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Research performed in the laboratory

Our aim is to better understand the molecular determinants of α-cell function and their potential alterations in diabetes, particularly in response to hyperglycemia and increased FFA (glucolipotoxicity). Over the last 20 years, we have focused our research on the determinants which regulate glucagon and insulin gene expression in the pancreatic islets of Langerhans. More recently we have analyzed the functional importance of specific transcription factors, such as Pax6 and Foxa1 and Foxa2, which are critical for glucagon gene expression (71-73). We have indeed attempted to better delineate the transcriptional network regulating glucagon gene expression, glucagon biosynthesis and glucagon secretion through the analyses of the gene targets of Pax6 as well as Foxa1 and Foxa2 using glucagon-producing cell lines and primary rat α cells. The α-cell transcriptional network of Pax6 and Foxa1/2 was investigated by specific inhibition of their gene expression through RNA interference experiments.

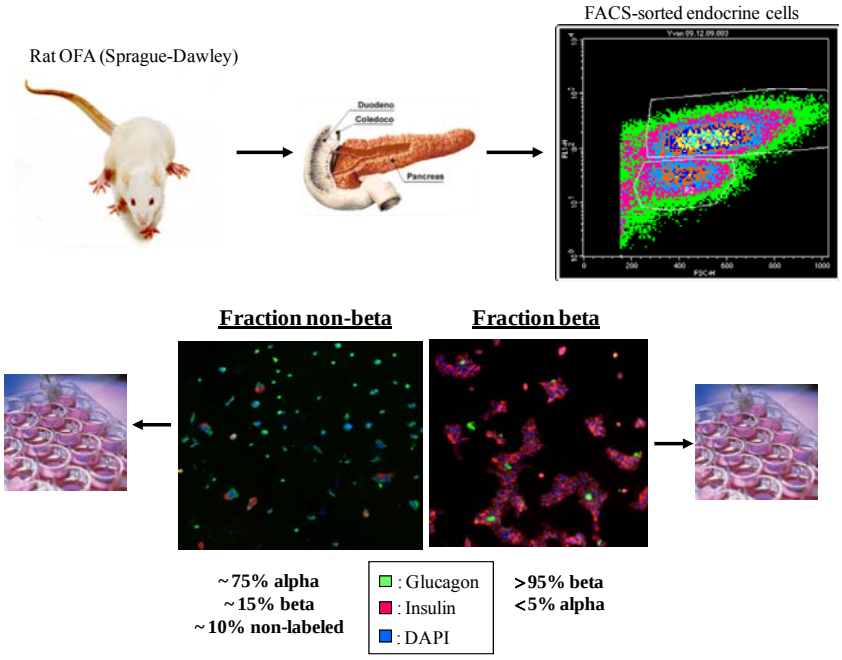


Figure 1: Rat primary α-cell model. *Primary rat α cells are prepared after islet isolation by collagenase digestion of rat pancreases followed by Ficoll purification as described (28). Non-β cells (including α cells) are separated from β cells by auto-fluorescence-activated sorting using a FACStar cell sorter. We then obtain 2 major cell populations characterized by immunocytochemistries, insulin-positive cells (upper fraction, >95 % pure) and an enriched α-cell fraction (lower fraction, 75 % +/- 5 %). We can isolate about 100'000 enriched α cells and 400'000 β cells per rat; these cells are then cultured on extracellular matrix-coated plates derived from 804 G cells (laminin-5-rich extracellular matrix).*

A- Role of Pax6 in α-cell function

The paired box homeodomain Pax6 is crucial for endocrine cell development and functions and plays an essential role in glucose homeostasis (71). Indeed, mutations of Pax6 are associated with a diabetic phenotype (74). Importantly, homozygous mutant mice for Pax6 are characterized by markedly decreased β and δ cells and absent α cells. Pax6 was previously shown to regulate the insulin, somatostatin and glucagon genes (75).

