal anti-53BP1	1:500	Santa Cruz Biotechnology, #sc-22760	IF
Mouse monoclonal anti-α-Tubulin	1:5000	Sigma-Aldrich, #T9026	WB
Mouse monoclonal anti-β-Actin (Clone AC-74)	1:5000	Sigma-Aldrich, #A2228	WB

Table 8: Secondary antibodies used for Immunofluorescence and Western blot (IF: Immunofluorescence; HRP: Horse Peroxidase Reaction; WB: Western blot).

Secondary antibodies	Dilution	Source	Technique IF/HRP/WB
Anti-goat IgG (whole molecule) peroxidase conjugated	1:5000	Sigma-Aldrich, #B8520	WB
Anti-rabbit IgG (whole molecule) peroxidase conjugated	1:5000	Sigma-Aldrich, #A0545	WB
Anti-mouse IgG (whole molecule) peroxidase conjugated	1:5000	Sigma-Aldrich, #A9044	WB
Anti-goat IgG (whole molecule) biotin conjugated	1:300	Sigma-Aldrich, #B7024	HRP
Anti-rabbit IgG (whole molecule) biotin conjugated	1:300	Sigma-Aldrich, #B8895	HRP
Anti-mouse IgG (whole molecule) biotin conjugated	1:300	Sigma-Aldrich, #B7264	HRP
Alexa Fluor 488 goat anti-mouse IgG (H+L)	1:800	Invitrogen, #A11001	IF
Alexa Fluor 488 donkey anti-goat IgG (H+L)	1:800	Invitrogen, #A11055	IF
Alexa Fluor 488 goat anti-mouse IgG (H+L)	1:800	Invitrogen, #A11001	IF
Alexa Fluor 488 goat anti-rabbit IgG (H+L)	1:800	Invitrogen, #A11008	IF
Alexa Fluor 568 goat anti-mouse IgG (H+L)	1:800	Invitrogen, #A11004	IF
Alexa Fluor 568 goat anti-rat IgG (H+L)	1:800	Invitrogen, #A11077	IF
Alexa Fluor 568 goat anti-rabbit IgG (H+L)	1:800	Invitrogen, #A11011	IF
Alexa Fluor 568 goat anti-guinea pig IgG (H+L)	1:800	Invitrogen, #A11075	IF

4.7. BrdU proliferation assay:

Human islets proliferation was evaluated by measuring the incorporation of 5-bromo-2'-deoxyuridine (BrdU) (Sigma-Aldrich, #B5002), a synthetic nucleoside that can be incorporated into newly synthesized DNA, replacing thymidine during cell replication. Forty human islets were seeded per condition into 96-well Nunc tissue culture plates and incubated with BrdU (10 µM) at 37ºC for the last 24 hours of prolactin treatment. Proliferation rates were estimated using the BrdU-cell

proliferation ELISA (Roche Applied Science, # 11647229001) following manufacturer´s protocol. Briefly, after medium removal, human islets were fixed using FixDenat solution and incubated for 30 min at room temperature. After removing FixDenat solution, anti-BrdU antibody conjugated with peroxidase (anti-BrdU-POD) was added and incubated for 90 min at room temperature. Following this, cells were washed and incubated with the substrate solution TMB. After incubation, absorbance of the samples was measured using a microplate reader (Infinite® M200 Pro, Tecan, Switzerland) at 370 nm and 492 nm every 5 min for 30 min.

4.8. Glucose-induced Insulin Secretion Stimulation Index:

Insulin secretion was measured over a period of 30 min. Groups of 10 mouse or human islets were washed in 0.5 mL Krebs-Ringer bicarbonate-HEPES buffer (KRBH) (140 mM NaCl, 3.6 mM KCl, 0.5 mM NaH_2PO_4, 0.5 mM $MgSO_4$, 1.5 mM $CaCl_2$, 2 mM $NaHCO_3$, 10 mM HEPES, 0.1% BSA) and pre-incubated at 37°C for 45 min in the same buffer supplemented with 2.5 mM glucose. Then, islets were centrifuged and KRBH buffer was discarded. Subsequently, media was replaced by 0.5 mL KRBH supplemented with 16.8 mM glucose and cells were incubated for another 30 min at 37°C. Insulin was measured by using mouse insulin enzyme immunoassay kit (Mercodia AB, #10-1247-01 and #10-1113-01) according to the manufacturer´s instructions. Stimulation index was expressed as the ratio of insulin levels at 16.8 mM glucose divided by insulin levels at 2.5 mM glucose.

4.9. Apoptosis assay:

Apoptosis was measured using the TUNEL assay (In Situ Cell Death Detection Kit, Roche Applied Science, #11544675001) following the manufacturer's protocol. Alternatively, the Cell Death Detection ELISAPLUS (Roche) was used to quantify the degree of cytoplasmic histone associated-DNA-fragments. Results of ELISA are presented as a percentage of apoptotic enrichment compared to untreated islets.

5.0. MTT assay:

Groups of 35 mouse and human islets were handpicked to determine the metabolic activity of islets using Cell Proliferation Kit I (MTT, Roche Applied Science, #11465007001) according to the manufacturer´s protocol. Briefly, islets were incubated with 0.5% MTT for 4 hours. Then, blue formazan crystals were solubilized overnight with 100 µL of solubilization solution. Optical density was determined at 550 nm with a reference wavelength of 650 nm using a Varioskan Flash spectrophotometer (Thermo Scientific).

5. MICROARRAY OF PAX4 TRANSGENIC MICE:

Islets from Pax4-rtTA mice and Pax4R129W-rtTA treated or not with 1g/L doxycycline for 1 month in the drinking water after weaning were used to study the gene expression alterations due to the Pax4 overexpression. In order to decrease the eventual natural variation in gene expression, RNA samples were generated from

pooled islet preparations. Each sample was prepared from approximately 200 islets using a pool of three to four animals per group. RNA was extracted as described in *section 3.1*. RNA samples with RNA integrity Number (RIN) greater than 7 was accepted for further analysis. Each experimental condition was analyzed in triplicates using the Affymetrix Gene Chip platform Mouse Gene 1.0 ST array, which provide coverage of the transcribed genome containing 28,853 transcripts and variants. 770.317 total probes (11 probes per gene) each of them possessing 25-mer enlarging the reproducibility and the specificity. Microarray analysis was performed in the Genomics facility at CABIMER using the Affymetrix standard protocol. Briefly, 500 ng of total RNA from each pool was used as template for to prepare biotinylated fragmented cRNA using a two-cycle amplification step, according to the GeneChip Expression Analysis Technical Manual (Affymetrix, Inc., Santa Clara, CA). GeneChip Mouse Gene 1.0 ST Array was hybridized for 16 h in a 45 ºC incubator, rotated at 60 rpm. According to the GeneChip Expression Analysis Technical Manual (Affymetrix, Inc., Santa Clara, CA), the array was then washed and stained using the Fluidics Station 450. Finally, scanning was carried out with the GeneChip Scanner 3000 7G. Image analysis, quality control and quantification of fluorescence data were performed using the specific software GCOS (*CEL raw data).

The biostatistic data analysis included processing of fluorescence data(raw data), data normalization by RMA, exchange value of expression of a condition regarding control, statistical parameters appropriate to establish a degree of credibility (p-value) and notes on the transcripts (Affymetrix identification number, gene symbol, gene name and description with the access number corresponding transcript). Biostatistic analysis of microarray data was conducted by two independent groups, the Genomics platform at CABIMER and in collaboration with Dr. Javier Santoyo López and Javier Pérez Florido, at Genomic and Bioinformatic Platform of Andalusia (GBPA, Sevilla, Spain).

Genes differentially expressed were identified using Student's t-test with fold change >2 or <−2 and p-value ≤0.05. These genes were associated with regulatory pathways represented in the Kyoto Encyclopedia of Genes and Genomes (KEGG Pathways). Raw and processed data were deposited in the GEO database with the accession number GSE62846.

6. PAX8 LENTIVIRAL APPROACH:

Overexpression of mouse Pax8 and human PAX8 were conducted by the generation of a dual-promoter lentivirus, pHRSIN-DUAL-GFP (also known as pHRSIN-CSGWdINotI_pUb_Em) kindly provided by Dr. Pintor-Toro (CABIMER, Seville, Spain).

The pHRSIN-DUAL-GFP lentiviral vector is a 2nd generation 12 kb lentivector (offers a high level of safety) based on human immunodeficiency virus, HIV-1, and composed of the following components:1) Native 5'LTR consistedin the U3, R and U5

regions of HIV-1 and the 5´part of the gag gene; 2) Rev-responsive element (RRE);3) Spleen Focus-Forming Virus (SFFV) promoter which improves levels and stability of the transgene expression and was preceded for EcoRII restriction site; 4) Internal expression cassette containing the transgene flanked for the restriction enzymes BamHI and NotI; 5) Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element (WPRE) to increase titer and transgene expression; 6) Ubiquitingene promoter (UBIQ); 7) Green fluorescent protein marker (GFP) flanked for MluI and XhoI restriction enzymes and 8) 3'LTR consisted in the R and U5 regions of HIV-1, but not the U3 region creating the so-called self-inactivating (SIN) LTR in which a large deletion in this 3'LTR makes the virus replication incompetent (Figure 18).

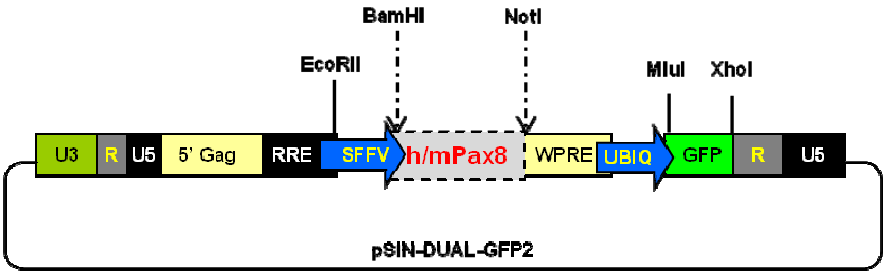


Figure 18: Schematic representation of lentiviral vector pHRSIN-DUAL-GFPoverexpressing h/mPax8.

The HIV packaging (pCMVDR8.91) plasmid that overexpress HIV-1 *gag/pol, tat,* and *rev* genes and the envelope VSVG (pMDG) plasmid were also kindly provided by Dr. Pintor-Toro (CABIMER, Seville, Spain). These accessory vectors were indispensable for the generation of the lentiviral particles (Figure 19).

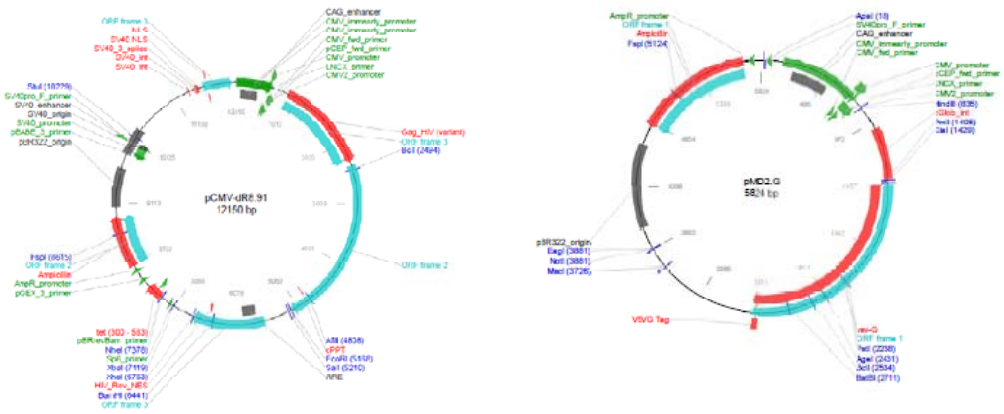


Figure 19: Schematic representation of lentiviralaccessory vector pCMVDR8.91 and pMDG (from Addgene).

The cloning strategy, for mouse and human Pax8 insertion into pHRSIN-DUAL-GFP lentivirus is described below:

A. pHRSIN-DUAL-GFP vector:

A.1. Linearization of PHRSIN-DUAL-GFP: the lentivector was linearized using the unique restriction sites flanking the cloning site for our genes of interest (mouse Pax8 and human PAX8), EcoRV on the 5´and XhoI on the 3´end. For DNA linearization, 4 µg DNA was digested with 30 units (U) of BamHI (New England Biolabs, # R0136) and NotI (New England Biolabs, #RR0189) in 2µl buffer 3 NEB and 2µl 1X BSA in a final volume of 20µl. Restriction analysis was performed in double (BamHI+NotI). Single cuts for each restriction enzyme were performed as digestion controls. The restriction samples were incubated for 2 hours at 37ºC in a water bath. Digestions were analyzed by electrophoresis in a 0.5% agarose gel (Pronadisa; #8014) dissolved in TBE 1X (Tris-Borate-EDTA, Sigma-Aldrich; #93290) and containing 0.1 µg/ml ethidiumbromide (Sigma-Aldrich, #E1510) for 45 min at 65V. Afterwards, the fragments were photographically visualized and documented under UV light. This results in one band of 12,000 bp.

A.2. Purification of linearized PHRSIN-DUAL-GFP: DNA fragment was purified two times. First, DNA band was isolated from gel and purified using QIAEX II (Qiagen; #20021) which is based on solubilization of agarose and selective adsorption of nucleic acids onto QIAEX II silica-gel particles in the presence of chaotropic salt. QIAEX II separates DNA from salts, agarose, polyacrylamide, dyes, proteins, and nucleotides without phenol extraction or ethanol precipitation. Thereafter, the elution was purified by column using QIAquick PCR Purification Kit (Qiagen; #28104) based on a silica membrane that binds DNA in high-salt buffer and elution with low-salt buffer or water, according to the manufacturer´s instructions. The purification procedure removes primers, nucleotides, enzymes, mineral oil, salts, agarose, ethidium bromide, and other impurities from DNA samples. For corroborating the correct DNA fragment at 12 Kb, 0.5% agarose gel electrophoresis was performed for 45 min at 65W.

B. Human and mouse Pax8: Pax8 cDNAs cloned unidirectionally into the EcoRI and SalI sites of the mammalian expression vector pCMV6 were obtained from Origene (TrueClone, Origene). Human PAX8 (NM_003466.2) was inserted into pCMV6-XL5 vector, while mouse Pax8 cDNA (NM_011040.4) (Figure 20).

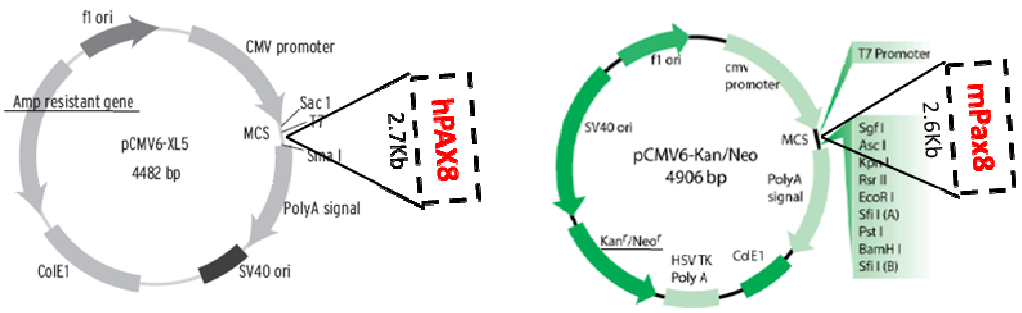
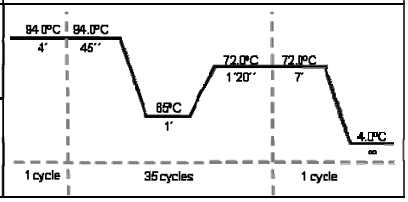


Figure 20: Schematic representation of Pax8 cDNAs expression vectors (modified from Origene).

B.1. Insertion of new restriction sites in human and mouse Pax8 coding regions: the purpose of this PCR were amplified the coding region of human and mousePax8 adding new restriction sites, BamHI on the 5´and NotI on the 3´end. Forty ng/µl of h/mPax8-pCMV6 were amplified by standard PCR with 0.7 µl High fidelity expand Taq polymerase (Roche; #1173 264 1001). Primers were designed for the coding region of our genes of interest adding the sequences of the restriction enzymes to include in the corresponding location.The sequences of the gene-specific primers synthesized by Sigma-Aldrich and the PCR protocol are outlined in Table 9 and Figure 21:

Table 9: Primer sequences in mouse and human Pax8 CDS.

Primers		PCR conditions
Human PAX8	BamHI-CDS human PAX8 _Fw 5'-CCGTTATggatccatgcctcacaactccatcag-3' CDS human PAX8-NotI_Rv 5'-CGGTATAgcggccgctacagatggtcaaaggc-3'	
Mouse Pax8	BamHI-CDS mouse Pax8 _Fw 5'-CGGTATggatccatgcctcacaactcgatcag-3' CDS mouse Pax8-NotI _Rv 5'-CGGTATAgcggccgctacagatggtcaaaggc-3'	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CGGTTATggatccatgcctcacaactcgatcag
mPax8      ATGCCTCACAACTCGATCAGATCCGGCCATGGAGGGCTGAATCAACTAGGAGGGGCCTTT 60
hPax8      ATGCCTCACAACTCCATCAGATCTGGCCATGGAGGGCTGAACCAGCTGGGAGGGGCCTTT 60
           ************* ******** ********** ** ** ************

CGGTTATggatccatgcctcacaactccatcag

mPax8       GTGAATGGCAGGCCTCTGCCAGAAGTTGTACGTCAACGCATTGTGGACTTGGCCCACCAG 120
hPax8       GTGAATGGCAGACCTCTGCCGGAAGTGGTCCGCCAGCGCATCGTAGACCTGGCCCACCAG 120
            ********** ******** ***** ** ** ** ***** ** *** ***********

mPax8       GGCGTGAGGCCCTGTGATATTTCTCGCCAGCTCCGTGTCAGCCATGGCTGTGTAAGCAAG 180
hPax8       GGTGTAAGGCCCTGCGACATCTCTCGCCAGCTCCGCGTCAGCCATGGCTGCGTCAGCAAG 180
            ** **.******** ** ** ************* ************* ** ******

mPax8       ATCCTTGGCAGGTACTACGAGACTGGCAGCATCCGGCCTGGAGTGATAGGGGGCTCCAAG 240
hPax8       ATCCTTGGCAGGTACTACGAGACTGGCAGCATCCGGCCTGGAGTGATAGGGGGCTCCAAG 240
            ************************************************************

mPax8       CCCAAGGTGGCCACCCCCAAGGTGGTGGAGAAGATAGGAGACTACAAGCGGCAGAACCCT 300
hPax8       CCCAAGGTGGCCACCCCCAAGGTGGTGGAGAAGATTGGGGACTACAAACGCCAGAACCCT 300
            ************************************ ******** ** *********

mPax8       ACCATGTTTGCTTGGGAGATCCGGGACCGGCTCCTGGCAGAAGGCGTTTGTGACAATGAC 360
hPax8       ACCATGTTTGCCTGGGAGATCCGAGACCGGCTCCTGGCTGAGGGCGTCTGTGACAATGAC 360
            ********** ********** ********** ***** ************

mPax8       ACTGTCCCCAGTGTCAGCTCCATCAACAGAATCATCCGGACCAAAGTGCAGCAGCCATTC 420
hPax8       ACTGTGCCCAGTGTCAGCTCCATTAATAGAATCATCCGGACCAAAGTGCAGCAACCATTC 420
            ***** ********** ** ************************ ******

mPax8       AACCTCCCCATGGATAGCTGTGTGGCCACCAAGTCTCTGAGCCCAGGACACACACTGATC 480
hPax8       AACCTCCCTATGGACAGCTGCGTGGCCACCAAGTCCCTGAGTCCCGGACACACGCTGATC 480
            ******** ***** ***** ********** ***** ** ******** ******

mPax8       CCCAGCTCAGCTGTAACACCCCCAGAGTCACCCCAGTCGGATTCCTTGGGCTCTACCTAC 540
hPax8       CCCAGCTCAGCTGTAACTCCCCCGGAGTCACCCCAGTCGGATTCCCTGGGCTCCACCTAC 540
            ********** ***** ********** ******* ******

mPax8       TCTATCAACGGGCTCCTAGGAATTGCTCAGCCTGGCAATGACAACAAGAGAAAGATGGAT 600
hPax8       TCCATCAATGGGCTCCTGGGCATCGCTCAGCCTGGCAG---CGACAAGAGGAAAATGGAT 597
            ** ***** ******** ** ** ********** * ******** ** ******

mPax8       GATAGTGACCAGGACAGCTGTCGGCTAAGCATCGACTCACAGAGCAGCAGCAGTGGTCCT 660
hPax8       GACAGTGATCAGGATAGCTGCCGACTAAGCATTGACTCACAGAGCAGCAGCGGACCC 657
            ** ***** ***** ***** ** ******** ********** ** **
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Figure 21: Primer localization in mouse and human Pax8 CDS.

PCR products were runned in a gel of 1.5% agarose dissolved in 1X TBE containing 0.1 µg/ml ethidiumbromide for 40 min at 100W. After electrophoretic separation, the fragment of interest was visualized under UV light obtaining 1.4Kb products that coincided with the size of amplified DNA.

B.2. Purification of BamHI-h/mPax8 CDS-NotI: PCR products were purified two times. Bands were isolated from gel and purified using QIAEX II (Qiagen; #20021) and, lately, the elution products were purified by column using QIAquick PCR Purification Kit (Qiagen; #28104) according to the manufacturer´s instructions. For corroborating the correct PCR amplification products at 1.4 Kb, 1.5% agarose gel electrophoresis was performed for 45 min at 90W.

B.3 Digestion of purified PCR products BamHI-h/mPax8 CDS-NotI: in order to obtain complementary ends, the total volume of purification products from PCR to amplify the CDS of h/mPax8 and to insert the new restriction sites for BamHI and NotI

were digested with 30U of these enzymes, BamHI and NotI in 2µl buffer 3 NEB and 2µl 1X BSA in a final volume of 20 µl for 2 hours at 37ºC in a water bath.

B.4. Purification of digested BamHI-h/mPax8 CDS-NotI: digestions were purified two times. Bands were isolated from gel and purified using QIAEX II and, after, the elution products were purified by column using QIAquick PCR Purification Kit according to the manufacturer´s instructions. For corroborating the correct PCR amplification products at 1.4 Kb, 1.5% agarose gel electrophoresis was performed for 45 min at 90W.

C. Creation of pPSD-h/mPax8 lentiviral vector:

C.1. Ligation of BamHI-pPSD-NotI with BamHI-h7mPax8-NotI: recombination of lentiviral vector pHRSIN-DUAL-GFP with our genes of interest, h/mPax8, were performed by ligation of their BamHI and NotI complementary overhangs. For ligation, 17.5ng of insert were ligated with 50ng of vector following a molar ratio of 1:3. Reaction was catalyzed by 1 µl T4 DNA ligase (Promega; #M180A) promoting the formation of phosphodiester bonds between the fragments under ATP consumption, and 1µl buffer ligase 10X (Promega; #C126B) in a final volume of 10µl. DNA was ligated at 16ºC over night and used directly for transformation of competent bacteria.

C.2. Transformation of pPSD-h/mPax8 ligation: to amplify plasmid DNA through cellular replication, 5µl DNA of the ligation mix was added to 50 µl of DH5α competent cells (Invitrogen, #18265017) for transformation. After a incubation for 30 min on ice, to heat-shock the bacteria cells were incubated at 42 °C for 20 sec in a water bath and cooled on ice for 2 min. Afterwards, 950 µl of Luria Broth (LB, Sigma-Aldrich; #L3022) medium was added and the suspension was incubated for 30 min at 37°C at 225 rpm. Cell suspension was centrifuged for 2 min at 1,000 rpm and 850 µl supernatant was discarded. The bacterial suspension was plated on LB plates containing 1 mg/ml ampicillin (Sigma-Aldrich, #A9518). When the vector is taken up by the bacteria, they receive an ampicillin resistance, which was used for selection. The plates were incubated at 37 °C overnight.

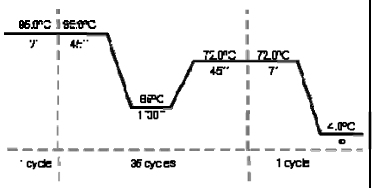
C.3. Purification of DNA from pPSD-h/mPax8: for preparation of plasmids pPSD-h/mPax8 from transformed bacteria, single colonies were transferred to 3 ml LB medium containing 1 mg/ml ampicillin and incubated at 37ºC over night. Next day, bacteria were harvested at 3000 rpm for 10 min at 4ºC. The resulting pellet was used for the purification of plasmid DNA according to the manufacturer´s instruction of the Qiaprep Spin MiniPrep kit (Qiagen; #27106) which is based on alkaline lysis of bacterial cells followed by adsorption of DNA onto silica in the presence of high salt. DNA concentration was measured using a NanoDrop (NanoDrop 2000c; Thermo Scientific).

C.4. Confirmation of creation of pPSD-h/mPax8 lentiviral vector: insertion and orientation of m/hPax8 were confirmed by restriction enzyme digestion analyses, standard PCR and sequencing.

C.4.a. Digestion of pPSD-h/mPax8 vector: to corroborate the correct bands in the purified plasmid pPSD-h/mPax8, restriction analysis was performed in double digestionusing the enzymes BamHI + NotI and single digestion for each restriction enzyme (only BamHI or NotI). 500ng of DNA were digested with 0.5 U of enzymes in 2µl buffer 3 NEB and 2µl 1X BSA in a final volume of 20µl for 2 hours at 37ºC in a water bath. Digestions were analyzed by electrophoresis in a gel of 0.75% agarose dissolved in 1X TBE and containing 0.1 µg/ml ethidiumbromide (EtBr) for 45 min at 65W. Afterwards, the fragments were photographically visualized and documented under UV light. Results coincided with the correct bands that were to appear, one band at 12kb and another at 1.4kb.

C.4.b. PCR to amplify pPSD-h/mPax8: toconfirm the insertion and the correct orientation of m/hPax8 into pPSD vector, 40 ng of DNA were amplified using the BioTaq DNA polymerase (Bioline; # 21040). The sequences of the gene-specific primers and the PCR protocol are outlined in Table 10:

Table 10: Primer sequences for h/mPax8 amplification.

Primers			Fragment size	PCR conditions
Human PAX8	SFFV promoter_Fw Internal human PAX8_Rv	5'-CAATTCCTGCAGCCCCCGGGCGACTCAG-3' 5'-GTGGAGAAGATTGGGGACTACA-3'	1.48kb	
	BamHI-CDS human PAX8_Fw CDS human PAX8-NotI_Rv	5'-CGGTTATggatccatgcctcacactccatcag-3' 5'-CGGTATAgcggccgcctacagatgg.caaaggc-3'	1.1kb	
Mouse Pax8	SFFV promoter_Fw Internal mousePAX8_Rv	5'-CAATTCCTGCAGCCCCCGGGCGACTCAG-3' 5'-AGATAAAGAGGAACGGGTGGAG-3'	1.48kb	
	BamHI-CDS mouse Pax8_Fw CDS mouse Pax8-NotI_Rv	5'-CGGTTATggatccatgcctcacaactcgatcag-3' 5'-CGGTATAgcggccgcctacagatggtcaaaggc-3'	1.4kb	

PCR products were runned in a gel of 1% agarose dissolved in 1X TBE containing 0.1 µg/ml ethidiumbromide for 40 min at 100W. After electrophoretic separation, the fragments of interest were visualized under UV light obtaining the correct size of amplified DNA.

C.4.c. Sequencing of pPSD-h/mPax8: nucleic acid sequencing was performed at the company STAB VIDA (Caparica, Portugal). For this purpose, DNA samples containing 1.5 µg plasmid DNA were sent together with the primers listed in Table 11 in a concentration of 10pmol/µl to the company. DNA sequence results were analysed using DNA Dynamo Sequence Analysis Software (United Kingdom) and Serial Cloner 1.3 Software.

Table 11: Primer for sequencing mouse and human Pax8 CDS.

Primers		
Human PAX8	BamHI-CDS human PAX8 _Fw	5'-CGGTTATggatccatgcctcacaactccatcag-3'
	CDS human PAX8-NotI_Rv	5'-CGGTATAgcggccgcctacagatggtcaaaggc-3'
	Internal human PAX8_Fw	5'-AGATAAAGAGGAAGGGGTGGAG-3'
	Internal human PAX8_Rv	5'-GTGGAGAAGATTGGGGACTACA-3'
Mouse Pax8	BamHI-CDS mouse Pax8 _Fw	5'-CGGTTATggatccatgcctcacaactcgatcag-3'
	CDS mouse Pax8-NotI_Rv	5'-CGG ATAgcggccgcctacagatggtcaaaggc-3'
	Internal mouse Pax8_Fw	5'-AGTGTCAGCTCCATCAACAGAA-3'
	Internal mouse Pax8_Rv	5'-AGA AAAGAGGAAGGGGTGGAG-3'

C.5. Large purification of pPSD-h/mPax8 DNA: once we had obtained the correct lentiviral plasmids for pPSD-h/mPax8, the positive bacterial cloneswere transferred to 100 ml LB medium containing 1 mg/ml ampicillin and incubated at 37°C over night with rotation. The bacteria culture was centrifuged at 4,000 rpmfor 10 minat 4 °C. Bacterial pellet was then lysated and plasmid isolated using the Qiagen Plasmid Plus Midi kit (Qiagen; #12943) according to manufacturer´s instructions and based on a modified alkaline lysis procedure, followed by binding of plasmid DNA to QIAGEN Anion-Exchange Resin under appropriate low-salt and pH conditions. The DNA concentration was measured using a NanoDrop (NanoDrop 2000c; Thermo Scientific).

D. Production of pPSD-h/mPax8 lentiviral particles: For lentivirus production, 3×10^6 293T cells (passage ≤ 20) were seeded onto a 10-cm Petri dish and transfected 24 hours later with CalPhos Mammalian Transfection kit (BD Biosciences Clontech, #631312) according to the manufacturer´s instructions using 15 µg of pPSD-h/mPax8 construct, 10 µg of pCMVDR8.91 and 5 µg of pMDG. The transfection medium was replaced 24 hours post transfection by complete DMEM. Lentiviruses were harvested from the media 72 h post-transfection. Media was collected and passed through a 0.45 µm PVDF filters Millex-HV (Merck Millipore, #SLHV033RS) and concentrated by ultracentrifugation at 22 000 rpm for 90 min at 4ºC. Virus particles were resuspended in serum-free DMEM medium and stored at − 80 °C.

E. Titration of lentiviral particles pPSD-h/mPax8: To determine the quality of the virus preparation, 1×10^5 293T cells were were seeded in 6 well plates and incubated over night. Different volumes of lentiviral particles were added to medium: 1 µl, 2 µl, 5 µl, 10 µl and 20 µl of virus and no infected cells as a negative control. Cells were harvested 72 h post-infection and the percentage of GFP positive cells determined by flow cytometry. Untransduced cells were used to define the negative population. The cell size was determined with the forward scatter (FSC), the inner complexity and granularity of the cell was determined with the sideward scatter (SSC) and the green fluorescence, derived from GFP, with the FL1 channel. The data was analysed using CellQuest Pro software (BD Biosciences FACS Systems, San Jose, CA). To calculate

titers, the number of target cells was multiplied by the percentage of GFP positive cells divided by the volume of the input virus.

F. Lentiviral infection protocol:

F.1. Cell lines lentiviral infection: For infection with the lentiviral stock, INS-1E or MIN6 cells were seeded at $3x10^5$ cells in 6-well plates (for FACS, western blot and QT-PCR) or 10^4 cells onto poly-L-lysine treated coverslips in 24-well plates (for inmunofluorescence) 24 hour prior to infection. Cells were transduced at 30-40% confluence. Lentiviral particles (pPSD-h/mPax8 or pPSD-GFP) were added in a multiplicity of infection (MOI) equal to 7 at 37ºC over night (approximately 16 hours). Infection was performed in serum-free cell culture medium. Media were changed to complete growth media for the rest of the experiment. The transduction efficiency was measured 72 hours post infection by FACS analysis to determine the GFP positive cells; by QT-PCR to validate the transcription levels of h/mPax8; by western blot and immunofluorescence to measure h/mPax8 protein expression.

F.2. Intact Human and Mouse islets lentiviral infection *(Jiménez-Moreno CM et al., manuscript in review)*: For lentiviral infection of mouse and human islets, islets were maintained in culture in complete medium for 24 hours prior to infection to allow their recovery. After islets were recovered, a mild trypsinization were performed using 0.5X trypsin (Gibco; #15400) diluted in 1X Hanks Balanced Solution (HBSS) (Gibco; #15400054) for 2 min at 37ºC and, lately, pipetting up and down 3 times (use 1000 µl points for micropipette). Islets were resuspended in serum-free medium for the infection. Media were changed to complete media for the rest of the experiment:

F.2.a. Monitoring GFP expression signal in an *in vivo* system, ImagExpressMycro System (High content screening system, Molecular Devices): Approximately 15 mouse or human islets were plated on µ-Plate 96 welllibiTreat (IBIDI, #89626) in a final volume of 150 µl of complete media. Islets were cultured for 4 days at 37ºC as shown in Figure 22. Daily fluorescence images were acquired using the ImagExpress system (Figures 22).

F.2.b. Measure transduction efficiency by Flow cytometry using a FACSCalibur (BD Biosciences): Approximately 50 mouse or human islets were transferred into 5ml polystyrene Round-bottom tube (BD Falcon; #352058) in a final volume of 500 µl complete media. Islets were incubated for 4 days at 37ºC as shown in Figure 23. Islets were disaggregated and the % GFP positive as compared to non-infected cells was estimated by flow cytometry (Figures 22).

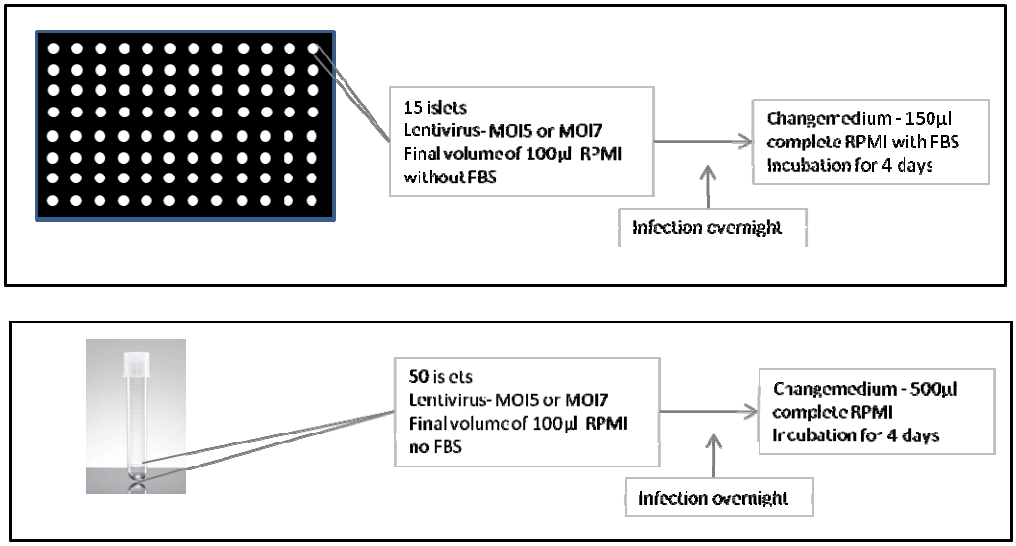


Figure 22: Schematic representation of the essential steps of infection for imaging and flow cytometry.

7. MICROARRAY OF PAX8-OVEREXPESSING MOUSE ISLETS:

Primary mouse islets were transduced using the infection protocol developed in our laboratory *(Jiménez-Moreno et al., manuscript accepted in Gene Therapy, pending revision)*. The lentiviral vector pHRSIN DUAL-GFP has been employed to overexpress mouse Pax8 gene. This construct also harbored the Green Fluorescent Protein (GFP) for technical purposes. Viruses that allow expression of GFP were used as negative control. Two hundred islets from 3-months-old male C57BL/6J mice were transduced according our infection protocol (0.5X trypsinization/ MOI 20/ 4 days post-infection) for each experimental group to study the gene expression alterations due to the Pax8 overexpression. RNA was extracted as described in *section 3.1*. RNA samples with RNA integrity Number (RIN) greater than 7 was accepted for further analysis. Each experimental condition was analyzed in triplicates using the Affymetrix Gene Chip platform Mouse Gene 1.0 ST array in Genomics facility at CABIMER. Microarray analysis was conducted as previously described in *section 4*. To determine the modulation of the transcriptional profile in pancreatic islet caused by the overexpression of Pax8, data analysis of transduced islets was performed using Ingenuity Pathway Analysis Platform (IPA).

8. STATISTICAL ANALYSIS

Results are expressed as mean ± SEM. Statistical differences was estimated by Student's *t*-test or ANOVA with Bonferronipost hoc test where appropriate * and ** indicate statistical significance with $p<0.05$ and $p<0.01$, respectively.

CHAPTER IV_PAX4 PROJECT RESULTS

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4.1. In Vivo Conditional Pax4 Overexpression in Mature Islets β-cells Prevents Stress-Induced Hyperglycemia in Mice

OBJECTIVE

This study focuses on deciphering the *in vivo* physiological role of the transcription factor Pax4 and its diabetes-linked mutant variant Pax4R129W in pancreatic islets proliferation and survival in response to metabolic stress situations and its impact on blood glucose homeostasis.

RESULTS and CONCLUSIONS

Our results demonstrated that conditional overexpression of Pax4, but not the mutant variant Pax4R129W, in adult β-cells protects transgenic animals against streptozotocin (STZ)-induced hyperglycemia and isolated islets against cytokines-induced apoptosis by suppressing selective NF-κB target genes such as IL-1β transcript levels. In addition, Pax4-overexpressing islets induced expression Bcl-2 that blunted cytochrome C release. Conversely, Pax4R129W islets exhibited elevated basal levels of IL-1β along with NOS2 failing to avoid cytochrome C release. Remarkably, long-term overexpression of Pax4 in animals increased levels of the pro-proliferative genes c-myc and Cdk4 promoting proliferation of a $Pdx1^{+}$/$Insulin^{-}$ subpopulation. These cells displayed impaired glucose-induced insulin secretion (GSIS). Indeed, overexpression of either Pax4 or Pax4R129W markedly results in a reversible repression of MafA, Glut2 and insulin suggesting a dedifferentiated state of these β-cells.

CONTRIBUTION

My contribution in this article has been participating in the planning, design and execution of the experiments corresponding to different figures. Therefore, my work consisted in collaborating in the management of Pax4 and Pax4R129W mice colony and in the administration of doxycycline in drinking water for 1 month starting at weaning. I also cooperated in the STZ studies. Blood glucose levels were measured three times per week (Figure 2B). Furthermore, I actively participated in immunohistochemical analysis of Pax4 and Pax4R129W pancreas (Supplementary figure 2A) and in the morphometry analysis of the different experimental groups (Supplementary figure 2B). I also performed the inmunohistochemical staining of c-myc (Figure 1C), MafA and Glut2 (Figure 5B and C) in pancreatic sections of Pax4 transgenic animals. Furthermore, I contributed in the mRNA profile measurement by QT-PCR of different targets of Pax4, COX2 and NOS2 on isolated mouse islets overexpressing Pax4 or Pax4R129W depicted in Figure 4C. These results confirmed that Pax4, but not Pax4R129W, prevented STZ-induced hyperglycemia and cytokine-mediated cell death. Likewise, I actively participated in the discussion of the results of the project.

ORIGINAL ARTICLE

In Vivo Conditional Pax4 Overexpression in Mature Islet β-Cells Prevents Stress-Induced Hyperglycemia in Mice

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OBJECTIVE—To establish the role of the transcription factor Pax4 in pancreatic islet expansion and survival in response to physiological stress and its impact on glucose metabolism, we generated transgenic mice conditionally and selectively overexpressing Pax4 or a diabetes-linked mutant variant (Pax4R129W) in β-cells.

RESEARCH DESIGN AND METHODS—Glucose homeostasis and β-cell death and proliferation were assessed in Pax4- or Pax4R129W-overexpressing transgenic animals challenged with or without streptozotocin. Isolated transgenic islets were also exposed to cytokines, and apoptosis was evaluated by DNA fragmentation or cytochrome C release. The expression profiles of proliferation and apoptotic genes and β-cell markers were studied by immunohistochemistry and quantitative RT-PCR.

RESULTS—Pax4 but not Pax4R129W protected animals against streptozotocin-induced hyperglycemia and isolated islets from cytokine-mediated β-cell apoptosis. Cytochrome C release was abrogated in Pax4 islets treated with cytokines. Interleukin-1β transcript levels were suppressed in Pax4 islets, whereas they were increased along with NOS2 in Pax4R129W islets. Bcl-2, Cdk4, and c-myc expression levels were increased in Pax4 islets while MafA, insulin, and GLUT2 transcript levels were suppressed in both animal models. Long-term Pax4 expression promoted proliferation of a Pdx1-positive cell subpopulation while impeding insulin secretion. Suppression of Pax4 rescued this defect with a concomitant increase in pancreatic insulin content.

CONCLUSIONS—Pax4 protects adult islets from stress-induced apoptosis by suppressing selective nuclear factor-κB target genes while increasing Bcl-2 levels. Furthermore, it promotes dedifferentiation and proliferation of β-cells through MafA repression, with a concomitant increase in Cdk4 and c-myc expression. *Diabetes* **60:1705–1715, 2011**

Diabetes is a disease characterized by high levels of circulating blood glucose. The etiology involves insufficient release of insulin from pancreatic islet β-cells and resistance of target tissues to the action of the hormone. The two most common forms of diabetes are type 1 diabetes characterized by a destruction of β-cells (1) and type 2 diabetes typified by β-cell failure combined with insulin resistance (2). Factors such as the environment and genetic predisposition are key determinants that influence development and progression of the disease. Genetic studies including linkage analysis, candidate gene approaches, and more recently, genome-wide association studies (GWAS) have identified at least 40 loci affecting risk of type 1 diabetes and 27 type 2 diabetes susceptibility genes (3–5). Although GWAS have been a powerful approach to yield new diabetogenes, susceptible gene loci for which functions may be altered by environmental factors such as pregnancy and obesity remain to be identified.

One such susceptibility gene locus not highlighted by GWAS encodes the islet β-cell transcription factor Pax4. Expression of the *pax4* gene is mandatory for the development and maturation of β-cells (6). Although detectable, *Pax4* expression was found to be low in adult β-cells (7). Forced expression of *Pax4* in embryonic α-cells induced a complete phenotypic change toward β-cells indicating that Pax4 is a master regulator of the β-cell genetic program (8).

Mutations and polymorphisms in the *pax4* gene have been associated with both type 1 and type 2 diabetes in several populations, contrasting with other diabetogenes for which association has only been linked to one or the other form of diabetes (7,9). Interestingly, we found that Pax4 expression is increased in type 2 diabetic islets, an effect that is most likely mediated by high blood glucose levels (10). Together, these studies suggest that Pax4 may function as a survival and/or proliferation gene allowing mature islets to adapt in response to physiological cues. Consistent with this premise, Pax4 mRNA levels were increased in islets cultured in the presence of glucose, betacellulin, activin A, and glucagon-like peptide-1 (10). Ectopic expression of mouse Pax4 in human or rat islets and in the mouse MIN6 cell line conferred protection against cytokine-mediated cell death and promoted replication (11,12). A diabetes-linked mutant variant R121W, identified in the Japanese population (13,14), was less efficient in protecting human islets against cytokines (11).

Although these in vitro studies suggest a fundamental role of Pax4 in β-cell survival and replication, the impact of Pax4 in vivo and its relation to diabetes remains to be established. Herein, we have generated two transgenic mouse lines that conditionally express Pax4 or its mutant variant R121W (PAX4R129W in mice) in β-cells. Our results demonstrate that conditional overexpression of Pax4 in adult β-cells protects transgenic animals against streptozotocin (STZ)-induced hyperglycemia and isolated islets against cytokines, while animals expressing the mutant variant were susceptible to developing hyperglycemia

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P.I.L. and T.B. contributed equally to this study.

and β-cell death by both treatments. Long-term expression of Pax4 in animals repressed MafA and insulin, resulting in blunted glucose-induced insulin secretion suggesting de-differentiation of β-cells.

