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Pancreatic somatostatin expressing-cells: an untapped source for β -cell regeneration ?

Noémie Druelle

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UNIVERSITE COTE D'AZUR – UFR SCIENCES
Ecole Doctorale des Sciences de la Vie et de la Santé (ED85)

THESE

Pour l'obtention du titre de
Docteur en Sciences de la Vie et de la Santé

Spécialité : Recherche Clinique et Thérapeutique

Présentée et soutenue par
Noémie DRUELLE

Pancreatic somatostatin expressing-cells: an untapped source for β -cell regeneration?

Soutenue publiquement le 2 décembre 2016 devant le jury composé de :

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elles sont les charmants jardiniers par qui nos âmes sont fleuries.*

Marcel Proust

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INTRODUCTION

I. Diabetes Mellitus

Affecting approximately 422 million people worldwide (“Worldwide trends in diabetes since 1980,” 2016), diabetes mellitus figures among the most common metabolic conditions. The prevalence of diabetes mellitus is increasing and predicted to rise to 552 million affected people by 2030 (Whiting et al., 2011). Despite therapies and prevention strategies, diabetes is one of the four main chronic diseases and was the direct cause of approximately 1.5 million deaths in 2012 (“WHO | *Global report on diabetes*”).

Due to a failure of insulin production or action, diabetes is responsible for long-lasting elevated blood glucose levels, such chronic hyperglycemia being associated with various complications. Indeed, diabetes is reported to be the main cause of blindness and increases the risk of cardiovascular diseases, nephropathy, stroke and amputation (Alwan, 2010; Morrish et al., 2001; Pascolini and Mariotti, 2012). Diabetes patients are also at risk of dying prematurely (Manuel and Schultz, 2004; Narayan et al., 2003). In addition to health concerns, diabetes care represents a substantial financial burden with an annual worldwide cost of \$825 billion (“WHO | *Global report on diabetes*”).

With chronic hyperglycemia as a common feature, diabetes mellitus is classified into different forms, described hereafter.

i. **Type 1 Diabetes Mellitus (T1DM)**

Formerly known as juvenile diabetes or insulin-dependent diabetes, Type 1 Diabetes Mellitus (T1DM) represents approximately 10% of all diabetic patients. Two peaks of disease onset have been reported: the first between 6 and 15 years of age and the second later in adolescence, typically before 30 years (Herold et al., 2013). T1DM is a polygenic disorder characterized by a cytolytic T-cell mediated autoimmune-destruction of the insulin-producing β -cells (Bottazzo GF, Florin-Christensen A, 1974; Gepts, 1965; Kolb et al., 1995), such selective loss leading to a lack of insulin secretion subsequently resulting in high blood glucose levels (**Figure 1A-B**).

While the exact causes for this autoimmune-mediated cell destruction are not yet known, a genetic component in the development of T1DM is well documented. The first described susceptibility locus associated with T1DM was the human leukocyte antigen (HLA) gene (Nerup et al., 1974; Singal and Blajchman, 1973). Other genes outside the HLA locus and strongly associated with T1DM have also been identified. Indeed, polymorphisms in the insulin (Rotwein et al., 1986), PTPN22 (Bottini et al., 2004), CTLA-4 (Ueda et al., 2003) and IL-2RA (Maier et al., 2009) genes are frequently associated with T1DM. In addition to genetic influences, the rapid rise in T1DM incidence suggests the contribution of environmental (Atkinson et al., 1994; Gillespie et al., 2004; Ziegler et al., 2011) or viral (Ginsberg-Fellner et al., 1985; Honeyman et al., 2000) stimuli in the development of this metabolic disease.

The progressive destruction of the β -cells by the immune system of T1DM patients leads to the production of autoantibodies directed towards several antigens, including GAD65 (Baekkeskov et al., 1990; Solimena et al., 1988; Towns and

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Pietro Paolo, 2011), insulin (Palmer et al., 1983; Wegmann et al., 1994) and proinsulin (Kuglin et al., 1988), ICA512 (Rabin et al., 1992) and ZNT8 (Wenzlau et al., 2007). Easily detectable in the serum, autoantibodies represent robust hallmarks for T1DM diagnosis (Bingley et al., 1997; Riley et al., 1990; Verge et al., 1996).

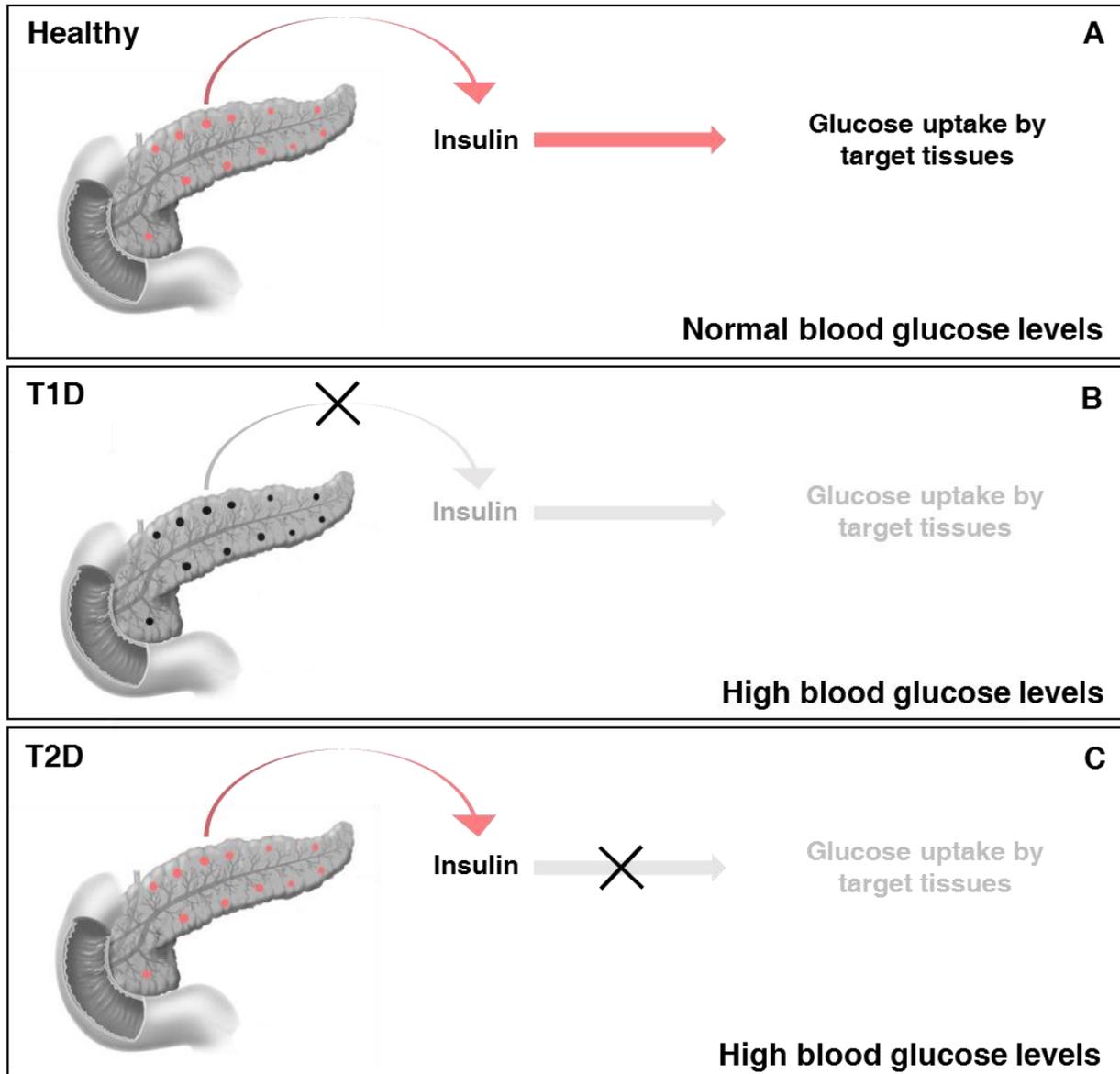


Figure 1: The two main types of diabetes – Type 1 and Type 2. In healthy subjects, insulin is released from pancreatic β -cells, leading to glucose uptake by target tissues and normoglycemia (A). In type 1 diabetic patients, the loss of β -cells in pancreatic islets induces a deficiency in insulin secretion and a lack of glucose uptake, further resulting in high blood glucose levels (B). In type 2 diabetic patients, insulin is secreted but a defect in its action on glucose uptake by target tissues results in hyperglycemia (C).

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Even though symptoms usually do not appear before at least 80% of the β -cell mass has been destroyed (Gillespie, 2006), the final absolute destruction of these cells leads to the dependence on exogenous insulin administration for T1DM patient survival.

ii. **Type 2 Diabetes Mellitus (T2DM)**

Type 2 Diabetes Mellitus (T2DM), previously known as adult-onset diabetes or non-insulin dependent diabetes, is the most common type of diabetes. Traditionally associated with adults, T2DM is a progressive condition characterized by insulin resistance due to defective insulin action on peripheral target tissues (liver, adipose tissue, muscles - **Figure 1A, C**). This sustained insulin resistance is accompanied by a β -cell compensation in order to increase insulin secretion. However, such adjustments can result, in time, in a failure in β -cell function (Kahn, 2000), further contributing to the loss of β -cells and impairment of insulin secretion. The subsequent reduced β -cell mass in T2DM patients highlights such failure in β -cell compensation (Klöppel et al., 1985).

T2DM is a complex disease caused by genetic variations acting in concert with environmental factors. Indeed, the IRS-1 and PPAR γ genes are highly polymorphic in patients suffering from T2DM (Andrulionytè et al., 2004; Kahn et al., 1996). Furthermore, several environmental factors influence T2DM prevalence (Hu FB, Li TY, Colditz GA, Willett WC, 2003; Murea et al., 2012), among which obesity-triggered inflammation, which was proposed to be the major risk factor in this disease (Haffner, 1998). In addition, endoplasmic reticulum stress, due to high fat diet, has

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been shown to have an important impact in insulin signaling impairment and in β -cell dysfunction (Cnop et al., 2012; Eizirik and Cnop, 2010; Eizirik et al., 2008).

Treatment of T2DM mostly focuses on lowering blood glucose levels by lifestyle adjustments, such as an adapted diet combined with increased physical activity (Eriksson and Lindgärde, 1991; Pan et al., 1997). Although less effective than lifestyle changes, oral hypoglycemic agents can also be used to manage T2DM hyperglycemia (Knowler et al., 2002; Ripsin et al., 2009). Insulin therapy can eventually be considered, if previously mentioned strategies are not effective or in advanced stages of this disease.

iii. **Gestational diabetes**

Gestational diabetes is a metabolic disorder with a prevalence of 9.2% (DeSisto et al., 2014) during the 3rd trimester of pregnancy. Reported risk factors include maternal age and obesity (Solomon, 1997). During gestation, the action of human placental lactogen and other hormones, such as prolactin, may interfere with insulin signaling (Carr and Gabbe, 1998; Sorenson and Brelje, 1997) leading to insulin resistance. Moreover, although insulin does not pass through the placental barrier, glucose does and can stimulate the fetus to produce the required insulin. This excess of insulin can result in neonatal hypoglycemia while glucose surplus can lead to macrosomia (Wendland et al., 2012; Wong et al., 2013). In addition, the incidence of obesity and T2DM is increased in these children (Dabelea et al., 2008).

Although gestational diabetes is reversed within 48 hours after delivery for 90% of women (Kahn, 1998), it increases the risk of the mother developing T2DM (Bellamy et al., 2009). Moderate physical exercise, improved diet and eventually,

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exogenous insulin injections can be used to prevent and control gestational diabetes (Alwan et al., 2009).

iv. **Monogenic diabetes**

As described above, Type 1, Type 2 and gestational diabetes are polygenic diseases. However, rare monogenic forms of diabetes exist and represent 1-2% of diabetes cases (Eide et al., 2008). MODY (Maturity Onset Diabetes of the Young) represents the main subtype of monogenic diabetes and generally occurs before 25 years of age (Tattersall and Fajans, 1975). This condition can be due to inherited or spontaneous mutations in different genes, such as *HNF-4 α* , *HNF-1 α* or *HNF-1 β* (Ellard, 2000).

Neonatal diabetes mellitus (NDM), another monogenic form of diabetes, commonly occurs before 6 months of age (Schwitzgebel, 2014). It can be transient or permanent and is generally due to mutations in the *ABCC8* and *KCNJ11* genes, involved in insulin signaling pathway (Flanagan et al., 2007).

II. **Pancreas anatomy and function**

Apparently initially discovered by Herophilus, a Greek anatomist, in approximately 300 BC, the organ was named “pancreas” by Ruphos of Ephesus around the first century AD. Translated “all flesh” from the Greek [*pan*: all and *kreas*: flesh], probably due to the consistency of the tissue (Busnardo et al., 1983), the pancreas has also been called “the hermit organ” because of its hidden location (Gavaghan, 2002).

i. **Gross anatomy of the pancreas**

The pancreas is a deep abdominal gland, located behind the stomach and connected to the duodenum of the small intestine (**Figure 2**).

The human pancreas is divided into different anatomic parts: the head, the body and the tail (Bockman, 1993). While the head of the pancreas is situated near the duodenum, the tail part lies close to the hilum of the spleen. The body segment stretches beneath the stomach (Dolensek et al., 2015 - **Figure 2**). Of note, the mouse pancreas is more diffuse within the abdomen and is segmented into the duodenal, splenic and gastric lobes (Dolensek et al., 2015).

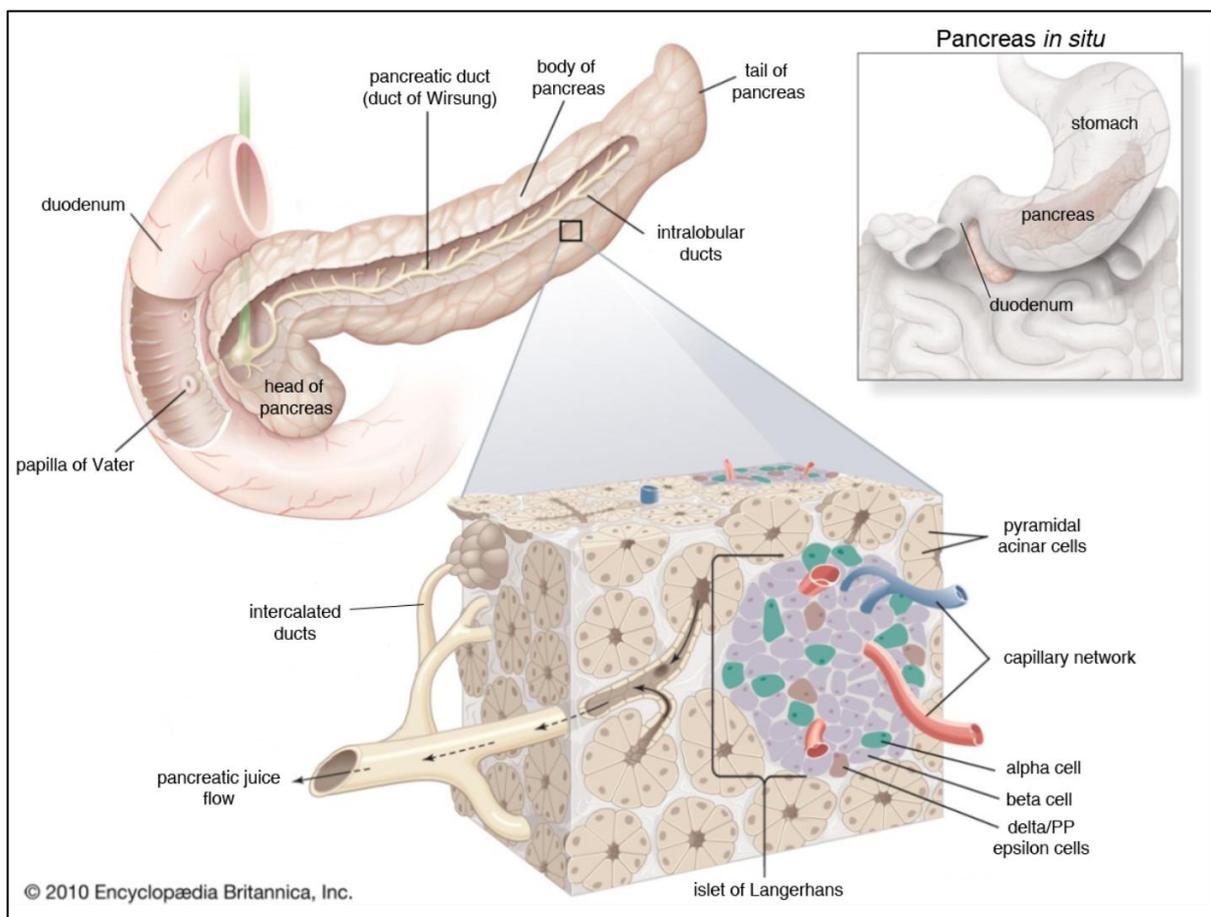


Figure 2: Human pancreas anatomy and histology (adapted from *Encyclopaedia Britannica, 2010*).

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The pancreas is an amphicrine gland, thus composed of two compartments: the exocrine tissue and the endocrine tissue, each with distinct structures and functions (**Figure 2**). Responsible for the secretion of various enzymes and hormones, it has a preponderant role in food digestion and glucose homeostasis (Cano et al., 2007; Slack, 1995).

ii. **Organization and function of the exocrine tissue**

Representing the major component of the gland, the exocrine compartment accounts for approximately 95% of the total organ (Rahier et al., 1981). The exocrine pancreas is involved in two physiological processes: the regulation of gastric acid and chemical digestion. It is mostly composed of serous acinar cells and ductal cells forming a branched network throughout the gland (Edlund, 2002).

a. **Acinar cells**

Pancreatic acini are made of pyramidal acinar cells containing zymogen granules within secretory vesicles. These exocrine acinar cells produce and secrete a juice composed of several enzymes (i.e. lipases, amylase, trypsin) involved in food digestion (Tan, 2005). Combined, these enzymes hydrolyze complex nutrients, such as polysaccharides, triglycerides and proteins (Whitcomb and Lowe, 2007). Pancreatic enzymes are released as pancreatic juice into the duodenum through the branched ductal network.

b. Duct cells

Pancreatic ductal cells are organized into a complex network running throughout the entire pancreas. Acinar cell secretions are collected by intercalated ducts and siphoned into intralobular ducts; this pancreatic juice is drained into the duodenum at the papilla of Vater through the main pancreatic duct also called duct of Wirsung (Reichert and Rustgi, 2011 - **Figure 2**).

To regulate duodenal acidity, ductal cells produce water and bicarbonate which is added to the enzyme mixture, thus providing optimal conditions for enzyme activity (Githens, 1988).

iii. Organization and function of the endocrine tissue

The endocrine compartment of the pancreas is composed of small functional units named islets of Langerhans. Originally described by Paul Langerhans in his thesis in 1869 (Langerhans, 1869), the islets represent about 4-5% of the pancreatic mass and are found scattered throughout the exocrine tissue (Ionescu-Tirgoviste et al., 2015; Rahier et al., 1981). However, regional islet distribution in the different parts of the pancreas is subject to controversy. Indeed, while reports suggest the highest number of mouse or human islets in the pancreatic tail (Wang et al., 2013), other studies point to a similar enrichment in the head of the pancreas (Hörnblad et al., 2011).

Islets of Langerhans are arranged into clusters containing five hormone-secreting cell types: β -cells, α -cells, δ -cells, PP-cells and ε -cells; respectively responsible for the secretion of insulin, glucagon, somatostatin, pancreatic

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polypeptide (PP) and ghrelin (Adrian et al., 1978; Prado et al., 2004; Roncoroni et al., 1983 - **Figures 2-3**). Interestingly, variations in the microarchitecture of islets have been observed between species. Indeed, while a central core of β -cells surrounded by a mantle of non- β -cells is typical for mouse islets (**Figure 3B**), human islets appear more unorganized with non- β -cells dispersed within the β -cell mass (Brissova et al., 2005; Cabrera et al., 2006; Steiner et al., 2010 - **Figure 3A**).

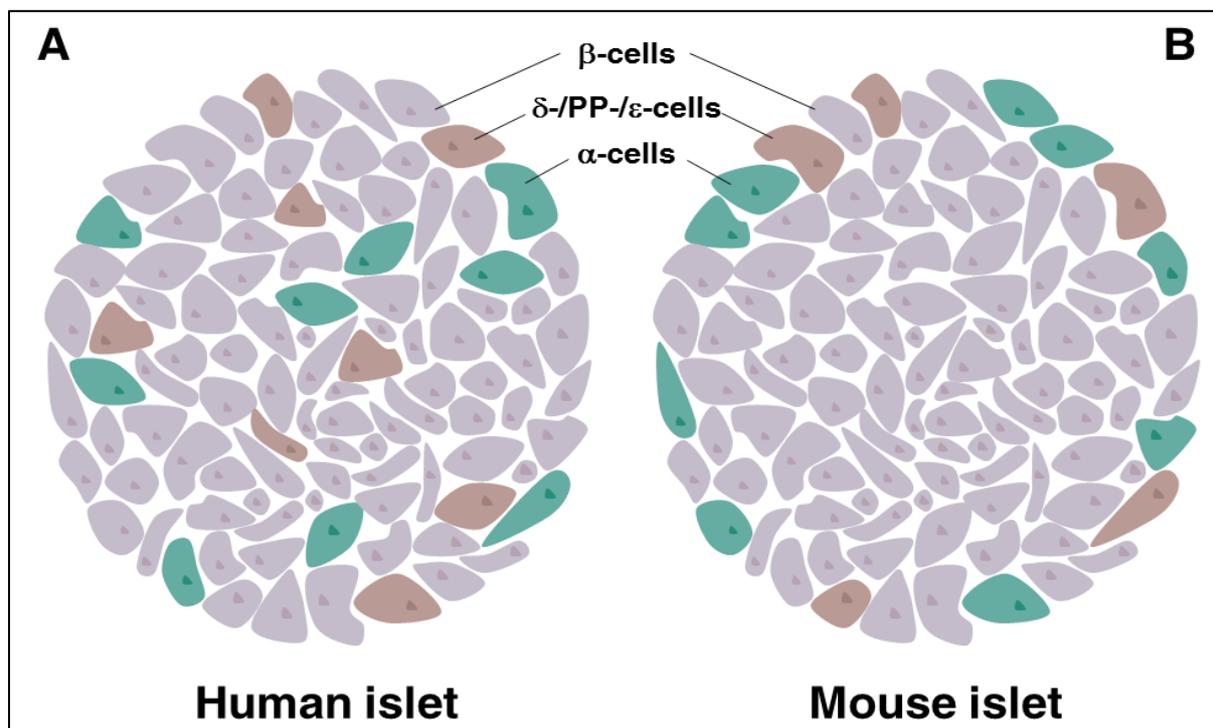


Figure 3: Microarchitecture of human and mouse islets. Architecture of the human islet with non- β -cells scattered within β -cell mass (**A**). Mouse islets display a mantle of non- β -cells at the periphery and a core typically constituted of β -cells (**B**).

Of note, recent reports argue the possibility of finding human islets displaying mouse islet organization and *vice versa*, depending on physiological variations, such as the increase in insulin demand observed during pregnancy (Kharouta et al., 2009; Kilimnik et al., 2012; Kim et al., 2009). Other variations, such as endocrine cell proportions within the islets, are also observed between organisms. Indeed, while human islets contain approximately 50-70% β -cells, 20-40% of α -cells and less than

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5% of δ -cells, mouse islets display 60-80%, 10-20% and less than 5% β -, α - and δ -cells respectively (Andralojc et al., 2009; Steiner et al., 2010 - **Figure 4**).

