NON-BETA-CELL PROGENITORS IN PREGNANT MICE AND THE ORIGIN AND FUNCTIONALITY OF BETA-CELLS AFTER DIABETIC RECOVERY IN A C-MYC ABLATION MODEL

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Declaration

The results presented in this thesis were obtained by the author, unless specifically indicated in the text.

The use of animals in this study has been approved under the Animals (Scientific Procedures) Act 1986 (Project Licence number: 40/2675). The author is also in possession of a personal individual licence (No. PIL 40/7545) to carry out regulated procedures on living animals under the aforementioned project licence.

All sources of information have been acknowledged by means of reference.

None of the work contained in this thesis has been previously used to apply for a degree.

Summary

The debate regarding the contribution of adult stem/progenitor cells during normal growth and beta-cell regeneration is far from being resolved. Therefore, we addressed in two distinct situations the origin of new beta-cells. We exploited a Cre/loxP lineage tracing system to efficiently label beta-cells in double transgenic mice (Z/AP; RIPCreER^{TAM}) to address the origin of new beta-cell during the beta-cell mass expansion in response to one and two pregnancies. Similarly, we examined origin of new beta-cell after diabetic recovery in triple transgenic mouse (Z/AP; RIPCreER^{TAM}; pIns-c-MycER^{TAM}). Finally we evaluated the functionality of regenerated beta-cells after diabetic recovery in the single pIns-c-MycER^{TAM} mouse model by microfluorimetry, in collaboration with Dr P. Squires. We showed that the beta-cell functionality in the pIns-c-MycER^{TAM} line was abnormal. Second, we showed that the human placental alkaline phosphatase label (HPAP) in the double and triple transgenic mice was 1) specific to beta-cells, 2) irreversible and heritable and 3) tamoxifen dose-dependant. Third, the analysis of the proportion of beta-cells labelled for HPAP in one pregnancy, showed that the HPAP labelling index of the non-pregnant animals (0.44±0.05) was greater that in the pregnant group (0.33±0.06),(paired two-tailed t-test, P-value 0.021), indicating a dilution of the label in pregnant animal pancreata. Furthermore the combined results of the mean HPAP labelling index in non-pregnant animals (0.44±0.12) and pregnant animals (0.33±0.09) in one and two pregnancies reinforced our results above by indicating that the difference between the two groups was considered extremely significant (paired, two-sided student t-test, P-value 0.0007). Likewise, we showed that two to three months after the tamoxifen pulse, beta-cells do not fully lose differentiation or transdifferentiate into other lineages of either endocrine or exocrine compartment. In conclusion, we demonstrated for the first time that non-beta-cell progenitors contribute significantly to the increase of the beta-cell mass in response to pregnancy in combination with pre-existing beta-cell self-duplication.

Abbreviations

$[Ca^{2+}]_i$: intracellular free Ca^{2+} concentration

Cre: Cre-recombinase

DAPI: 4',6-diamidine-2-phenyl indole

E. X: Embryonic day X

ER: Oestrogen receptor

FCS: Foetal calf serum

FITC: Fluorescein isothiocyanate

Glut2: glucose transporter-2

HBSS: Hanks balanced salt solution

hESC: Human embryonic stem cell

HPAP/AP: Human placental alkaline phosphatase

HSP: Heat shock protein

iPS: Induced pluripotent stem

IPGTT: Intraperitoneal glucose tolerance test

Lac Z: Beta-galactosidase

Ngn3: Neurogenin 3

4-OHT: 4-hydroxytamoxifen

Pdx1: Pancreatic duodenal homeobox 1

RIP: Rat insulin promoter

TAM/TM: Tamoxifen

CHAPTER 1

INTRODUCTION

Introduction

Diabetes mellitus is characterized by abnormal glucose homeostasis (namely elevated blood glucose), which is associated with cardiovascular disease, retinopathy, neuropathy and nephropathy. It is estimated to affect currently 150 million people worldwide and this figure is predicted to double by year 2025 (Prentki and Nolan, 2006). Two major types of diabetes are recognised, type 1 results from autoimmune destruction of beta-cells and type 2 (representing 90% of cases) resulting from a combination of impaired peripheral action of insulin (insulin resistance) and inadequate compensatory adaptation of beta-cells resulting at the later stage of the disease to a significant decrease of beta-cell mass.

Currently available drugs do not prevent the decline in beta-cell mass and function so that most patients will require increased doses of drugs and eventually insulin to control their blood glucose levels. In fact, at present the only hope is to prevent diabetes by encouraging healthy eating, weight control and exercise or, in established diabetes, to replace missing beta-cells by transplantation. Islet transplantation is proven to normalize patient's blood glucose level for several years. Unfortunately the shortage of donor human pancreatic islets severely limits the number of diabetic people able to benefit from it.

Therefore, faced with such a rising problem, a large research effort is directed at developing alternative sources of beta-cells for transplantation or to develop new strategies to maintain/restore the endogenous pancreas beta-cell mass. At least in some respects, regeneration of beta-cells in the adult recapitulates elements of beta-cell developmental ontogeny. So first, I will describe what is known about pancreas development and second how this knowledge can be translated to ex and in vivo strategies to artificially yield unlimited sources for new beta-cells. Finally, I will introduce the relevance of my research in relation to the debate concerning the elusive adult pancreatic progenitor or stem cell.

1.1 Mouse pancreas and beta-cell development

The growing interest for pancreas development derives from diseases related to this organ such as diabetes and to a lesser extent, cancer. Pancreas development represents an experimental system to investigate cellular and molecular pathways involved in pancreas and beta-cell ontology during normal conditions. Knowledge gained from this model could be applied to regenerate or stimulate an endogenous functional beta-cell mass in patients, to direct glucose-responsiveness beta-cell-like phenotype formation from embryonic cells or other cell types. Likewise, it could allow understanding mechanisms responsible for pancreas homeostasis or beta-cell regulation during development and postnatal life. Because Piper et al. (Piper et *al*., 2004) reported that human pancreas development resembles that of mouse, we will describe processes regulating pancreas development specifically in the mouse owing to the great amount of available information provided from this species in this field. Even though, Bonner-Weir et *al*. (Bonner-Weir et *al*., 2004) mentioned some differences in major pathways in mouse and human for new beta-cells in postnatal life.

1.1.1 Pancreas anatomy and function

The pancreas is composed of three integrated tissue-types, which are the exocrine, endocrine and ductal components. The exocrine compartment forms the bulk of this organ, around ninety to ninety-five percent of the pancreatic mass, and it is organized into acini which develop at the termini of ducts. The exocrine compartment is a branched, lobulated organ in which cells grouped in the acinus secrete digestive enzymes important for food processing in the gut (lipases, proteases, nucleases) in an inactive form in the intestine via an elaborated pancreatic ductal network. Duct cells secrete bicarbonate ions and electrolytes. These enzymes become active only in the digestive tract, and the secretory process of the acini is tightly regulated by hormone stimulation. The endocrine gland, by contrast, represents one to two percent of the organ, is organized into islets of Langerhans which are compact globular clusters of cells embedded in the exocrine component. Five endocrine cell-types compose the islets of Langherans: beta-cells, secreting insulin and also an insulin antagonist amylin, alpha-cells, secreting glucagon, delta-

cells, secreting somatostatin, PP-cells, secreting pancreatic polypeptide (PP), and the recently described epsilon-cells producing ghrelin. Overall, beta-cells form the core of the islets, alpha-cells are located at the periphery of the islets, while somatostatin and PP-cells are scattered within or at the mantle of islets. Insulin has a binary action, it possesses both mitogenic and metabolic (specifically by promoting glucose uptake) effects on its target tissues (Slack, 1995, Murtaugh, 2007, Collombat et al., 2006).

1.1.2 Pancreas ontology

The pancreas is a complex organ composed of exocrine and endocrine tissues that fulfil distinct functions. The processes coordinating pancreagenesis imply complex molecular interplays which are becoming understood due to identification of key signalling pathways and transcription factors controlling pancreatic development.

1.1.2.1 Outline of mouse pancreas development

During mammalian development (Figure 1), the definitive endoderm, in addition to the two others germ layers (i.e. ectoderm and mesoderm) is formed and specified during gastrulation, in an area named the primitive streak. Molecular factors such as Wnt/beta-catenin, Nodal, GATA-4/6, FoxA2 (forkhead box A2), Sox17 (SRY-box containing gene 17), and Mix are required for definitive endoderm formation. The definitive endoderm subsequently develops into the primitive foregut epithelium which will become committed to a pancreatic fate around E.8.5 (embryonic day eight) with the expression of the pancreatic duodenal homeobox 1 (Pdx1) transcription factor. The thickening region of the posterior foregut endoderm, around E.9-9.5, gives rise dorsally and ventrally to one and two buds respectively in the mouse embryo. But later, one of the ventral primordia will regress. Subsequently as embryogenesis proceeds, the two pancreatic buds expand, and branch into the surrounding mesenchyme. Then consecutive rounds of branching, further growth and differentiation result in the formation of a fully mature single organ prior to birth, the

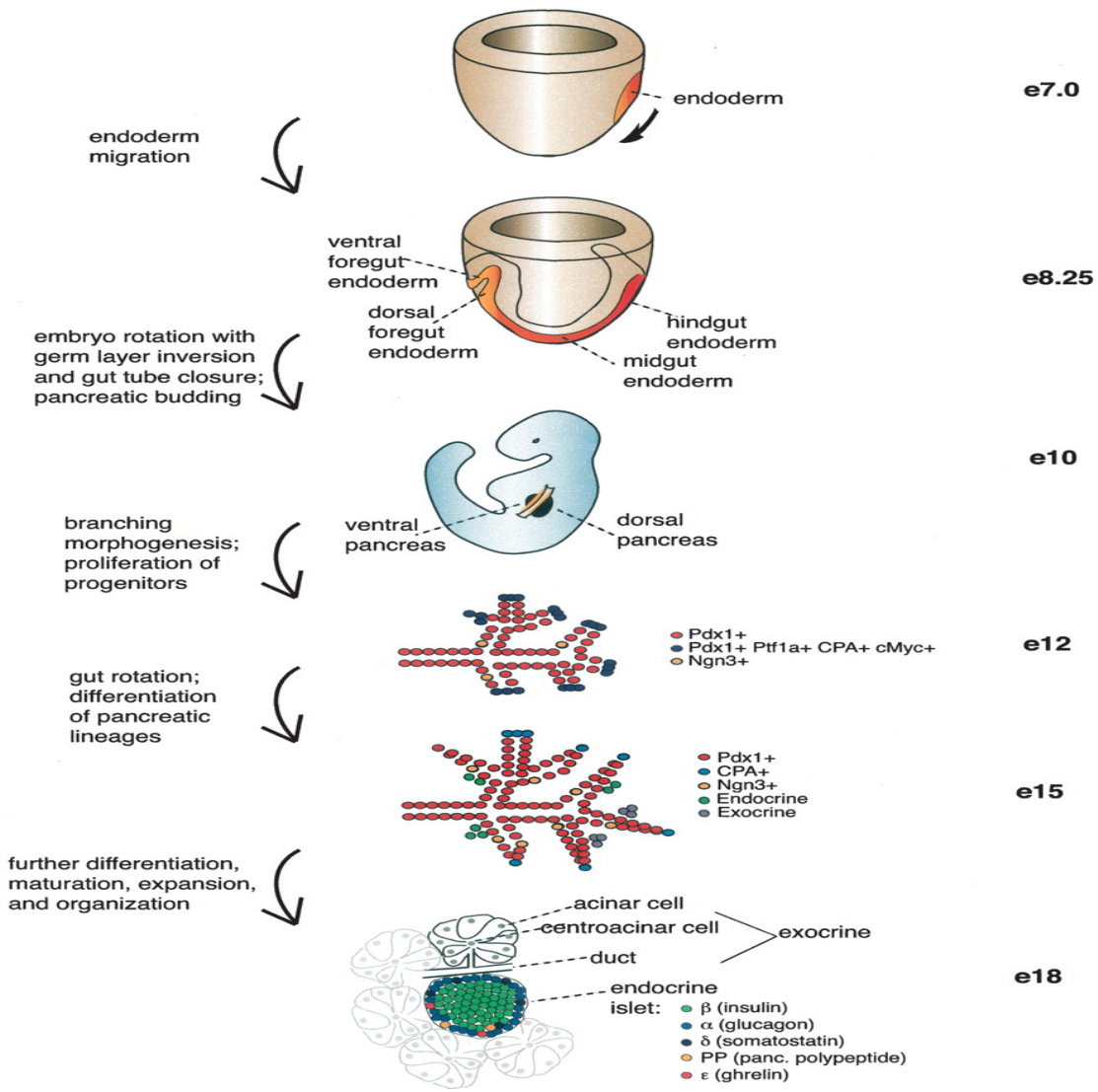


Figure 1 **Overview of mouse pancreatic organogenesis**. Mouse embryonic development is shown from early primitive streak stage (E.7) through endoderm migration and specification, pancreatic budding, branching morphogenesis, and differentiation of pancreatic lineages. Multipotent progenitors that give rise to all pancreatic lineages express Pdx1, Ptf1a, CPA, and c-Myc in early pancreas development, while exocrine progenitors express CPA during mid-pancreatic development. At all stages, endocrine progenitors express Ngn3. (Oliver-Krasinski and Stoffers, 2008).

ventral and dorsal pancreas having been brought into contact by the rotation of the stomach and duodenal region at E.12.5 (Slack, 1995, Murtaugh, 2007, Zhou et *al*., 2007, Murtaugh and Melton, 2003, Offield et *al*., 1996, Jensen, 2004, Oliver-Krasinski and Stoffers, 2008, Guo and Hebrok, 2009, Ackermann and Gannon, 2007)

At the earliest stage of pancreagenesis called "the primary transition", around E.9.5, epithelial cells express endocrine hormones but the glucagon secreting-cells are certainly the most predominant. We note in passing that the order of appearance of the endocrine lineages during the early stage of pancreatic development is still uncertain (Herrera et *al*., 1991, Gittes and Rutter, 1992, Herrera, 2000). Subsequently, during the period called "the second transition", around E13.5-15.5, endocrine cells of the five endocrine lineages differentiate and proliferate exponentially, and beta-cells secreting insulin become the majority lineage from this stage onwards. Then, the new endocrine cells detach from the epithelium to aggregate into islet clusters. Deltour et *al*. (Deltour et *al*., 1991) have demonstrated that these endocrine cells composing the islets are of polyclonal origin. What's more, during this time-window, amylase-expressing acinar cells begin to differentiate. At the final stage of islet formation and maturation, vascularisation and innervation of the islets are observed. In addition to key transcription factors required for pancreatic growth, morphogenesis and differentiation during development, soluble and membrane-bound factors from the surrounding tissue are crucial for directing these cascades of events; particularly the interplays between the mesenchyme and the pancreatic epithelium (Zhou et *al*., 2007, Murtaugh and Melton, 2003, Maestro et *al*., 2003, Oliver-Krasinski and Stoffers, 2008, Guo and Hebrok, 2009, Edlund, 2002).

A recent study from Zhou and collaborators (Zhou et *al*., 2007) reported multipotent progenitors positive for Pdx1, Ptf1a, c-Myc and carboxipeptidase A1 (CPA1). They are localized at the tips of the epithelium branches within the early pancreatic primordia, while the trunks of the branches are formed of endocrine and ductal cells (Figure 1 and 2). The authors have shown, by cell-lineage tracing, that this multipotent progenitor, positive for CPA1, gives rise to all pancreatic cell types. Of note, CPA1 during postnatal life is restricted to acinar cells. Subsequently, the proliferation of the multipotent progenitors positive for CPA1 at the tips of branches, leads to the elongation of the finger-like epithelium

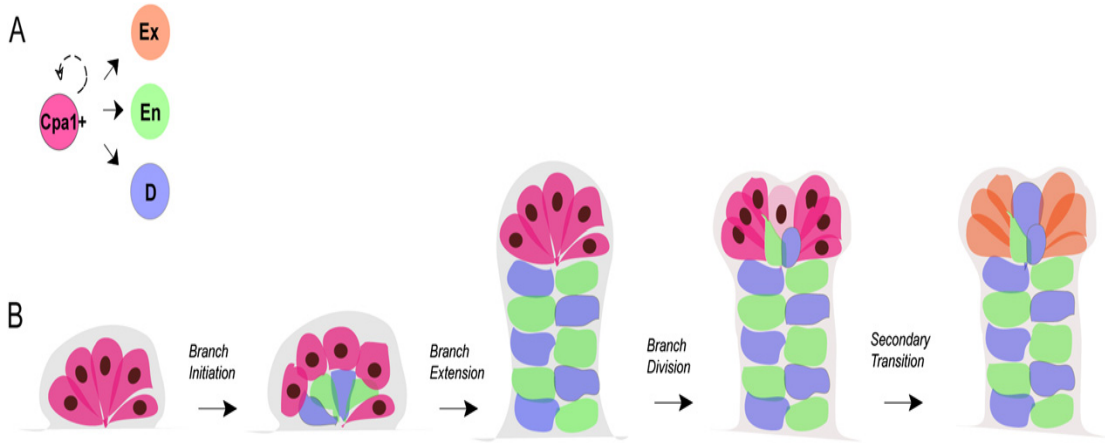


Figure 2 **Multipotent Progenitors Guide Pancreatic Organogenesis**.(A) CPA1^{+} multipotent progenitors give rise to exocrine, endocrine, and duct cells in vivo and may undergo limited self-renewal. (B) Early pancreatic buds are composed primarily of multipotent progenitors. At the onset of branching morphogenesis (E.12), the multipotent cells may divide asymmetrically such that they are propelled away from the centre of the pancreatic buds, thus producing branches. Continued fast proliferation and differentiation of these progenitors into endocrine and duct cells generate the trunk of the branches. When the branching tip divides, CPA1 is down regulated in the cleft region. Around E.14, CPA1^{+} tip cells restrict to exocrine fate during the secondary transition (Zhou et *al*., 2007).

(the branch) within the pancreatic anlagen, and the cells left behind the tips become more differentiated and later will be converted into endocrine and ductal cells which make up the trunks of the branches. Ultimately, the endocrine cells differentiate and migrate away from the epithelium, leaving the trunks to be composed entirely of duct cells. Then, during midgestation, at a developmental stage termed "second transition", the multipotent progenitors confined to the branch tips differentiate into exocrine cells.

1.1.2.2 Pancreas and beta-cell development

Key transcription factors critical for pancreas organogenesis at different stages of development have been defined by genetic lineage tracing methods and the use of mutant animals for the loss and gain of function studies of these transcription factor genes. Thus, it has been shown that the transcription factors Pdx1 and Ptf1a are expressed in cells that are progenitors giving rise to all pancreatic cell types (Offield et *al*., 1996, Kawaguchi et *al*., 2002, Ahlgren et *al*., 1996). Pdx1 and Ptf1a are expressed at the early stage of pancreas development around E.8.5 and E.10.5 respectively and are important for pancreatic specification, and subsequently for pancreatic growth and differentiation of all pancreatic lineages. Kawaguchi and collaborators have shown, by lineage tracing, that Ptf1a is required not only for the establishment of the exocrine lineage but for all pancreatic cell types. Likewise, Offield et *al*. and Ahlgren et *al*. (Offield et *al*., 1996, Ahlgren et *al.*, 1996) reported that Pdx1 was not essential for the initial pancreatic bud formation since the *Pdx1*$^{-/-}$ null mice displayed a rudimentary pancreatic anlage, but it is required rather for the specification of the early pancreatic epithelium and subsequently for pancreatic growth, proliferation and differentiation of all pancreatic lineages. Thus the requirement of other factors upstream of Pdx1 is critical for the initiation of pancreas organogenesis (Oliver-Krasinski and Stoffers, 2008, Edlund, 2002). These studies as well suggest that the programs governing dorsal and ventral pancreatic formation have distinct pathway requirements (Kumar and Melton, 2003, Guo and Hebrok, 2009).