RESEARCH DESIGN AND METHODS

Transgenic animals. The pIRES2-DsRedexpress (Clontech) vector was used for the generation of the inducible Pax4 or the mutant variant Pax4R129W cDNA cassette. The final construct contained the tetracycline responsive promoter, the rabbit β-globin intron followed by the Pax4 or Pax4R129W coding sequence. A myc-epitope and polyhistidine tag were added for detection purposes. DsRedexpress was included in the constructs in order to follow Pax4 induction using noninvasive in vivo imaging. Pax transgenic animals were crossed to RIPrtTA mice to generate double transgenic descendants with conditional expression of Pax4 or Pax4R129W specifically in β-cells. Induction of transgene expression was achieved by providing 1 g/L of doxycycline (Sigma-Aldrich) in the drinking water. The GLUT2 knockout mouse has been described elsewhere (15). The Geneva Veterinary Cantonal Office and the CABIMER animal committee approved all experiments.

Glucose and insulin measurements. Animals fasted overnight or for 4 h and were injected intraperitoneally with 2 g glucose/kg body wt or with 1 unit insulin/kg body wt Actrapid (Novo Nordisk Pharma). Blood was collected from tail vein, and glucose levels were measured with a Precision Xceed glucometer. Plasma insulin levels were measured at 0 and 15 min using a mouse insulin ELISA kit (Mercodia, Uppsala, Sweden). Pancreatic insulin content was determined using an insulin enzyme immunoassay kit (SPI-BIO; Bertin Pharma Biotech Division, Brunschwig, Basel, Switzerland).

STZ treatment. STZ was prepared in 0.1 mmol/L sodium citrate at pH 4.5 and administered by intraperitoneal injection (200 mg/kg body wt). Blood glucose was determined before STZ injection and then three times per week.

Mouse islet isolation. Pancreatic islets were isolated by collagenase (Roche, Switzerland) digestion, handpicked and maintained in 11.1 mmol/L glucose/RPMI-1640 (Invitrogen, Switzerland) supplemented with 10% FCS (Brunschwig), 100 units/mL penicillin, and 100 mg/mL streptomycin (Sigma, St-Gallen, Switzerland).

Quantitative real time PCR. Total RNA from islets was extracted using the RNeasy Micro Kit (Qiagen), and quantitative real time PCRs (Q-RT-PCRs) were performed as previously described (16). Primer sequences were designed using the Primer Express Software (Applera Europe) and can be obtained upon request.

Transfection studies. Transient transfections were performed as previously described (11). The luciferase reporter contructs, pFOX-Luc and pFOX-MafAR3-Luc (region 3 of the MafA promoter specifying β-cell expression), were provided by Dr. R. Stein (Vanderbilt University, Nashville, TN).

Cytokine treatment, apoptosis, and nitrite measurements. Islets were isolated from transgenic animals that had received 1 g/L doxycycline in drinking water for 1 month. Islets were cultured for 48 h in the presence of 1 ug/L doxycycline prior to the addition of 0.25 ng/mL interleukin (IL)-1β, 9.1 ng/mL tumor necrosis factor-α, and 100 ng/mL γ-interferon (R&D Systems). Apoptosis was evaluated 24 h posttreatment using the Cell Death ELISA Plus kit (Roche). Alternatively, cell death was estimated by cytochrome C release. Dispersed islets were concentrated by cytospin onto glass slides, and immunofluorescence was performed using anticytochrome C serum (BD Biosciences) and antiserum for the mitochondrial marker TOM 20 (Santa Cruz). Nitrite production in culture media was determined using the Griess reaction (Sigma).

Proliferation assay. Mice were injected with 1 mL BrdU solution/100 g body wt (Roche); 24 h later, they were killed. Proliferation was detected by immunohistochemistry on pancreatic sections.

Immunohistochemistry. For paraffin sections, pancreata were dissected and fixed in 4% paraformaldehyde. Dehydration, embedding, and sectioning were performed at either the Geneva or CABIMER Histology platform. Sections were rehydrated in ethanol and blocked in PBS containing 1% BSA and 0.1% Tween. The following primary polyclonal antibodies were used: guinea pig anti-insulin, mouse anti-glucagon, rabbit anti-pdx1 (1:200; provided by C. Wright, Vanderbilt University), rabbit anti-GLUT2 (1:200), and rabbit anti-MafA (1:200; Bethyl). A mouse monoclonal BrdU was also used. The following secondary antibodies were added: Alexa Fluor 568 goat anti-guinea pig, Alexa Fluor 488 goat anti-mouse, and Alexa Fluor 488 goat anti-rabbit. For cryostat sections, pancreata were frozen in liquid nitrogen prior to sectioning and mounting. Sections were fixed in 4% paraformaldehyde, and immunodetections were performed with the following primary polyclonal antibodies: rabbit anti-Pax4 (provided by Dr. Sosa-Pineda) and guinea pig anti-insulin. Secondary antibodies used were as follows: Alexa Fluor 568 goat anti-rabbit and Alexa Fluor 488 goat anti–guinea pig.

Immunoblot analysis. Islets were harvested in lysis buffer (0.05 mol/L TRIS-HCl, pH 7.5, and 62.5 mmol/L EDTA), cells disrupted by sonication, and proteins resolved by 10% PAGE. Gels were processed for immunoblotting using standard procedures. The following primary polyclonal antibodies were used: rabbit anti-Pax4, goat DsRed (Santa Cruz), and rabbit TFIIE-α (Santa Cruz). A goat anti-rabbit IgG antiserum conjugated to horseradish peroxidase (GE Healthcare) was then added, and immunoreactive products were visualized by chemiluminescence (Pierce, Rockford, IL).

Statistical analysis. Results are expressed as means ± SEM. Statistical differences were estimated using the unpaired *t* test, ANOVA with Bonferroni post hoc test, or nonparametric Mann-Whitney test.

RESULTS

Pax4 overexpression in adult pancreatic β-cells does not impact glucose homeostasis. To study in vivo the role of Pax4 and the impact of a mutant variant associated with diabetes in β-cell plasticity, we generated inducible double transgenic mouse lines expressing RIP-rtTA along with either Pax4/DsRedexpress or Pax4R129W/DsRedexpress. For simplicity, double transgenic rtTA/Pax4 and rtTA/Pax4R129W animals are denoted as Pax4 and Pax4R129W, respectively, while control animals comprising single transgenic littermates are referred to as ST. Q-RT-PCR revealed that both Pax4 and Pax4R129W transcripts were expressed to similar levels exclusively in islets of doxycycline-treated transgenic Pax4 animals, whereas expression of the transgenes was not detected in either control ST mice or in double transgenics that had not been provided with doxycycline (Fig. 1*A*). Western blotting confirmed expression of Pax4 and DsRedexpress in islets of the doxycycline-treated animals (Fig. 1*B*). Immunohistochemistry using either Pax4 or c-myc antisera further substantiated specific expression of Pax4 in islets and revealed colocalization with insulin in most but not all β-cells, suggesting β-cell–restricted expression of the transgene (Fig. 1*C* and *D*).

In parallel, induction of DsRedexpress was monitored by noninvasive in vivo imaging in Pax4 transgenic animals. Fluorescence was detected in the abdominal region of anesthetized living animals treated with doxycycline for 48 h (Supplementary Fig. 1*A*). Imaging of extracted organs confirmed expression of DsRedexpress specifically in the pancreas of treated animals, while other organs exhibited background fluorescence (Supplementary Fig. 1*B*).

Pax4 transgenic animals that received doxycycline for 1 month exhibited normal glucose tolerance and insulin sensitivity (Fig. 1*E* and *F*). Consistent with this finding, glucose-induced insulin secretion was normal in islets isolated from Pax4-expressing mice compared with control ST islets (245 ± 56 vs. 246 ± 55 ng/mL). Similar results were obtained with Pax4R129W animals (data not shown). These results demonstrate that conditional expression of Pax4 can be specifically achieved in islet β-cells and that its forced expression does not alter glucose homeostasis in double transgenic animals.

Overexpression of Pax4 but not Pax4R129W protects mice against STZ-induced hyperglycemia. To assess the protective role of Pax4 in vivo, doxycycline-treated Pax4 or ST animals were challenged with a single high dose of STZ to destroy β-cells. ST animals rapidly developed hyperglycemia while Pax4-overexpressing animals remained normoglycemic throughout the experiment (Fig. 2*A*). For confirmation that Pax4 was mediating the protection against STZ-induced hyperglycemia, doxycycline was removed on day 27 and animals in group A received, 14 days later, a second STZ injection. Group A animals developed hyperglycemia similar to that observed in ST animals, whereas Pax4 animals that did not receive a second STZ injection (group B) remained normoglycemic (Fig. 2*A*). In an independent set of experiments, animals continuously exposed to doxycycline and rechallenged

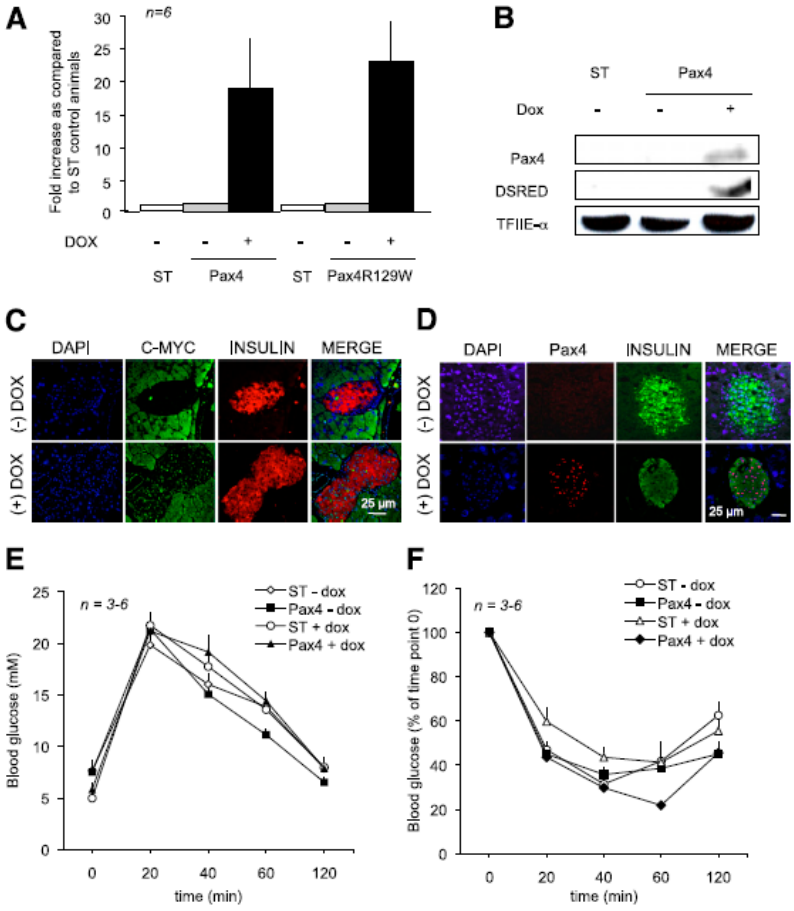

FIG. 1. Selective conditional overexpression of either Pax4 or Pax4R129W in mouse β-cells. *A*: Double transgenic animals bearing either the pTRE-Pax4 or Pax4R129W-myc-His-IRES-DsRedexpress construct along with the RIP-rtTA transgene (denoted as Pax4 or Pax4R129W) were provided with 1 g/L doxycycline (DOX) in drinking water for 1 month. Pax4 and Pax4R129W transcript levels were then evaluated by Q-RT-PCR. Relative mRNA levels were normalized to the transcript levels of the housekeeping genes cyclophilin and/or *rps29*. Data were calculated as fold change compared with ST animals. *B*: Doxycycline-induced Pax4 and DsRedexpress proteins were confirmed by Western blot analysis. Immunofluorescence analysis using antimyc (*C*) or anti-Pax4 (*D*) sera revealed specific expression of the Pax4 protein only in the pancreatic islet β-cells of animals that received doxycycline. Of note, the intense green staining within the islet in the c-myc (−) doxycycline image is nonspecific. *E*: Glucose tolerance tests were performed on control ST animals and Pax4 animals treated or not with doxycycline. *F*: Insulin tolerance tests were also performed on the same animals. Data are expressed as means ± SEM from 3–6 animals per group. (A high-quality digital representation of this figure is available in the online issue.)

with a second high dose of STZ did not develop hyperglycemia, confirming Pax4-dependent protection even after two high doses of STZ (Fig. 2*B*). Pax4R129W animals challenged with a single dose of STZ developed an intermediate level of hyperglycemia, indicating that the diabetes-associated mutant is less efficient in protecting mice against stress-induced hyperglycemia (Fig. 2*C*). Immunohistochemistry combined with morphometry analysis revealed a clear reduction in insulin$^+$ cells, with a concomitant increase in glucagon$^+$ cells in STZ-injected ST animals compared with what was observed in untreated mice (Fig. 2*D* and Supplementary Fig. 2*A* and *B*). In contrast, islet morphology and cell composition in Pax4-overexpressing animals challenged with either one or two doses of STZ were similar to those of islets from control animals (Fig. 2*D* and Supplementary Fig. 2*A* and *B*). Islets from Pax4R129W mice exhibited less insulin staining, suggesting a partial loss of β-cells correlating with intermediate hyperglycemia (Fig. 2*D*). These results show that overexpression of Pax4 protects β-cells against multiple STZ challenges and prevents development of hyperglycemia, whereas the mutant is less effective.

Pax4 but not Pax4R129W protects islets against cytokine-induced apoptosis. For further assessment of the protective action of Pax4, islets isolated from either Pax4 or Pax4R129W transgenic animals were treated with cytokines. Pax4 islets were protected against cytokine-induced cell death compared with ST control animals that displayed a threefold increase in cell death in the presence of cytokines (Fig. 3*A*). Islets isolated from Pax4R129W animals exhibited a similar degree of apoptosis compared with cytokine-treated ST animals (Fig. 3*A*).

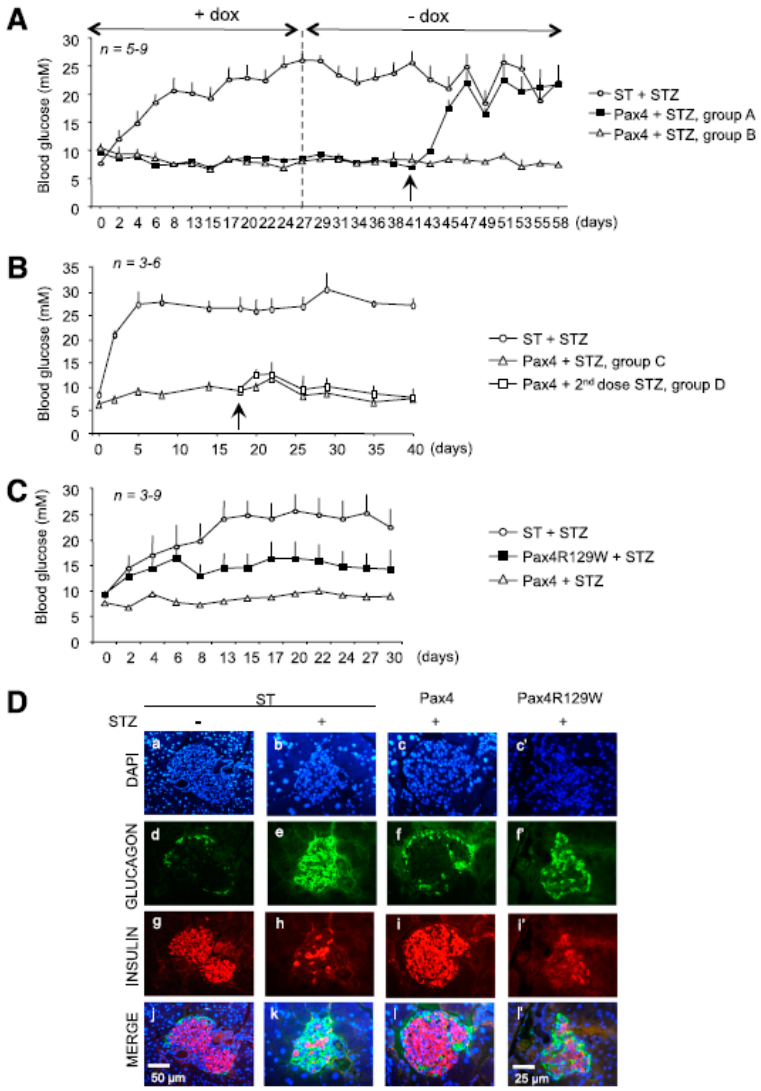

FIG. 2. Pax4-overexpressing animals are protected against STZ-induced hyperglycemia. *A*: A single high dose of STZ (200 mg/kg body wt) was injected in control (ST) and Pax4 animals treated with doxycycline (dox) (groups A and B). Blood glucose levels were measured three times per week for up to 58 days. All animals prior to STZ injection received doxycycline in drinking water for 1 month starting at weaning. For further assessment of the protective role of Pax4, doxycycline was removed from the drinking water at day 27. Blood glucose levels were followed for 2 weeks before a second injection of STZ on day 41 was administered to group A (arrow) while group B received only the vehicle. *B*: Alternatively, Pax4 animals maintained on doxycycline (group D) were challenged with a second high dose of STZ (arrow) and glycemia was monitored for an extra 22 days. Group C animals were sustained on doxycycline without an STZ rechallenge. *C*: Pax4, Pax4R129W, and ST animals were injected with STZ; glycemia was then monitored for 30 days. Data are expressed as the means ± SEM from 3–9 animals per group. Statistical difference was tested by the nonparametric Mann-Whitney test (significance from $P < 0.05$). *D*: Immunohistochemical analysis of glucagon (green) and insulin (red) on representative pancreatic paraffin sections from doxycycline-treated ST, Pax4, and Pax4R129W mice challenged or not with STZ. Animals were killed on day 27, and paraffin sections were prepared from ST animals that received citrate buffer injection (a, d, g, and j) or challenged with STZ (b, e, h, and k) and from Pax4 (c, f, i, and l) or Pax4R129W (c', f', i', and l') animals injected with STZ injection. Bars, 50 µm for a, d, g, and j and 25 µm for remaining panels. (A high-quality digital representation of this figure is available in the online issue.)

Exogenous IL-1β was shown to induce endogenous levels of IL-1β and to lead to human islet β-cell death (17). Interestingly, IL-1β transcript levels were suppressed by 50% in islets isolated from Pax4-overexpressing animals. In contrast, Pax4R129W mutant mice exhibited a twofold increase in IL-1β transcript levels compared with control untreated ST islets (Fig. 3*B*). A parallel modulation in transcript levels of the IL-1β antagonist was observed in both transgenic animal models, whereas expression levels of the IL-1β receptor remained unaltered (Fig. 3*B*).

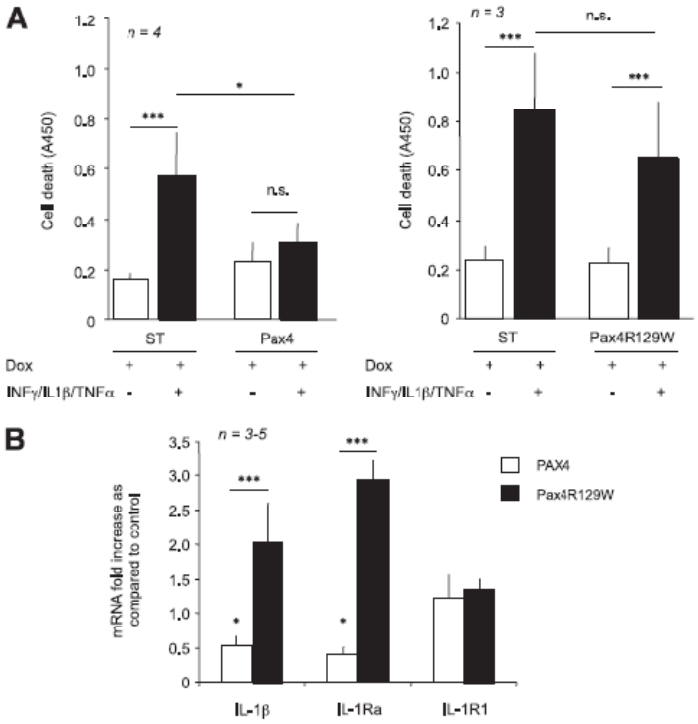

FIG. 3. Isolated islets from Pax4 transgenic mice are protected against cytokine-induced apoptosis. *A*: Freshly isolated islets from ST animals and double transgenic animals (Pax4, *left panel*, and Pax4R129W, *right panel*) were cultured with 1 μg/L doxycycline for 48 h and subsequently challenged with a mix of IL-1β, γ-interferon (INFγ), and TNF-α. Cell death was measured using a cell death detection ELISA kit. Results are expressed as means ± SEM of three or four independent experiments relative to nontreated islets. *B*: IL-1β, IL-1Ra, and IL-1R1 mRNA expression levels were measured by Q-RT-PCR in either Pax4- or PaxR129W-overexpressing animals. Relative mRNA levels were normalized to the transcript levels of the housekeeping gene cyclophilin. Data were calculated as fold change compared with ST animals that received doxycycline (dashed line) and expressed as means ± SEM from 3–5 animals per group. Statistical difference was tested by one-way ANOVA with Bonferroni post hoc test. *$P < 0.05$ and ***$P < 0.001$.

Mitochondrial cytochrome C release is blunted in Pax4-overexpressing islets treated with cytokines. A hallmark of cytokine-mediated cell death is the activation of the nuclear factor (NF)-κB pathway, which leads to enhanced NOS2 and COX2 expression and the downstream increase in nitric oxide (NO) production, ultimately resulting in cytochrome C release from mitochondria (18). Consistent with this model, islets isolated from Pax4 mice and exposed to cytokines in the absence of doxycycline exhibited a 2.5-fold increase in cytochrome C release. In contrast, cytochrome C release was abrogated in doxycycline-treated Pax4 islets, whereas Pax4R129W-overexpressing islets displayed a threefold increase compared with noncytokine-treated islets (Fig. 4*A* and *B*). Interestingly, NOS2 transcript levels were only significantly elevated in Pax4R129W islets, whereas COX2 levels were augmented in both mutant and wild-type Pax4 islets (Fig. 4*C*). Despite differences in NOS2 expression, both Pax4 and Pax4R129W islets presented a significant increase in cytokine-induced nitrite production, an indicator of NO production (Fig. 4*D*). However, Pax4 islets displayed a trend toward a small decrease in nitrite production compared with non–doxycycline-treated islets.

Overexpression of Pax4 alters expression of MafA, GLUT2, insulin, and Bcl2. To further delineate the mechanism by which Pax4 protects β-cells from either STZ- or cytokine-induced apoptosis, we determined mRNA expression levels of key β-cell markers and those of antiapoptotic genes in islets of Pax4- and Pax4R129W-overexpressing animals (Fig. 5*A*). Transcript levels of Pdx1 and Pax6 were not altered in Pax4- or Pax4R129W-overexpressing islets, whereas MafA, GLUT2, and insulin levels were decreased in both animal models. Immunohistochemistry confirmed decreased MafA and GLUT2 protein levels in islets of Pax4-expressing mice (Fig. 5*B* and *C*). Bcl-2 but not Bcl-xL expression levels were increased threefold specifically in Pax4 islets compared with those in control animals without doxycycline administration (Fig. 5*A*).

Because insulin and GLUT2 are target genes of MafA (19,20) and because a Pax4 consensus sequence was predicted in region 3 of the MafA promoter (21), we investigated whether Pax4 could directly regulate MafA transcription. Pax4 and Pax4R129W dose dependently inhibited a luciferase reporter construct driven by region 3 of the MafA promoter, reaching 50% inhibition at the highest dose (Fig. 5*D*). Repression was specific; Pax4 was unable to inhibit the control pFOX-Luc construct. However, Pax4 failed to interact with the putative binding site identified in this MafA region (MafAR3Pax4), whereas it displayed specific binding to the G3 element of the glucagon promoter (data not shown) (22).

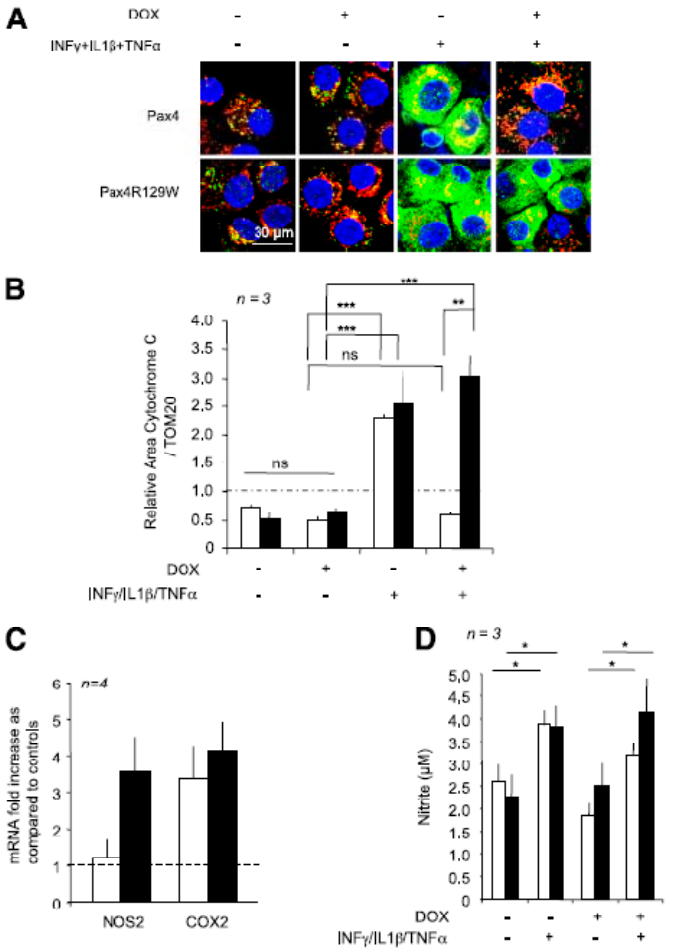

FIG. 4. Pax4 overexpression protects from cytokine-induced β-cell death by impeding mitochondrial cytochrome C release. *A*: **Immunofluorescent localization of cytochrome C (green), TOM-20 (red), and DAPI (blue) within islet cells treated with or without cytokines in the presence or absence of either Pax4 (□) or Pax4R129W (■) expression.** *B*: **Quantification of cytochrome C release from the mitochondria. Metamorph software was used to measure the area of red (TOM-20) and green (cytochrome C) fluorescence. A total of 20 images, each containing 4–5 cells, were taken per condition and per mouse in three independent experiments. Total green fluorescence (cytochrome C) area was then divided by total red fluorescence (TOM-20) area. Values >1 indicate that cytochrome C is external to the mitochondria, and values ≤1 indicate that it is inside the organelle.** *C*: **Q-RT-PCR analysis of nos2 and cox2 transcript levels in islets of Pax4 and Pax4R129W transgenic animals treated with doxycycline. Relative mRNA levels were normalized to the transcript levels of the housekeeping gene cyclophilin. Data were calculated as fold change compared with ST animals that received doxycycline (represented by dashed line) and expressed as the means ± SEM from four animals per group.** *D*: **Nitrite release in culture media was estimated using the Griess reagent. Data were expressed as means ± SEM from three independent experiments. Statistical difference was tested by one-way ANOVA with Bonferroni post hoc test. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. INFγ, γ-interferon. (A high-quality digital representation of this figure is available in the online issue.)**

GLUT2 heterozygous mice are protected against STZ-induced hyperglycemia. STZ is selectively taken up by β-cells through GLUT2 (23). Because GLUT2 levels were decreased in Pax4-overexpressing islets, we determined whether such inhibition would be sufficient to protect β-cells against STZ. To this end, the GLUT2 heterozygous mouse model was used in order to directly measure the impact of the glucose transporter in STZ-mediated cell death. Transcript levels of the transporter were reduced by 60% in heterozygous GLUT2 mice (Fig. 6*A*). Similarly to Pax4 mice, these animals were refractory to STZ-induced β-cell apoptosis, whereas control wild-type littermates were sensitive to the toxic agent (Fig. 6*B*). These results suggest that the protective effect of Pax4 against STZ is potentially partially conveyed by reduced GLUT2 expression. However, despite similarly lower levels of GLUT2, Pax4R129W mice are only partially protected against STZ, suggesting that alternative detrimental processes are associated with the mutant phenotype.

Transient overexpression of Pax4 improves total pancreatic insulin content after 4 months. Adenoviral-mediated overexpression of Pax4 in cultured rat islets was shown to hamper insulin secretion (11). Although this effect was not observed in Pax4 transgenic animals treated

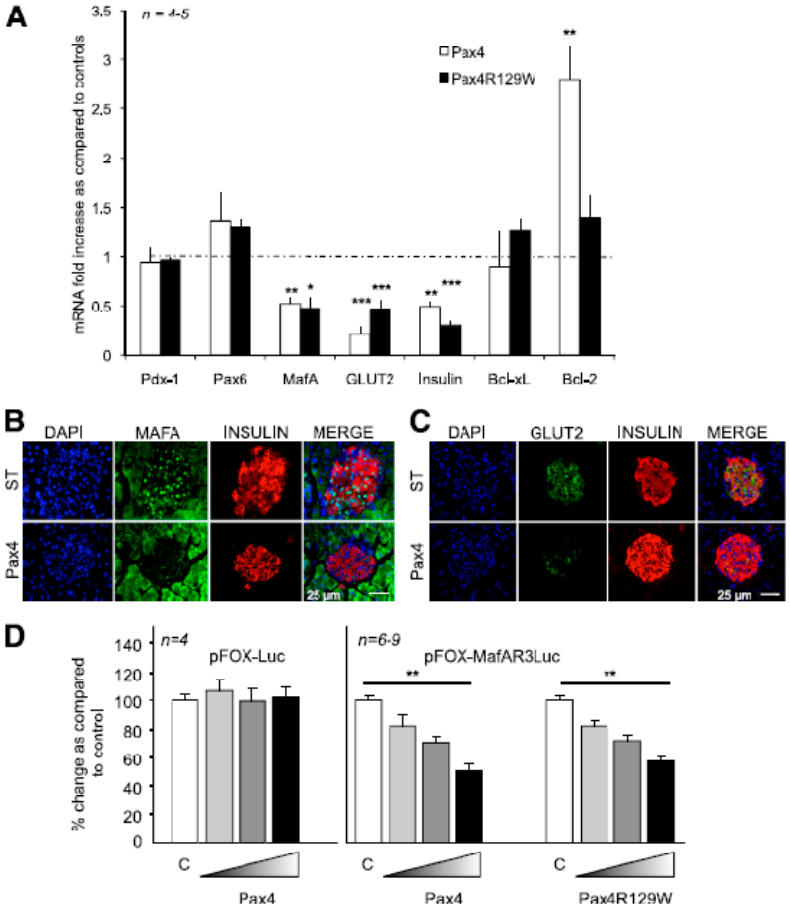

FIG. 5. Induction of Pax4 in islets suppresses MafA, insulin, and GLUT2 expression, whereas it increases Bcl-2. *A*: Q-RT-PCR was performed on islets isolated from doxycycline-treated Pax4 and Pax4R129W animals. Relative mRNA levels were normalized to the transcript levels of the housekeeping genes cyclophilin and/or *rps29*. Data were calculated as fold change compared with ST animals that received doxycycline (dashed line) and are expressed as means ± SEM from 4–5 animals per group. Statistical difference was tested by one-way ANOVA with Bonferroni post hoc test (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$). Immunohistochemistry analysis of MAFA (*B*) and GLUT2 (*C*) was performed on pancreatic sections from Pax4-overexpressing animals and in control ST animals treated with doxycycline. *D*: Transient cotransfection studies using INS-1E cells were performed with increasing amounts of either Pax4 or PaxR129W expression vectors (0.25, 0.50, 0.75, and 1 mg) along with 500 ng of either pFOX-Luc or pFOX-MafAR3-Luc. Data are presented as fold change of basal luciferase activity and expressed as the means ± SEM of three experiments (*$P < 0.05$). (A high-quality digital representation of this figure is available in the online issue.)

with doxycycline for 1 month, plasma insulin levels in response to glucose were greatly reduced in animals treated for 4 months (Fig. 7*A*). This was accompanied by glucose intolerance in these animals (Fig. 7*B*). Furthermore, morphological analysis of pancreas isolated from Pax4-expressing mice revealed altered islet architecture typified by an increased number of enlarged intercellular spaces, reminiscent of capillaries detected in control islets (Supplementary Fig. 3). To determine whether this effect was reversible, we removed doxycycline from the drinking water of 4-month–treated animals to repress Pax4 expression and reevaluated plasma insulin levels 1 month later. Consistent with our premise, the rise in plasma insulin levels in response to glucose was rescued in transgenic animals subsequent to removal of doxycycline (Fig. 7*C*). Total pancreatic insulin content was significantly increased in Pax4 mice after removal of doxycycline (Fig. 7*D*).

Long-term (4 months) but not short-term (1 month) Pax4 overexpression increases islet β-cell replication. The increase in insulin content observed after doxycycline removal (Fig. 7*D*) suggests that long-term expression of Pax4 may induce β-cell replication. Consistent with this premise, islets isolated from Pax4 mice that underwent 4-month but not 1-month doxycycline treatment showed reduced glucose-induced plasma insulin levels (Fig. 7*A*) and exhibited increased BrdU labeling of β-cells compared with control ST islets (Fig. 8*A*). This was accompanied by an overall nonsignificant increase in the proportion of β-cells (Fig. 8*B*). A $BrdU^+$/insulin$^-$ cell subpopulation was also detected specifically in Pax4-overexpressing islets (Fig. 8*A*). These cells were also negative for glucagon and

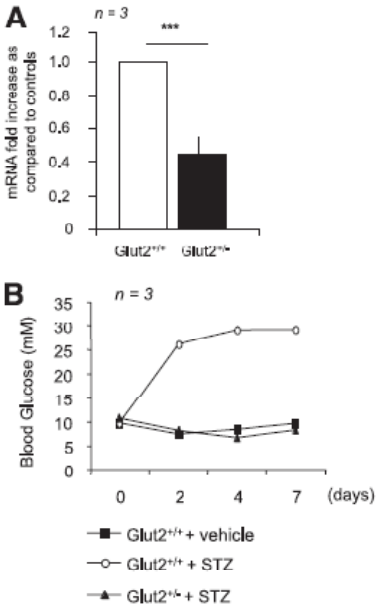

FIG. 6. GLUT2 heterozygous mice are protected against STZ-mediated hyperglycemia. *A*: GLUT2 diabetes mRNA expression levels were measured by Q-RT-PCR in isolated islets from GLUT2$^{+/+}$ and GLUT2$^{+/-}$ mice. Relative mRNA levels were normalized to the transcript levels of the housekeeping gene cyclophilin. Data were calculated as fold change compared with GLUT2$^{+/+}$ mice and expressed as means ± SEM from three animals per group. Statistical difference was tested by unpaired *t* test (*$P < 0.001$). *B*: GLUT2$^{+/+}$ and GLUT2$^{+/-}$ male mice were injected with a single dose of STZ (200 mg/kg body wt), and glycemia was monitored for up to 7 days. Data are expressed as means ± SEM from three animals per group. Statistical difference was tested by the nonparametric Mann-Whitney test.**

somatostatin staining. Given that Pax4 overexpression impedes insulin transcription (Fig. 5*A*), we reasoned that these cells were likely β-cells with low levels of insulin. Therefore, Pdx1 was used as an alternative β-cell marker. Most BrdU$^+$ cells were also Pdx1$^+$, and the total percentage of these cells was equivalent to the sum of the insulin$^+$ and insulin$^-$ cell subpopulations that were BrdU$^+$ (compare Fig. 8*A* and *C*). Despite a significant increase in overall proliferation (Fig. 8*C*), we did not detect an increase in the total population of Pdx1$^+$ cells (Fig. 8*D*) or an increase in islet mass due to a low number of proliferating cells. Consistent with increased long-term cell replication, c-myc and Cdk4 but not Cdk2 or 6 were increased in Pax4 overexpressing islets (Fig. 8*E*).

DISCUSSION

Our study highlights the physiological role of Pax4 in vivo as a regulator of mature islet β-cell survival and proliferation. Pax4 prevented development of hyperglycemia in animals exposed to environmental insults such as repeated STZ challenges and protected islets against cytokine-mediated apoptosis. The diabetes-associated R129W mutation was less efficient in protecting animals against STZ-induced hyperglycemia. The detrimental effect of the mutation was only revealed in the presence of harmful environmental cues highlighting the interplay between genetic determinants and the environment in the development of hyperglycemia (24). Thus, in response to physiological demand or pathophysiological situations, Pax4 expression may be transiently induced to protect and to compensate β-cell mass, whereas the mutant variant would be unable to achieve this, resulting in the gradual loss of β-cells and ultimately diabetes. The regulation of Pax4 expression by environmental factors provides a likely explanation for the failure of GWAS to identify *Pax4* as a diabetic susceptibility gene as well as for discrepancies in different studies to validate the association of Pax4 polymorphisms with type 2 diabetes in various ethnic populations (7).

One mechanism by which Pax4, but not Pax4R129W, protects β-cells against STZ- and cytokine-induced cell death may, in part, be connected to the regulation of selective target genes of the NF-κB signaling cascade (25). Indeed, we found that transcript levels of IL-1β, the predominant cytokine implicated in the activation of the NF-κB pathway, were decreased in Pax4-expressing islets, whereas they were increased in Pax4R129W islets. Consistent with NF-κB activation, transcript levels of its target gene *nos2* were selectively higher in mutant animals, whereas levels of the target gene *cox2* were increased in both Pax4 and Pax4R129W. NOS2 is involved in NO production and downstream stimulation of apoptosis (25), whereas the COX2-generated metabolite prostaglandine E_2 was found to be antiapoptotic (26). As IL-1β was shown to autostimulate expression levels of endogenous IL-1β (17) and to induce β-cell death in human islets (27), Pax4-mediated inhibition of IL-1β transcription with a concomitant increase in COX2 levels may confer increased resistance to apoptosis. In contrast, elevated basal levels of IL-1β and NOS2 in Pax4R129W-overexpressing mice may sensitize islet cells to NO-induced apoptosis. Furthermore, because NO can also be released from islets exposed to STZ (28), we suggest that Pax4R129W mice are more prone to cell death than GLUT2 heterozygous mice because of an increased predisposition to NO-mediated apoptosis. Interestingly, the levels of the IL 1β antagonist, IL 1Ra, were also increased in mutant islets, suggesting restoration of an IL-1β–to–IL-1Ra ratio that prevents activation of the apoptotic program (29). Consistent with this model, several putative Pax4 binding sites were predicted in both the *il-1β* and *nos2* gene promoters.

Despite differences in Il-1β levels, both Pax4- and Pax4R129W–expressing islets exhibited similar levels of NO production upon exposure to cytokines. This indicates that the NF-κB signaling pathway can still be fully activated upon strong exogenous stimulation and that the protection conveyed by Pax4 is relayed through additional gene product that blocks the NF-κB apoptotic pathway. One such candidate gene could be *Bcl-2*, which regulates the mitochondrial response to proapoptotic signals, preventing the release of cytochrome C and the subsequent activation of the cell death program (30). Indeed, we found that Pax4- but not Pax4R129W-overexpressing islets exhibited increased expression of Bcl-2 with blunted cytokine-mediated cytochrome C translocation. Corroborating our results, Bcl-2 overexpression was shown to protect mouse and human islets against cytokine-induced apoptosis (31,32). In contrast to our previous study in rat islets overexpressing Pax4 (11), the increased expression of Bcl-xL in mouse islets was not detected, possibly as a result of species differences.

Overexpression of either Pax4 or Pax4R129W markedly perturbed expression of MafA and two of its target genes, *glut2* and *insulin*, which are key markers of mature

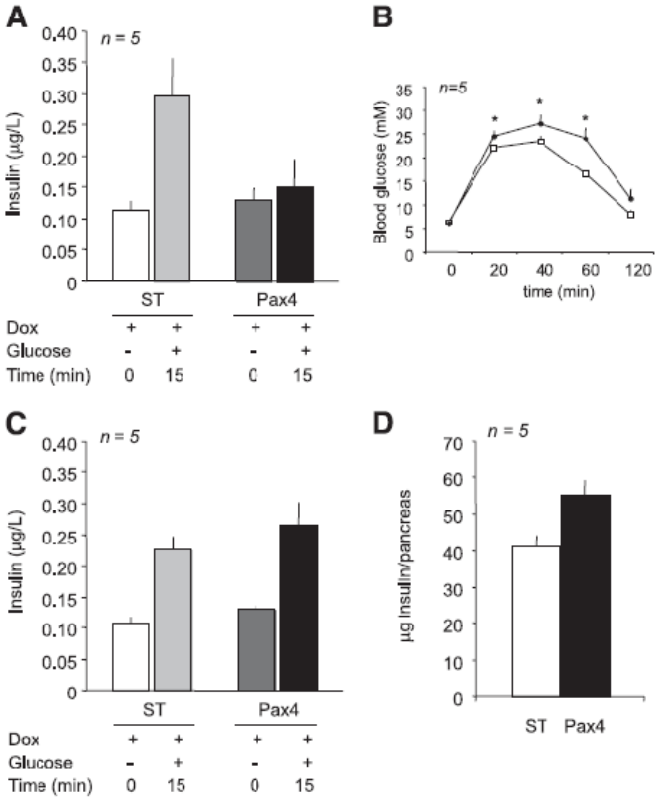

FIG. 7. Transient overexpression of Pax4 improves glucose tolerance and total pancreatic insulin content after 4 months. *A*: Plasma insulin levels, measured 15 min after an injection of 2 g glucose/kg body wt in Pax4-overexpressing animals and in ST animals that were maintained on doxycycline (Dox) for 4 months. *B*: Glucose tolerance tests were performed in the same animals (Pax4-overexpressing [●] and ST [□] animals that were maintained on doxycycline for 4 months). *C*: After doxycycline treatment was abolished for 1 month, plasma insulin levels were reevaluated as in *A*. *D*: Total pancreatic insulin content was measured as indicated in RESEARCH DESIGN AND METHODS in Pax4 animals that received doxycycline for 4 months and then were not treated for 1 month. Data are expressed as means ± SEM from five animals per group. Statistical difference was tested by *t* test (*$P < 0.05$).

β-cells. Although both Pax4 proteins suppressed the activity of a luciferase construct harboring the MafA gene promoter region 3, we could not detect Pax4 binding to a putative binding site within this region. Preliminary ChIP assay results revealed no convincing interaction of Pax4 with region 3, suggesting an indirect effect of Pax4 on MafA expression. However, we have previously demonstrated that Pax4 impairs glucagon and insulin gene transcriptions through direct protein interaction with Pax6 (22). Given that Pax6 was recently shown to bind to the *mafa* gene promoter and to regulate transcription (33), we speculate that Pax4 may be inhibiting MafA expression by a mechanism similar to the one delineated for glucagon and insulin. Inhibition through protein-protein interaction, and not direct DNA binding activity, would explain the inhibitory effect observed with the weaker DNA-interacting mutant variant.

Long-term overexpression of Pax4 resulted in a blunted glucose-induced rise in plasma insulin levels—an effect reversible upon inhibition of Pax4. A recent study also reported impaired glucose-induced insulin secretion in an animal model in which Pax4 was constitutively overexpressed in α-cells. These cells were converted to β-cells that remained functional for up to 4 weeks before an unexpected decline in insulin secretion occurred. Animals ultimately died of hyperglycemia (8). We would therefore like to propose that Pax4-dependent impaired insulin secretion is mediated by the long-term repression of the mature β-cell marker MafA and insulin.