1- Pax6 controls glucagon biosynthesis

To better understand the critical role of Pax6 in glucagon-producing cells, we developed Pax6-deficient models using primary rat α cells and stable clones of InR1G9 glucagon-producing cells expressing constitutively a dominant-negative form of Pax6. To study the transcriptional network of Pax6 in adult and differentiated α cells, we generated Pax6-deficient primary rat α cells using specific small interfering RNA (siRNA). We found that Pax6 controls the transcription of the Proglucagon and processing enzyme PC2 and 7B2 genes (76,77). Prohormone convertase 2 (PC2) is

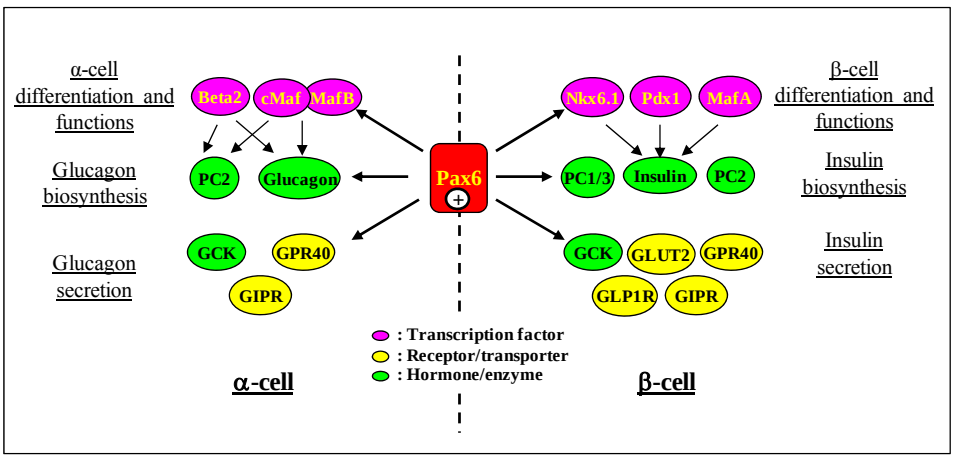
the main processing enzyme in pancreatic α cells, where it processes pro-glucagon to produce glucagon under the spatial and temporal control of 7B2, which functions as a molecular chaperone. Pax6 is thus critical for glucagon biosynthesis and processing by activation on the glucagon gene promoter, as well as the PC2 and 7B2 genes. We also identified in rat primary α cells three new target genes coding for MafB, cMaf and Beta2 (28), which are all critical for glucagon gene transcription and α-cell differentiation. Finally our results suggest that the critical role of Pax6 action on α-cell differentiation is independent of those of Arx and Foxa2, two transcription factors that are necessary for α-cell development (73,78). We conclude that Pax6 is critical for α-cell function and differentiation through the transcriptional control of key genes involved in glucagon gene transcription, proglucagon processing and α-cell differentiation.

2- Pax6 controls glucagon secretion

We then investigated the role of Pax6 in glucagon secretion using Pax6-deficient primary rat α cells. We first showed that Pax6 knockdown decreases glucagon release (16). The decrease of Pax6 affects specifically acute glucagon secretion in primary α-cells in response to glucose, palmitate, and glucose-dependent insulinotropic peptide (GIP) but not the response to arginine and epinephrine. We identified genes involved in glucagon secretion such as the glucokinase (GCK), G protein-coupled receptor 40 (GPR40), and GIP receptor (GIPR) genes as well as the corresponding proteins which were significantly decreased whereas the insulin receptor (IR), Kir6.2/Sur1 and glucose transporter 1 (Glut1) genes were not affected. Through in vitro experiments, we demonstrated that Pax6 acts on the regulation of glucagon secretion at least through the transcriptional control of GCK, GPR40 and GIPR (16). We propose that Pax6 is not only critical for glucagon biosynthesis but also for glucagon secretion particularly in response to nutrients.