Examination of *Ngn3*-null mice combined with lineage tracing studies has revealed that neurogenin3 (Ngn3) is a key transcription factor required for endocrine cell differentiation (Guo and Hebrok, 2009, Gradwohl et *al*., 2000, Schwitzgebel et *al*., 2000). It is transiently expressed during development (it peaks at E.15.5), it is positively regulated by Hfn6 (Hepatic Nuclear Factor6) (Maestro et *al*., 2003), and its expression marks endocrine precursor cells that give rise to all islet cells, but this progenitor cell is not self-renewing. Ngn3-expressing cells characterize the immediate precursors for the endocrine cells in the developing organ. Experiments in which Ngn3 was misexpressed have demonstrated that this transcription factor per se is not sufficient to induce the differentiation of beta-cells. Therefore additional signals are required to induce the beta-cell lineage (Schwitzgebel et *al.*, 2000). Indeed, Ngn3 activates a set of genes including Isl1 (Lim homeodomain factor), neurod1 (neurogenic differentiation1) and Insm1/IA1 (insulinoma-associated1) that are essential for the development of islet cell lineages. Then, a combination of additional factors expressed in endocrine precursors further restricts the latter into a specific endocrine lineage. Pax4 (pair box gene 4) and Arx (aristaless related homeobox) are Ngn3 target genes, and precursor cells that express either Pax4 or Arx later will develop into beta-cells or alpha-cells respectively (Collombat et *al*., 2003). Thus, beta-cell specification and maturation requires additional transcription factors, including Pax4, Pax6, Nkx2.2 , Nkx6.1, MafB, Pdx1, Hlxb9 reviewed by Murtaugh and Melton, 2003, Guo and Hebrok, 2009, Spence and Wells, 2007, Collombat et *al*., 2006 and presented in figure 3. A model becomes apparent regarding the role of the bi-potential Pdx1 progenitor cells. In the presence of notch signalling and mitogenic signals, either the Pdx1 progenitor pool grows and multiplies to be available later for endocrine and exocrine differentiation, or if it escapes these factors, then Ngn3 is up-regulated and this progenitor undergoes endocrine differentiation (Murtaugh and Melton, 2003, Ackermann and Gannon, 2007). Finally, Gu and colleagues (Gu et *al*., 2002) demonstrated by a lineage tracing method, that Ngn3-expressing cells function also as islet-progenitors in adult mice during renewal and maintenance, recapitulating in this respect the mechanism used to produce endocrine cells during pancreas organogenesis.

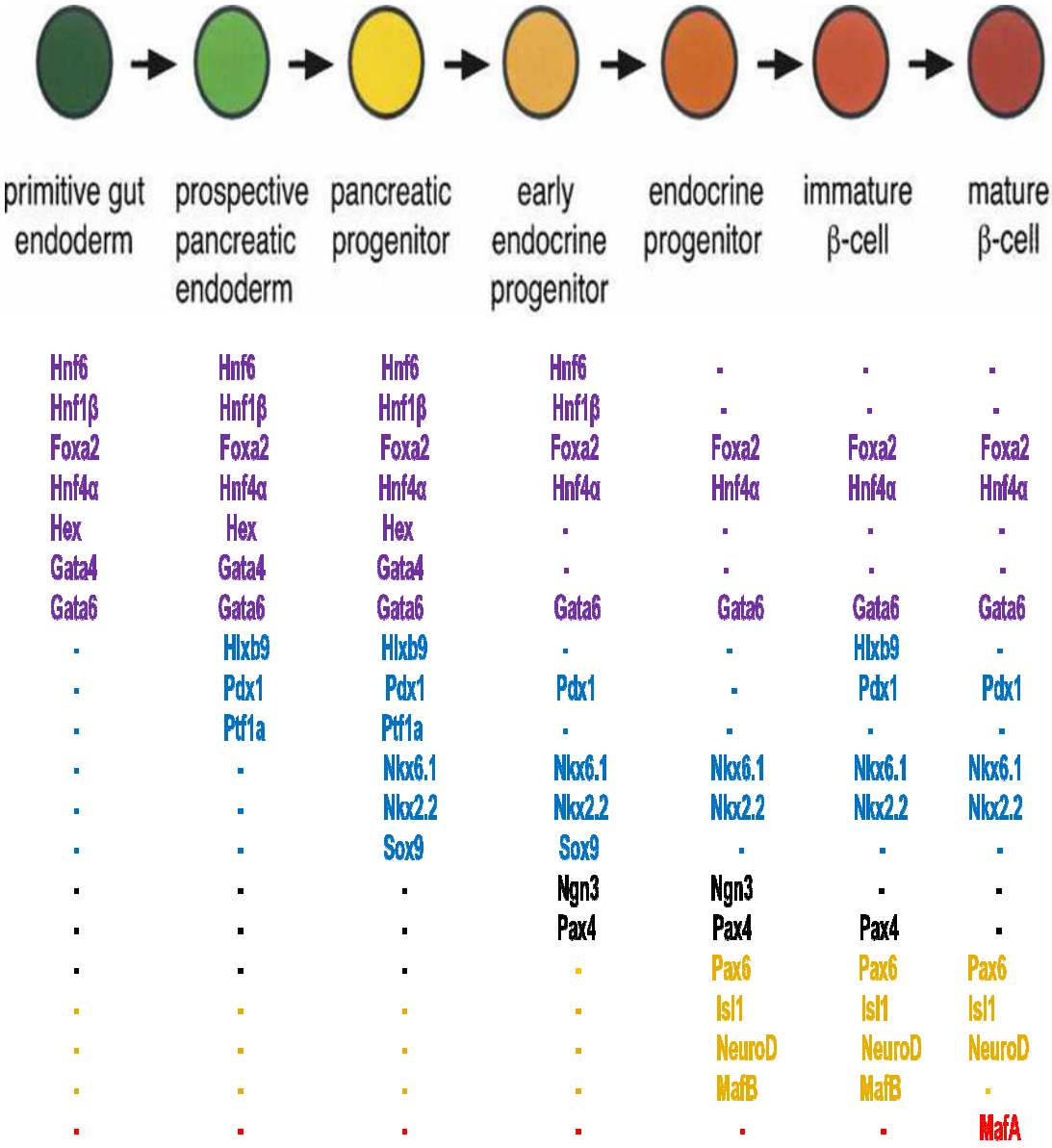


Figure 3 **Transcription factor profile during stages of cell formation from endodermal derivatives.** The diagram indicates transcription factors expressed at each stage of differentiation. Factors initially expressed at a particular stage are colour-coded as follows: gut endoderm (purple), pancreatic endoderm progenitor (blue), early endocrine progenitor (black), endocrine progenitor (orange), cell (red). (Adapted from Oliver-Krasinski and Stoffers, 2008).

1.1.3 Signalling pathways and transcription factors in the developing pancreas

Studies dedicated to the appreciation of events directing pancreagenesis have demonstrated that the presence of signal transduction molecules and transcription factors in tissues prefigure the processes that govern pancreas development. Thus, spatio-temporal expression of key transcription factors and signalling pathways can be an indicator predicting the fate of undifferentiated cells in the developing pancreas. In the following, I will describe the main transcription factors and transduction pathways important for pancreatic organogenesis because they could be instrumental in understanding how to generate ex-vivo beta-cell-like cells.

1.1.3.1 Signalling pathways

Pancreas development is conducted by extracellular factors that are part of signalling pathways, and which are secreted by the surrounding environment. Thus, factors secreted by mesodermal tissues such as the notochord and, later, the dorsal aorta are critical for dorsal pancreatic specification. The lateral plate mesoderm that is positioned underneath the presumptive ventral pancreatic endoderm provides factors that initiate the ventral pancreatic region, likewise the dorsal aorta or endothelial cells (Kumar and Melton, 2003). Many signalling pathways including Hedgehog, Fgf (fibroblast growth factor), Notch, Wnt/beta-catenin, and TGF-beta (transforming growth factor) transduction pathways, regulate important phases of pancreas development. I will describe succinctly their functions, although this list is not exhaustive, reviewed by authors in brackets (Oliver-Krasinski and Stoffers, 2008, Murtaugh and Melton, 2003, Guo and Hebrok, 2009, Edlund, 1998, Edlund, 2002, Kim and Hebrok, 2001, Dhawan et *al*., 2007, Spence and Wells, 2007).

<u>Hedgehog signalling</u>

Studies of members of the Hedgehog family (interacting with the Patched receptor), particularly sonic hedgehog, have shown that the expression of the latter is excluded from the dorsal and ventral pancreatic anlagen. This exclusion by inhibition of sonic hedgehog within the two anlagen is crucial for Pdx1 expression and the

specification of both pancreatic primordia, at an early stage of pancreas organogenesis. The dorsal endoderm at an early stage of gut formation is normally in contact with the notochord, a transitory mesodermal structure that secretes soluble factors such as members of TGF/ activin and Fgf family, before the two structures will be separated around E.8.5 by the midline fusion of the paired dorsal aortas. And these notochord-derived-signals (including activin beta B and Fgf2) prevent sonic hedgehog expression. Since the notochord is not in close proximity to the ventral part of the endoderm, these signals blocking hedgehog expression in the ventral part of the primitive gut might be secreted by other tissues, such as the lateral plate mesoderm. By repressing sonic hedgehog, activin and Fgf induce expression of pancreatic genes. Thus the down regulation of sonic hedgehog before pancreas organogenesis later inhibits intestinal differentiation and promotes pancreatic fate in the posterior foregut endoderm.

Endothelial signals

Aorta-derived endothelial cells in contact with dorsal endoderm induce in this region, the expression of Ptf1a and maintain Pdx1 expression. Thus, the endothelium has a critical role for pancreatic specification and beta-cell differentiation and proliferation.

Fgf signalling

In addition to Fgf2, members of the Fgf family play an active role in initiating the pancreatic program. Fgf10 expressed by the dorsal and ventral mesenchyme from E.9.5 to E.12.5 is also involved in pancreatic specification by promoting expression of Pdx1 and the maintenance of Ptf1a. During later stages, Fgf10 stimulates division of pancreatic progenitors positive for Pdx1, and consequently inhibits their differentiation into endocrine cells, through activation of Notch signalling and Hes1 (hairy and enhancer of split family) expression. Thus, this preserves the pancreatic progenitor pool for early differentiation. Studies focused on the Fgf signalling will help deciphering the mesenchyme-to-epithelium critical signalling for pancreatic growth, morphogenesis and differentiation.

Notch signalling

Interaction of the membrane-bound ligand Delta or Jagged/Serrate with the Notch receptor on neighbouring cells leads to cleavage of the Notch intracellular domain (NICD) resulting in its translocation in the nucleus where its transcription factor property activates genes such as Hes1. In turn Hes1, by inhibiting Ptf1a activity and Ngn3 expression in these cells, prevents early differentiation of pancreatic progenitors into exocrine and endocrine cells respectively. Thus, Notch signalling at the early stage of pancreas organogenesis has a critical role for maintaining the pool of pancreatic progenitors in an undifferentiated state.

Wnt signalling

The Wnts bind to Frizzled receptors and LRP5/LRP6 coreceptor in the plasma membrane. Upon translocation into the nucleus, beta-catenin cooperates with transcription factors such as TCF/LEF to activate transcription of genes. Wnt/beta-catenin signalling plays a role in the maintenance of Ptf1a-positive exocrine progenitor cells. There is evidence that Wnt signalling plays a role in the endodermal versus mesodermal fate choice.

TGF-beta signalling

TGF-beta signalling is required for specification and branching of the pancreatic epithelium. Elements composing TGF-beta signalling were detected in developing pancreatic epithelium and mesenchyme. Nodal, a member of this family, plays a role in promoting endodermal and mesodermal formation, and high dose of this factor might be important to direct an endodermal fate. Activin and growth differentiation factor, also members of the TGF family, are involved in the endocrine and exocrine lineage development. Other members are TGF-beta, inhibin, Bone Morphogenetic Protein (BMP), ligand antagonist such as follastin, and the intracellular mediators Smads. BMP regulates, with retinoic acid, global anterior-posterior patterning of the gut and these signals and others specify the endoderm at gastrulation to induce a pancreatic fate or make it competent to respond later to other factors

1.1.3.2 Transcription factors during pancreas and beta-cell development

Studies using genetically modified mouse technology have led to considerable progress in understanding the sequential activation of signal transduction pathways and the expression of transcription factors critical for pancreas development. Below is a current list of transcription factors known to be involved in pancreas development and elegantly reviewed by authors in brackets (Spence and Wells, 2007, Murtaugh and Melton, 2003, Guo and Hebrok, 2009, Edlund, 1998, Edlund, 2002, Dhawan et al., 2007, Jensen, 2004, Slack, 1995, Kim and Hebrok, 2001, Habener et al., 2005, Chakrabarti and Mirmira, 2003). This list is of course not exhaustive.

Endodermal establishment and the core endodermal program

FoxA2 (HnF3β) is a key protein in endodermal development and required to generate midgut and foregut endoderm in most species. It is expressed at first in embryo at E.6.5, then in the foregut endoderm, notochord and floor plate prior to pancreas formation. In mature beta-cells it controls insulin secretion.

HnF4α is expressed throughout pancreatic development and is important for adult beta-cell function.

HnF1α is expressed at E.10.5, and important for mature beta-cell function and abundant in adult endocrine cells.

HnF1β seems to play a role in the establishment of the endodermal program, later in the epithelium of both pancreatic buds and it becomes restricted to ductal cells cell by E.14, even though it continues to be express in endocrine cells at a low and detectable level. Also it might be required for activating pancreatic (Ptf1a, Hfn6) and endocrine (Ngn3) gene expression and for regulating the generation of endocrine precursors. HnF1β positive cells may be the immediate precursors of Ngn3 positive progenitor cells.

GATA-6 is expressed at E.9.5, present in developing ducts, endocrine precursors. Later it is restricted to endocrine cells.

HnF6 has a role in pancreatic determination and it is essential to the generation of Ngn3 positive progenitor cells. It is expressed before Pdx1 activation in the prospective pancreatic domains and it is capable of activating the Pdx1 promoter.

Nrsa2 it is expressed in early endoderm development in liver and pancreas. It is expressed in pancreatic ductal and exocrine cells in the mature organ.

Pancreatic determination and progenitors

Pdx1/IPF1 expression begins at E.8.5 in the dorsal and ventral gut epithelium endoderm region that give rise to pancreatic primordia. Cell expressing Pdx1 and Ptf1a are progenitors giving rise to all pancreatic cell types. The early glucagon producing endocrine cells do not express Pdx1. Progressively Pdx1 becomes restricted to beta-cells and a subset of delta and PP-cells. Its cofactors such as Pbx and Meis2 in the pre-pancreatic domain by regulating the action of Pdx1, might result in directing a subpopulation of the Pdx1-positive endoderm cells towards a pancreatic fate. Pdx1 plays a role in several aspects of adult beta-cell function.

Ptf1a protein is detected in the early pancreatic epithelium at E.10.5 (*Ptf1a* mRNA at E. 9.5). Cells expressing Ptf1a in the developing pancreas are progenitors of all pancreatic cells and not only those of the acinar lineage. It is expressed only in foregut cells that become devoted to pancreatic fate and decides whether cells allocated to the pancreatic anlagen proceed towards pancreagenesis or revert back to duodenal fate. Ptf1a determines which cells positive for Pdx1 in the wide Pdx1 population will undergo pancreatic differentiation. Later it is restricted to adult acinar cells.

Sox17 is early marker of the definitive endoderm and it is required for pancreas formation

Sox9 is detected in the pancreatic epithelium of progenitor cells expressing Pdx1 at E.9-9.5. It plays a role in the maintenance of the pancreatic progenitor pool. It is co-expressed with Ngn3 progenitor cells, and in vitro studies suggest that Sox9 positively regulates Ngn3 gene.

Hlxb9 (homeobox b9) it is expressed throughout the early dorsal and ventral pancreatic domain and the notochord but its expression in the dorsal pancreatic primordium precedes Pdx1. It is present in pancreatic progenitor cells. Later it has a role in adult beta-cells.

Islet progenitors

HnF1β function was described above.

Ngn3 expression is detected first in the embryonic pancreatic epithelium at E.9.5 at a high level, peaks at E.15.5, then, declines progressively so that it is no longer detectable at birth and during the postnatal life. It is expressed in the immediate precursors of endocrine cells, and cells expressing Ngn3 give rise to all endocrine cells. It is positively regulated by Hnf6 and possibly by Sox9. HnF1β positive cells might be the immediate precursors of Ngn3 positive cells. In progenitors expressing Ngn3, it is not sufficient to promote a beta-cell fate; other competent factors are required. This suggests that the islet cell type decision is made before the expression of the hormone.

Pax4 expression is visible at E.9.5 in both pancreatic anlagen but later it is restricted to the developing beta-cells but not present in the adult. Lineage tracing experiments have demonstrated that similar to Ngn3 positive cells, Pax4 positive cells contribute to all endocrine lineages and might mark endocrine precursors. Ngn3 seems to activate Pax4 promoter directly and it is upstream of Pax4. Another Ngn3 target gene, the transcription factor Arx appears to co-express with Pax4. When both Pax4 and Arx begin to repress the expression of the other, then two populations of cells develop, one expressing Pax4 and the other Arx. Subsequently both cell populations will differentiate into beta/delta-cell or alpha-cells respectively.

<u>Lineage specification and beta-cells differentiation</u>

Nkx2.2 it is detected around E.8.75-9.5 in the pancreatic epithelium and by E.15.5 becomes restricted to endocrine alpha, beta and PP-cells. Nkx2.2 acts as a repressor for alpha and beta-cells formation during development and as an activator for beta-cell terminal differentiation. Later this transcription factor is expressed in adult beta-cells and plays a role as activator in beta-cell function.

Nkx6.1: it is expressed in the dorsal and ventral pancreatic epithelium buds at E.10.5. As development proceeds, Nkx6.1 is expressed in Ngn3 positive endocrine precursors, and become restricted to the developing and adult beta-cells. Nkx.6.1 represses glucagon expression and regulates insulin secretion in adult beta-cells. Also, Nkx.6.1 is required only for the second peak of beta-cell differentiation during the period called “the second transition”, around E13.5-15.5.

Maf A expression begins at E.13.5 in the beta-cell population and later is expressed in adult beta-cells. Maf A is positioned downstream of Nkx6.1. In adult beta-cells, Maf A promotes glucose-induced insulin released and interacts directly with Pdx1 and NeuroD1/Beta2 to activate insulin gene transcription.

Maf B is expressed in both beta- and alpha-cells beginning at E.12.5 and is restricted to alpha-cells during postnatal life. Maf B plays a role in beta and alpha-cell differentiation and maturation.

Forkhead family transcription factors includes FoxA1 (Hfn3alpha), FoxA2 (Hfn3beta), FoxA3 (Hfn3gamma). In mature beta-cells FoxA2 regulate insulin secretion.

Exocrine differentiation

PTF1a gene encodes P48 which plays a role in uncommitted pancreatic precursors and does not program the exocrine fate simply by its expression.

MIST1 is strongly expressed in pancreas. It is required for maintaining exocrine cell identity and it is restricted to exocrine cells.

In conclusion, during the last two decades, results from targeted gene disruption and enrichment in mice, and from genetic lineage tracing experiments, have established the role, and the spatial and temporal organization of transcription factors and signals indispensable for pancreas organogenesis. By collective efforts from different research groups, the cascade of events governing pancreas development can be better understood. However, although a more coherent description of the different phases of pancreagenesis has emerged, many important questions in this area still have to be examined, such as the cross-regulation between signalling pathways or transcription factors, to refine our understanding of the overall mechanisms directing pancreas formation. Thus, the current understanding of the genetic networks that govern pancreatic development can be applied to identify the genetic signature of pancreatic or endocrine progenitors during development, and as an instrumental tool to trace potential progenitors in postnatal pancreas. This genetic network and signalling pathways may be also useful to transdifferentiate non-beta-cell adult cells into functional beta-cell-like cells or to derive a glucose responsiveness-beta-cell-like phenotype from embryonic stem cells.

1.2 Human embryonic stem cells as a potential unlimited source for beta-cells

Human embryonic stem cells (hESC) derived from the inner cell mass of human blastocysts (Thomson et *al*., 1998) are capable of both unlimited self-renewal and differentiation into all cell lineages of the body. Consequently differentiation of hESCs into functional insulin-producing cell population, if achieved, could represent a promising source of expandable surrogate beta-cells for cell-based therapies to treat diabetes (Spence and Wells, 2007).

Progress in this fast moving field was made recently by developing protocols in which the processes of hESC differentiation recapitulate in some aspect those of embryonic pancreas development to generate hESC-derived insulin-producing cells. In this regard D'Armour and colleagues (D'Amour et *al.*, 2006) have proposed a cell differentiation protocol that successfully converted hESC into endocrine islet cells. Furthermore, the in vitro step-wise process conducting the hESC differentiation into endocrine cells appeared to mimic the different stages of embryonic pancreagenesis.