Aberrant expression of *Pax* genes were shown to convert cells to a less differentiated state and to promote self-renewal and increasing survival (34). Consistent with this premise, a subpopulation of replicating $Pdx1^{+}$/$insulin^{-}$ cells was identified in Pax4-overexpressing islets. These cells were also negative for both glucagon and somatostatin, suggesting that they likely represent insulin-deprived β-cells resulting from repression of MafA and insulin transcription by Pax4. A similar β-cell subpopulation was recently characterized in vitro (35). These cells displayed impaired glucose-induced insulin secretion and were prone to proliferation in response to activin A (36). More importantly, MafA and GLUT2 transcript levels were lower in this subpopulation, whereas Pax4 levels were higher. Taken together, these results suggest that Pax4 under physiological

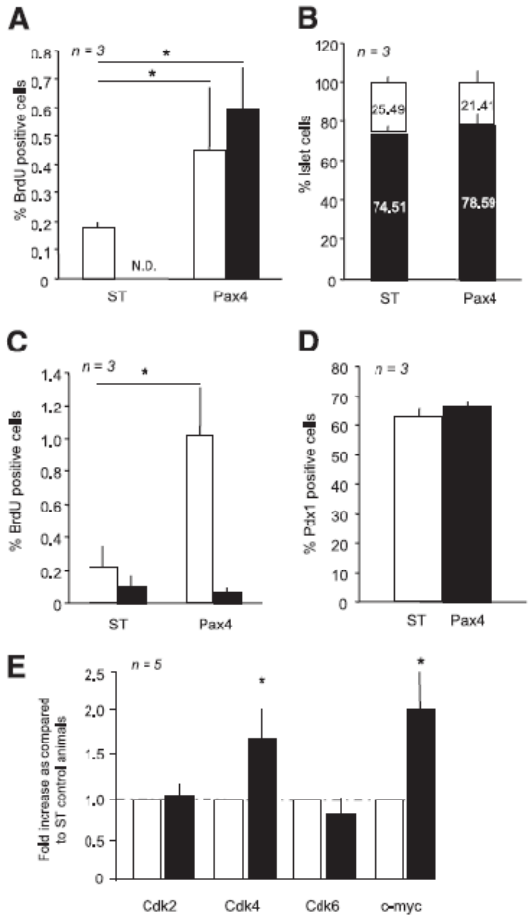

FIG. 8. Long-term expression of Pax4 increases β-cell proliferation. *A*: Pancreatic sections from 3 months' Pax4-overexpressing animals were coimmunostained for BrdU and insulin. Approximately 8,000 β-cells were counted from five different pancreatic sections separated 50 μm from each other. □, BrdU$^+$/insulin$^+$; ■, BrdU$^+$/insulin$^-$. ND, not detected. *B*: The proportion of insulin$^-$ (□) and insulin$^+$ (■) cells was counted on the slides from *A*. *C*: Pancreatic sections from 3 months' Pax4-overexpressing animals were coimmunostained for BrdU and Pdx-1. Cell counting was performed as in *A*. □, BrdU$^+$/Pdx-1$^+$; ■, BrdU$^+$/Pdx-1$^-$. *D*: Total number of Pdx-1$^+$ cells was counted on the slides from *B*. □, BrdU$^+$/Pdx-1$^+$; ■, BrdU$^+$/Pdx-1$^-$. *E*: Transcript levels of proliferative genes were assessed by Q-RT-PCR on freshly isolated islets from Pax4-overexpressing animals (■). Relative mRNA levels were normalized to the transcript levels of the housekeeping gene cyclophilin. Data were calculated as fold change compared with ST animals (□) that received doxycycline (dashed line) and are expressed as means ± SEM from five animals per group. Statistical difference was tested by *t* test (*$P < 0.05$).

conditions is most likely expressed in a subpopulation of β-like cells prone to proliferation. Substantiating this hypothesis, Cdk4 and c-myc transcript levels were increased in Pax4-overexpressing islets. Adenovirus-mediated Cdk4 expression was shown to increase mouse islet cell proliferation (37), whereas its repression causes insulin deficiency and hyperglycemia (38). Similarly, β-cell–specific overexpression of c-myc caused downregulation of insulin gene expression with a concomitant increase in proliferation and apoptosis (39). That a fraction of islet β-cells responds to proliferation would provide a mechanism by which a pool of mature cells can rapidly undergo regulated replication to compensate for lost cells while quiescent cells retain the ability to regulate blood glucose.

In summary, in vivo overexpression of Pax4 reveals an important role of the transcription factor in islet β-cell proliferation and survival. The detrimental impact of Pax4 mutations is only revealed upon environmental stresses, which reinforces the critical role of gene-environment interaction in the development of diabetes.

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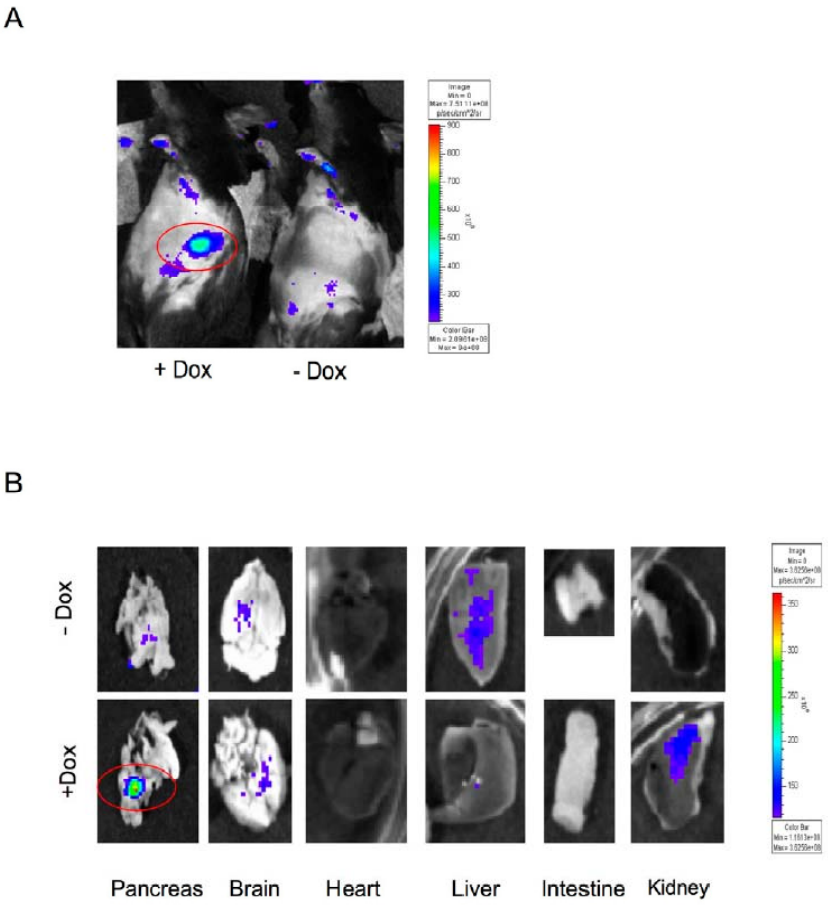
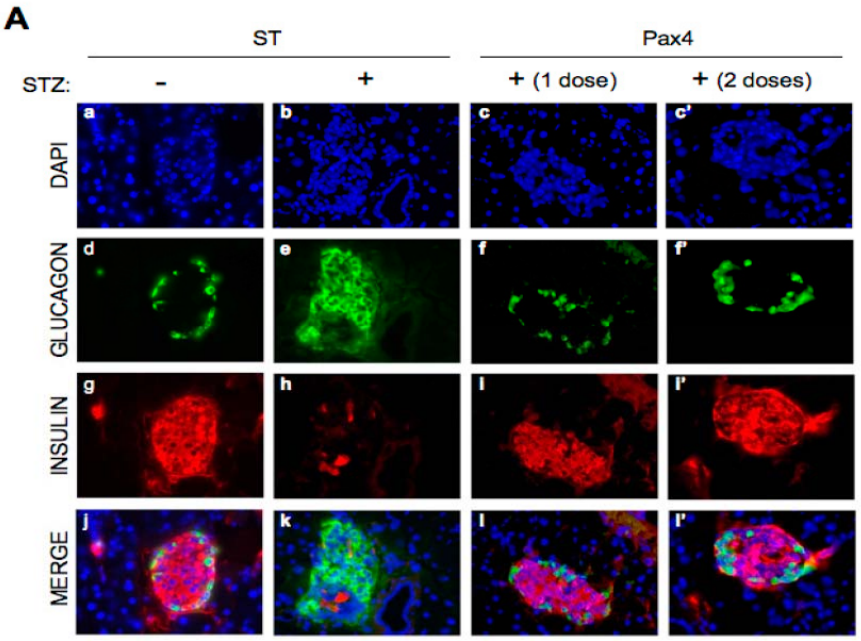
SUPPLEMENTARY DATA

Supplementary Figure 1. Non-invasive in vivo imaging through DsRedexpress fluorescence monitor pancreatic-specific induction of Pax4 expression after doxycycline induction. (A) animals were given, or not, 1 g/L oral doxycycline for 48 hours. Animals were then anesthetized imaged for DsRedexpress fluorescence using the Xenogen IVIS 200 imaging system (Caliper Sciences AG, Oftringer, CH). Animals were shaved to minimize background emission. Doxycy treated animals (Left panel), but not control untreated (right panel), emitted fluorescence i anatomical region corresponding to the pancreas. (B) Animals were sacrificed and individual o isolated and imaged for fluorescence. Only the pancreas of doxycycline-treated animals exh specific DsRedexpress fluorescence.



SUPPLEMENTARY DATA

Supplementary Figure 2. Pancreatic islet morphology in Pax4 overexpressing transgenic animals is preserved subsequent to two consecutive high dose-streptozotocin treatments. (A) Immunohistochemistry on pancreatic paraffin sections from mice sacrificed at day 40 post STZ-treatment, revealed intact islets in Pax4 overexpressing animals; (a, d, g, j) are sections from control ST animals that received citrate buffer injection; (b, e, h, k) are sections from control ST animals that received STZ injection; (c, f, i, l) are sections from Pax4 animals that received 1 dose of STZ and (c', f', i', l') are sections from Pax4 animals that received 2 doses of STZ. Images were acquired using a Zeiss epifluorescence microscopy. (B) Summary of the morphometry analysis performed on the various experimental groups. Images were acquired using a TCSSP 5 Leica confocal microscope and analyzed using the MetaMorph software (version 7.5.1.0, Molecular Devices). Two thousand islet cells were counted on average per group and results are presented as percentage of either insulin or glucagon cells over the total number of DAPI positive cells per islet.



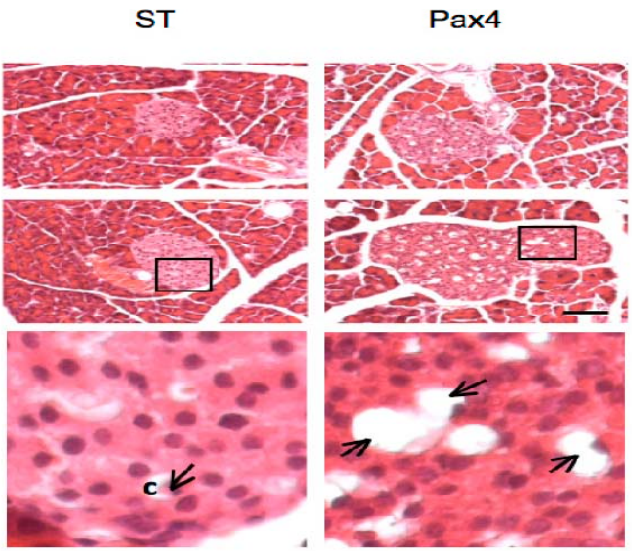
B

	ST		Pax4	
STZ:	-	+	+ (1 dose)	+ (2 doses)
Glucagon (%)	23.9 ± 3.8	40.5 ± 5.7	18.3 ± 6.2	25.6 ± 3.3
Insulin (%)	75.7 ± 2.8	18.3 ± 4.3	79.4 ± 3.5	71.6 ± 3.9

Data are means ± SEM

SUPPLEMENTARY DATA

Supplementary Figure 3. Long-term Pax4 overexpression alters islets architecture. Hematoxylin-eosin staining of paraffin sections derived from either control (ST) or Pax4 transgenic animals treated for 4 months with DOX. Islets isolated from Pax4 overexpressing animals display large opened vessels reminiscent of capillaries (c) detected in control islets (arrows). The bottom panels are enlargements of the islet areas boxed in the middle panels. Bar, 25 m.



4.2. Regulation of ER Homeostasis and Cell Cycle by Pax4 Thrives a Healthy β-cell Mass and Protects Against Experimental Autoimmune Diabetes

OBJECTIVE

This study investigates the role of the transcription factor Pax4 and its diabetes-linked mutant variant Pax4R129W in β-cell regeneration and survival in a pernicious environment of autoimmunity using the transgenic RIP-B7.1 mouse model of experimental autoimmune diabetes (EAD).

RESULTS and CONCLUSIONS

Our results reported that overexpression of Pax4, but not the T2DM-linked mutant variant Pax4R129W, in adult β-cells avoid the development of hyperglycemia in the RIP-B7.1 mouse model of T1DM through reduced insulitis, decreased β-cell apoptosis correlating with diminished DNA damage and increased proliferation. Transcriptome profiling revealed up regulation of genes involved in immunomodulation, cell cycle and ER homeostasis in islets overexpressing Pax4 as compared to the Pax4R129W overexpressing islets. Pax4 but not Pax4R129W protected islets from thapsigargin-mediated ER-stress apoptosis. We conclude that Pax4 regulated important pathways related to maintain healthy β-cells mass under adverse environmental conditions of autoimmunity.

CONTRIBUTION

My contribution in this article has been participating in the planning, design and execution of the experiments corresponding to different figures. Therefore, my work consisted in collaborating in the management of Pax4 and Pax4R129W mice colony and in the administration of doxycycline. I have conduted the compilation of islets samples of the different experimental groups, as well as the RNA extraction and analysis of microarray data using several softwares such as KEGG, GeneOntology and IPA for the transcriptome profile study (Figure 7A, B and C). Furthermore, I actively participated in immunohistochemical staining and analysis of DNA damage in Pax4 overexpressing animals in two independent models, STZ and EAD (Figure 5C). I also monitored Pax4 expression by DsRed fluorescence using the ImagExpress MycroSystem in living isolated islets from Pax4 transgenic mice (Figure 8A). Additionally, I performed the thapsigargin-induced ER stress apoptosis experiment in isolated mouse islets overexpressing Pax4 or Pax4R129W depicted in Figure 8B. These results confirmed that Pax4, but not Pax4R129W, favored DNA repairing, modulated genes involved in cell cycle and ER homeostasis and protected against thapsigargin-induced apoptosis resulting in preserved β-cell mass. Likewise, I actively participated in the discussion of the results of the project.

Regulation of ER Homeostasis and Cell Cycle by Pax4 Thrives a Healthy β-cell Mass and Protects Against Autoimmune Diabetes

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Running title: Pax4 and Experimental Autoimmune Diabetes

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SUMMARY

A hallmark of both Type 1 and Type 2 Diabetes Mellitus (T1DM and T2DM) is the gradual deterioration of the functional β-cell mass resulting in hyperglycemia. Strategies to enhance islet β-cell survival and regeneration while refraining inflammation through manipulation of molecular targets would provide means to normalize glucose homeostasis. Herein we report that over expression of the islet enriched transcription factor Pax4 refrains development of hyperglycemia in the RIP-B7.1 mouse model of T1DM through reduced insulitis, decreased β-cell apoptosis correlating with diminished DNA damage and increased proliferation. Transcriptome profiling revealed up regulation of genes involved in immunomodulation, cell cycle and endoplasmic reticulum homeostasis in islets over-expressing Pax4 as compared to the T2DM-linked mutant variant Pax4R129W. Pax4 but not Pax4R129W protected islets from thapsigargin-mediated ER-stress apoptosis. We conclude that, under pathophysiological conditions, the coordinated regulation of distinct cellular pathways by Pax4 conveys adaptive properties to islets, not attained by the diabetes-linked mutant variant Pax4R129W.

INTRODUCTION

The islet of Langerhans is the core unit of the endocrine pancreas that regulates blood glucose homeostasis. This physiological task is achieved by the release of insulin from β-cells in response to increasing levels of glucose and secretion of glucagon from α-cells in fasting conditions. Imbalance in this intricate circuitry leads to either hyperglycemia, the hallmark of Diabetes Mellitus (DM), or to fatal hypoglycemia. Loss of β-cell function coupled to insulin resistance of target tissues nornally associated with obesity and to a chronic low grade inflammation defines Type 2 DM (T2DM) (Donath, 2014), while T-lymphocytes autoimmune-mediated destruction of β-cells, is characteristic of Type 1 DM (T1DM) (Tisch and Wang, 2008). Genetic as well as environmental factors contribute to the loss of a functional β-cell mass, which is common to both T1DM and T2DM (Tuomi et al., 2014).

Emerging evidence suggests that alterations in the function of the endoplasmic reticulum (ER) induced by the diabetic milieu, contribute, together with the increased production of ROS and DNA damage to the cytokine-induced loss of β-cells in both T1DM and T2DM (Papa, 2012). In an attempt to re-establish ER functionality, all cell types including β-cells will mount the unfolded protein response (UPR) operated through activation of the ER membrane bounded inositol- requiring protein-1α (IRE1α), activating transcription factor-6 (ATF6) and protein kinase RNA (PKR)-like endoplasmic reticulum kinase (PERK). Pending on whether the UPR is successful in restoring ER homeostasis the two possible outcomes are survival or death (Cao and Kaufman, 2014). This delicate balance in cell faith decision is particularly well illustrated in the human Wolfram and Wolcott-Rallison syndromes, in which mutations in UPR genes result in unresolved ER-stress, favoring β-cell death that ultimately leads to early-onset DM (Delepine et al., 2000; Inoue et al., 1998). In addition, transcription factors such as Pdx1 and HNF-1α, for which gene mutations cause different forms of maturity onset diabetes of the young (MODY) also regulate UPR-associated genes (Kirkpatrick et al., 2011; Sachdeva et al., 2009). These clinical conditions suggest that transcription factors abundant in pancreatic islets may stimulate insulin biosynthesis and secretion, while preserving the β-cell mass by limiting ER stress.

The family of paired box (*PAX*) genes encodes transcription factors that are critical for tissue development and cellular differentiation, by promoting cell proliferation, migration and survival (Robson et al., 2006). In the pancreas, Pax4 is necessary for the generation of islet cell progenitors and for the subsequent differentiation of β-cells (Greenwood et al., 2006; Sosa-Pineda et al., 1997). Mutations and polymorphisms in the gene coding for this transcription factor have been associated with both T1DM and T2DM in several ethnic populations, indicating a key role of Pax4 during development and in the maintenance of the β-cell mass in mature islets (Brun and Gauthier, 2008).

Accordingly, the conditional over-expression of Pax4 in adult β-cells blocked the development of hyperglycaemia in mice exposed to repeated streptozotocin (STZ) challenges, and protected islets against cytokine-mediated apoptosis, whereas animals conditionally expressing the T2DM-linked variant Pax4R129W (R121W in humans) were more susceptible to develop hyperglycemia as a result of increased sensitivity of β-cells to the cytotoxic effect of STZ (Hu He et al., 2011a). Strikingly, and despite differences in the levels of Il-1β and nitric oxide synthase 2 (NOS2), both Pax4- and Pax4R129W-expressing islets exhibited similar levels of NO production upon exposure to cytokines, indicating that the NF-kB signalling pathway was fully activated, implying that the protection conveyed by Pax4 is relayed through additional gene products that blocks the NF-kB pro-apoptotic pathway. (Hu He et al., 2011a).

Herein, we investigated whether Pax4 and Pax4R129W could promote β-cell survival and prevent the development of hyperglycemia in the transgenic RIP-B7.1 mouse model of experimental autoimmune diabetes (EAD). This model, in which hyperglycemia is triggered by immunization with a preproinsulin cDNA encoding plasmid (Karges et al., 2002), was preferred to the NOD model (Anderson and Bluestone, 2005) because of a higher incidence of CD8 T-cell autoimmune-mediated β-cell destruction, in both male and female mice. Importantly, composition of the insulitis resembles by large the human phenotype (Karges et al., 2007). We show that immunized RIP-B7.1 animals, which conditionally over-express Pax4, develop hyperglycemia less frequently than both Pax4R129W-expressing and control immunized mice. Insulitis, apoptosis and DNA damage were reduced while islet cell proliferation was increased in Pax4-expressing mice. Transcriptome profiling of Pax4 and Pax4R1291W over-expressing islets revealed differences in genes related to immune-modulation, cell cycle and ER-homeostasis. Consistent with these genetic changes, islets over-expressing Pax4 were protected against thapsigargin-induced ER-stress apoptosis. Our results demonstrate that Pax4 fosters β-cell fitness in the face of an autoimmune attack, by stimulating cell proliferation and maintaining ER-homeostasis under inflammatory conditions.

RESULTS

Characterization of the BPTL Transgenic Mouse Model

The RIP-B7.1 animal model of EAD was selected to assess the role of Pax4 in protecting islets against autoimmune-mediated destruction. In order to monitor changes in β-cells mass using non-invasive *in vivo* imaging technology, we generated the BPTL mouse which bears four transgenes (Supplemental Figure 1A and B): 1) RIP-B7.1, a construct coding for the *cd80* gene under control of the rat insulin promoter (RIP); 2) TRE/CMV Pax4, a strong tetracycline-inducible promoter driving Pax4 expression; 3) RIP-rtTA, a construct which allows for the selective expression of Pax4 in β-cells exposed to doxycycline (DOX); 4) MIP-Luc, a construct which codes for the selective expression of luciferase in β-cells under control of the mouse insulin promoter (MIP).

We first monitored *in vivo* the bioluminescence (BLI) emitted by β-cells of BPTL mice from 4 to 65 weeks of age (Figure 1A and Supplemental Figure 1C). In 4 week-old mice, the BLI signal was 2 fold higher in male than female animals (Figure 1A). Consistent with a decrease rate of islet growth around weaning (Scaglia et al., 1997), both genders displayed, between the 4^{th} and 9^{th} week, a drastic decline in the BLI signal (Figure 1A). Thereafter, this signal did not significantly change with the age of the animals, up to the end of the experiment (Figure 1A). The parallel morphometric evaluation of the β-cell mass in sections of pancreas showed that the volume density of β-cells was similar in animals aged from 4 to 16 weeks (Figure 1B). The data indicate that by the age of 9 weeks the BLI signal reflects the mass as well as the function of β-cells, which we hereafter refer to as the functional β-cell mass.

We next assessed whether the BPTL mouse behave as the parental RIP-B7.1 animal as a model of EAD. Non-immunized BPTL mice were normoglycemic for up to 35 days (Figure 1C), consistent with a control content of insulin-containing β-cells (data not shown). In contrast, immunized BPTL mice developed hyperglycemia within 21 days, due to the gradual loss of insulin-containing cells (Figure 1D). These data validate the BPTL mouse as a model of EAD in which the functional β-mass can be monitored by non-invasive *in vivo* imaging.

The Conditional Over-expression of Pax4 Preserves the β-cell Mass in Immunized BPTL Mice and Delays the Development of Hyperglycemia

Five-week old BPTL mice were treated with DOX for 4 weeks prior to immunization, in order to induce Pax4 expression. Compared to islets of control mice, islets isolated from treated mice revealed a 10-fold increase in Pax4 expression (Figure 2A), in spite of a constant β-cell mass (Figure 2C), whereas transcript levels of CD80 were unchanged (Figure 2B). Nine-week old, untreated animals (non-immunized and without DOX administration) remained normoglycemic and featured no significant variation of the BLI signal for up to 28 days (Figure 3A). In the absence of Pax4

induction, immunized BPTL mice revealed a rapid decrease in BLI signal, reaching undetectable levels by day 28 post-immunization coinciding with sustained hyperglycemia (Figure 3B, left panel). The escalation in blood glucose levels observed by 21 days post-immunization correlated with a 60% decrease in BLI intensity, suggesting that 40% of the functional β-cell mass is required to sustain normoglycaemia (Figure 3B, left panel). DOX-induced Pax4 over-expression preserved both normoglycemia and most of the BLI signal in immunized BPTL mice (Figure 3B right panel). By extending the observation period, we found that these animals became hyperglycemic by day 63, at the time the BLI signal was 40% of the initial value (Figure 3C, right panel). At the same time point, control animal maintained normal blood glucose levels, in spite of a 40% decrease in BLI (Figure 3C, left panel). Morphometry revealed that the functional β-cell mass of BPTL mice, which did not receive DOX, was reduced by 40 and 80 % 21 and 28 days post-immunization, respectively (Figure 4A and 4B). Such a change was not observed in DOX-treated mice (Figure 4A and B). By day 63, the latter animals retained about 50% of the original functional β-cell mass (Figure 4A and 4B). The data demonstrate that Pax4 over-expression concomitantly delays the onset of hyperglycemia and the loss of the functional β-cell mass in BPLT mice.

Pax4 Over-expression Decreases Insulitis and Enhances β-cell Fitness in Immunized BPTL Mice

Morphometry performed 28 days after immunization revealed that 65% of islets of DOX-treated BPTL mice were insulitis free (grade 0), whereas 90% of islets derived from non-DOX-treated BPTL mice displayed severe insulitis (grades 2-4) (Figure 5A). Of note, more than 50% of the islets of DOX-treated BPLT mice displayed no (grade 0) or mild insulitis (grade 1) 63 days after immunization (Figure. 5A). Consistent with the preserved β-cell mass and decreased insulitis, DOX treatment also significantly diminished the percentage of cleaved caspase-3 positive islet cells up to 63 days after immunization (Figure 5B and Supplemental Figure 3A). Double-strand DNA damage induced by NO and ROS was recently shown to be a major factor contributing to β-cell death in both T1DM and T2DM (Oleson et al., 2014; Tornovsky-Babeay et al., 2014). To assess whether Pax4 over-expression reduced DNA damage in immunized BPTL mice, we immunostained 53BP1, a protein recruited to sites of double-strand DNA breaks, in pancreatic sections. Fourteen days after immunization, a 2-fold decrease in the average number of foci per β-cell was observed in BPTL mice over-expressing Pax4 (Figure 5C). Of note, later time points failed to reveal double strand DNA breaks in immunized and (-) DOX treated mice suggesting that by day 21 the apoptotic program most likely prevailed over DNA repair (data not shown). As positive control, we also assessed 53BP foci formation in islets of either Pax4 over-expressing or control mice treated for 48 hours with STZ, an alkylating compound that cause DNA damage (Hu He et al., 2011a; Lenzen, 2008). Consistent with its action, STZ induced a robust DNA

damage in β-cells, which was significantly blunted by Pax4 over-expression (Figure 5C). The data indicate that Pax4, in two independent models of experimental diabetes, suppresses double strand DNA breaks and blunts apoptosis. These changes were paralleled by an increase in cell proliferation in islets of DOX-treated BPLT animals, which decreased by day 63 after immunization (Figure 5D and Supplemental Figure 3B).

Transcriptome Profiling of Islets Over-Expressing Pax4 Reveals the Up-regulation of Genes Implicated in Cell Cycle and ER homeostasis

To elucidate the genes involved in the Pax4-dependent β-cell survival and proliferation, we conducted a RNA microarray analysis of islets derived from mice over-expressing either wild type Pax4 or its diabetes-linked variant Pax4R129W (mouse equivalent to the human R121W mutation) (Hu He et al., 2011a). We initially evaluated the protective effect of Pax4R129W in immunized RIP-B7.1 (mutBPTL) as compared to Pax4 (BPTL). As expected, all BPTL mice which were not treated with DOX developed severe hyperglycemia by the 4th week after immunization, while BPTL mice treated with DOX to over-express Pax4 remained normoglycemic up to the same time point (Figure 6). The incidence of hyperglycemia in mutBPTL mice treated with DOX to induce the over-expression of the variant Pax4R129W was identical to that of the immunized BPTL animals, which were not treated with DOX, up to the 3rd week after immunization (Figure 6). Noticeably 75% of mutBPTL animals developed hyperglycemia at 4 weeks post-immunization (Figure 6). The data establish that over-expression of the Pax4R129W variant as compared to Pax4 very mildly protect animals against hyperglycemia induced by immunization. Thus comparing and contrasting the transcriptome signature of Pax4 to Pax4R129W over-expressing islets may highlight sets of genes for which the differential expression could account for the degree of protection. Islets exhibiting a similar 3-fold increase in either Pax4 or Pax4R129W transcript levels subsequent to 1 month DOX treatment were processed for RNA microarray profiling and analysis (data not shown).Differential gene expression assessment using *limma* moderated t-statistics (raw p-value < 0.05) revealed that 770 transcripts were up-regulated and 449 were down-regulated in islets over-expressing Pax4, whereas 1437 genes were up-regulated and 1136 down-regulated in islets over-expressing the Pax4R129W mutant variant. Among these genes, those showing the largest changes, as compared to the levels evaluated in islets of control mice, which were not treated with DOX, are listed in Supplemental Table 1. Of note, the genes highly modified by the over-expression of wild type Pax4 are different from those that appear regulated after over-expression of Pax4R129W (Supplemental Table 1).

KEGG Functional enrichment analysis further highlighted the pathways that were significantly enriched by either Pax4 or Pax4R129W (Supplemental Tables 2 and 3). Cell cycle and endoplasmic reticulum (ER) processing of proteins were among the top up-

regulated KEGG pathways in islets over-expressing wild type Pax4 (adjusted p-value<0.05), but were among the most significantly down-regulated pathways in islets over-expressing in Pax4R129W (adjusted p-value<0.05) (Figure 7A, B and C and Supplemental Tables 2 and 3). To contrast the direct expression of individual genes contributing to these two pathways, we generated heat maps amenable to statistical analysis (raw p-value<0.05). Consistent with the enhanced proliferation of β-cells in DOX-treated BPTL mice, a considerable number of genes associated with cell cycle were found increased after over-expression of Pax4 (Figure 7B). Most of these genes were either unchanged or decreased in islets expressing Pax4R129W (Figure 7B). Similarly, numerous genes encoding proteins involved in peptide folding (*Hspa5/Bip, Calr*), ER-Golgi translocation (*Lman1*, *Lman2*, *Sec23b* and *Plaa*) and ER-associated degradation (*Ufd1l*, *Derl3*, *Pdia4*, *Ssr3*, *Syvn1* and *Dnaja2/Hsp40*) were up-regulated after over-expression of wild type Pax4, but were down-regulated after over-expression of the variant Pax4R129W (Figure 7C). The data indicate that over-expression of wild type and variant Pax4 differentially regulate a set of distinct genes. To validate this premise we assessed by quantitative PCR (Q-RT-PCR) expression levels of galectin-9 (*Lgals9*) (Supplemental Table 1), a gene involved in immune-modulation and Calreticulin (*Calr*), an ER chaperone (Figure 7C). Consistent with microarray data, both galectin-9 and Calreticulin expression levels were increased in Pax4 expressing islets whereas decreased in islets expressing Pax4R129W (Figure 7D).

Interestingly, *Mbtps2* (a gene coding for a protease that cleaves the ER-membrane bound ATF6), *Eif2ak3* (also known as *Perk*) and *Mapk10* (also known as *Jnk3*) were up-regulated in islets over-expressing Pax4, but were marginally altered in islets over-expressing Pax4R129W (Figure 7C). As most of these Pax4-regulated genes are key to the maintenance of ER homeostasis through activation of the UPR, we assessed whether Pax4 and Pax4R129W could protect against ER stress-induced β-cell apoptosis. Exposure to thapsigargin of isolated islets over-expressing either Pax4 or Pax4R121W, as monitored by DsRed fluorescence (Figure 8A), revealed that the inhibitor of ubiquitous ER Ca^{2+}-ATPases increased by about 2 fold the β-cell apoptosis in islets of mice that had not received DOX (Figure 8B). Administration of DOX significantly reduced the thapsigargin-induced apoptosis in islets over-expressing Pax4 but not in islets expressing the Pax4R129W variant (Figure 8B). The data suggest that the beneficial effects of Pax4 over-expression are at least partly mediated by a preserved function of β-cell ER.

DISCUSSION

T1DM and T2DM are both characterized by β-cell failure and death, predominantly by apoptosis. Since there is yet no 'unifying hypothesis' about the mechanisms triggering β-cell deterioration (Cnop et al., 2005; Donath and Halban, 2004), it is surmised that deciphering the molecular roadmap regulated by factors whose mutations are linked

to both forms of DM may help identifying common pathways. This is the case of the transcription factor Pax4 (Brun and Gauthier, 2008). Over-expression of Pax4, but not of the T2DM-associated Pax4R129W variant, preserves the islet β-cell mass through proliferation, resistance to apoptosis, and α-cell trans-differentiation thereby protecting animals against chemical-induced hyperglycemia (Collombat et al., 2009; Hu He et al., 2011a). We now demonstrate that over-expression of Pax4, but not Pax4R129W, also preserves the mass of β-cells and delays the development of hyperglycemia in the RIP-B7.1 animal model of T1DM. The data provide the proof-of-concept that Pax4 protects β-cells against a chronic, *in vivo* autoimmune attack.

Consistent with previous reports exploiting this model, 90-100% of immunized non-DOX treated RIP-B7.1 mice bearing either the Pax4 or Pax4R129W transgene (non-DOX-treated BPTL or mutBPTL) developed hyperglycemia within 3 to 4 weeks correlating with massive insulitis and β-cell destruction (Karges et al., 2007). Induction of EAD was strictly conditional on immunization and independent of gender far exceeding expectations of the conventional NOD mouse model of T1DM in which only 50% of animals, predominantly females, developed spontaneous diabetes with age (Anderson and Bluestone, 2005). Doxycycline-mediated induction of Pax4 completely restrained development of hyperglycemia in BPTL animals up to 4 weeks post immunization whereas the induction of the Pax4R129W variant reduced the hyperglycemia in only a minority of mutBPTL mice. These data establish that over-production of Pax4 delays hyperglycemia in an autoimmune context, in contrast to several other factors such as Bcl-2, caspase-3-generated RasGAP N-terminal fragment (fragment N) or the Cytokine response modifier A (CrmA) which could not extend the normoglycemic period in various animal models of T1DM, despite increased β-cell survival (Allison et al., 2000; Bulat et al., 2011; Millet et al., 2006). Since these factors inhibit β-cell apoptosis as we report here, the data imply that Pax4 is involved in additional regulatory pathways, possibly including immune-modulation. In this context, insulitis was significantly reduced after Pax4 over-expression, an effect not attributable to a non-specific repression of the CD80 transgene that facilitates the immune response. Our observations extend the somewhat analogous findings that inhibition of vascular endothelial growth factor receptor-2 (VEGFR-2) and anti-CD3 monoclonal antibody administration in NOD mice efficiently reversed hyperglycemia by abrogating insulitis and restoring islet function (Chatenoud et al., 1994; Villalta et al., 2013). Although the mechanism by which Pax4 acts at the interface of islet β-cells and the immune system to blunt insulitis and potentially improve islet recovery remains to be fully delineated, our transcriptome profiling analysis combined with Q-RT-PCR revealed that *Lgals9* was up regulated in islets over-expressing Pax4. This gene induces in many tissues the apoptosis of terminally differentiated Th1 cells (Zhu et al., 2005), and its experimental over-expression in NOD mice reduced insulitis and hyperglycemia (Chou et al., 2009), prolonging the survival of grafts (Chou et al., 2013).

It is therefore plausible that, by enhancing the expression of *galectin-9*, Pax4 may down-regulate Th1 function, partially impeding the development of insulitis, and resulting in extended survival of β-cells.

Our RNA profiling analysis further establishes that Pax4 over-expression enriches several transcripts involved in stimulating β-cell growth (Hu He et al., 2011a) including Cyclin D3 which also promotes β-cell survival (Saavedra-Avila et al., 2014). In spite of these effects, we did not observed an aberrant islet over growth, possibly because the expression of the cyclin-dependent kinase inhibitor 1A (*Cdkn1a*, also known as *p21*), a molecular brake for pro-proliferative pathways (Cozar-Castellano et al., 2006) was also up regulated by Pax4. Our transcriptome analysis further revealed that Pax4 is a key regulator of ER function, by a combined targeting of genes involved in UPR, Ca^{2+} homeostasis, ER-Golgi translocation, and ERAD. The functional importance of the transcriptional changes of these genes was validated by the capacity of Pax4 to block the apoptosis of β-cells exposed to thapsigargin, which is mediated by ER stress (Cardozo et al., 2005), consistent with the finding that Pax4 binding sites are enriched within the promoter region of palmitate-modified ER stress response genes (Cnop et al., 2014). These data document that the efficacy of Pax4 in protecting β-cells against apoptosis is due to a parallel action on several branches of the UPR.

Strikingly, most of the genes that were up-regulated by Pax4 were down-regulated by the diabetes-linked Pax4R129W variant, in spite of a comparable expression levels of both transgene transcripts providing some molecular insights about the negative pathogenic effect of the R129W mutation. Specifically, the T2DM-associated cyclin-dependent kinase inhibitor 2A, which strongly inhibits the proliferative kinase cdk4 (*Cdkn2a*)(Scott et al., 2007) was enriched in islets expressing Pax4R129W, but decreased in those over-expressing Pax4. Conversely, genes involved in ER homeostasis, which were enriched in islets over-expressing Pax4, were repressed in islets expressing Pax4R129W. The production of NO and ROS induced by inflammatory cytokines in the diabetic environment also promotes β-cell apoptosis by a variety of mechanisms, including the induction of irreversible double strand DNA breaks (Cardozo et al., 2005; Oleson et al., 2014). We found that Pax4 blunts DNA damage in two models of experimental DM, pointing to a general protective mechanism, possibly by up-regulating the transcription of *Calreticulin* (*Calr*), which, again, was down-regulated in islets expressing Pax4R129W (Figure 6D). Calreticulin is a major Ca^{2+} chaperone of the ER, which contributes to the quality control of protein folding (Wang et al., 2012). Over-expression of *Calr* in MIN6 cells enhances ER Ca^{2+} stores and prevents NO-induced apoptosis (Oyadomari et al., 2001), and inhibition of calcineurin, a downstream target of calreticulin, reduces β-cell death (Tornovsky-Babeay et al., 2014). The data indicate that, by promoting *Calr* expression Pax4 helps preserving ER Ca^{2+} homeostasis under stress conditions. Thus, an important aspect of Pax4 function

is to regulate the transcriptome of islet cells, to promote genes enhancing β-cell survival, proliferation, function and DNA repairing, all positive changes which are opposed by specific mutations in the transcription factor.

Still, this tentative scenario does not provide a direct explanation for why the effect of Pax4 over-expression faded after weeks, leading to hyperglycemia of the DOX-treated BPTL mice approximately 2 months after triggering of the autoimmune attack. At this time, β-cell proliferation declined, while β-cell apoptosis and insulitis increased. Previous experiments have shown that long-term Pax4 over-expression alters β-cell function, by a mechanism which can be rescued by interrupting the over-production of the transcription factor (Hu He et al., 2011a), and that *Cyclin D3* is repressed by insulitis (Saavedra-Avila et al., 2014). Furthermore, the up-regulation of cyclin-dependent kinase inhibitor 2A was reported to limit, with time, the regeneration of aging β-cells (Krishnamurthy et al., 2006). The data are consistent with a dual, temporal effect of Pax4, whose favourable effects may not be sustainable during a continuous, long-term over-expression.

We conclude that Pax4 favors β-cell survival and regeneration in various deleterious environments, including autoimmunity, through the coordinated regulation of immune-modulation, cell cycle, cell survival, ER-homeostasis and DNA repairing. While both Pax4 and Pax4R129W modulate these pathways, it is the wild type transcription factor, which conveys pro-survival properties by increasing the expression of selected, adaptive genes. Given that the Pax4-regulated networks contribute to the pathogenesis of both T1DM and T2DM, the possibility of a therapeutic strategy should be considered, which first implies the identification of 'druggable' Pax4 targets.

MATERIALS AND METHODS

Mice

All mice experiments were approved by the CABIMER Animal Committee and performed in accordance with the Spanish law on animal use RD 53/2013. Quadruple transgenic BPTL mice were generated as followed (Supplemental Figure 1A): Pax4 transgenic mice (CMV-Pax4) bearing a Pax4/DsRed-Express construct under the transcriptional control of the CMV promoter were mated with RIP-rtTA mice to engender PT bigenic mice, as previously described (Hu He et al., 2011a). PT mice were then crossed to MIP-LUC-VU animals (Park et al., 2005) to generate PTL triple transgenic mice. The RIP-B7.1 animal model of EAD (Karges et al., 2002) was then mated to PTL animals to derive BPTL quadruple transgenic mice that were used for the current study. A similar approach was used to generate quadruple mice bearing the Pax4 diabetes-linked mutant variant Pax4R121W (mutBPTL) (Hu He et al., 2011a). Mice were genotyped with the REDExtract-N-Amp kit (Sigma-Aldrich, Madrid, Spain), using

specific primers for each transgene that can be obtained upon request (Supplemental Figure 1B). All mice were maintained on a C57BL/6 background. Induction of Pax4 or Pax4R129W expression was achieved by providing 1 g/L of DOX (Sigma-Aldrich) in the drinking water, starting at 5 weeks of age and lasting for the duration of the experiment. Induction of EAD was achieved by *i.m.*immunization of 9-week-old BPTL animals with 50 µg a pC1/ppins plasmid DNA (1 µg/ µL) encoding the murine preproinsulin II. Ultra pure plasmid DNA isolation was contracted to the Plasmid factory GmbH (Bielefeld, Germany). Non-fasting blood glucose levels were measured weekly from tail vein samples, using a Precision Xceed glucometer (Abbott, Madrid, Spain).

Bioluminescence Imaging (BLI)

Beetle luciferin potassium salt (150 mg/Kg; Promega Biotech Ibérica S.L., Alcobendas, Spain) dissolved in PBS was administered *i.p.*to BPTL animals. Anaesthesia was then induced and maintained with 2.5 % isofluorane in 97.5 % O_2. Bioluminiscence was subsequently assessed using a Xenogen IVIS 50 imaging system (Caliper Life Sciences, Hopkinton, USA). Images were recorded from 3 consecutive 2-min periods and the integrated measurements were corrected for the background emission from an area of equal size. Images were analyzed using the Living Image software version 4.1 (Caliper Life Sciences, HopkintonUSA).

Mouse Islet Isolation and Treatment

Mouse islets were isolated as previously described (Hu He et al., 2011a). To assess Pax4, CD80, Calreticulin and galectin-9 transcript levels, total RNA was extracted from BPTL islets treated or not with DOX, using the RNeasy Micro Kit (Qiagen) and Q-RT-PCR was performed as described previously (Gauthier et al., 2004). Primer Express Software (Life Technologies, Alcobendas, Spain) was used to design the primer sequences and can be obtained upon request. For studies on thapsigargin-induced ER-stress and apoptosis, islets isolated from either Pax4/rtTA or Pax4R129W/rtTA transgenic mice were initially treated or not with 1µg/L DOX for 96 h to induce Pax4 overexpression (Hu He et al., 2011a). DsRed fluorescence correlating with Pax4 expression was then monitored in groups of 10 islets plated on µ-Plate 96 welllibiTreat (IBIDI, Martinsried, DE) in a final volume of 150 µl complete medium; RPMI-1640 (Invitrogen S.A., Barcelona, ES) supplemented with 10% fetal calf serum (Sigma-Aldrich Quimica S.A.), 100 Units/ml penicillin and 100 mg/ml streptomycin (Sigma-Aldrich Quimica S.A.), and 2mM glutamine (GlutaMAX; Invitrogen S.A.). Images were captured using an ImageXpress micros system (Molecular devices, Spain). For thapsigargin-induced cell death determinations islets were incubated or not with 1µM thapsigargin for 48 h. Apoptosis was then assessed using the Cell Death Detection Elisa Plus kit (Roche Diagnostics, Mannheim, Germany).

Immunohistochemistry and Morphometry

For paraffin sections, pancreata were dissected and fixed in 4% paraformaldehyde. Dehydration, embedding and sectioning were performed at the CABIMER Histology Core Facility. Sections of 7 µm thickness were rehydrated by immersion in decreasing concentration of ethanol. Heat induced antigen retrieval was performed in 0.01M Sodium Citrate Buffer (pH6). In the case of Ki67 and cleaved caspase-3 antigen retrieval was carried out using a pressure cooker. Thereafter, all sections were maintained overnight at 4ºC in the presence of primaries antibodies at the indicated dilutions (Table S4). Subsequently, corresponding secondary antibodies (Table S4) were added for 1 hour at room temperature, followed by DAPI-nuclear staining (Life Technologies), Slides were finally cover slipped using a fluorescent mounting medium (DAKO, Barcelona, Spain). The β-cell mass and islet cell number were assessed in three insulin-stained pancreatic sections from 5 to 6 mice per group, using the Metamorph software package (Molecular Devices, St Gregoire cédex, France) as described elsewhere (Mellado-Gil et al., 2011). Results are expressed as percentage of control, untreated mice. Apoptosis, proliferation and DNA damage was quantified by counting cleaved caspase-3, Ki67 and 53BP1 positive cells, respectively, in at least 20 to 50 islets from three independent pancreatic sections of 3-4 mice per group. Cell death and proliferation are presented as percent positive cells of the total islet cell number while DNA damage is provided as foci percentage per β-cell. Insulitis scoring was performed as previously outlined (Bulat et al., 2011), using a score from 0 to 4 (grade 0, islet free of insulitis; grade 1, <10% islet area invaded by immune cells; grade 2, 3 and 4, >55%; 55-75%; and >75% of islet area invaded by immune cells) on either DAPI or H&E-stained pancreas sections. Results are expressed as percent infiltration of islet areas (Bulat et al., 2011).