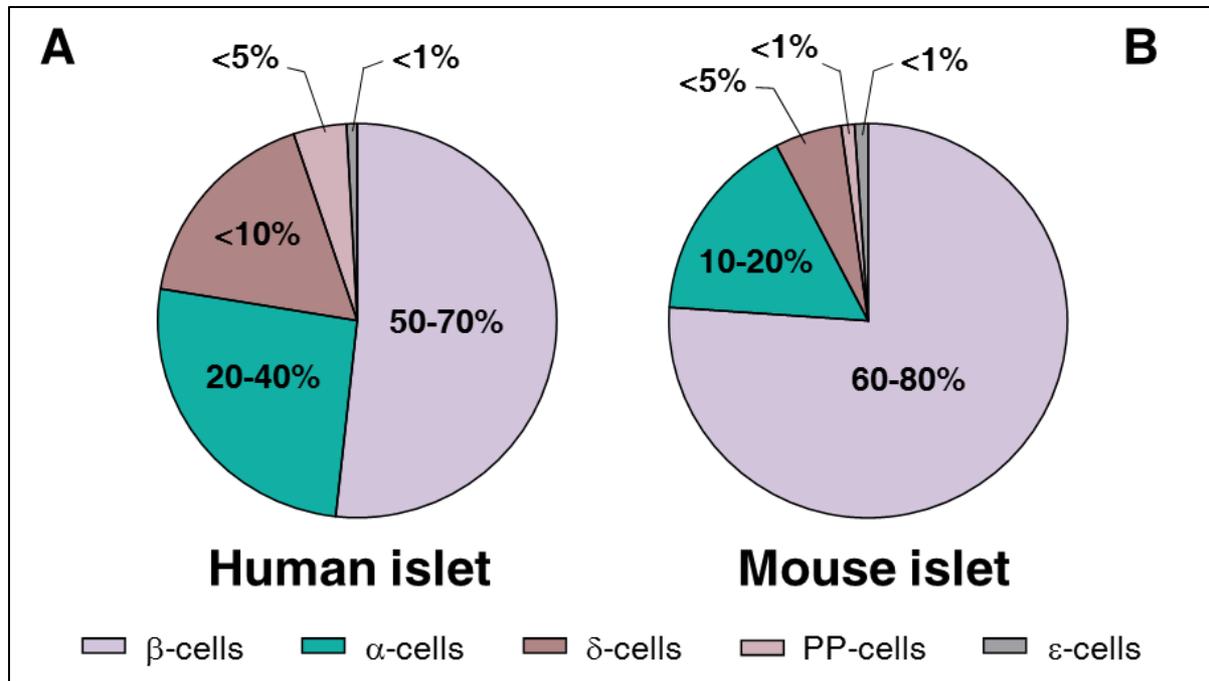


Figure 4: Endocrine cell proportions of human and mouse islets. Endocrine proportions of human islets showing a majority of β - and α -cells (A). Differences in proportions in mouse islets are observed although β - and α -cells remain the main endocrine cells (B).

Critical for glucose homeostasis regulation, pancreatic islets are densely vascularized allowing the direct secretion of hormones into the blood circulation (Brissova et al., 2006; Lifson et al., 1985). Blood glucose levels are finely tuned to be maintained within normal ranges, corresponding to <6.1 mmol/l in a fasted state (**Table1**).

	Non diabetic	Diabetic
Fasting	< 6.1 mmol/l	≥ 7 mmol/l
2 hour after meal	< 7.8 mmol/l	≥ 11.1 mmol/l

Table 1: Blood glucose levels in non-diabetic and diabetic patients (adapted from the global diabetes community website <http://www.diabetes.co.uk/>).

a. Insulin-secreting β -cells

Pancreatic β -cells represent the most abundant cell type in islets with 50-70% in humans and 60-80% in mice (Dolensek et al., 2015 - **Figure 4**). Synthesizing and secreting the hypoglycemic hormone insulin, they play a key role in regulating glucose homeostasis. Insulin is a protein of 51 amino acids, composed of two polypeptide chains linked by disulfide bonds resulting from the cleavage of proinsulin by the action of prohormone convertases PC1/3 and PC2 (Steiner et al., 1967; Weiss et al., 2014). Stored as granules in secretory vesicles, insulin is secreted upon high glucose blood levels. Indeed, glucose enters β -cells via the low affinity GLUT2 transporter expressed at the cell membrane, its assimilation leading to an increase in intracellular ATP concentrations. This rise causes the closure of K_{ATP} channels, membrane depolarization and the opening of Ca^{2+} channels. The influx of Ca^{2+} leads to the release of insulin by exocytosis into the blood (Ashcroft et al., 1984; Larsson et al., 1996; Nichols et al., 1996 - **Figure 5**).

Insulin travels to its target tissues through the bloodstream (liver, adipose tissue, skeletal muscles) where it interacts with its receptor (IR; insulin receptor) present at the membrane of target cells and activates its own signaling pathway. Activation of the insulin signaling pathway leads to the recruitment to the plasma membrane of the glucose transporters to facilitate uptake and storage of circulating glucose. In this manner, insulin stimulates glucose absorption by muscle and adipose tissue cells but also glycolysis and glycogenesis in the liver to store glucose as glycogen and decrease glycemia. In addition, insulin inhibits hepatic glycogenolysis and gluconeogenesis to block glycogen catabolism and the synthesis of glucose.

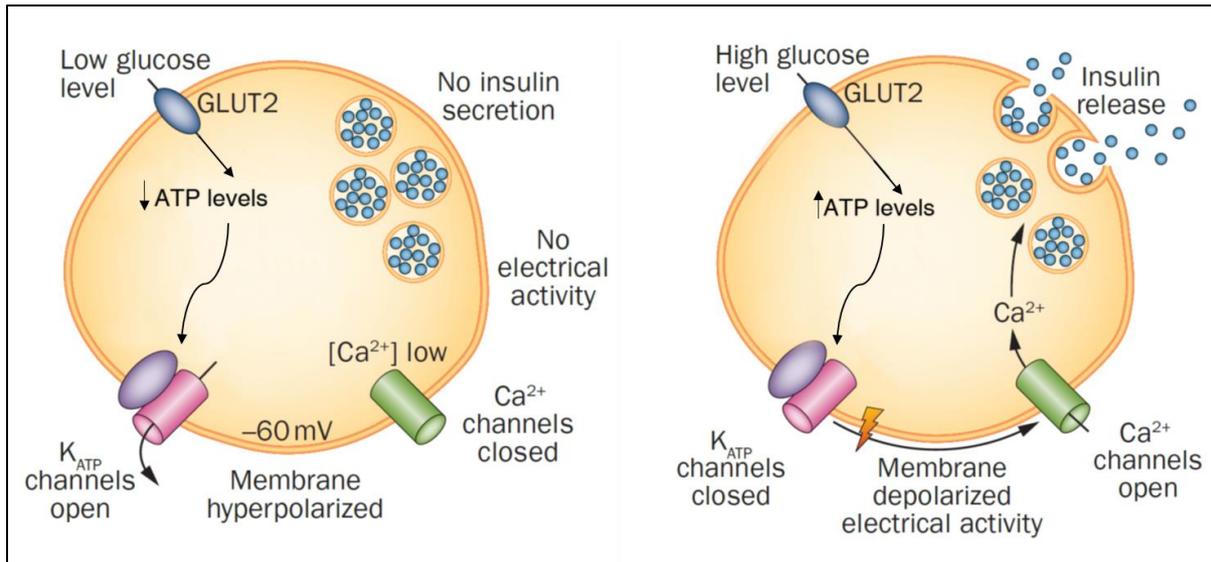


Figure 5: Insulin release from pancreatic β -cells (adapted from Ashcroft and Rorsman, 2013). Insulin secretion is inhibited at low glucose concentrations. At high glucose levels, glucose enters the β -cell through its receptor GLUT2, increasing intracellular ATP concentration further leading to the closure of K_{ATP} channels, the depolarization of the plasma membrane and the opening of the Ca^{2+} channels. As a result, the intracellular concentration of Ca^{2+} increases causing the exocytosis of insulin granules.

Although glucose is the main stimulus involved in insulin secretion, other molecules have been shown to induce insulin release, such as amino acids, acetylcholine or cholecystokinin (Hermans et al., 1987; Karlsson and Ahrén, 1989; Zhang and Li, 2013). Moreover, somatostatin has been shown to inhibit insulin secretion (Hauge-Evans et al., 2009).

b. Glucagon-secreting α -cells

Representing 20-40% and 10-20% of human and mouse total endocrine cell type respectively (Dolensek et al., 2015), α -cells secrete the hormone glucagon, a critical actor in the regulation of glucose homeostasis. Indeed, as a hyperglycemic hormone, glucagon plays the antagonist role compared to insulin in the maintenance

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of blood glucose levels within normal ranges (Unger, 1971). In accordance with a common feature of hormones, glucagon is originally synthesized as a precursor named proglucagon. This precursor will be processed in pancreatic α -cells to produce the 29-amino acid peptide glucagon (Furuta et al., 2001), stored as granules in secretory vesicles.

The exocytosis of glucagon is induced when blood sugar levels drop, during fasting or increased energy expenditure, to promote target tissues to mobilize glucose and therefore increase glycemic levels. In the liver, glucagon interacts with its membrane-bound receptor - a G-protein coupled receptor - (Authier and Desbuquois, 2008; Brubaker and Drucker, 2002) to promote hepatic gluconeogenesis and glycogenolysis, therefore stimulating the breakdown of glycogen (Habegger et al., 2010).

Regulation of glucagon secretion is driven by various signals. Glucagon release is inhibited by glucose, insulin and somatostatin (Hamaguchi et al., 1991; Hauge-Evans et al., 2009; Kisanuki et al., 1995). Moreover, other stimuli such as circulating amino acids (Pagliara et al., 1974) and fatty acids (Bollheimer et al., 2015) have been shown to impact the secretion of glucagon.

c. **Somatostatin-secreting δ -cells**

Pancreatic δ -cells make up less than 10% and 5% of endocrine cell types in human and mouse islets, respectively (Dolensek et al., 2015). δ -cells secrete somatostatin (SST), also known as growth hormone-inhibiting hormone (GHIH) or somatotropin release-inhibiting factor (SRIF).

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Somatostatin exists as two isoforms, somatostatin-14 (SST-14) and somatostatin-28 (SST-28), resulting from different cleavage of the prosomatostatin (Andrews and Dixon, 1986). Binding somatostatin receptors (members of the G-protein coupled receptor family which comprise five subtypes - SSTR1-5 - Yamada et al., 1993), SST-14 and SST-28 are able to exert a wide range of effects.

Typically, somatostatin is described as a paracrine and endocrine peptide as it can induce the inhibition of the secretion of numerous hormones of the gastrointestinal tract, the nervous system and the pancreas (Dollinger et al., 1976). In addition to pancreatic δ -cells, somatostatin is synthesized by cells in the central nervous and gastrointestinal system (Reichlin, 1983). While tissue distribution of SST-14 and SST-28 is still debated, it has been suggested that the two peptides have different inhibitory potentials. SST-14 strongly inhibits glucagon secretion through SSTR2 while SST-28 mostly blocks insulin release through SSTR5 (D'Alessio et al., 1989; Mandarino et al., 1981; Mitra et al., 1999). However, recent analyses suggested that SSTR5 is not expressed in islet endocrine cells (DiGruccio et al., 2016). Various stimuli induce somatostatin secretion, such as low concentrations of glucose, fatty acids, HCL and urocortin 3 (Rouiller et al., 1980; Schusdziarra et al., 1978; van der Meulen et al., 2015).

d. Pancreatic polypeptide-secreting PP-cells

Representing less than 5 and 1% of human and mouse total endocrine cells respectively, PP-cells are more abundant in the islets located in head region of the pancreas (Baetens et al., 1979; Gingerich et al., 1978). PP-cells secrete pancreatic polypeptide (PP), a peptide of 36 amino acids and a member of the neuropeptide Y

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(NPY) family (Holzer et al., 2012). Pancreatic polypeptide is initially synthesized as a precursor named pre-pancreatic polypeptide before further processing to produce the hormone (Boel et al., 1984; Leiter et al., 1984).

Principally secreted postprandially (Batterham et al., 2003), PP is involved in gastrointestinal motility, food intake, energy metabolism and gastric secretions (Asakawa et al., 2003; Katsuura et al., 2002; Lin et al., 1977). The precise role of PP in the pancreas is not yet evident, although its implication in the regulation of pancreatic exocrine secretions is well documented (Lonovics et al., 1981; Louie et al., 1985; Putnam et al., 1989). Moreover, it has been suggested a role for PP in the stimulation of insulin secretion via the inhibition of somatostatin release (Kim et al., 2014).

e. Ghrelin-secreting ϵ -cells

Pancreatic ϵ -cells are abundantly present in the fetal pancreas (Rindi et al., 2002; Wierup et al., 2004) but constitute less than 1% of the endocrine cells in adult human and mouse islets (Andralojc et al., 2009; Dolensek et al., 2015). Ghrelin, the hormone secreted by the pancreatic ϵ -cells, is a 28 amino acid peptide resulting from two successive cleavages of the precursor pre-proghrelin and is mainly produced and secreted by gastrointestinal cells upon food intake (Cummings et al., 2001). This hormone is involved in numerous physiological processes, such as regulation of appetite, body weight, gastrointestinal motility, gastric secretions and control of anxiety (Müller et al., 2015).

The role of ghrelin in the pancreas and on insulin secretion is still debated as it has been shown to stimulate or suppress insulin release depending on its plasma

concentration (Granata et al., 2010; Salehi et al., 2004). Subject to controversy is the hypothesis that ghrelin could influence somatostatin, glucagon and PP secretion (Arosio et al., 2003; Egido et al., 2002; Salehi et al., 2004). However, a recent report suggested a role of ghrelin in potentiating glucose-stimulated somatostatin secretion in mouse and human islets (DiGruccio et al., 2016).

III. Mouse pancreatic development

Our current knowledge of pancreatic development mostly results from information gleaned using loss- and/or gain-of-function mouse models and genetic lineage-tracing experiments. It is important to mention that, while sharing similarities, rodent and human pancreas development also show divergences (Sarkar et al., 2008). Pancreas development is a highly complex process, requiring diverse interactions between tissues, genes and soluble factors. An elegant approach using whole-mount immunofluorescence experiments and global gene expression analyses on endodermal domains suggested that pancreas organogenesis is mostly dependent on combinations of large cohort of transcription factors (TFs) and signaling pathways (Sherwood et al., 2009). Of note, epigenetic modifications collaborate with transcription factors to determine pancreatic cell fates (Haumaitre et al., 2008; Xu et al., 2011), although this aspect will not be discussed in this manuscript.

Here, we will describe key events and TFs involved in mouse pancreas organogenesis.

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i. **Early development**

a. **Endoderm patterning**

During embryogenesis, following fertilization, the zygote undergoes multiple cycles of mitotic divisions until the formation of the blastocyst. The blastocyst will implant into the uterine wall at which point the process of gastrulation begins. In vertebrates, gastrulation leads to the formation of three germ layers identified as ectoderm, mesoderm and endoderm, each layer giving rise to distinct organs. In mice, at embryonic day (E) 6-7.5, the endoderm initially appears as a flat single-cell layer epithelium which will then, 2 days later (E8.5), internalize and fold to form the primitive gut tube.

b. **Gut tube patterning and pancreas budding**

Upon stimulation by soluble factors originating from the adjacent germ layers, particularly the mesoderm, the primitive gut tube will be regionalized into different segments along the anterior-posterior and dorsal-ventral axes, later giving rise to all endoderm-derived organs (Zorn and Wells, 2009 - **Figure 6**). This induction appears to be directed by numerous factors, in particular fibroblast growth factor (FGF)-4 (Bayha et al., 2009; Hebrok, 2003; Wells and Melton, 2000).

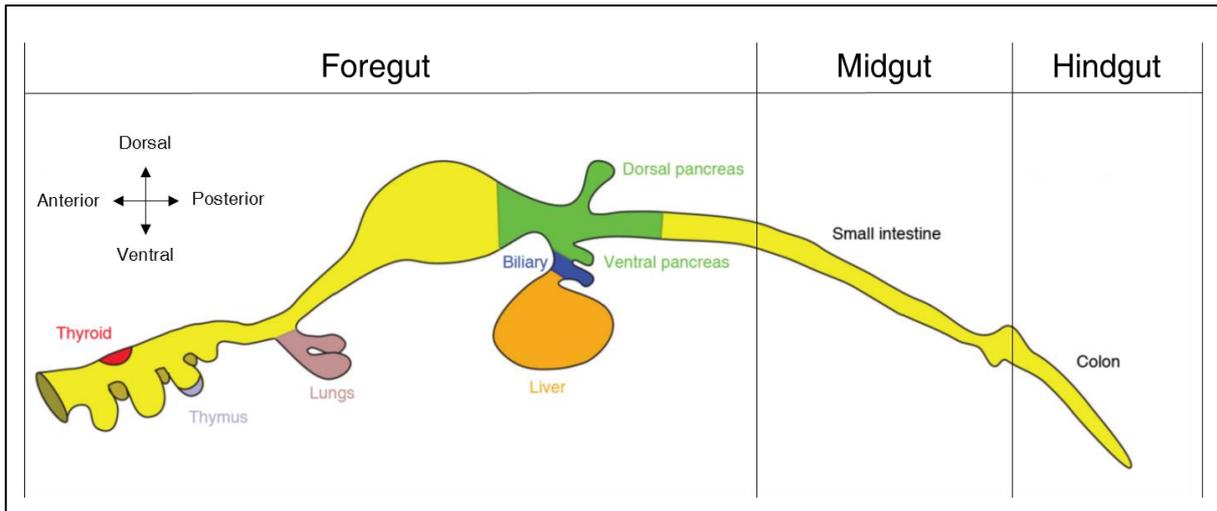


Figure 6: Gut tube patterning and endoderm-derived organs (adapted from Zorn and Wells, 2009). Schematic representation of the gut tube of a mouse embryo at E10.5. The gut tube is divided into Foregut, Midgut and Hindgut segments through anterior-posterior and dorsal-ventral axes and will give rise to digestive and respiratory tracts and associated organs.

In vertebrates, the pancreas emerges from ventral and dorsal domains of the foregut endoderm (E9.5) (Slack, 1995). As a result of their localization along the gut tube, the two buds are not surrounded by the same tissues and are therefore subjected to different signals (**Figure 7**). The dorsal outgrowth of the pancreas receive permissive signals from the notochord and the dorsal aorta (M. Hebrok et al., 1998), whereas the ventral bud is subjected to cardiac mesoderm and septum transversum mesenchyme signals (Kumar et al., 2003).

Signals from the notochord, such as FGF, activin ligands and retinoic acid (RA), direct the adjacent endoderm to develop into the pancreatic dorsal bud (Martín et al., 2005), by repressing sonic hedgehog (Shh) expression (Kim et al., 1997), an activator of hedgehog (Hh) pathway (**Figure 7A**). Repression of the signaling factor Shh results in the expression of *Pdx1* (Hebrok et al., 1998), a key regulator of pancreas development. In addition, FGFs and BMPs signals from the cardiac

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mesoderm and the septum transversum mesenchyme are required for *Pdx1* expression and ventral pancreatic bud specification (Kumar et al., 2003; Wandzioch and Zaret, 2009 - **Figure 7B**).

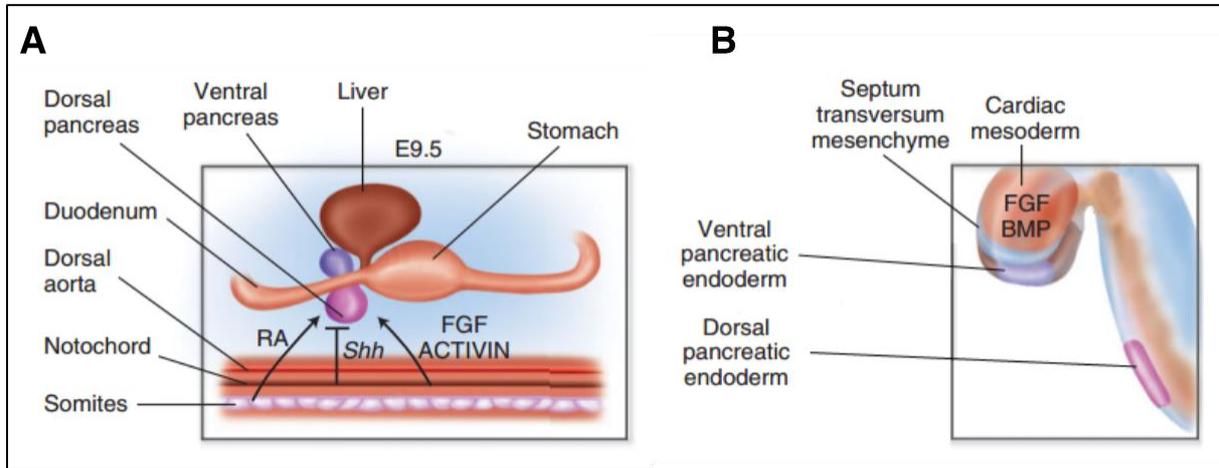


Figure 7: Ventral and dorsal pancreas specification (adapted from Mastracci and Sussel, 2012). Most signals received by the dorsal pancreas originate from the notochord (A), while the ventral pancreas is subjected to septum transversum mesenchyme and cardiac mesoderm signals (B).

ii. Pancreas specification

Ventral and dorsal buds are morphologically detectable around E9.5 in mice. However, as early as E8.5 and prior the outgrowth of these two primordia, the ventral and dorsal prepancreatic regions are specified. Indeed, Wessells and Cohen demonstrated the ability of cells in the foregut endoderm to differentiate into all pancreatic lineages (Wessells and Cohen, 1967). It has also been suggested that such prepancreatic epithelium contains multipotent pancreatic progenitors (MPCs) able to differentiate into all pancreatic cells (Gu et al., 2002). Of note, pancreatic anlage specification is a dynamic process involving several transcription factors acting in coordination (**Figure 9**).

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Pdx1 (pancreatic and duodenal homeobox 1) is a key regulator of pancreas development, its expression in progenitor cells (at E8.5) driving the differentiation of all exocrine and endocrine pancreatic cells. Mice lacking *Pdx1* display bud formation but pancreas agenesis, demonstrating that *Pdx1* is necessary for bud growth but not for their initial induction (Jonsson et al., 1994; Offield et al., 1996; Stoffers et al., 1997), suggesting that pancreatic development is initiated before *Pdx1* expression. Motor neuron and pancreas homeobox 1 (*Mnx1*), also known as Homeobox HB9 (*HLXB9*) precedes *Pdx1* in the dorsal pancreatic endoderm as it is expressed on embryonic day 8 (Harrison et al., 1999; Li et al., 1999). Its expression is necessary for dorsal bud induction, as *HLXB9*-deficient mice displayed dorsal lobe agenesis due to a lack of *Pdx1* induction. In addition to *Pdx1* and *Mnx1* expression, early pancreatic specification requires numerous additional transcription factors, including *Ptf1a* (Burlison et al., 2008; Kawaguchi et al., 2002; Krapp et al., 1998), *Sox9* (Seymour et al., 2007) and *Hnf1 β* (De Vas et al., 2015; Haumaitre et al., 2005). Indeed, the mice deficient for each of these genes display varying degrees of pancreas hypoplasia or agenesis.

Importantly, mouse pancreas organogenesis is separated into two major waves : a primary and a secondary transitions (Pictet et al., 1972; Rutter et al., 1968).

a. Primary transition

The primary transition begins around E9.5 with the thickening of the foregut endoderm, which mainly contains MPCs. The shift from a monolayer to a multilayer

epithelium induces the invasion of the surrounding mesenchyme and the formation of morphologically detectable ventral and dorsal buds (Pictet et al., 1972 - **Figure 8A**).