Thus D'Armour's protocol consists in converting first hESC into definitive endoderm (DE). This was achieved by inducing the expression of Nodal expression in those cells and other markers of embryonic definitive endoderm such as Sox17, CXCRA (chemokine receptor), CER (the mouse Cerberus homolog) or FoxA2, using a high concentration of activin A. One has to stress that the differentiation of hESC into the DE is a critical step to later yield endocrine cells efficiently. The second step is to differentiate the definitive endodermal cells into a phase that resembles the embryonic primitive gut cells, this after activin A removal. At this stage the hESC-derived cells are like primitive gut, expressing markers similar to the embryonic primitive gut, such as Hnf1beta and Hnf4A. The third step is to differentiate the hESC-derived cells resembling primitive gut into posterior foregut endoderm, after addition of retinoic acid (RA), FGF10 and KAAD-cyclopamine (an inhibitor of hedgehog signalling). The posterior foregut expresses at this stage a combination of high level of Pdx1 and Hnf6 while the level of Hnf1beta and Hnf4A is maintained or increased. In later stages, cell were cultured with exendin 4, insulin-

like growth factor 1 and hepatocyte growth factor (HGF) to differentiate the posterior foregut endodermal cells to pancreatic endodermal cells, pancreatic and endocrine precursors which give rise to endocrine islet cells expressing insulin, glucagon, somatostatin, pancreatic polypeptide and ghrelin hormones.

Unfortunately, with this protocol of hESC differentiation the insulin-producing cells were not glucose responsive, suggesting that those cells were still immature. However, another protocol of hESC differentiation developed by Jiang et *al*. (Jiang et al., 2007a) successfully converted hESCs into glucose-responsive insulin-secreting cells. They exposed a monolayer of hESCs (a two-dimensional culture system) with low activin and sodium butyrate to generate definitive endoderm. The latter was cultured in suspension (a three-dimensional culture system) with FGF, noggin and EGF to promote the formation of Pdx1 positive cells and their further differentiation into endocrine cells with addition of nicotinamide, insulin-like growth factor 2. Also, ductal cells were evident in the final stage of the culture as well as amylase mRNA, a marker of exocrine cells.

Thus, the spatial arrangement of the cells during the various steps of differentiation may be an important element to consider to efficiency convert hESCs to definitive endoderm and later into mature endocrine cells (Spence and Wells, 2007).

The next step to achieve for realistic progress regarding cell-based therapies is to make certain that the hESC-derived beta-cells are capable of correcting diabetes in animals after transplantation, and do not have any adverse effects. Indeed, in the past previous protocols used to derive hESC into insulin-producing cells did not recapitulate important elements of embryonic pancreatic development. As a result the insulin-positive cells transplanted in diabetic animals induced by streptozotocin, failed to restore euglycemia in those mice and furthermore formation of teratomas was observed (Spence and Wells, 2007).

Kroon and colleagues (Kroon et al., 2008) used an improved protocol of D'Armour to drive hESCs into definitive endodermal cells. Then, the hESC-derived definitive endodermal cells were transplanted into immune-compromised mice to promote their differentiation into mature endocrine cells. Subsequently, the latter protected the mice from streptozotocin-induced hyperglycaemia, indicating that the

hESC-derived insulin secreting cells were fully functional. However some mice developed teratomas due to hESC-derived non-pancreatic endoderm, suggesting the need of a step of purification to discard such unwanted cells. The formation of teratomas, if it not prevented, could seriously undermine the utility of such an approach for clinical application. In another study, the authors (Jiang et *al*., 2007b) developed another differentiation protocol for hESC which led to the generation of hESC-derived glucose responsive insulin-secreting cells in a step-wise process mimicking embryonic pancreas organogenesis. They showed that the hESC-derived functional insulin-secreting cells transplanted in streptozotocin-treated nude mice protected them from hyperglycaemia. Importantly, the mice did not develop a teratoma. However, the number of engrafted mice investigated in this report was only nineteen compared to the number of forty-six grafts observed in Kroon's report (Kroon et *al*., 2008).

In conclusion, collectively, these studies underscore the requirement to use a differentiation process that recapitulates pancreas development to guide hESC differentiation into fully mature surrogate beta-cells and to eliminate any residual hESC capable of forming teratomas (Spence and Wells, 2007, Oliver-Krasinski and Stoffers, 2008, Guo and Hebrok, 2009).

Despite the fact that hESC hold great hope to derive expandable surrogate beta-cells, a hurdle to its widespread clinical application relates to the problem of rejection associated to allograft transplantation even though important progress has been made by continuously optimizing immunosuppressant drugs. However, a breakthrough that might represent an alternative to hESC, is the reprogramming of somatic cells into pluripotent stem cells induced by defined transcription factors (Park et *al*., 2008), and virus-free (Woltjen et *al*., 2009). The reprogramming of somatic cell into induced pluripotent stem cells (iPS) promises to revolutionize regenerative medicine because the use of patients' own cells will possibly abrogate the pitfall of graft rejection that impedes cell-base therapies, and because adult cells that are easily available and in great amount could be utilized. Importantly, similar to hESCs, iPS cells can differentiate into the three embryonic germ layers, so it will be of interest to see whether in the future, differentiation protocols for hESC can be applicable for iPS cells (Best et *al*., 2008, Guo and Hebrok, 2009).

1.3 Transdifferentiation as a potential source for beta-cells

Internal organs such as the liver, gut or pancreas share a common endodermal origin during development. Based on this observation and others, some groups intended to transdifferentiate liver or gut cells into a beta-cell fate using mice model such as Hes1 knockout mice (Burke and Tosh, 2005) or engineering approaches (Nir and Dor, 2005). In theory an advantage of this strategy is the availability of these types of autologous cells in patients.

Also in the pancreas, exocrine tissue has long been the centre of attention as a source for new beta-cells. Bertelli and colleagues (Bertelli and Bendayan, 1997) reported that after duct ligation in rats, intermediate endocrine-acinar pancreatic cell phenotype appeared co-expressing insulin and amylase which may represent an intermediate step in a transdifferentiating process. However, recently, Desai and colleagues demonstrated by direct lineage tracing experiments that exocrine cells do not give rise to new beta-cells during regeneration, at least in mice after inducing various methods of pancreatic injury including duct ligation (Desai et *al*., 2007). This result does not signify that exocrine cells are not able to transdifferentiate under other circumstances. Indeed, Baeyens and colleagues (Baeyens et *al*., 2005) reported the conversion of cultured exocrine cells into beta-cells under specific conditions. This result was confirmed using direct lineage tracing by two other reports (Minami et *al*., 2005, Okuno et *al*., 2007). Furthermore Minami et al. showed that the cultured acinar cells also expressed markers recapitulating some aspects of pancreas development, suggesting that the acinar cells in culture were able to revert into immature pancreatic cells. These results demonstrate that exocrine cells are indeed capable of sufficient cellular plasticity in vitro when exposed to specific signals. One must stress, as Minami et *al*. in a review (Minami and Seino, 2008) reported, that the acinar cell-derived insulin-producing cells are still immature and so full differentiation of these insulin-positive cells has yet to be achieved to be really instrumental for clinical application. In another recent report (Zhou et *al*., 2008), the authors succeeded in reprogramming exocrine cells into beta-cells in mice, by delivering key transcription factors controlling cell fate during pancreas development. The reprogrammed beta-cells could partially correct hyperglycaemia in streptozotocin-treated mice. This report is a perfect example of how reprogramming

of differentiated adult cells might be used in the future to generate beta-cells or other cell types using accessible patient cells.

1.4 Diffentiated beta-cells as a resource for new beta-cells

Replication of differentiated beta-cell during postnatal period could prove to be effective either for replacing missing beta-cells in patients or for propagating donor islet beta-cells ex vivo. The adult pancreatic cell mass was considered for a long time as quiescent, but recent studies have shown that actually beta-cell mass is dynamic and can fluctuate under normal and patho-physiological conditions during postnatal life (Bouwens and Rooman, 2005). There is an abundant literature dedicated to beta-cell mass regulation during adulthood, in which copious reports reviewed by Cozar-castellano et *al*., Heit et *al*., Vasavada et *al*., Elghazi et *al*., Bouwens and Rooman, Ackermann and Gannon (Cozar-Castellano et *al*., 2006a, Heit et *al*., 2006, Vasavada et *al*., 2006, Bouwens and Rooman, 2005, Ackermann and Gannon, 2007, Elghazi et *al*., 2006, Lee and Nielsen, 2009) support the view that beta-cell proliferation is essential for the maintenance and generation of postnatal beta-cell mass.

Most of these studies used animal models in which cell-cycle machinery or elements of signalling pathways were altered (Fatrai et *al*., 2006, Marzo et *al*., 2004, Zhong et *al*., 2007, Zhang et *al*., 2005a, Buteau et *al*., 2001, Nguyen et *al*., 2006, Rulifson et *al*., 2007, Cozar-Castellano et *al*., 2006b, Karnik et *al*., 2005, Krishnamurthy et *al*., 2006, Garcia-Ocana et *al*., 2000, Kushner et *al*., 2005, Lindberg et *al*., 2005, Georgia and Bhushan, 2004). Kushner et *al*., Georgia and Bhushan (cited above) showed that deletion of Cyclin D2 in mice was accompanied with a dramatic beta-cell mass decrease during the postnatal life. Thus the authors demonstrated that Cyclin D2, a component of the cell cycle, plays a critical role in beta-cell mass expansion and maintenance during postnatal life. Marzo et *al*. showed how a mutation of the *Cdk4* gene encoding for Cdk4 another component of the cell cycle (Marzo et *al*., 2004) could also disturb beta-cell mass growth. And many other studies regarding alterations of other components of the cell-cycle showed similar effects on the postnatal beta-cell mass development (see references above).

Collectively these reports, have positioned cycling-beta-cells as the primary mechanism for maintaining postnatal beta-cell mass and growth. Definitive proof came from a pulse-chase lineage tracing experiment to follow the fate of beta-cells during postnatal life, performed by Dor et *al*. (Dor et *al*., 2004). The author and co-workers demonstrated for the first time with a genetic lineage tracing system that pre-existing beta-cells were the main source for new beta-cells during beta-cell self maintenance and expansion in adult mice. This lineage tracing experiment result was reinforced by another study employing a DNA analogue-based lineage tracing system (Teta et *al*., 2007). Collectively, these results indicate that pre-existing beta-cells exhibit a remarkable proliferative capacity that could be exploited as a potential source for regenerative medicine (Dor, 2006). However, Teta et *al*. in a study in which they investigated beta-cell replication turnover in adult mice discovered that beta-cell of old mice have extremely low rates of replication (Teta et *al*., 2005), suggesting that beta-cell replication can be possibly a rare event in mammals and it is tightly regulated (Kushner, 2006).This result was reinforced by Krishnamurthy et *al* (Krishnamurthy et *al*., 2006) showing that regenerative potential of islets was age-dependent in mice.

However, in contrast with these observations, there are circumstances in which cell cycle restriction can be relaxed. For instance, in pregnancy, beta-cells show remarkable proliferative capacity to expand the maternal beta-cell mass to accommodate the increased insulin demand (Kargar and Ktorza, 2008). Thus Karnick et *al*. and Gupta et *al* (Karnik et *al*., 2007, Gupta et *al*., 2007) demonstrated that Menin and HNF-4alpa play a critical role in the regulation of beta-cell replication in response to new metabolic demand characteristic of pregnant states. The inability of these factors to coordinate the cell cycle pathway results in a failure of the maternal beta-cell mass to expand properly in response to the increased metabolic demand. Therefore, taken together, these works suggest that manipulation of factors providing the right signals to proliferate might be used as a strategy to force beta-cells to re-enter the cell-cycle and then expand beta-cell mass in diabetic patients. Consequently this source may be seen as a promising avenue to expand beta-cell mass in vivo and in vitro for future transplantation. Nevertheless, there are important concerns that could limit this approach. First the promotion of beta-cell replication using factors might induce unwanted neoplastic effects in other tissues in

patients. Therefore these target molecules will have to be thoroughly tested before any clinical trials. Second, in vitro expansion of islet beta-cells was proven to be very difficult because of the loss of markers of differentiation over time in cultured beta-cells (Guo and Hebrok, 2009, Limbert et *al*., 2008, Weinberg et *al*., 2007). But, Ouziel-Yahalom and co-workers (Ouziel-Yahalom et *al*., 2006) described a culture condition to propagate adult human pancreatic islets cells ex vivo, and allowing their subsequent redifferentiation. At the same time Path and co-workers (Path et *al*., 2006) reported the successful expansion of human islets over-expressing a nuclear protein P8, without, in this case evidence of dedifferentiation. Thus, optimization of islet culture conditions might be decisive for future application of donor pre-existing-beta-cell propagation.

1.5 Adult pancreatic progenitor or stem cells as a source for new beta-cells: the debate continues

Researchers have been fascinated during the last two decades by two major questions related to adult pancreatic progenitor or stem cells. First, do adult pancreatic progenitor or stem cells exist and second, do they contribute to beta-cell mass renewal or growth during adulthood? Thus, the elusive progenitor or stem cell to maintain endocrine lineages in postnatal life is a subject of ongoing debate in this area of research which moreover does not seem to converge towards a general consensus (Bonner-Weir, 2000a, Ackermann and Gannon, 2007, O'Neill et *al*., 2008, Lee and Nielsen, 2009, Bonal et *al*., 2008, Xia et *al*., 2009, Guo and Hebrok, 2009, Levine and Itkin-Ansari, 2008, Zhang et *al*., 2005b, Bonner-Weir and Weir, 2005, Hanley et *al*., 2008, Limbert et *al*., 2008, Bonner-Weir et *al*., 2004).

It is well-established that endocrine lineages during pre-natal development are the progeny of progenitor cells (neogenesis) within the primitive gut epithelium that begin to express Pdx1 protein around E.8.5 (Gu et *al*., 2002). In postnatal development, on the other hand, the picture is far less clear and the existence of progenitors giving rise to pancreatic islet or beta-cells in adults is a highly contentious issue. The first reports suggesting the existence of such a pancreatic bona fide progenitor are related mostly to models of pancreatic regeneration caused

by pancreatic injury, and histological observations. Regular analysis of human donor pancreata examined by Bouwens and Pipeleers (Bouwens and Pipeleers, 1998), revealed that single beta-cell units are frequently associated with ductules from which they seemed to emerge by budding, as seen in the foetal and neonate pancreas. These observations led the authors to suggest that beta-cell neogenesis might be reliant on duct cell replication and differentiation. In models of pancreatic injury, the majority of reports if not almost all, strongly suggested the existence of such elusive progenitors within the duct, exocrine or endocrine compartments. Bonner-Weir and co-workers developed (Bonner-Weir et *al*., 2004) solid argumentations in favour of adult pancreatic ductal progenitors that regress to a less differentiated phenotype with replication, before functioning as endocrine progenitors. Their results largely came from the study of a model of ninety percent pancreatectomy, well-known to induce pancreatic regeneration. Briefly, they showed that twenty four hours after pancreatectomy, there was an increased proliferation in the duct compartment, followed forty eight hours later by expression of the transcription factor Pdx1 in this same component, a molecule has been suggested by others as a marker for adult progenitor cells. Also analysis of the gene expression profile of isolated pancreatic ducts, showed that ductal genes were preferentially expressed in newly formed islets from the focal region of proliferating ducts but not in pre-existing pancreatic islets, both from regenerated pancreas after partial pancreatectomy**.** Wang et *al* (Wang et *al*., 1995) reported that partial duct ligation in adult rats resulted in islet cell neogenesis that could be activated by stimulation of pancreatic ductal cells. In models of chemical injury inducing specifically beta-cell ablation by toxic agents such as streptozotocin or alloxan (Bonal et *al*., 2008), several authors also suggested that the regenerative process after the insults was in part due to beta-cell neogenesis from precursors residing in the duct or islet compartment (De Haro-Hernandez et *al*., 2004, Yamamoto et *al*., 1997, Yamamoto et *al*., 2000, Li et *al*., 2004, Fernandes et *al*., 1997, Guz et *al*., 2001). Other authors showed similar results in animals infused with glucose (Lipsett and Finegood, 2002, Jetton et *al*., 2008), or in adult human pancreatic islets treated with a combination of EGF (epidermal growth factor) and Gastrin in vivo and in vitro (Suarez-Pinzon et *al.*, 2005). Furthermore, authors who have studied models of transgenic mice expressing IFN-g (interferon-gamma) or expressing a combination of Gastrin and TGFalpha (transforming growth factor alpha) also suggest that islet neogenesis could be reactivated in the pancreatic duct

epithelium of adult mice (Wang et *al*., 1993, Gu et *al*., 1994, Gu and Sarvetnick, 1993). What's more, in vitro studies aiming to isolate and propagate pancreatic progenitors and culture them in appropriate medium, came to similar conclusions that a bone fide progenitor might exist in adult pancreas (Suzuki et *al.*, 2004, Seaberg et *al*., 2004, Zulewski et *al*., 2001, Yatoh et *al*., 2007, Peck et *al*., 2002).

Collectively, these reports strongly suggest the existence of putative pancreatic progenitors that could be reactivated in certain circumstances or can be isolated and propagate in appropriate in vitro setting. Even though these conclusions are very convincing, none of these studies used a direct lineage tracing approach to conclusively demonstrate the existence of the elusive adult pancreatic progenitors.

To confirm the existence of such adult pancreatic progenitors suggested by authors above and others, Dor et *al*. (Dor et *al*., 2004), designed an elegant lineage tracing system for beta-cells in order to trace beta-cell fate in adult mice. Unexpectedly, they demonstrated that replication of pre-existing beta-cells was the main mechanism that give rise to new beta-cells during the mouse lifespan and even after partial pancreatectomy. This publication has had a massive impact in this field and has cast doubt at its extreme on the existence of putative progenitors residing within adult pancreas and the contribution of such progenitors during beta-cell renewal and maintenance in adulthood.

These results were reinforced by two other studies. One, using the same lineage tracing system in a model of transgenic mouse expressing the diphtheria toxin in beta-cells after diabetic recovery (Nir et *al*., 2007). The other employing a DNA analogue-based lineage tracing system (Teta et *al*., 2007).

But ultimately, very recently, because this field is moving fast, three reports and a review, one after the other (Xu et *al*., 2008, Inada et *al*., 2008, Collombat et *al*., 2009, Liu and Habener, 2009), have reinstated adult pancreatic progenitors on the map. They have conclusively demonstrated that adult progenitors within the pancreas do exist, are of ductal origin, and contribute significantly to the formation of new beta-cells shortly after birth or during beta-cell regeneration after duct ligation in mouse. First, Xu and colleagues (cited above), used a direct approach to track down the origin of new beta-cells in response to duct ligation in mice, a well-establish model of pancreatic injury and regeneration. They showed that new beta-

cells were the progeny of Ngn3 positive endocrine precursors and the cultured endocrine precursors were able to differentiate into islet cells in appropriate conditions. This report was the first to demonstrate incontrovertibly that adult progenitors do exist and reside in the pancreatic duct. Furthermore, if given the right signals they could be activated to give rise to endocrine lineages in vivo and in vitro. Second, Inada and co-workers (cited above), using a distinct direct approach, demonstrated that ductal cells engender endocrine and exocrine lineages during the neonate period, and in response to duct ligation, in mice. They generated mice expressing the Cre-recombinase under the control of an adult ductal marker promoter, that of the *carbonic anhydrase II* gene. Third, Collombat et *al*. (cited above) very recently published an article in which they reported the certainty of adult progenitors in pancreas and possibly residing in the ductal component. They demonstrated, using a lineage tracing system that forced expression of Pax4 in glucagon-deficient mice, the continual conversion of glucagon-expressing alpha-cells into insulin-expressing beta-cells. Importantly, the generation of the alpha-cells was dependent of Ngn3-positive progenitor cells.

Taken together, these reports indicate, in the context of regenerative medicine that adult pancreatic progenitors could be manipulated in vitro to yield sufficient surrogate beta-cells suitable for transplantation into diabetic patients. Additionally, given the right factors, these adult progenitors may be activated to beget new beta-cells in situ in patients.

In the light of new findings arising from the advent of novel genetic cell-lineage techniques, undeniably there is a resurgence of the debate regarding the mechanism by which new beta-cell arise in normal and patho-physiological conditions. It is reasonable to address the cellular origin of new beta-cells in other situations involving different types of stimuli. Pregnancy is an appealing model of beta-cell mass expansion in which the origin for new beta-cells could be specifically investigated.