RNA Microarray and Bioinformatic Analysis.

Labeled cRNA samples were prepared from pools of at least 100 islets isolated from either Pax4/rtTA or Pax4R121W/rtTA transgenic animals (8-week old females) treated or not with DOX as previously described (Hu He et al., 2011a). Three independent preparations of cRNA per group were then hybridized to the GeneChip Mouse Gene 1.0 ST Array chip (Affymetrix, Santa Clara, CA) using standard protocols of the Genomic Core Facility of CABIMER. The Robust Multiarray Analysis (RMA) method was then applied on a per-chip basis for background correction (Irizarry et al., 2003). Subsequent normalization across arrays and summarization were performed using a quantile algorithm and median-polish, respectively via *oligo* package from Bioconductor (http://www.bioconductor.org). A differential gene expression analysis was then performed using the *limma* package (Smyth, 2004). Computed p-values were corrected using the widely accepted False Discovery Rate (FDR) method to harmonize for the multiple comparisons for all the genes (Benjamini and Hochberg, 1995). In this method, the p-values are ranked, giving value 1 to the smallest and N to the largest. Each p-value is then multiplied by the number N and divided by its rank number to

produce the adjusted p-value. Thus, to reduce the number of false positives only genes with an adjusted p-value less than 0.05 are chosen as statistically significant. Gene set analysis was achieved for KEGG pathways using the logistic regression model (Montaner and Dopazo, 2010) while KEGG annotations for genes in the microarrays were extracted from the Reactome database (Matthews et al., 2009). Analysis methods and databases were used as implemented in Babelomics web tools (Medina et al., 2010). Heatmap displaying t-statistic values of differential expression analyses for Pax4/rtTA (+DOX versus -DOX) and Pax4R121W (+DOX versus -DOX) were then generated with selected genes of either Pax4 or Pax4R121W associated with the statistically enriched (p-adjusted<0.05) cell cycle and protein processing in ER KEGG pathways. Raw data are accessible in the Gene Expression Omnibus database under accession number GSE62846.

Statistical Analysis

Results, excluding the ones presented in Figure 6A, B and C are expressed as mean + SEM. Statistical differences between 2 conditions were estimated using the unpaired Student t test. One-way ANOVA was used for comparison of more than 2 groups with Bonferroni post hoc test or non-parametric Mann-Whitney test (**p<0.05, **p<0.01*).

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ONLINE SUPPLEMENTAL MATERIAL

Supplemental information includes 2 figures and four tables and can be found with this article online at http:

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FIGURE LEGENDS

Figure 1: Validation of the BPTL Mouse as a Model of EAD.

(A) The BLI signal over time was assessed in male (*n=5*) and female (*n=5*) BPTL mice. Animals were injected with D-luciferin, anesthetized and monitored for emitted photons using a Xenogen IVIS 50 imaging system. Values are presented as means ± SEM. (B) Quantification of β-cells as a percentage of total islet cells was assessed after insulin immunostaining. DAPI-nuclear staining was performed to determine total islet cell number. Five to 6 animals were used per age group with at least 25 to 50 islets counted from 3 pancreatic sections of each mouse. Values are presented as means + SEM. (C) Nine week-old quadruple transgenic BPTL mice were immunized via i.m.injection of a pC1/ppins plasmid DNA encoding murine preproinsulin II. Blood glucose levels were measured for up to 35 days from tail vein blood samples. Values are derived from 3 animals per time point and are presented as means + SEM. (D) Pancreatic sections of BPTL mice at the indicated time after immunization were co-immunostained for glucagon (GCG, green) and insulin (INS, red). Nuclei were stained with DAPI (blue). Representative single-channel fluorescence images are presented both individually and merged. Scale bar: 25 μm for all panels.

Figure 2: Doxycycline Treatment Prior to Immunization Induces Pax4 Expression Without Altering CD80 Expression and β-cell Mass in BPTL mice.

Five week-old BPTL animals received 1g/L DOX in drinking water for one month, sacrificed and RNA isolated from pancreatic islets. (A) Pax4 and (B) CD80 transcript levels were then determined by Q-RT-PCR. Relative mRNA levels were normalized to the transcript levels of the housekeeping gene *β-actin* and/or *rps29*. Data were calculated as percent change compared to the values in control, non-DOX treated animals. Values are presented as means + SEM. Statistical difference was tested by one-way ANOVA with Bonferroni post-hoc test (**p<0.01). (C) Quantification of β-cell mass was assessed by insulin immunostaining in an independent group of BPTL mice. Four to 6 animals were used per age group with at least 25 to 50 islets counted from 3 independent pancreatic sections. Results are presented as percent change compared to non-DOX treated matched control animals.

Figure 3: Immunized BPTL Mice Overexpressing Pax4 Display a Delayed Loss of Both BLI Signal and Hyperglycemia.

BLI signal and blood glucose levels were measured weekly in: (A) Control untreated BPTL mice (*n=4*) for up to 28 days, (B) Immunized BPTL animals without (-DOX; *n=6*) or with (+DOX; *n=6*) Pax4 overexpression for up to 28 days and (C) Control untreated BPTL (*n=4*) and immunized BPTL animals with (+DOX; *n=5*) Pax4 overexpression for an additional 35 days. BLI results are presented as percent change compared to day 0 (t=0) while blood glucose levels are expressed as means + SEM.

Figure 4: Pax4 Overexpression Preserves a Sizable Functional β-cell Mass in Immunized BPTL mice.

(A) Immunohistochemical analysis of glucagon (GCG, green) and insulin (INS, red) on pancreatic paraffin sections from BPTL mice treated or not with DOX and sacrificed at day 0, 21 and 28 post-immunization (IMM) as well as day 63 for DOX treated animals only. Nuclei were stained with DAPI (blue). Representative single-channel fluorescence images are presented individually and merged. Scale bar: 25 μm for all panels. (B) Quantification of β-cell mass was then assessed using images as shown in (A). Five to 6 animals were used per time point with at least 25 to 50 islets counted from 3 independent pancreatic sections. Results are presented as percent change compared to control BPTL mice sacrificed at each time point. As similar results were obtained for day 28 and 35, the data was combined as a single time point. Statistical difference was tested by one-way ANOVA with Bonferroni post-hoc test (*p<0.05, **p<0.01). §, All animals not treated with DOX died or were sacrificed for ethical reason by this time point.

Figure 5: BPTL Islets Overexpressing Pax4 Display Reduced Insulitis Correlating with Decreased Apoptosis and DNA Damage and Increased Proliferation.

(A) Insulitis scoring was performed according to a grade of 0 to 4 on either DAPI or H&E-stained pancreas sections obtained from immunized (IMM) and DOX-treated or not BPTL mice sacrificed at days 0 (*n=5*), 28 (*n=6*) and 63 (*n=5*). Results are expressed as percent infiltration of total islet area. Statistical difference was tested by one-way ANOVA with Bonferroni post-hoc test (*p<0.05 vs IMM day 0 and (-) DOX group; #p<0.05 vs IMM day 28 and (-) DOX group). §, not detected. (B) Pancreatic sections from immunized BPTL mice treated (+) or not (-) with DOX and sacrificed at 0 (*n=5*), 14 (*n=5*), 21 (*n=5*), 28/35 (*n=6*) and 63 days (+DOX only, *n=5*) were co-immunostained for Caspase-3 and Insulin. Apoptosis was quantified by counting cleaved caspase 3-positive cells using images as shown in Supplemental Figure 2A. Results are presented as percent cleaved caspase-3 positive cells of the total islet cell number. As similar results were obtained for day 28 and 35, the data was combined as a single time point. Statistical difference was tested by one-way ANOVA with Bonferroni post-hoc test between (+) vs (-) DOX groups within each time point (*p<0.05, **p<0.01). (C) Pancreatic sections from immunized BPTL mice or streptozotocin-treated Pax4 over-expressing mice, treated (+) or not (-) with DOX and sacrificed at 14 days post-immunization or 2 days after streptozotocin treatment (Hu He et al., 2011a) were co-immunostained for p53BP1 and Insulin. The number of 53BP1 foci was counted and results are presented as the percent of foci per β-cell. Statistical difference was tested by unpaired students t-test (*p<0.05 vs untreated mice; #p<0.05 vs DOX-untreated immunized or STZ- treated mice, respectively). (D) Pancreatic sections from immunized BPTL mice treated (+) or not (-) with DOX and sacrificed at 0 (*n=5*), 14 (*n=5*), 21 (*n=5*), 28/35 (*n=6*) and 63 days ((+) DOX only, *n=5*) were co-immunostained for Ki67 and

Insulin. Proliferating cells assessed by Ki67 positivity were then counted using images as shown in Supplemental Figure 2B. Results are presented as percent Ki67 positive cells of the total islet cell number. Statistical difference was tested by one-way ANOVA with Bonferroni post-hoc test between (+) vs (-) DOX groups within each time point (*p<0.05, **p<0.01).

Figure 6: MutBPTL Mice are More Susceptible to Develop Immunization-Induced hyperglycemia than BPTL Mice.

The incidence of hyperglycemia was determined weekly for up to 5 weeks by measuring blood glucose from a tail vein blood samples collected from four cohorts of mice: Control mice immunized (IMM) BPTL animals treated or not with DOX and immunized/DOX-treated mutBPTL mice. Data are presented as the percentage of hyperglycemic (postprandial blood glucose $\geq$ 13.8mmol/L) mice per group.

Figure 7: KEGG pathways Related to Cell Survival and Proliferation are Enriched in Pax4 Overexpressing Islets.

DNA microarray profiling was performed on Pax4 and Pax4R121W islets after *in vivo* treatment or not with DOX for 1 month. (A) List of the most statistically significant enriched KEGG pathways (adjusted p-value <0.05) altered by Pax4 overexpression (+DOX) as compared to control islets (-DOX) from gene set analysis. KEGG Pathways were divided as either up (upper panel) or down regulated (lower panel) as compared to control conditions. The dotted line indicates the threshold of significance. Complete lists of significantly enriched KEGG pathways (adjusted p-value<0.05) in both Pax4 and Pax4R129W expressing islets are provided in Supplementary Tables S2 and S3. (B) Heatmap displaying t-statistic values of cell cycle KEGG pathway genes modulated in either Pax4 (Pax4-overexpressing islets versus control) or Pax4R121W (Pax4R121W-overexpressing islets versus control) contrasts from differential expression analysis. Selected genes are those with a no adjusted p-value<0.05 in either Pax4 or Pax4R121W contrast and associated with the statistically enriched) cell cycle KEGG pathway (p-adjusted<0.05). (C) Heatmap displaying t-statistic values of protein processing in the ER KEGG pathway genes modulated in either Pax4 (Pax4-overexpressing islets versus control) and Pax4R121W (Pax4R121W-overexpressing islets versus control) contrasts from differential expression analysis. Selected genes are those with a no ajdusted p-value<0.05 in either Pax4 or Pax4R121W contrast and associated with the statistically enriched protein processing in ER KEGG pathway (p-adjusted<0.05). Colors in each heatmap display the t-statistic values of all genes within the corresponding KEGG pathway estimated using the t-statistic value from differential expression analysis; green indicates the highest t-statistic value, black indicates t-statistic value close to zero and red indicates the lowest t-statistic value. (D) Galectin-9 and Calreticulin transcript levels were determined by Q-RT-PCR in islets isolated from Pax4 or Pax4R129W transgenic mice treated or not with DOX. Relative mRNA levels were

normalized to the transcript levels of the housekeeping gene *β-actin*. Data were calculated as percent change compared to the values in control, non-DOX treated animals. Values are presented as means + SEM. Statistical difference was tested by one-way ANOVA with Bonferroni post-hoc test (*p<0.05, **p<0.01).

Figure 8: Pax4 Protects Islets Against Thapsigargin-Induced ER-Stress Apoptosis
(A) Islets freshly isolated from double transgenic mice harboring either Pax4 or Pax4R121W along with the RIP-rtTA were treated in culture with DOX (1μg/l) for 96 hours. Induction of Pax4 expression was then qualitatively assessed through DsRed fluorescence acquired using an imageXpress Micro system. Of note, Pax4 and Pax4R129W along with DsRed are contained within a trans bicistronic construct that allows imaging of islet. Non-DOX treated Pax4 or PaxR121W islets were used as controls. Scale bar: 10 μm for all panels.(B) Islets were then challenged or not with the ER-calcium depleting drug thapsigargin (1μM) that induces ER stress. Apoptosis was then measured using a cell death detection ELISA kit. Results are the average of 5 independent experiments and expressed as percent change as compared to control non-thapsigargin treated islets. Statistical difference was tested by one-way ANOVA with Bonferroni post-hoc test (*p<0.05).

Figure 1: Jiménez-Moreno CM *et al.*

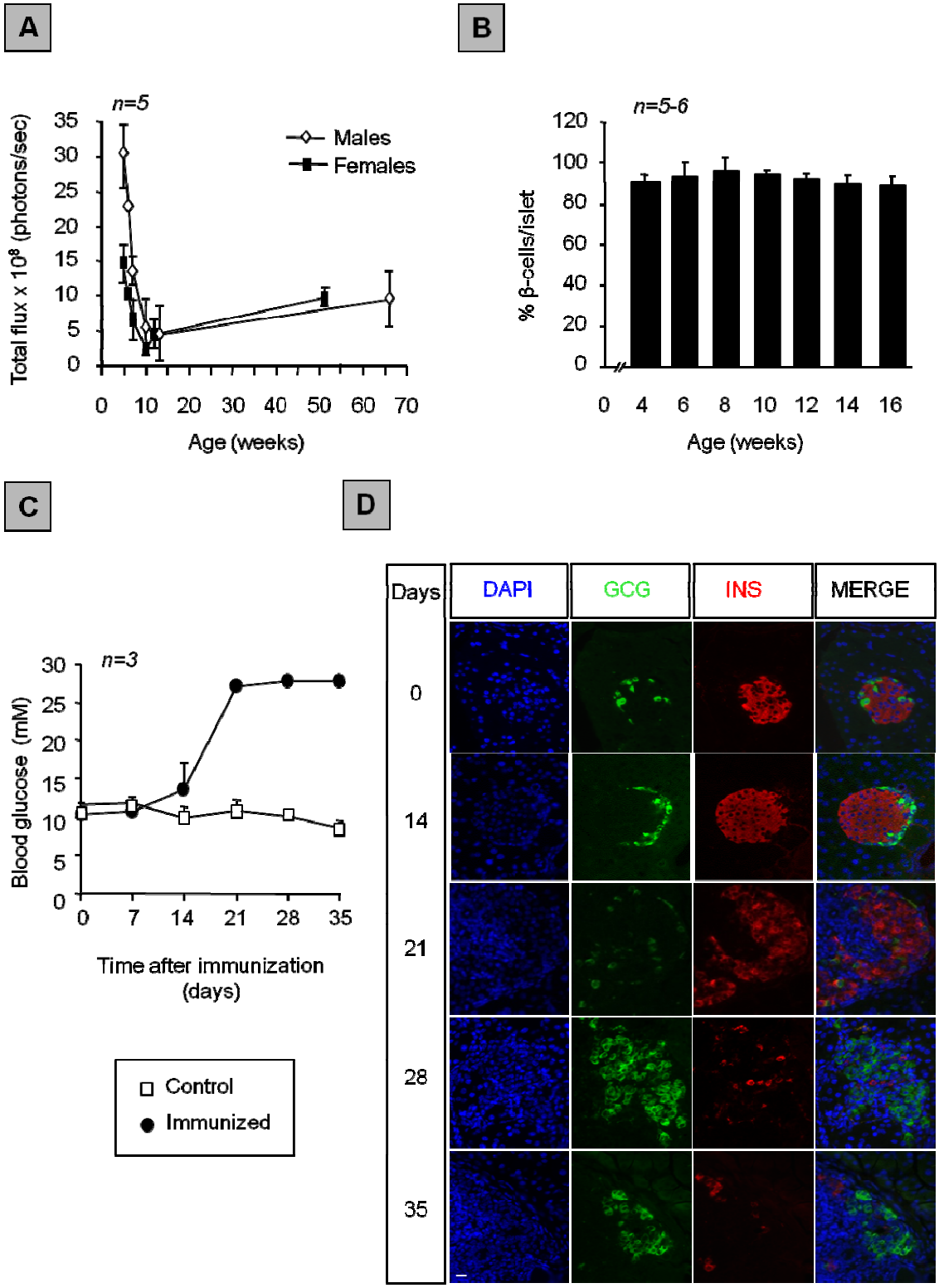
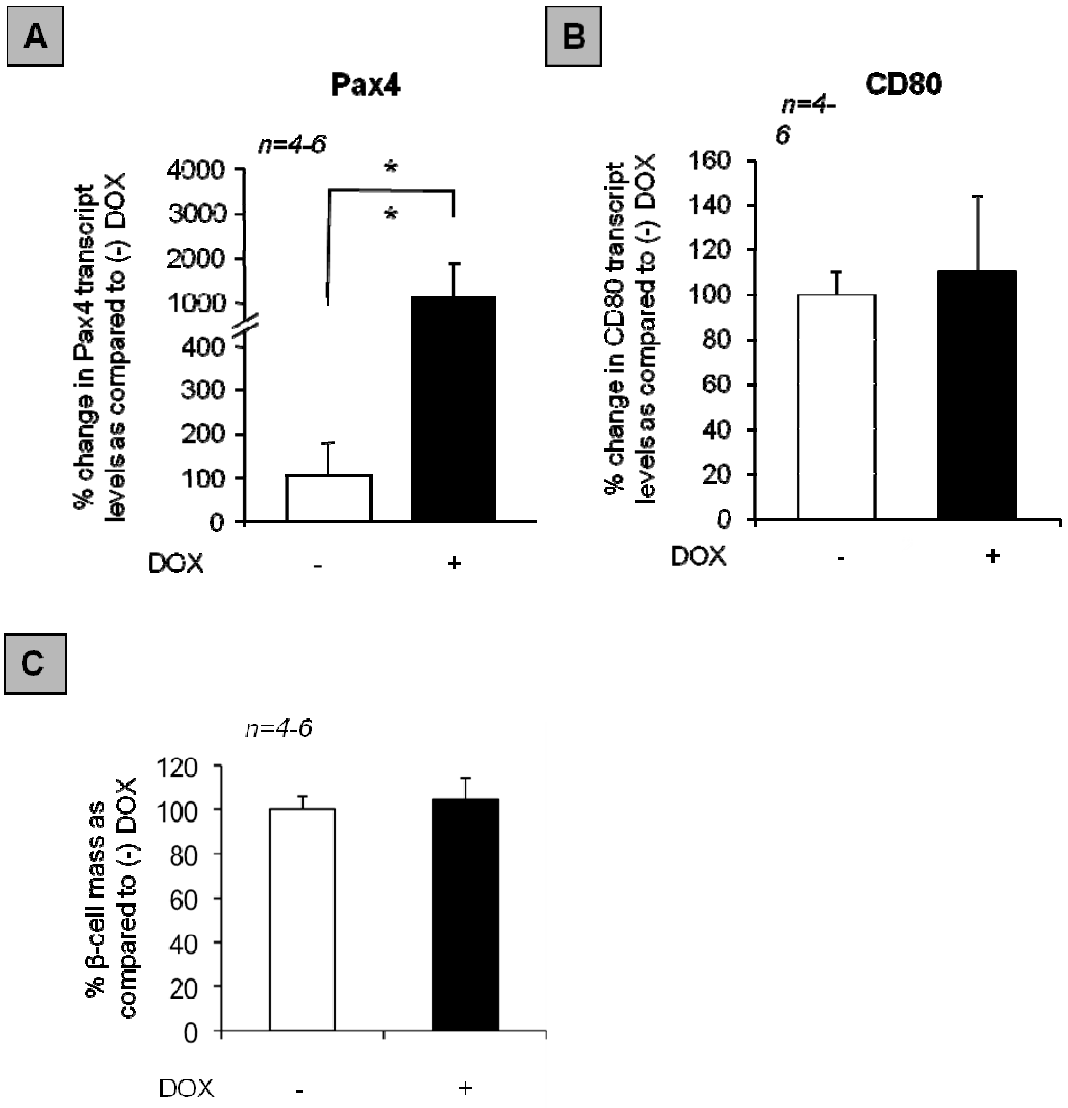


Figure 2: Jiménez-Moreno CM *et al.*



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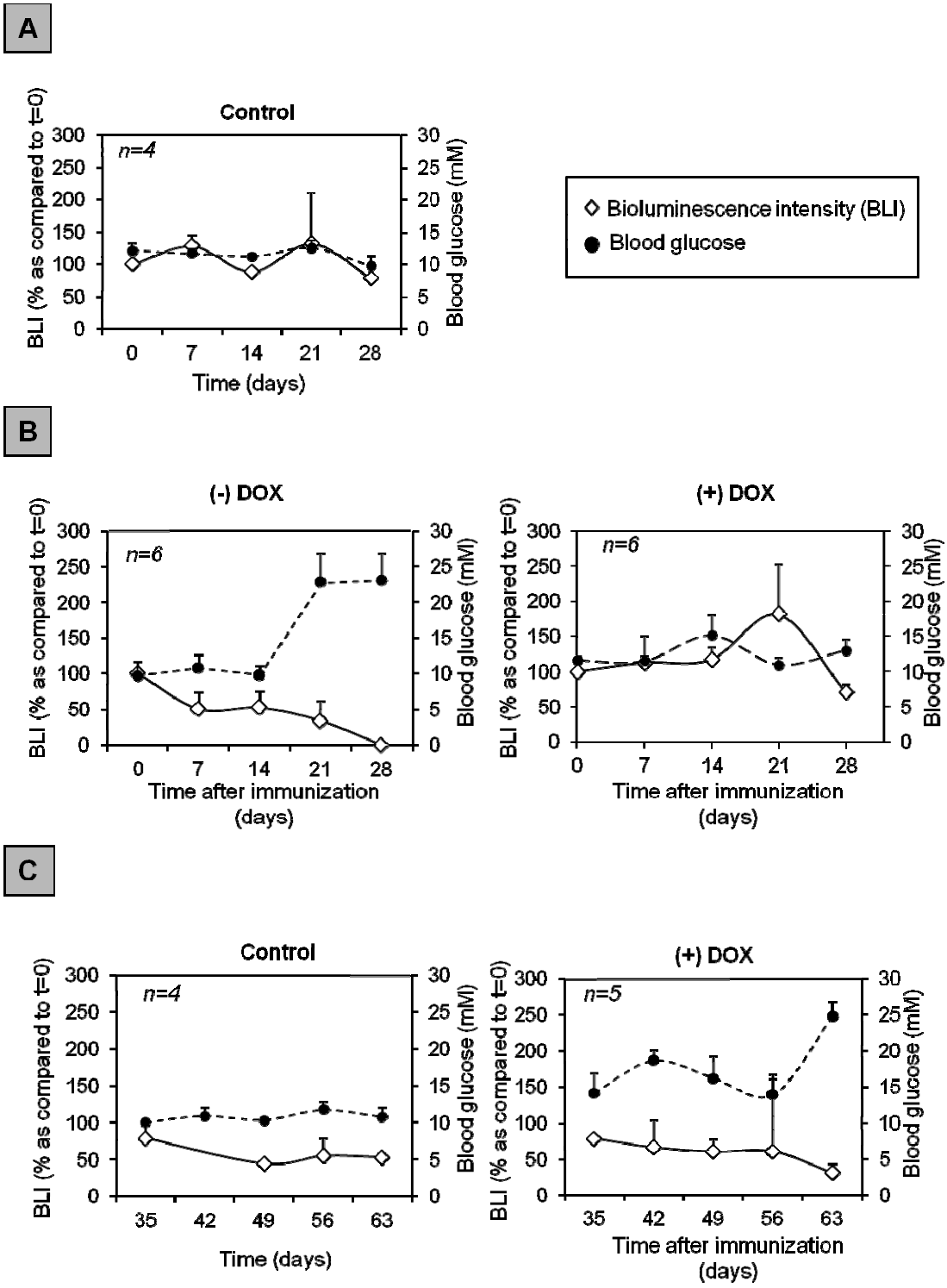
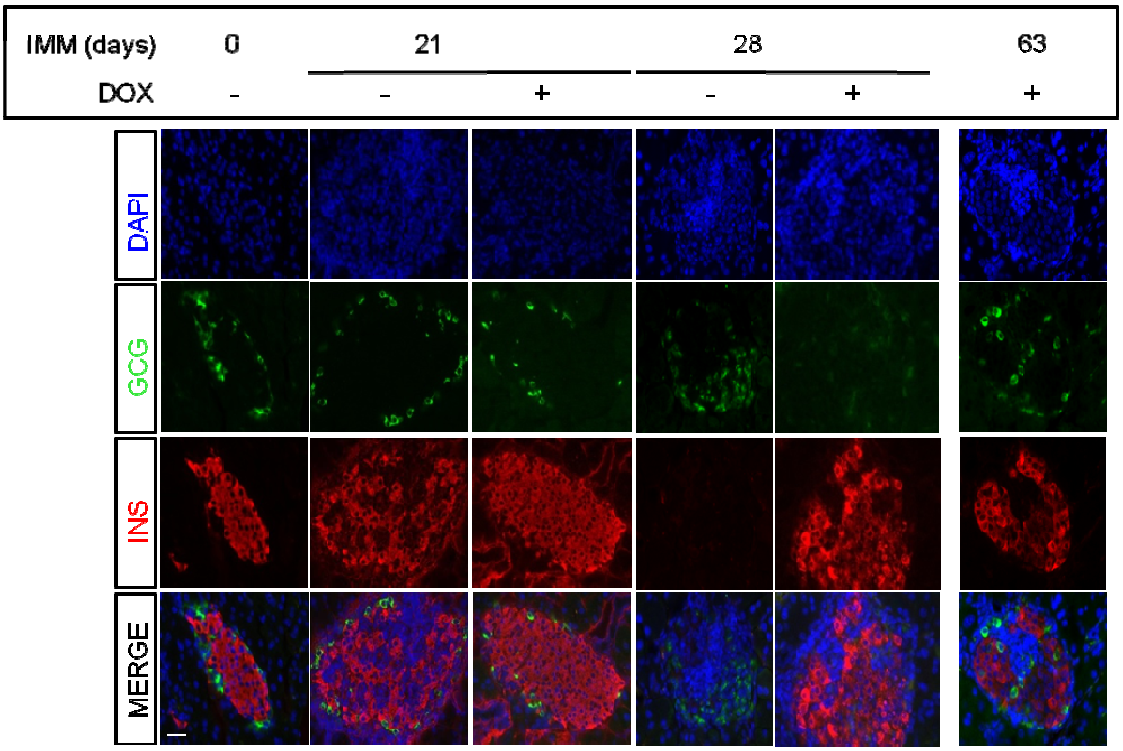


Figure 4: Jiménez-Moreno CM *et al.*

A



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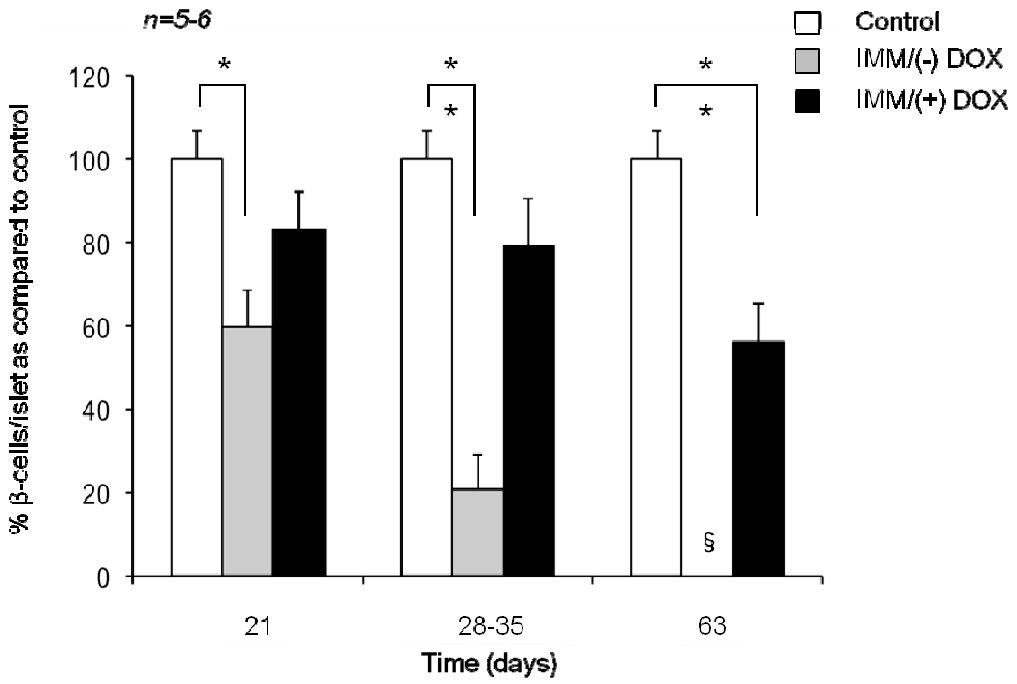


Figure 5: Jiménez-Moreno CM *et al.*

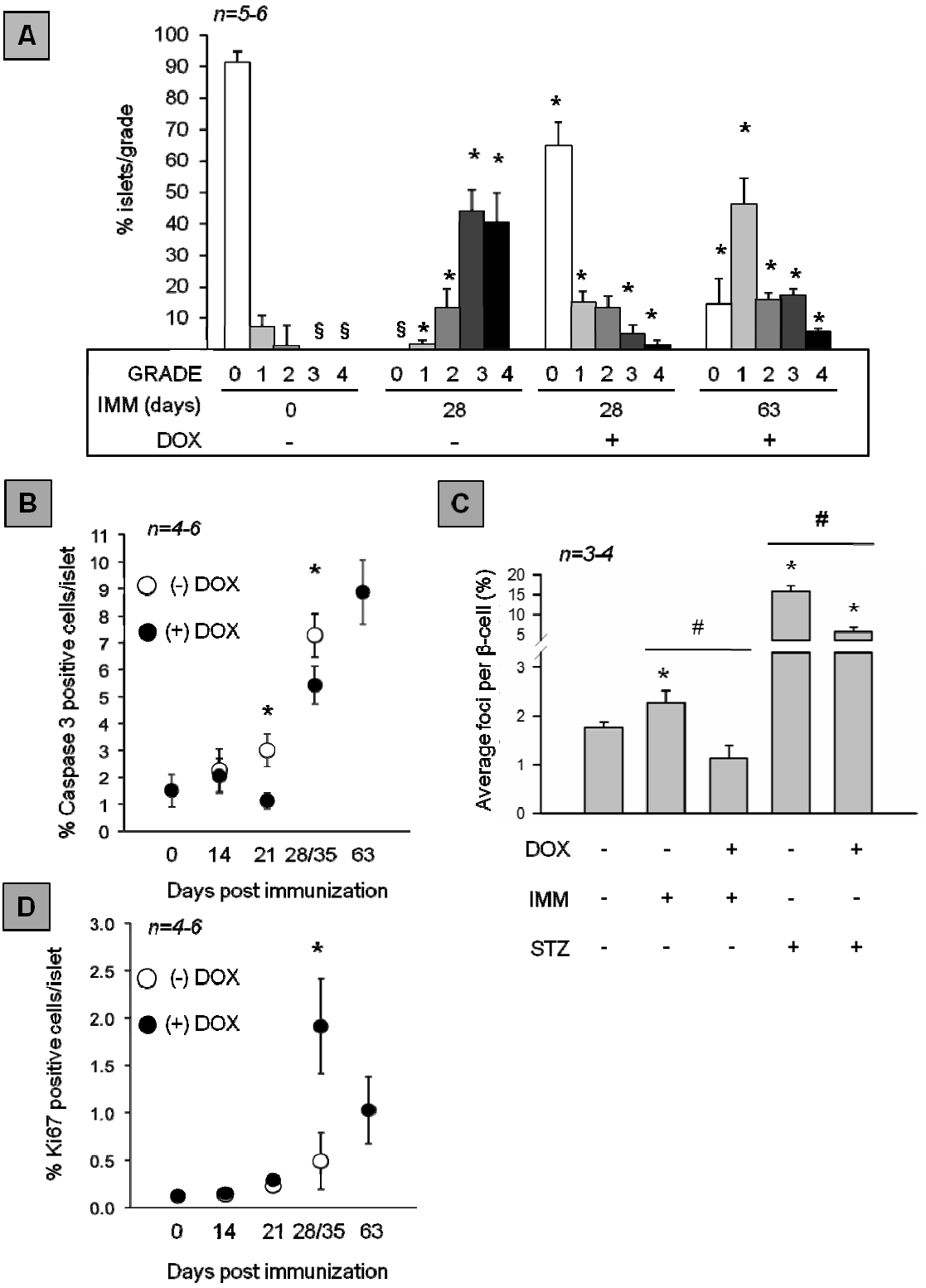
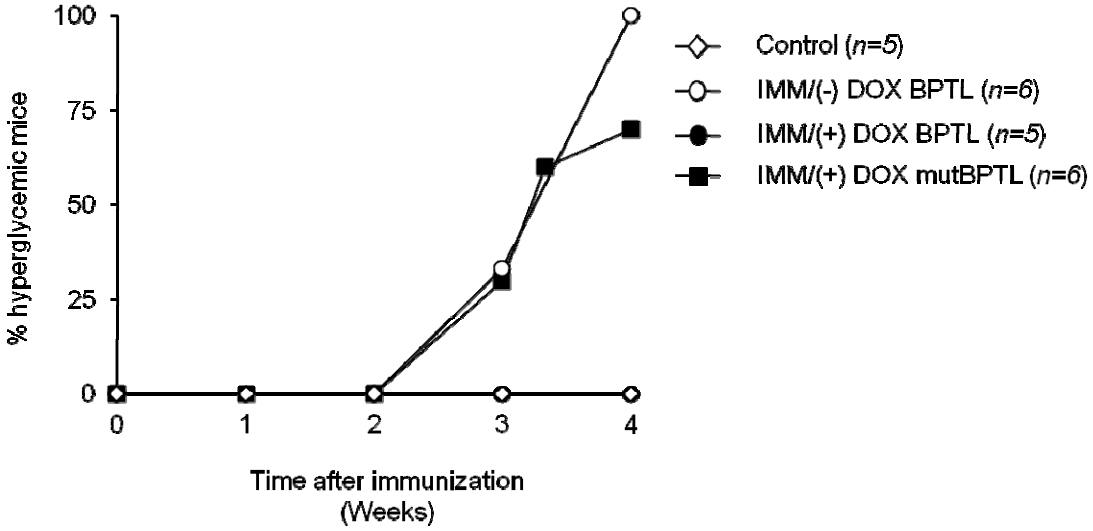


Figure 6: Jiménez-Moreno CM *et al.*



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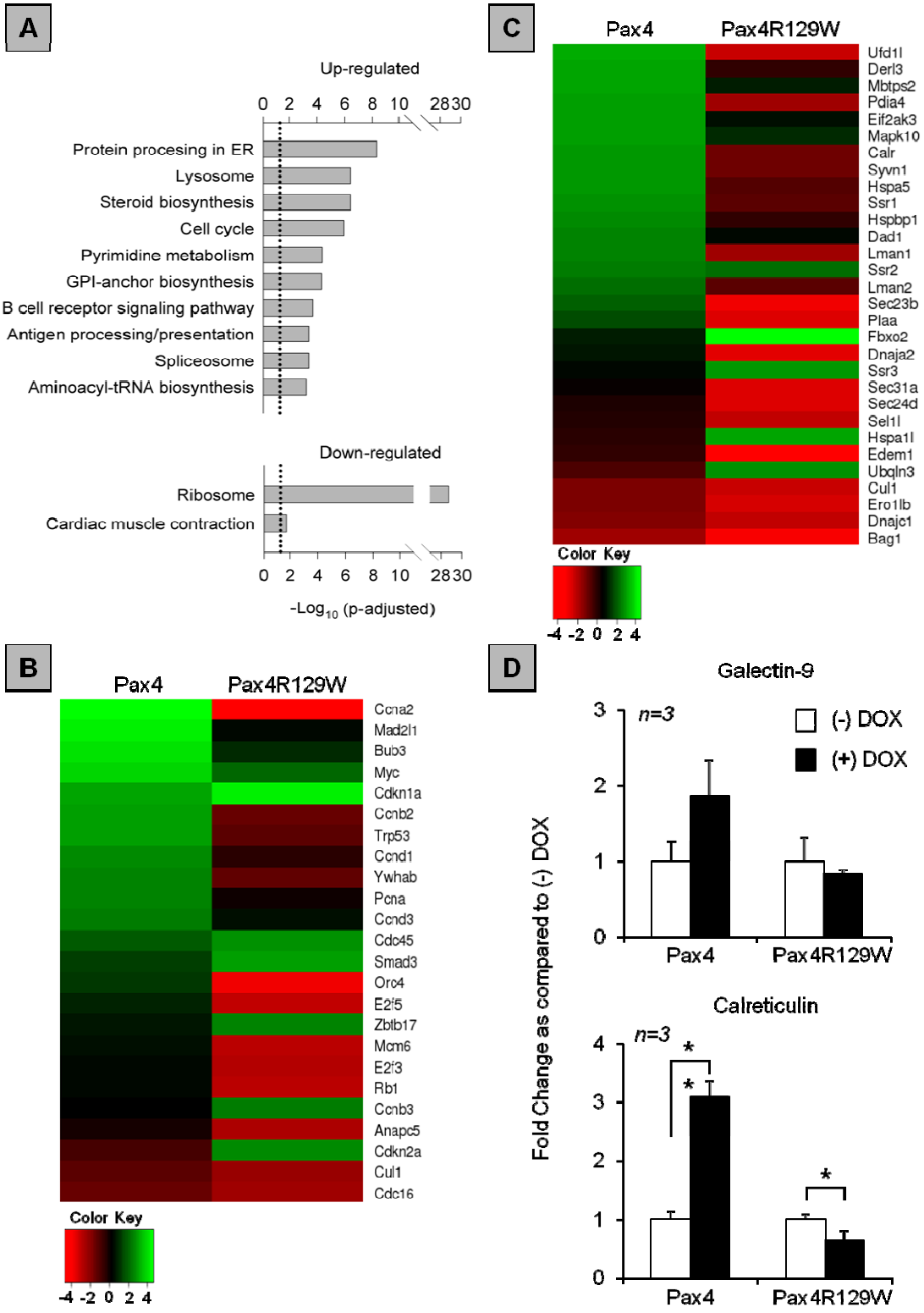
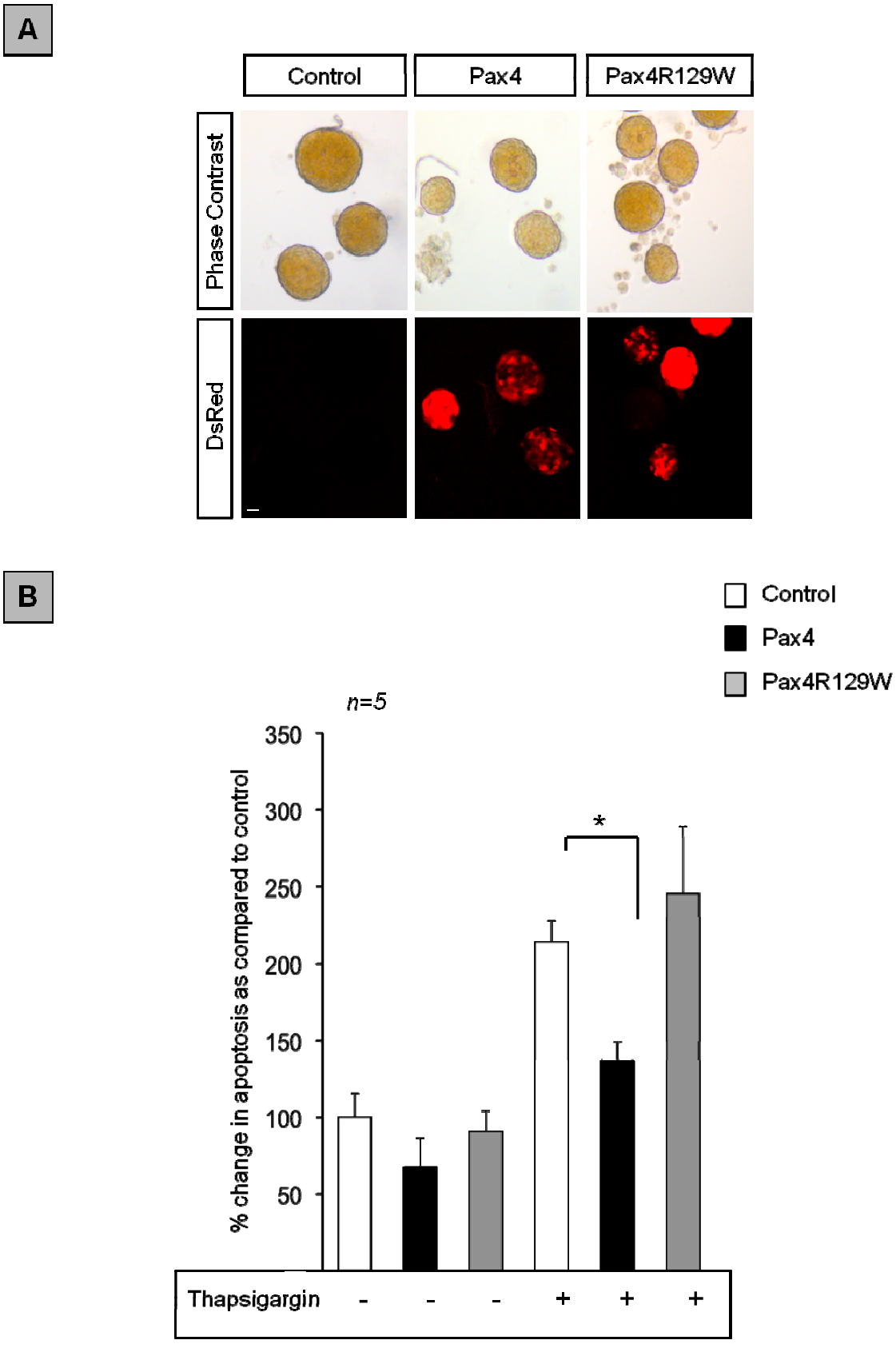
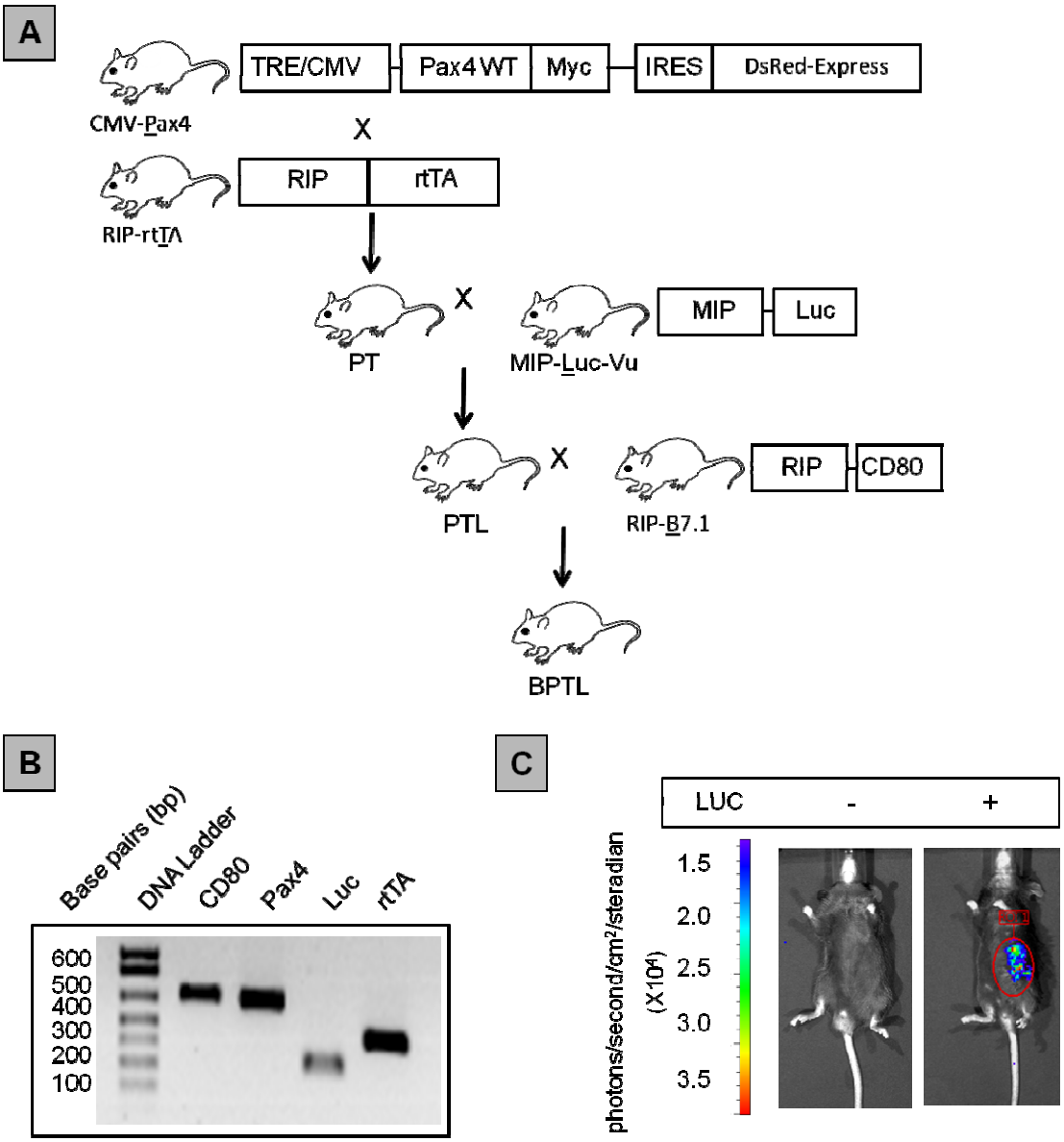
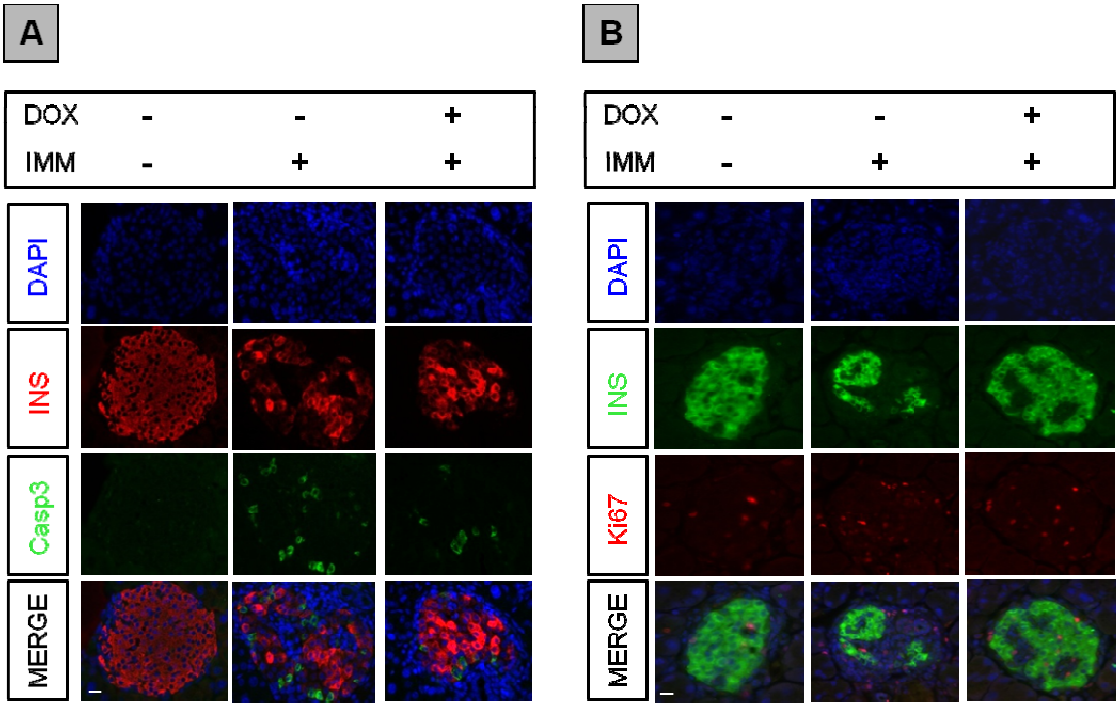


Figure 8: Jiménez-Moreno CM *et al.*





Supplemental Figure 1: Breeding Scheme to Generate BPTL Mice and Validation of Bioluminescence Emission.
(**A**) Pax4 transgenic animals (CMV-Pax4) bearing a Pax4/DsRed-Express construct under the transcriptional control of the CMV promoter were mated with RIP-rtTA mice to engender PT bigenic mice. These mice were then crossed to MIP-LUC-VU animals to generate PTL triple transgenic mice. RIP-B7.1 animals were then mated to PTL animals to derive the BPTL,quadruple transgenic mice that were used in this study. All animals were maintained on a C57BL/6 background. (**B**) Genotyping of BPTL mice by PCR. PCR was used to amplify DNA from individual animals, using primer sets for CD80, Pax4, Luc and rtTA. Samples, and a 100 bp DNA ladder were analyzed on a 1% agarose gel stained with ethidium bromide. (**C**) Representative bioluminescence imaging of control non-transgenic (left image) and BPTL (right image) mice. Circle delineates area quantified for bioluminescence intensity, measured as photons emitted per second per square cm per steradian (surface area of a sphere).