3- Pax6 is critical for β-cell function

Since mutations of Pax6 are associated with a diabetic phenotype and a drastic decrease of insulin-positive cell number, we also wanted to analyze the gene targets of Pax6 in β cells and compare the functional role of this transcription factor in both α and β cells. Our aim was to better define the β-cell Pax6 transcriptional network and thus provide further information concerning the role of Pax6 in β-cell function. We generated a Pax6-deficient β-cell model using rat primary β cells (Figure 1) with siRNA leading to a 75% knockdown of Pax6 expression (79). Through a candidate gene approach, we confirmed that Pax6 controls the mRNA levels of the insulin 1 and 2, Pdx1, MafA, GLUT2 and PC1/3 gene in β-cells. Importantly, we identified new β-cell Pax6 target genes coding for GCK, Nkx6.1, cMaf, PC2, GLP-1 and GIP receptors (GLP-1R and GIPR) which are involved in β-cell function. We also demonstrated that Pax6 knockdown led to decreases in insulin cell content, in insulin processing and a specific defect of glucose-induced insulin secretion as well as a significant reduction of GLP-1 action in rat primary β-cells. Our results strongly suggest that Pax6 is crucial for β-cells through transcriptional control of key genes coding for proteins that are involved in insulin biosynthesis and secretion as well as glucose and incretin actions on β-cells.



Our work demonstrate that Pax6 is critical for α- and β- cell functions through the control of glucagon and insulin biosynthesis as well as nutrients and incretin's effects on

glucagon and insulin secretion.

B- Role of Foxa1 and Foxa2 in α-cell function

The Foxa group of the Forkhead family comprising Foxa1, Foxa2 and Foxa3 is essential for the regulation of hepatocyte-specific expression of several target genes (80). They share a high homology in the Forkhead box protein sequences. Expressed in the embryonic endoderm, the Foxa genes contribute to the specification of the pancreas and have a great importance in metabolic regulation. They may be required for the regulation of the glucagon gene in pancreatic α cells. Indeed, gene ablation studies revealed a critical role for Foxa1 and Foxa2 in the regulation of glucose homeostasis and pancreatic islet function. In fact, Foxa1-deficient mice have a 70 % decrease in circulating glucagon levels (72). Foxa2 has a critical role in the regulation of the terminal differentiation steps and maturation of glucagon-producing α cells inasmuch as Foxa2-deficient mice have no α cells (73). By contrast, no essential function of Foxa3 for the control of islet or intestinal proglucagon gene expression has been found. Glucagon secretion is comparable between wild-type and Foxa3-deficient mice (81). Foxa1 and Foxa2 have critical roles in glucose homeostasis both in the liver and pancreas by activating genes coding for proteins involved in maintaining glucose homeostasis and protection against hypoglycemia. Among these genes, Foxa proteins activate the glucagon gene in α-cell lines and are, along with Pax6, the major activators of proglucagon gene transcription.

Our aim has been to investigate the role of Foxa1 and Foxa2 in α cells and to define their target genes to better understand their respective effects in the function and dysfunction of the pancreatic α cells.

We first wanted to study the role of Foxa1/Foxa2 in glucagon gene transcription and precisely identify their cognate DNA elements. We found that Foxa1, Foxa2 and Foxa3 are all expressed in α and β cells of the endocrine pancreas. In vivo, both factors bind to the promoter. In vitro experiments revealed that both Foxa1 and Foxa2 are independent activators of glucagon gene transcription acting only through G2; in addition, we found that Foxa1 does not regulate Foxa2 gene expression and vice versa. Our studies thus indicate that both Foxa1 and Foxa2, independently, are critical regulators of glucagon gene transcription through G2. Along with other studies in the liver, these data strengthen the concept that Foxa1 and Foxa2 are involved in glucose homeostasis and in the protection against hypoglycemia.