1.6 Maternal adaptation in response to pregnancy

The general mechanisms of maternal adaption to accommodate the new metabolic demand during pregnancy, were studied by several authors in specific situations reviewed by Sorenson and Brelje (Sorenson and Brelje, 2009), but none of these reports used a direct approach to investigate the cellular lineages responsible for the increased beta-cell mass. Still, it is established that pregnancy is accompanied by a near doubling of the beta-cell mass (Scaglia et *al*., 1995, Blondeau et *al*., 1999, Avril et *al*., 2002, Huang et *al*., 2009, Aerts et *al*., 1997, Gupta et *al*., 2007, Karnik et *al*., 2007) mostly due to beta-cell cycling (Gupta et *al*., 2007, Karnik et *al*., 2007, Scaglia et *al*., 1995, Avril et *al*., 2002, Brelje et *al*., 1993, Brelje et *al*., 2008, Sorenson et *al*., 1993, Huang et *al*., 2009, Vasavada et *al*., 2000, Parsons et *al*., 1992). Studies in pregnant rodents have shown that prolactin or placental lactogens are responsible for up-regulation of islets during pregnancy (Sorenson and Brelje, 2009). The beta-cell mass adaptation to pregnancy seems to be regulated through the prolactin receptor signal transduction pathways (Figure 5) (Brelje et *al*., 2004, Sorenson and Brelje, 2009). Recently, Karnik et *al*. and Gupta et *al*. (Karnik et *al*., 2007, Gupta et *al*., 2007) showed that beta-cell proliferation is largely accountable for the beta-cell mass expansion in response to pregnancy, this through the repression of the *Men1* gene (which encodes a protein component of a histone methyltransferase) and its targets (Karnik et *al*., 2007) or Hnf-4alpha (hepatocyte nuclear factor-4alpha) (Gupta et *al*., 2007). Other adaptations during pregnancy include a slight beta-cell hypertrophy (Huang et *al*., 2009), an increase in glucose-induced insulin secretion, and a decrease of the threshold of the amount of glucose that induces insulin secretion (Parsons et *al*., 1992). Also a substantial increase in peripheral insulin resistance combined with a slightly lower blood glucose level and elevated serum insulin level are observed during this state (Sorenson and Brelje, 2009). However, there is little information about whether beta-cell neogenesis takes place during pregnancy (Parsons et *al*., 1995). For this reason, it will be of great interest to use a direct approach to examine the contribution of non-beta-cell progenitors during the beta-cell mass expansion in response to pregnancy.

1.7 Toxic-genetic model of beta-cell ablation and regeneration to address origin of new beta-cells in patho-physiological conditions

Identification of cellular sources capable of generating new beta cells in the endogenous postnatal pancreas, or for transplantation, could have important implications for developing new therapeutic strategies to treat diabetes more efficiently.

In this context we employed a mouse model (pIns-c-MycER^{TAM}) of toxic-genetic ablation-regeneration of beta-cells, developed by Pelengaris and co-workers (Pelengaris et *al*., 2002), to address the origin of new beta-cells after diabetic recovery. In this model, beta cells are depleted by apoptosis after activation of the c-Myc protein upon tamoxifen exposure. This results in the development in the mouse of a diabetic phenotype, which, can be reversed by beta cell regeneration upon tamoxifen withdrawal. The latter point constitutes the originality of this mouse model which can be exploited to investigate the cellular sources for new beta-cells after diabetic recovery, in combination with a cell-lineage tracing system.

1.8 Thesis aim

Thus, since the debate regarding the contribution of adult stem/progenitor cells as a legitimate cellular source for new beta-cells, is far from being resolved, it is justifiable to address the origin of new beta-cell in different circumstances where other types of stimuli might be involved. This is the subject of my thesis. Indeed, we exploited a Cre/loxP lineage tracing system to efficiently label β-cells to examine, 1) the origin of new beta-cells during the beta-cell mass expansion in response to pregnancy, and 2) in a specific model of pancreatic regeneration, the origin of new beta-cells after toxic-genetic beta-cell ablation in transgenic mice over-expressing c-Myc (Pelengaris et *al*., 2002).

Finally, a further important question relates to the functionality of such newly formed beta-cells subsequent to beta-cell mass depletion. Therefore, in parallel, we evaluated the functionality of regenerated beta-cells in a mouse model of toxic-genetic ablation cited above by single-cell microfluorimetry, in collaboration with Dr P. Squires.

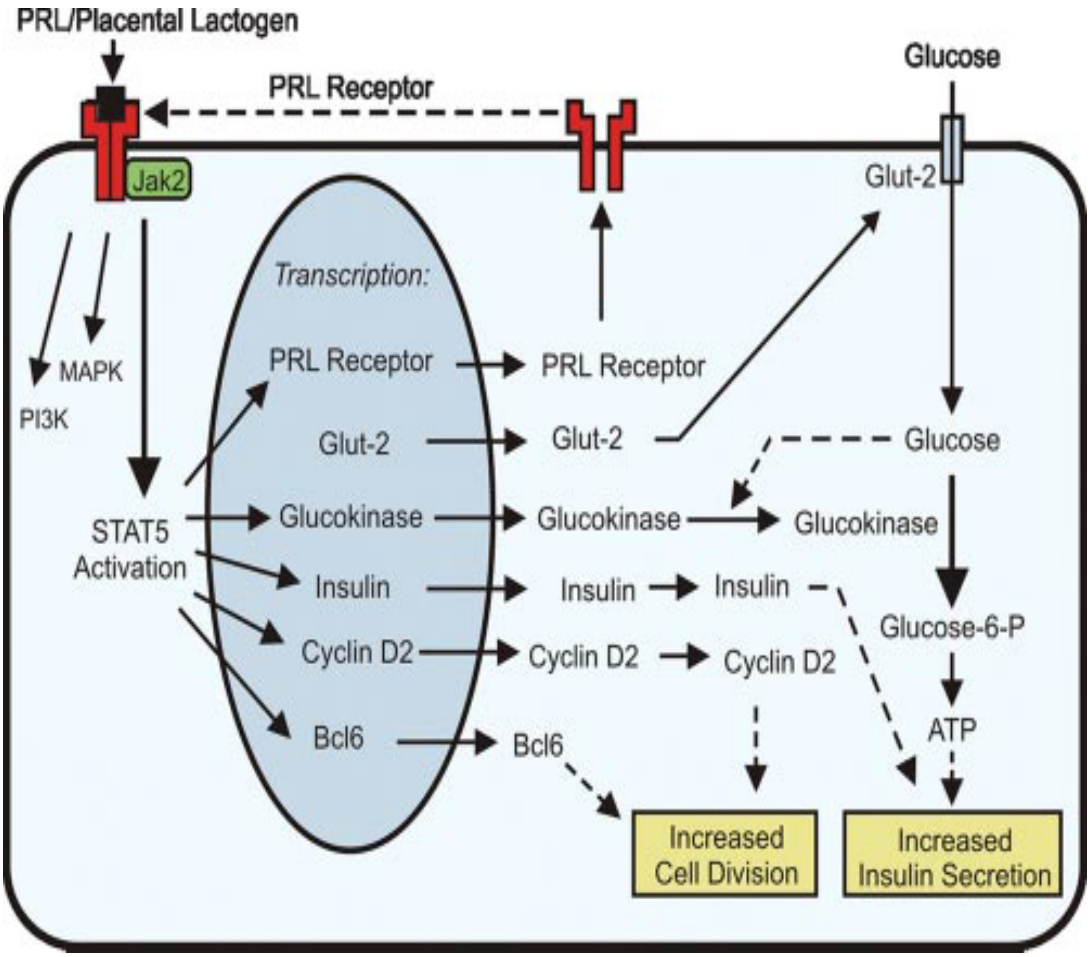


Figure 4 **During pregnancy, islet mass increases and insulin secretion becomes increasingly sensitive to glucose stimulation**. The figure highlights cellular events that occur in islet cells as they adapt to pregnancy. These were determined by comparing the results of lactogen-treated islets with those observed in islets during pregnancy. This has led to the hypothesis that cell PRL receptors have a central role in the adaptation of islets to pregnancy. Glut-2, Glucose transporter-2; Jak2, Janus kinase 2; PI3K, phosphatidylinositol 3-kinase. (Sorenson and Brelje, 2009).

CHAPTER 2

MATERIALS AND METHODS

Materials and Methods

2.1 Animal care

The three single strains, RIP-CreER^{TAM}, pIns-c-MycER^{TAM}, and Z/AP transgenic mice were maintained by crossing with CBAxC57BL/6 F1 background. The double transgenic mice (RIP-CreER^{TAM}; Z/AP) were generated by crossing the two single transgenic mice RIP-CreER^{TAM} and Z/AP, while the triple transgenic mice (Z/AP; RIPCreER^{TAM}; pIns-c-MycER^{TAM}) were generated by crossing the double transgenic mice (RIP-CreER^{TAM}; Z/AP) with the single transgenic pIns-c-MycER^{TAM}. Single and multiple transgenic mice were housed under a 12 hour light/dark cycle and received food and water *ad libitum* until sacrifice. Animal care and experiments were carried out according to UK Home Office regulations.

2.2 Animal genotyping

2.2.1 PCR genotyping

2.2.1.1 DNA extraction from mouse ear disc

First, two reagents are prepared, one (hotshot reagent) at pH 12, made with 25mM NaOH, 0.2mM disodium EDTA and the second (Neutralising reagent) at pH 5, made with 40mM Tris-HCl (Tris-HCl is dissolved in water without adjusting pH). Of note, a neutral pH should result from a 1:1 mix of the two solutions. Then, 75µl of hotshot reagent is added to the tube containing the mouse ear disc, after that, the tube containing the ear disc plus the reagent is heated at 95°C for 10min to 1hour (30min is sufficient). Afterwards 75µl of neutralising agent is added in the tube with the ear sample. Subsequently, after cooling at 4°C for 1 hour (for longer term storage, the sample can be frozen at -20°C), 1 to 5µl of the final preparation of the DNA extract could be used for PCRing.

2.2.1.2 PCRing procedure for RIP-CreER^{TAM} strain genotyping

In a PCR tube is added, 5µl of the final preparation of the DNA extract above from one mouse ear disc or distilled water, followed by 0.75µl of $MgCl_2$ at 50mM (Invitrogen kit), 2.5µl of 10xPCR reagent (Invitrogen kit), 1µl of each, reverse and forward primers at 10picomol/ul, 0.25µl of dNTP (deoxyribonucleotide triphosphate) at 10mM (Invitrogen kit), 0.25µl Taq at 500 units (Invitrogen kit), and finally, 14.25µl of distilled water. The tube with all reagents is then ready to be processed in a PCR machine using the appropriate PCR program.

2.2.1.3 PCR procedure pINS-c-MycER^{TAM} strain genotyping

In a PCR tube is added, 3µl of the final preparation of the DNA extract above from one mouse ear disc or distilled water, followed by 0.75µl of $MgCl_2$ at 50mM (Invitrogen kit), 2.5µl of 10xPCR reagent (Invitrogen kit), 0.5µl of each, reverse and forward primers at 10picomol/µl, 0.25ul of dNTP (deoxyribonucleotide triphosphate) at 10mM (Invitrogen kit), 0.25µl Taq at 500 units (Invitrogen kit), and finally, 17.25µl of distilled water. The tube with all reagents is then ready to be processed in a PCR machine using the appropriate PCR program.

2.2.1.4 PCR product analysis

When the PCR is completed, the PCR product size is analysed, after migration in a 1 to 1.5% percent of agarose gel containing ethidium bromide (0.2mg/µl). DNA PCRing products from transgenic, control animals positive and negative for the target gene and the appropriate DNA ladder are loaded in distinct wells in the gel at the same time, for subsequent analysis. For this purpose, prior to loading, 25µl of each DNA PCRing sample is mixed with 5µl of 6X gel-loading buffer, while 1µl of a 1kb ladder solution (1µg/µl) is mixed with 9µl of distilled

water and 2µl of 6X gel-loading buffer. Before loading the different samples in the wells, the gel is place in a tank gel containing 0.5% of TBE (Tris/Borate/EDTA) buffer.

2.2.1.5 Primers and PCR program used for the RIP-CreER^{TAM} strain genotyping

RIP-CreER^{TAM} strain was genotypes by PCR using the following set of primers which detect part of the Cre-recombinase gene sequence (forward 5'ACGGCGCTAAGGATGACTCT3' and reverse 5'CCACCAGCTTGCATGATCTC3'), and PCR program (step 1: 94°C for 30 seconds, step: 2: 57°C for 30 seconds, step: 3: 72°C for 1 minute, then step 1 to 3 are repeated for 35 cycles). The product size expected is 124 base pairs. The Cre-recombinase PCR assay using these Cre primers is illustrated in Appendix1.

2.2.1.6 Primers and PCR program used for the pINS-c-MycER^{TAM} strain genotyping

pIns-c-MycER^{TAM} strain was genotypes by PCR using the following set of primers, the forward MERTM (5')(5' CCAAAGGTTGGCAGCCCTCATGTC 3')and reverse Myc (3') (5' AGGGTCAAGTTGGACAGTGTCAGAGTC 3') which detect part of the Mus musculis oestrogen receptor and Homo sapiens c-Myc gene respectively and PCR program (step 1: 94°C for 2 minutes, step: 2: 94°C for 1 minute, step: 3: 57°C for 1 minute, step 4: 72°C 2 minutes, then step 2 to 4 are repeated for 30 cycles, and step 6: 72°C for 10 minutes). The product size expected is 413 base pairs.

2.2.2 Enzymatic genotyping

The Z/AP double reporter strain was genotyped by enzymatic staining using ear biopsy from transgenic mice mixed with a solution containing 5bromo-4chloro-3indolybetagalactopyranoside (X-gal, Sigma). In a tube containing a mouse ear disc, 50µl of a fresh made X-gal solution is added, the mixture is then incubated at 37°C for 1 and half to 2 hours. Ear discs which turn blue during this period of incubation will be scored positive for the Z/AP target gene. The X-gal solution is prepared as follows. In an universal is added, 125µl of potassium ferrocyanide at 100mM ($K_4Fe(CN)_6$, H_20), 125µl of potassium ferricyanide at 100mM ($K_3Fe(CN)_6$), 5µl of $MgCl_2$ at 1M, 50µl of X-gal reagent at 50mg/ml which was first dissolved in DMSO or dimethylformamide (the X-gal stock solution is wrapped in aluminium foil and kept at -20°C), and finally 2183µl of PBS (phosphate buffered-saline) at 100mM pH 7.3. After gentle homogenising, the solution is ready to use. All the reagents are prepared with phosphate buffered-saline pH 7.3 (PBS) except the X-gal solution.

2.2.3 Solution content

1l of 10X TBE (Tris/Borate/EDTA) is made with Tris (108g), boric acid (55g), 40ml of EDTA at 0.5M.

6X gel-loading buffer is made with 0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water

2.3 Preparation of tamoxifen and 4-hydroxytamoxifen (4-OHT)

Tamoxifen and 4-hydroxytamoxifen (4-OHT) emulsions were prepared by sonication in peanut oil. The compound or the peanut oil was administrated intraperitoneally in the animals.

2.4 Intraperitoneal glucose tolerance test (IPGTT)

Subsequent to a 16 hour fast, the animals were injected intraperitoneally with 30% D-glucose in PBS (2mg/g body weight). Blood glucose levels were monitored

prior to, and 10, 30, 60 and 120 minutes after glucose injection using an Advantage glucose meter (Roche Diagnostics). For measurement 2 to 3 µl of blood are collected from the tip of the animals' tail.

2.5 Tissue processing and immunohistochemistry.

Pancreata from animals were dissected out and fixed in 4% paraformaldehyde for 2 hours at room temperature, then transferred to 30% sucrose overnight at 4°C before embedding in OCT mounting medium (Tissue Tek).One block for each pancreas was sectioned for immunohistochemistry. Five sections per pancreas, representing different parts of the organ and separated at least by 500 to 1000µm from each other, were immunostained with an insulin polyclonal guinea-pig anti-swine (DAKO), a polyclonal sheep anti-human placental alkaline phosphatase (ARP American Research Products, Inc) or a polyclonal rabbit anti-human placental alkaline phosphatase (Accurate Chemical & Scientific Corporation). Vectashield mounting medium with DAPI (Vector laboratories) was used to stain the nucleus. DAPI (diamidino-2-phenylindole) is a blue fluorescent dye that fluoresces brightly when it is selectively bound to double stranded DNA

The secondary antibodies used to reveal the primary antibodies were Alexa 633-conjugated goat anti-guinea-pig (Invitrogen), FITC-conjugated donkey anti-sheep (Jackson ImmunoResearch laboratories) and FITC- conjugated goat anti-rabbit (Vector laboratories). All the antibodies were diluted in phosphate buffered saline (PBS) containing 0.1% Triton X-100 and 2% normal donkey serum (Jackson ImmunoResearch laboratories).

The antibody working dilution was 1/100 for the insulin polyclonal guinea-pig anti-swine, 1/1000 for the sheep and rabbit anti-human placental alkaline phosphatase, and 1/200 for the secondary antibodies. Briefly, the sections were stained sequentially with, i) the polyclonal sheep anti-human placental alkaline phosphatase overnight at 4°C, ii) the FITC labelled anti-sheep for 30min at room

temperature, iii) the polyclonal guinea-pig anti insulin for 1hour at room temperature, iv) the Alexa 633 goat anti-guinea-pig for 30min at room temperature.

Then slides were mounted with Vectolab mounting medium with DAPI. The sections were washed 3 times with PBS containing 0.1% Triton X-100 before adding the next antibody.

2.6 Morphometric analysis and calculation

The images of the whole pancreatic tissue sections, immunostained and DAPI-stained, were captured using a x5 objective using a Leica confocal microscope while the total pancreatic sections immunostained for insulin and HPAP (human placental alkaline phosphatase) used a x40 objective. Slides were coded and scored 'blind' as to their experimental origin. Systematically, all single islets and beta-cell clusters found in each section were photographed and 5 sections representing different regions of the pancreas were analysed. After adjusting a threshold separately for each image, ImageJ was then used to count the number of pixels of cross-sectional area stained for HPAP and insulin in each picture. Alternatively, beta-cells positive for HPAP or insulin were counted manually in each picture using ImageJ. Each such measurement was repeated, to obtain three separate counts for each image. Most of the time, these images showed only one islet. For the measurement of the all pancreatic tissue cross-sectional area positive for DAPI we first processed the pictures using the Gaussian blur option before proceeding with the count. This option blurs the areas positive for DAPI, so that all nuclear area positive for DAPI meet and this allow a better estimation of the tissue area.

The beta-cell mass was calculated by forming the ratio of the total number of pixels of insulin cross-sectional area to the total number of pixels of the pancreatic tissue cross-sectional area in the five sections in each pancreas, multiplying by the pancreas weight of the given mouse. Because the pancreatic tissue area was captured with the 5x objective, its area values were first multiplied by a 64 correction factor before proceeding with the calculation and comparison with images collected with 40x objective.

Similarly, the proportion of beta-cell area positive for HPAP or the HPAP labelling index was calculated by forming the ratio of total number of pixels of cross-sectional area immunostained for HPAP to total number of pixels cross-sectional area immunostained for insulin, in all five sections in the given mouse from the x40 images.

Alternatively, the HPAP labelling index was also calculated by forming the ration of total number of beta-cells positive for HPAP over total number of beta-cells positive for insulin in all five sections in the given mouse from the x40 images.

The beta-cell area was calculated by forming the ratio of total number of pixels cross-sectional area positive for insulin over total number of beta-cell positive for insulin, in all five sections in the given mouse from the x40 images

2.7 Origin of new beta-cell in response to pregnancy in the double transgenic (RIP-CreER^{TAM}; Z/AP) mice

2.7.1 Induction of beta-cell labelling and one cycle of pregnancy

The study was performed on eight RIP-CreER^{TAM}; Z/AP double transgenic female mice, weighing 16.4±1.9g, aged 9 weeks ± 4 days and paired at the time of their first pulse of tamoxifen. Beta-cell labelling with the human placental alkaline phosphatase (HPAP) reporter was carried out by injecting in each pair, 5 doses of 4 mg of tamoxifen (Sigma), diluted in peanut oil, over a period of 2.5 weeks.