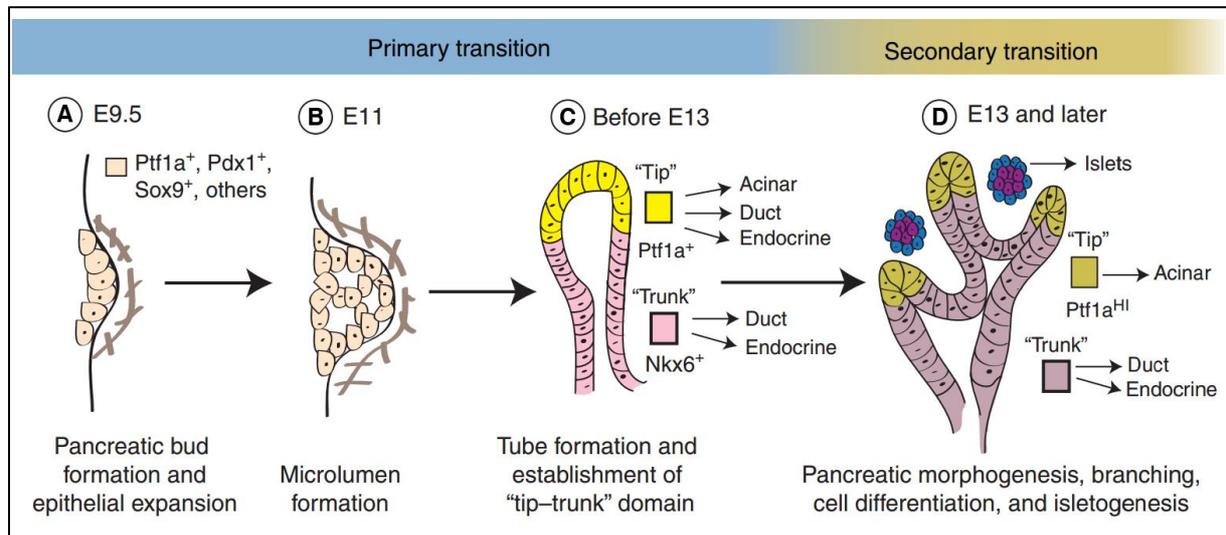


Figure 8: Primary and secondary transitions (from Benitez et al., 2012). Mouse pancreas development is characterized by two overlapping transitions from E9.5 to birth. MPCs proliferation induces the thickening of the foregut endoderm therefore leading to pancreatic bud emergence(A) and microlumen formation (B). The onset of the secondary transition is marked by the segregation of two domains known as tip/trunk compartmentalization with Ptf1a and Nkx6.1 as key regulators (C). The bipotent “trunk” cells express Nkx6.1 and will give rise to endocrine and ductal cells while the multipotent “tip” domain contains MPCs capable of differentiating into acinar, ductal and endocrine cells, quickly restricted to acinar progenitors (D).

By E10.5, both ventral and dorsal epithelium undergo extensive growth and active proliferation of the MPCs, inducing epithelium stratification further resulting in the formation of microlumens (Villasenor et al., 2010 - **Figure 8B**). The following coalescence of these microlumens and branching morphogenesis will give rise to a large tubular network. At E12.5, as the gut rotates, the ventral and the dorsal bud fuse into a single organ (Jørgensen et al., 2007). Following buds fusion (by E12.5), the pancreatic epithelium undergoes dramatic morphogenetic reorganization, known as tip/trunk compartmentalization, leading to the formation of two domains (**Figure**

8C). Of note, Notch signaling appears to play a key role in the tip/trunk segregation (Qu et al., 2013).

b. Secondary transition

The secondary transition begins around E12.5-E13.5 and is marked by an extensive reorganization due to a massive growth and branching of the pancreatic epithelium, concomitant to a major wave of endocrine and exocrine cell differentiation.

The bipotent “trunk” region contains cells that will give rise to endocrine and ductal cells while the multipotent “tip” domain contains MPCs capable of differentiating into acinar, ductal and endocrine cells. The multipotent tip progenitors undergo a developmental switch at around E13-E14 and are then restricted to acinar progenitors (Zhou et al., 2007). The specification of the different cell fates is driven by numerous genes and transcription factors, with *Ptf1a* and *Nkx6.1* as key regulators of tip/trunk segregation (**Figure 8D**). *Ptf1a* is expressed in the tip cells while *Nkx6.1* is exclusively expressed in trunk bipotent cells. Importantly, these two transcription factors mutually inhibit each other, favoring one or the other domain. It has been suggested that *Ptf1a* represses *Nkx6.1* to block endocrine differentiation at the expense of acinar development (Schaffer et al., 2010).

iii. Exocrine specification

a. Acinar cell fate

Acinar cells arise from MPCs located in the tip cells of the pancreatic epithelium. This differentiation occurs between E14.5 and E15.5 in mice and acini are morphologically visible by E15.5.

Acinar cell specification is regulated by several transcription factors (**Figure 9**), including *Ptf1a* and *Nr5a2* (Esni, 2004; Rose et al., 2001). As previously mentioned, *Ptf1a*-null mice display a pancreas agenesis. Interestingly, a total loss of the exocrine tissue is observed in these mice, while few endocrine cells remain (Krapp et al., 1996). In addition, the loss of *Nr5a2* in adult mice results in impaired exocrine enzymes production and secretion (Holmstrom et al., 2011). Moreover, *Mist1* is required for acinar fate maintenance and complete differentiation. Although *Mist1*-deficient mice display acinar cells, these cells present defective apical-basal polarity leading to a massive disorganization of the exocrine tissue (Direnzo et al., 2012; Pin et al., 2001). Interestingly, carboxypeptidase (*Cpa*) is expressed by the forming acini at E12.5 and is considered to be an early exocrine marker (Jørgensen et al., 2007). Finally, recent studies described *c-Myc* as another acinar differentiation factor, as its inactivation in pancreatic progenitors leads to acinar cell hypoplasia (Bonal et al., 2009; Nakhai et al., 2008).

b. Ductal cell fate

The mechanisms involved in ductal cell fate specification are poorly understood. However, as previously mentioned, the “trunk” region contains bipotent

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cells able to give rise to endocrine and ductal cells. It has been suggested that the switch that induces trunk cells to derive into duct cells is mostly driven by Notch pathway (Figure 9).

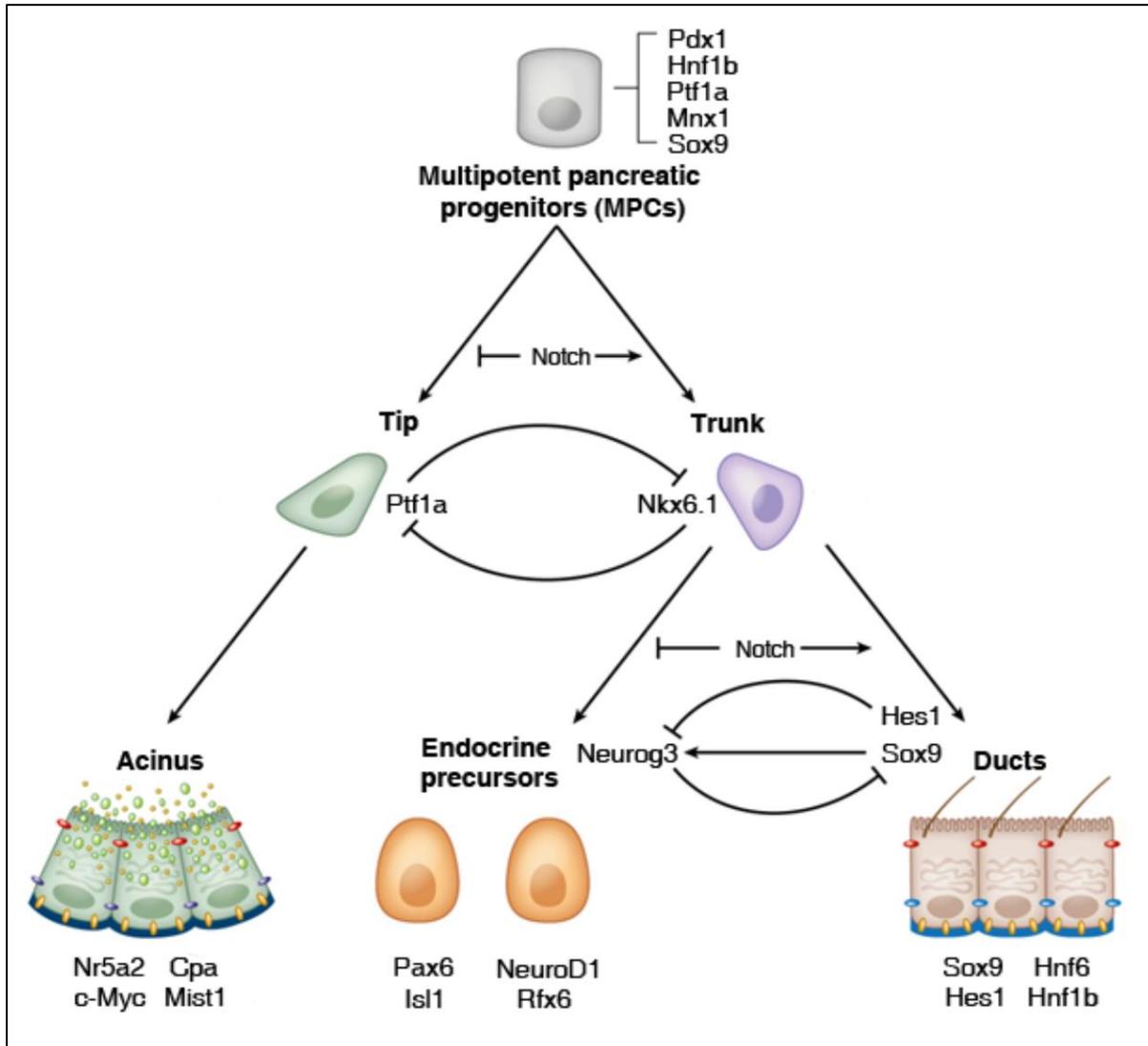


Figure 9: Simplified model of pancreatic cell fate determination (adapted from Shih et al., 2013). Multipotent pancreatic progenitors (MPCs) located in the prepancreatic epithelium express a set of TFs, key regulators of pancreas development (including *Pdx1*, *Hnf1 β* , *Ptf1a*, *Mnx1* and *Sox9*). At the onset of the secondary transition, the tip/trunk segregation appears dependent on the Notch signaling pathway. The specification of the acinar, ductal and endocrine cell lineages is governed by additional TFs, acting in synergy and described in the main text.

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The activation of the Notch signaling pathway induces the expression of a target gene *Hes1*. Importantly, *Hes1* has been found to act as a repressor of the pro-endocrine gene *Neurogenin 3 (Neurog3)*. Indeed, *Neurog3*-deficiency is proposed to induce ductal cell specification (Jensen et al., 2000; Magenheim et al., 2011) while *Hes1* mutant mice display an augmented differentiation of endocrine cells (Jørgensen et al., 2007). Moreover, the ductal epithelium expresses *Sox9* (Delous et al., 2012), *Hnf6* (Pierreux et al., 2006) and *Hnf1 β* (De Vas et al., 2015), these transcription factors forming a transcriptional network that has been involved in duct cell specification. Of note, the deletion of the corresponding genes leads to the impairment of duct morphogenesis.

iv. Endocrine specification

Endocrine allocation and maintenance of the different endocrine cell lineages requires the sequential and stage-dependent activation or repression of specific genes. Indeed, endocrine cell specification is controlled by a network of transcription factors acting in specific combinations (**Figure 9**). The master gene involved in endocrine fate determination is *Neurog3*. *Neurog3* is a proendocrine gene required for the development of all the endocrine cells, as *Neurog3*-deficient mice do not display any hormone-expressing endocrine cells (Gradwohl et al., 2000). Both activation and repression of *Neurog3* expression have been suggested to be dependent on Notch signaling. A recent study demonstrated that high Notch activity induces the expressions of *Hes1* and *Sox9*, leading to *Neurog3* inhibition and the subsequent blockade of endocrine differentiation. However, intermediate Notch

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activity leads to the expression of *Sox9* alone and the derepression of *Neurog3* expression (Shih et al., 2012).

Neurog3 expression begins at E8.5 and peaks at E15.5, corresponding to the major wave of endocrine cell differentiation with little or no of this gene in hormones-expressing cells (Schwitzgebel et al., 2000). Previous reports demonstrated that *Neurog3* initiates endocrine specification but acts upstream of numerous transcription factors important for endocrine cell differentiation/maintenance. Indeed, using gene-deficient mouse models and further genetic analyses, it has been shown that *Insm1*, *Rfx6*, *Isl1*, *NeuroD1*, *Pax6*, *Myt1* are required for proper endocrine-cell differentiation. The deletion of one of these gene impact in different manner final islet-cell differentiation (Ahlgren et al., 1997; Mellitzer et al., 2006; Naya et al., 1997; Sander et al., 1997; Smith et al., 2010; Soyer et al., 2010; Wang et al., 2008 - **Figure 9**).

v. Differentiation of endocrine cells

As previously mentioned, *Neurog3*-expressing cells generate all the five endocrine cell types: α -, β -, δ -, PP- and ϵ -cells. While early expression of *Neurog3* exclusively gives rise to α -cells (E8.5), ϵ -, β -, PP- and δ -cells appear at E10.5, E11.5, E12.5 and E14.5 respectively (Johansson et al., 2007; Prado et al., 2004). Endocrine cells subsequently migrate and emerge from the ductal epithelium and aggregate to eventually form the islets of Langerhans around E18.5 (Bouwens and De Blay, 1996).

The specification of the different hormone-secreting cells largely depends on the sequential activation/repression of TFs. Importantly, these TFs control the

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differentiation, as well as the maintenance and the function of endocrine cell subtypes.

Here we will focus on α -, β - and δ -cell determination/maintenance.

a. α -cells – glucagon

Arx (aristaless-related homeobox) plays a key role in the determination of the α - and PP-cell lineages, later being restricted to mature glucagon-expressing cells where it is involved in maintaining their identity. Indeed, *Arx* mutant mice display severe hypoglycemia caused by a loss of α -cells associated with a proportional increase in β - and δ -cell counts (Collombat et al., 2003).

Although *Pax6*, *NeuroD1* and *Nkx2.2* are not specific to α -cells, their deletion induces glucagon-expressing cell deficiency (Naya et al., 1997; Sander et al., 1997; Sussel et al., 1998). *Brn4* (also known as POU3F4) is exclusively expressed by α -cells. Although not necessary for α -cell development, *Brn4* acts as a transcriptional regulator of glucagon expression (Heller et al., 2004; Hussain et al., 1997).

b. β -cells – insulin

A number of TFs involved in early pancreatic development are also found to be important in β -cell identity maintenance.

Pax4 is critical for β -cell determination and is exclusively expressed in β -cells in the adult pancreas. Accordingly, *Pax4*-deficient mice develop severe hyperglycemia and die shortly after birth. Analysis of their pancreas revealed the absence of β - and δ -cells, concomitant with an increase in α -cell numbers (Sosa-

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Pineda et al., 1997). Together with *Pax4*, *Pdx1* and *Nkx6.1* are major β -cell determinants. Indeed, β -cell-specific inactivation of *Nkx6.1* or *Pdx1* leads to their reprogramming into δ - and α -cells (Gao et al., 2014; Schaffer et al., 2013). Moreover, the loss of one allele of *Pdx1* results in impaired β -cell function and glucose intolerance (Brissova et al., 2002).

MafA is expressed exclusively in β -cells and regulates the expression of genes involved in insulin synthesis and secretion. Its deletion causes impaired insulin secretion and abnormal islet architecture (Olbrot et al., 2002; Zhang et al., 2005). Interestingly, *MafA* was found to regulate insulin gene expression together with *Pdx1* and *NeuroD1* (Zhao et al., 2005). While not expressed exclusively in β -cells, *Nkx2.2* plays a role in the differentiation and maturation of insulin-producing cells, as its deletion induces severe hyperglycemia in mice (Sussel et al., 1998). Similarly, *NeuroD1* and *Pax6* appear to be involved in establishing and maintaining β -cells as their loss results in reduced β -cell numbers (Gu et al., 2010; Naya et al., 1997; Sander et al., 1997).

c. δ -cells – somatostatin

Somatostatin-producing δ -cell specification during pancreas development is not well understood, mostly due to a lack of identification of δ -cell-specific transcription factors.

As previously mentioned, total depletion of *Pax4* expression leads to an absence δ -cells (Sosa-Pineda et al., 1997). Conversely, Ganon et al. observed increased numbers of δ -cells after specific the deletion of *Pdx1* in β -cells, such supplementary somatostatin-expressing cells not arising from the conversion of

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Pdx1-null- β -cells. The authors therefore suggested that the increased δ -cell number is due to either their augmented proliferation or the reallocation of endocrine progenitors toward a δ -cell fate (Gannon et al., 2008).

In addition, an increase in somatostatin-positive cells was observed in mice deficient for *Arx* (Collombat et al., 2003), as well as in *Arx/Pax4* double mutant mice (Collombat et al., 2005). Moreover, pancreata of mice lacking *Arx* and *Nkx2.2* displayed an increase in δ -cell numbers, a significant number of such cells expressing the hormone ghrelin (Kordowich et al., 2011). These studies strongly suggest an antagonist effect of *Arx* on somatostatin-expressing cell development.

The first transcription factor involved in pancreatic somatostatin-cell development has been recently characterized. In addition to its critical role in ventral pancreas organogenesis (Bort et al., 2004), it has been shown that *Hhex* is essential for the differentiation and the maintenance of δ -cells (Zhang et al., 2014). In this elegant study, *Hhex* deficiency in mouse pancreas was found to induce a massive loss of δ -cell numbers concomitant with a decrease in somatostatin secretion. Interestingly, a recent study showed a β -to- δ cell conversion upon the inactivation of *Mnx1* in endocrine precursors. The authors further suggested that *Mnx1* acts as a repressor of δ -cell specification through the direct or indirect inhibition of *Hhex* expression (Pan et al., 2015).

Finally, α -endosulfine, *PCSK9* and *Ptch1* receptor were found to be specifically expressed in δ -cells in rat-, human- and mouse-islets; but no investigation concerning their role in δ -cell fate specification has been undertaken (Grieco et al., 2011; Gros et al., 2002; Langhi et al., 2009).

IV. T1DM actual therapies & current research

As previously mentioned, diabetes patients are exposed to various cardiovascular complications due to chronic increased blood glucose levels and are at risk of dying prematurely. Thereby, doctors and researchers are acting collectively in an attempt to manage or even reverse this hyperglycemia.

i. Management of T1DM

The priority in the management of T1DM is to monitor blood glucose levels to strictly maintain glycemia within normal ranges (**Table 1**), thereby preventing hyperglycemia- and hypoglycemia-associated complications.

a. Exogenous insulin administration

To compensate the inability of the pancreas to secrete insulin, type 1 diabetic patients are dependent on exogenous insulin administration, such compensation being indispensable for their survival. Although efficient, chronic insulin administration through subcutaneous daily injections results in difficulties to strictly regulate glycemic levels (due to fluctuations in diet, physical exercises or age) and induces glycemic variability (alternations between hyper- and hypoglycemia) (Group., 1993). The more recent implementation of subcutaneous pumps allows sustained delivery of insulin and appears to be more efficient at maintaining normoglycemia (Shetty and Wolpert, 2010).

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Another or an additive strategy involves the use of a combination of different types of insulin with distinct timing of actions in lowering glucose levels. Mixed preparations with long-, regular or short-acting and rapid-acting insulin can be used for an improved delivery of insulin and therefore glycemic regulation. Long-acting insulin acts several hours after injection and for a period of 24 hours while regular or short-acting insulin acts within 30 minutes and is effective for 3 to 6 hours. Finally, rapid-acting insulin requires only 15 min to lower blood glucose levels and is efficient for up to 4 hours (McCall and Farhy, 2013). Such preparations afford a better glycemic control, depending on the need and eating habits of patients, and can be used through injections or via pumps.

Recently developed, the artificial pancreas (AP) is based on a closed-loop control system consisting of a continuous subcutaneous glucose sensor and insulin pump. This allows constant glucose-monitoring and insulin delivery. Although not yet available, clinical trials demonstrate that this approach offers significant advantages as it manages blood glucose levels with less variations and presents less daily stress for the patients (Kovatchev et al., 2016; Kropff et al., 2015). However, hypoglycemia remains a risk that should be dealt prior to a putative application.

b. Total pancreas and islets transplantation

As previously mentioned, exogenous insulin administration represents an imperfect treatment for diabetes due to its inability to finely regulate blood glucose levels. Thus, two alternatives aiming at replacing the damaged β -cell mass in diabetic patients exist: whole pancreas or islets transplantation. Whole pancreas transplantation often also includes kidney transplants, as the organs could have been

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damaged due to hyperglycemia-associated complications. However, pancreas transplantation is a heavy burden for diabetic patients, which is why islets transplantation has been favored for the past decade. The Edmonton protocol (Shapiro et al., 2000) has played a key role in islet transplantation from cadaveric donors. It is of importance to note that these grafts involve lifelong immunosuppressant treatment to avoid rejection and new β -cell destruction, such treatment rendering the patients susceptible to other infections.

Nonetheless, total pancreas or islet transplantation normally result in a rapid glycemia normalization and exogenous insulin independence for up to 5 years, further improving the quality of life of the patients (Domínguez-Bendala et al., 2016; Sureshkumar et al., 2006). However, these medical procedures are mostly used for type 1 diabetic patients with serious complications.

Although efficient, these therapies face the shortage of organ donors and the associated side-effects of immunosuppressive drugs (Bruni et al., 2014).

ii. **Current axes of research**

Despite the help offered by the different strategies available to physiologically mimic the action of insulin, the difficulties in strictly regulating glycemic levels, discomfort due to daily injections or pump replacement and financial limitations, have encourage researchers to investigate alternatives. Consequently, current research focuses on the replacement of the injured- β -cell-mass in diabetic patients using several approaches and cell sources. The common goal of these diverse strategies is to generate cells synthetizing and secreting appropriate amounts of insulin under physiological conditions (**Figure 10**).

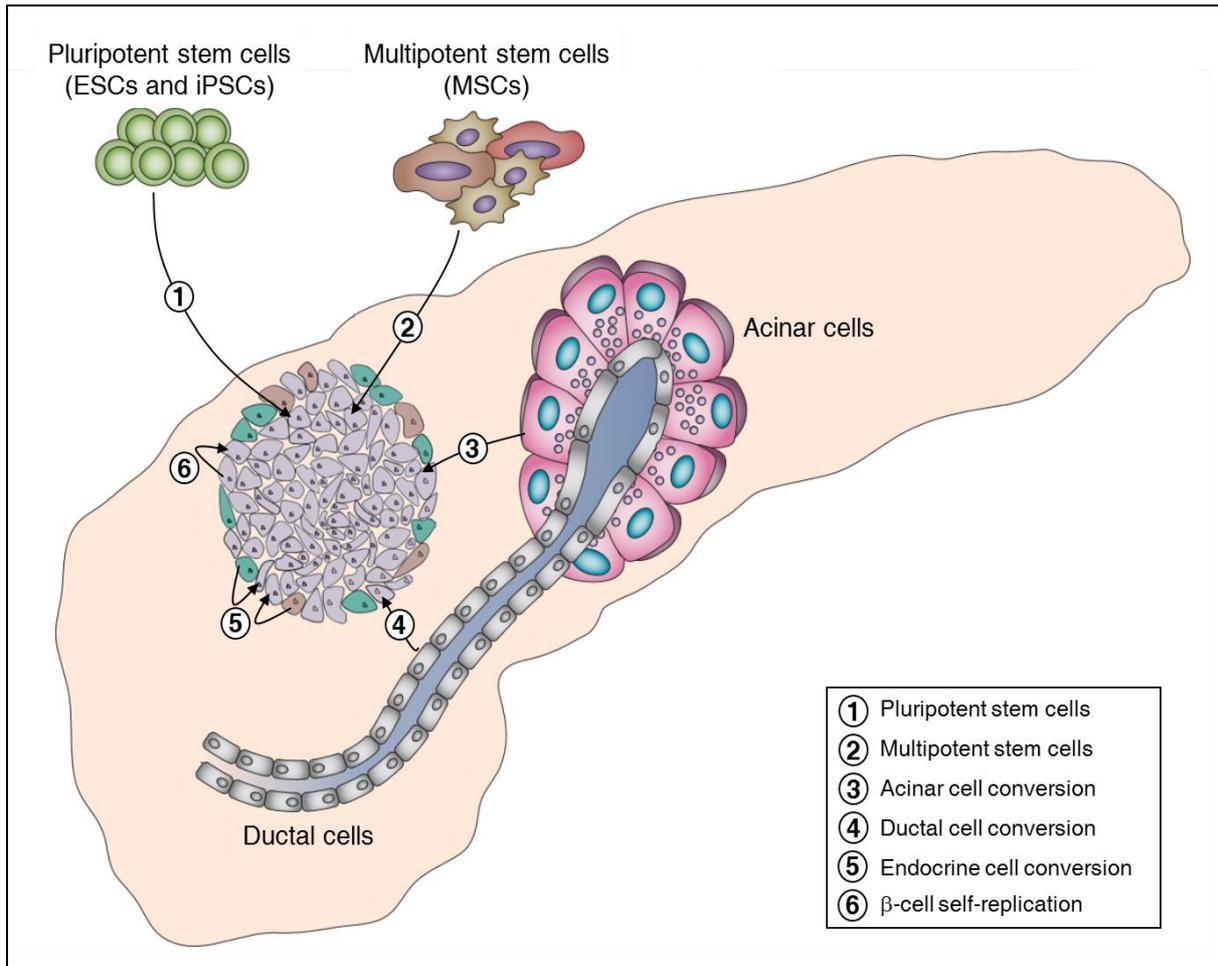


Figure 10: Cell sources used in the replacement of β -cells (adapted from Bouwens et al., 2013). Able of long-term self-renewal, pluripotent (1) or multipotent stem cells (2) are used for directed differentiation into insulin-secreting cells. Multiple strategies using endogenous pancreatic cells for β -cell replacement involve acinar (3), duct (4) or endocrine cell conversion (5) or β -cell self-replication (6).

a. β -cell mass replacement from stem cells

With their capacity of long-term self-renewal while maintained in an undifferentiated state and their pluripotency (able to differentiate into progenitors of the three germ layers: ectoderm, mesoderm et endoderm - Thomson, 1998), stem cells are widely used in regenerative research (Colman and Dreesen, 2009).