Similarly to the work already presented for the characterization of Pax6-target genes, we hypothesized that Foxa1 and Foxa2 may regulate directly or indirectly a number of genes which are important for α-cell differentiation and function. We selected the same list of genes studied for the Pax6 experiments, but also genes which have been shown in very elegant studies to be regulated by Foxa2 in β cells (82,83). Foxa2 is essential for the physiology of mature β-cells, the oscillation of nutrients-stimulated Ca^{2+} and the machinery of exocytosis. Indeed, inducible gene ablation of Foxa2 in mature β cells induces hyperinsulinemic hypoglycemia. The mutant β cells exhibits a markedly increased pool of docked insulin granules, engaged in exocytosis, consistent with an increased first phase glucose-stimulated insulin secretion. Multiples genes coding for enzymes, ion channels or receptors, such as the genes coding for the subunits of ATP-dependent K^+ channel Kir6.2 and Sur1, GCK, hexokinase I and II, G-protein-coupled receptor 43 (GPR43), short-chain L-3-hydroxyacyl-coenzyme A dehydrogenase (Hadhsc), dopa decarboxylase (Ddc), deoxycytidine kinase (Dck) and pyruvate dehydrogenase kinase 1 (Pdk1) as well as genes involved in vesicular trafficking, membrane targeting and fuel-dependent secretion pathways, such as chromogranin B, membrane targeting C2 domain (Mtac2d1), myosin heavy chain 10 8Myh10), vesicle-associated membrane protein B (Vapb), RAS guanyl releasing protein 1 (Rasgrp1), and Rab6 are affected by Foxa2 deficiency (83,84).

Since Foxa2 is involved in the protection of hypoglycemia acting both on the β cell, by refraining insulin secretion, but also on the α cell by activating glucagon gene transcription, we hypothesized that Foxa2 may also affect the glucagon secretory pathways by activating rather than repressing genes coding for proteins involved in the regulation of secretion through ion channels, receptors, enzymes and docking proteins in a mirror image compared to β cells. To assess this hypothesis, we used Foxa1/2-deficient α cells using siRNAs against Foxa1 and Foxa2 in rat primary α cells.

The following genes were found to be down-regulated with either Foxa1 or Foxa2 gene inhibition: Glucagon and PC2 (glucagon biosynthesis), Kir6.2 and Sur1 (glucagon secretion),Arx, MafB, HNF4α, Nkx2.2, Isl1 and Brain 4 (α-cell differentiation and function).

We also selected genes shown to be Foxa2 targets in β-cells, such as the AKAP1, MYH10, RAB6 and VAPB genes. However, none of these genes were found to be targets of Foxa2 in α cells. These results indicate that Foxa2 regulates different genes in α and β cells, although some genes, such as Nkx2.2, Kir6.2 and Sur1, HNF4α are targets of Foxa2 in both cell types ***(Manuscript in preparation)***.

C- Role of sumoylation in α-cell function

To better understand the differential effects of glucose and Foxa1/2 on α- and β-cell functions, we studied the role of protein sumoylation, previously shown to be a "brake" of insulin secretion particularly in response to glucose. ***We thus attempted to study the implications of sumoylation in the regulation of key genes involved in α- and β-cell functions and observe whether differential sumoylations between these two cell types could explain the opposite response from the same stimuli.***

Sumoylation of specific proteins in β cells such as MafA, Pdx1 as well as the GLP-1 receptor and synaptatogamin VII are associated with the control of insulin secretion and hypersumoylation leads to a decrease of insulin release and specific alterations of the glucose response. By contrast to β cells, our preliminary results in α cells suggest that the consequence of sumoylation leads to an increase of glucagon secretion. We performed in vitro secretion tests on rat primary FACS-sorted α and β cells in the presence or absence of NEM (N-ethylmaleimide, pre-incubation of 30 mins) known to stabilize the sumoylated and ubiquitinated forms of proteins mimicking a hypersumoylated and hyperubiquitinated state. The results obtained for glucose-induced insulin secretion were consistent with the results obtained when Sumo1 was over-expressed in insulin cell lines (21), reflecting a negative regulation by sumoylation on the glucose-induced insulin secretion at 16,7mM glucose. The NEM pretreated α cells showed a significant increase in glucagon secretion at 1mM glucose and no inhibitory effect of glucose (7mM) was observed. These results point out to a differential regulation of α- and β-cell functions by sumoylation. We are currently employing a specific strategy to assess the implication of sumoylation on α-cell function by targeting specifically Sumo1, 2 and 3 using siRNAs.

The importance of sumoylation on β-cell physiology is becoming clearer and its possible implication in diabetes leads us to address the question of the importance of this modification on glucagon-secreting α cell functions as well as its potential role in α-cell alterations observed in diabetes.