One animal in each pair was either mated for pregnancy or maintained in a non-pregnant state. As soon as the females in each pair were fertile again, that is 1.5 to 2.5 months after the last pulse of tamoxifen, they were mated until visibly pregnant (around 15 to 18 days of pregnancy). Both groups (non-pregnant control and pregnant females in the same pair) were sacrificed at the same time. Pancreata were excised for analysis. Tissue processing and analysis were carried out together.

The mean weight of the pups at time of pregnant animal sacrifice was 1.05±0.22g.

4 pregnant and 4 non-pregnant animals in total were used for the whole experiment.

2.7.2 Induction of beta-cell labelling and two cycle cycles of pregnancy

The study was executed on 6 double transgenic (Z/AP; RIPCreER^{TAM}) female animals, age and weight-matched. They were paired and animals in each pair were injected together with 25mg of tamoxifen to label beta-cells with HPAP, for a period of 2.5 weeks. They weighed 16.8±1.7g and were aged 9 weeks ± 3 days at the start of the first tamoxifen injection.

As soon as the females were fertile again, one animal in each pair was either mated for pregnancy or maintained in a non-pregnant state. After a first pregnancy, the same animals were mated yet again ten days after giving birth. Then, during the second pregnancy (around 15 to 18 days), the pregnant animals were euthanized concurrent with the non-pregnant control females in the same pair and their pancreata were excised, processed and analysis contemporaneously.

The mean weight of the pups at time of pregnant animal sacrifice was 1.1±0.3g.

Three pregnant and three non-pregnant animals in total were used for the whole experiment.

One pair of animals was injected with tamoxifen from the same batch as animals from the experiment above, while the two other pairs of animals were injected with tamoxifen from a new batch.

2.8 Origin of new beta-cell after diabetic recovery in the triple transgenic (Z/AP; RIP-CreER^{TAM} ; pINS-c-MycER^{TAM}) mice

2.8.1 Induction of beta-cell labelling and diabetes

The study was carried out with 9 triple transgenic (Z/AP; RIPCreER^{TAM} ; pIns-c-MycER^{TAM}) mice about seven weeks of age at the start of the 4-hydroxytamoxifen (4-OHT) pulse.

4-OHT and tamoxifen compounds have similar effects when injected in animals, but tamoxifen is far less expensive and easier to prepare in peanut oil. Of note, at the time I started this experiment, 4-OHT was still used routinely in my group to treat the single pIns-c-MycER^{TAM} transgenic mice.

Beta-cell labelling with the human placental alkaline phosphatase (HPAP) reporter, and the toxic genetic beta-cell ablation by activation of c-Myc to induce diabetes, were carried out by injecting animals daily with 1ml of 4-OHT dissolved in peanut oil (1mg/ml) for ten consecutive days.

The animals were divided in 3 groups of 3. Both the control vehicle group (n=3) injected daily with 1ml of peanut oil for 10 days, and 3 animals treated with the drug, were euthanized 28 days after the last injection, while 3 other animals treated with the drug were euthanized just after the last day of injection. Pancreata of all animals were excised for analysis.

The fed blood glucose level was monitored in animals during the course of the whole experiment i.e. before, during, after 4-OHT injections, and at the time of sacrifice.

2.9 Beta-cell functionality after diabetic recovery in the single transgenic (pINS-c-MycER^{TAM}) mice

2.9.1 Diabetic induction

Single transgenic pIns-c-MycER^{TAM} (n=7) and wild-type mice (n=4) about three months of age were daily injected with 1ml of 4-OHT (1mg/0.2ml) for six consecutive days, while other pIns-c-MycER^{TAM} mice (n=3) from the same age were injected with peanut oil, the control vehicle.

9 months after the last pulse of 4-OHT or peanut oil, pIns-c-MycER^{TAM} mice were aged 12 months at the time of euthanizing while the wild-type animals were aged 3 months, but all groups were sacrificed contemporaneously for islets.

The islets from pIns-c-MycER^{TAM} and wild-type mice were isolated, cultured and analysed to examine their beta-cell functionality using the single-cell microfluorimetric method performed by Dr Paul E. Squires.

Also, we isolated islets from three month old pIns-c-MycER^{TAM} control (n=3) and wild-type mice (n=3) that did not received any treatment, for additional control tests of functionality.

Fed blood glucose levels and glucose tolerance tests were performed on the animals prior to, during, just after the last 4-OHT injection and before euthanizing the mice for islet isolation.

2.10 Pancreatic islet isolation

2.10.1 Preparation of solutions

_HBSS 1 medium is made with 500ml HBSS (Hanks Balanced Salt Solution), 2.4ml of $NaHCO_3$ at 7.5%, 10ml of HEPES at 1M pH 7.4 and1.4ml of a mixture of antibiotics containing Penicillin (10000IU/ml) and Streptomycin (10mg/ml).

_HBSS1-10%FCS medium is made with the medium above with 10% (v/v) of foetal calf serum (FCS).

_RPMI 1 medium is made with 500mls RPMI 1640 pH7.4, 0.9g of glucose (final concentration at 10mM), 1.5ml of the same antibiotic solution cited above, BSA (bovine serum albumin) at 5g/l and 5ml of L-glutamine.

_Collagenase solution is made with 9ml of HBSS 1 medium and 10mg of collagenase with protease inhibitor (1mg/ml, Type V, Sigma-Aldrich)

2.10.2 Islet isolation procedure for one pancreas and culture

The islet isolation procedure is described as follow.

1) A sterile universal with 10ml of HBSS1 medium is placed in a waterbath at 37°C prior to pancreas dissection.

2) An empty sterile universal and the collagenase solution are placed on ice.

3) After euthanizing the animal, its abdomen is incised.

4) 2.5mls of the cold collagenase solution is aspirated into 5 ml syringe, and slowly is injected first into the pancreatic duct then into all the part of the pancreas. The latter must be inflated.

5) The pancreas is excised gently in one piece and placed immediately in the empty cold universal. The rest of the procedure should be carried out in a class II cabinet.

6) 10mls of the warmed HBSS1 medium (in the waterbath at 37°C) is added to the universal containing the pancreas, then, the latter is placed in a waterbath at 37°C for 18 minutes to digest the pancreas.

7) The warm HBSS1 is discarded and replaced with 10mls of cold HBSS1 medium (without FCS) to stop the digestion, then the universal containing the digest is kept on ice.

8) The pancreas tissue is disrupted in the universal by mild hand shaking for 1 min.

9) The digest is poured through a 500µ mesh and the supernatant is collected, the tissue left on mesh is washed by pouring again 10mls of HBSS1 -10%FCS medium through the mesh, then supernatant is collected.

10) The supernatant is equally split into 2 conical 50ml tubes.

11) The conical tube containing the supernatant is centrifuged at 1200rpm for 1 min at 4°C. The medium is discarded and the pellet is resuspended in fresh HBSS1-10% FCS medium (10mls), then this step is repeated twice.

12) The pellet is resuspended this time in10mls of RPMI 1 medium, then, centrifuged at 1200rpm for 1 min. The medium is discarded, and fresh medium is added to resuspend the pellet. This step is repeated a second time.

13) The pellet is resuspended in 10mls of RPMI 1medium, then, pooled in a non-coated Petri-dish.

14) Islets are purified by hand picking under a dissecting microscope.

Then, subsequent to islet purification, the islets were cultured on 3-aminopropyltriethoxysilane-coated glass coverslips in a Petri dish containing 10ml of RPMI 1 medium. They were incubated at 37°C in the presence of 5% CO_2 for two to three days prior to analysis. The calcium microfluorimetric measurements to study beta-cell functionality were performed by Dr Paul E. Squires according to the protocol described by Squires et *al*. (Squires et *al*., 2000).

2.11 Statistical analysis

The results are presented as MEANS±SD. Pregnant and non-pregnant animals were derived from pairs of age and weight-matched animals injected with tamoxifen at the same time, the pairs of animals were killed contemporaneously, and their pancreata were processed and analysed in pairs. Therefore, the paired 2-tailed student *t* test was used for statistical analysis using the GraphPad InStat3 software. A P value <0.05 was considered as significant. For the other experiments, the unpaired 2-tailed student *t* test was used for statistical analysis using the GraphPad InStat3 software. A P value <0.05 was considered as significant.

CHAPTER 3

ORIGIN OF NEW BETA-CELLS DURING A SINGLE PREGNANCY

Origin of new beta-cells during a single pregnancy

3.1 Introduction

One key feature of the multiple physiological changes that characterize the pregnant state is the near doubling of the maternal beta cell mass, in a short period of time, to adapt to new metabolic challenges. Therefore, pregnancy is an appealing condition to understand beta-cell mass regulation, and specifically to examine the cellular sources that give rise to new beta cells.

To address this question about where new beta cells arise during this normal physiological situation, we used an inducible Cre/Lox based direct cell-lineage tracing system developed by Dor et *al*. (Dor et *al*., 2004). In double transgenic mice (Z/AP; RIP-CreER^{TAM}) beta-cells can be heritably marked.

3.2 Genetic cell-lineage tracing for beta cells and experimental design

This Cre/LoxP cell-lineage tracing system in the double transgenic mice presents three fundamental features. The first property is the ability to actively label beta-cells in adult mice during a predetermined time-window, called the pulse period. The two other properties concern the heritability and the constitutive or permanent expression of the label once the cells are labelled. Put simply, when using this labelling system, 1) one can fix the appropriate time to induce beta-cell labelling *in vivo*, and 2) only beta cells marked during the pulse phase will express the label subsequently, during the chase period (time following the pulse), as will their progeny, even if they arise some time after the pulse period (Nir et *al*., 2007).

By tracking the fate of labelled beta-cells and their progeny one can use the system to infer the source of new beta-cells in differing conditions *in vivo*.

3.2.1 Genetic construct and mechanism

The pulse chase strains are generated by the simple expedient of crossing the Z/AP double reporter mice with RIP-CreER^{TAM} mice to yield the necessary double transgenic mice required. The double transgenic mice carry an inducible Cre/LoxP system in which the Cre-recombinase (Cre), after its activation excises, at the LoxP sites, stop signal sequences upstream of a reporter gene. This leads to the expression of the reporter in beta-cells.

The system to be functional requires two distinct DNA constructs. The first genetic construct RIP-CreER^{TAM} (Dor et *al*., 2004) comprises a rat insulin promoter (RIP) driving the expression of a Cre-recombinase (Figure 5) exclusively in insulin-secreting cells, but in the absence of tamoxifen the recombinase enzyme is inactive.

The second DNA construct, the double reporter Z/AP (Lobe et *al*., 1999), comprises a general CMV/beta-actin promoter (Figure 5), (further details regarding this DNA construct can be found in appendix 2) driving the expression of either beta-galactosidase, the first reporter gene (Lac Z), or the human placental alkaline phosphatase (AP or HPAP), the second reporter gene, which is the label of interest in our case. The cassette composed of the Lac Z gene and the stop signal sequences, is flanked by two LoxP sites. As one will see further, the position of the LoxP sites in the second construct is crucial to the expression of the HPAP label.

How to induce the labelling of beta-cells for HPAP in double transgenic mice (Z/AP; RIP-CreER^{TAM})?

The Cre-recombinase is a chimerical protein fused with a truncated form of the oestrogen receptor (ER). In absence of tamoxifen, (Figure 6), the heat shock proteins (HSP) bind to the altered oestrogen receptor domain of the enzyme, creating a steric obstruction that prevents the enzyme being active and translocated into the nucleus. The Cre-recombinase is confined to the cytoplasmic compartment. As a result, at this stage (Figure 9A); the HPAP label (green staining) is not expressed in the beta-cells (red staining) while the beta-galactosidase (Lac Z) is expressed in all the cells of the rodent, including the beta-cells (Lobe et *al*., 1999).

By contrast, in presence of tamoxifen (TAM) (Figure 7), the drug binds to the altered oestrogen receptor domain of the Cre-recombinase in place of the HSP. This leads to the activation of the enzyme and its translocation in the nucleus.

Once the active Cre-recombinase is within the nuclear compartment (Figure 8), it catalyses deletion in the second DNA construct. After recognition of two LoxP sites, the lac Z gene and the stop signal sequences upstream to the HPAP gene are deleted, by recombination. This simultaneously results in the constitutive expression of the HPAP label (green staining, Figure 9B) specifically in beta-cells (red staining) and the abolition of the beta-galactosidase expression in these same beta-cells (Lobe et *al*., 1999).

Thereby, the Cre-recombinase-mediated recombination at the LoxP sites has a binary effect in the second transgene by replacing the Lac Z expression with the HPAP which is expressed restrictively in beta-cells.

The success of the labelling relies on the ability of the Cre-recombinase to efficiently remove the stop signal sequence upstream to the HPAP label gene by recombination. This leads one to anticipate that there should be a positive curvilinear relationship between the percentage of active Cre-recombinase molecules and the percentage of beta-cells that become labelled, which is another property of this system that deserves to be examined.

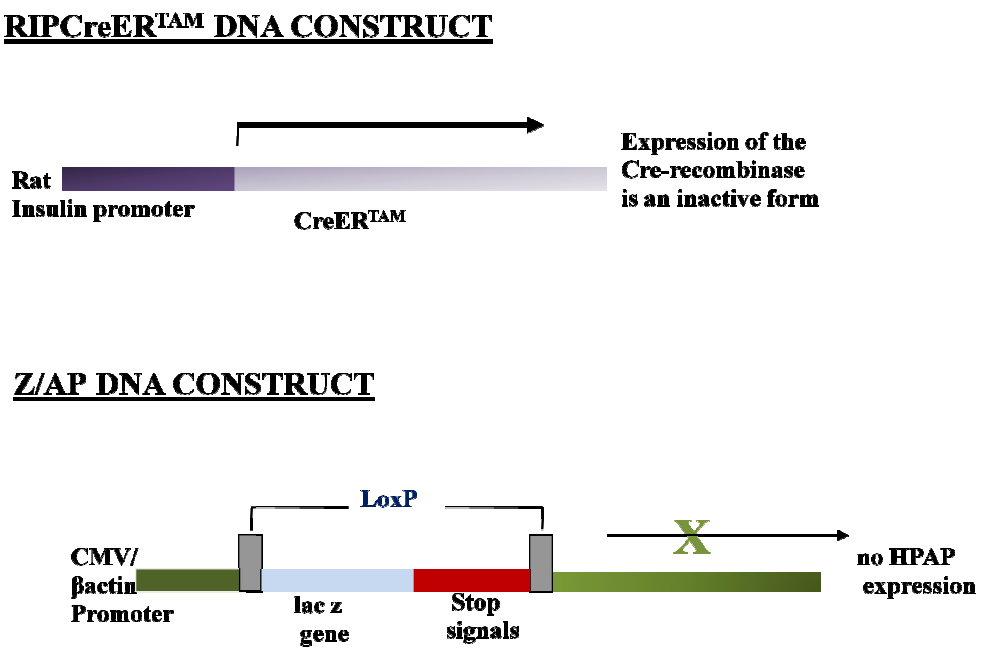


Figure 5 **Lineage tracing system DNA constructions in the double transgenic mice**. The double transgenic mice include a RIP-CreER^{TAM} DNA construct in which the rat insulin promoter (RIP) drives the expression of the Cre-recombinase in mature beta-cells, but it is inactive in absence of tamoxifen. The second DNA construct includes, Z/AP or the reporter gene and a generally active promoter (CMV/beta actin) which leads to the expression either of the beta galatosidase (Lac Z) or the human placental alkaline phosphatase, the label (HPAP or AP). Furthermore, in the second transgene, the cassette formed by the Lac Z gene and the stop signals, is flanked by two LoxP sites.

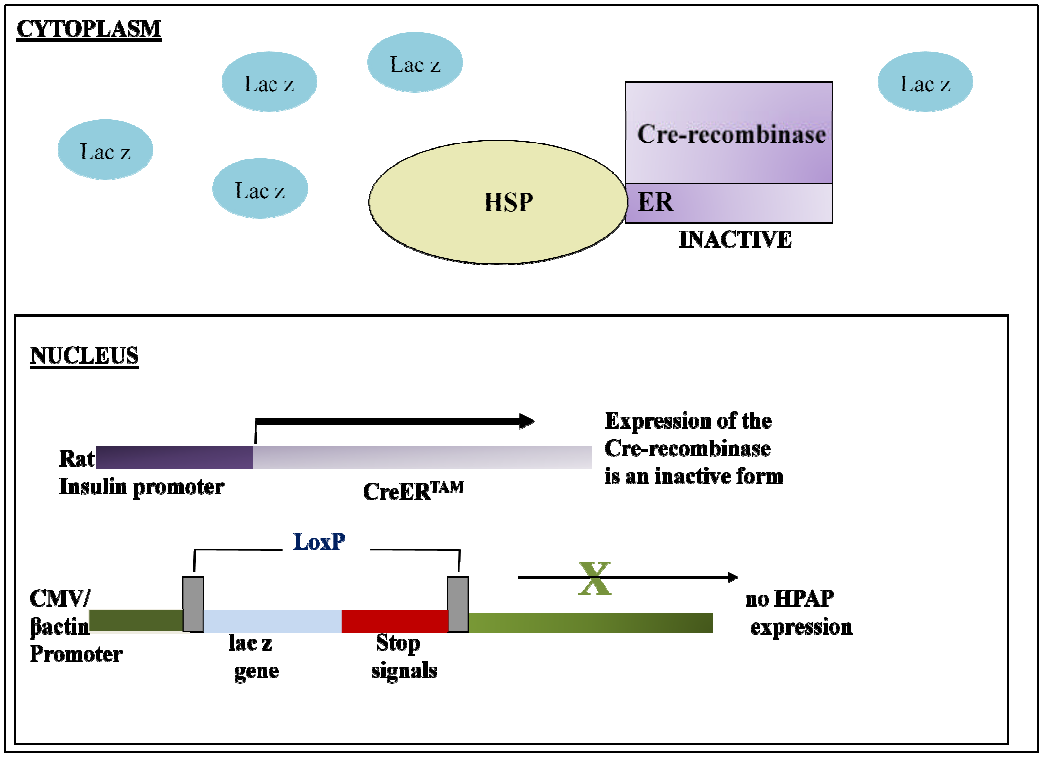


Figure 6 **Beta-cell labelling mechanism in the double transgenic mice**. In absence of tamoxifen, the Cre-recombinase is expressed only in mature beta-cells, but is inactive due to its sequestration by HSP (heat shock protein). As a result the HPAP label is not expressed while the beta galactosidase is expressed in the entire animal including the beta-cells.

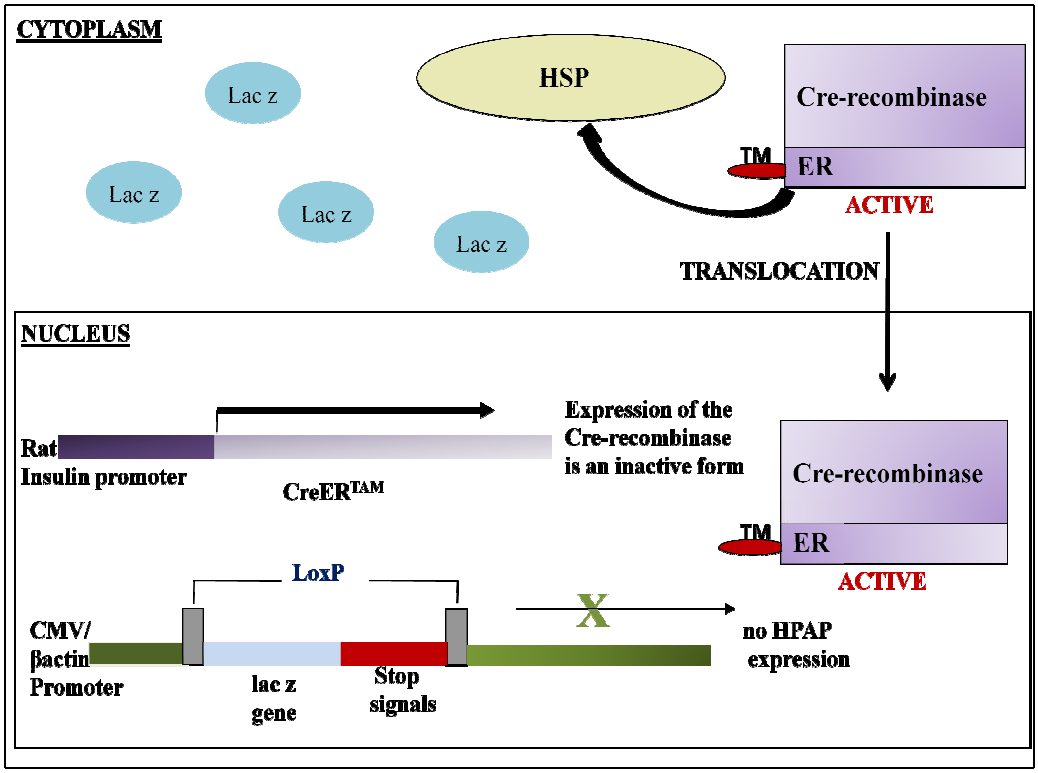


Figure 7 **Beta-cell labelling mechanism in the double transgenic mice**. Upon tamoxifen administration, the drug binds to the Cre-recombinase by displacing the HSP. As a result, the Cre-recombinase becomes active and translocates from the cytoplasm to the nucleus.