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Several sources of stem cells exist and are used in diabetes research for directed differentiation into insulin-secreting cells *in vitro* and subsequently *in vivo*.

1) Pluripotent stem cells (PSCs)

Stem cells are pluripotent, thus, under specific conditions, they are able to specialize into progenitors of almost all cell lineages.

Embryonic stem cells (ESCs) are isolated from the inner cell mass (ICM) of a developing blastocyst in early embryogenesis. The induction of their differentiation into pancreatic progenitors and insulin-secreting cells is mainly based on developmental studies and therefore aims at using strategies to modulate signaling pathways and genes involved in pancreatic development. Numerous studies have reported the differentiation of ESCs into insulin-producing cells. In 2000, Soria *et al.* reported the restoration of chemically-induced diabetes by the injection of genetically reprogrammed ESCs into β -cells in mice. The construct used allowed the expression of an antibiotic resistance gene under the control of human insulin gene (Soria *et al.*, 2000). Later, D'Amour *et al.* derived human embryonic stem cells (hESCs) into pancreatic progenitors after a five-step protocol using sequential exposure of various inducing agents and growth factors (D'Amour *et al.*, 2006). Although this study was the first demonstration of human-derived- β -cells, the resulting insulin-secreting cells were glucose unresponsive. Later, the optimization of the previous protocol resulted in hESC-derived-insulin-producing cells able to respond to glucose following their transplantation and further differentiation in mice (Kroon *et al.*, 2008). However, all these protocols led to cell producing too low amounts of secreted insulin mostly due to incomplete maturation. Recent studies have developed protocols that have had

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more success. Indeed, Rezanian *et al* published a seven-stage protocol leading to the differentiation of hESCs into β -like cells, which were responsive to glucose and able to restore chemically-induced diabetes *in vivo* after transplantation into immunodeficient mice (Rezanian *et al.*, 2014). In addition, Pagliuca *et al.* described a promising protocol allowing the production of large numbers of insulin-secreting cells from human pluripotent stem cells, such cells preventing hyperglycemia following transplantation into Akita immunodeficient mice (Pagliuca *et al.*, 2014). In 2015, a further improved protocol was published, leading the production of glucose responsive β -cells from hESCs *in vitro* and *in vivo* (Russ *et al.*, 2015).

Although representing a promising therapy with clinical trials currently ongoing (Viacyte, Inc.), the use of ESCs faces several obstacles. Indeed, the use of hESCs requires embryos at the blastocyst stage to create ESC lines, therefore leading to ethical concerns with the use of human embryonic cells (De Wert and Mummery, 2003; McLaren, 2001). Another barrier is that transplanted exogenous ESCs can trigger host immune rejection, thus requiring lifelong immunosuppressive treatments.

Induced pluripotent stem cells (iPSCs) represent a second type of PSCs, exhibiting similar properties to ESCs. They are obtained from the reprogramming of somatic cells into pluripotent cells able to self-renew. By expressing 4 specific transcription factors, *Oct4*, *Sox2*, *Klf4* and *c-Myc*, fibroblasts were reprogrammed into pluripotent stem cells (Takahashi and Yamanaka, 2006; Yu *et al.*, 2007). Later, iPSCs were obtained by reprogramming other cell types, such as neuronal progenitor cells (Kim *et al.*, 2008), keratinocytes (Aasen *et al.*, 2008), hepatocytes and gastric epithelial cells (Aoi *et al.*, 2008). Recently, pluripotent stem cells were induced from mouse somatic cells using chemicals that could improve reprogramming efficiency or replace one or all previously mentioned transcription factors (Bar-Nur *et al.*, 2014;

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Hou et al., 2013; Ye et al., 2016). Using different strategies, such as chemically-induced differentiation, iPSCs can be directed to differentiate into any cell type including β -cells (Alipio et al., 2010; Zhang et al., 2009). The use of iPSCs in diabetes therapy therefore presents advantages when compared to hESCs. Indeed, the reprogrammed cells avoid ethical issues of hESCs and graft rejection as they are obtained from the differentiation of adult patients somatic cells.

Although promising, the use of embryonic stem cells and induced pluripotent stem cells in diabetes therapy still needs further investigation. It is important to note that ESCs and iPSCs possess oncogenic properties, as undifferentiated or not fully differentiated stem cells transplanted can form teratomas (tumors containing cells from 3 germ layers) (Hentze et al., 2009). In addition, autoimmunity in type 1 diabetic patients can trigger the destruction of newly-implanted β -cells.

2) Tissue stem cells

Tissue stem cells are found in various tissues including bone marrow (Jiang et al., 2002), gastric epithelium (Bjerknes and Cheng, 2002), liver (Overturf et al., 1997), brain (Reynolds and Weiss, 1992), adipose tissue (Zuk et al., 2002) and umbilical cord (Prindull et al., 1978). Most stem cells found in post-natal tissues are multipotent, therefore able to develop into different cell types. Although highly debated, they represent another putative source for β -cell regeneration.

Reports have shown the differentiation of bone marrow stem cells into insulin-producing cells (Ianus et al., 2003; Karnieli et al., 2007), whereas a following study failed to reproduce these findings (Taneera et al., 2006). Stem cells from human umbilical cord were also suggested to be able to derive into islet cells

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(Pessina et al., 2004; Sun et al., 2007). In addition, human adipose tissue stem cells were induced to differentiate into pancreatic endocrine cells (Timper et al., 2006) and into functional insulin-producing cells in mice (Kajiyama et al., 2010). Liver stem cells have also been suggested to possess the ability to differentiate into insulin-secreting cells (Ferber et al., 2000; Yang et al., 2002).

Excluding ethical concerns and risk of immune rejection, the use of tissue stem cells offers promising perspectives for diabetes therapy, although more investigation is required to fully restore β -cell mass function in diabetic patients using these cell sources.

b. β -cell mass replacement from pancreatic cells

With a rising number of reports, reprogramming/transdifferentiation of adult pancreatic cells into functional β -cells is an exciting topic but still highly debated. Even though pancreas plasticity has mostly been described in experimental diabetes models in mice, several approaches including genetic or chemical manipulations, have been used to induce acinar-, ductal- or endocrine-cell conversion into insulin-producing cells. Also, the development of lineage tracing tools has resulted in large progress in this field.

1) From acinar cells

Abundant in the pancreatic tissue, exocrine acinar cells represent more than 95% of the gland, thus they constitute an interesting source for β -cell replacement.

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An elegant approach by Zhou *et al.* demonstrated that adenovirus-mediated ectopic expression of *Pdx1*, *Neurog3* and *MafA* (all three preponderantly involved in pancreatic development, see section III), resulted in the direct conversion of acinar cells into insulin-secreting cells able to improve hyperglycemia in diabetic mice (Zhou *et al.*, 2008). However, these cells were not fully efficient as they did not cluster into organized islets. Another study suggested that cytokine stimulation with EGF (epidermal growth factor) and CNTF (ciliary neurotrophic factor) of chemically-induced diabetic mice induces the restoration of a functional β -cell mass through acinar-to- β -cell conversion via *Neurog3* activation (Baeyens *et al.*, 2013). Interestingly, using genetic manipulation or chemical exposure, recent publications have reproduced this conversion *in vitro* and *ex vivo* using human exocrine cells (Klein *et al.*, 2015; Lemper *et al.*, 2015; Lima *et al.*, 2013). However, the study undertaken by Desai *et al.* invalidates the theory of acinar cells ability to convert into β -like cells in mice, leaving the topic contested (Desai *et al.*, 2007).

2) From ductal cells

As previously discussed (see section III), endocrine cells emerge from the ductal epithelium and aggregate into islets of Langerhans during development. Such observations led to several theories, one suggesting that such endocrine cells could arise from pancreatic progenitors in the ducts (Bonner-Weir *et al.*, 2008; Bonner-Weir *et al.*, 2004). Heimberg and colleagues demonstrated the recruitment of ductal progenitors in the pancreatic injury model of PDL (partial pancreatic duct ligation) in mice. These cells were found able to convert into islet cells, this conversion being mediated by *Neurog3* expression (Xu *et al.*, 2008). Partial pancreatectomy (Ppx) is

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another model of pancreatic injury widely used in pancreatic cell conversion studies. While Lee *et al.* demonstrated that β -cell regeneration does not require the reactivation of *Neurog3* expression in the pancreas after 50% Ppx (Lee et al., 2006), a following study suggested transient reexpression of the pro-endocrine gene only after 90% Ppx (Li et al., 2010). Despite the observed β -cell regeneration, ductal cell plasticity was challenged by two studies affirming that ductal cells can derive into endocrine cells only before birth (Kopp et al., 2011; Solar et al., 2009).

Still controversial and non-confirmed, one recent hypothesis suggests the existence of different ductal cell populations with only a fraction of these able to convert into endocrine cells (Grün et al., 2016).

3) From endocrine cells

The increase of the β -cell mass from the interconversion between the different endocrine cell types or pre-existing β -cells expansion is a widely investigated field.

Aside from specific conditions, such as pregnancy or insulin resistance, islet β -cells are mostly quiescent with a low replication rate of 0.2% per day (Teta et al., 2005). Inducing β -cell self-replication using specific mitogenic factors, such as exendin-4 (Xu et al., 1999) or IGF-1 (Agudo et al., 2008), has been shown to be efficient to increase the β -cell mass. Similar results using human islets demonstrated that the modulation of cell cycle genes in human β -cells (Fiaschi-Taesch et al., 2013) or the use of HGF (hepatocyte growth factor - Beattie et al., 2002) induce their proliferation. An alternative approach used by Cano *et al.* demonstrated the regeneration of the β -cell mass through inactivation of *c-Myc* expression specifically in insulin-expressing cells. The authors showed that this regeneration occurred via

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β -cell replication (Cano et al., 2008). Inducing β -cell self-replication is therefore of interest but unsuitable in advanced stages of T1DM where very few insulin cells remain following autoimmune destruction.

Another potential source of β -cells was revealed with the discovery of α -cell plasticity. Several experimental models contributed to the demonstration of the ability of α -cells to convert into insulin-producing cells. Using transgenic mice expressing the diphtheria toxin receptor (DTR) specifically in insulin-cells, Thorel *et al.* showed that near total β -cell loss forced the transdifferentiation of adult α -cells into insulin-producing cells able to restore normoglycemia (Thorel et al., 2010). Chung et al. also described α -to- β -conversion following the combination of PDL and alloxan-mediated β -cells destruction (Chung et al., 2010). Other approaches involving genetic manipulations demonstrated the ability of α -cells to transdifferentiate into β -like cells upon ectopic expression of *Pdx1* in *Neurog3*-positive cells (Yang et al., 2011) or through epigenetic modifications (Bramswig et al., 2013). Moreover, α -to- β -like cell conversion was demonstrated upon *Pax4* misexpression/*Arx* loss-of-function (further detailed below).

V. *Pax4*-mediated β -cell regeneration

As discussed in section III, *Pax4* is a key transcription factor in specifying and maintaining β -cell identity. It was shown that the ectopic expression of *Pax4* in adult or embryonic α -cells induces their conversion into β -like cells (Al-Hasani et al., 2013; Collombat et al., 2009). Conversely, the specific loss of *Arx* in glucagon-expressing

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cells similarly triggers their conversion into functional insulin-producing cells (Courtney et al., 2013).

Equally important was the finding that the α -to- β -like cell conversion observed in both models, induces the re-expression of *Neurog3* in ductal cells and their differentiation into endocrine cells through the reawakening of the epithelial-to-mesenchymal transition (EMT). The newly formed α -cells being yet again converted into β -like cells upon α -cell-specific *Pax4* misexpression/*Arx* loss-of-function, such a cycle of conversion and neogenesis results in β -like cell hyperplasia over time (**Figure 11**). Importantly, such β -like cells are functional and can revert the consequences of chemically-induced diabetes.

It is worth noting that in these different models, in addition to glucagon-expressing cells regeneration, an increase in the number of somatostatin-expressing δ -like cells was consistently observed (**Figure 11**). Considering the re-expression of the pro-endocrine gene *Neurog3* in the ductal lining of these transgenic mice, one could assume a continuous neogenesis of these somatostatin-expressing cells. Unexpectedly, while being continuously produced, these δ -cells were not found to be accumulated over time.

Based on these observations and considering that δ -cells are closely linked to β -cells during development, as they share the same lineage, we sought to determine whether adult δ -cells could not be induced to adopt a β -like cell identity.

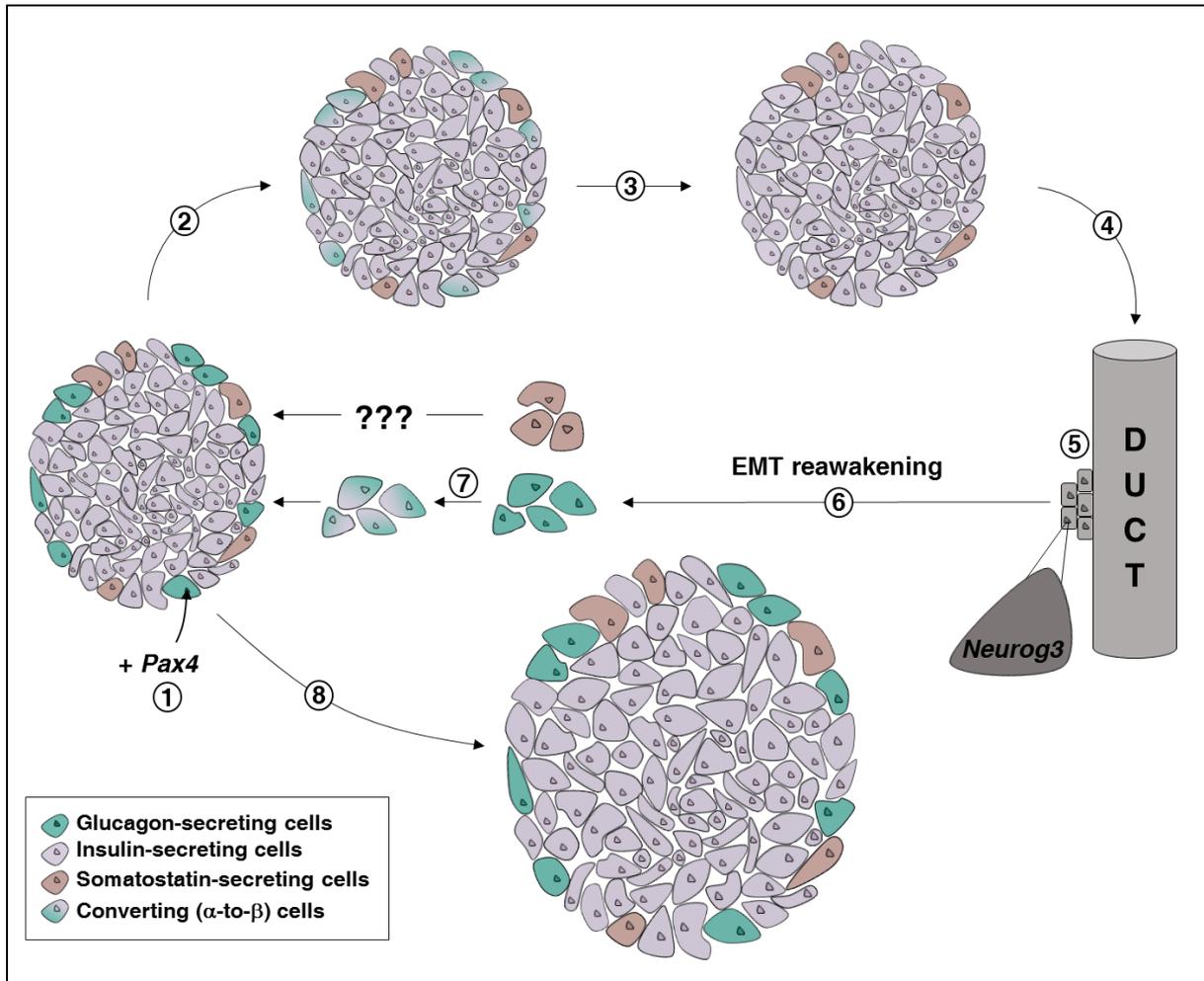


Figure 11: *Pax4* misexpression in adult or embryonic α -cells induce their neogenesis and conversion into β -like cells. Following *Pax4* misexpression in α -cells (1), these can be converted into β -like cells (2,3). This leads to a shortage in glucagon (4) responsible for the mobilization of ductal precursor cells, these re-expressing the pro-endocrine gene *Neurog3* (5), prior to undergo EMT (6) and adopt an endocrine cell identity (7). Such a continuous cycle of conversion/regeneration results in insulin⁺ cell hyperplasia (8).

MATERIAL AND METHODS

I. Animal procedures

All mice were housed on a standard 12:12h light:dark cycle, with standard diet food and water *ad libitum*. Animal care was conducted according to French ethical regulations. This project received the approval from our local ethics comity (NCE/2011-22). 129/Sv wildtype mice were obtained from Taconic laboratories. Experiments were performed on transgenic and control males only. Controls include wildtype mice or transgene-negative littermates.

i. Generation of transgenic mouse line

Using the Cre-LoxP system to investigate δ -cell plasticity in pancreatic islets, we generated Sst-Cre::Pax4-OE double transgenic animals allowing both the conditional expression of *Pax4* in somatostatin⁺ cells and their lineage tracing (**Figure 12** – For details, see the results section). Importantly, Sst-Cre::Pax4-OE transgenic mice were found to be viable, fertile, with no premature death being observed.

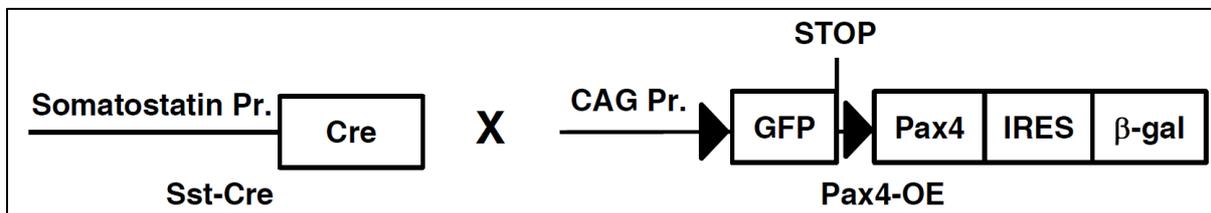


Figure 12: Transgenic mouse line allowing *Pax4* misexpression in somatostatin-expressing cells

Material and Methods

ii. Genotyping

Genotyping was performed on small ear punch biopsies using the KAPA Express DNA Extraction Kit (KK7103, Kapa Biosystems). Briefly, the digestion of each sample was performed with 100 μ L of KAPA Express Extract lysis buffer (10 μ L 10X KAPA Express Extract Buffer; 2.0 μ L 1 U/ μ L KAPA Express Extract Enzyme; 88 μ L PCR-grade water), followed by an incubation in a thermomixer for 10 min at 75°C with shaking at top speed. Heat-inactivation of the thermostable KAPA Express Extract protease was achieved after incubation for 5 min at 95°C with shaking and samples were centrifuged at 14000 rpm for 1 min to pellet debris. The DNA-containing supernatant was transferred to a fresh tube and Polymerase Chain Reaction (PCR) was then used to assess the genotype of our mice.

PCRs were performed by combining in each reaction tube 15 μ L of 2X GoTaq® G2 Hot Start Green Master Mix (M7423, Promega); 0.08 μ L of both forward and reverse primers (**Table 2**) and 13.84 μ L H₂O.

Gene	Forward primer	Reverse primer
Cre	ATGCTTCTGTCCGTTTGCCG	CCTGTTTTGCACGTTACCG
Lac	TTGGCGTAAGTGAAGCGAC	AGCGGCTGATGTTGAACTG

Table 2: Primer sequences used for genotyping

PCR results were analyzed by using the QIAxcel Advanced System (QIAGEN) and the QIAxcel DNA Screening Kit (929004, Qiagen). The presence of the GFP gene was assessed by observation of our mice under blue light while wearing goggles equipped with green filters.

II. Mice treatments

i. Proliferation assessment

5-bromo-2'-deoxyuridine, BrdU, is a synthetic analogue of thymidine which is incorporated into DNA during the S phase (part of the cell cycle when the DNA is replicated). 5-iodo-2'-deoxyuridine (IdU) and 5-chloro-2'-deoxyuridine (Cldu), are modified forms of BrdU. To assess cellular proliferation, animals were treated with IdU, CldU, or BrdU (MP Biomedicals – **Figure 13**) in drinking water for different durations prior to examination (1 mg/mL). The solution was changed every two days and protected from light. IdU-positive cells were detected using a mouse anti-BrdU primary antibody while CldU-positive cells were detected using a rat anti-BrdU antibody.

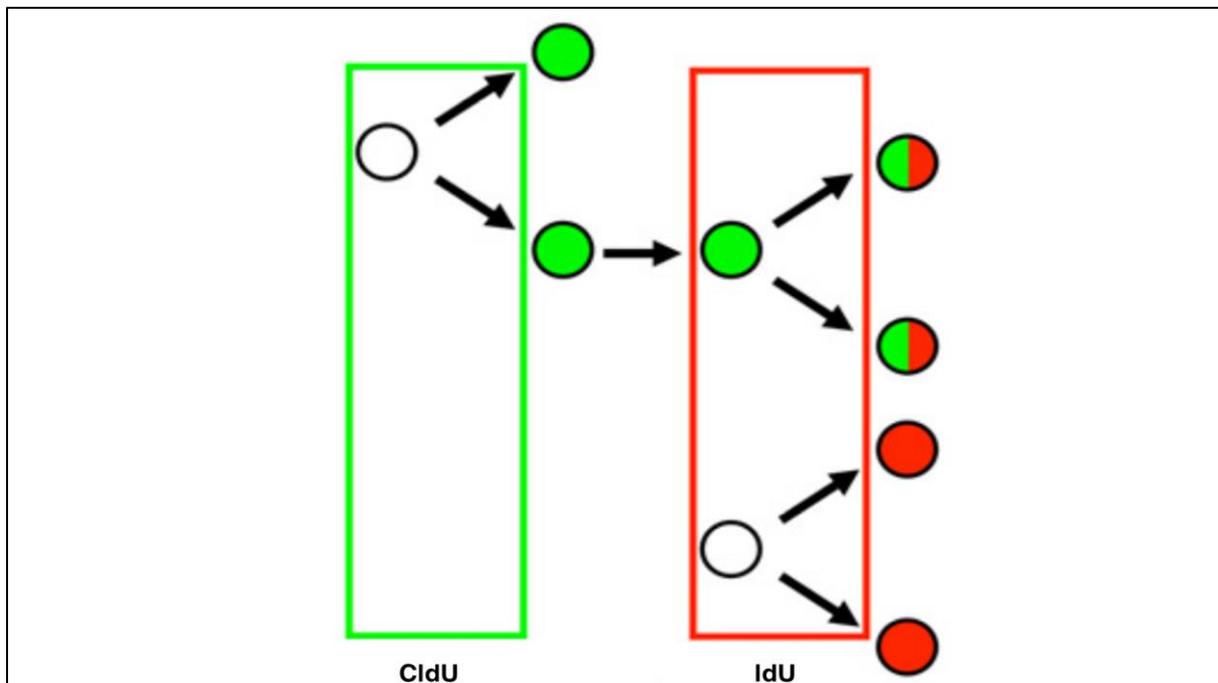


Figure 13: Cldu and IdU labelling. The first cell division is labeled with CldU (here in green) and the second cell division with IdU (here in red). Therefore, sequential cell division results in co-labeled cells with both CldU and IdU (green/red).