Supplemental Figure 2: Assessment of Islet-Cell Apoptosis and Proliferation in BPTL Mice by Immunohistochemistry.
(A) Apoptosis detection by immunohistochemical analysis of Caspase-3 (Casp3, green) and Insulin (red) on pancreatic paraffin sections from control, immunized (IMM) or immunized and DOX-treated BPTL mice. Nuclei were stained with DAPI (blue). Representative images correspond to animals sacrificed 28 to 35 days post immunization. (B) Cell proliferation assessment by immunohistochemical analysis of Ki67 (red) and Insulin (green) on pancreatic paraffin sections from control, immunized (IMM) or immunized and DOX-treated BPTL mice. Nuclei were stained with DAPI (blue). Representative images correspond to animals sacrificed 28 to 35 days post immunization. Scale bar: 25 µm for all panels in (A) and (B).

Table S1: List of the 20 most highly up- and down-regulated genes (p-value < 0.05) in islets over-expressing Pax4 or Pax4R129W as sorted using the t-statistic algorithm.

Symbol	Pax4 versus Control	Symbol	Pax4R129W versus Control
Lgals9	6.43	4933402P03Rik	7.37
Pax4	6.38	Scn5a	5.93
Nnat	6.05	Olfr1045	5.84
Soat1	5.82	Ttll11	5.54
Nsdhl	5.39	Hapln3	5.44
Pdyn	5.29	Proca1	5.43
Rab8a	5.25	Nudt11	5.34
Cyb5b	5.11	Esyt3	5.31
Mrps21	5.09	Tmem200a	5.24
Rit2	4.98	Edn2	4.93
Rpl37	-4.12	Spry1	-4.90
Rps21	-4.15	Meg3	-4.99
Rps28	-4.27	Snapc3	-5.13
Inmt	-4.28	2610005L07Rik	-5.36
Snord8	-4.36	6820431F20Rik	-5.37
Snora16a	-4.45	6720401G13Rik	-5.39
Snord35b	-5.25	Ddx17	-5.87
4931414P19Rik	-5.40	Ankrd12	-6.16
Snora34	-5.64	Ddx26b	-6.31
Snord104	-8.10	Snora23	-6.33

Table S2: List of significant enriched KEGGs terms (adjusted p-value < 0.05) classified as up regulated in islets either overexpressing Pax4 or Pax4R129W.

Pax4 versus Control		Pax4R129W versus Control	
KEGG Term	-log10 (adjusted p-value)	KEGG Term	-log10 (adjusted p-value)
Protein processing in endoplasmic reticulum	8.32	Neuroactive ligand-receptor interaction	7.85
Lysosome	6.43	Cardiac muscle contraction	2.62
Steroid biosynthesis	6.43	Parkinson's disease	1.49
Cell cycle	5.93	Oxidative phosphorylation	1.42
Pyrimidine metabolism	4.38		
Glycosylphosphatidylinositol (GPI)-anchor biosynthesis	4.34		
B cell receptor signaling pathway	3.69		
Antigen processing and presentation	3.38		
Spliceosome	3.38		
Aminoacyl-tRNA biosynthesis	3.21		
DNA replication	2.29		
Cysteine and methionine metabolism	2.28		
Fc gamma R-mediated phagocytosis	2.27		
Hepatitis C	2.27		
Phagosome	2.27		
MAPK signaling pathway	2.27		
Glutathione metabolism	2.19		
RNA transport	2.08		
Biosynthesis of unsaturated fatty acids	2.08		
Glycosaminoglycan degradation	1.97		
Phenylalanine metabolism	1.85		
Thyroid cancer	1.85		

N-Glycan biosynthesis	1.85		
Leishmaniasis	1.76		
Fc epsilon RI signaling pathway	1.73		
Prion diseases	1.73		
One carbon pool by folate	1.71		
Insulin signaling pathway	1.68		
Endocytosis	1.66		
Glycerophospholipid metabolism	1.63		
Chronic myeloid leukemia	1.63		
Wnt signaling pathway	1.63		
Neurotrophin signaling pathway	1.61		

Pax4 versus Control		Pax4R129W versus Control	
KEGG Term	-log10 (adjusted p-value)	KEGG Term	-log10 (adjusted p-value)
Where is Progesterone-mediated oocyte maturation? Purine metabolism	1.61		
Osteoclast differentiation	1.61		
Rheumatoid arthritis	1.56		
p53 signaling pathway	1.55		
Drug metabolism - other enzymes	1.50		
Toxoplasmosis	1.47		
Proteasome	1.43		
Glycolysis / Gluconeogenesis	1.43		
Pentose phosphate pathway	1.42		
Jak-STAT signaling pathway	1.42		
Mismatch repair	1.36		
Citrate cycle (TCA cycle)	1.36		
Nucleotide excision repair	1.35		
Acute myeloid leukemia	1.35		

Table S3: List of significant enriched KEGGs terms (adjusted p-value < 0.05) classified as down regulated in islets overexpressing Pax4 or Pax4R129W.

Pax4 versus Control		Pax4R129W versus Control	
KEGG Term	-log10 (adjusted p-value)	KEGG Term	-log10 (adjusted p-value)
Ribosome	28.73	Ubiquitin mediated proteolysis	7.88
Cardiac muscle contraction	1.73	Spliceosome	7.80
		RNA transport	4.40
		Protein processing in endoplasmic reticulum	3.74
		Ribosome biogenesis in eukaryotes	3.74
		Aminoacyl-tRNA biosynthesis	3.00
		mRNA surveillance pathway	2.89
		Prostate cancer	2.62
		Phosphatidylinositol signaling system	2.62
		Oocyte meiosis	2.62
		Inositol phosphate metabolism	2.33
		Chagas disease (American trypanosomiasis)	2.20
		Endocytosis	1.90
		Lysosome	1.77
		NOD-like receptor signaling pathway	1.77
		Progesterone-mediated oocyte maturation	1.75
		Cell cycle	1.75
		Bacterial invasion of epithelial cells	1.75
		Pancreatic cancer	1.50

Table S4: Antibodies used in this study.

Primary Antibody	Vendor	Cat. No.	Fold Dilution
Mouse anti-insulin	Sigma	I2018-.2ML	500
Rabbit anti-glucagon	Cell Signalling	2760S	200
Mouse anti-glucagon	Sigma	G2654-.2ML	200
Rabbit anti-cleaved caspase-3	Cell Signaling	9661	150
Rabbit anti-Ki67	Thermo Scientific	RM-9106-SO	150
Mouse anti-53BP1	Santa Cruz Biotechnology	Sc-22760	500
Secondary Antibody	Vendor	Cat. No.	Fold Dilution
Goat anti-mouse IgG Alexa Fluor 568	Invitrogen	A11004	1000
Goat anti-rabbit IgG Alexa Fluor 488	Invitrogen	A11008	500
Donkey anti- goat IgG Alexa Fluor 488	Invitrogen	A11055	500
Donkey anti-mouse IgG Cy3	Jackson ImmunoResearch	715-175-151	800

CHAPTER V_PAX8 PROJECT RESULTS

CHAPTER V_PAX8 PROJECT RESULTS

5.1. Immunohistochemical Assessment of Pax8 Expression During Pancreatic Islets Development and in Human Neuroendocrine Tumors

OBJECTIVE

The transcription factor Pax8 has recently been reported in adult human pancreatic islets and in pancreatic neuroendocrine tumors (PNETs) *(Haynes et al., 2011; Laury et al., 2011; Long et al., 2010; Ozcan et al., 2011; Sangoi et al., 2011)*. The aim of this work was to analyze the expression of Pax8 during pancreogenesis and in mature mouse and human islets. Ultimately, this study evaluates Pax8 detection as a potentially valuable diagnostic marker of PNETs.

RESULTS and CONCLUSIONS

Pax8 transcript levels were not detected in neither developing nor mature mouse islets, whereas in human islets were almost undetectable. Our results demonstrated that the most commonly used polyclonal Pax8 antibody cross-reacts with the islet-enriched Pax6 protein. A novel monoclonal Pax8 antibody was used to re-evaluate Pax8 expression in islets and PNETs corroborating our QT-PCR findings. In conclusion, Pax8 is not expressed in mature islets under normal physiological conditions. Additionally, Pax8 is not a reliable marker for PNETs highlighting the importance of validating the antibodies used for clinical studies.

CONTRIBUTION

My contribution in this article is the participation in the planning, design and execution of all results of the entire project. Therefore, my work consisted in the isolation, mRNA extraction and quantification by QT-PCR of Pax8, Pax4 and Pax6 transcript levels in mouse and human islets, kidney, liver and several cell lines (Figure 1 and Figure 4A). Furthermore, I performed cell culture, transient transfection, protein extraction and western blot analysis of cell extracts using different Pax8 antibodies (Figure 4C and Figure 5A). I performed immunohistochemical analysis of developing pancreas (Figure 2A and Figure 7), adult mouse and human islets (Figure 3, Figure 6 and Figure 7), various cell lines (Figure 4B and Figure 5B) and in pancreatic neuroendocrine tumors, human kidney and liver (Figure 8). These results indicate that Pax8 is neither expressed in normal pancreatic islets nor in PNETs discarding its potential use as a tumoral marker. Likewise, I actively participated in the discussion of the results of the project.

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ORIGINAL PAPER

Immunohistochemical assessment of Pax8 expression during pancreatic islet development and in human neuroendocrine tumors

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Abstract The paired box transcription factor Pax8 is critical for development of the eye, thyroid gland as well as the urinary and reproductive organs. In adult, Pax8 overexpression is associated with kidney, ovarian and thyroid tumors and has emerged as a specific marker for these cancers. Recently, Pax8 expression was also reported in human pancreatic islets and in neuroendocrine tumors, identifying Pax8 as a novel member of the Pax family expressed in the pancreas. Herein, we sought to provide a comprehensive analysis of Pax8 expression during pancreogenesis and in adult islets. Immunohistochemical analysis using the most employed Pax8 polyclonal antibody revealed strong nuclear staining in the developing mouse pancreas and in mature human and mouse islets. Astonishingly, Pax8 mRNA in mouse islets was undetectable while human islets exhibited low levels. These discrepancies raised the possibility of antibody cross-reactivity. This premise was confirmed by demonstrating that the polyclonal Pax8 antibody also recognized the islet-enriched Pax6 protein both by Western blotting and immunohistochemistry. Thus, in islets polyclonal Pax8 staining corresponds mainly to Pax6. In order to circumvent this caveat, a novel Pax8 monoclonal antibody was used to re-evaluate whether Pax8 was indeed expressed in islets. Surprisingly, Pax8 was not detected in neither the developing pancreas or in mature islets. Reappraisal of pancreatic neuroendocrine tumors using this Pax8 monoclonal antibody exhibited no immunostaining as compared to the Pax8 polyclonal antibody. In conclusion, Pax8 is not expressed in the pancreas and cast doubts on the value of Pax8 as a pancreatic neuroendocrine tumor marker.

Keywords Pancreatic islet · Transcription factor · Pax genes · Neuroendocrine tumors · Antibody cross-reactivity

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Introduction

A complex network of transcription factors controls pancreatic islet development as well as its subsequent mature function responsible for the exquisite maintenance of blood glucose homeostasis (Servitja and Ferrer 2004). Of particular interest is the family of paired box (Pax) genes, which encode key regulators of tissue development and cellular differentiation. The Pax transcription family comprises 9 members divided into 4 groups based on the

specific assembly of 3 structural domains; the highly conserved 128 amino acid DNA binding paired domain, the octapeptide and a complete or residual DNA interacting homeodomain. Combinations of individual Pax members are spatially as well as temporally regulated during development and contribute to the formation of diverse organs (Lang et al. 2007).

Two members of this family, Pax6 and Pax4, comprising group IV and typified by the lack of the octapeptide domain, were shown to be essential for pancreatic islet organogenesis and function. Indeed, deletion of Pax4 in mice results in the absence of both β- and δ-cells with a concomitant increase in α-cells (Sosa-Pineda 2004). In contrast, deletion of Pax6 results in the absence of α-cells together with a decrease in the other types of pancreatic endocrine cells (St-Onge et al. 1997). Although β-, δ- and PP-cells are still present, they fail to form distinct islets and remain disorganize throughout the exocrine pancreas, suggesting that Pax6 is not only essential for α-cell differentiation but also for cell migration necessary for islet formation (St-Onge et al. 1997). Animals with both Pax6 and Pax4 ablation completely lack pancreatic islets demonstrating the absolute requirement of both Pax factors for islet generation.

Surprisingly Pax8, which is essential for development of the eye, thyroid gland as well as the urinary and reproductive organs, has recently emerged as a new member of the Pax family involved in islet physiology. Indeed, although previous reports have not detected Pax8 expression in pancreatic islets (Goode and Elgar 2009), more recent studies claim strong Pax8 immunostaining in pancreatic neuroendocrine tumors (Haynes et al. 2011; Long et al. 2010; Sangoi et al. 2011) as well as in normal human islets. Reported Pax8 expression in human pancreas is restricted to the endocrine compartment, as exocrine acinar and ductal cells exhibited no staining (Ozcan et al. 2011; Sangoi et al. 2011). Moreover, transcript expression profiling performed on islets isolated from pregnant mice revealed a robust induction of Pax8 expression levels during gestation (Rieck et al. 2009). In addition, a genome-wide linkage and admixture mapping study has linked Pax8 to Type 2 diabetes in African American families (Elbein et al. 2009). Taken together, these studies prompt us to investigate whether Pax8 could also be expressed during pancreatic islet development pinpointing to a potential new role of this transcription factor in islet physiology. To address this question, we analyzed Pax8 expression during development using a polyclonal Pax8 antibody previously utilized in several clinical studies that clearly demonstrated Pax8 staining in pancreatic neuroendocrine tumors (Haynes et al. 2011; Long et al. 2010; Sangoi et al. 2011). Consistent with these studies, strong Pax8 immunostaining was detected in the developing pancreas as well as in adult mouse and human islets of different ages. In contrast, Pax8 mRNA levels in both human and mouse islets were low to undetectable. In an attempt to confirm and validate the specificity of this Pax8 antibody, we found that it cross-reacted with the more abundant islet enriched Pax6 protein. In order to circumvent this caveat, we used a monoclonal antibody against Pax8 and re-evaluated whether Pax8 was indeed detected in islets. Astonishingly, we were unable to confirm expression of this Pax member neither in the developing pancreas, islets nor in pancreatic neuroendocrine tumors. Therefore, our results cast serious doubts on the value of Pax8 as a pancreatic neuroendocrine tumor marker and call for vigilance in the use of non-validated antibodies for clinical studies.

Materials and methods

Islet isolation and, culture

Pancreatic mouse islets were isolated by collagenase (Sigma-Aldrich Quimica S.A., Madrid, ES) digestion, handpicked and maintained in 11.1 mM glucose/RPMI-1640 (Invitrogen S.A., Barcelona, ES) supplemented with 10% fetal calf serum (Sigma-Aldrich Quimica S.A.), 100 Units/ml penicillin and 100 mg/ml streptomycin (Sigma-Aldrich Quimica S.A.), and 2 mM glutamine (GlutaMAX; Invitrogen S.A.). Isolated human islets obtained from the Cell Isolation and Transplantation Center, Geneva University Hospitals, were maintained in CMRL 1066 medium w/o phenol red (Sigma-Aldrich Quimica S.A.) supplemented with 10% fetal calf serum (Sigma-Aldrich Quimica S.A.), 100 Units/ml penicillin and 100 mg/ml streptomycin (Sigma-Aldrich Quimica S.A.), 100 mg/ml gentamycin (Sigma-Aldrich Quimica S.A.) and 2 mM glutamine (GlutaMAX, Invitrogen S.A.).

Tissue samples and preparation

Embryonic, pup and adult mouse tissues were dissected and fixed in 4% paraformaldehyde overnight at 4°C, embedded in paraffin and sectioned at 7 µm. Sections from biopsies of human pancreatic neuroendocrine tumor were kindly provided by the Hospital Universitario Virgen del Rocio (Sevilla, Spain). Sections included both neuroendocrine tumor and neighbouring non-neoplastic areas.

Quantitative real time PCR (Q-RT-PCR)

Total RNA from islets was extracted using the RNeasy Micro Kit (Qiagen, Madrid. ES) and Q-RT-PCRs performed as described previously (Hu He et al. 2011). Briefly, 2 µg RNA were used to synthesize first-strand

cDNA using Superscript II (Invitrogen S.A.) in a total volume of 20 µL. 3 µL of diluted cDNA samples were used as template for the Q-RT-PCR using SYBR green (Roche Farma S.A., Madrid, ES). Q-RT-PCR data were normalized using the $2^{-\Delta Ct}$ method for transcripts levels evaluated in different tissues. In situation in which transcript levels were derived from the same tissue, the $2^{\Delta\Delta Ct}$ method was employed and normalized to Pax6 expression levels. Briefly, the ΔΔCt is calculated as the Pax6 ΔCt minus the ΔCt of the gene of interest. The ΔCt is calculated as the crossing point of the housekeeping gene (cyclophilin) minus the crossing point of the gene of interest. Primer sequences listed in Table 1 were designed using the Primer3 Software (http://frodo.wi.mit.edu/primer3/).

Transient transfection and immunoblot analysis

Transient transfection of human Pax4 (Brun et al. 2008), Pax6 and Pax8 (Origene Technologies, Rockville USA) expressing plasmids were performed using Lipofectamine 2000 (Invitrogen S.A.) following the manufacturer's protocol. Briefly, MCF-7 cells plated on six multiwell plates were transfected with 4 µg DNA. 24 h after transfection cells were harvested and whole-cell extracts were prepared by lysing the cells at 4°C for 1 h in WCB (20 mM Hepes (pH 7.7), 300 mM NaCl, 0.2 mM EDTA, 0.1% Triton X-100, 1.5 mM MgCl2, 0.5 mM DTT) supplemented with protease inhibitors. 15 µg of protein extracts were resolved in SDS-PAGE gels and transferred to PVDF membranes. Membranes were blocked with 5% milk in Tris-buffered saline—0.1% Tween (TBST) and subsequently incubated overnight at 4°C with the primary antibody in TBST containing 3% BSA. The membranes were then incubated with the corresponding secondary antibody in TBST-3% nonfat milk for 1 h at room temperature and the immunoreactive bands were visualized using the enhanced chemiluminescence detection system (ECL) (GE-Healthcare, Barcelona ES). Specific antibodies were used as follow: Polyclonal Pax8 (No 10336-1-AP ProteinTech Group, Chicago, USA) 1:500 dilution; monoclonal Pax8 (No ab53490, Abcam, Cambridge UK) 1:100 dilution; monoclonal Pax6 (DSHB-University of Iowa, USA) 1:1,000 dilution; c-Myc (Sigma-Aldrich Quimica S.A.) 1 µg/mL; α-tubulin (Sigma-Aldrich Quimica S.A.) 1:5,000 dilution.

Table1 Sequence of the primers used in the study

Gene		Primers (5′–3′)
Human		
Pax4	Forw.	ACCCCACCTAAAGCCTGTCT
	Rev.	AGGCAAAGCAGTCCTGAGTC
Pax6	Forw.	CTCGGGCACCACTTCAACA
	Rev.	TCCGGGAACTTGAACTGGAA
Pax8	Forw.	CCCCCTACTCCTCCTACAGC
	Rev.	GGCCTTGATGTGGAACTGTAA
Cyclophilin	Forw.	TACGGGTCCTGGCATCTTGT
	Rev.	CCATTTGTGTTGGGTCCAGC
Mouse		
Pax4	Forw.	TCGAATTGCCCAGCTAAAGG
	Rev.	TTCAGTGCAAAGCTGGTGTTG
Pax6	Forw.	CAACCTGGCTAGCGAAAAGC
	Rev.	TCTGCCCGTTCAACATCCTT
Pax8	Forw.	GTTTGAGCGGCAGCATTAC
	Rev.	GTAAGGGCAGTGGGTACAGC
Cyclophilin	Forw.	ATGGCAAATGCTGGACCAA
	Rev.	GCCATCCAGCCATTCAGTCT

Immunostaining

Immunohistochemistry analysis was performed as previously described (Rojas et al. 2009). Briefly, subsequent to antigen retrieval by autoclaving in citrate buffer for mouse tissues or in EDTA buffer using PT-Link (DAKO, Barcelona, ES) for human sections; tissue samples were blocked in PBS-3% BSA for 1 h. Overnight incubation with primary antibodies (rabbit polyclonal Pax8 antibody at 1:400 dilution, mouse monoclonal Pax8 at 1:10 dilution and monoclonal Pax6 antibody at 1:800 dilution) was performed at 4°C. Corresponding biotinylated secondary antibodies (dilution 1:300) were used for 1 h at room temperature and DAB (Vector Lab, Barcelona, ES) was used for signal development. Counterstaining with Hematoxilin (Panreac, Barcelona, ES) was performed.

Alternatively, for immunofluorescence, cells grown on coverslips were fixed 10 min at room temperature in 4% paraformaldehyde and methanol for 2 min at −20°C. Cells were then permeabilized with PBS containing 0.1% Tween-20 and 2% BSA and blocked in 5% BSA in TBS-0.1% Triton X-100. Primary antibodies, rabbit polyclonal Pax8 antibody at 1:800 dilution, a mouse monoclonal Pax8 at 1:10 dilution and a monoclonal Pax6 antibody at 1:800 dilution, were used overnight at 4°C. Secondary antibodies used were either rabbit or mouse Alexa Fluor 568 and Alexa Fluor 488. Counterstaining with DAPI was performed in order to reveal nuclei. The slides were analyzed using epifluorescence microscopy (Leica DM6000B, Leica).

Results

Pax8 transcript levels are low to undetectable in adult pancreatic islets

Recent studies revealing Pax8 staining in human pancreatic neuroendocrine tumors as well as in normal adult islets

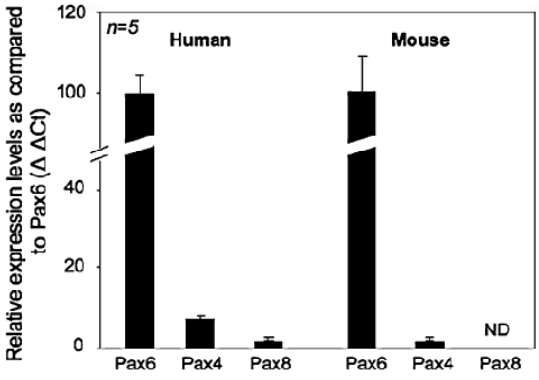

Fig. 1 Pax8 transcript levels are low to undetectable in adult human and mouse islets as compared to Pax6 and Pax4. Quantitative RT-PCR using RNA purified from freshly isolated mouse and human islets were performed on Pax8, Pax6, Pax4 and the housekeeping gene cyclophilin. The Pax4 and Pax8 relative mRNA abundance levels were established using the $2^{\Delta\Delta Ct}$ method and compared to the expression levels of Pax6 (arbitrarily indicated as 100). Results represent the average of at least five experiments performed in duplicate ± SE (*ND* non detected)

prompted us to evaluate expression levels of this transcription factor in human and mouse islets as compared to Pax6 and Pax4, two Pax family members expressed in the endocrine pancreas. Consistent with previous reports (Brun et al. 2004; Hu He et al. 2011; Kutlu et al. 2009), Pax6 was highly expressed in both human and mouse islets whereas Pax4 levels were much lower, nonetheless detectable (Fig. 1). In contrast, the Pax8 transcript was not detected in mouse islets whereas human islets exhibited low levels as compared to Pax6 and Pax4 (Fig. 1). As several Pax8 splice variants have been reported (Kozmik et al. 1993), various combinations of primers were evaluated to eliminate the possibility that only a subset of these spliced transcripts were expressed in islets. Astonishingly, identical results were obtained independently of the primer pairs used (Data not shown).

A Pax8 polyclonal antibody reveals staining in the developing mouse pancreas and in neonatal islets

Low levels of the Pax8 transcript in adult islets led us to investigate whether Pax8 expression could be temporally expressed during development. To address this question, we performed immunohistochemical analysis on kidney, pancreas and liver of mouse embryos at embryonic stage (E)15.5 and E17.5 as well as newborn pups using the most cited Pax8 polyclonal antibody. We opted for this approach in order to accurately determine whether Pax8 is specifically expressed in the developing pancreas. Consistent with a previous study detecting Pax8 transcript (Plachov et al. 1990), strong Pax8 nuclear immunostaining was observed in the endodermal compartment of both the developing and newborn kidney (Fig. 2A; a, d and g). In the liver, Pax8 staining was only detected in scattered cells (Fig. 2A; c, f and i). Consistent with our hypothesis, the developing pancreas also exhibited Pax8 staining in the endocrine compartment while the surrounding exocrine tissue displayed no expression of the transcription factor (Fig. 2A; b and e). Intriguingly, Pax8 immunostaining also persisted in the majority of islets cells of neonatal animals (Fig. 2A; h). These findings were substantiated by immunofluorescence analysis (Fig. 2B).

The Pax8 polyclonal antibody reveals immunostaining in the adult pancreas

Detection of Pax8 positive cells in neonatal islets using a polyclonal antibody prompt us to investigate whether the protein could also be detected in adult islets. Consistent with the immunostaining observed in embryos and newborn mice, Pax8 was also detected in most cells of adult mouse and human islets (Fig. 3A). Surprisingly, these results are in sharp contrast to our gene expression analysis in which the Pax8 transcript was not detected in adult mouse islets (Fig. 1). In order to verify these results, immunohistochemistry analysis were repeated using a recently commercialized Pax8 monoclonal antibody generated against the C-terminal region of Pax8. In contrast to the polyclonal antibody, the monoclonal Pax8 antibody revealed no staining in either islets from adult mice or human (Fig. 3B). Discrepancies in the immunohistochemical data combined with undetectable Pax8 transcripts in islets raise the possibility that the Pax8 polyclonal antibody may cross-react with other pancreatic islet-enriched Pax members.

The polyclonal Pax8 antibody cross-reacts with the islet-enriched Pax6 family member

To authenticate or rebut the specificity of the Pax8 polyclonal antibody, we performed Western blotting analysis on cells transfected with either Pax6 or Pax4 expressing vectors. These two Pax members are expressed in both the developing pancreas as well as in mature islets (Brun et al. 2004, 2008; Sosa-Pineda et al. 1997; St-Onge et al. 1997) (Fig. 1). In order to identify the most suitable experimental cell model and exclude cross reactivity with other potential proteins, we initially screened several cell lines for the expression of Pax8 using Q-RT-PCR. The Pax8 transcript was not detected in the human epithelial carcinoma Hela cell line, the human breast cancer MCF7 cell line or in the rat insulinoma INS-1E cell line (Fig. 4A). However, immunofluorescence and Western blot analysis using the polyclonal Pax8 antibody revealed strong positive staining

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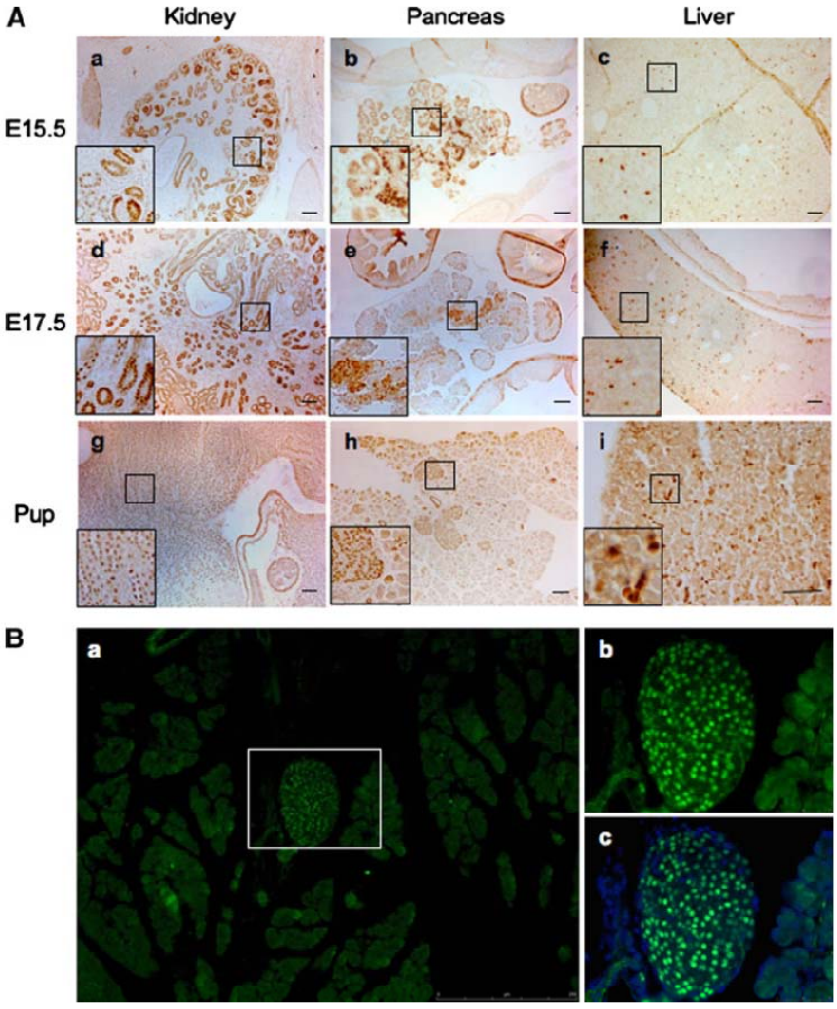

Fig. 2 A polyclonal Pax8 antibody reveals expression of the transcription factor in kidney, pancreas and liver during development. A Immunohistochemistry staining using a polyclonal Pax8 antibody shows strong nuclear staining in kidney and pancreas and in scattered cells in liver during embryonic development (*a–f*) and in new born pups (*g–i*) (*scale bar* 50 μm). B Immunofluorescence images of pancreatic tissue from neonatal animal incubated with polyclonal Pax8 (*green*) exhibiting strong Pax8 nuclear staining in the islets while the surrounding exocrine tissue is negative. *b* and *c* are the enlarged detail of the islet indicated in *a*, showing in *c* the co-staining with DAPI (*blue*) to reveal the totality of islet cells

in both HeLa and INS-1E, further indicating a possible non-specificity of the polyclonal antibody (Fig. 4B, C). In contrast, MCF7 cells were negative for Pax8 staining (Fig. 4B, C) and we therefore selected this cell line to further study the specificity of the polyclonal Pax8 antibody. Protein extracts derived from MCF7 cells transfected with human Pax4, Pax6 or Pax8 expression vectors were blotted with an anti-myc (Pax4) and an anti-Pax6 serum as well as with the polyclonal Pax8 antibodies. The anti-myc and anti-Pax6 sera specifically detected Pax4 and Pax6, respectively. However, although the polyclonal Pax8 antibody revealed a strong signal in Pax8-tranfected cells, it also recognized, albeit with lower intensity, a band of similar molecular weight in Pax6-transfected MCF7 cells (Fig. 5A). In contrast, the Pax8 monoclonal antibody exclusively recognized a band in MCF7 cells transfected with Pax8 (Fig. 5A). Immunofluorescence analysis performed on Pax-transfected MCF7 cells confirmed that the polyclonal Pax8 antibody recognized both Pax6 and Pax8 while the monoclonal antibody was specific to Pax8 (Fig. 5B).

The polyclonal Pax8 antibody detects Pax6 in islets as well as in the developing pancreas

Our findings indicating cross-reactivity of Pax8 polyclonal antibody with Pax6 raise the possibility that the signal detected in islet is not Pax8 but Pax6. To verify this possibility, co-immunofluorescence studies were performed on both human and mouse disaggregated islets using the

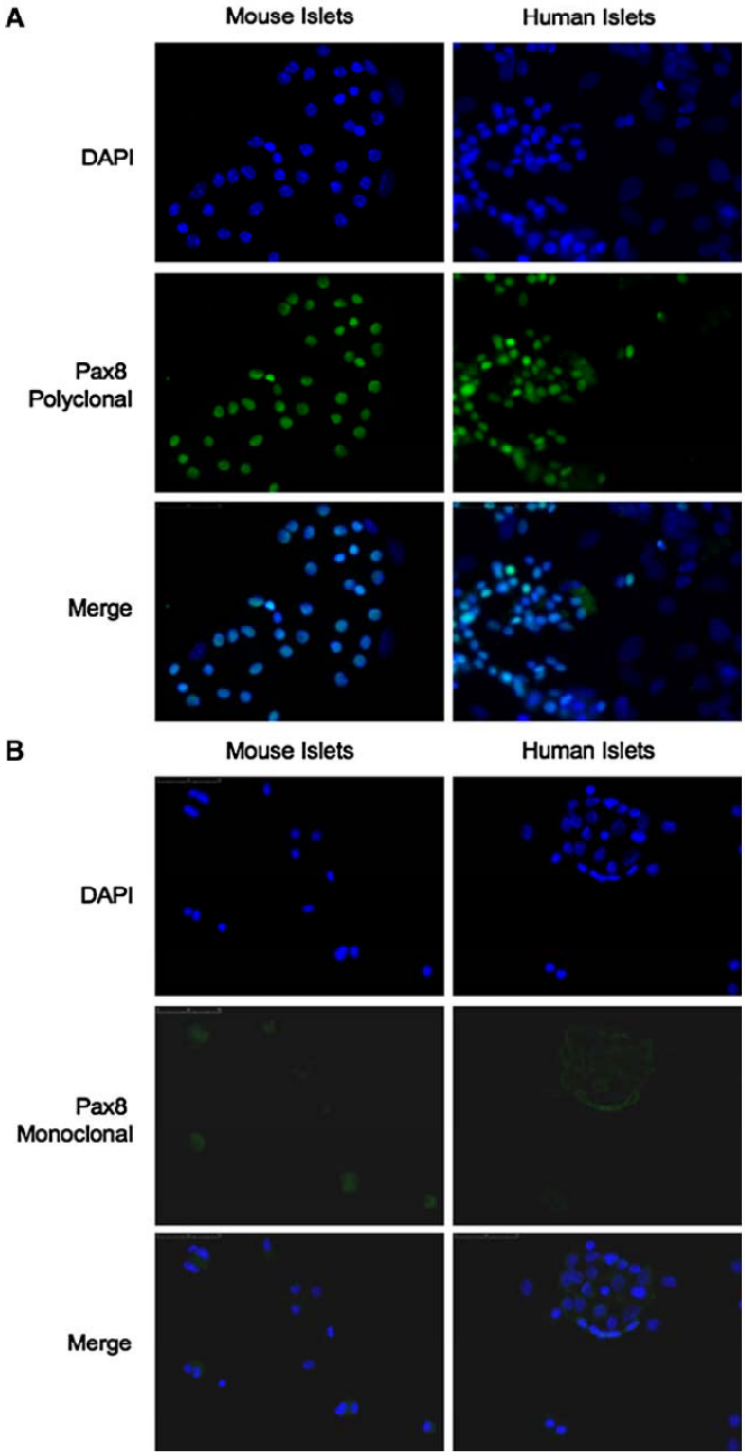

Fig. 3 A polyclonal but not a monoclonal antibody against Pax8 immunostains adult mouse or human islets. **A** Immunofluorescence analysis of disaggregated mouse (*left panels*) and human (*right panels*) islet cells (×400) using a polyclonal Pax8 antibody (*green*) shows, in the majority of islet cells, a strong nuclear Pax8 staining. **B** No immunostaining was detected either in mouse (*left panels*) or human (*right panels*) islets when a monoclonal Pax8 antibody (*green*) was used (×400). For the visualization of all cells, counterstaining with DAPI (*blue*) was performed

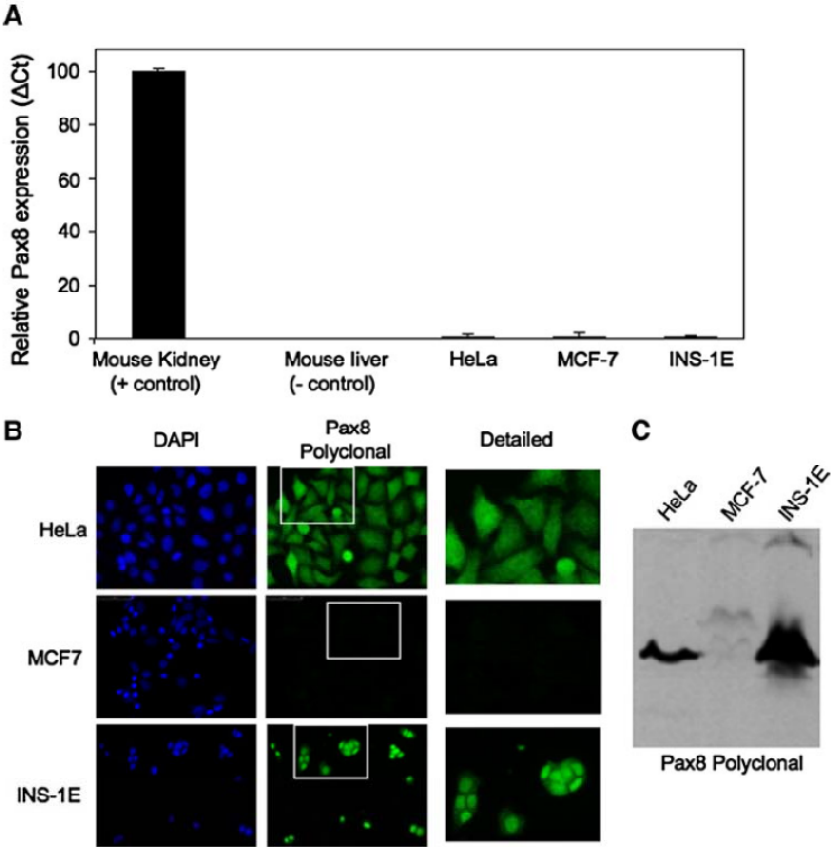

Fig. 4 The Pax8 protein but not the transcript is detected in various cell lines. **A** Q-RT-PCR analysis shows nearly non-detectable expression levels of Pax8 in HeLa, MCF-7 and INS-1E cell lines. The graphic represents the relative abundance of Pax8 mRNA in the different cell lines as compared to the expression levels in kidney (arbitrarily indicated as 100). **B** Immunofluorescence analysis of the same cell lines shows that only MCF-7 cell line is negative for immunostaining when using the Pax8 polyclonal antibody (*green*) (*middle panel*). Counterstaining with DAPI (*blue*) is shown in the left panels (×400). A detailed image corresponding to an enlargement of the indicated area is showed in the *right panels*. **C** Western blot analysis of cell extracts using the polyclonal Pax8 antibody revealed the presence of an immunoreactive band of approximately 48 kD (corresponding to the molecular weight of Pax8) in all cell lines except MCF-7 cells

polyclonal Pax8 antibody together with the anti-Pax6 sera. Consistent with our hypothesis, all Pax8-positive cells were also Pax6-positive and single staining for either Pax8 or Pax6 was never observed in either human or mouse islets (Fig. 6). These results suggest that Pax8, as initially reported (Plachov et al. 1990), is most likely not expressed in the mature pancreas. The latter conclusion, prompt us to re-appraise whether Pax8 was expressed during pancreas development. Consecutive sections from E17.5 embryos as well as from newborn pups and adult pancreas were analyzed by immunohistochemistry using the polyclonal and monoclonal Pax8 antibodies or the Pax6 monoclonal antibody. Strikingly, overlapping expression patterns within the developing pancreas and islets were again observed with the polyclonal Pax8 and Pax6 antibodies (Fig. 7, compare images from a, d and g to b, e and h). In contrast, the Pax8 monoclonal antibody revealed no signal either in the developing pancreas or in adult islets (Fig. 7c, f, i). These results clearly demonstrate that the Pax8 polyclonal antibody cross-reacts with Pax6.