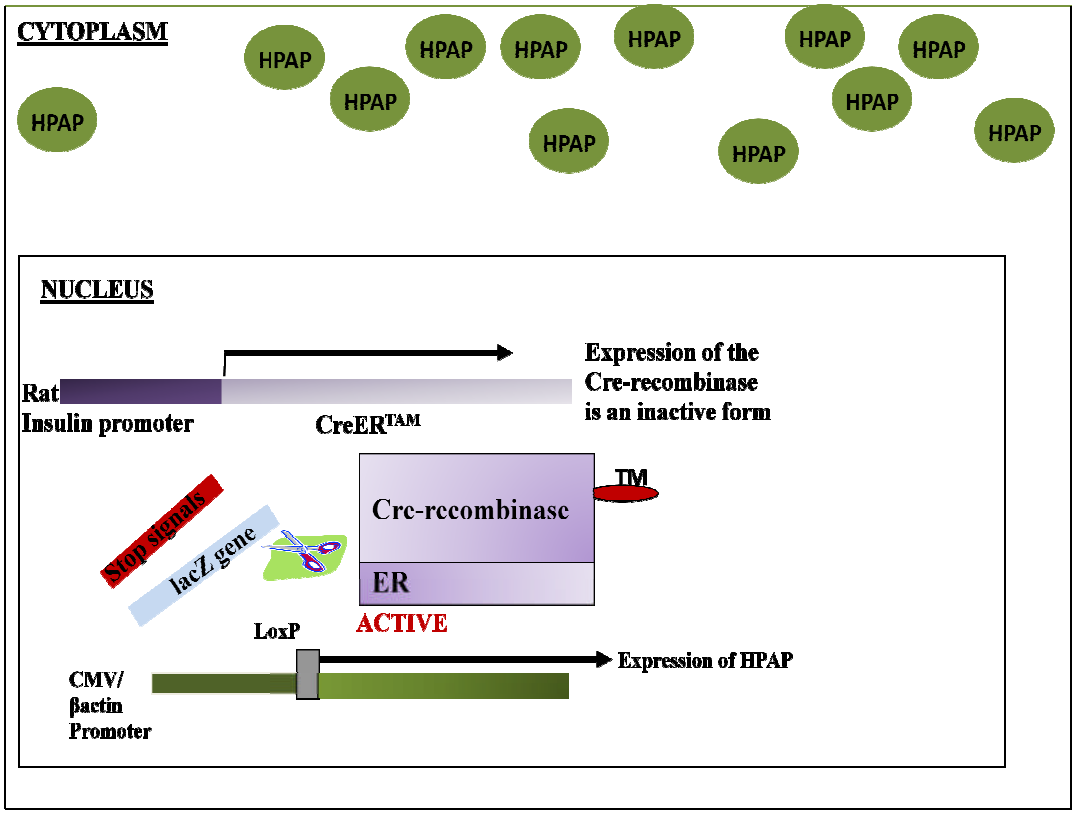


Figure 8 **Beta-cell labelling mechanism in the double transgenic mice**. After migration into the nucleus, the active Cre-recombinase mediates recombination at the LoxP sites, hence causing the deletion of the Lac Z gene and the transcriptional and translational stop signals upstream to the HPAP reporter gene. As a result, the beta-cells are irreversibly and heritably labelled by HPAP while the expression of the beta galactosidase (Lac Z) is irreversibly abolished in these same beta-cells.

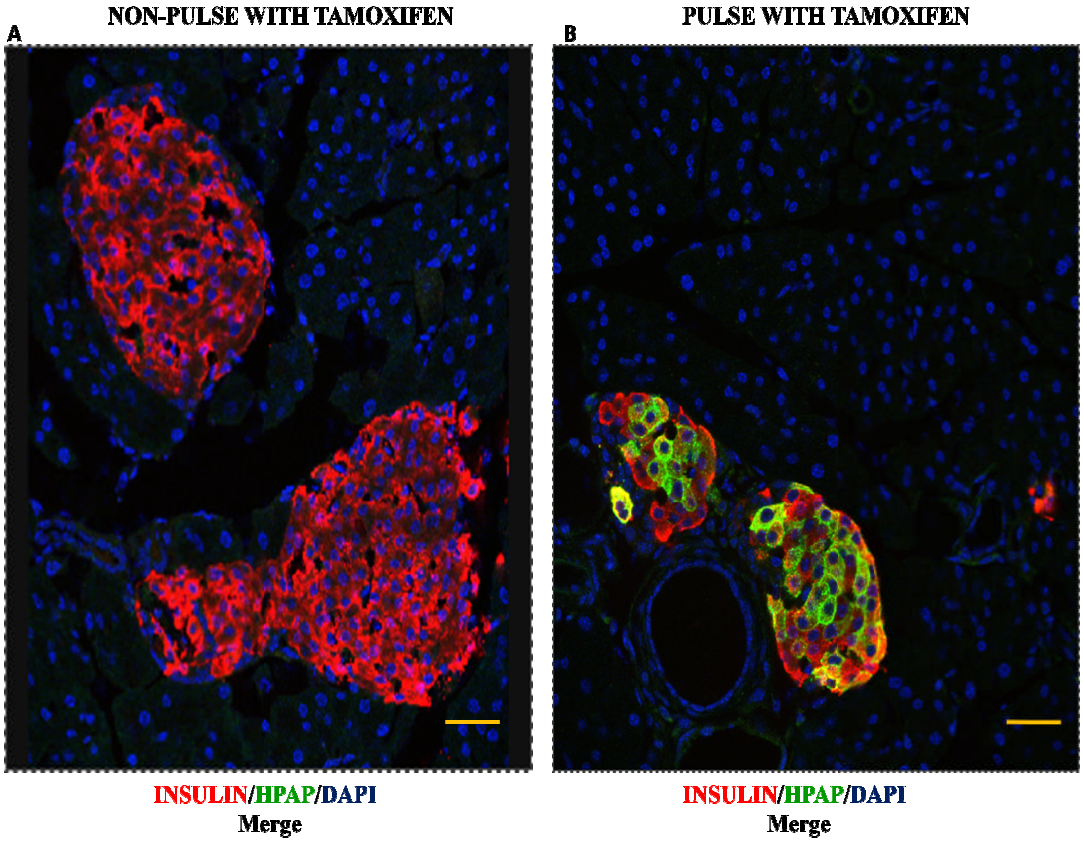


Figure 9 **Beta-cell labelling mechanism in the double transgenic mice**. (A) Pancreatic section of mouse not pulsed with tamoxifen. The image shows no HPAP (green staining) expression in beta-cells (red staining). (B) Pancreatic section of a mouse pulsed with tamoxifen. The image shows HPAP expression in the beta-cells. The nucleus is stained with DAPI (blue). The scale bar corresponds to 40 µm.

3.2.2 Other properties associated with the labelling system which have been investigated

If the percentage of beta-cell labelled does depend on the rate of the active Cre-recombinase, one would expect that the tamoxifen dose administered to the animal might modulate the amount of active Cre-recombinase and, consequently the proportion of labelled beta-cells.

To test this hypothesis, we injected two female double transgenic animals of similar age and weight with 20 or 25mg of tamoxifen. The percentage of HPAP labelled beta-cells was 49% and 66% respectively (Figure 10), leading us to assume that the labelling is sensitive to tamoxifen or tamoxifen dose-dependent, as originally suggested by Dor et *al*. (Dor et *al*., 2004).). Previous works carried out by Norhayati, Ahmad who worked in our group on the same double transgenic mice (Z/AP; RIP-CreER^{TAM}, the data can be found in her thesis) clearly demonstrated dependency of recombination frequency on tamoxifen dosage.

On the other hand, in absence of tamoxifen, the proportion of beta-cells labelled for HPAP was zero, even though we observed, occasionally in older animals, a few islets containing one to two beta-cells labelled for HPAP.

Moreover, the beta-cell labelling in the pancreas of animals injected with the same dose of tamoxifen (20mg) but sacrificed after different times, two or eleven weeks after the tamoxifen pulse (Figure 11) showed no sign of dilution. This indicates that the labelling of the beta-cells is irreversible. This system is a genetic lineage tracing system.

Also, preceding work by N. Ahmad showed that a high proportion of labelled beta-cells in the pancreas could be obtained by pulsing the double transgenic mice (Z/AP; RIP-CreER^{TAM}) with a total amount of 40mg of tamoxifen. Using this dose of tamoxifen, we found that 90% of beta-cells in the animal pancreata were labelled for HPAP.

Unfortunately, for our current experiment it was not feasible to inject a total amount of 40mg of tamoxifen into our female animals. This tamoxifen dose was lethal for the majority of them. One explanation may be related to the small size of

our 9 week old female mice. We could not use older animals because the fertility of the female mice decreases when they get older.

However, after optimisation of the tamoxifen dose, we found that half of the amount of tamoxifen (that is 20mg) was sufficient to label efficiently the beta-cells in the mice without compromising subsequent fertility. Also, using this quantity of tamoxifen, we observed a significant improvement of the survival rate of the animals. Consequently, this dose of tamoxifen was used to inject our mice to perform the present study.

However, despite the fact that many of our mice could tolerate a total quantity of 20mg of tamoxifen, nonetheless this amount was still high for animals of low weight. This explains in part, why the number of animals at the end of the experiment was low and so the number of replicates in the study. Limitations related to this type of experiment were 1) the permanent loss of the fertility of some female mice due to the dose of tamoxifen administrated and 2) the low ratio of female double transgenic mice expected to be of the right genotype.

To sum up, we are confident to state that our lineage tracing system is 1) specific to beta-cells, 2) the labelling is irreversible 3) heritable and 4) the percentage of Cre-recombinase induced recombination is tamoxifen dose-dependent. Consequently, it could be used in pulse-chase experiment to determine the origin of new beta-cells.

3.2.3 Pulse-chase experiment and analyses of beta-cell origin

The double transgenic mice (Z/AP; RIP-CreER^{TAM}) can be employed in pulse-chase experiments to predict where new beta-cells arise during pregnancy or other conditions.

The methodology is presented in Figure 12. The first step is to mark beta-cells with the HPAP label, in our case by injecting 20mg of tamoxifen in female double transgenic animal mice for two and half weeks; a period called the pulse.

It is important to point out that before starting the tamoxifen treatment, age and weight-matched female mice were paired. Each pair was treated the same way during the whole experiment. Likewise their pancreas were excised, processed and analysed contemporaneously.

Importantly, the average percentage of labelled beta-cells after completion of the pulse of tamoxifen was 44%. Thereafter, during the chase period, in each pair, pregnancy was induced in one animal while the other female was maintained in a non-pregnant state. Thenceforth, when the pregnancy was visible, around 15 to 18 days of gestation, the pregnant and non-pregnant control female paired mice were euthanized contemporaneously and their pancreata collected for analyses.

According to the percentage of labelled beta-cells measured in the pancreas of pregnant animals, one can deduce the cell origin for newborn beta-cells during pregnancy. At least three likely possibilities must be considered concerning the contribution of stem/progenitor cells (neogenesis) and/or pre-existing beta-cells (mitogenesis) to the formation of new beta-cells in these studies.

Hypothesis 1, the percentage of labelled beta-cells in the pancreas of the pregnant animal is the same as in the non-pregnant control, suggesting that new beta-cells originate from pre-existing differentiated beta-cells. Beta-cells give rise to fresh new beta-cells by self-duplication.

Hypothesis 2, the percentage of label in pregnant animals is diluted, suggesting that an unlabelled pool of cells of non beta-cell lineage, are contributing significantly to the formation of new beta-cells, in combination or not with differentiated beta-cells, hinging on the degree of dilution. These cells can be either stem/progenitor cells or differentiated cells which are not beta-cells, or simply both.

Hypothesis 3, whole new islets devoid of any marked beta-cells are formed in the pregnant female pancreas, resulting in an increase of the percentage of these types of islets. If this happened, one could conclude that these newly formed islets derive from beta-cells generated from non-beta-cell progenitors.

The quantification of the percentage of the beta-cells labelled for HPAP is an important question since our conclusions depend on the accuracy of these data. So

we defined a reliable method to measure the HPAP and insulin area. This is the subject of the next section and merits further discussion.

3.2.4 HPAP labelling index and quantification

With a view to determining the percentage of labelled beta-cells in the pancreas of double transgenic mice, we quantified the area positive for HPAP or insulin in multiple digitally recorded images of pancreatic sections using ImageJ software. First of all, it was necessary to put into place a method to process the pancreas and to carry out the HPAP and insulin measurement so as to produce reliable data.

First, we needed to employ an appropriate protocol to take into account the complex and heterogeneous organization of islets within the pancreas (Alanentalo et *al*., 2007, Avril et *al*., 2002).

Thus, specific regional adaptation of the rat pancreas has been shown during pregnancy, which include an increased replication rate in the head of the pancreas (region adjacent to the duodenum) compared to the tail (region of the pancreas adjacent to the spleen). Although, this might not necessarily reflect what occurs in mouse, we decided to roll up the excised pancreas in a flat spiral, and to section it in the same plane as the flat spiral, namely, along a z-axis (Figure 13). Thus, the different anatomical parts of the pancreas (tail, body, and head) will be on the same section and each section will represent different levels of the organ.

Secondly, to avoid bias in sections chosen for analysis, we numbered our sections sequentially by ascribing the number one to the first microtome section, the number two to the second and so forth. In total from five to seven hundred sections per pancreas were cut but only six sections per level were collected. One level corresponds to ten abutting sections. As the thickness of the section was ten micrometers, one level represented a thickness of approximately one to two hundred micrometers, about the size range of a typical intermediate islet (Jo et *al.*, 2007).

Given the great number of sections produced per pancreas, it was not feasible to process every single one of them. For this reason, five sections representing different regions or levels of the pancreas along a z-axis, and separated from each other by at least five hundred micrometers to minimize the sampling of the same islet twice, were chosen at random.

To recapitulate, with the objective to extract the greatest amount of practicable information from the pancreas, first, we embedded the harvested organ in a flat spiral. Then, it was sectioned along a z-axis and each section was numbered appropriately to ensure that in the selected analysed sections the same islets were not scored twice.

The second criterion was to consider the HPAP and insulin area measurement procedure. In order to quantify the HPAP and insulin area in the section using ImageJ, we captured images of every immunostained pancreatic cross-sectional area of single beta-cells, clusters of beta-cells, and islets which were present in each section. Five sections per pancreas were entirely scanned using a confocal microscope (Leica, SP2).

An average, two hundred and twenty one photos of islets or beta-cell clusters per pancreas were captured, but four hundred and forty two images per pancreas or in the whole five sections were analysed, employing the measurement software, because the quantification of HPAP and insulin cross-sectional area had to be performed on separate images after immunostaining (i.e. red and green channels). Overall, three thousand five hundred and thirty six images were processed for the whole experiment (eight harvested pancreata in total).

The area evaluation was performed on pancreatic sections immunostained with anti-HPAP and anti-insulin antibodies (as described in Materials and Methods). Fluorescein and Alexa fluo 633, which are fluorescent dies bound to secondary antibodies, were used to reveal the primary antibodies respectively.

Using ImageJ, the HPAP and insulin area can be measured either automatically or manually after adjusting an image intensity threshold with a slider. In our case, the threshold was set manually for each picture, as it proved more accurate for distinguishing positive HPAP or insulin immunoreactivity

from“background”. After adjusting the threshold, the HPAP or insulin area was measured. The adjustment of the threshold was repeated three times on the same object, so that three counts per object were carried out and the three computed values were averaged.

It is of interest to point out that ImageJ measures the area by counting the number of pixels constituting the object on the picture. Thus, the HPAP and insulin area determination represent the total number of pixels counted.

Importantly, all such measurements were conducted blind. The images used to quantify the HPAP or insulin area were all anonymously coded. The experimenter had no knowledge of the condition being observed, meaning if the pictures analysed were from a pregnant or non-pregnant animal or from a given replicate.

Using this tool (ImageJ), we were able to evaluate the HPAP and insulin area in each beta-cell cluster and islet, and to calculate the proportion of HPAP labelled beta-cells, the “HPAP labelling index” in the pancreas of each mouse. The HPAP labelling index was computed by forming the ratio of the total pixel cross-sectional area positive for HPAP over the total pixel cross-sectional area positive for insulin in the whole five sections of each animal. We applied the same method to measure the cross-sectional area for insulin and the pancreatic tissue to calculate the beta-cells mass in the pregnant and non-pregnant mice (Huang et *al*., 2009).

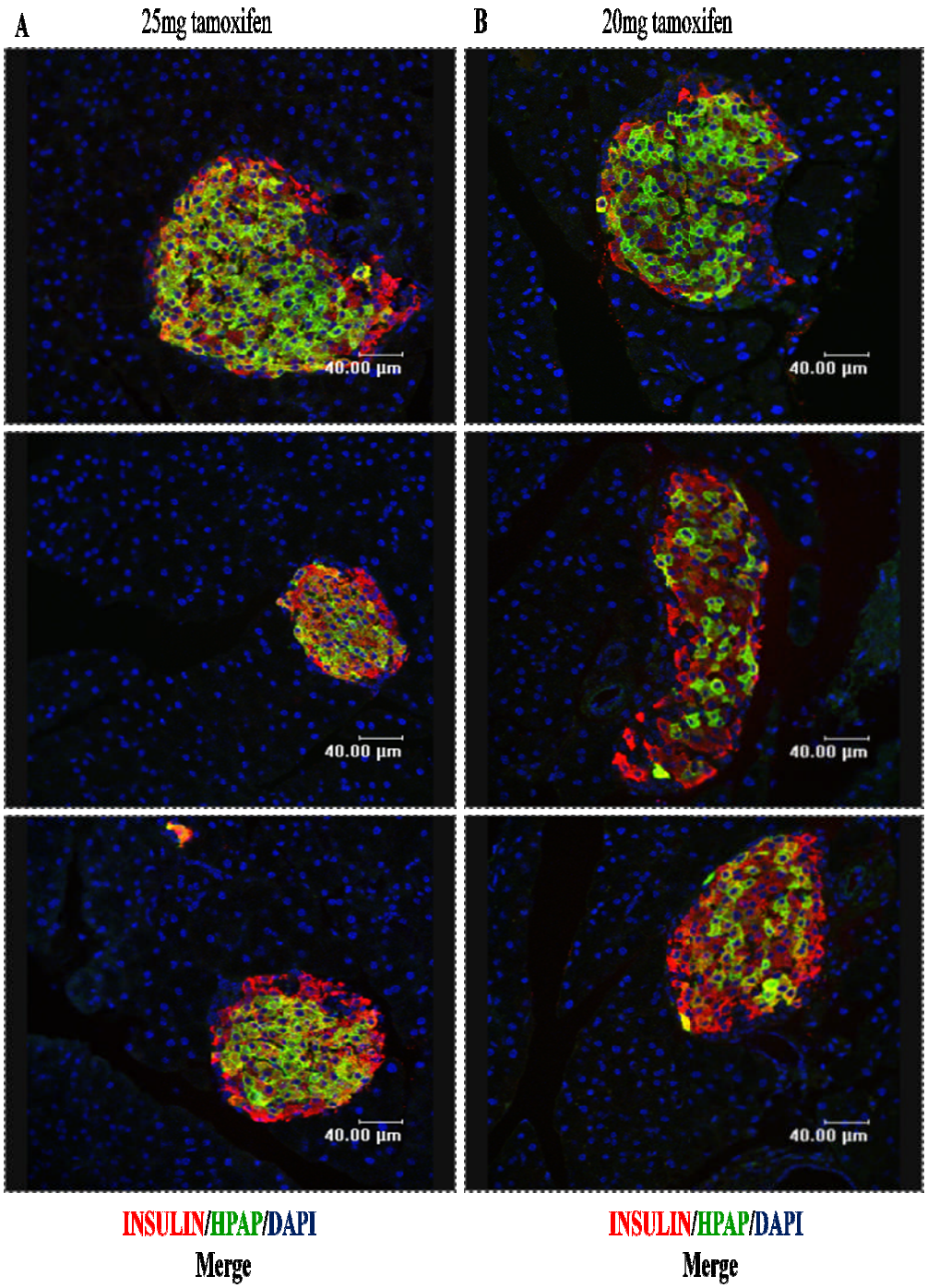


Figure 10 **Lineage tracing system is tamoxifen dose dependent**. Pancreatic sections from mice pulsed with 25mg (A) or 20mg (B) of tamoxifen. The sections were immunostained for HPAP (green), insulin (red staining) and the nuclei were stained by DAPI (blue).