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ii. Induction of streptozotocin-mediated hyperglycemia

Streptozotocin is a glucosamine-nitrosourea compound causing DNA damage. Its chemical structure is similar to glucose which allows it to be transported specifically into the β -cell via the glucose transporter GLUT-2. To induce hyperglycemia, streptozotocin, (STZ; S0130, Sigma) was dissolved in 0.1M sodium citrate buffer (pH 4.5), and a single dose was administered intraperitoneally (115 mg/kg) within 10 min after dissolution. Diabetes progression was assessed by monitoring the blood glucose levels and/or survival rates of mice. Glycemia was measured with a ONETOUCH glucometer (Life Scan, Inc., CA).

III. Tolerance tests and blood glucose level measurements

For intraperitoneal glucose tolerance tests (ipGTT) and intraperitoneal insulin tolerance tests (ipITT), animals were fasted for 16-18 hours and injected intraperitoneally with 2g/kg of bodyweight of D-(+)-glucose (G7201, Sigma) or 0.75U/kg of insulin (Novo Nordisk A/S). Blood glucose levels were measured at the indicated time points post-injection with a ONETOUCH glucometer (Life Scan, Inc., CA).

IV. RNA analyses

i. RNA extraction from total pancreas

Mice were euthanized by cervical dislocation and the pancreatic tissue was isolated and placed in cold RNA lysis solution (AM7024, Fisher Scientific) for 48 hours at 4°C. A small piece of pancreas was taken and homogenized in lysis buffer (10µL of β-mercaptoethanol in 10mL of RLT buffer from Qiagen) with a tissue disruptor. Total pancreas RNA was extracted using Rneasy Mini Kit (Qiagen) according to the manufacturer's instructions.

ii. cDNA synthesis

RNA concentration and quality were determined using an Agilent 2100 Bioanalyzer system (Agilent Technologies). 10µL of H₂O containing 1µg of RNA was then processed for first strand synthesis mixing 1µL 10mM dNTP and 1µL 50mM oligodT. This reaction was then incubated 5' at 65°C, then on ice for at least 1 min. For cDNA synthesis, 10µL of DNA synthesis mix (2µL 10x RT buffer; 4µL 25 mM MgCl₂; 2µL 0.1 M DTT; 1µL Rnase out; 1µL Superscript III) was added to each sample and incubated for 50 min at 50°C, 5 min at 85°C then chilled on ice. Finally, 1µL of Rnase H was added and samples were incubated for 20 min at 37°C.

iii. qPCR analyses

Quantitative RT-PCR reactions were carried out with the QuantiTect SYBR Green RT-PCR kit (Roche) and Qiagen-validated primer probes on a LightCycler 480® instrument (Roche Life Science). The program used for the RT-PCR was the following (**Table 3**), the fluorescence being monitored during each amplification PCR cycle. *Neurog3* primer sequences used were the following: 5'-AAACTTCGAAGCGAGCAGAG-3' and 5'-CATCCTGAGGTTGGGAAAAA-3'. The housekeeping gene GAPDH (validated primers from Qiagen) was used as an internal control to normalize transcript expression.

qPCR reactions were performed in tubes containing 5µL 2x SYBR Green Supermix, 0.5µL Primer Assay, 3µL H₂O and 1.5µL of 1/20 diluted cDNA.

Step	Temperature	Time	No of cycle
Pre-Incubation	95°C	10 min	1
Amplification	Denaturation	95°C	10 sec
	Annealing	60°C	45 sec
	Elongation	72°C	1 sec
	Cooling	40°C	10 sec
Melting curves	95°C		1
Cooling	40°C	10 sec	

Table 3: RT PCR program used

V. Pancreatic tissue processing and sectioning

i. Paraffin embedding – Microtome sectioning

Mice were euthanized by cervical dislocation and the pancreatic tissue was isolated and placed in cold PBS. Tissue was fixed for 30 min at 4°C in Antigenfix (paraformaldehyde solution pH 7,2-7,4; Microm Microtech France), washed 5 x 20 min in cold PBS and incubated 1 hour in 0.86% saline. Following dehydration through a series of increasing ethanol dilutions (50%, 70%, 80%, 90%, and 100%), the pancreas was treated with isopropanol and toluene, a hydrophobic agent, to remove alcohol from the tissue. Finally, the organ was incubated in clean paraffin baths (1 hour, 3 hours and overnight) and embedded in paraffin in metal molds on a cold plate. Paraffin blocks were stored at 4°C prior to sectioning.

8µm sections were cut using a rotary microtome (Leica) and placed on polarized glass slides (Fisher Scientific). Paraffin sections were allowed to dry 45 min at 42°C into a heating block and kept at 37°C overnight. Slides were subsequently stored at 4°C until staining.

ii. OCT embedding – Cryostat sectioning

Mice were euthanized by cervical dislocation and the pancreatic tissue was isolated and placed in cold PBS. Tissue was fixed for 30 min at 4°C in a fixative solution (1.35mL 37% paraformaldehyde; 400µL 25% glutaraldehyde; 100µL 10X NP40; 5mL 10X PBS; H₂O to 50mL) and washed 6 x 20 min in cold PBS. To remove the water and protect the tissue from freezing damage, the sample was incubated

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overnight at 4°C in a 25% sucrose solution diluted in PBS. Finally, the organ was washed 2 x 1h in Jung freezing medium (Leica Biosystems) and embedded in plastic molds in Jung freezing medium on dry ice. Cryo blocks were stored at -80°C prior to sectioning.

16µm sections were cut using a Cryostat (Leica) with the following settings: object temperature of -14°C and chamber temperature -19°C. Sections were placed on polarized glass slides (Fisher Scientific) and dried for 45 min at 42°C into a heating block. Slides were subsequently stored at -80°C until staining.

VI. Staining of pancreatic sections

i. Immunohistochemistry (IHC)

Paraffin sections were deparaffinized 3 x 3 min in xylene, rehydrated in decreasing ethanol dilutions (5 min in 2 x 95%; 5 min in 80%; 5 min in 60%; 5 min in 30%), and finally rinsed twice for 5 min in ddH₂O. To reveal epitopes that could have been masked during the tissue preparation, antigen retrieval was performed by boiling the tissue for 1 min at 140°C in 1.6L ddH₂O with 15mL Antigen Unmasking solution (Citric acid based pH6, Vector) in a pressure cooker. Slides were then transferred into tap water for approximately 30 min. Of note, this antigen retrieval step results in the denaturation of GFP, subsequently leading to its quenching.

Tissue sections were then washed 3 x 5 min in PBS, kept in a blocking solution for 45 min at room temperature (PBS-FCS 10% - Gibco) to block any nonspecific binding of the antibodies and incubated overnight in a humid chamber at 4°C with primary antibodies (**Table 4**) diluted in PBS-FCS 10%.

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The samples were rinsed 3 x 5 min in PBS before application of the appropriate secondary antibodies (**Table 5**) for 45 min at room temperature in a humid chamber.

Antigens	Species	Dilutions	Sources
Insulin	Guinea Pig	1:500	DAKO
Glucagon	Rabbit	1:500	R&D systems
Somatostatin	Goat	1:500	Santa Cruz
Glut-2	Rabbit	1:500	Chemicon
PC1/3	Rabbit	1:500	Millipore
Nkx6.1	Rabbit	1:3000	Novo Nordisk
Pdx1	Guinea Pig	1:1000	C. Wright (Vanderbilt)
DBA		1:400	Vector
Pax6	Rabbit	1:500	BioLegend
Pax4	Rabbit	1:500	B. Sosa-Pineda
NeuroD1	Rabbit	1:500	Millipore
β -galactosidase	Rabbit	1:8000	MP-Cappel
Vimentin	Chicken	1:4000	Millipore
BrdU	Mouse	1:50	Roche
BrdU	Rat	1:50	Abcam
Ngn3	Mouse	1:250	DSHB

Table 4: Primary antibodies used for IHC

Finally, slides were washed 3 x 5 min in PBS, dried, and mounted with coverslips and Vectashield HardSet Mounting Medium (Vector) with 4', 6-diamidino-2-phenylindole (DAPI) as a nuclear counterstain.

Slides were kept at 4°C prior to observation. Pictures were processed using ZEISS Axioimager Z1 equipped with the appropriate filter sets and a monochrome camera, with Axiovision software from ZEISS.

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ii. IHC on cryosections

Cryosections were washed from the embedding Jung freezing medium for 3 x 15 min in PBS and blocked in a PBS-FCS 10% blocking solution for 45 min at room temperature in a humid chamber. The sections were then incubated overnight in a humid chamber at 4°C with primary antibodies (**Table 4**) diluted in PBS-FCS 10%. The samples were rinsed 3 x 5 min in PBS before application of the appropriate secondary antibodies (**Table 5**) for 45 min at room temperature in a humid chamber. Finally, slides were washed 3 x 5 min in PBS, dried, and mounted with coverslips and Vectashield HardSet Mounting Medium (Vector) with 4', 6-diamidino-2-phenylindole (DAPI) as a nuclear counterstain. Slides were kept at 4°C prior to observation.

Antigens	Conjugation	Dilutions	Sources
Rabbit/Rat/Guinea Pig/Mouse/Goat	Alexa-488	1:1000	Invitrogen
Rabbit/Rat/Mouse/Goat	Alexa-594	1:1000	Invitrogen
Rabbit/Mouse/Goat	Alexa-647	1:1000	Invitrogen
Guinea Pig	Cy3	1:1000	Jackson ImmunoResearch
Rat/Guinea Pig	Cy5	1:1000	Jackson ImmunoResearch
Chicken	Alexa-488	1:1000	Invitrogen
Chicken	Alexa-594	1:1000	Invitrogen

Table 5: Secondary antibodies used for IHC

VII. Quantification analyses

Quantification were performed on pictures from Zeiss Axioimager Z1 with Axiovision software and an automatic mosaic acquisition system (Zeiss). The entire pancreata of at least 3 mice per genotype and per condition were serially cut in 8 μm thick sections, applied to glass slides and every 5 to 10 pancreatic sections were processed for immunohistochemistry.

For cre recombinase efficiency and β -galactosidase expressing cells, positive cells were counted manually. IdU and BrdU counting was assessed manually counting proliferative cells in the different pancreatic compartments. For hormones quantifications, widefield multi-channels tile scan images treatment and analysis were done using a Fiji (Schindelin et al., 2012) home-made semi-automated macro. Briefly, the overall pancreas slice area was determined and measured using a low intensity based threshold on the insulin or glucagon or somatostatin labelling channel. Islets were then segmented and counted on the insulin labelling, applying a high intensity threshold. The density of islets per pancreas section was obtained reporting the number of islets to the pancreatic area. In a second step, the respective area of each segmented islet was measured. The corresponding contours were then transferred to the somatostatin or glucagon images. Finally, the signal coverage of either glucagon or somatostatin was measured and then normalized to the total area of the corresponding islet.

VIII. Data analyses and statistics

All values are depicted as mean \pm SEM of at least data from 3 animals. Data were analyzed using GraphPrism 6 software. Normality was tested using D'Agostino-Pearson omnibus normality test and appropriate statistical tests were performed. Results are considered significant if $p < 0.0001$ (****), $p < 0.001$ (***), $p < 0.01$ (**), and $p < 0.05$ (*).

RESULTS

I. Sst::Cre-Pax4-OE double transgenic mouse line generation and phenotype

Aiming to determine whether the sole misexpression of *Pax4* in δ -cells could alter their phenotype/identity *in vivo*, we first crossed Sst-Cre animals (harboring a transgene encompassing the *somatostatin* promoter driving the expression of the phage P1 *Cre recombinase* - Taniguchi et al., 2011 - **Figure 14A**) with the ROSA26- β -gal mouse line (harboring a transgene containing the *Rosa26* promoter upstream of a loxP-Neomycin resistance-Stop-loxP- β -galactosidase cassette - Soriano, 1999 **Figure 14A**). Our analyses of the pancreata from the resulting Sst-Cre::ROSA26- β -gal double transgenic mice validated the specificity of Cre expression solely in somatostatin-producing cells (**Figure 14C**).

Subsequently, Sst-Cre animals were mated with Pax4-OE mice (whose transgene encompasses the ubiquitous CAG promoter upstream of a “GFP-STOP” cassette flanked by LoxP sites and followed by the *Pax4* cDNA in front of an IRES and the β -galactosidase reporter - Collombat et al., 2009 - **Figure 14B**). In the resulting Sst-Cre::Pax4-OE double transgenic animals, *Pax4* misexpression was solely detected in Cre-expressing somatostatin⁺ cells. Accordingly, quantitative immunohistochemical analyses confirmed such specificity with an ectopic expression of *Pax4* in $66\pm 3.09\%$ of somatostatin-expressing cells (**Figure 14D-E**). Importantly, Sst-Cre::Pax4-OE transgenic mice were found to be viable, fertile, with no premature death being observed. Along the same line, no statistical difference was observed in the glycemia of control and transgenic animals of matching ages (**Figure 14F**), demonstrating that *Pax4* misexpression in somatostatin⁺ cells does not impact basal glycemia levels.

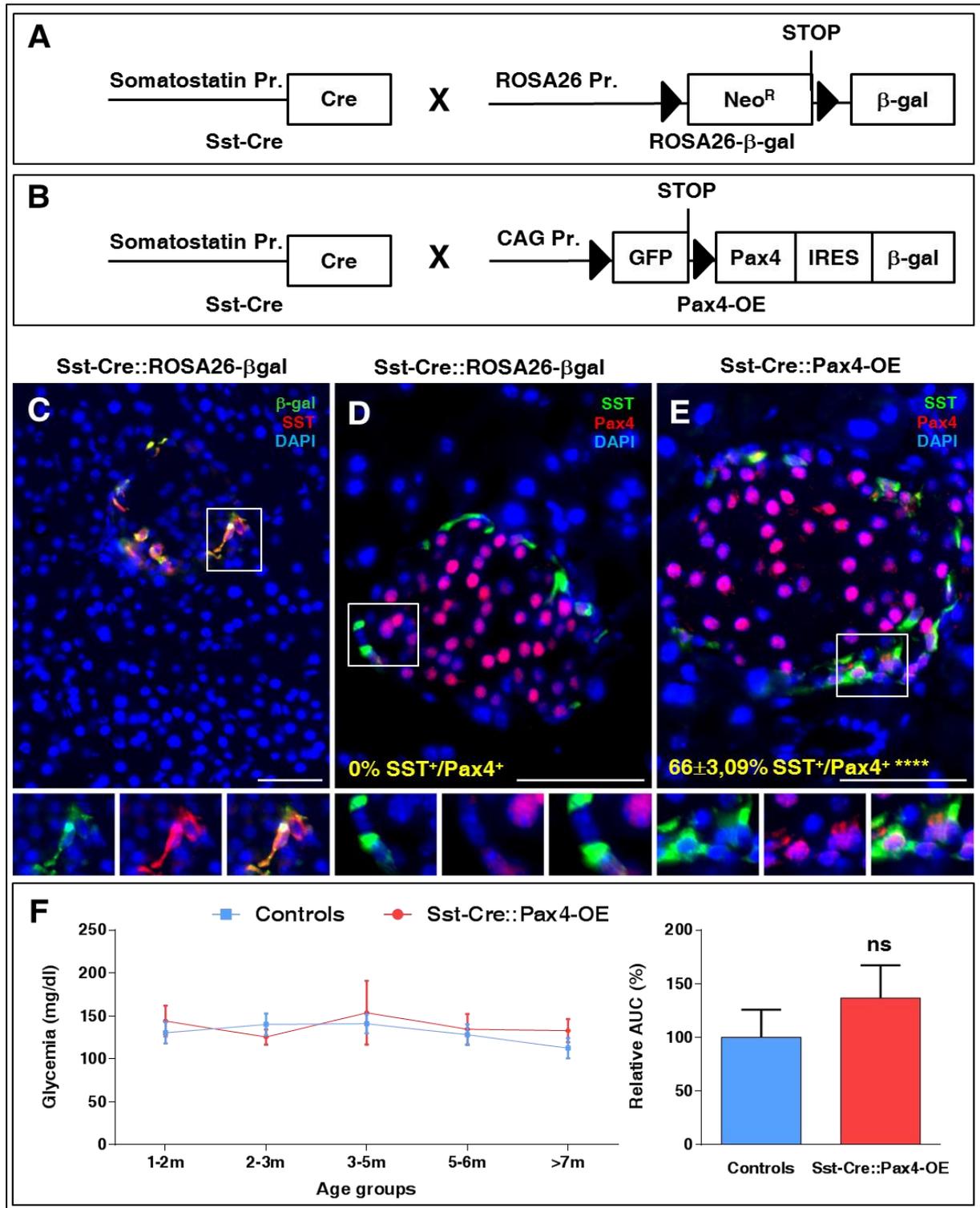


Figure 14: Transgenic mouse line allowing *Pax4* misexpression in somatostatin-expressing cells. Control Sst-Cre::ROSA26-β-gal double transgenic mice were obtained by crossing Sst-Cre animals with the ROSA26-β-gal line (in which the *Rosa26* promoter is upstream of a Neomycin resistance cassette and a STOP codon flanked by LoxP sites and followed by the β-galactosidase reporter) (A). Sst-Cre mice were also crossed with Pax4-OE animals (in which a CAG promoter is upstream of the GFP gene and a STOP codon flanked by LoxP sites and followed by the *Pax4* and the β-galactosidase cDNA sequences) (B). In

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the resulting Sst-Cre::Pax4-OE bitransgenic mouse line, somatostatin expression drives the expression of the Cre recombinase and allows the excision of the region between the two LoxP sites thereby promoting the expression of *Pax4* and β -galactosidase. β -galactosidase and *Pax4* immunodetection in Sst-Cre::ROSA26- β -gal double transgenic mice confirmed Cre expression specifically in somatostatin-expressing cells (**C-D**). In Sst-Cre::Pax4-OE islets, *Pax4* is detected in $66\pm 3.09\%$ of the somatostatin-expressing cells (**E**). Glycemia of non-fasted control and transgenic mice of different ages (**F**). The area under the curve (AUC) was measured and demonstrated no statistical differences between both groups. For Cre recombinase efficiency, the p-value was calculated by one sample *t*-test. Statistics for AUC were determined using Mann-Whitney test. **** $p < 0.0001$, ns $p > 0.05$. Scale bars, 20 μ m.

II. *Pax4* misexpression in δ -cells results in progressive islet hypertrophy and insulin-producing cell hyperplasia

The pancreata of Sst-Cre::Pax4-OE animals and age-/sex-matched controls were analyzed by immunohistochemistry at 2-, 5-, and 8-months of age. Importantly, an increase in islet number and size was demonstrated already in 2-month old Sst-Cre::Pax4-OE animals compared to controls (**Figure 15A-B**). As interesting were the further augmentations in both islet number and size observed in 5- and 8-month old Sst-Cre::Pax4-OE pancreata relative to controls (**Figure 15C-D**), suggestive of progressive processes. Quantitative analyses corroborated these results with the demonstration of a significant (2.38 ± 0.17 -fold) increase in islet counts and a 1.48 ± 0.07 -fold augmentation in islet size when comparing 2-month old Sst-Cre::Pax4-OE pancreata with controls (**Figure 15E-F**). Importantly, quantification of insulin⁺ area per pancreas demonstrated that islet hypertrophy mostly resulted from a significant insulin⁺ cell hyperplasia (**Figure 15G**).

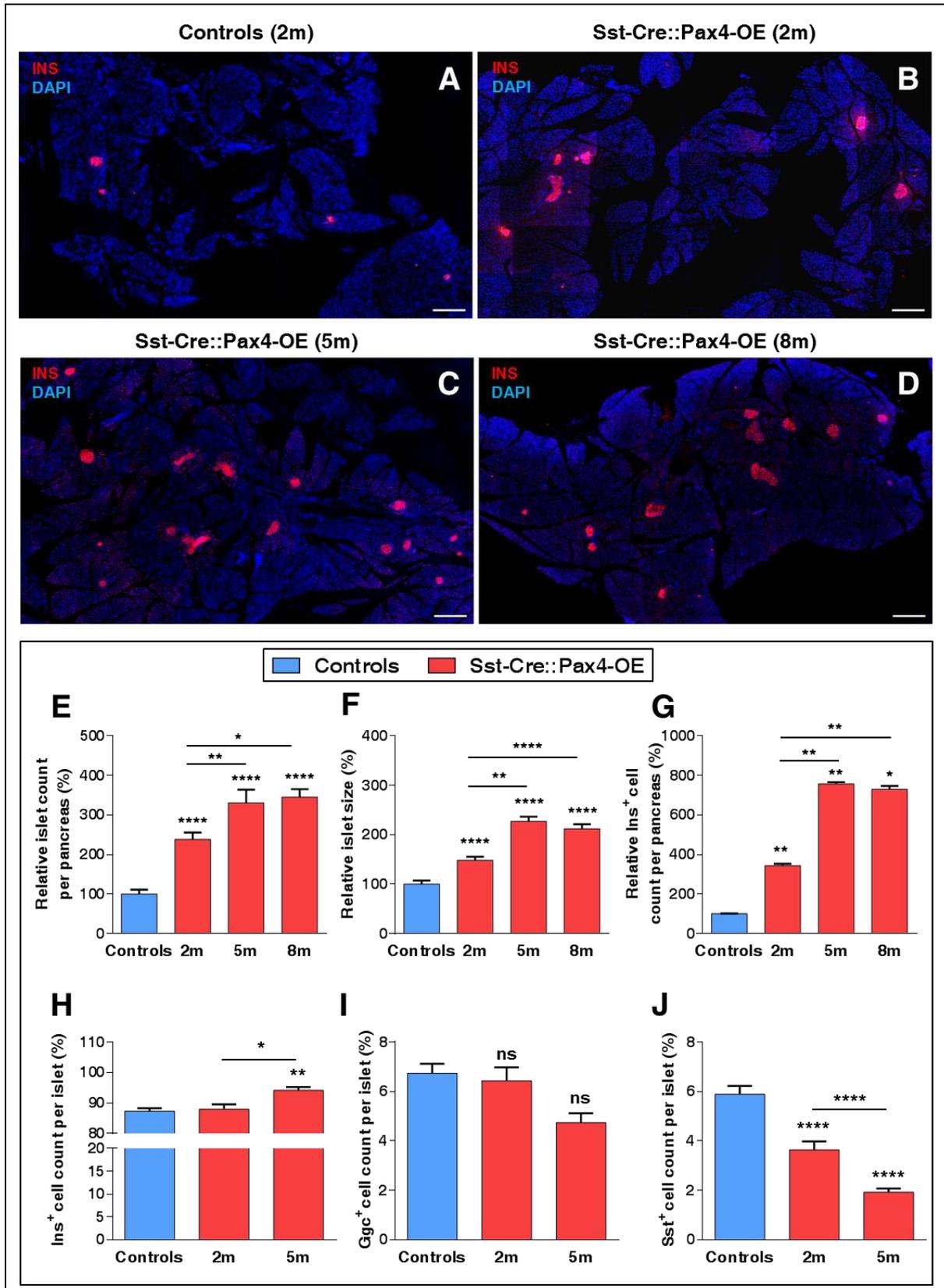


Figure 15: Pax4 misexpression in δ -cells leads to islet hypertrophy and β -cell hyperplasia. Representative pictures of insulin (red) immunohistochemical analyses on pancreas sections of 2- (B), 5- (C) and 8- (D) month old transgenics and controls (A) showing a dramatic increase in islet number and size. Quantitative analyses of at least 3 animals from each group revealed a ~1.5x increase in islet size (F) and 2x in islet number (E)

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in 2-month old transgenic pancreata. Quantitative analyses of insulin⁺ area in 2-month old mice outlined a ~4x increase respectively, consistent with the observed increased islet number and counts (**G**). At 5- and 8-months of age, an average of 3x, 2x, and 8x increase in islet counts (**E**), size (**F**), and insulin⁺ area respectively, was quantified in Sst-Cre::Pax4-OE pancreata as compared to age-matched controls. Quantification of insulin⁺ cell proportions demonstrated an increase in 5-month old transgenic animals compared to controls (**H**). Quantification of islet glucagon⁺ cell content in 2- and 5-month old mutant islets did not reveal any differences compared to controls (**I**). A significant decrease in somatostatin⁺ proportions was observed in 2- and 5-month old mutant islets compared to controls (**J**). All values are depicted as mean \pm SEM of $n>3$ independent animals. Statistics for insulin⁺ area, islet size, glucagon and somatostatin contents were performed with Mann-Whitney test. For islet number p-values were calculated by unpaired t test with Welch's correction. **** $p<0.0001$, ** $p<0.01$, * $p<0.05$. Scale bars, 500 μ m.