Pax8 is not expressed in pancreatic neuroendocrine tumors

A crucial question that stems from our study is whether Pax8 is truly expressed in pancreatic neuroendocrine tumors as claimed by several independent studies, all using the same Pax8 polyclonal antibody (Haynes et al. 2011; Long et al. 2010; Sangoi et al. 2011). To address this important issue, serial sections of pancreatic neuroendocrine tumors obtained from 9 independent donors were immunostained with Pax8 antibodies (polyclonal and monoclonal) as well as with the anti-Pax6 sera. Consistent with previous reports, the Pax8 polyclonal antibody revealed positive staining in some but not all tumors. Indeed, seven out of nine tumors were positive using this antibody (two representative tumors are shown in Fig. 8A). However, this staining was not confirmed using the more specific monoclonal Pax8 antibody (Fig. 8A, compare a and d, to c and f). In parallel, human kidney and liver samples were used as positive and negative controls for Pax8 expression. Both Pax8 antibodies

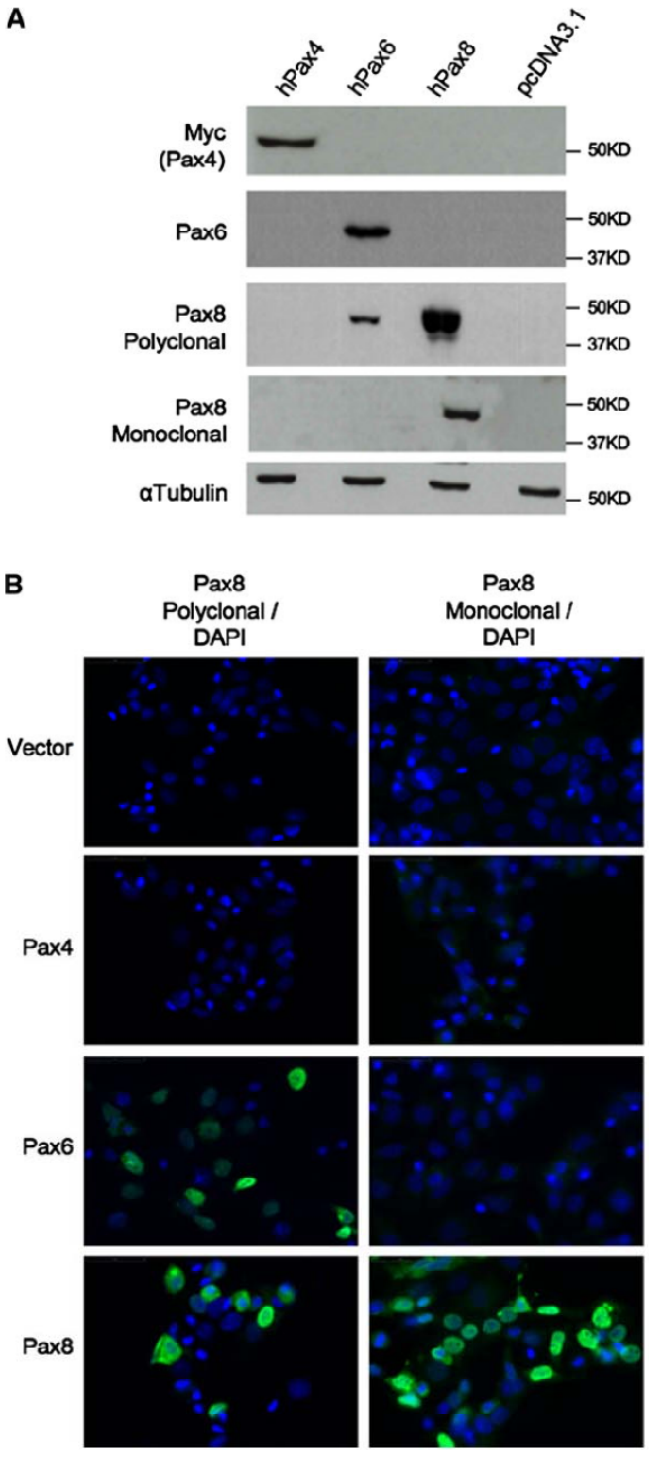

Fig. 5 The polyclonal Pax8 antibody cross-reacts with Pax6. **A** Western blot analysis of extracts from MCF-7 cells transfected with Pax4, Pax6 or Pax8 expression vectors using c-Myc (for the detection of Pax4), Pax6, polyclonal Pax8 and monoclonal Pax8 antibodies. Pax8 polyclonal antibody detects not only Pax8 (*lane 3*) but also Pax6 (*lane 2*), indicating a cross-reactivity of this antibody with Pax6. The other three antibodies used, including a new monoclonal Pax8 antibody, are specific. α-tubulin staining was used for loading control. **B** Immunofluorescence analysis of transfected MCF-7 cells from **A**, using either the polyclonal (*left panels*) or a new monoclonal (*right panels*) Pax8 antibodies (*green*). No immunostaining was detected in Pax4-transfected MCF-7 cells with any of the Pax8 antibodies. However, polyclonal Pax8 antibody is able to detect exogenous Pax6, indicating the existence of cross-reactivity of polyclonal Pax8 antibody with Pax6 protein. Counterstaining with DAPI (*blue*) is shown to visualize also the non-transfected cells

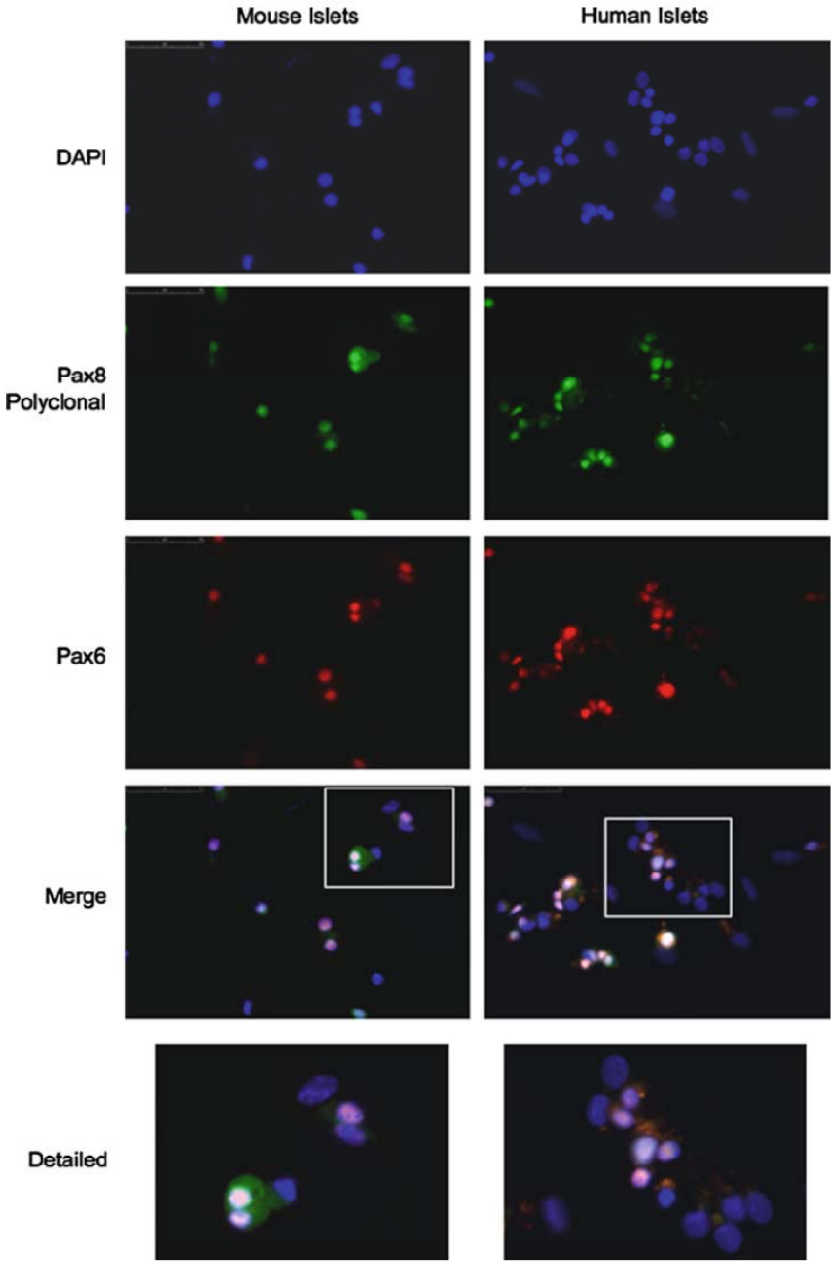

Fig. 6 The Pax8 polyclonal antibody immunostains Pax6 in both human and mouse adult islets. Immunofluorescence analysis of disaggregated mouse (*left panels*) and human (*right panels*) pancreatic islet cells co-immunostained with polyclonal Pax8 (*green*) and Pax6 (*red*) antibodies, shows an overlap in the staining both in human and in mouse islets (×400). The enlargement of the indicated area (*lower panels*) shows this overlap. Counterstaining with DAPI (*blue*) is also shown

exhibited strong nuclear staining in kidney sections while no signal was detected in liver sections (Fig. 8B, C). Interestingly, 3 out of 7 samples displayed strong positive signal for Pax8 polyclonal antibody as well as for Pax6 antibody suggesting that the signal given by the Pax8 polyclonal antibody is due, at least in part, to the recognition of the endogenous Pax6 protein (Fig. 8A, upper panels). However, in the four remaining tumors that reacted with the Pax8 polyclonal antibody, no staining was detected for Pax6 (Fig. 8A, compare d and e).

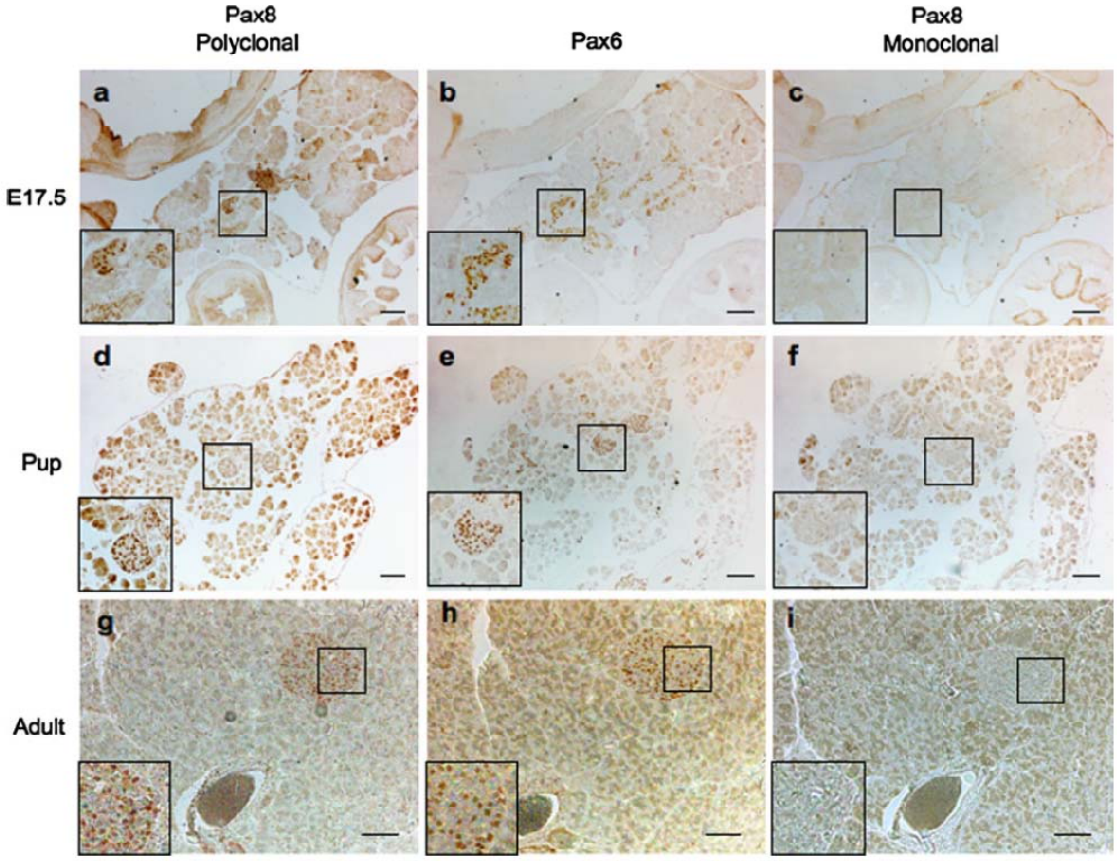

Fig. 7 Pax8 is not expressed in the developing mouse pancreas or in mature islets. Immunostaining of pancreatic consecutive sections using either the polyclonal (*left panels*) or monoclonal (*right panels*) Pax8 antibody, or the Pax6 antibody (*middle panels*) reveals an overlapping signal between Pax6 and the polyclonal Pax8 antibodies. The monoclonal Pax8 antibody does not stain mouse islets at any studied age. **a**–**c** E17.5, **d**–**f** Pup and **g**–**i** adult mice. *Scale bar* 50 μm

Discussion

The aim of the current study was to establish whether Pax8 was expressed during pancreas development. The rational was to complement recent clinical studies demonstrating the presence of this transcription factor in normal human islets (Ozcan et al. 2011; Sangoi et al. 2011) as well as in pancreatic neuroendocrine tumors (Haynes et al. 2011; Long et al. 2010; Sangoi et al. 2011). However, our Q-RT-PCR data does not substantiate these studies as Pax8 transcript was not detected in mouse islets and was barely detectable in human islets. Furthermore, our results reveal that the most commonly used Pax8 polyclonal antibody cross-reacts with Pax6 in normal islets and pancreatic neuroendocrine tumors. The lack of Pax8 immunodetection in the developing pancreas, islets or pancreatic neuroendocrine tumors using a more specific Pax8 monoclonal antibody suggest that this Pax family member may not be expressed in the pancreas under normal physiological or pathophysiological conditions. More importantly, our results highlight the essentiality of validating the specificity of antibodies before embarking into large immunohistochemical studies such as the ones performed for Pax8 (Haynes et al. 2011; Long et al. 2010; Sangoi et al. 2011). Our results also cast serious concerns on the reliability of using Pax8 as a marker to identify pancreatic neuroendocrine tumors as proposed by Sangoi et al. (2011).

What could be the cause of such cross-reactivity? A plausible explanation may be linked to the peptide antigen used to generate the Pax8 polyclonal antibody. Indeed this antibody was raised against a 212 amino acids (aa) polypeptide encompassing the N-terminal region of Pax8. This region includes the notoriously conserved 128 aa paired DNA binding domain found in all members of the *pax* gene

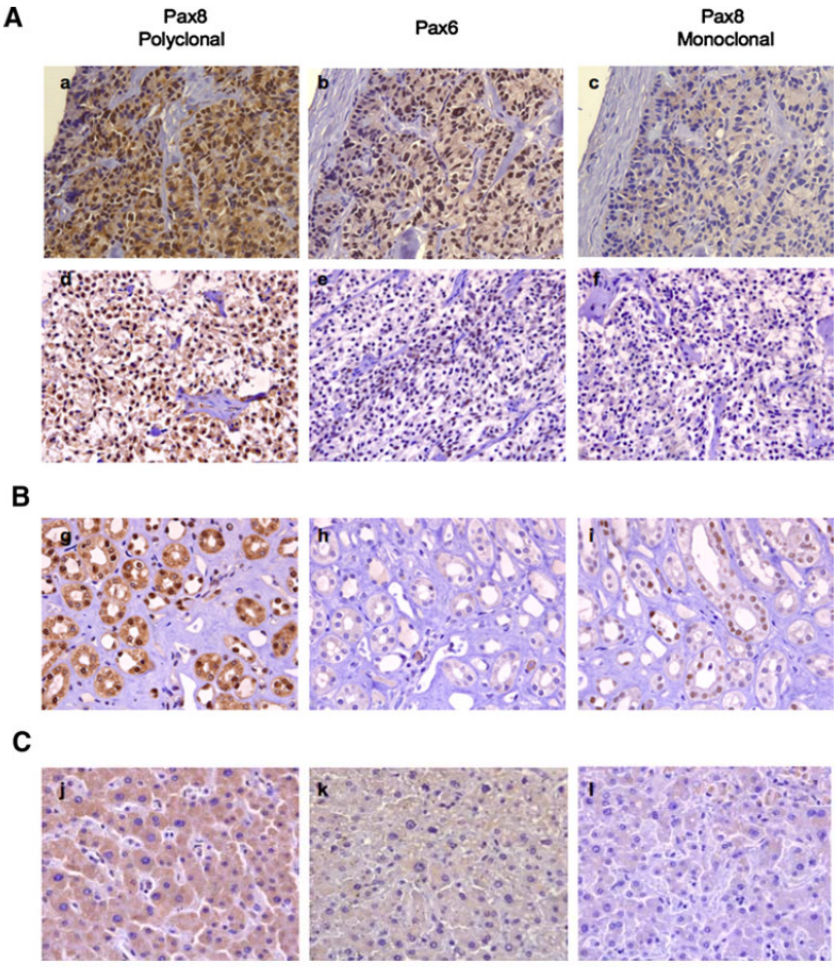

Fig. 8 Pax8 is not expressed in human pancreatic neuroendocrine tumors. **A** Pax8 or Pax6 immunostaining in two different pancreatic neuroendocrine tumors using polyclonal (*a*, *d*) or monoclonal (*c*, *f*) Pax8 antibody, or Pax6 antibody (*b*, *e*) reveals the lack of Pax8 staining when Pax8-specific monoclonal antibody is used. Of note, the lower panel tumor is also negative for Pax6. **B** Pax8 or Pax6 immunostaining in human kidney and **C** liver using polyclonal (*g*, *j*) or monoclonal (*i*, *l*) Pax8 antibody, or Pax6 antibody (*h*, *k*). An overlap in the immunostaining signal between the two Pax8 antibodies in kidney tubule epithelia, indicates the expression of Pax8 in this tissue. No nuclear immunostaining for Pax6 was detected in kidney. Pax8 or Pax6 antibodies revealed no signals in the liver

family (Lang et al. 2007; Robson et al. 2006). Thus, immunization using this region will likely produce a multitude of antibodies that will recognize several Pax members, including Pax6. Further substantiating the promiscuity of the Pax8 polyclonal antibody, we also found that this antibody but not the Pax6 or Pax8 monoclonal antibodies immunostained four tumor samples suggesting cross reactivity with yet another potential Pax member. As Pax8 along with Pax5 and Pax2 define group II of the Pax family with over 95% protein homology in the common paired DNA binding domain, it is tempting to speculate that Pax5 or Pax2 are potential culprits. Consistent with this premise, Sangoi and colleagues recently reported Pax8 immunoreactivity in hematopoietic neoplasms, which they attributed to cross-reactivity with Pax5, a well-defined marker of B-lineage hematologic malignancies (Sangoi et al. 2010). Interestingly, we detected Pax8 staining in the developing mouse liver using the polyclonal antibody (Fig. 2c, f, i). This organ is the major site of hematopoiesis during murine embryonic development (Johnson and Moore 1975) and therefore expresses high levels of Pax5. The latter may therefore offer an explanation for false Pax8 staining in the liver. A similar case of promiscuity in Pax family immunoreactivity was recently described for a Pax5 monoclonal antibody that was found to cross-react with its close relative Pax2 in nonhematopoietic tissues (Morgenstern et al. 2010). Interestingly, we have previously demonstrated that Pax2 is expressed in the endocrine pancreas (Ritz-Laser et al. 2000) raising the possibility that the Pax8 polyclonal antibody may also recognize this Pax member. Thus, therefore, a panel of antibodies against different Pax members should be screened to determine which Pax proteins are aberrantly expressed in pancreatic neuroendocrine tumors. Moreover, validation of the specificity with at least two antibodies raised against different epitopes would be highly recommended prior to launching large-scale clinical studies.

In contrast to the Pax8 polyclonal antibody, the immunogen that was used to create the monoclonal antibody was taken from aa 318–426 of the transcription factor. This region, located at the carboxy-terminal end of the protein, is highly divergent among Pax members and should therefore exhibit target-specific recognition. Nonetheless, despite the lack of Pax8 immunodetection in normal pancreatic islets using this monoclonal antibody, Pax8 transcripts were detected, albeit at low levels, in human islets suggesting a possible involvement of this transcription factor in islet physiology. Similar to Pax4, Pax8 expression may be induced in response to physiological situations that requires β-cell mass expansion (Brun and Gauthier 2008; Hu He et al. 2011). Consistent with this idea, expression levels of Pax8 were shown to increase during pregnancy in mice (Rieck et al. 2009).

In summary, this study highlights pitfalls of using ill-defined antibodies as markers to either characterize or classify human tumors. It is of utmost importance to validate the specificity of antibodies generated against proteins, which have high homology with other members of the same family. In this regard, caution should be taken on whether Pax8 is a reliable marker for pancreatic neuroendocrine tumors.

Acknowledgments We acknowledge the technical assistance of J. Vallejo Ortega. This work was supported by grants from the Fundacion Progreso y Salud (to B.R.G.), the Spanish Ministry of Science and Innovation, Instituto de Salud Carlos III (PI10/00871 to B.R.G) and the Juvenile Diabetes Research Foundation (9-2004-384 to E.C.I.T Plan Nacional de I+D+I 2008-2011, ISCIII (PI08/0018) co-funded by Fondos FEDER (to A.R.) and Consejería de Salud, Junta de Andalucía (PI0008 to A.R.).

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5.2. Pax8 Detection in Well-Differentiated Pancreatic Endocrine Tumors: How Reliable Is It?

OBJECTIVE

Several studies published in *American Journal of Surgical Pathology* refered Pax8 as a specific biomarkers for determining a pancreatic primary origin for both neoplasmic and metastatic WDNETs and distinguish them from neuroendocrine tumors of other anatomic sites in order to obtain a pronostic treatment for PNETs *(Haynes et al., 2011; Laury et al., 2011; Long et al., 2010; Ozcan et al., 2011; Sangoi et al., 2011)*. Moreover, Long et al. showed Pax8 expression in normal adult human islets *(Long et al., 2010)*, in contrast with previous data *(unpublished data from our group; (Goode and Elgar, 2009)*. The aim of this letter to the editor was to inform to readerships that these studies are potentially inaccurate.

RESULTS and CONCLUSIONS

Pax8 is not expressed in mature pancreatic islets under normal physiological conditions, neither in PNETs. Our findings demonstrated that the commonly used polyclonal Pax8 antibody recognized the islet-enriched Pax6 protein. A monoclonal Pax8 antibody was used to re-evaluate Pax8 expression in islets and PNETs corroborating our QT-PCR data. In conclusion, our results highlight the importante of antibodies proper validation to perform large-scale clinical studies. Additionally, we determined that Pax8 is not a reliable molecular marker for PNETs.

CONTRIBUTION

My contribution in this article is the participation in the planning, design, execution and discussion of the results of the project. I performed the immunohistochemical analysis of the human pancreas sections obtained from Hospital Virgen del Rocío patients in collaboration with Dr. Rocío García-Carbonero and Dr. Lourdes Gómez-Izquierdo (Figure 1). These results indicate that Pax8 is not expressed in adult islets and is not a potential tumoral marker for PNETs.

Pax8 Detection in Well-Differentiated Pancreatic Endocrine Tumors: How Reliable is it?

To the Editor:

One of the most challenging areas in pancreatic endocrine tumor (PET) biology is the identification of specific biomarkers that can morphologically and histochemically distinguish well-differentiated PETs from other types of neuroendocrine tumors (NETs). Novel markers could improve diagnosis accuracy and could consequently lead to a more appropriate and tailored treatment and therefore to a better prognosis. This is highly relevant from the clinical point of view as at least 10% of patients with NETs have a widespread disease of unknown primary origin, and available antineoplastic agents are fundamentally effective only on tumors of pancreatic origin.[2,3] Unfortunately, current markers including synaptophysin and chromogranin A are unable to distinguish whether a particular NET originated initially from the endocrine pancreas or from other sites such as the gastrointestinal tract or the bronchopulmonary system. In this regard, a recent study published in the *American Journal of Surgical Pathology* has identified the transcription factor Pax8 as a potentially new biomarker capable of performing such a task.[7] Although previously not detected in the pancreas,[4] Long and colleagues reported strong Pax8 immunostaining in normal human islets and have suggested that this transcription factor could be a specific marker of mature pancreatic endocrine cells and could even be implicated in terminal differentiation of islet cells during development. This premise was corroborated by a second *American Journal of Surgical Pathology* publication confirming the expression of Pax8 in healthy human islets.[6] The discovery of Pax8 expression in the endocrine pancreas led Long and colleagues to assess whether Pax8 was expressed in PETs and in other well-differentiated NETs and to evaluate its potential value as a specific biomarker. They showed that a significant percentage of primary well-differentiated PETs displayed strong Pax8 staining, whereas ileal and pulmonary carcinoid tumors were negative. Furthermore, liver metastases of PET origin were often Pax8 positive. The investigators concluded that Pax8 could be a useful marker to determine the primary origin site of metastatic well-differentiated PETs lodged within the liver.[7] Interestingly, an independent study substantiated these findings and proposed a flowchart in which Pax8 was included in a panel of immunohistochemical stainings to determine the site of origin of metastatic well-differentiated NET in the liver.[11]

Unfortunately, we would like to communicate to the *American Journal of Surgical Pathology* readership that the conclusion of these studies is potentially inaccurate and that Pax8 proteins are not detectable in either healthy islets or PETs.[8] Indeed, subsequent to the publication by Long et al[7] we embarked on a study to determine whether Pax8 could also be expressed during pancreatic islet development, thereby pinpointing to a yet unknown function of this transcription factor in islet physiology. To this end, we analyzed Pax8 expression during mouse pancreatic development and in adult islets by immunohistochemistry using the same Pax8 polyclonal antibody (ProteinTech Group Inc., Chicago, IL; Cat. No. 10336-1-AP) that Long et al[7] used in their study. It is noteworthy that this polyclonal antibody was also used for most, if not all, clinical studies that demonstrated Pax8 staining in PETs.[5,6,11] Consistent with these published reports we were successful in detecting Pax8 immunostaining in mature mouse and human islets and in the developing mouse pancreas. However, in an attempt to complement our immunohistochemical data with transcript expression profile, we unexpectedly found that Pax8 mRNA levels in both human and mouse islets were low to undetectable. These findings led us to question the specificity of the Pax8 polyclonal antibody. Indeed, as the 9 Pax members share a high degree of homology at the N-terminal paired domain, the region included in the peptide use for originating the antibody, a potential cross-reactivity of the antibody to another Pax member could be envisaged. This premise was indeed confirmed by demonstrating that the polyclonal Pax8 antibody also recognized the islet-enriched Pax6 protein both by Western blotting and by immunohistochemistry.[8] To circumvent this caveat, a novel Pax8 monoclonal antibody from Abcam (No. ab53490) generated against the more variable C-terminal region of Pax8 was used to reevaluate the expression pattern of Pax8 during development and in islets. This antibody recognizes the 2 predominant isoforms of Pax8 expressed in most tissues and tumors.[10] Consistent with our QT-RT-PCR data, neither the developing pancreas nor mature islets exhibited staining with this novel Pax8 monoclonal antibody as compared with the polyclonal antibody.[8]

These results led us to reassess whether Pax8 was truly present in PETs. To this end, serial sections of PETs were immunostained with Pax8 antibodies (polyclonal and monoclonal) and with anti-Pax6 sera (Fig. 1).[8] Immunohistochemical analysis revealed positive staining both in non-neoplastic islets and in tumor areas of pancreas sections when the Pax8 polyclonal antibody was used, whereas no staining was revealed with the Pax8 monoclonal antibody (Figs. 1A, B). An immunostaining pattern similar to that of the Pax8 polyclonal antibody was discerned with the Pax6 antibody, substantiating cross-reactivity of the commonly used Pax8 polyclonal antibody with Pax6 and possibly with other members of the Pax family (Fig. 1C).[8] Thus, in contrast to the study by Long et al,[7] we

Am J Surg Pathol • Volume 35, Number 12, December 2011 Letter to the Editor

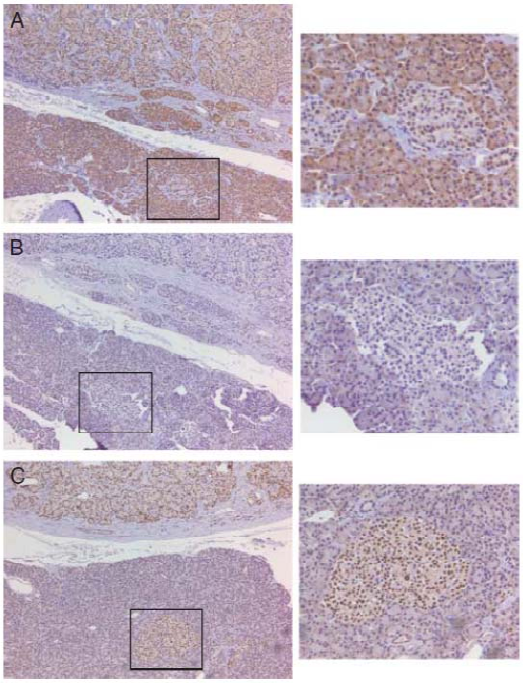

FIGURE 1. Pax8 is expressed neither in non-neoplastic islets nor in PETs. Immunohistochemistry analysis performed on sections of a representative PET using either the polyclonal (A) or monoclonal (B) Pax8 antibody and the Pax6 antibody (C). Using identical immunohistochemical conditions, no staining in either islets (enlarged inset) or tumor areas is detected using the specific Pax8 monoclonal antibody as compared with the polyclonal Pax8 antibody. The latter displays both nuclear and diffused nonspecific cytoplasmic staining. It is noteworthy that Pax6 immunostaining reveals a similar pattern as the Pax8 polyclonal antibody, suggesting cross-reactivity of the latter with Pax6. Magnification ×10.

believe that Pax8 is not expressed in the endocrine pancreas under normal physiological conditions and is most likely not an accurate biomarker for diagnosis of well-differentiated PETs.[8] This conclusion is further substantiated by an earlier study in which Pax8 was not identified as a molecular marker specific for PET by genetic profiling of core biopsies.[1]

Our findings highlight the pitfalls of using non-properly–validated antibodies to perform large-scale clinical studies, which may result in flawed diagnostics. Moreover, antibody promiscuity in the Pax family has previously been reported,[9] and therefore it is of utmost importance to ensure specificity. Thus, although the Pax8 polyclonal antibody may remain clinically useful to "query" the expression of any Pax protein, we propose that various antibodies against different Pax members should be evaluated to establish which Pax proteins are reliably expressed in PETs.

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The authors have disclosed that they have no significant relationships with, or financial interest in, any commercial companies pertaining to this article.

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Erratum

Clinicopathologic analysis of solid papillary carcinoma of the breast and associated invasive carcinomas: Erratum

The article appeared on page 501 of the April 2006 issue of the journal and has the third author listed incorrectly. The correct listing of the author's name is N. Volkan Adsay. The correct citation for the author's name is Adsay NV.

REFERENCE

Nassar H, Qureshi H, Adsay NV, et al Clinicopathologic analysis of solid papillary carcinoma of the breast and associated invasive carcinomas. *Am J Surg Pathol*. 2006;30:501–507.

5.3. Role of Pax8 in Human and Mouse Pancreatic Islets Physiology

5.3.1. Pax8 is induced *in vivo* in mouse pancreatic islets during pregnancy

In our previous studies, we postulated that Pax8 expression could be induced under certains metabolic demands *(Lorenzo et al., 2011; Moreno et al., 2011)*. Indeed, a recent study revealed upregulation of Pax8 in adult mouse pancreatic islets during pregnancy *(Rieck et al., 2009)*, in contrast to previous reports that never detected this member of Pax family in the endocrine pancreas *(unpublished data from our group; (Goode and Elgar, 2009; Lorenzo et al., 2011))*. These discrepancies prompted us to evaluate whether truly this transcription factor is expressed in islets under this specific circumstances of metabolic stress. In order to investigate the Pax8 expression during pregnancy, twelve-week old C57BL/6J mice were selected to assess Pax8 expression levels in islets during gestation using QT-PCR. Pancreatic islets from pregnant females were isolated at 10.5, 14.5, 16.5 and 18.5 days of gestation as well as from control non-pregnant mice. All animals used were normoglycemic during gestation (data not shown). Analysis of Pax8 mRNA levels in islets of pregnant mice showed that its expression was transiently induced during pregnancy, reaching maximum transcript levels 5-fold higher at day 14.5 as compared to islets from control non-pregnant mice (Figure 23A), in which Pax8 expression was almost undetectable in control non-pregnant islets consistent with previous studies *(Lorenzo et al., 2011; Moreno et al., 2011)*. This transient increase in mRNA expression correlates with the described pattern of β-cell proliferation and, consequently, β-cell mass expansion *in vivo* during pregnancy *(Rieck and Kaestner, 2010)*. Transcript levels of the islet-enriched transcription factors Pdx1 and Tph1 were also analyzed as controls. Intriguingly, Pdx1 expression shows a 5-fold suppression in mouse islets during pregnancy progression as compared to its levels in islets from control non-pregnant mice (Figure 23B). This result leads us to speculate whether decreased Pdx1 expression levels could be related with islets growth control since repression of this factor has been reported to induce genes involved in replication *(Gauthier BR, 2004; Johnson, 2003)*. Consistent with recent studies *(Kim et al., 2010b; Rieck et al., 2009)*, the positive control gene activated during pregnancy, Tph1, exhibited a 5-fold increase from day 10.5 of gestation in islets of pregnant females (Figure 23C).

To accurately determine whether Pax8 protein is specifically expressed in islets during pregnancy, we performed immunohistochemical analysis of the pancreas of pregnant mice at different days of gestation. Based in our previous results with different Pax8 antibodies *(Lorenzo et al., 2011; Moreno et al., 2011)*, we used a mouse monoclonal Pax8 antibody (clone BC12) from Abcam *(Tacha et al., 2013)* to avoid any cross-reactivity with other Pax genes. Kidney samples were employed as positive control for Pax8 expression. Our data reveals a strong nuclear Pax8 immunostaining in pancreatic islets of pregnant mice at days 10.5-14.5 of gestation (Figure 23D). Remarkably, Pax8 mRNA levels peak at day 14.5 of gestation, whereas maximal Pax8

protein expression was detected at day 10.5 of gestation. These discrepant data may be due because the relative Pax8 transcript levels derived from six experiments, while protein levels were obtained from a single experiment. Unfortunately, we were unable to reproduce protein detection using several immunohistochemical approaches due to the low expression levels of this transcription factor in pancreatic islets. Interestingly, only a subpopulation of islet cells was Pax8 positive in these pregnant mice. Additionally, islet cells proliferation was assessed using the proliferation marker Ki67. The number of proliferating cells augmented at day 14.5 of gestation (Figure 23D) as previously reported *(Rieck and Kaestner, 2010)*. Our findings suggest that Pax8 expression is induced during pregnancy in mice coinciding with the maximal *in vivo* islets proliferation.

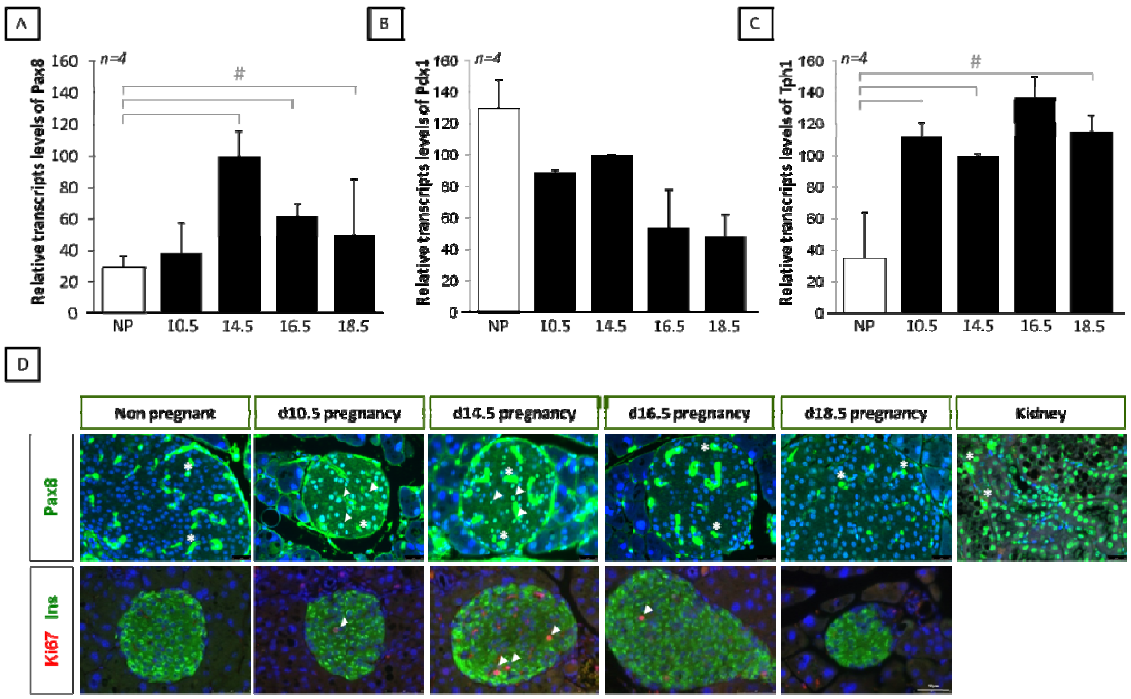

Figure 23: Pax8 expression is induced in mouse islets during pregnancy. (A) Pax8, (B) Pdx1 and (C) Tph1 relative transcript levels in islets isolated at various time points during gestation. Time point with maximal relative transcript levels is relativized to 100. n=4 per group (D) Representative immunostaining images of mouse islets and kidney (positive control) for Pax8 (green, upper panel), Insulin (green, bottom panel) and Ki67 (red, bottom panel) at different days of gestation. * indicates non-especific signal in blood cells of Pax8 antibody. Images at 40X augmentation. n=1 per group. **#** p< 0.01 versus control non-pregnant islets

5.3.2. PAX8 is induced *in vitro* in human pancreatic islets during prolactin treatment

To substantiate our data in mice to humans and study the expression pattern of Pax8 during human pregnancy, we performed *in vitro* treatment of human islets with prolactin, the most important hormone during of human gestation, to reproduce *in vitro* the pregnancy environment. Isolated human islets were cultured with 200 ng/mL prolactin. This concentration of the hormone is within the normal physiological concentration found at third trimester of human pregnancy. Prolactin was added daily, due to its short half-life, for up to 96 hours of treatment. Prior to prolactin treatment, human islets were cultured with medium supplemented with a charcoal-treated serum to remove any traces of others hormones for 48 hours (Figure 24). Human islets from six independent donors were employed for the experimental procedure to minimize the possible differences between individuals (Table 10).

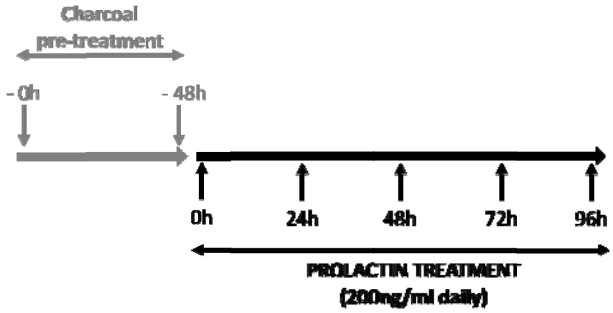

Figure 24: Experimental procedure for reproducing *in vitro* pregnancy conditions in isolated human islets by daily addition of 200 ng/ml prolactin treatment.

Table 10: Human islets´ donors information.

	Age	Sex	BMI (Kg/m2)	IEQs	Viability	Purity	Cause of death
Donor 1	54	M	26.2	20000	92	50	Cerebral hemorrhage
Donor 2	26	M	28.0	20000	90	40	Cerebral hemorrhage
Donor 3	50	F	33.3	30000	96.7	50	Iliac angioplasty
Donor 4	38	M	22.5	20000	88.8	50	Suicide
Donor 5	54	F	28.5	30000	75	60	Meningeal hemorrhage
Donor 6	61	F	25.4	13000	96	55	Cerebrovascular stroke

Importantly, we found that human islets treated with 200 ng/ml prolactin daily displayed a 5-fold increase in PAX8 expression after 72 hours of treatment (Figure 25A). In parallel, TPH-1 (direct target of prolactin) expression was induced by approximately 2-fold at 48 hours of treatment emphasizing the ability of prolactin to partially recapitulate pregnancy conditions *in vitro* (Figure 25C). Furthermore, PDX1 transcript levels were 2-fold lower after 24 hours of treatment similarly to those in mouse islets during pregnancy (Figure 25B). Proliferating islet cells were quantified by chemiluminescence measurement of bromodeoxyuridine (BrdU) incorporation after

entire human islets were incubated for 24 hours in the presence of 10 µM BrdU. *In vitro* prolactin treatment evoked a transient increase in the proliferation of human islets reaching maximal levels of 2-fold after 24 hours of treatment (Figure 25D). Intriguingly, this *in vitro* approach showed that the peak of Pax8 expression at 72 hours did not coincide and, indeed, occurs after the peak of proliferation, suggesting that PAX8 may not be implicated in β-cell replication. Notwithstanding this discrepancy, these data indicate that under specific physiological conditions, such as pregnancy, PAX8 expression is induced in human islets.

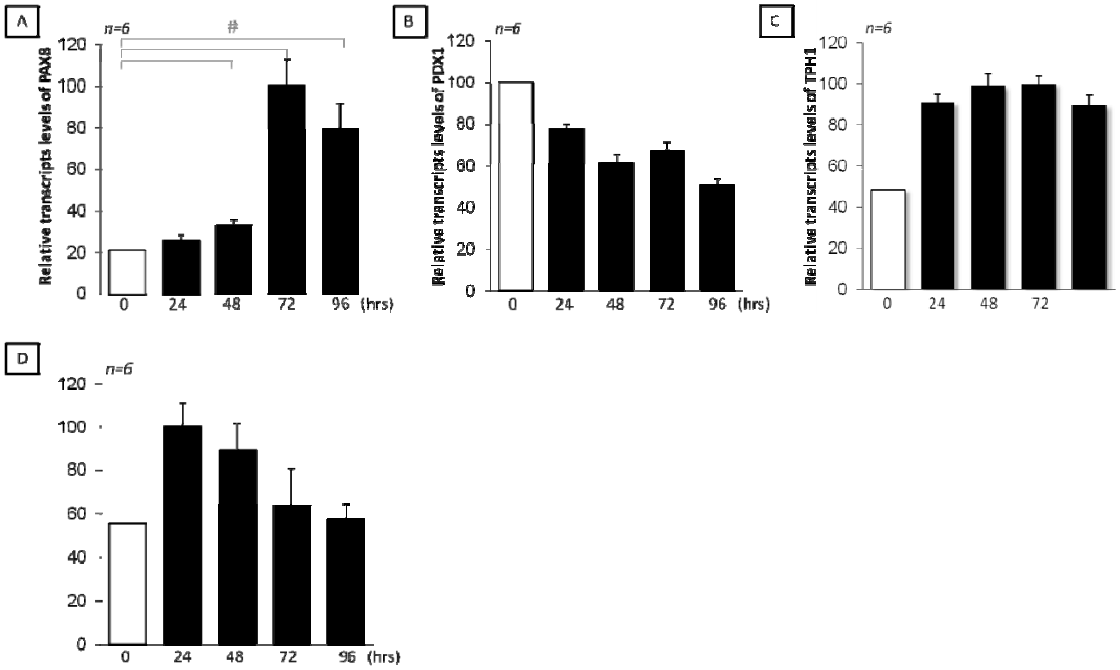

Figure 25: PAX8 is induced in prolactin-treated human islets. (A) PAX8, (B) PDX1 and (C) TPH1 relative transcript levels in human islets treated with prolactin for the indicated time. Time point with maximal relative transcript levels is relativized to 100. (D) Proliferation of human islets cells treated with prolactin at various time points. n=10 per group. **#** p< 0.05 versus control non-treated islets.