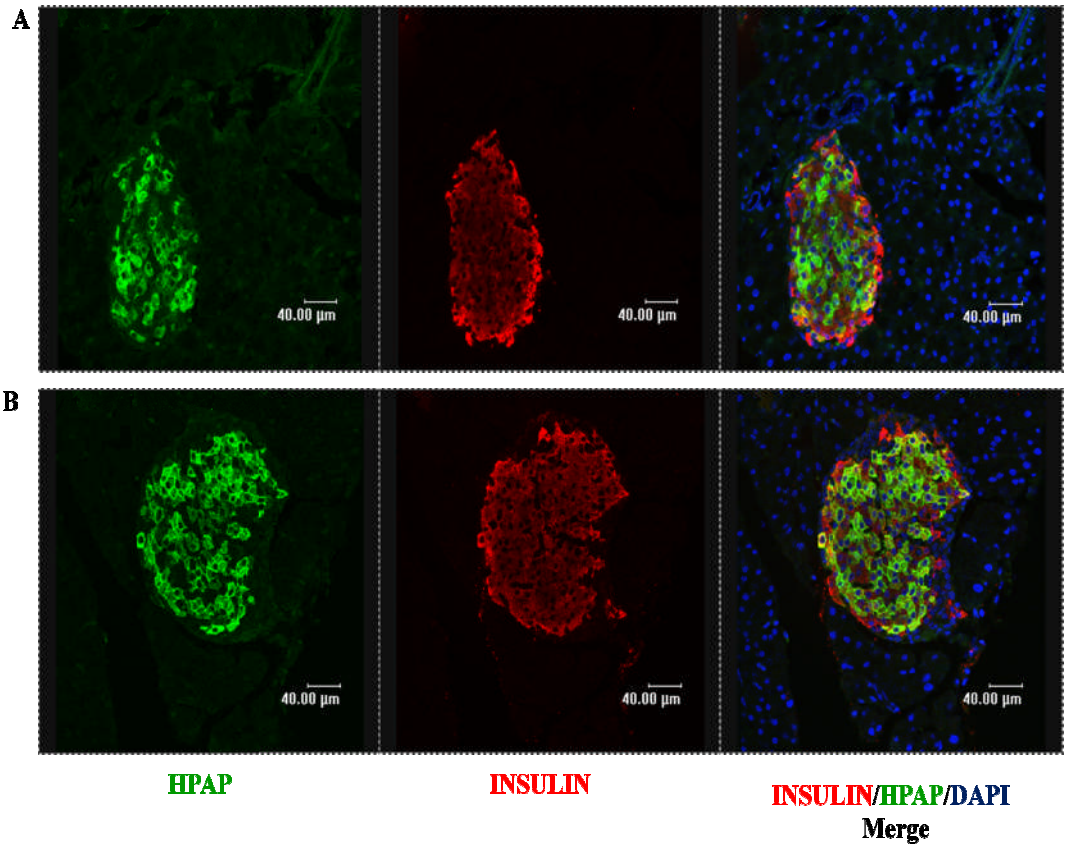


Figure 11 **Irreversibility of the label in the beta-cells**. Pancreatic sections from mice pulsed with 20mg of tamoxifen but euthanized two weeks (A) or eleven weeks (B) after the pulse. The sections were immunostained for HPAP (green), insulin (red staining) and the nuclei were stained by DAPI (blue). The scale bar corresponds to 40µm

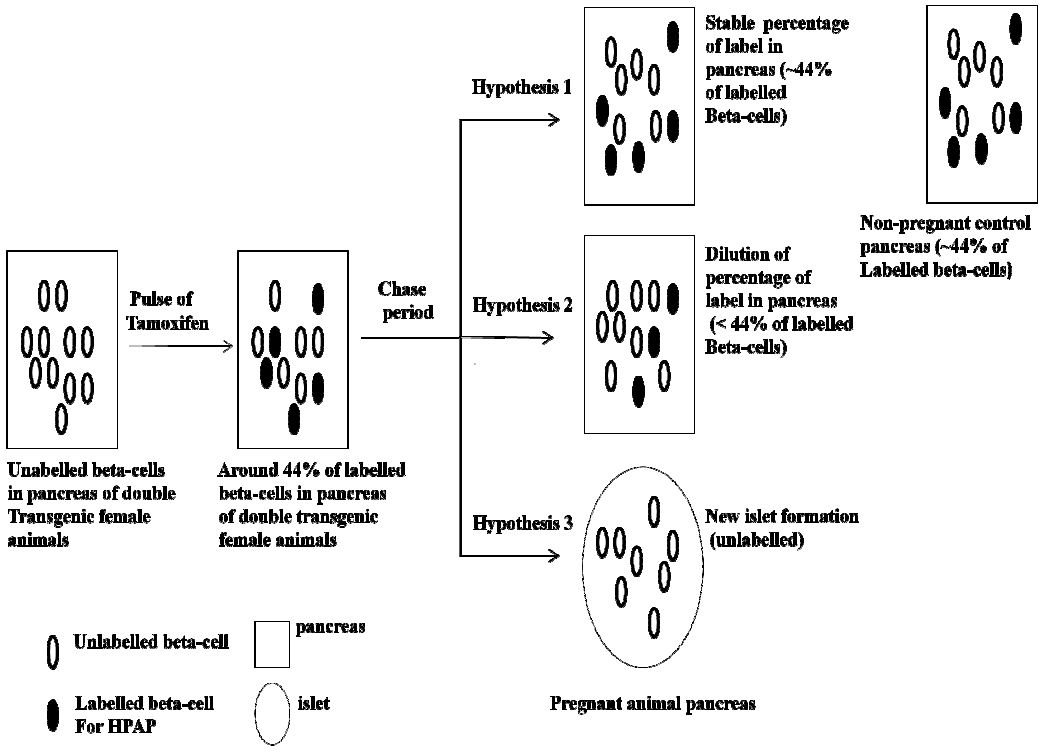


Figure 12 **Analysing the origin of new beta-cells during pregnancy**. Double transgenic female mice (Z/AP; RIP-CreER^{TAM}) were pulsed with tamoxifen to label their beta-cells. Then during the chase period, pregnancy was induced in only one group of mice while the other animals were assigned to the non-pregnant control group. After the chase period, if the percentage of beta-cells positive for HPAP in pancreas of the pregnant animals is identical to the non-pregnant controls we conclude that new beta-cells are the progeny of pre-existing beta-cells (Hypothesis 1). However, if there is a dilution of the percentage of HPAP-labelled beta-cells in the pregnant animals, we conclude that a non-beta-cell source gives rise to new beta-cells, possibly in combination with a contribution from pre-existing beta-cells (Hypothesis 2). Finally, if the percentage of islets totally devoid of any label is higher in pregnant animals than in the controls, one can deduce that whole new islets arise from non-beta-cell progenitors (Hypothesis 3).

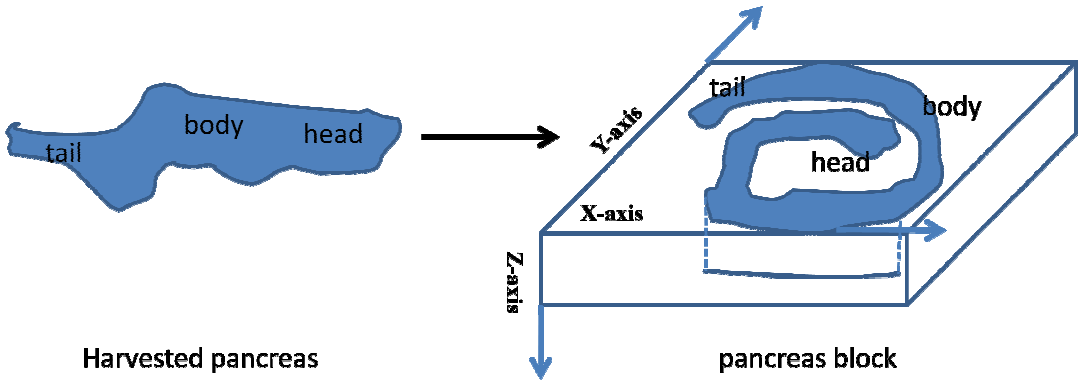


Figure 13 **Pancreas orientation before embedding and sectioning**. The pancreas is a heterogeneous organ which is divided in three regions, the head, body and tail. In order to extract information from this organ with little bias, the pancreata were rolled up in flat spiral before embedding and sectioning in the same plane as the flat spiral, i.e. along the z-axis.

3.3 Results

3.3.1 Experiment outline

The Cre/LoxP lineage tracing system was exploited to analyse the origin of new beta-cells induced by a single cycle of pregnancy. In transgenic animals (Z/AP; RIP-CreER^{TAM}) beta-cells and their progeny were marked with HPAP in response to tamoxifen-induced activation of the Cre-recombinase in beta-cells.

To maximize the potential of the lineage tracing system technique for detecting any dilution of the labelling during the chase period, we aimed to use a dose regime of tamoxifen that gave a relatively high proportion of labelling in the range of 40-50% in the transgenic animals, without compromising subsequent fertility.

For this purpose age and weight-matched female animals were paired and injected together with a total of 20mg of tamoxifen, about 9 weeks of age, over a period of two and half weeks, to label beta-cells for HPAP. Thereafter, one female animal in each pair was either mated for pregnancy or maintained as a virgin in a non-pregnant state. Pregnant animals between fifteen to nineteen days of gestation and non-pregnant controls from the same pair, were euthanized contemporaneously for examination of the pancreata. Each pair (a total of four pairs were set up for the whole experiment) was treated the same way, and their pancreata processed and analysed similarly and at the same time.

We suspected (see below) that a variable in the lineage-tracing is the preparation of the tamoxifen solution for injection, and the paired design should minimize this source of variation, as both animals were injected with the same preparation of the drug. This is consistent with our observations on the sensitivity of the dose-response to tamoxifen.

3.3.2 Response to pregnancy

In line with another study, pregnancy was associated with an increase of the body weight (Karnik et *al*., 2007) and pancreas weight. At the time of the experiment we observed a 45% increase of the mean body weight (Figure 14A) and a 51% increase of the mean pancreas weight (Figure 14B) in pregnant animals compared with the age-matched non-pregnant controls. In the same way, we determined the beta-cell mass by morphometric analysis as described in Materials and Methods, in the animals. We observed a 91% increase of the beta-cell mass in the pregnant animals compared to the age-matched non-pregnant controls (Figure 14C). This result corroborates previous studies (Scaglia et *al*., 1995, Aerts et *al*., 1997, Huang et *al*., 2009, Avril et *al*., 2002, Blondeau et *al*., 1999, Karnik et *al*., 2007, Parsons et *al*., 1995, Parsons et *al*., 1992, Teta et *al*., 2007) indicating that maternal adaptation to pregnancy was accompanied by a substantial beta-cell mass increment of about fifty to a hundred percent, compared to non-pregnant control animals. Importantly, the authors above have demonstrated that increase in beta-cell numbers was mainly responsible for the maternal beta-cell mass increase with slightly or no contribution from beta-cell hypertrophy (i.e. increase in beta-cell size). Therefore, we could deduce that the increased beta-cell mass observed in pregnant animals in our study, was the result of an increase of the beta-cell population. Consequently, our next question was to examine where the newly formed beta-cells arose.

3.3.3 Dilution of HPAP labelling index during a single cycle of pregnancy

The double transgenic female mice were paired for age and weight, and injected with tamoxifen. At the start of the study, 17 animals were involved, but due to the experimental difficulties mentioned above, only 8 animals could be analysed. Following the pulse, after the mice recovered their fertility, pregnancy was induced in one animal in each pair while the other one in the same pair was maintained in a non-pregnant state. At the end of pregnancy, the pancreata were collected for analysis.

We observed that the mean HPAP labelling index (Figure 15A) in the non-pregnant control group was greater (0.44±0.05) than in the pregnant group (0.33±0.06). Also, we draw attention to the fact that the HPAP labelling index, when

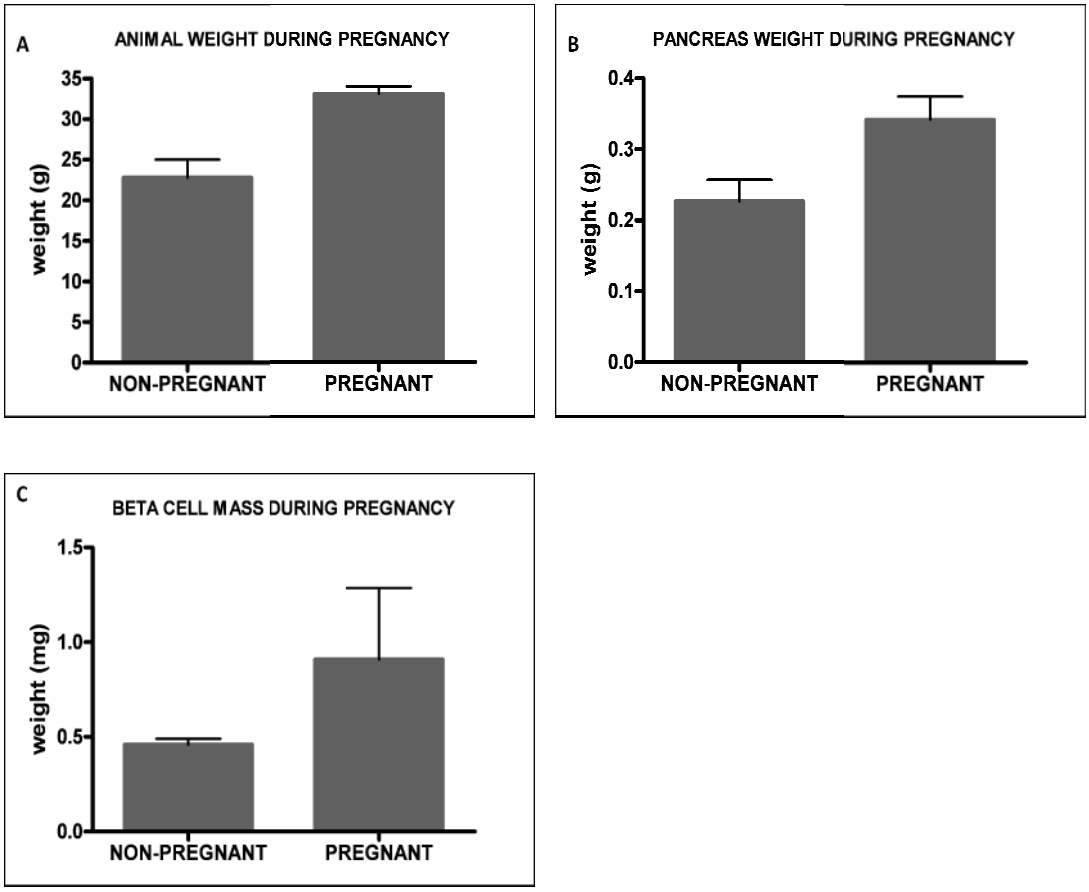


Figure 14 **Responses to pregnancy**. During pregnancy, the mean body weight increased by 45% (A), and the mean pancreas weight by 51% (B), as compared with their age-matched non-pregnant control counterparts. Mean beta-cell mass in pregnant animals increased by 91% (C).

analysed individually shows a dilution of the label systematically in the pregnant animal within each pair (Figure 15C). It is worth reminding that animals in each pair were treated and analysed identically and contemporaneously to minimize variations other than differences between animals. The difference between the two groups was statistically significant (P=0.021, paired two-tailed t-test).

This dilution leads to rejection of hypothesis 1 and indicates for the first time, the contribution to new beta-cells from a non-beta cell source during the beta cell mass expansion in response to pregnancy. Accordingly, the data are consistent with hypothesis 2.

One potential mechanism for beta-cell expansion is the creation of new islets. To investigate this possibility, we focused on determining the frequency of islet or beta-cell clusters whose sectioned area appeared totally devoid of any HPAP label, in pancreata of pregnant and non-pregnant animals.

The appearance of beta-cell clusters or small sized-islets after certain types of pancreatic injury or under specific conditions is frequently reported as neogenic beta-cells or islets (Wang et *al*., 1995, Yamamoto et *al*., 1997, Yamamoto et *al*., 2000, Lipsett and Finegood, 2002, Aerts et *al*., 1997, Blondeau et *al*., 1999, Huang et *al*., 2009, Parsons et *al*., 1995).

To validate hypothesis 3, that is if new whole islets are formed by neogenesis during pregnancy, we counted islet cross-sectional area for islets appearing as totally negative for HPAP in pancreatic section of non-pregnant control and pregnant animals.

We noted in our studies that amongst small islet cross-sections, several did not contain any labelled beta-cells, whereas, by contrast, intermediate and large islets cross-sections always contained HPAP labelled beta-cells.

This observation regarding the small islets cross-sections devoid of HPAP immunoreactivity, supports the idea that small islets sizes or beta-cell clusters might represent formation of new islets by neogenesis.

Alternatively, these label-free small islets cross-sections, might represent glancing sections or edges of larger islets (Figure 16).

The proportion of islet cross-sectional area HPAP negative in the two groups was computed by forming the ratio of total number of islet cross-sectional area negative for HPAP over the overall number of islet cross-sectional area positive or not for HPAP, in the whole five sections for each pancreas.

We found that the mean proportion of islets negative for HPAP (Figure 15B) was greater (0.19±0.06) in the pregnant mice compared to this in the control non-pregnant group (0.11±0.04). Likewise, the analysis of the proportion of islets HPAP negative in each animal (Figure 15D), shows a rise of these specific islets or beta-cell clusters, systematically in the pregnant animals in each pair. The difference between the two groups was not statistically significant (P=0.093, paired two tailed t-test).

It is still tempting to speculate that there might be formation of whole new islets during pregnancy. Yet, small islet cross-sections might represent glancing sections of larger islets and not solely representative sections of genuine islet of small mass.

Numerous reports showed that during pregnancy, islets grew bigger (Aerts et *al*., 1997, Blondeau et *al*., 1999, Parsons et *al*., 1995), So it is possible that the dilution within such enlarged islets will increase the number of small cross-sections lacking HPAP immunoreactivity in pregnant animals. Eventually, the increased number in label-free small cross-sections in pregnancy, supports our results above regarding the diminution of the HPAP labelling index in this condition.

To conclude, we have demonstrated for the first time that non-beta-cell progenitors contributed significantly to the beta-cell mass expansion during pregnancy. The next question is: where do these new beta-cells come from?