Additional analyses performed in 5-month old animals ascertained a further increase reaching 3.30 ± 0.33 -fold in the islet count, 2.27 ± 0.09 -fold in islet size and 7.57 ± 0.08 -fold in insulin⁺ cell counts as compared to controls (**Figure 15E-G**). Similar augmentations were also observed in 8-month old animals with a 2.12 ± 0.09 -fold increase in islet size, a 3.45 ± 0.19 -fold increase in islet number and an increase in insulin⁺ area of 7.29 ± 0.17 -fold versus age-/sex-matched controls (**Figure 15E-G**), suggestive of a plateau in islet growth and multiplication after 5 months of age. Focusing our quantitative studies on relative islet cell proportions, the insulin⁺ cell counts were found increased in 5-month old Sst-Cre::Pax4-OE islets ($+ 7\pm 1.09\%$ - **Figure 15H**), whereas the glucagon⁺ cell proportions were found unchanged (**Figure 15I**), suggesting an increase in α -cell counts following faithfully the overall endocrine cell hyperplasia. Surprisingly, the somatostatin⁺ cell contents was 1.61 ± 0.34 -fold reduced in 2-month old Sst-Cre::Pax4-OE islets and further diminished (3.05 ± 0.15 -fold) in their 5-month old counterparts (**Figure 15J**).

In addition to the evidently altered endocrine cell count observed in Sst-Cre::Pax4-OE pancreata, an abnormal positioning of non- β -cells was noted within the

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islets. Indeed, while glucagon- and somatostatin-expressing cells were predictably found uniformly distributed at the periphery of control islets (**Figure 16A-B**), a preferential localization at a pole of the islets, close to adjacent ducts, was evidenced for these non- β -cells in 2-month old Sst-Cre::Pax4-OE islets (**Figure 16C-D**).

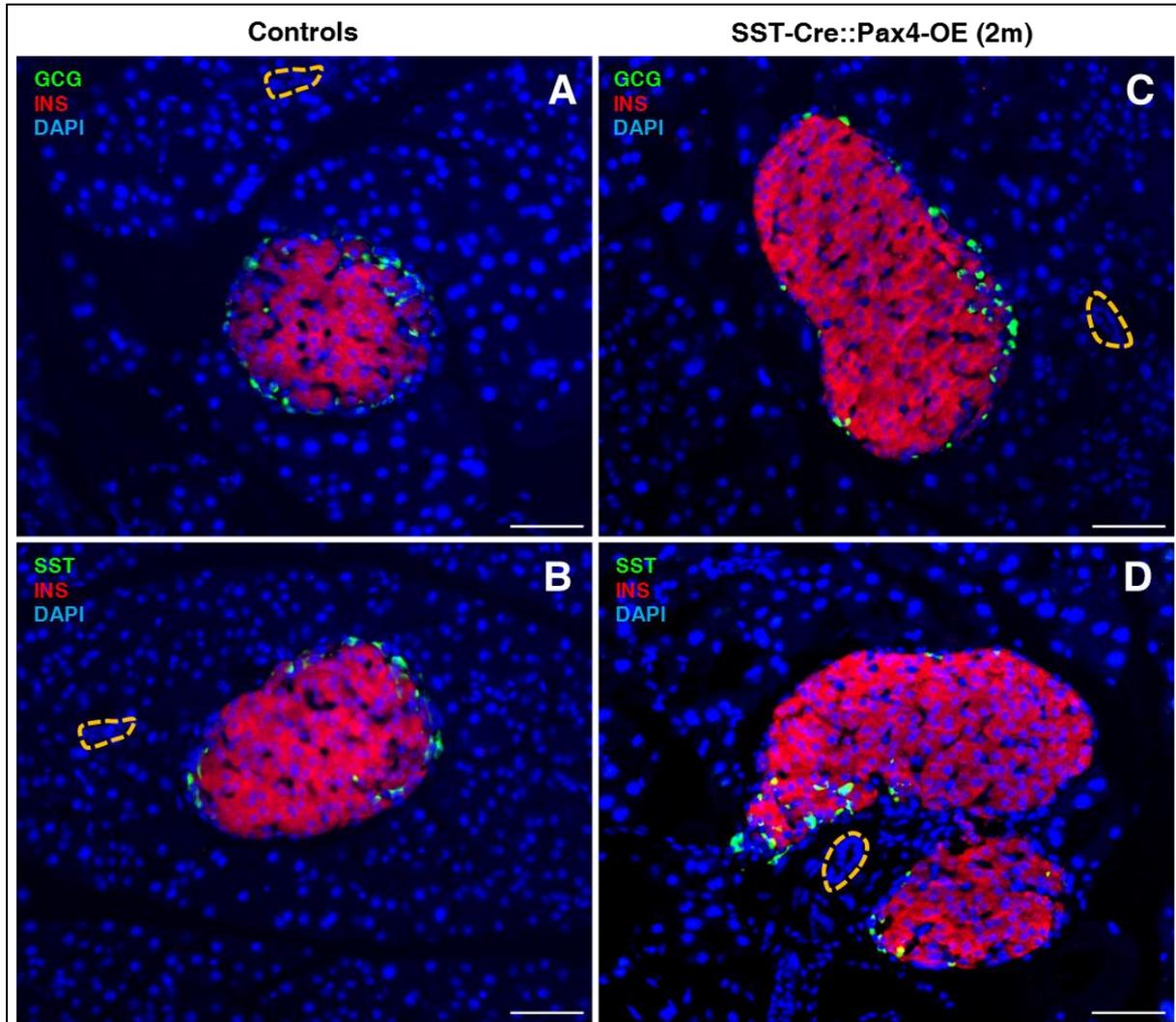


Figure 16: Abnormal positioning of non- β -cells within the islets of Sst-Cre::Pax4-OE transgenic mice. Representative photographs of somatostatin-, glucagon- and insulin-expressing cells within control islets (**A-B**) or 2-month old transgenic islets (**C-D**). While somatostatin- and glucagon-positive cells were found distributed in the mantle of control islet (**A-B**), a preferential localization close to adjacent ducts of such cells was observed in Sst-Cre::Pax4-OE islets (**C-D**). For the purpose of clarity, pancreatic ducts are outlined in dashed orange lines. Scale bars, 50 μ m.

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Together, these results suggest that the misexpression of *Pax4* in δ -cells promotes both an increase in islet numbers and size. Interestingly, the islet hypertrophy appears to result from an insulin⁺ cell hyperplasia and, to a lesser extent, from an increase in glucagon⁺ cell counts, indicative of neogenesis processes. However, somatostatin⁺ cells are found underrepresented, suggesting that the neogenesis processes observed in Sst-Cre::*Pax4*-OE pancreata could involve their conversion into alternative cell subtypes.

III. *Pax4* misexpression in somatostatin⁺ cells induces their conversion into β -like cells

To investigate the fate/destiny of *Pax4* misexpressing δ -cells in Sst-Cre::*Pax4*-OE pancreata, further immunohistochemical analyses were undertaken. Interestingly, a fraction of somatostatin-positive cells were found to ectopically express insulin in 2-month old Sst-Cre::*Pax4*-OE islets (**Figures 17B, 18A-B**), such bi-hormonal cells being expectedly absent in control pancreata (**Figure 17A**). As these results suggested a putative conversion of somatostatin⁺ cells into insulin⁺ cells, we investigated the consequences of *Pax4* misexpression in δ -cells using lineage tracing. The expression of the β -galactosidase tracer was therefore monitored comparing Sst-Cre::ROSA26- β -gal double transgenics with Sst-Cre::*Pax4*-OE animals.

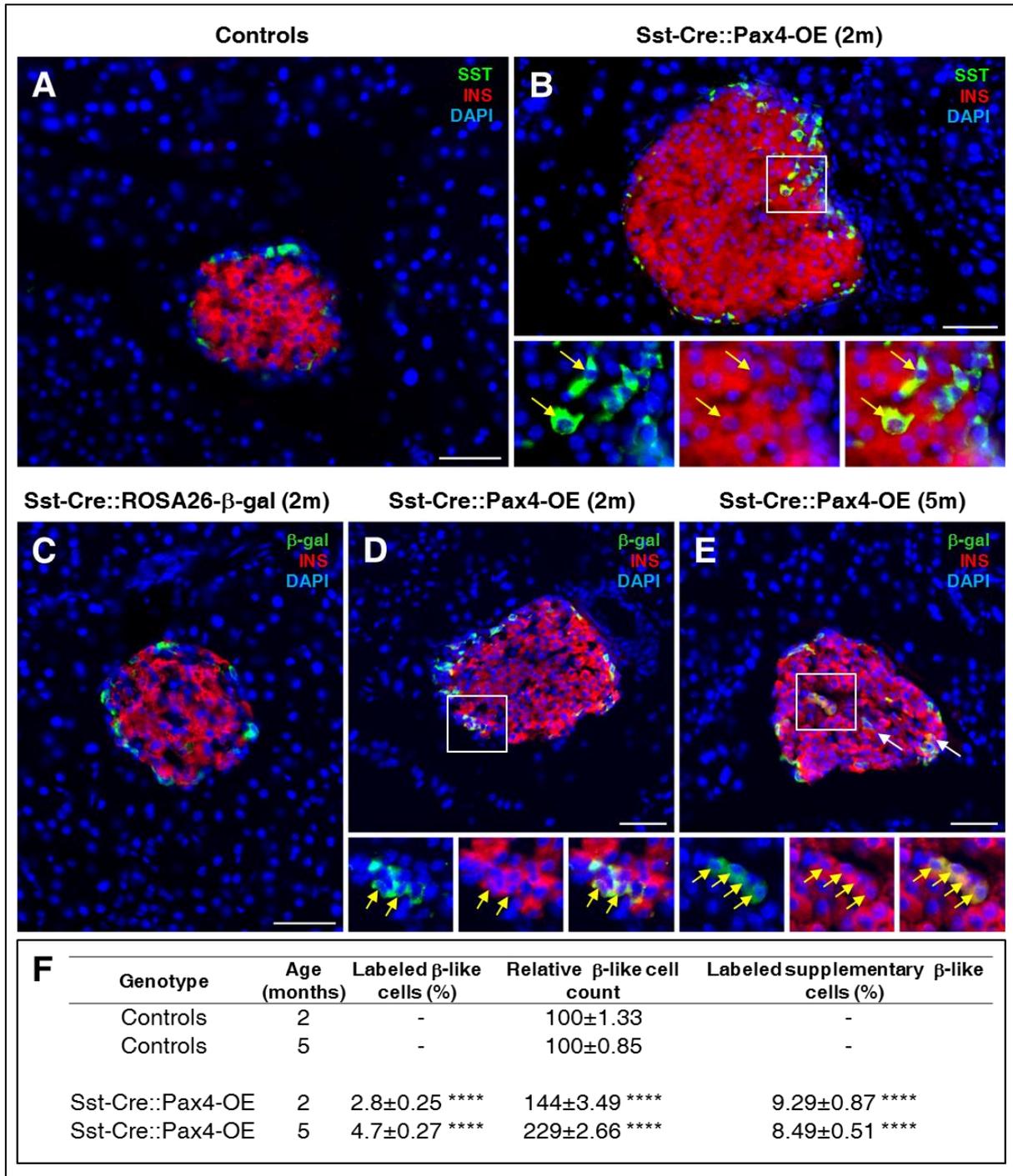


Figure 17: Conversion of adult δ -cells into insulin-producing cells upon *Pax4* misexpression. Immunohistochemical analyses on pancreas sections of 2-month old mice revealing cells co-expressing somatostatin and insulin in transgenics (inlet and arrows in **B**), such cells being absent in control islets (**A**). Representative images of lineage tracing experiments of somatostatin-expressing cells performed on pancreas sections of 2- (**D**) and 5- (**E**) month old transgenics and controls (**C**). While β -galactosidase⁺ cells did not label insulin⁺ cells in control islets (**C**), immunohistochemical analyses showed several insulin⁺ cells marked by the β -galactosidase tracer in *Sst-Cre::Pax4-OE* pancreata (inlets and arrows in **D-E**) indicative of a conversion of δ -cells into insulin⁺ cells. Further examinations revealed that 2.8 \pm 0.25% of insulin⁺ cells expressed β -galactosidase in 2-month old transgenics, such

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proportion increasing to $4.7\pm 0.27\%$ in 5-month old Sst-Cre::Pax4-OE animals (**F**). For the proportion of supplementary β -like cells labeled with the β -galactosidase reporter, the following formula was used: $(\% \text{ of labeled } \beta\text{-like cells} \times \text{relative } \beta\text{-like cell count}) / (\beta\text{-like cell increase} - 100)$. A similar proportion of $9\pm 0.87\%$ of supplementary β -like cells appeared β -galactosidase⁺ was found in the different age groups. Statistics were determined using Mann-Whitney test **** $p < 0.0001$. Scale bars, $50\mu\text{m}$.

In control Sst-Cre::ROSA26- β -gal pancreata, β -gal⁺ cells were found, as expected, in the mantle of the islets, where somatostatin-expressing cells are normally located (**Figure 17C**). While, no insulin/ β -gal double positive cells could be found in control islets (**Figure 17C**), several insulin⁺/ β -galactosidase⁺ cells were observed both in the core and the mantle of 2-month old Sst-Cre::Pax4-OE pancreata (**Figure 17D, 18C-D**). Interestingly, similar lineage tracing experiments performed on 5-month old transgenics revealed an increased number of insulin⁺ cells labeled with β -galactosidase (**Figure 17E, 18E-F**). These results were further confirmed using quantitative analyses. Indeed, while no insulin⁺ cells expressed β -galactosidase in control islets, $2.8\pm 0.25\%$ and $4.7\pm 0.27\%$ of insulin⁺ cells expressed β -galactosidase in 2- and 5-month old transgenic islets respectively (**Figure 17F**). Importantly, a proportion of $9\pm 0.87\%$ of supplementary β -like cells appeared β -galactosidase⁺ in 2-month old Sst-Cre::Pax4-OE mice, this proportion being maintained in older transgenics. Together, these findings demonstrate the conversion of somatostatin-expressing cells into insulin⁺ cells upon the sole misexpression of *Pax4*. Remarkably, the proportion of insulin⁺ cells from a δ -cell origin faithfully follows the observed islet size increase. This suggests continuous processes of conversion of δ -cells into insulin⁺ cells.

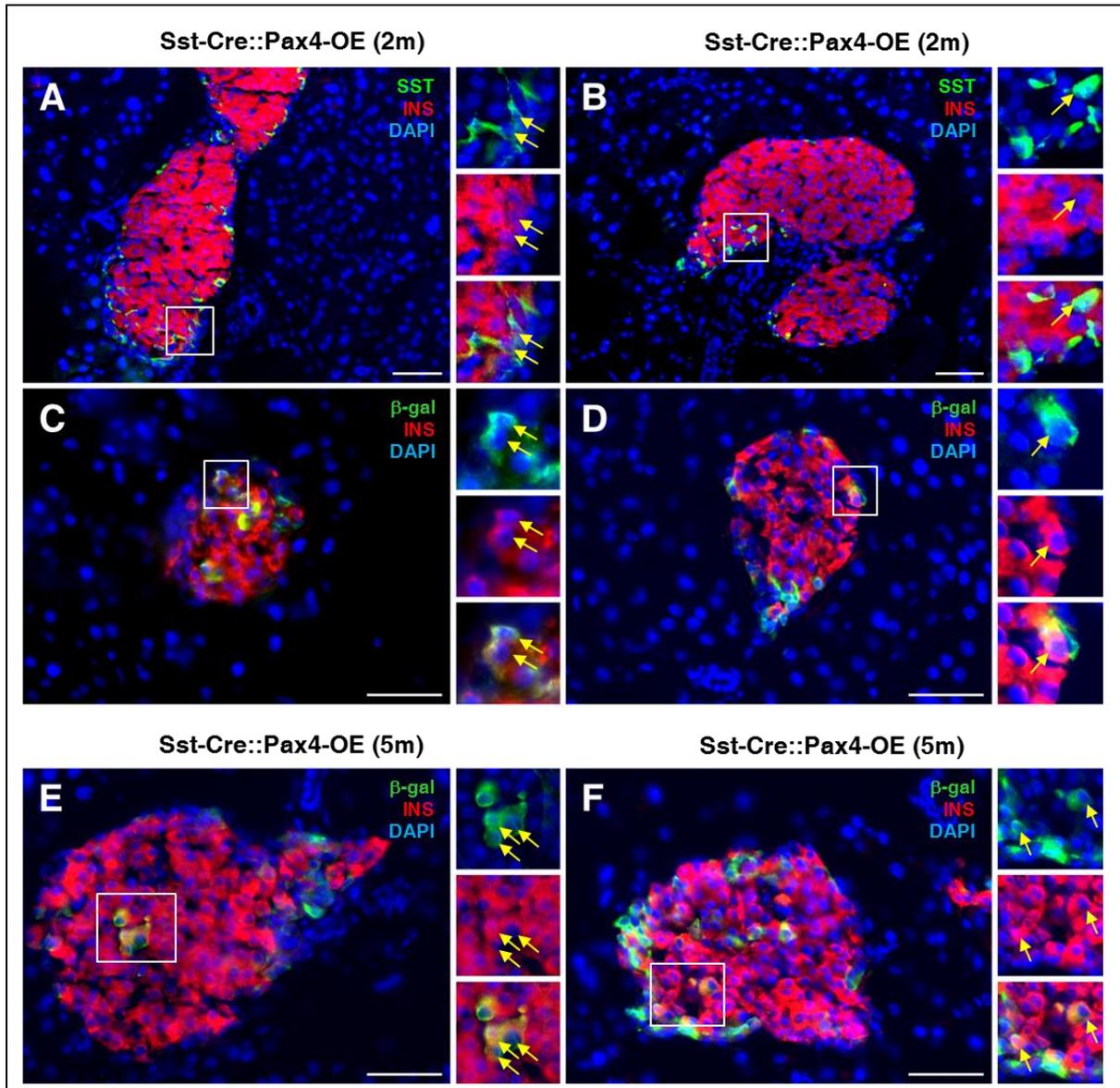


Figure 18: Additional examples illustrating the conversion of δ -cells to insulin-producing cells upon *Pax4* misexpression. Immunohistochemical analyses on pancreas sections of 2-month old mice revealing cells co-expressing somatostatin and insulin-cells in transgenics (inlets in **A**, **B**), such cells being absent in control islets. Representative images of lineage tracing experiments of somatostatin-expressing cells performed on pancreas sections of 2- (**C-D**) and 5- (**E-F**) month old transgenics, suggesting a δ -to-insulin⁺ cell conversion. Scale bars, 50 μ m.

IV. *Pax4* misexpression in δ -cells results in increased proliferation within the ductal epithelium

Our results demonstrate that the misexpression of the *Pax4* gene in δ -cells is sufficient to induce their reprogramming into cells displaying a β -like cell identity. However, this conversion can clearly not account for the massive endocrine cell hyperplasia observed in Sst-Cre::*Pax4*-OE animals, suggestive of the involvement of additional processes.

Thus, to investigate whether the latter could also be caused by an increase in cell replication, cell proliferation rates were assayed in Sst-Cre::*Pax4*-OE transgenics and age-matched controls treated with either Idu or BrdU for different durations. Importantly, after short Idu pulse (3 days), a significant augmentation in replicating cell numbers was observed in the pancreas of 2-month old transgenic mice as indicated by increased Idu⁺ cell numbers (**Figure 19A, D**). Strikingly, most proliferating cells were not located within the islets of Langerhans but rather within the ductal epithelium and lining (**Figure 19D**). Interestingly, longer Idu treatment (10 days) revealed numerous Idu⁺ cells again in the ductal epithelium and lining (**Figure 19B-C, E**), such Idu⁺ cells being then also found within the islets of Sst-Cre::*Pax4*-OE mice (**Figure 19B-C, F**), suggesting that proliferating cells progress from the ductal compartment to the islets of Langerhans. Quantitative analyses verified these observations and outlined a 5.73 ± 0.38 -fold increase in the numbers of Idu⁺ cells within the ductal lining or epithelium after short-term Idu treatment comparing Sst-Cre::*Pax4*-OE pancreata versus their control counterparts (**Figure 19G**).

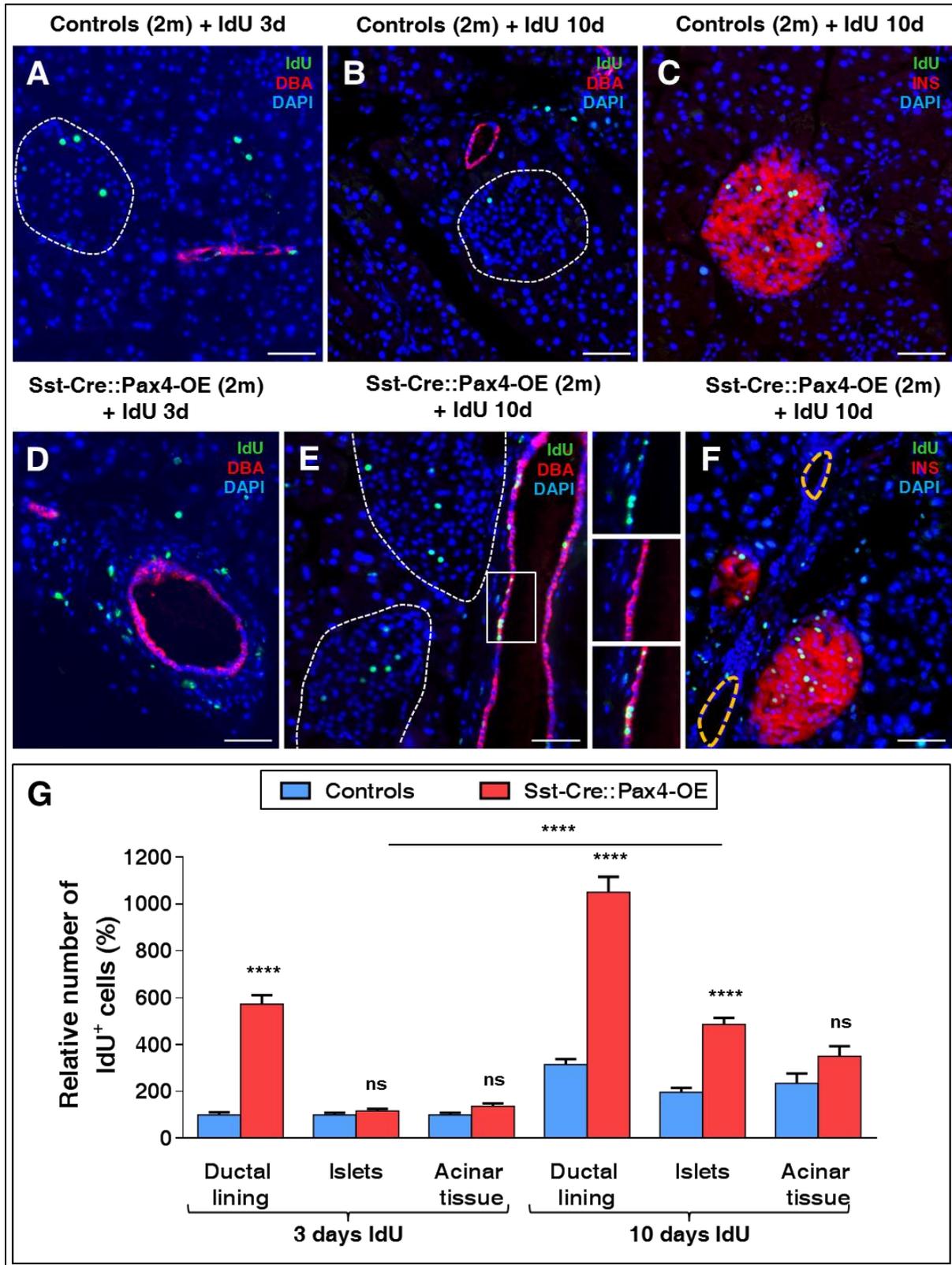


Figure 19: Increased cell proliferation within the ductal epithelium of Sst-Cre::Pax4-OE pancreata. Cell proliferation was assessed evaluating IdU incorporation (detected with a BrdU antibody) in 2 month-old transgenics and controls after short- (3 days) or long- (10 days) term IdU pulse. Representative images of immunohistochemical analyses on pancreas sections of control (A-C) and Sst-Cre::Pax4-OE (D-F) mice. An increase in proliferation was observed in the ductal lining of 2-month old transgenics after a short-term IdU pulse (A, D).