5.3.3. Development of a lentiviral approach for Pax8 overexpression in mouse and human pancreatic islets

Due to the discrepancies related to human and mouse Pax8 expression pattern and the peak of islets proliferation, we decided to generate a lentiviral system to overexpress mouse and human Pax8 genes in adult pancreatic islets in order to study the direct action of this transcription factor in islets physiology. For this purpose, we overexpressed Pax8 in the insulinoma cell line INS-1E and in isolated mice islets using a lentiviral vector in which Pax8 expression is controlled by the SFFV promoter and GFP by the constitutive ubiquitin promoter pPSD-mPax8 and pPSD-hPAX8). Viruses that express only GFP were used as negative control (pPSD-GFP) (see epigraph *"Chapter III_Materials and Methods: Pax8 lentiviral approach"*). To validate that our lentiviral approach is working properly, the insulinoma cell line INS-1E was transduced using an intermediate MOI7 for 16 hours. Four days post-infection, transduction efficiency was measured by western blot and immunofluorescence for Pax8 protein expression and by FACS to determine the GFP positive cells. Pax8 protein was detected achieving approximately 90% of transduced cells (Figure 26A). Nevertheless, lentiviral infection of intact isolated mice islets to overexpress Pax8 reported low transduction efficiency when we monitored GFP expression in living islets using the MycroExpress system and by FACS (Figure 26B). Therefore to the islet tridimendional structure that hampers the insertion of lentiviral particles in the core of mouse islets, where insulin-producing cells reside, less than 69% of islet cells were infected and, these transduced cells were located exclusively in the periphery of the micro-organ This problem prompts us to develop a transduction protocol for lentiviral infection of intact mouse and human islets described in the next section *(section 5.3.4)* *(Jiménez-Moreno et al., manuscript accepted in Gene Therapy, pending revision).*

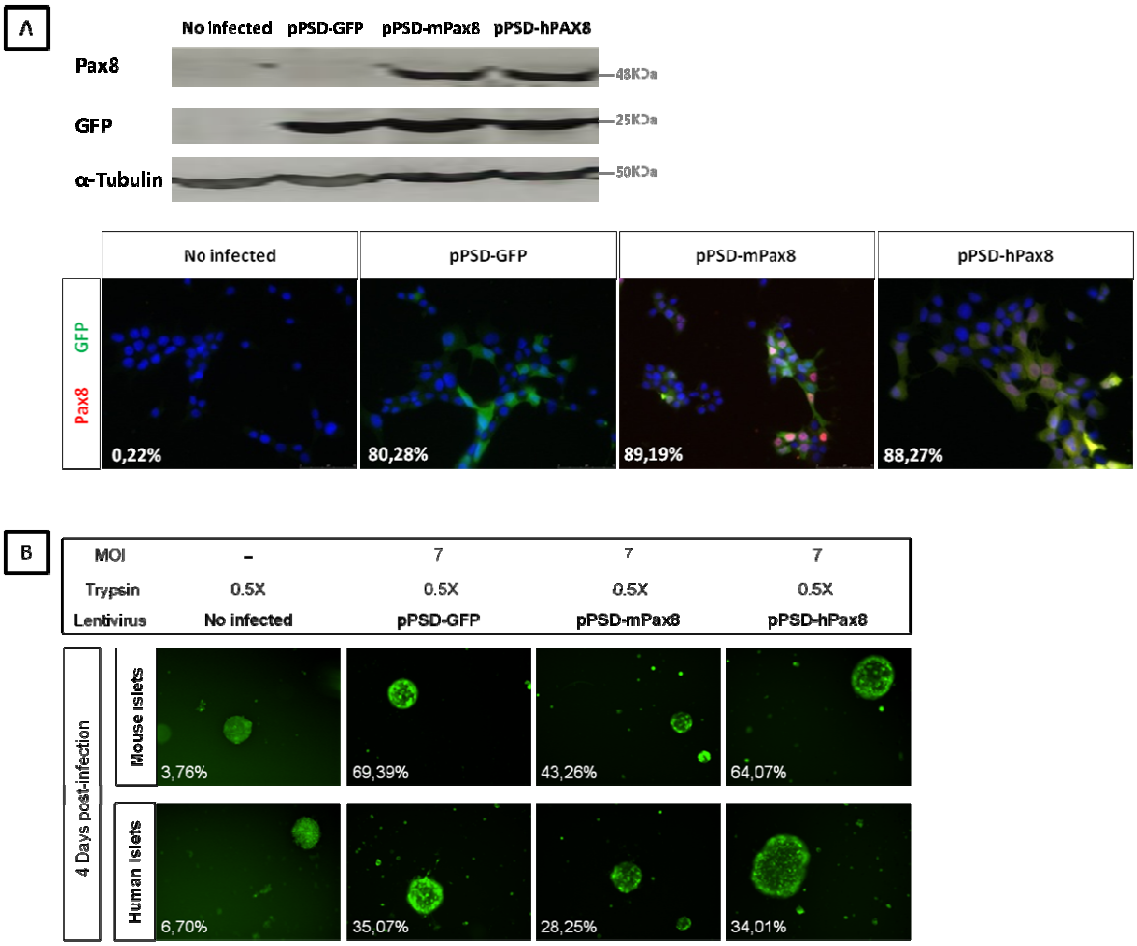

Figure 26: Lentiviral infection of INS-1E and mouse and human islets. (A) Representative western blot (upper panel) and immunostaining images (bottom panel) of INS-1E cell line for Pax8 (red) and GFP (green) with the different lentiviral vectors generated. Images at 40X augmentation. Percentage of infected cells, estimated by measurement of GFP expression levels by FACS, is shown at bottom left corner (B) Representative images of transduced mouse and human islets. Isolated mouse and human islets were treated with 0.5X trypsin prior to lentiviral infection using either an MOI=7 as indicated on the Figure. Daily fluorescence images were acquired using the ImagExpress system. At 4 days post-infection, islets were disaggregated and the % GFP positive as compared to non GFP cells was estimated by flow cytometry. Percentage is shown at bottom left corner.

5.3.4. A Simple High Efficiency Intra-Islet Transduction Protocol Using Lentiviral Vectors

OBJECTIVE

Gene therapy is one of the most promising approaches to circumvent the major problems of pancreatic islets transplantation procedures aiming to restore the functional β-cell mass in T1DM patients. Unfortunately, due to the tridimensional complexity of islets, current gene modulation therapies are technically challenging resulting in low and transient gene transfer efficiencies and compromising islets health, limiting their applicability. The objective of this work is developing an optimal and universal lentiviral-mediated gene transfer method to improve islet function for the treatment of T1DM and to allow long-term insulin independency.

RESULTS and CONCLUSIONS

We have generated a reliable and easy-to-use lentiviral-mediated gene transfer protocol that allows the transduction of approximately 80 % of mouse and human islet cells while preserving islet architecture, metabolic function and glucose-dependent stimulation of insulin secretion, therefore not compromising islet health. Our protocol provides the dual purpose of studying the impact of therapeutic genes in entire pancreatic islet physiology and, ultimately, facilitating the universalization of a stable islet-gene modulation prior to human islets transplantation.

CONTRIBUTION

My contribution in this article is the participation in the planning, design, execution and discussion of the results of the project. Firstly, my work consisted in the generation and production of the lentiviral vector used in our transduction protocol (see epigraph *"Chapter III_Materials and Methods: Pax8 lentiviral approach"*). I developed the infection protocol in mouse and human islets (Figure 1 and Box 1) being in charge of isolation, processing, infection and validation of others and our protocol for islet samples. Furthermore, I performed the bright field images of infected islets to show their estructure and viability (Figure 2A, 3A, 4A, 4C and 5A). I also monitored GFP fluorescence from living transduced islets using the ImagExpress MycroSystem (Figure 2A, 3A, 4A, 4C and 5A). I conducted the GFP measurement by flow cytometry (Figure 2B, 3B, 4B and 5B). I activately participated in MTT (Figure 2C, 3C, 4E and 5C) and, GSIS (Figure 4F) assays to assess the viability and functional integrity of islets. I contributed in the immunohistochemical analysis of embedded islet sections to report a normal insulin and glucagon staining, as well as the transduction of the majority of cells residing in the core of entire islets (Figure 2D, 4D and 5D). These results have led to the generation of a simple, reliable and easy-to-use transduction protocol of entire human and mouse pancreatic islets for xenotransplantation therapies to T1DM patients.

A Simple High Efficiency Intra-Islet Transduction Protocol Using Lentiviral Vectors

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Running title: An efficient protocol for intact islet infection

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ABSTRACT

Successful normalization of blood glucose in patients transplanted with pancreatic islets isolated from cadaveric donors established the proof-of-concept that Type 1 Diabetes Mellitus is a curable disease. Nonetheless, major caveats to the widespread use of this cell therapy approach have been the shortage of islets combined with the low viability and functional rates subsequent to transplantation. Gene therapy targeted to enhance survival and performance prior to transplantation could offer a feasible approach to circumvent these issues and sustain a durable functional β-cell mass *in vivo*. However, efficient and safe delivery of nucleic acids to intact islet remains a challenging task. Here we describe a simple and easy-to-use lentiviral transduction protocol that allows the transduction of approximately 80 % of mouse and human islet cells while preserving islet architecture, metabolic function and glucose-dependent stimulation of insulin secretion. Our protocol will facilitate to fully determine the potential of gene expression modulation of therapeutically promising targets in entire pancreatic islets for xenotransplantation purposes.

INTRODUCTION

Type 1 Diabetes Mellitus (T1DM) is one of the most common multifactorial endocrine and metabolic diseases in childhood resulting in persistent hyperglycaemia. Currently, approximately 490,000 children have been diagnosed with T1DM and 78,000 children under the age of 15 are estimated to develop T1DM annually worldwide [1]. More alarmingly, a recent epidemiological study has revealed that the incidence rate of T1DM in children in the United Sates has increased dramatically by 29% between 1985 and 2004 surpassing by 18 times the incidence of Type 2 DM (T2DM) in the white population [2]. The most common form of T1DM results from the breakdown of β-cell-specific self-tolerance by T-lymphocytes precipitating an autoimmune attack and eradication of insulin-producing cells [3]. Strong genetic and environmental factors contribute to the onset of T1DM [4]. Existing treatments for T1DM are primarily focused on insulin supplementation. However, despite the beneficial effects of life-long insulin therapy on glucose homeostasis, insulin administration does not eliminate severe diabetic complications such as retinopathy, nephropathy as well as cardiovascular and cerebrovascular diseases [5].

In the past 10 years, clinical islet transplantation has gain much attention as a cell replacement therapy for restoring the functional β-cell mass. Unfortunately, the limited supply of islets from donors has failed to meet demands imposed by the ever-growing number of T1DM patients. An additional major hurdle has been the lack of durability of islet function with insulin independency in less than 10% of patients 5 years after transplantation [6, 7]. Furthermore, several post-transplant events, such as instant blood mediated inflammatory reaction and cytokine cascade, seriously affect the long-term functionality of islets [8-11]. *Ex vivo* genetic modifications of islets to enhance cell function and survival prior to transplantation has been successfully demonstrated in animal models [12, 13]. This strategy can ultimately increase islet viability and performance providing a tangible approach to improve human islet transplantation and long-term insulin independence. Although protocols designed to modulate gene expression have been extensively used in single cells, the complexity of pancreatic islets has impeded successful gene delivery. Indeed, due to its tridimensional structure, β-cells embedded within the core of islets are sequestered from any significant contact with the remote environment [14-19].

The use of lentiviral vectors in gene therapy has become a powerful tool to safely deliver genetic material with the purpose to rectify molecular defects, enhance functional performance or increase viability of cells. Major advantages of lentiviral vectors include the capacity to infect both dividing and non-dividing cells using repeated dosing, genome integration and long-term expression as well as low immunogenicity [20]. Currently, 89 gene therapy clinical trials using lentiviral vectors are ongoing [21] focusing predominantly on the treatment of primary

immunodeficiencys [22]. Given the tremendous attributes of lentiviral vectors combined with their current use in clinical trials, we set out to develop an optimal transduction protocol permitting efficient gene delivery in islets prior to transplantation without compromising integrity and functionality.

MATERIALS AND METHODS

Consumables. Reagents and materials used in this study along with reference numbers and companies of purchase are outlined in Table 1.

Animals. Male mice (c57bl/6, 12 week-old) were purchased from Janvier Labs (Le Genest-Saint-Isle, France). Mice experimentations were approved by the CABIMER Animal Committee and performed in accordance with the Spanish law on animal use RD 53/2013.

Islets procuration and culture. Mice were sacrificed by cervical dislocation and pancreatic islets were isolated using the collagenase digestion procedure with subsequent handpicking as previously described [23]. Prior to culture islets were washed with Phosphate Buffered Saline (PBS) containing 100 U/ml penicillin and 100 µg/ml streptomycin to minimize post-isolation contaminations. Subsequently islets were cultured in mouse Complete Media (CM) comprised of RPMI 1640 supplemented with 10 % FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, 1 mM sodium pyruvate, 50 µM β-mercaptoethanol and 10 mM HEPES. Isolated human islets were kindly provided the Cell Isolation and Transplantation Centre (Geneva, Switzerland). Islets were cultured in human Complete Media (CM) composed of CMRL-1066 supplemented with 10 % FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 100 µg/ml gentamycin.

Lentivirus generation. We opted for the dual-promoter lentivirus, pHRSIN DUAL-GFP also known as pHRSIN-CSGWdINotIpUbEm (kindly supplied by Dr. Pintor-Toro, CABIMER, Spain) to conduct our studies [24]. This vector allows the cloning and expression of a Gene Of Interest (GOI) under the control of the SFFV promoter while the constitutive *Ubiquitin* (Ubi) promoter regulates expression of the reporter GFP. Lentivirus amplification and purification was performed by seeding 5×10^6 Hek293T cells into a 100 mm Petri dish and subsequently transfected 24 hours later with: 1) 15 µg of vector, 2) 10 µg the HIV packaging plasmids pCMVDR8.91 and 3) 5 µg of HIV packaging plasmids pVSVG (also known as pMDG). Transient DNA transfection was performed using the CalPhos transfection mammalian kit according to the manufacturer's recommendations. Viral particles were harvested 72 hours post-transfection, purified using a 0.45 µm Millex-HV filter, and concentrated by ultracentrifugation in an Optima™ L-100K ultracentrifuge at 87300 x g for 90 minutes at 4º C in a swinging bucket rotor SW-28 (Beckman-Coulter, Spain). Virus particles were resuspended in serum-free DMEM (Invitrogen), distributed in aliquots, snapped frozen in liquid nitrogen, and stored at −80 °C. Viral titer was estimated by transducing Hek293T cells with increasing amounts of pHRSIN DUAL-GFP followed by flow cytometry (FACSCalibur, BD Biosciences, Spain) analysis to determine the Multiplicity Of Infection (MOI) based on GFP emission.

Live imaging and flow cytometry. An ImageXpress Micro System (Molecular Devices) was used to monitor GFP fluorescence in living islets. To this end, approximately 20 transduced human or mouse islets were seeded in µ-Plate 96 welllibiTreat plate in a final volume of 200 µl of CM. Islets were cultured for 4 days at 37º C and images (fluorescence or phase contrast) were automatically captured daily and processed using the MetaXpress software. In parallel, islet transduction efficiency was estimated by flow cytometry. In brief, approximately 20 islets were transferred into 5 ml polystyrene Round-bottom tube in a final volume of 50 µl of CM. Islets were disaggregated using1 X trypsinization for 5 minutes at 37º C and subsequently centrifuged at 200 x g for 5 minutes. Cells were resuspended in 300 µl of PBS and the number of GFP positive cells was estimated as compared to non-infected cells.

Islet processing and immunocytochemistry. Islet embedding was performed according to the protocol developed by Cozar-Castellano *et al*. [25]. In brief, approximately 200 human or murine islets were fixed in 10 % formaldehyde at room temperature for 48 hours. Islets were then washed three times in distilled water prior to adding warm (70° C) HistoGel containing100 µl of 150-300 µm diameter Affi-Gel blue beads. After cooling, Histogel containing the islet-bead mixture was embedded in paraffin following the standard procedures of the CABIMER Histology Core Facility.Paraffin blocks were sectioned (5 µm thickness) using a microtome Leica RM 2255 (Leica Microsystems, Spain). Sections were mounted on SuperFrost Plus slides. After every 10 sections, a slide was stained with hematoxylin-eosin to confirm islet integrity and presence of islets. Sections were deparaffinized/rehydrated at 60˚ C for 20 minutes followed by immersion in decreasing concentrations of ethanol (Xylene 5 minutes/2 x; Ethanol 100 % 1 minute/2 x; Ethanol 96 % 1 minute; Ethanol 80 % 1 minute; Ethanol 70 % 1 minute; Distilled water 2 minutes/2 x). After deparaffinization and rehydration, sections were subjected to heat-induced antigen retrieval using 10 mM sodium citrate buffer (pH 6.0) in the microwave in 3 cycles of 3 minutes at 800 W avoiding boiling of the buffer, with 2 minutes at room temperature between heating cycles. Samples were cold down in the same solution for 20 minutes at room temperature. After washing with PBS, samples were incubated in PBS + 0.5 % Triton X-100 and then washed again with PBS. Blocking was performed with PBS + 0.2 % Triton X-100 containing 1 % Bovine Serum Albumin (BSA) and 3 % Donkey serum for 1 hour at room temperature. Primary antibodies (Table 1) at the indicated dilutions were added in PBS + 0.1 % Triton X-100 containing 1 % BSA and 3 % Donkey serum and incubated overnight at 4˚ C in a dark humid chamber. Subsequently, sections were washed with PBS for 5 minutes, PBS + 0.2 % Triton X-100 for 5 minutes and PBS for 5 minutes. Samples were then incubated with secondary antibodies (Table 2) diluted in PBS + 0.2 % Triton X-100 containing 0.1 % BSA for 1hour at room temperature in a dark humid chamber. Nuclear counterstaining was performed by DAPI staining diluted 1:1000 in PBS for 5 minutes at

room temperature. Finally, samples were washed three times with PBS for 5 minutes each and sections were mounted using DAKO fluorescent mounting medium.

Viability and functional assay. Islet viability subsequent to transduction was assessed in groups of 35 islets using the Cell Proliferation Kit I (MTT) according to the manufacturer´s recommendations (Roche, Spain). Optical density was determined at 550 nm with a reference wavelength of 650 nm using a Varioskan Flash spectrophotometer (Thermo Scientific, Spain). In parallel, glucose stimulated insulin secretion (GSIS) was performed to assess the functional integrity of islets. Groups of 10 islets were washed in 500 µL of Krebs-Ringer bicarbonate-HEPES buffer (KRBH) (140 mM NaCl, 3.6 mM KCl, 0.5 mM NaH2PO4, 0.5 mM MgSO4, 1.5 mM CaCl2, 2 mM NaHCO3, 10 mM HEPES, 0.1% BSA) and pre-incubated at 37° C for 45 minutes in 300 µl of the same buffer. Islets were then centrifuged and KRBH buffer was discarded. Subsequently, fresh KRBH supplemented with 2.5 mM glucose was added and islets were incubated for 30 minutes. Next, buffer was harvested (basal insulin secretion) and 500 µL of KRBH supplemented with 16.8 mM glucose was added. Islets were incubated for an additional 30 minutes at 37° C and then buffer was harvested (induced insulin secretion). Insulin levels were measured using a mouse insulin enzyme immunoassay kit (Mercodia AB, Spain) according to the manufacturer´s instructions. Stimulation index was expressed as the ratio of insulin levels at 16.8 mM glucose divided by insulin levels at 2.5 mM glucose.

Statistical analysis. Results are expressed as the mean± SEM. Statistical differences were estimated by two-tailed unpaired student's *t*-test. * indicates statistical significance, p value <0.05.

RESULTS

Elaboration of a high efficiency transduction protocol in mouse islets

Modulation of gene expression has been particularly challenging in the context of whole pancreatic islets has compared to cell lines due to their three dimensional structure composed of approximately 1000 to 2000 compacted cells. Sophisticated protocols such as *in vivo* perfusion or microporation using adeno and lenti viruses claim to have successfully and homogenously transduced up to 70% islet cells [14, 26]. As these protocols may be cumbersome to carry out and simply not always feasible (i.e. *in vivo* perfusion of human islets) we sought to develop a readily accessible and friendly user lentiviral protocol (BOX 1 and Fig. 1). Consistent with previous reports, the mere exposure of islets to increasing MOI of pHRSIN DUAL-GFP resulted in enhanced GFP fluorescence from living islets corroborating with a greater number of islet cells expressing GFP, as assessed by flow cytometry (Fig. 2A-B). However, the MOI 100 that achieved 80% infection efficiency also considerably reduced islet viability (Fig. 2C) with the appearance of necrotic cells in the islet core (Fig. 2A, arrows). Intriguingly, immunocytochemistry revealed that even at high MOI only cells at the periphery were GFP positive predominantly co-localizing with glucagon-expressing α-cells while β-cells randomly co-stained with GFP (Fig. 2D). In an attempt to increase accessibility to cells sequestered within the core to viral particles without compromising viability, we mildly loosen up islet cells using either 1X (500 mg/L trypsin; 0.96 mM EDTA) or 0.5X (250 mg/L trypsin; 0.48 mM EDTA) trypsin-EDTA for 3 minutes prior to transduction. Both trypsin concentrations improved the number of GFP-expressing islet cells at either MOI 5 or 20 (Fig. 3A). Flow cytometry confirmed that the number of GFP-positive cells infected at MOI 5 increased from ~30% in control islets to ~50% in islets pre-treated with trypsin independent of its concentration (Fig. 3B). Similarly, a MOI 20 resulted in 80% of cells expressing GFP independent of trypsin concentrations (Fig. 3B). Unexpectedly, 1X trypsin jeopardized viability of cells in all conditions (Fig. 3C). We next sought to determine the temporal evolution of GFP expression subsequent to transduction using 0.5X trypsin. In order to expose islets to minimal amount of viral particles, we also assessed the transduction efficiency of MOI 7 and 10. A progressive increase in the percentage of GFP-positive cells with time was observed at all MOI (Fig. 4A-B). However, by day 10 post infection, islet architecture was strongly compromised with signs of necrosis as compared to islets 4 days post infection (Fig. 4C). In some instances, bacterial contamination was also observed 10 days after transduction (data not shown). Islets transduced at MOI 20 consistently presented the highest infection efficiency, reaching 80% of islet cells at day 4 post-infection as compared to all other MOI tested (Fig. 4B). Although, GFP expression as assessed in live islets appeared to be more intense at day 10 as compared to day 4 post transduction (Fig. 4C), the percentage of GFP-positive cells were similar at both time points (Fig. 4B) suggesting that 4 days is sufficient to attain maximal transduction efficiency of the entire islet. Consistent with this premise, GFP immunostaining was detected homogeneously

throughout the islet co-localizing with both insulin as well as glucagon-positive cells (Fig. 4D). More importantly at this time point, neither islet viability (Fig. 4E) nor function as measure by glucose-induced insulin secretion (Fig. 4F) were altered at MOI 20 as compared to MOI 5, 7, 10 or control non-transduced islets. In summary, our data indicate that 80% of mouse islet cells express GFP 4 days after exposure to a short and mild trypsin treatment and to a viral dosage of MOI 20.

Transduction protocol validation in human islet.

We next validated our transduction protocol in human islets. Live human islets revealed intense GFP expression without apparent ultra structural abnormalities 4 days post transduction (Fig. 5A). Consistent with mouse islets, approximately 70 to 80% of islet cells were GFP-positive (Fig. 5B) while viability was not altered (Fig. 5C). Finally, GFP immunostaining was detected homogeneously throughout islets co-localizing with insulin and glucagon (Fig. 5D). Taken together, our data indicate that the proposed protocol is easy, reliable and allows the transduction of the majority of cells residing in entire islets from murine and human origin.

DISCUSSION

Given the indispensable role of pancreatic islets in glucose homeostasis, the modulation of gene expression in transplanted islets could be a promising approach to boost islet performance and durability for the treatment of T1DM [27, 28]. In this context, non-viral strategies, such as electroporation, gene gun particle bombardment, cationic liposomes and polymeric particles, have been developed for genetic modification of islet cells [15, 19, 29, 30]. Unfortunately, these techniques provide only low gene transfer efficiencies, limiting their applicability. In contrast, published adenovial-based infection protocols claim to have successfully transduced up to 90 % of islet cells using high viral doses [14, 15, 31-34]. Although promising, these protocols are technically challenging to perform resulting in only transient expression of the transgene as well as often inducing cell toxicity. Alternatively, lentiviral vectors have emerged as a promising strategy to modulate gene expression in intact islets. Up to 50 % of β cells in intact islets have been efficiently transduced without adverse viability effects [14, 16-18, 35-37]. Based on these initial successes, we have devised an easy-to-use and reproducible protocol that bestows a significant improvement of murine and human islet transduction efficiency. Three non-mutually exclusive parameters were considered: 1) MOI, 2) islet architecture, and 3) time post-transduction. Consistent with other reports, we found that high MOI (e.g. MOI 100 or greater) increased transduction efficiency but to the detriment of islet cell function and survival [14, 32, 33]. The negative impact of high virus dosage has also been substantiated in vivo xenotransplantation studies [15]. We established that a MOI 20 was the optimal viral dosage reaching 50% cell infection in intact islets without jeopardizing either viability or function. This MOI is substantially lower to those (100-1000 MOI) previously

utilized in another published protocol [32]. Addition of a mild 0.5 X treatment to facilitate core accessibility greatly improved transduction efficiency while preserving islet health and function. Interestingly, 1X trypsin-EDTA affected cell viability. Pro-distension agents such as collagenase and triton-X-100 were also found to increase infection efficiency yet compromised islet functionality [14, 34]. Thus, although these treatments seemingly appear to be beneficial, it is of utmost importance to verify that islet function and viability are preserved post-treatment. We also found that time post infection was another critical parameter to the successful outcome of the experiment. Indeed, we established that islet integrity and health is maintained up to 4 days post transduction.

Although islet cell transplantation has demonstrated many clinical successes to date, more work is necessary to further improve its efficacy and universalize this treatment to the vast majority of T1DM patients and to allow long-term insulin independency. From the results shown in this report, we speculate that human islets infected with our protocol may provide a venue to improve health and function prior to transplantation and prevent post-transplantation dismay. Indeed, human islets presented marked insulin and glucagon expression and normal islet architecture, suggesting that the proposed protocol for islet infection does not compromise human islet health. Therefore, lentiviral-mediated gene expression modulation using this protocol could be therapeutically promising to generate a functional and stable islet transplanted mass in humans.

CONCLUSION

Here we present a protocol that represents a reliable easy-to-use procedure to transduce efficiently human and mouse islets with the dual purpose of studying the impact of therapeutic genes in islet physiology and ultimately facilitating the universalization of islet infection prior transplantation. The stable integrating nature of lentiviral vectors, supports the notion that lentiviral-mediated gene transfer might be an optimal method to improve islet function for the treatment of T1DM [38]. In this sense, the value of potential benefits based on the modulation of gene expression in entire islets warrants further experimentation to determine the applicability of our protocol for islet infection prior transplantation.

LIST OF ABREVIATIONS

Bovine Serum Albumin (BSA)
Complete Media (CM)
Gene Of Interest (GOI)
Glucose Stimulated Insulin Secretion (GSIS)
Krebs-Ringer bicarbonate-HEPES buffer (KRBH)
Multiplicity Of Infection (MOI)
Phosphate Buffered Saline (PBS)
Type 1 Diabetes Mellitus (T1DM)
Type 2 DM (T2DM)
Ubiquitin (Ubi)

COMPETING INTERESTS

The authors have no competing interests to declare.

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CM J-M, IG H-G, L L-N, PI L, N C-V, E F-M, JM M-G and A M-M performed the experiments; G P and D B isolated and provided human pancreatic islets. CM J-M, BR G and A M-M designed the study and wrote the manuscript.

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FIGURE LEGENDS

Fig. 1. Optimized protocol for lentiviral-mediated islet infection. Summarized scheme of the transduction protocol described in Box 1.

Fig. 2. High pHRSIN DUAL-GFP MOI levels compromise islet viability with sub-optimal islet transduction efficiency. Freshly isolated murine islets were exposed to increasing MOI of pHRSIN DUAL-GFP. Non-transduced islets (Mock) were used as control. (A) Representative images of *ex vivo* cultured entire live transduced islets. Top; GFP expression was assessed by fluorescence acquisition using an ImageXpress Microsystem. Low; Bright field images. Images were captured at 4 days post-infection. Arrows indicate necrotic areas. Scale-bars indicate 100 µm. *n=4* experiments per condition. (B) Transduction efficiency, defined as the percentage of islet cells expressing GFP, was determined by flow cytometry in disaggregated islets at 4 days post-infection. *n=6* per condition. (C) Determination of islet metabolic activity using the MTT assay at 4 days post-infection *n=4-6* per condition. (D) Representative immunofluorescence images of Affi-Gel bead-embedded pancreatic islets 4 days post-infection. Antibodies against GFP (green), insulin (red) and glucagon (cyan) were employed. Of note, in some instances the Affi-Gel beads, emitted a non specific fluorescent signal along with GFP (Green). Scale-bars, 50 µm. *n=3* per condition. Data are represented as the mean ± SEM. * p< 0.05 versus control non-transduced islets.

Fig. 3. A Mild Trypsin-EDTA treatment increases transduction efficiency in murine islets. Freshly isolated murine islets were treated or not with two concentrations of trypsin-EDTA prior transduction or not with pHRSIN DUAL-GFP. (A) Representative images of live islets exhibiting GFP fluorescence subsequent to treatment: Top; GFP expression was assessed by fluorescence acquisition using an ImageXpress Microsystem. Low; Bright field images. Images were captured at 4 days post-infection. Arrows indicate necrotic areas. Scale-bars 100 µm. *n=4* experiments per condition. (B) Transduction efficiency in trypsin-EDTA treated islets was determined by flow cytometry at 4 days post-infection. *n=3-8* per condition. (C) Determination of islet metabolic activity using the MTT assay 4 days post-infection. *n=4* per condition. 0 X: Untreated; 0.5 X: 0.5 X trypsin-EDTA treatment (250 mg/l trypsin; 0.48 mM EDTA); 1 X: 1 X trypsin-EDTA treatment (500 mg/l; 0.96 mM EDTA). Data are represented as the mean ± SEM. * $p < 0.05$ versus control non-transduced trypsin-EDTA untreated islets.

Fig. 4. Mild trypsinization combined with a MOI 20 represents the optimal infection protocol for murine islets. Freshly isolated murine islets were treated with 0.5 X trypsin-EDTA (250 mg/l; 0.48 mM EDTA) and subsequently exposed to increasing MOI of pHRSIN DUAL-GFP. (A) Representative images of GFP fluorescence emitted from live islets: Top; GFP expression was assessed by fluorescence acquisition using an

ImageXpress Microsystem. Low; Bright field images. Images were captured at 4 days post-infection. Scale-bars 100 µm. *n=4* experiments per condition. (B) Transduction efficiency in 0.5 X trypsin-EDTA treated islets at different days after transduction was determined by flow cytometry. *n=4* per condition. (C) Representative images of live islets exhibiting GFP fluorescence 4 and 10 days post-treatment: Top; GFP expression was assessed by fluorescence acquisition using an ImageXpress Microsystem. Low; Bright field images. Scale-bars 100 µm. *n=4* experiments per condition. (D) Representative immunofluorescence images of Affi-Gel bead-embedded pancreatic islets trypsin-treated and transduced or not with pHRSIN DUAL-GFP. Antibodies against GFP (green), insulin (red) and glucagon (cyan) were employed. Images were captured in samples fixed at 4 days post-infection. Filled arrows indicate transduced cells expressing insulin; Non-filled arrows indicate transduced cells expressing glucagon. Scale-bars 50 µm. *n=3* per condition. (E) Determination of islet metabolic activity subsequent to a 0.5 X trypsin-EDTA treatment followed by transduction with a MOI 20. A MTT assay was performed 4 days post-infection. *n=3-4* per condition. (F) Glucose-stimulated insulin secretion was assessed in islet treated with 0.5 X trypsin-EDTA followed by transduction with increasing amount of pHRSIN DUAL-GFP. Data are represented as the mean ± SEM of *n=3*. * $p < 0.05$ versus control non-transduced 0.5 X trypsin-EDTA treated islets.

Fig. 5. Human islets are efficiently transduced using the optimized protocol. Human islets obtained from cadaveric donors were initially treated with 0.5 X trypsin-EDTA (250 mg/l trypsin; 0.48 mM EDTA) and then transduced with pHRSIN DUAL-GFP at MOI 20. (A) Live imaging reveals GFP expression in human islets 4 days post-infection: Top; GFP expression, Bottom; Bright field images. Scale-bars 100 µm. *n=3* per condition. (B) Transduction efficiency in 0.5 X trypsin-EDTA treated islets was determined by flow cytometry at 4 days post-transduction with a MOI 20. *n=3* per condition. (C) Islet metabolic activity was assessed using the MTT assay. *n=3* per condition. (D) Co-immunostaining of GFP (green), insulin (red) and glucagon (cyan) was performed on sections from Affi-Gel bead-embedded human pancreatic islets subsequent to treatment. Images were captured in samples fixed at 4 days post-infection. Scale-bars 50µm. *n=3* per condition. Data are represented as the mean ± SEM. * $p < 0.05$ versus control non-transduced 0.5 X trypsin-EDTA treated islets.

Table 1. List of reagents and materials used in this study.

Product	Vendor	Catalog number
50 x 9 mm Petri dishes	BD Falcon	351006
Affi-Gel blue beads	Bio-Rad	153-7301
Bovine Serum Albumin	Sigma-Aldrich	A3294
CalPhos mammalian transfection kit	ClonTech	631312
CMRL-1066	Cellgro	99-663-CV
Collagenase	Roche	C9263
DAKO fluorescent mounting medium	Dako	S3023
DAPI	Sigma-Aldrich	32670
Donkey serum	Sigma-Aldrich	D9663
Fetal Bovine Serum	Sigma-Aldrich	F7524
Formaldehyde	Panreac AppliChem	252931
Gentamycin	Sigma-Aldrich	G1397
Glutamine	Sigma-Aldrich	G7513
Hanks Balanced Salt Solution 1X	Gibco	14170088
HEPES	Gibco	15630-056
HistoGel	Thermo Scientific	R904012
micro-Plate 96 welllibiTreat	IBIDI	89626
Millex-HV filter 0.45 µm	Merck Millipore	SLHV033RS
PBS	Sigma-Aldrich	P5368
Penicillin/Streptomycin	Sigma-Aldrich	P4333
Polystyrene Round-bottom tube	BD Falcon	352058
RPMI-1640	Sigma-Aldrich	R0883
Sodium pyruvate	Sigma-Aldrich	S8636
SuperFrost Plus slides	Menzel-Glaser	J1800AMNZ
Trypsin-EDTA 10 X	Gibco	15400054
β-mercaptoethanol	Gibco	31350-10

Optimized Islet infection protocol.

Step 1. Isolation of fresh murine islets by collagenase digestion (0.7-0.9 mg/ml) and subsequent handpicking (for human samples go to step 2).

Step2. Culture islets for 3 hours in 50 x 9 mm Petri dishes in 2.5 ml of complete RPMI. For human samples, place islets upon sample arrival in 100 x 9 mm Petri dishes and culture them over-night in 10 ml of complete CMRL-1066.

Step3. Collect medium and islets from the plate in a 15 ml falcon tube.

Step4. Centrifuge islets at 50 x g for 2 minutes and remove supernatant.

Step5. Incubate islets with 1000µl of warm (37° C) 0.5 X trypsin- Ethylenediaminetetraacetic acid (EDTA) (250 mg/l trypsin; 0.48 mM EDTA) for 3 minutes in a cell culture incubator (37° C, 5 % CO2). For trypsin-EDTA preparation: Aliquots of 0.5 % Trypsin-EDTA 10 X (5000 mg/l; 9.6 mM EDTA) are diluted in Hanks Balanced Salt Solution (HBSS) 1 X to obtain a final concentration of 0.5 X trypsin-EDTA (250 mg/l; 0.48 mM EDTA)).

Step6. Pipette up and down 3 times slowly and carefully with a 1000 µl tip using a micropipette and subsequently add 1000 µl of complete RPMI for murine islets or complete CMRL-1066 for human islets.

Step7. Centrifuge islets at 50 X g for 2 minutes and remove supernatant.

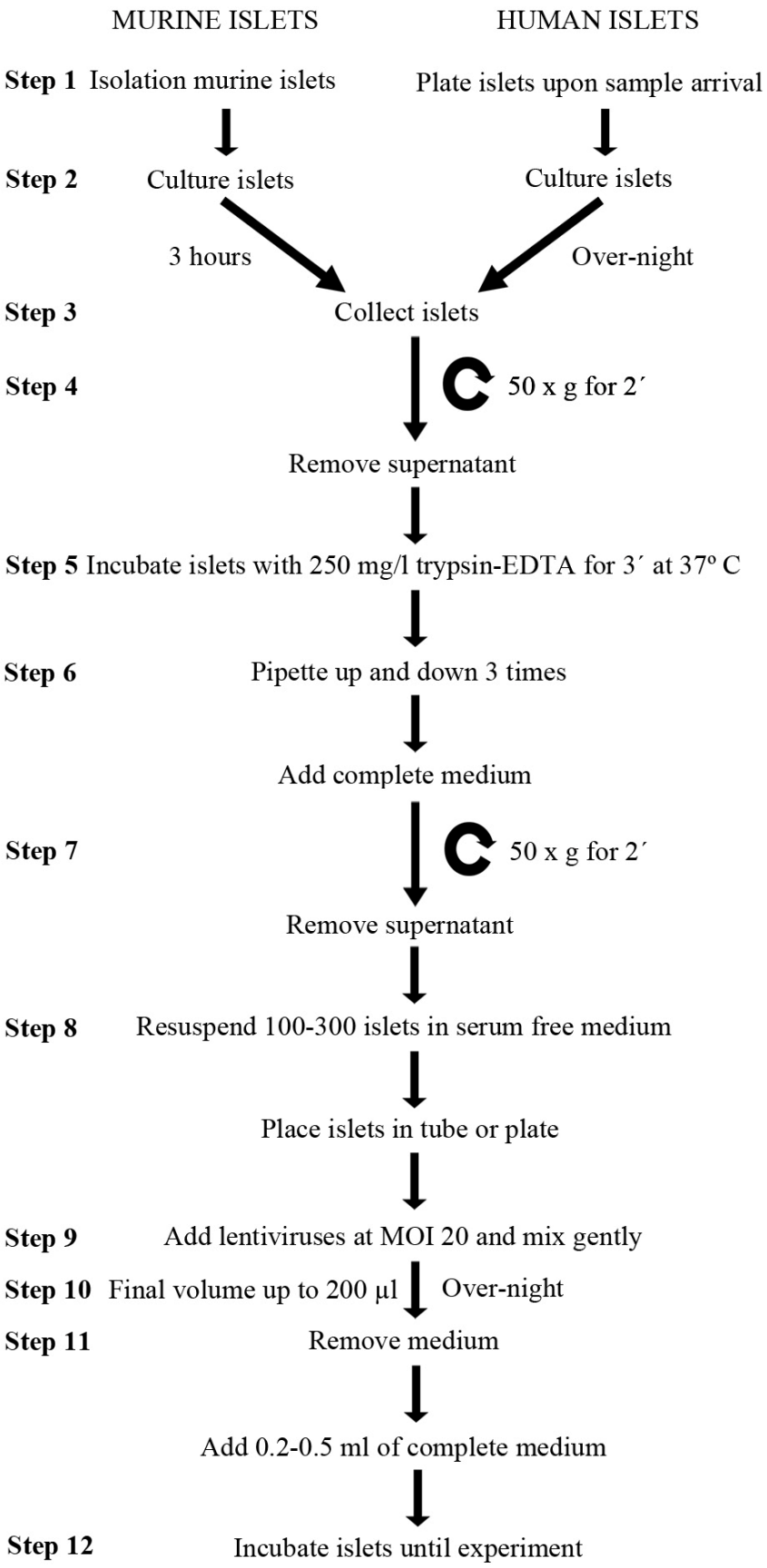
Step8. Resuspend 100-300 islets in serum free RPMI (for murine islets) or serum free CMRL-1066 (for human islets). Place islets in a polystyrene Round-bottom tube or in a µ-Plate 96 welllibiTreat culture plate depending on the desired experiment. Note that final volume must not exceed 200 µl.

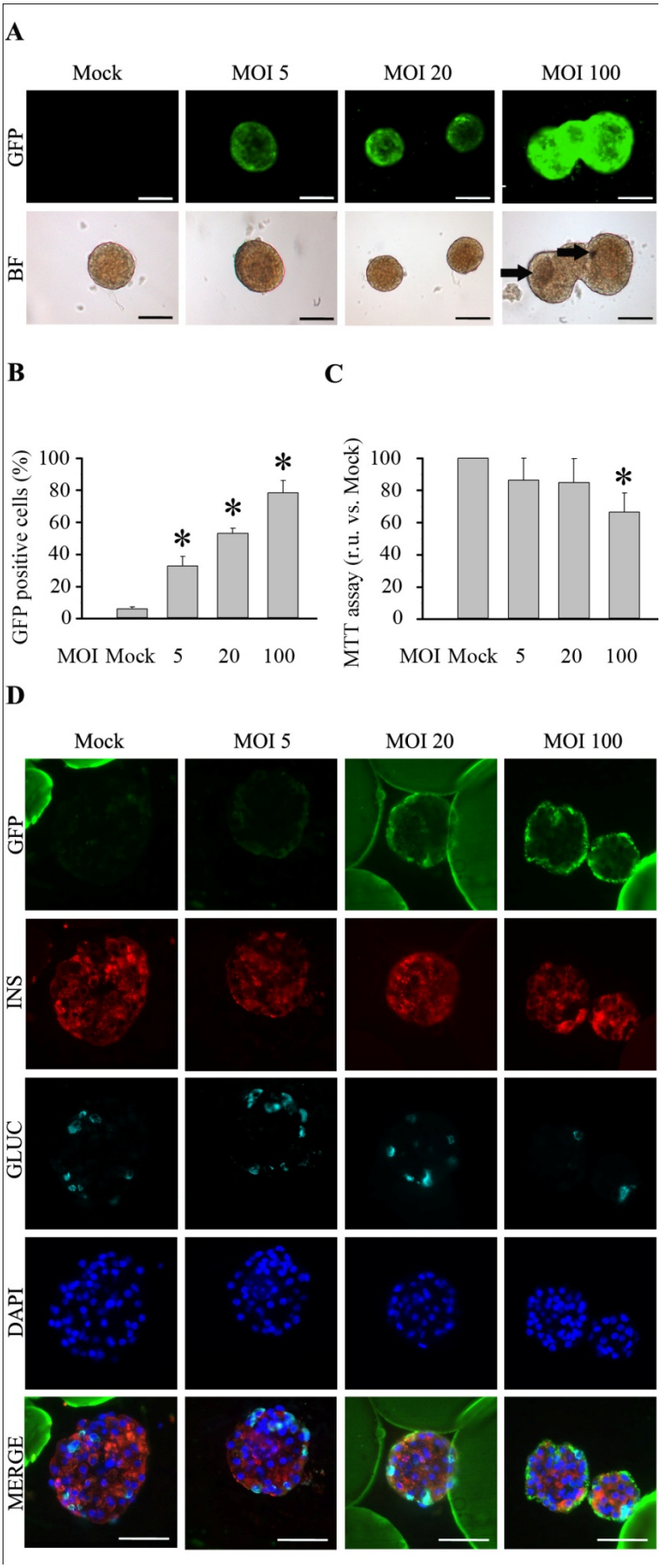
Step9. Add lentiviruses at Multiplicity Of Infection (MOI) 20, assuming that a single islet has 1000 cells. Note that final volume must not exceed 200 µl. Resuspend islets by gently tapping the polystyrene Round-bottom tube or the µ-Plate 96 welllibiTreat culture plate three times.