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After 10 days of IdU treatment, a further increase in proliferating cells was noted in the ductal epithelium (inlet in **E**) and islets (**F**) of transgenic mice as compared to controls (**B**, **C**). Quantitative analyses revealed a 5.73 ± 0.38 -fold increase in proliferating cell counts within the ductal lining or epithelium after short-term IdU (**G**). Interestingly, a 2.47 ± 0.13 -fold and a 3.34 ± 0.21 -fold increase in IdU⁺ cells is outlined in the endocrine compartment and in ductal lining, respectively, of Sst-Cre::Pax4-OE pancreata after long-term IdU treatment (**G**). For the purpose of clarity, in selected photographs, islets are outlined with dashed white lines. All values are depicted as mean \pm SEM of $n > 3$ independent animals. Statistics were performed Mann-Whitney test or unpaired t test with Welch's correction. **** $p < 0.0001$, ns $p > 0.05$. Scale bars, 50 μ m.

A 3.34 ± 0.21 -fold increase within the ductal lining or epithelium and a 2.47 ± 0.13 -fold increase in the numbers of IdU⁺ cells within the endocrine compartment were observed after 10 days of IdU treatment (**Figure 19G**). Of note, no difference in IdU⁺ cell counts were observed in the acinar tissue comparing both groups.

In order to determine whether proliferating cells went through one or more cell division in Sst-Cre::Pax4-OE pancreata, control and transgenic mice were sequentially treated with CldU and IdU for 10 days each (**Figure 20A**). Despite the fact that the number of proliferative cells was, as shown before, increased in transgenic islets, no IdU⁺/CldU⁺ cells were detected in Sst-Cre::Pax4-OE nor control islets (**Figure 20B-D**). Concomitant with the plateau in islet hypertrophy observed after 5-months of age, comparative quantifications of endocrine and non-endocrine cell proliferation rates in 5- and 8-month old mutants did not reveal any differences (**Figure 20E-F**). Thus, these data suggest that the observed augmentations in islet size and number involve increased cell proliferation essentially originating in the ductal lining/epithelium, these proliferating cells eventually sprouting from the ductal compartment to reach the islets of Langerhans.

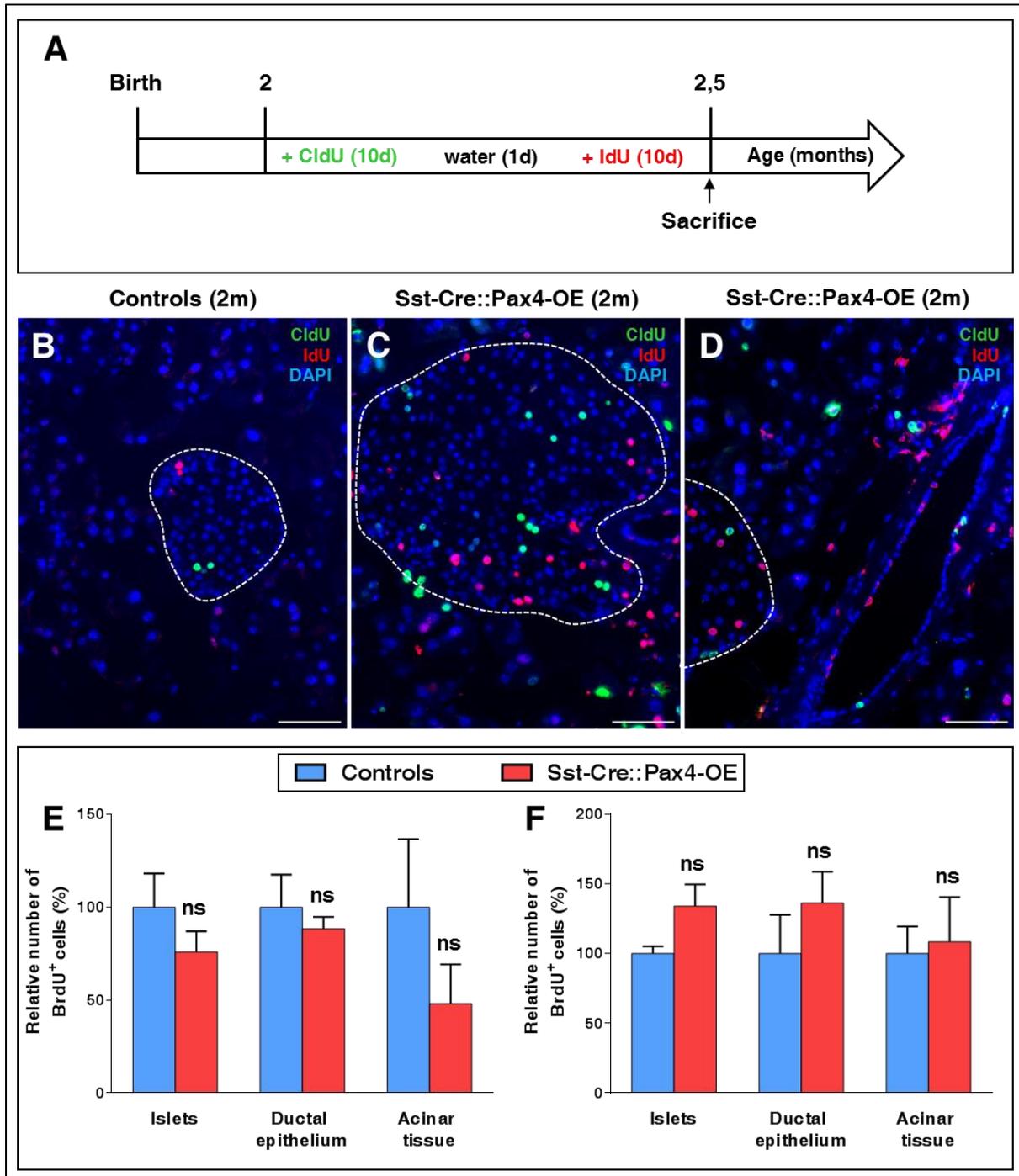


Figure 20: Increased proliferation within the ductal epithelium of Sst-Cre::Pax4-OE pancreata occurring before 2 month of age. CldU and IdU were administered consecutively for 10 days with a washout period of 24 hours (A). Representative pictures of immunohistochemical analyses on pancreas sections of 2-month old transgenics (C-D) and matched controls (B) showing no IdU/CldU double positive cells in both groups. Quantification of endocrine and non-endocrine BrdU⁺ cells in 5- (E) and 8- (F) month old mutants did not reveal any differences compared to controls. For the purpose of clarity, islets are outlined with dashed white lines. All values are depicted as mean \pm SEM of $n > 3$ independent animals. Statistics were performed with Mann-Whitney test. Scale bars, 50 μ m

V. Reactivation of EMT and *Neurog3*-controlled endocrine differentiation program in Sst-Cre::Pax4-OE pancreata

Aiming to gain further insight into the mechanisms underlying ductal cell proliferation and the resulting endocrine cell neogenesis observed in Sst-Cre::Pax4-OE pancreata, we wondered whether these processes may involve a re-awakening of the developmental endocrine differentiation program. Thus, to test this hypothesis, the expression of the pro-endocrine/developmental master gene, *Neurog3*, was investigated. Whereas *Neurog3* was expectedly not found expressed in the adult control pancreas (**Figure 21A**), a re-expression of this pro-endocrine gene was evidenced in a few ductal cells and within the ductal lining (**Figure 21B**). Due to known difficulties to detect *Neurog3* expression by immunohistochemistry and to ascertain the re-expression of this proendocrine gene, total pancreas quantitative RT-PCR analyses were performed. A 2.26 ± 0.30 -fold increase in *Neurog3* transcripts was demonstrated in 2-month old Sst-Cre::Pax4-OE pancreata, further confirming its re-expression in transgenic pancreata (**Figure 21C**). Combined, these observations support the notion of a reactivation of the expression of *Neurog3* in the ductal lining of adult animals misexpressing *Pax4* in δ -cells. Importantly, *Neurog3*⁺ cells give rise to all endocrine cells during development, further suggesting that the islet hypertrophy observed Sst-Cre::Pax4-OE is due to its re-expression leading to the neogenesis of insulin⁺, glucagon⁺ and somatostatin⁺ cells with the normal endocrine proportions.

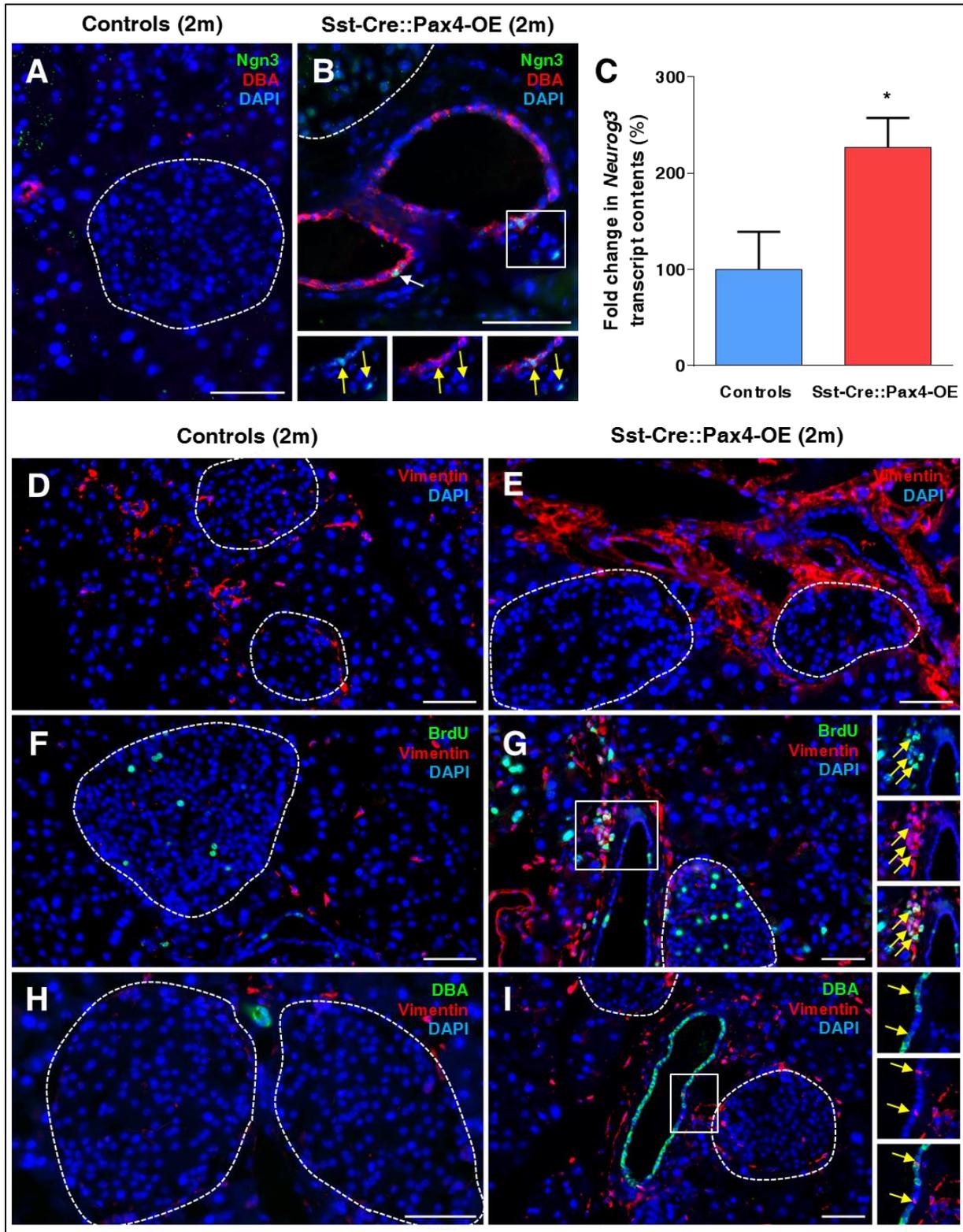


Figure 21: Re-expression of *Neurog3* in duct-associated cells upon *Pax4* misexpression in δ -cells and reawakening of epithelial-to-mesenchymal transition. Representative photographs of immunohistochemical analyses on pancreas sections of 2-month old transgenics (B, E, G, I) and matched controls (A, D, F, H). *Neurog3* expression was assessed in 2-month old controls (A) and transgenics (B). *Neurog3* was found re-expressed in the ductal lining (B inlet yellow arrow) and epithelium (B white arrow) of transgenic mice while being absent in controls (A). Accordingly, in Sst-Cre::Pax4-OE

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pancreata, quantitative RT-PCR analyses outlined an average 2.26 ± 0.30 -fold increase in *Neurog3* transcripts (**C**). Surprisingly, the mesenchymal marker *Vimentin* was found to be largely expressed in the ductal lining epithelium of 2-month old transgenic animals (**E, G, I**) while being weakly expressed in control mice (**D, F, H**), supporting the notion of an EMT reawakening following *Pax4* misexpression in somatostatin⁺ cells. Moreover, proliferating *vimentin*-expressing cells were detected in Sst-Cre::Pax4-OE pancreata (inlet in **G**), such cells being absent in matched controls (**F**). All values are depicted as mean \pm SEM of $n > 3$ independent animals. Statistics for *Neurog3* qPCR were performed with Mann-Whitney test. * $p < 0.05$. For the purpose of clarity, in selected photographs, islets are outlined with dashed white lines. Scale bars, 50 μ m.

Aiming to further ascertain a putative reenactment of endocrine developmental processes in Sst-Cre::Pax4-OE animals, we focused our analyses on the epithelial-to-mesenchymal transition (EMT), a key developmental process by which cells located within an epithelial layer acquire the ability to spread and migrate to a distant site to form new structures (Kang and Massague, 2004). EMT is orchestrated by a cascade of events, among which the loss of an epithelial cell identity (as outlined by the loss of E-cadherin), and the acquisition of a mesenchymal cell phenotype and associated markers (such as Vimentin and N-cadherin – Chiang and Melton, 2003). We therefore assessed the expression of *Vimentin* in 2-month old Sst-Cre::Pax4-OE pancreata versus control counterparts. Surprisingly, our analyses revealed a massive increase in the numbers of Vimentin⁺ cells in the ductal lining and close to adjacent islets of transgenic pancreata (**Figure 21D-E, H-I**). Of note, the examination of 10 days BrdU-treated Sst-Cre::Pax4-OE animals also unraveled a number of duct-lining BrdU⁺ cells expressing Vimentin (**Figure 21F-G**), such short-term lineage tracing suggesting that proliferating ductal cells undergo EMT prior to acquire an endocrine cell identity. These results indicate that *Pax4* misexpression in δ -cells eventually results in a reawakening of the *Neurog3*-controlled endocrine differentiation program and associated developmental processes, including EMT.

VI. Supplementary insulin-expressing cells in Sst-Cre::Pax4-OE pancreata display a β -cell phenotype and are functional

To further investigate the identity of the supplementary insulin-expressing cells observed in Sst-Cre::Pax4-OE pancreata, thorough marker gene analyses were performed in 2-, 5- and 8-month old animals. In all three instances, our data indicated that all insulin⁺ cells (pre-existing and supplementary) displayed a β -like cell phenotype. Indeed, these cells uniformly expressed the *bona fide* β -cell labels, such as *PC1/3* (Figure 22A-D), *Glut-2* (Figure 22E-H), *Nkx6.1* (Figure 22I-L), *Pdx1* (Figure 22M-P), and the pan-endocrine genes *NeuroD1* (Figure 22Q-T) and *Pax6* (Figure 22U-X).

To assess the function of these supplementary insulin⁺ cells, IPGTTs (Intraperitoneal Glucose Tolerance Tests) were performed on 2-month old Sst-Cre::Pax4-OE mice and matched controls. Interestingly, transgenic mice displayed an improved response with a lower peak in glycemia and a faster return to normoglycemia as compared to controls (Figure 23A), suggestive of an increased functional β -like cell mass. In addition, insulin tolerance tests (ITTs) revealed no significant difference between 2-month old Sst-Cre::Pax4-OE animals and matching controls (Figure 23B), indicating that, despite an increased β -cell content, Sst-Cre::Pax4-OE mice do not develop any insulin resistance.

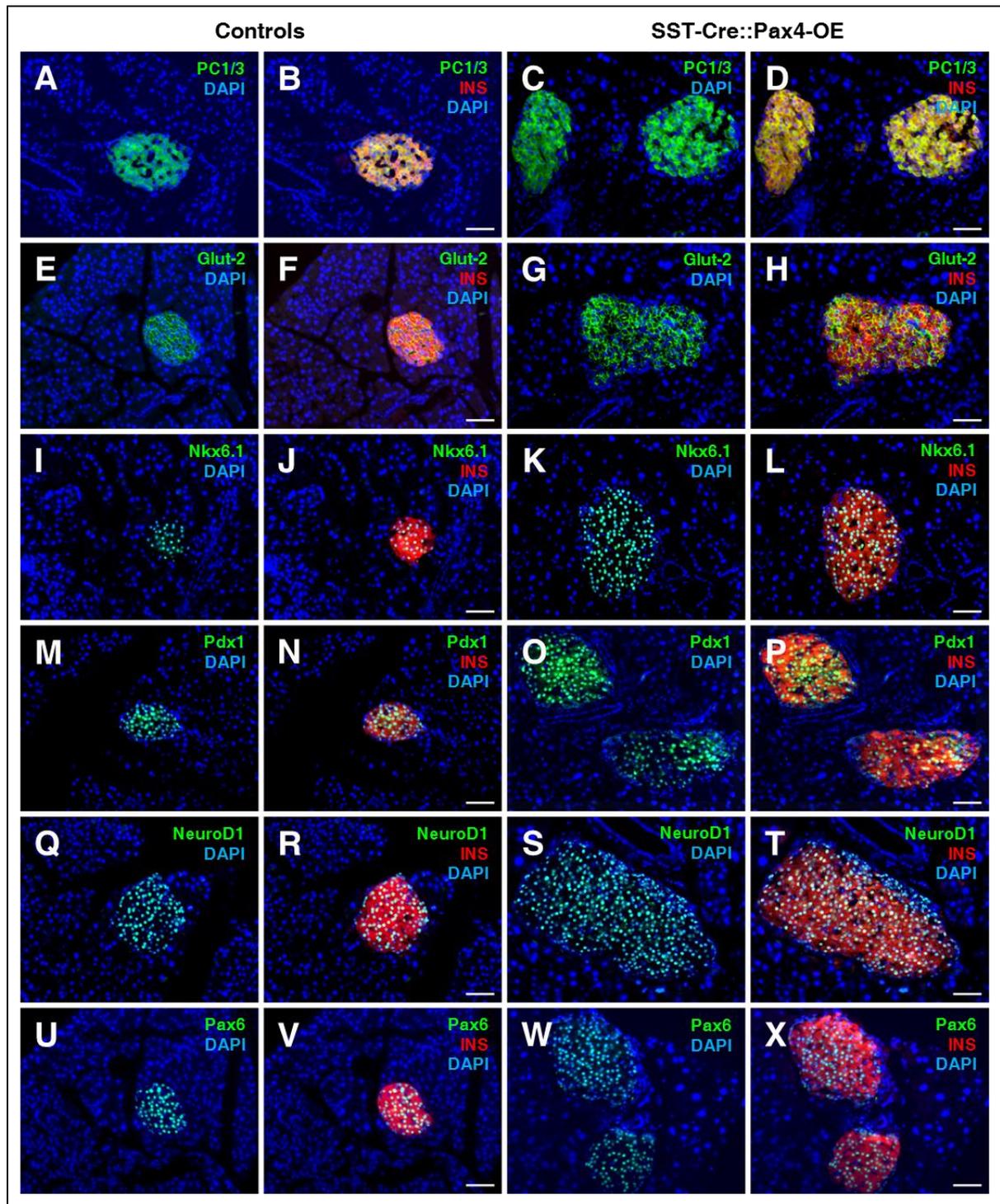


Figure 22: Sst-Cre::Pax4-OE endogenous and supplementary insulin⁺ cells display a β -cell phenotype. Representative photographs of immunohistochemical analyses on pancreas sections of controls (A-B, E-F, I-J, M-N, Q-R, U-V) and Sst-Cre::Pax4-OE (C-D, G-H, K-L, O-P, S-T, W-X). All insulin⁺ cells uniformly expressed the bona fide β -cell markers *PC1/3* (A-D), *Glut-2* (E-H), *Nkx6.1* (I-L), *Pdx1* (M-P) and the pan-endocrine markers *NeuroD1* (Q-T) and *Pax6* (U-X). Scale bars, 50 μ m.

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These analyses led us to conclude that the hyperplastic insulin-expressing cells observed following *Pax4* misexpression in somatostatin⁺ cells, exhibit a β -cell phenotype and are functional.