Step 10. Incubate islets over-night in cell culture incubator (37° C, 5 % CO2) for optimal lentiviral transduction.

Step 11. Remove medium and add 200-500 µl of complete RPMI (for murine islets) or CMRL-1066 (for human islets).

Step12. Incubate islets at 37° C in a cell culture incubator (37° C, 5 % CO2) until the optimal timing for the desired experiment is achieved.


MURINE ISLETS
HUMAN ISLETS
Step 1 Isolation murine islets
Plate islets upon sample arrival
Step 2
Culture islets
Culture islets
3 hours
Over-night
Step 3
Collect islets
Step 4
50 x g for 2´
Remove supernatant
Step 5 Incubate islets with 250 mg/l trypsin-EDTA for 3´ at 37º C
Step 6
Pipette up and down 3 times
Add complete medium
Step 7
50 x g for 2´
Remove supernatant
Step 8
Resuspend 100-300 islets in serum free medium
Place islets in tube or plate
Step 9
Add lentiviruses at MOI 20 and mix gently
Step 10 Final volume up to 200 µl
Over-night
Step 11
Remove medium
Add 0.2-0.5 ml of complete medium
Step 12
Incubate islets until experiment


A
Mock
MOI 5
MOI 20
MOI 100
GFP
BF
B
GFP positive cells (%)
100
80
60
40
20
0
MOI Mock 5 20 100
C
MTT assay (r.u. vs. Mock)
100
80
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MOI Mock 5 20 100
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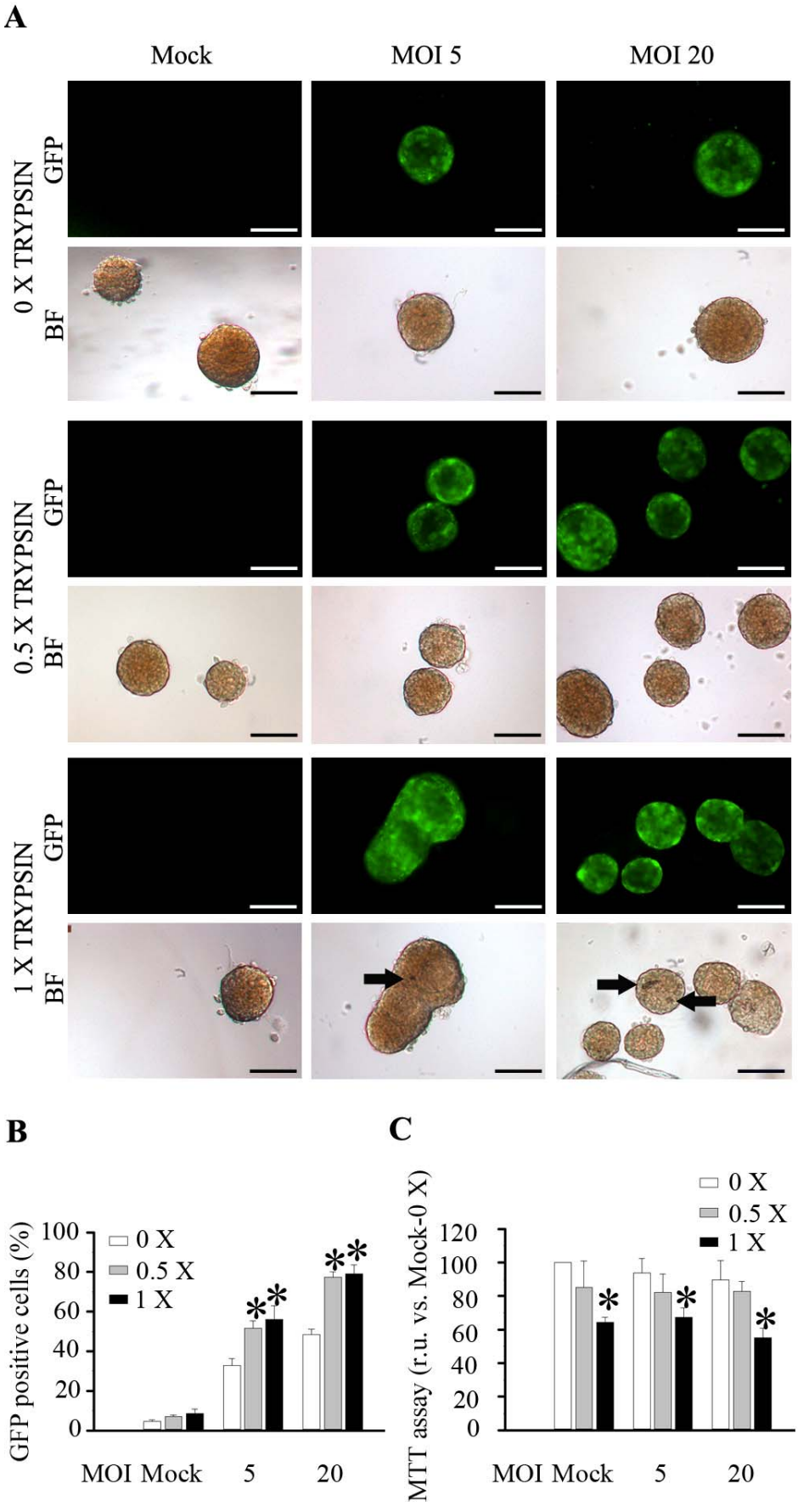
MOI 5
MOI 20
MOI 100
GFP
INS
GLUC
DAPI
MERGE


A
Mock
MOI 5
MOI 20
0 X TRYPSIN
GFP
BF
0.5 X TRYPSIN
GFP
BF
1 X TRYPSIN
GFP
BF
B
GFP positive cells (%)
100
80
60
40
20
0
0 X
0.5 X
1 X
**
**
MOI Mock 5 20
C
MTT assay (r.u. vs. Mock-0 X)
120
100
80
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40
20
0
0 X
0.5 X
1 X
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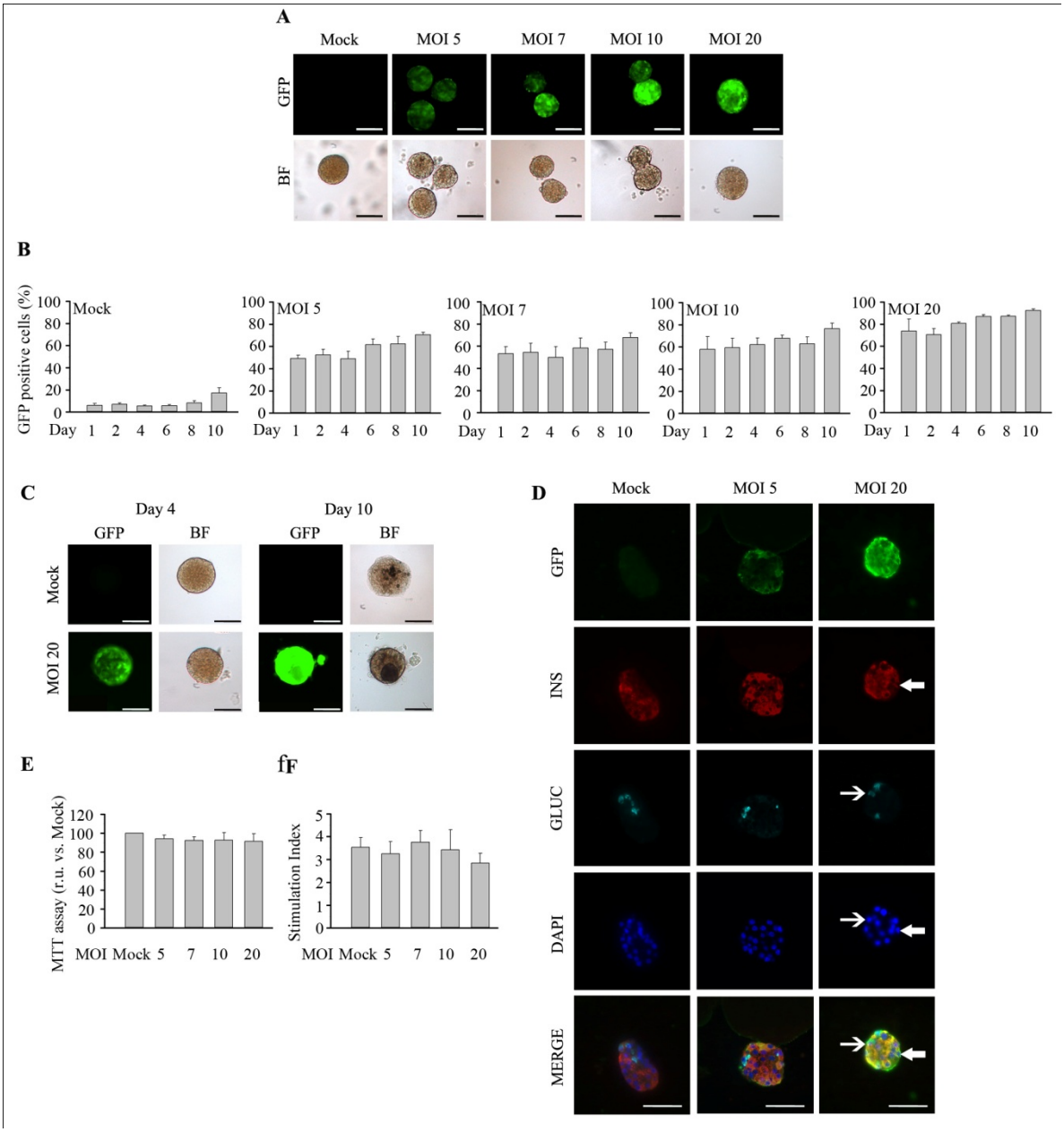
*
*
MOI Mock 5 20


A
Mock
MOI 5
MOI 7
MOI 10
MOI 20
GFP
BF
B
GFP positive cells (%)
Mock
MOI 5
MOI 7
MOI 10
MOI 20
Day 1 2 4 6 8 10
C
Day 4
Day 10
GFP
BF
Mock
MOI 20
D
Mock
MOI 5
MOI 20
GFP
INS
GLUC
DAPI
MERGE
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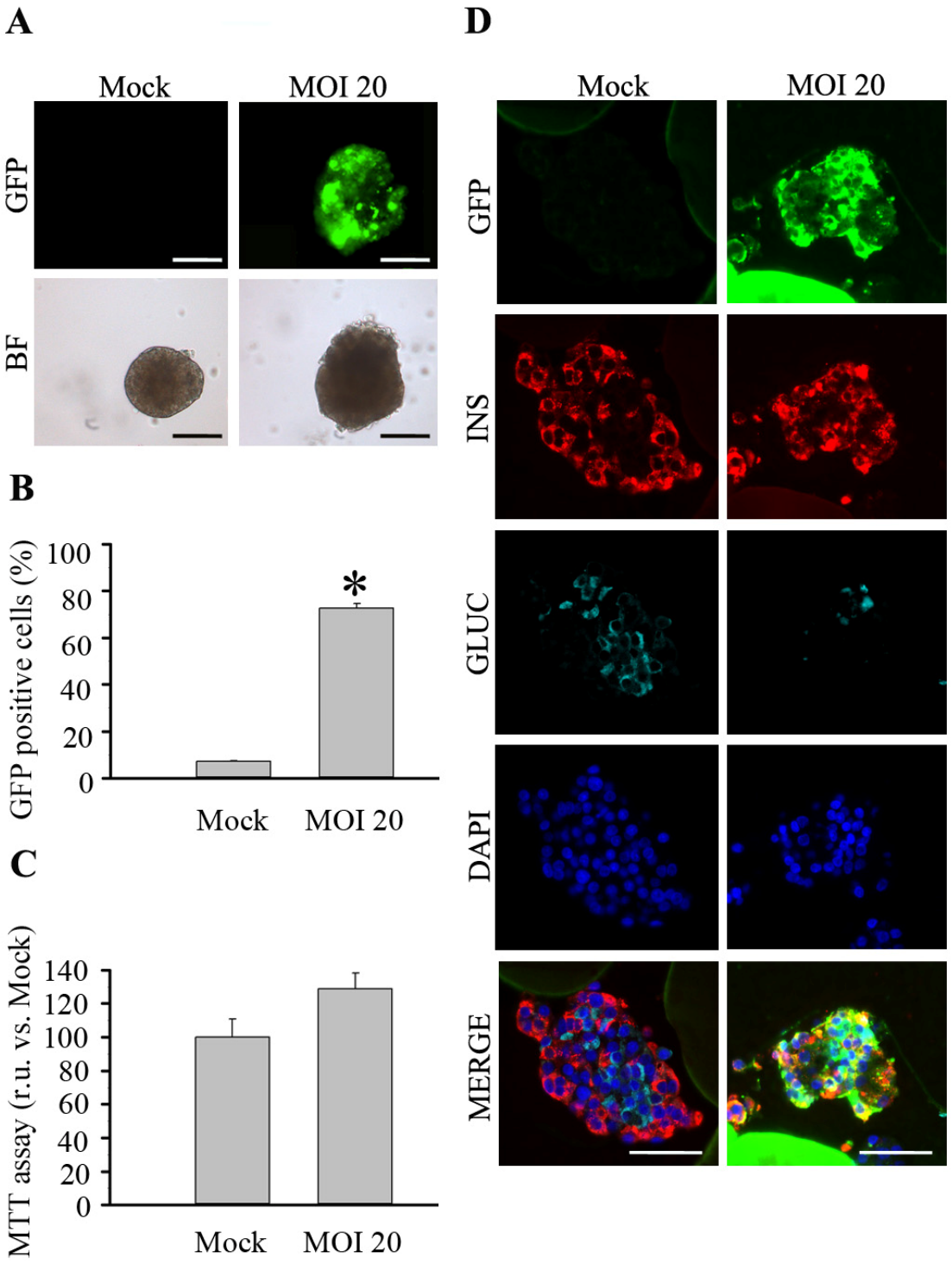
MTT assay (r.u. vs. Mock)
MOI Mock 5 7 10 20
fF
Stimulation Index
MOI Mock 5 7 10 20


A
Mock
MOI 20
GFP
BF
B
GFP positive cells (%)
100
80
60
40
20
0
*
Mock
MOI 20
C
MTT assay (r.u. vs. Mock)
140
120
100
80
60
40
20
0
Mock
MOI 20
D
Mock
MOI 20
GFP
INS
GLUC
DAPI
MERGE

5.3.5. Pax8 overexpression is involved in immunomodulation of mouse pancreatic islets during pregnancy

With the objective of determining the metabolic pathways controlled by Pax8 in pancreatic islets, primary mouse islets were infected using the infection protocol developed in our laboratory *(Jiménez-Moreno et al., manuscript in review)*. After infection, a transcriptome profiling analysis of Pax8-overexpressing islets was performed using RNA microarray. Analysis by Ingenuity Pathway Analysis Platform (IPA) found that overexpression of the transcription factor Pax8 in mouse pancreatic islets modulates genes mainly associated with processes related to the development and/or protection against diabetes and metabolic syndrome (Figure 27A). Our results reinforce a previous publication which linked Pax8 as a potential T2DM candidate gene in the Afro American population *(Elbein et al., 2009)* In addition, a comprehensive analysis of genes and metabolic pathways induced by Pax8 overexpression denoted that several components of the interferon pathway (related to the immune system) are up-regulated such as Ifit1, Ifit3 and Ifit44 among others (Figure 27B and 27C). We could hypothesize that Pax8 controls genetic networks that allow immunomodulation of pancreatic islets during pregnancy favoring its protection against immunological alterations required for a successful physiological condition in which the immune system undergoes great changes due to the presence of the fetus in the mother *(Arck et al., 2015; Jiang et al., 2014)*. Interestingly, cell cycle control replication pathway is also modulated by Pax8 overexpression indicating that this transcription factor is playing an important function in islet mass adaptation under specific metabolic circumstances. These data collectively indicate that Pax8 can be an essential component of a cell signaling pathway that promotes insulin production, proliferation and/or immunomodulation of β-cells during pregnancy.

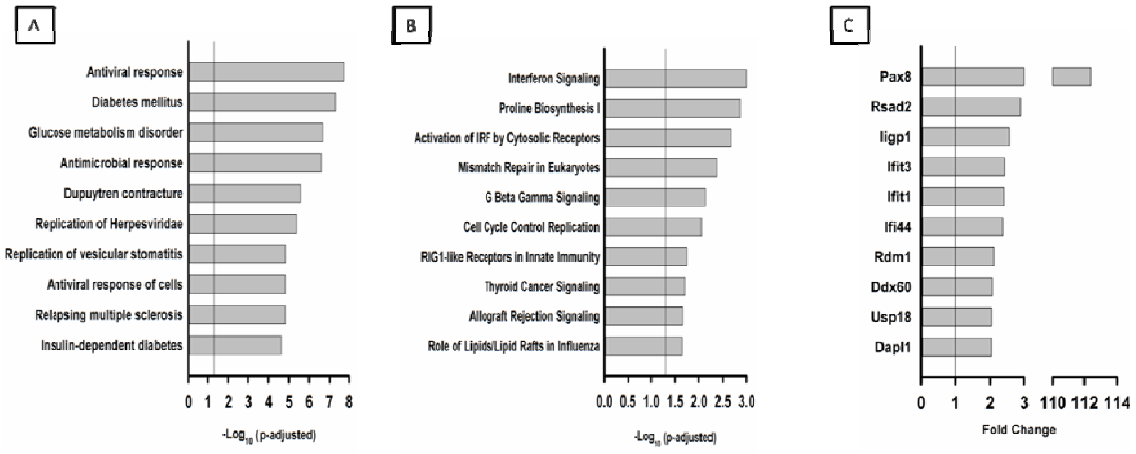


Figure 27: Transcriptome profile analysis of murine islets overexpressing Pax8 reveals up-regulation of immunomodulatory processes. Primary mouse islets were transduced with lentivirus allowing overexpression of Pax8 or GFP, used as a negative control. Then, RNA microarray was performed to determine the modulation of the transcriptional profile in pancreatic islet caused by the overexpression of Pax8. (A) Analysis by Ingenuity Pathway Analysis software determines the transcriptional profile of Pax8 overexpressing islets with development and/or protection against any disease. (B) Analysis by

Ingenuity Pathway Analysis software to determine the metabolic pathways modulated by overexpression of Pax8 gene. (C) List of the 10 most induced genes in islets overexpressing Pax8. As expected Pax8 gene is induced. n=3 per group.

CHAPTER VI_DISCUSSION AND PERSPECTIVES

CHAPTER VI_DISCUSSION AND PERSPECTIVES

Diabetes Mellitus is a pandemic disease characterized by a wide range of complications affecting seriously the health of patients. Therefore, the development of an effective treatment for this disease is an important worldwide objective. Main experimental therapies are focused in the preservation of β-cell mass to control glucose levels in the bloodstream. β-cell mass maintenance is regulated by a complex system of transcription factors. Two members of the Pax family, the transcription factors Pax4 and Pax8, are key players involved in the modulation of β-cell plasticity under specific metabolic circumstances conceiving them as potential therapeutic targets for DM treatments.

T1DM and T2DM are complex diseases characterized by a final common pathway: progressive β-cell failure and death, predominantly by apoptosis *(Cnop et al., 2005; Donath, 2004)*. β-cell apoptosis can be prevented via the activation of specific β-cell gene networks under the control of key transcription factors. Our studies evidence Pax4 as a master gene in β-cell plasticity in response to physiological and patophysiological situations through the protection of β-cell against apoptosis, favoring β-cell survival and proliferation in order to maintain a compensatory β-cell mass for an adequate control of glucose homeostasis. We wager on Pax4 as a first-class target candidate capable of unifying a mechanism through which it will be possible to develop an innovative therapeutic strategy for the treatment of T1DM and T2DM.

Our study emphasizes that Pax4 is a pivotal transcription factor in β-cell dynamics. Pax4 is implicated in maintaining mature islets homeostasis under detrimental metabolic conditions by 1) preventing hyperglycemia; 2) increasing β-cell survival; 3) stimulating β-cell proliferation *(Brun et al., 2008)* and 4) reducing the development of autoimmunity against β-cells.

1. Transient Pax4 overexpression prevents the development of hyperglycemia by preserving β-cell mass *in vivo* in animals exposed to environmental insults such as repeated STZ challenges. Noteworthy, the diabetes-linked mutant variant Pax4R129W (which has a decreased DNA binding activity) was less efficient in *in vivo* protection of mouse islets after STZ-induced hyperglycemia, resulting susceptible to present a mild hyperglycemia with a gradual β-cell loss. These data has been mimicked in the RIP-B7.1 animal model of experimental T1DM (BPTL) used in our project. Therefore, conditional overexpression of Pax4, but not the T2DM-associated Pax4R129W variant, preserves β-cell mass in immunized BPTL mice and delays the development of hyperglycemia.

2. Further investigation of the implication of Pax4 in β-cell survival has been performed. Pax4 overexpression *in vitro* protects isolated islets against the deleterious effects of cytokine-mediated apoptosis. Nevertheless, Pax4R129W variant was less effective in the protection of human islets *in vitro* against cytokines-induced cell death *(Brun et al., 2004)*. We hypothesize that a cytokine-induced “dialogue” between the

nucleus, mitochondria, and ER influences the decision of the β-cell to undergo apoptosis. Pax4 survival effects were shown to be mediated, at least in part, by regulation of the NF-κB signaling pathway. Several studies have confirmed that IL-1β is the predominant cytokine that activates the transcription factor nuclear factor (NF)-κB in rodent and human islet cells *(Eizirik and Darville, 2001; Eizirik and Mandrup-Poulsen, 2001)*. Prevention of NF-κB activation protects pancreatic β-cells against cytokine-induced apoptosis *(Cnop et al., 2005; Giannoukakis et al., 2000; Heimberg et al., 2001)*. Furthermore, it has been previously shown that low levels of IL-1β induce Pax4 expression *(Maedler et al., 2006)* suggesting that a reverse effect could be possible and high levels of Pax4 could repress endogenous IL-1β production. Indeed, our results have shown that transcript levels of IL-1β and its antagonist IL-1Ra were decreased to 50% in Pax4-overexpressing islets. Notably, Pax4-mediated inhibition of IL-1β with a concomitant augment in the NF-κB target gene Cox2, an antiapoptotic factor, confers increased resistance to death. In contrast, T2DM-associated Pax4R129W animals exhibit elevated basal levels of IL-1β, IL-1Ra and the proapoptotic gene Nos2 sensitizing these islets to NO-induced apoptosis.

In addition to the effects in NF-κB signaling pathway, Pax4 operate at different levels of the pro-apoptotic cascade. Remarkably, Pax4 expression, but not Pax4R129W, induced expression of the antiapoptotic gene Bcl-2 that blunted cytochrome C release into the cytosol and, therefore, prevent from cytokine-mediated apoptosis. Corroborating our data, previous studies reported that Bcl-2 regulate the mitochondrial response to pro-apoptotic signals *(Kuwana and Newmeyer, 2003)*, impeding release of mitochondrial proteins such as cytochrome C which sequentially activate caspase-9 and -3 and execute cell death *(Friedlander, 2003)*. In addition, it has shown that overexpression of Bcl-2 partially protects mouse *(Iwahashi et al., 1996)* and human *(Rabinovitch et al., 1999)* islets against cytokine-induced cell death.

Our RNA profiling analysis using KEGG (Kyoto Encyclopedia of Genes and Genomes) functional analysis elucidate that Pax4 overexpression, but not the T2DM-associated Pax4R129W variant, enriched the ER protein processing pathway stimulating β-cell survival and preventing apoptosis through the regulation of key genes involved in ER stress response. Disruption of ER homeostasis triggers accumulation of unfolded proteins and activation of ER stress response that, finally, leads to apoptosis *(Schroder and Kaufman, 2005)*. Because of their high rate of protein synthesis, β-cells are particularly susceptible to ER stress *(Harding et al., 2001)*. Our microarray data reveals that most of the up-regulated genes in Pax4-overexpressing islets are related to protein folding (Hspa5/Bip, Calr), ER-Golgi translocation (Lman1, Lman2, Sec23b and Plaa) and ER-associated degradation (Ufd1l, Derl3, Pdia4, Ssr3, Syvn1 and Dnaja2/Hsp40). Conversely, these transcripts involved in ER homeostasis were down-regulated after overexpression of the Pax4R129W variant. Indeed, we have demonstrated that Pax4 is able to protect β-cells against apoptosis by a parallel action on several branches of the UPR by blocking the thapsigargin-mediated apoptosis in

isolated islets. Thapsigargin is a chemical generally used to induce ER stress whose action consists in the inhibition of ubiquitous ER Ca^{2+}-ATPases (SERCA) that leads to the depletion of ER-Ca^{2+} stores *(Samali et al., 2010)*. Interestingly, the three most important proximal sensors of ER stress, Mbtps2 (ATF6), Eif2ak3 (Perk) and Mapk10 (Jnk3), were up-regulated in islets overexpressing Pax4, but were marginally altered in islets overexpressing Pax4R129W.

Interestingly, Pax4 overexpression reduced DNA damage and blunts β-cell apoptosis in two experimental model of diabetes, our immunized BPTL mice model and in a STZ chemical model, as showed by immunodetection of 53BP1 and cleaved caspase-3 proteins. Pax4 seems to present a protective mechanism from apoptosis induced by double-strand DNA breaks as a result of oxidative stress accumulation in the diabetic environment.

3. Of special interest in our project is the determination of the role of Pax4 in β-cell replication. Pax4 overexpression long-term (4 months) but not short-term (1 month) increases islet β-cell proliferation. A $BrdU^{+}/Pdx1^{+}/insulin^{-}$ cell subpopulation with impaired glucose-induced insulin secretion was detected specifically in Pax4-overexpressing islets. These cells were also negative for both glucagon and somatostatin. Given that Pax4 overexpression impedes insulin transcription by the repression of MafA, we hypothesize that these cells were likely β-cells with low levels of insulin prone to replication. Nonetheless, it is important to note that long-term expression of Pax4 may disrupt β-cell function as the cells revert to a dedifferentiated progenitor phenotype thereby losing their capacity to secrete insulin in response to glucose and promoting self-renewal and survival. These aberrant alterations may be pernicious due to the increased possibility of developing cancer. The latter is consisting with the concept that Pax genes are Pan genes that promote lineage commitment while simultaneously impeding full differentiation *(Robson et al., 2006)*. Strikingly, this effect was reversible and not harmful upon inhibition of Pax4 expression.

Consistent with increased long-term cell replication, Pax4-overexpressing islets displayed increased levels of the proproliferative genes c-myc and the cell cycle-dependent kinase, Cdk4. Importantly, the key markers of mature β-cells MafA and Glut2 were decreased in Pax4-overexpressing islets, whereas Pax4 levels were higher from 1 month DOX administration. Our results are in agreement with published data that described a mixed islet population composed by $Pdx1^{+}/insulin^{-}$ cells which express markers of undifferentiated β-cells and are more susceptible to proliferate *(Szabat et al., 2009)*. Remarkably, the transcript levels of the cell cycle-dependent kinases Cdk2 and Cdk6 were reduced. This could argue the low proliferation ratio detected in our $BrdU^{+}/Pdx1^{+}/insulin^{-}$ subpopulation, although the replication is higher that those usually observed in normal physiological circumstances in mature pancreatic islets.

To further investigate the Pax4 function as proliferative gene in β-cells, comparison of the transcriptome profile of Pax4 and Pax4R129W overexpressing islets reveals the functional enrichment of the cell cycle pathway, being among the top up-

regulated KEGG pathways in islets overexpressing the wild type Pax4, but was among the most significantly down-regulated pathways in islets overexpressing the T2DM-linked mutant R129W variant. In addition, a considerable set of genes associated to cell cycle were found increased after Pax4 overexpression such as the Cyclin D3 which possesses a double action: promotes proliferation and represses insulitis leading to enhancement of β-cell survival *(Saavedra-Avila et al., 2014)*; cyclin A2 (Ccna2); cyclin B2 (Ccnb2); cell division cycle protein 16 homolog (Cdc16) and origin recognition complex subunit 4 (Orc4) among others. Strikingly, most of these genes were unchanged or decreased in islets overexpressing Pax4R129W. Nevertheless, Pax4 overexpressing islets did not show an anomalous or increased growth after 1 month. This could be explained by our RNA microarray analysis which also reveals the expression of molecular cell cycle brakes such as cyclin-dependent kinase inhibitor 1A (Cdkn1a/p21) and cyclin-dependent kinase inhibitor 2A (Cdkn2a). In summary, we hypothesize that Pax4 is a master transcription factor which action is tilting the balance towards to β-cell proliferation or to the cell cycle molecular brakes depending of the metabolic circumstances. Notwithstanding, a second stimulus is needed for the inhibition of these brakes to allow β-cell proliferation.

4. Pax4 confers β-cell protection against an *in vivo* autoimmune attack blunting insulitis. Insulitis is characterized by the invasion of pancreatic islets by mononuclear cells leading to the loss of most β-cells by autoimmune attack after prolonged periods of disease *(Kloppel et al., 1985).* Of note, approximately 90% of non-Pax4 overexpressing BPTL mice suffered severe insulitis after 28 days post-immunization. In contrast, more than 50% of Pax4 overexpressing BPTL mice displayed no or mild insulitis even after 63 days of inducing the immune attack. In addition, our transcriptome profile analysis reveals a strong up-regulation of Lgals9 (galectin-9) in Pax4-overexpressing islets. In this context, Lgals9 overexpression, through attenuation of Th1 response, has been related to reduction of insulitis and hyperglycemia and prolongation of grafts survival in NOD mice *(Chou et al., 2013)*.

Taken these data together, we conclude that Pax4 overexpression in adult β-cells could be a relevant target for the development of novel β-cell regenerative strategies for T1DM and T2DM treatment as a result of its functions in β-cell survival, function and proliferation in response to physiological cues by modulating important regulatory pathways for islet plasticity, such as cell cycle, cell survival, ER-homeostasis, immune response and DNA repair.

Similar to Pax4, our studies reveal Pax8 as a potential master gene in the modulation of β-cell plasticity in response to physiological situations of increased insulin demand. We suggest that the transcription factor Pax8 is a master candidate target for anti-diabetic drug development capable of enhancing islets physiology and glucose homeostasis during adulthood.

Compilation of data about Pax8 has exposed that this unknown transcription factor in pancreatic islets physiology is in fact an emerging modulator of islets plasticity in circumstances of metabolic stress. Pax8 exerts broad functions in different organs *(De Felice and Di Lauro, 2011; Li et al., 2011; Mittag et al., 2007; Pfeffer et al., 1998; Plachov et al., 1990; Stoykova and Gruss, 1994; Tong et al., 2009)*. Nevertheless, previous studies did not detect expression of Pax8 in the endocrine compartment of the pancreas *(unpublished data from our group; (Goode and Elgar, 2009)*, which invalidated this transcription factor as a candidate for anti-diabetic treatments. Nonetheless, converging information from various studies gave hints about a potential role of Pax8 in pancreatic islets. First of all, recent clinical studies have demonstrated the presence of Pax8 in healthy human islets as well as in primary and metastatic PNETs suggesting that this transcription factor could be a specific marker for these pancreatic neoplasms *(Haynes et al., 2011; Laury et al., 2011; Long et al., 2010; Ozcan et al., 2011; Sangoi et al., 2011)*. Secondly, a transcript expression profile performed on islets from pregnant mice at different stages of gestation suggested a robust induction of Pax8 expression at day 14.5 of gestation coinciding with the peak of β-cell replication and, consequently, β-cell mass expansion *(Rieck and Kaestner, 2010; Rieck et al., 2009)*. Interestingly, in a Portuguese family of Azores with a newly identified PAX8 mutation *(Carvalho et al., 2013)*, the only pregnant female among the carriers of the mutation developed GDM *(Dr. Anselmo, personal communication)*. Moreover, a recent GWAS study has linked PAX8 with T2DM susceptibility in African Americans families *(Elbein et al., 2009)*. This information leads us to determine if Pax8 was really expressed in mature β-cells, and if it had an impact on islets physiology and patophysiology.

Based on the recent description of Pax8 expression in adult islets and PNETs, we analyzed the expression of this transcription factor in mouse and human islets. Astonishingly, our data did not support these reports, as Pax8 transcript was not detected in adult mouse islets and almost undetectable in human islets as compared to the expression levels in kidney, control organ abundant for Pax8. Curiously enough, all previous clinical studies have been performed only by immunostaining using the same polyclonal antibody *(Haynes et al., 2011; Laury et al., 2011; Long et al., 2010; Ozcan et al., 2011; Sangoi et al., 2011)*. In contrast, using a different monoclonal Pax8 antibody we were unable to confirm the expression of this gene in those tissues. Our results have demonstrated that the most commonly used Pax8 polyclonal antibody cross-reacts with Pax6 (Pax gene highly expressed in islets). Our data highlight that the detection observed in normal and developing islets as well as in PNETs was not Pax8 but Pax6 as demonstrated by immunochemical staining with different Pax8 and Pax6 antibodies. We have proposed that the cause of the cross-reactivity could be that PAX8 polyclonal antibody was raised against a 212-amino acid-long polypeptide encompassing the N-terminal region of PAX8 which includes the paired domain, structure highly conserved between the different members of Pax family. Interestingly,

one of these reports already pinpoints PAX8 detection in hematopoietic neoplasms suggesting that the inmunoreactivity of PAX8 may be attributed to cross-reactivity with other members of PAX family, notably PAX5. Indeed, PAX5 is well-characterized marker of B-cells and their associated hematologic malignancies, as well as a member of the same subgroup that PAX8 sharing over 95% sequence homology *(Sangoi et al., 2011)*. In fact, cross-reactivity between PAX2 protein, the third member of subgroup II, and PAX5 antibody has been previously reported *(Morgenstern et al., 2010)*. Additionally, following the publication of our study, other studies corroborate our findings showing that the aberrant detection by polyclonal PAX8 was partially caused by cross-reactivity with PAX5 and/or PAX6 *(Ordonez, 2012; Toriyama et al., 2014) (Moretti et al., 2012; Tacha et al., 2013)*. Taken together, our study highlights 1) the lack of Pax8 immunodetection in adult islets under normal physiological conditions and 2) Pax8 detection as a valuable diagnostic marker of PNETs, as it as been suggested, should be re-evaluated. This also highlights the pitfalls of using non-properly validated antibodies to perform large-scale clinical studies. However, despite these results, we cannot exclude the induction of Pax8 during pregnancy suggested by Kaestner group *(Rieck et al., 2009)*.

Our next step was to try to elucidate if Pax8 expression is activated in response to physiological situations in which the organism requires increased insulin demand and, consequently, a β-cell mass expansion such as pregnancy. Consisting with this idea, we initially confirmed that Pax8 expression is induced during pregnancy in mice reaching maximum transcript levels 5-fold higher than those of non-pregnant mice at day 14.5 of gestation that coincides with the peak of β-cell proliferation *in vivo (Rieck and Kaestner, 2010)*. In agreement with this, we also detected Pax8 expression by immunofluorescence in pancreatic islets of pregnant mice at days 10.5-14.5 of gestation. Furthermore, treatment of human islets in culture with prolactin, one of the main hormones produced during pregnancy, evoked a 5-fold increased in Pax8 expression 72-hours post-treatment. Surprisingly, $Pax8^{-}/^{-}$ mice displayed a dramatic decrease in prolactin expression *(Friedrichsen et al., 2004)* suggesting a close interaction between this transcription factor and the hormone lactotropes. Our findings were supported by microarray data from Beta Cell Gene Atlas *(Kutlu et al., 2009); http://www.t1dbase.org/page/AtlasView)* which confirm Pax8 expression in human and mouse pancreatic islets under physiological and patophysiological conditions. Interestingly, Pdx1 levels were suppressed in both mouse and human islets during pregnancy progression or prolactin treatment respectively. We speculate that decreased Pdx1 expression levels are related to the control of β-cell growth, function previously reported by global gene profile analysis of rat islets *(Gauthier et al., 2004; Johnson et al., 2003)*. This transcription factor is indispensable for both pancreas development and subsequent β-cell formation and function *(Hansen et al., 2000; Stoffers et al., 1997a)*. We postulate that similar to Pax4, Pax8 expression could lead to the emergence of a pro-proliferative $Pax8^{+}$ subpopulation in order to force islets

machinery in the replication process. Importantly, prolactin treatment induced a transient increase in the proliferation of human islets that reached maximum levels of 2-fold at 24-hours post-treatment. Intriguingly, our *in vitro* data indicate that proliferation precedes induction of Pax8 while in our *in vivo* data correlates in gestational circumstances. Notwithstanding these facts, Pax8 is clearly activated under specific physiological conditions such as pregnancy, although the implication of this transcription factor in regulating β-cell replication and/or survival remains to be established. Therefore, correlating with the novel PAX8 mutation responsible for congenital hypothyroidism in an Azores family, we could speculate a potential function of Pax8 in islets glucoregulation and/or islets maturation that, in close relation with thyroid dysfunction, leads to a specific genetic disorder that, finally, develops GDM. Deciphering the mechanism of action in which Pax8 controls the pancreatic islets physiology is crucial to discover novel therapies to treat DM.

The unexpected finding that Pax8 is induced during pregnancy reveals the presence of a new player in islets physiology that may play an important role in cell expansion in response to metabolic demands. In order to evaluate the potential direct functional implications of Pax8 on islets physiology we created a lentiviral vector for Pax8 overexpression. Unfortunately, the limitations of the efficiency and difficulty of current protocols for gene transfer in entire islets impeded us to study the role of Pax8 since it is of great importance the maintenance of the integrity of islets to conduct mechanistic studies. As a consequence of these complications, we have developed a simple, reproducible and easy-to-use transduction protocol that allows a significant improved efficiency, being able to infect approximately 72-80% of the cells of entire murine and human islets. Our protocol includes a mild trypsinization step (0.5X trypsin-EDTA for 3 minutes) and a lower virus dosage of MOI (Multiplicity Of Infection) 20 for up to 4 days post-transduction. Previous protocols that have shown similar infection efficiency required much higher virus dosage (MOI 100) and chemical agents that results detrimental for islets function and survival *(Barbu et al., 2006; Kvell et al., 2005; Rajalingam et al., 2001)*. Remarkably, our transduction protocol mantains 1) islets functionality in the absence of detrimental effects in glucose-induced insulin secretion; 2) islet integrity presenting normal islet architecture; 3) islets health with optimal insulin and glucagon expression in human and mouse islets and, finally, 4) normal metabolic activity of islets cells measured by NAD(P)H-oxidase activity. Singularly, our entire-islets transduction protocol supposes a reliable easy-to-use procedure that would facilitate and improve the current gene therapies prior islets transplantation to treat diabetes mellitus and so necessary to treat this pandemic disease.

Gene profiling analysis on mouse islets overexpressing murine Pax8 had allowed us to identify metabolic pathways and target genes modulated by Pax8 that may be useful for the development of novel anti-diabetic agents. Indeed, transcriptome profiling analysis determines that Pax8 overexpression is mainly related to processes associated with the development and/or protection against DM and

metabolic syndrome. Furthermore, Pax8 controls gene network connected with immunomodulation favoring protection against insulitis under circumstances of metabolic stress such as pregnancy in which the immune system undergoes great changes due to the presence of the fetus. Taken together, our data reveal Pax8 as a potential therapeutic gene involved in islets plasticity due its implication in a cell signaling pathway that promotes insulin production, proliferation and/or immunomodulation of β-cells during pregnancy.

To conclude, islet β-cell regeneration is a fascinating field with promising potential for the treatment of diabetes mellitus. The master transcription factors Pax4 and Pax8 play a crucial role in mature islets plasticity improving β-cell survival, function and proliferation under certain physiological and/or patophysiological situations of increased metabolic demands. Alternatively, these factors could also be useful for optimizing islet transplantation and post-transplantation processes blunting the immune response and favoring long-term islet function and survival. In perspective, Pax4 and Pax8 should be considered in future attempts to develop novel agents for the prevention and treatment of diabetes mellitus.

CHAPTER VII_CONCLUSIONS

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PAX4 PROJECT CONCLUSIONS:

- Pax4, but not the T2DM-linked mutant variant Pax4R129W, protects animals of the development of hyperglycemia by preserving β-cell mass in two *in vivo* models of β-cell destruction, in a chemical-STZ model and in a chronic *in vivo* autoimmune attack
- Pax4, but not Pax4R129W, overexpression *in vitro* protects isolated islets against cytokine-mediated β-cell apoptosis by suppressing IL1β expression levels and its antagonist IL-1Ra, while increasing Bcl-2 and Cox2 levels that blunted cytochrome C release.
- Pax4 overexpression decreases insulitis and enhances β-cell fitness in immunized BPTL mice**.**
- Pax4 overexpression reduced DNA damage in immunized BPTL mice and in STZ-challenged mice by suppressing double strand DNA breaks and blunting apoptosis.
- Transcriptome profiling of Pax4-overexpressing mouse islets reveals the upregulation of genes and implicated in ER homeostasis. The endoplasmic reticulum (ER) processing of proteins was among the top up-regulated KEGG pathways in islets overexpressing Pax4, but was among the most significantly down-regulated pathways in islets overexpressing Pax4R129W mutant variant.
- Pax4 overexpression long-term (4 months), but not short-term (1 month), increases islet β-cell proliferation through MafA, insulin and Glut2 repression, with a concomitant increase in Cdk4 and c-myc expression.
- Long-term Pax4 expression promoted a pro-proliferative $BrdU^{+}/Pdx1^{+}/insulin^{-}$ cell subpopulation correlating with impaired glucose-induced insulin secretion.
- Long-term expression of Pax4 disrupts β-cell function promoting a dedifferentiated state thereby losing their capacity to secrete insulin. Strikingly, this effect was reversible and not harmful upon inhibition of Pax4 expression.
- RNA profiling analysis establishes that Pax4 overexpression enriches several transcripts involved in stimulating β-cell growth including Cyclin D3, Cyclin B2 and Cyclin A2. Most of these genes were either unchanged or decreased in islets expressing Pax4R129W. Furthermore, cell cycle was among the top up-regulated KEGG pathways in islets overexpressing Pax4, but was among the most significantly down-regulated pathways in islets overexpressing Pax4R129W mutant variant.

PAX8 PROJECT CONCLUSIONS:

- Pax8 is not expressed in adult mouse islets and almost undetectable in human islets under normal physiological conditions.
- Pax8 is not detected in PNETs refusing its use as a valuable diagnostic marker for pancreatic neoplasm.
- Pax8 expression is induced during pregnancy in mice reaching maximum transcript levels at day 14.5 of gestation correlating with the peak of β-cell proliferation *in vivo*.
- Pax8 protein is detected in pancreatic islets of pregnant mice at days 10.5-14.5 of gestation suggesting the existence of a pro-proliferative $Pax8^{+}$ subpopulation.
- Human islets treated with prolactin (to mimick pregnancy) evoked a transient increased in Pax8 expression 72-hours post-treatment.
- Prolactin treatment induced an increase in the proliferation rate of human islets reaching maximum levels at 24-hours post-treatment. Proliferation precedes induction of Pax8 in human islets.
- Pax8 overexpression modulates genes associated with processes related to the development and/or protection against diabetes and metabolic syndrome that, finally, leads to GDM.
- Pax8 controls gene network connected with immunomodulation and cell cycle control replication. Transcriptome analysis reveals that several components of the interferon pathway are up-regulated such as Ifit1, Ifit3 and Ifit44 among others in Pax8-overexpressing islets.

CHAPTER VIII_REFERENCES

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