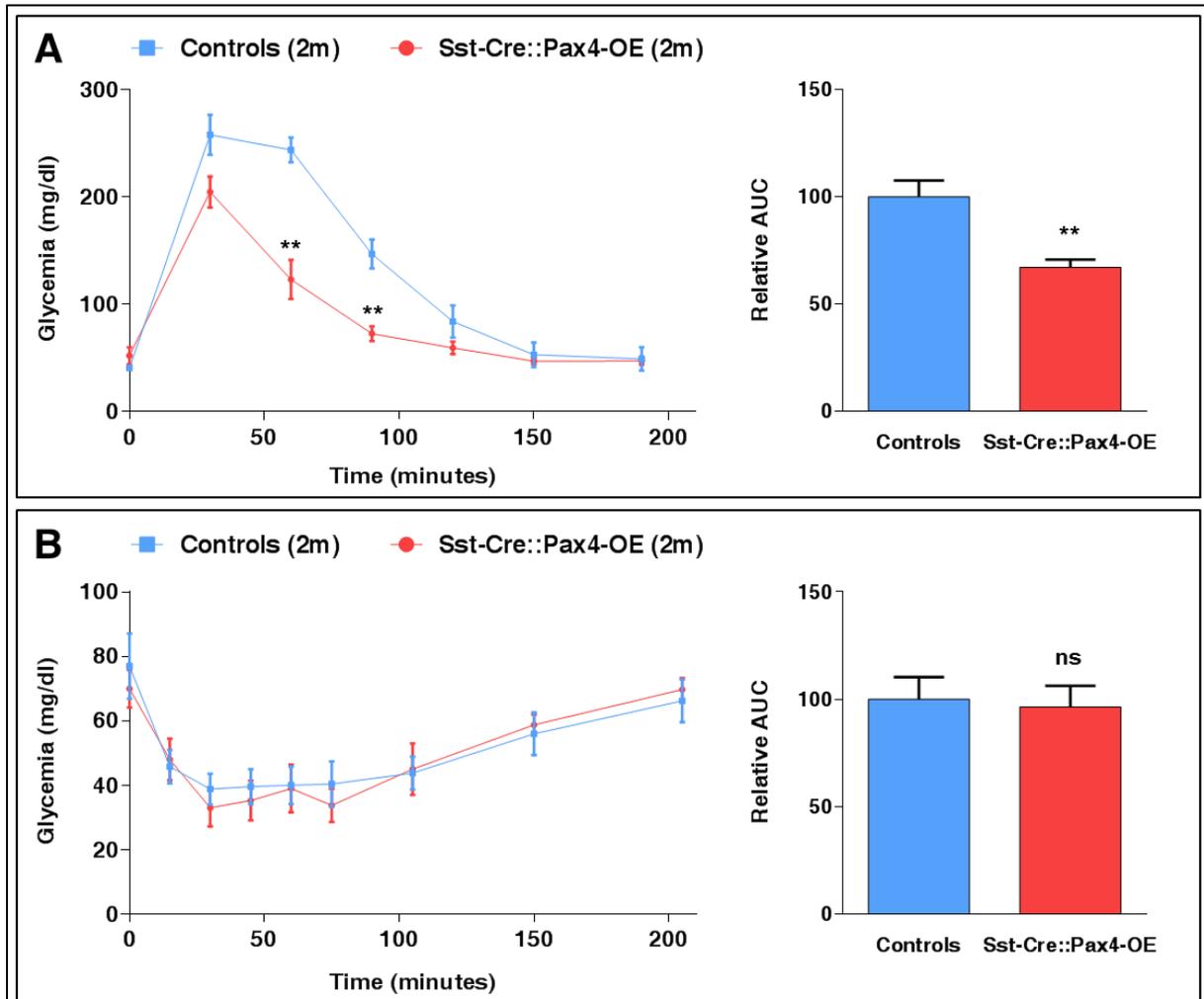


Figure 23: Improved β -cell function in transgenic Sst-Cre::Pax4-OE mice. 2 month-old transgenic and controls were challenged with glucose. Transgenic mice displayed an improved glucose clearance with a lower peak in glycemia and a faster recovery to euglycemia (**A**). The relative area under the curve (AUC) confirmed this improved glucose tolerance in 2 month-old Sst-Cre::Pax4-OE animals. Upon insulin tolerance tests (ITT), glycemia levels of 2 month-old transgenic and matched controls were monitored. Transgenic mice exhibited unaltered glycemia levels, indicative of a standard insulin sensitivity (**B**). AUC were indeed similar in both groups. The relative AUC are expressed as a percentage relative to the average AUC of the controls. All values are depicted as mean \pm SEM of $n > 3$ independent animals. Statistics were determined using Mann-Whitney test ** $p < 0.01$, * $p < 0.05$, ns $p > 0.05$.

VII. Adult somatostatin⁺ cells convert into insulin-producing cells upon *Pax4* misexpression following streptozotocin treatment

Adult Sst-Cre::Pax4-OE δ -cell plasticity was investigated in the context of chemically-induced β -cell ablation. The glycemia of these mice was monitored throughout the experiment, prior to sacrifice at different time points (**Figure 24A**). A single dose of streptozotocin (STZ – 115mg/kg) was administered to 2.5-month old transgenic mice and matching controls, the goal being to induce the destruction of a vast majority of β -cells without complete ablation to avoid predictable death (**Figure 24B, E**). As expected after specific β -cell loss, control and Sst-Cre::Pax4-OE mice developed hyperglycemia within 1 week post-injection (**Figure 24H**). 3 months post-injection, all control mice showed a glycemia >600mg/dl and a high rate of lethality (41%), while in contrast transgenic mice displayed an average glycemia of 350mg/dl (**Figure 24H**), suggesting a partial β -cell mass restoration and an extended lifespan. This is further supported by the detection of insulin-expressing cells in islets of control and transgenic mice, at different time after STZ-mediated β -cell ablation. Indeed, while after 1 week of STZ administration, control and Sst-Cre::Pax4-OE animals displayed a massive loss of insulin-expressing cells (**Figure 24B, E**), a progressive restoration of such cells was observed in transgenic mice (**Figure 24F, G**), such partial recovery being absent in matching controls (**Figure 24C, D**).

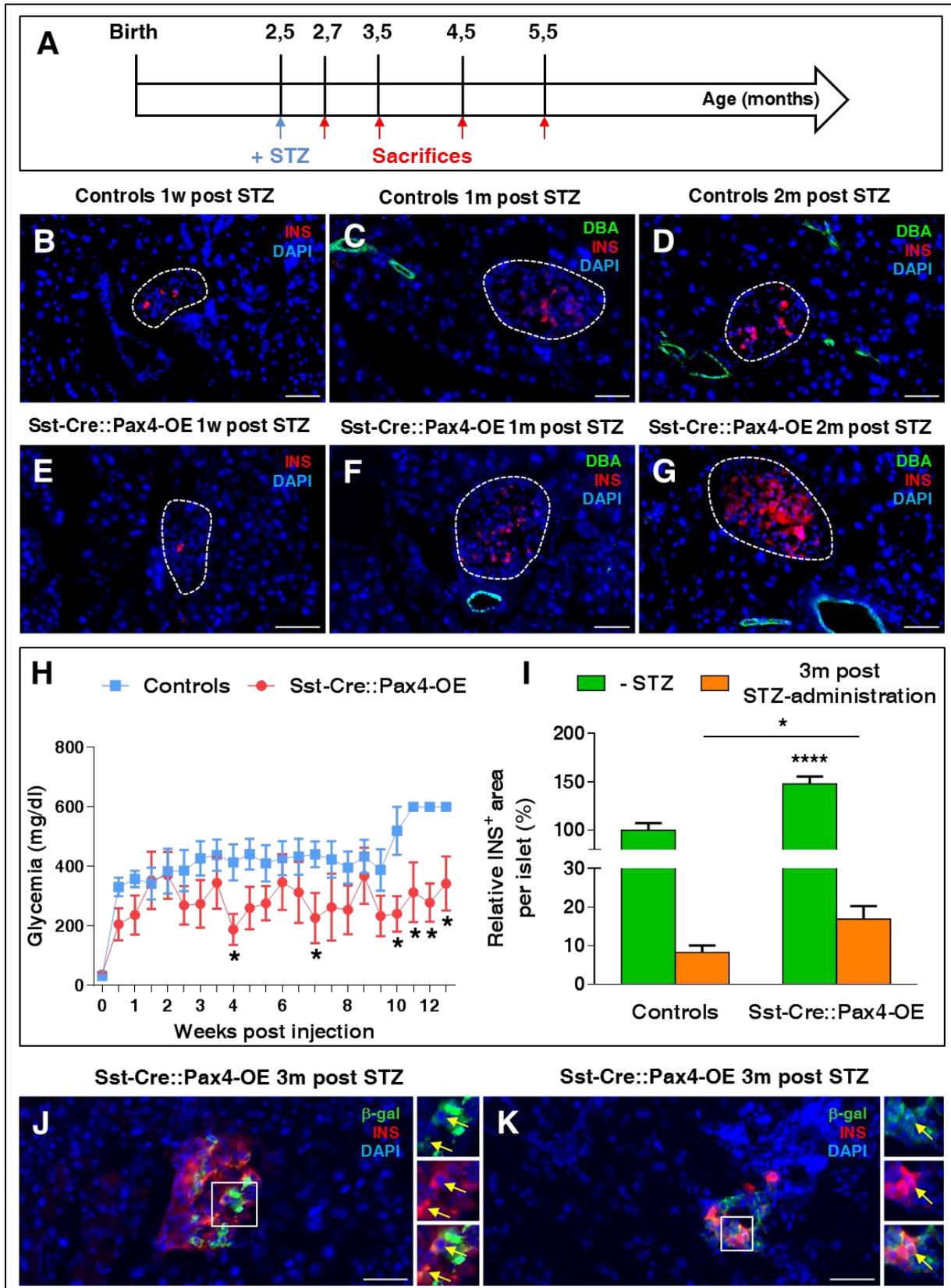


Figure 24: Pax4 misexpression in δ -cells can induce functional β -like cell neogenesis upon chemically-induced β -cell ablation. 2.5 month-old mice were treated with streptozotocin and sacrificed at different time points (A). Immunohistochemical analyses of insulin-expressing cells in control (B, D) or transgenic (E, G) islets 1 week, 1 month and 3

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months following STZ-mediated β -cell destruction. In Sst-Cre::Pax4-OE transgenic mice, a progressive recovery of the β -cell mass is observed. The monitoring of the glycemia revealed an expected peak in glycemic levels in both control and transgenic mice (**H**). 2.5 months following β -cell ablation, Sst-Cre::Pax4-OE mice displayed lowered glycemic levels, while control animals remained hyperglycemic. Quantitative analyses indicate an approximate doubling in the β -cell area in transgenic islets 3 months after STZ-administration (**I**). Lineage tracing experiments of somatostatin-expressing cells performed on pancreas sections of STZ-treated mice where β -gal⁺/insulin⁺ cells were detected in transgenic islets (inlets in **J-K**). For the purpose of clarity, in selected photographs, islets are outlined with dashed white lines. Scale bars, 50 μ m. All values are depicted as mean \pm SEM of $n > 3$ independent animals. Statistics were determined using Mann-Whitney test and multiple t -tests **** $p < 0.0001$, * $p < 0.05$.

To further assess these regeneration processes, the β -cell mass was quantified in control and transgenic pancreata 3-month post-STZ administration. Although decreased compared to their non-injected counterparts, STZ-treated Sst-Cre::Pax4-OE islets exhibited $16 \pm 3.40\%$ insulin-expressing cells compared to $8 \pm 1.77\%$ in treated-control islets (**Figure 24I**). This difference represents an average 2-fold increase in islet β -like cell content (**Figure 24I**). Immunohistochemical analyses and lineage tracing experiments were performed on STZ-injected control and transgenic pancreata to characterize the mechanisms underlying this partial β -cell mass renewal. We thereby detected insulin⁺/ β -galactosidase⁺ cells in Sst-Cre::Pax4-OE islets, 3 months following β -cell mass ablation (**Figure 24J-K**). However, the results presented here suggest that adult somatostatin-expressing cells, upon *Pax4* misexpression, are plastic and able to convert into insulin-producing cells also in the situation of a β -cell loss. Although encouraging, one would have expected a better recovery upon STZ-mediated β -cell destruction. Therefore, we initiated additional experiments using younger animals in order to take advantage of their improved β -cell neogenesis capability.

DISCUSSION

Here, we report that *Pax4* misexpression in δ -cells leads to their conversion into insulin-producing β -cells, further inducing an increase in islet number and islet hypertrophy provoked by an insulin⁺ cell hyperplasia. Interestingly, the latter is associated with an improved glucose tolerance. Our data show that these effects essentially appear before 5-months of age and do not alter the relative endocrine cell composition as the respective endocrine cell proportions are maintained. Importantly, these processes involve the recruitment of putative endocrine progenitor cells located within the ductal lining or epithelium, such cells reactivating the *Neurog3*-controlled differentiation program prior to their delamination by EMT reawakening. Moreover, we demonstrate here that adult δ -cells are plastic and capable of converting into β -cells following chemically-induced diabetes upon the sole misexpression of *Pax4*.

I. *Pax4* misexpression in δ -cells induces their conversion into β -cells

Our results demonstrate that the misexpression of *Pax4* in δ -cells is sufficient to induce their conversion into functional β -like cells, further resulting in a massive insulin-producing cell hyperplasia. Indeed, we show that 2.8% of insulin⁺ cells arise from δ -cells in 2-month old transgenics, such amount increasing to 4.7% in 5-month old Sst-Cre::*Pax4*-OE animals, suggesting continuous processes of conversion of δ -cells into insulin⁺ cells. According to previous studies, near-total β -cell ablation in mice induces pancreatic δ -cells to convert into insulin-producing cells, such conversion only occurring in juveniles, before 2 months of age (Chera et al., 2014). Here, we confirm these results but also demonstrate that somatostatin-expressing

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cells retain the ability to be converted into β -like cells until at least 5 months of age. In addition, we provide evidence that δ -cell plasticity does not require β -cell ablation but the sole misexpression of *Pax4*. Finally, the absence of further augmentations in 8-months old Sst-Cre::*Pax4*-OE animals further suggest that δ -cells might lose their plasticity and ability to convert into insulin⁺ cells after 5 months of age, possibly due to aging processes.

While these results are of interest, the mechanisms by which *Pax4* induces such δ -to- β -like cell conversion remain to be elucidated. Accordingly, the identification of *Pax4* targets would provide a better understanding of the mechanisms involved. Interestingly, *Hhex* could represent a reasonable target as it is required for δ -cell specification. Surprisingly, the *Hhex* locus, in both humans and mice, appears to contain binding sites for *Pax4*. As demonstrated in this work, *Pax4* misexpression in δ -cells induce their reprogramming toward a β -like cell identity. One hypothesis could be a role for *Pax4* in *Hhex* inhibition to promote the acquisition of a β -like cell phenotype. *Hhex* expression was investigated in Sst-Cre::*Pax4*-OE transgenics but none of available *Hhex* antibodies worked in our hands. Another legitimate alternative would be *Mnx1* as its inactivation in endocrine cells induces a β -to- δ -cell conversion (Pan et al., 2015). Interestingly, the *Mnx1* locus was also found to contain binding sites for *Pax4*. One could therefore assume that *Pax4* could be an indirect or a direct activator of *Mnx1* expression, its ectopic expression in δ -cells leading to their reorientation toward a β -cell phenotype. In addition, Chera *et al.* suggested that adult δ -cells can convert into β -like cells upon FoxO1 inhibition. In all three instances, it would be of interest to investigate the involvement of these

putative targets in the processes underlying the reprogramming of δ -cells into β -like cells.

II. Recruitment of *Neurog3* expressing ductal cells and EMT reawakening

In this study, we demonstrated that a proportion of 9% of supplementary β -like cells appeared to arise from δ -cells in 2-month old Sst-Cre::Pax4-OE mice, this proportion being maintained in older transgenics. Therefore, the observed conversion rate of δ -cells misexpressing *Pax4* cannot explain such increase in islet count and size, suggestive of the involvement of additional processes.

As previously mentioned, *Neurog3* is a pro-endocrine gene only expressed in endocrine progenitors during development, and is responsible for the generation of the main endocrine cell types. Here, we demonstrate the re-expression of *Neurog3* within or close to the ductal lining epithelium upon *Pax4* misexpression in somatostatin-expressing cells. Emphasizing that *Neurog3* is responsible for the specification of all the endocrine cell lineages following specific ratios, the generation of endocrine cell types from the adult ductal epithelium would respect the standard endocrine proportions (80% of β -cells/10% of α -cells/10% of δ -cells). Our analyses of islets from animals misexpressing *Pax4* in δ -cells support this hypothesis with a clear favoring toward β -like cells neogenesis. Accordingly, our results demonstrate an increase in α -cell contents perfectly matching the progressive islet hypertrophy.

Moreover, it has been shown that during mouse pancreas morphogenesis, *Neurog3* induces the delamination of progenitors from the ductal epithelium through

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EMT processes (Gouzi et al., 2011). Interestingly, our data show a massive increase in the expression of mesenchymal markers within the ductal lining of Sst-Cre::Pax4-OE transgenics, further supporting the notion of an endocrine cell neogenesis from the ductal epithelium.

Finally, the proliferation of ductal cells and the re-expression of *Neurog3* were previously noted in regeneration processes (Criscimanna et al., 2011). While ductal cells exhibit low proliferation rates in normal conditions (Kopp et al., 2016), our analyses reveal a significant augmentation in cell proliferation within the ductal epithelium, a number of these proliferating cells eventually acquiring a mesenchymal phenotype. Together, these results clearly suggest that *Pax4* misexpression in δ -cells induces their conversion into β -like cells and a subsequent cycle of endocrine cell neogenesis through recapitulation of developmental processes.

Our analyses also demonstrate that *Pax4* misexpression in δ -cells induces their conversion into β -like cells following chemically-induced β -cell ablation, resulting in a partial β -cell mass restoration. Although not exhibiting euglycemia as expected, Sst-Cre::Pax4-OE transgenics displayed an average glycemia of 350mg/dl and an extended lifespan. Therefore, as δ -cells plasticity seems to decline with age, it would be of interest to induce β -cell ablation before 2 months of age. Accordingly, additional experiments putting to the test δ -cells plasticity at 1 month of age are currently ongoing.

Moreover, to definitively ascertain the requirement of *Neurog3* for the endocrine cell neogenesis observed in Sst-Cre::Pax4-OE transgenics, it would be of interest to use an inducible knock down of *Neurog3* within the ductal epithelium.

CONCLUSION / PERSPECTIVES

I. Somatostatin: a signal inducing β -cell regeneration?

We hypothesized that the *Pax4*-mediated conversion of somatostatin-expressing cells induces a disruption of islet homeostasis, further leading to the recruitment of *Neurog3*-reexpressing ductal precursor cells adopting an endocrine cell identity to counteract the loss of δ -cells. However, further investigations are required.

Thus, the treatment of Sst-Cre::Pax4-OE mice with a somatostatin agonist or antagonist would allow for a better comprehension of such mechanisms. More specifically, the supplementation of Sst-Cre::Pax4-OE transgenics with a somatostatin agonist is expected to prevent *Neurog3* re-expression and therefore β -like cell hyperplasia. In this context, one could wonder whether somatostatin antagonist administration to wild-type mice would induce such regeneration. However, somatostatin exerts a wide-range of effects and its inhibition could lead to undesirable side effects. In addition, the precise expression of the different SST receptors within endocrine cells remains controversial. Therefore, inhibition of one or several of these receptors could have an effect on other islet cells, provoking uncontrolled side-effects. Several studies focusing on the effects of somatostatin receptor deficiency demonstrated changes in insulin secretion and alterations in islet architecture depending on the isoform deleted. SSTR2 loss leads to increased glucagon secretion while no difference in insulin levels was observed (Strowski et al., 2000). Ablation of SSTR1 or SSTR5 mostly results in glucose intolerance with decreased numbers of somatostatin-expressing cells being observed in SSTR1 deficient mice (Wang et al., 2006; X. P. Wang et al., 2005). Remarkably, double mutant mice for SSTR1 and 5 show improved glucose tolerance, increased insulin

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secretion and islet cell hyperplasia (Wang et al., 2004). Although intriguing, understanding such results is challenging due to possible compensation in the expression of the different receptor subtypes. Moreover, the ability for somatostatin receptor to heterodimerize adds further difficulties in the interpretation (Pfeiffer et al., 2001; Wang et al., 2006; X. P. Wang et al., 2005).

In addition, the production of SST-28 was found increased in type 1 diabetic patients and a δ -cell hyperplasia was observed (Xaio Ping Wang et al., 2005). Understanding the role of SST and its receptors in the context of type 1 diabetes would therefore be of great interest. Importantly, we strongly believe that islet homeostasis is not only preserved by the hormones secreted by the different endocrine cell types, but also by cellular interactions, integrity and cohesion.

II. Model proposed

The aim of this study was to investigate the plasticity of δ -cells, aiming to eventually determine whether the sole misexpression of *Pax4* in δ -cells could induce their conversion into insulin-producing cells *in vivo*. To address this question, we generated *Sst-Cre::Pax4-OE* double transgenic animals allowing the misexpression of *Pax4* specifically in δ -cells. In this model, we demonstrated the conversion of adult somatostatin-expressing cells into insulin⁺ cells upon the sole misexpression of *Pax4*. Our data suggest that such conversion induces a disruption of islet homeostasis, leading to a reawakening of *Neurog3*-controlled endocrine differentiation program and associated developmental processes, including EMT (**Figure 25**). Importantly, we observed a progressive islet neogenesis and islet hypertrophy together with an

increase in endocrine cell numbers, these augmentations plateauing after 5 months of age.

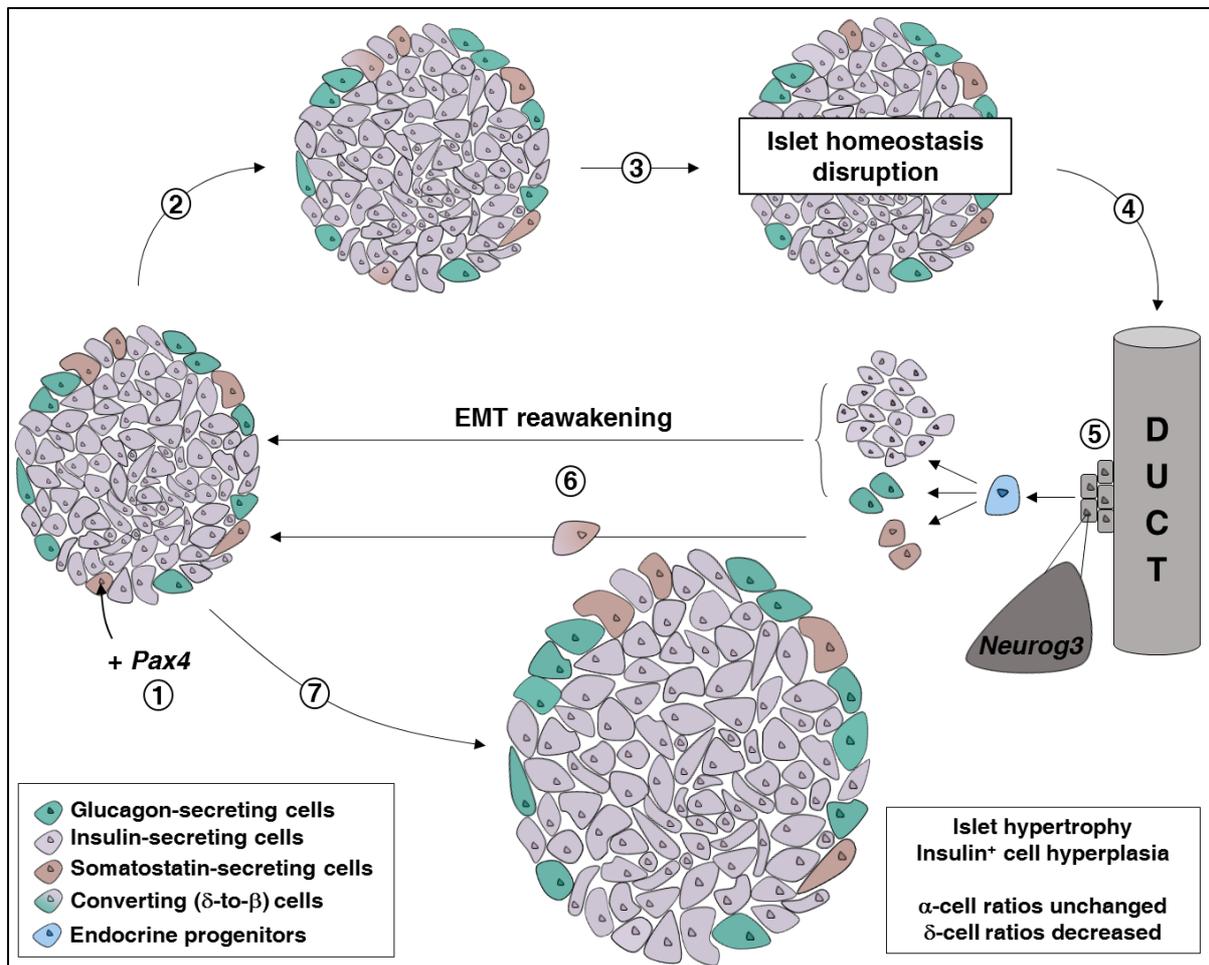


Figure 25: *Neurog3*-mediated EMT reawakening upon *Pax4* misexpression in δ -cells. Following *Pax4* misexpression in δ -cells (1), these can be converted into β -like cells (2,3). This leads to the disruption of islet homeostasis responsible for the mobilization of ductal precursor cells (4), these re-expressing the pro-endocrine gene *Neurog3* (5). *Neurog3*-endocrine progenitor cells give rise to insulin⁺, glucagon⁺ and somatostatin⁺ cells following normal endocrine endogenous proportions prior to undergo EMT (6). Such a continuous cycle of conversion/regeneration results in insulin⁺ cell hyperplasia and islet hypertrophy (7).

To conclude, these findings demonstrate a hitherto unrecognized plasticity of adult δ -cells, such cells potentially representing a new source for β -cell regeneration therapies. Considering that the misexpression of *Pax4* in α - (Al-Hasani et al., 2013; Collombat et al., 2009) and δ - cells induces their continuous regeneration and

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conversion into β -like cells, this defines *Pax4* as a considerable target for diabetes therapies.

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