Thus, we next sought to examine the identity of the cell-lineage contributing to beta-cell neogenesis. Based on extensive literature surrounding the beta-cell differentiation, obvious candidates were progenitors associated with the pancreatic duct epithelium

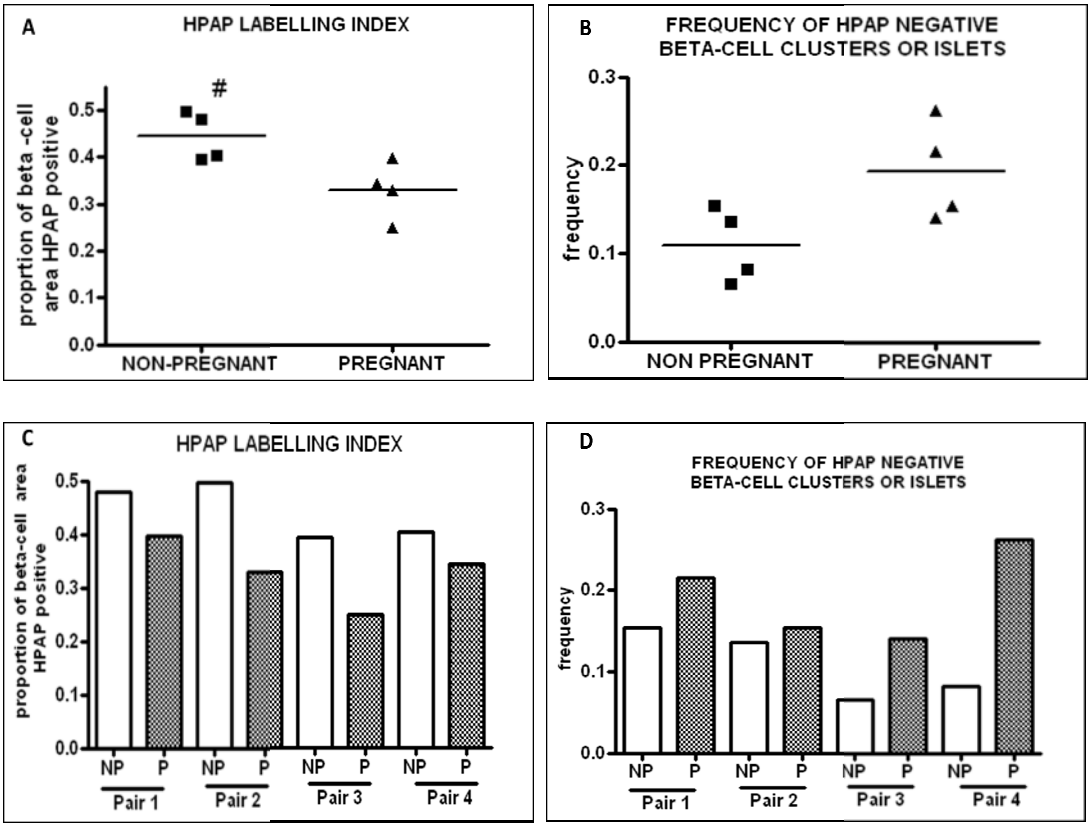


Figure 15 **Lineage tracing in pregnancy**. (A), The HPAP labelling index in non-pregnant control and pregnant female animal pancreata was calculated by forming the ratio of total number of pixels of cross-sectional area immunostained for HPAP to total number of pixels cross-sectional area immunostained for insulin, using Image J. The mean labelling index in the pancreata of non-pregnant animals is higher (0.44±0.05) than in the pregnant mice (0.33±0.06). (B) The proportion of islet sections completely negative for HPAP is calculated by forming the ratio of the HPAP-negative islet cross-sectional area to the total islet cross-sectional area in the whole of 5 sections for each pancreas. The mean proportion of islet sections HPAP negative in the pregnant group is (0.19± 0.06) is higher than in their age-matched, non-pregnant control counterparts (0.11±0.04) as seen in the graph. (C) and (D) represent the HPAP labelling index and the frequency of HPAP negative beta-cell cluster or islets in pregnant and non-pregnant animals respectively in each pair. # designates a P-value <0.05 by the paired two-tailed student t-test. Non-pregnant (NP), Pregnant (P).

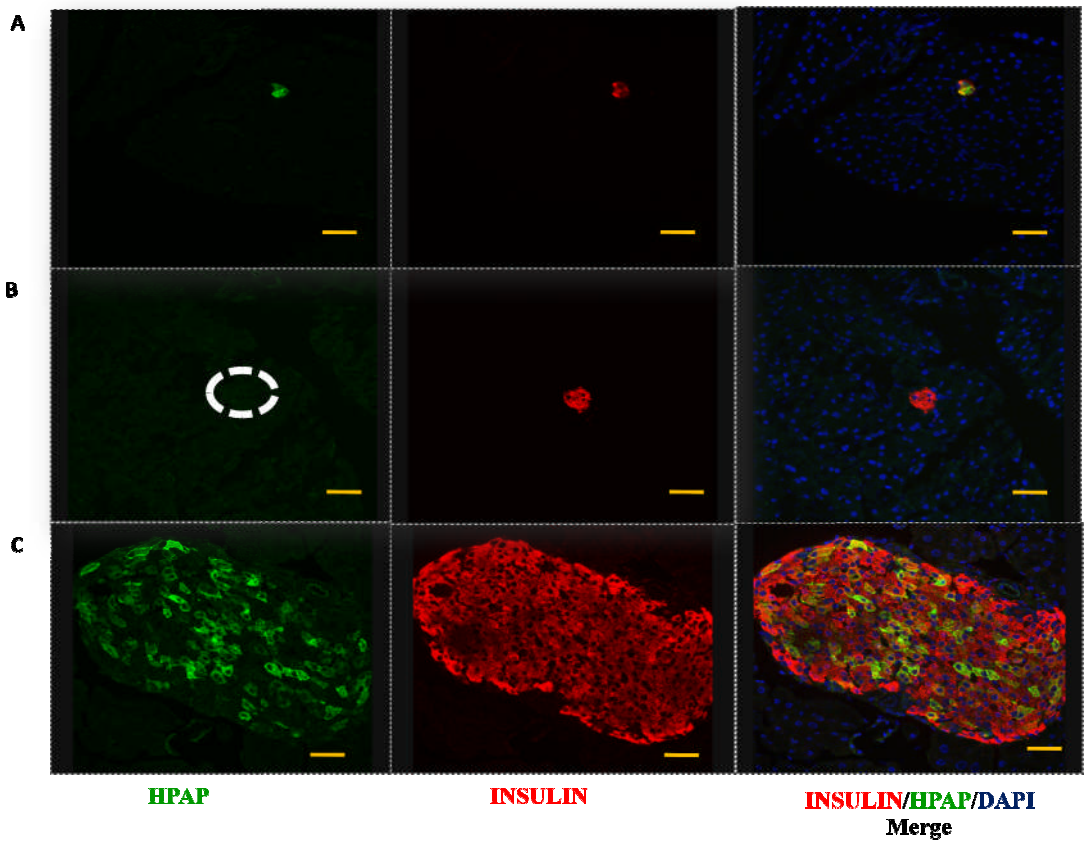


Figure 16 **Beta-cell clusters negative for HPAP**. Photomicrographs of pancreatic sections from pregnant animals stained for insulin (red), HPAP (green), and nuclei (blue, DAPI). Small islet sections are either partly or wholly HPAP-positive (A), or completely HPAP-negative (dashed circle, B). Small islet sections represent either genuinely small islets or glancing sections of larger islets (C). Scale bar corresponds to 40µm.

3.3.4 Beta-cell clusters associated with the pancreatic duct

In numerous studies, the pancreatic duct epithelium has been identified as a potential location for islet beta-cells progenitors in adult pancreas both during normal growth and beta-cell regeneration.

A program of beta-cell neogenesis that might be activated in the adult pancreatic ductal epithelium has been well-documented in the literature. Studies analysing beta-cell regeneration, subsequent to pancreatic injury (Wang et *al*., 1995, Waguri et *al*., 1997, Thyssen et *al*., 2006, Yamamoto et *al*., 1997, Yamamoto et *al*., 2000, Sharma et *al*., 1999), in transgenic mice over-expressing INFα (Gu and Sarvetnick, 1993) or TGFα and gastrin (Wang et *al*., 1993), showed that regenerated beta-cell mass was associated with an increase of single beta-cells, beta-cell clusters or small sized-islets closely in contact with the ductal structure.

Furthermore, the studies revealed that the activation of this intricate program of neogenic beta-cells required, in this sequence, a burst of proliferation in the pancreatic ductal component, then the expression of endocrine markers within this compartment (Wang et *al*., 1995, Yamamoto et *al*., 2000, Bonner-Weir et *al*., 2004, Bouwens and Pipeleers, 1998, Suarez-Pinzon et *al*., 2005, Holland et *al*., 2005, Dutrillaux et *al*., 1982, Gu et *al*., 1994, Sharma et *al*., 1999).

These observations led the authors to conclude that the actual adult pancreatic ductal epithelium might contain islet cell precursors that could be re-activated in specific situations.

More recently Inada et *al*. (Inada et *al*., 2008) demonstrated, using a duct cell-lineage tracing system, that duct progenitors were indeed accountable for the generation of new islet-cells and acinar cells shortly after birth, and after pancreatic injury induced by duct ligation.

Also, the abundant literature concerning putative islet progenitors residing in the ductal epithelium, reported by studies above and reviewed in others reports

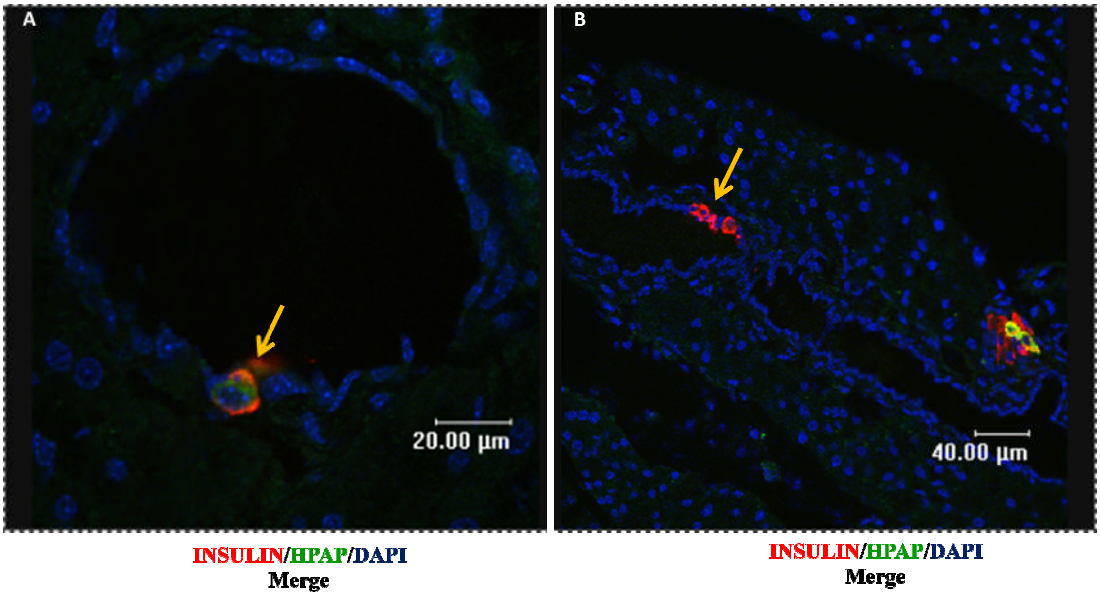


Figure 17 **Insulin-positive cells associated with the ductal epithelium**. Pancreas sections from pregnant female animals, stained for insulin (red), HPAP (green), and nuclei (blue, DAPI), show examples (yellow arrows) of HPAP-positive beta-cells (A), or HPAP-negative beta-cells (B), associated with duct epithelium. Scale bars correspond to 20µm (A) and 40µm (B).

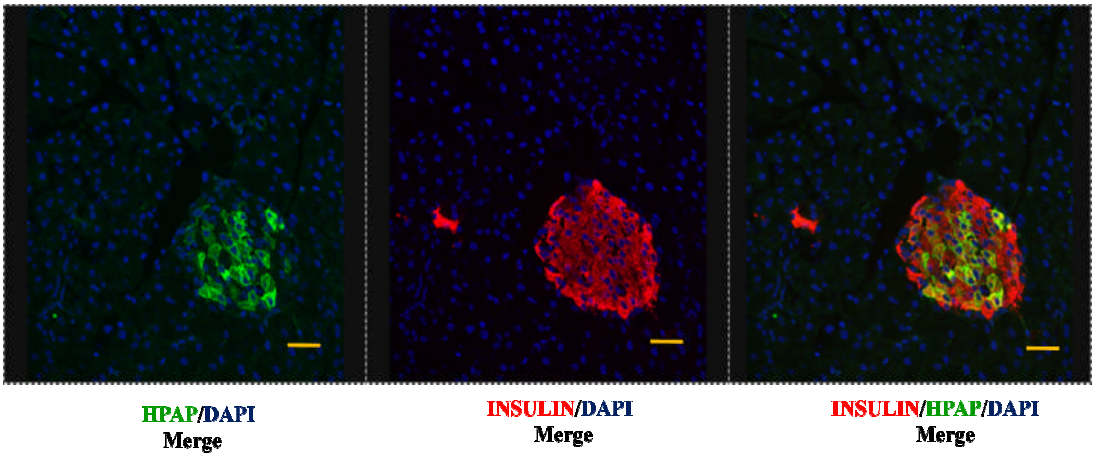


Figure 18 **Beta-cells do not transdifferentiate into other cell-lineages**. Pancreatic sections from non-pregnant control females animals stained for insulin (red), HPAP (green), and nuclei (blue, DAPI), showing that the HPAP label is restricted to beta-cells, at two and half months following the pulse of tamoxifen. Scale bar corresponds to 40µm.

(Zhang et *al*., 2005b, Levine and Itkin-Ansari, 2008, Bonner-Weir, 2000b, Bonner-Weir et *al*., 2004, Banerjee et *al*., 2005, Xia et *al*., 2009), led us to postulate that beta-cell precursors in the ductal compartment might also be re-activated in pregnancy. In other words, cells associated with the ductal system might represent a preferred source for new beta-cells. If this assumption is true one should expect a decrease of the proportion of beta-cells labelled for HPAP in the ductal structure with respect to the islet compartment.

To investigate if this is the case, first we examined the presence of cells positive for insulin within the pancreatic ductal system in pancreata of pregnant animals. We observed, as reported in others studies, increased numbers of insulin-producing cells abutting or within the ductal epithelium (Figure 17). We counted the number of the insulin-producing cells positive or not for HPAP, located in, or adjacent to the duct and in islets in the same pancreatic section, to derive the HPAP labelling indices for the islets and ductal compartment. For comparison, the labelling index for insulin-producing cells in the duct was 0.21 (n=57 cells, representing 4 replicates) and for the islets 0.31 (n=7053 cells, representing 4 replicates). We note in passing that the labelling index (0.31) determined by cell counting is close to the value noted above (0.33) which was obtained by morphometric analysis based upon cells areas determined by pixel numbers. The labelling indices of insulin-positive ductal cells and islets are not significantly dissimilar (2x2 contingency table, the chi-square approximation, two-sided, P= 0.15).

On the assumption that the number of insulin-positive cells in the ductal component increases substantially during pregnancy, the similar HPAP labelling indices in the ductal component and in the islets are not consistent with the duct being a “ preferential” source of new beta-cells during pregnancy.

3.3.5 Beta-cells do not transdifferentiate into other cell types.

An interesting aspect of cell plasticity is the ability to transdifferentiate into other cell types. Having confirmed that the HPAP-labelling was initially specific for beta-cells, we examined whether beta-cells are able to transdifferentiate into other

(insulin-negative) cells in the endocrine or exocrine components of the pancreas during pregnancy. We therefore looked for cells positive for HPAP, and negative for insulin, in the pancreata of pregnant and non-pregnant animals after the chase period (about two and half months after the last pulse of tamoxifen). We did not find any example of cells positive for HPAP and negative for insulin in the pancreatic sections of pregnant and non-pregnant female mice (Figure 18).

This analysis indicates that during this time-window, beta-cells do not fully lose differentiation or transdifferentiate into other cell types of either endocrine or exocrine tissue. This is similar to conclusion reached after induction of acute pancreatitis (Strobel et *al*., 2007).

However, we cannot exclude that transdifferentatiation could take place during a much longer period following the tamoxifen pulse, or self-evidently under different conditions not examined here, for example following pancreatic injury and regeneration.

3.3.6 Conclusion

We investigated the origin of new beta-cells in response to a first cycle of pregnancy using a cell-lineage tracing for beta-cells in pulse-chase experiments.

We first showed that the labelling system in the double transgenic mice (Z/AP; RIP-CreER^{TAM}) was 1) specific to beta-cells, 2) irreversible and heritable and 3) tamoxifen dose-dependant.

Secondly, we showed that in response to pregnancy, 1) the mean body and pancreatic weight increased about 45 and 51% respectively in pregnant animals compared to the aged-matched non-pregnant control animals, 2) the mean beta-cell mass increased around 91% in pregnant mice compared with the age-matched non-pregnant control mice.

The analysis of the proportion of beta-cells labelled for HPAP, showed that the labelling index of the non-pregnant animals (0.44±0.05) was greater than in the pregnant group (0.33±0.06). The difference between the two groups was statistically

significant (P=0.021, paired two-tailed t-test). This result is consistent with hypothesis 2 and indicates that non-beta-cell progenitors contribute significantly to the increase of the beta-cell mass in response to pregnancy.

The analysis of the proportion of the beta-cell clusters or small sized-islets devoid of HPAP, showed that the means of the beta-cell clusters or small islets negative for HPAP was greater in the pregnant group (0.19±0.06) than in the non-pregnant controls (0.11±0.04). The difference between the two groups was not statistically significant (P=0.093, paired two tailed t-test). However, there is a trend suggesting that either new islets are formed during pregnancy or alternatively, these results might reflect the dilution of the label in expanded islets.

The analysis of the HPAP labelling indices in the beta-cells in the lining of the ductal component (0.21, n=57 cells, representing 4 replicates) and in the islet compartment in the same sections (0.31, n=7053 cells, representing 4 replicates) showed that the two indices are not significantly dissimilar (2x2 contingency table, chi-square approximation, two-sided, P= 0.15) These results indicate that the duct compartment is not “a preferential” source for new beta-cells during the beta cell mass expansion occurring to adapt to the new insulin demand during pregnancy.

Finally, we did not observe any examples of cells positive for HPAP and negative for insulin in the pancreatic sections of non-pregnant and pregnant animals, indicating that even after a lengthy chase period, beta-cells do not fully lose differentiation or transdifferentiate into other lineages of either endocrine or exocrine compartment.

In conclusion, using a cell-lineage tracing system for beta-cells, we demonstrated, for the first time, that non-beta-cell progenitors contribute significantly to the beta-cell mass increment in response to pregnancy, in addition to the role of pre-existing beta-cells in beta-cell duplication.

What might be the other potential lineages of the non-beta-cell progenitors during pregnancy?

It is a question that we will discuss in the last discussion chapter.

In this chapter we demonstrated that non-beta-cell precursors can differentiate into insulin-producing cells during a first pregnancy. We aimed to repeat this experiment during a second cycle of pregnancy after the beta-mass reversed subsequent to the parturition period, to reinforce our previous finding. Thus, we wish to ask if there is a more pronounced dilution of the HPAP label in the pancreas of pregnant animals that have undergone two consecutive pregnancies, compared to those that undergo only one cycle of pregnancy. This is the subject that we have explored in the following chapter.

CHAPTER 4

ORIGIN OF NEW BETA-CELLS IN TWO PREGNANCIES

Origin of new beta-cell in two pregnancies

4.1 Introduction

Heretofore, we showed that the proportion of beta-cells positively labelled for HPAP decreased significantly during a first cycle of pregnancy. This dilution led us to conclude that a substantial population of new beta-cells were the progeny of non-beta-cell progenitors in pancreas of pregnant animals. We wished to explore this result and ask whether non-beta-cells progenitors contribute significantly to the beta-cell mass expansion occurring in pregnant animals, but this time during two cycles of pregnancy. We employed the same cell-lineage tracing system for beta-cells to address the role of non-beta-cell precursors during two pregnancies.

4.2 Experimental design

For this purpose, age and weight-matched double transgenic female animals (Z/AP; RIP-CreER^{TAM}),were paired and injected together with 25mg of tamoxifen to mark beta-cell with HPAP, around nine weeks of age for a period of two and half weeks. At the start of the study, 14 animals were involved, but due to the experimental difficulties mentioned in the previous chapter, only 6 animals could be analysed. Thereafter, one animal in each pair was either mated for pregnancy or maintained in a non-pregnant state. After a first pregnancy, the same animals were mated yet again ten days after giving birth. Then, during the second pregnancy, between fifteen to nineteen days of gestation, the pregnant animals were euthanized concurrent with the non-pregnant control females and their pancreata were excised for analysis.

Three pairs of animals were studied for the whole experiment and their pancreata were processed and analysed, as previously described, to determine the proportion of HPAP labelled beta-cells by using cross sectional area and also by determination of cell numbers positive for insulin or HPAP.

The counting of labelled or non-labelled beta-cells in sections were performed manually and the HPAP labelling index related to this analysis, was derived by forming the ratio of total number of beta-cells positive for HPAP over the total number of beta-cells positive for insulin in the whole five sections per pancreas and per animal. The counting of number of beta-cells or the measurement of area positive for HPAP or insulin was carried out simultaneously on the same set of sections for subsequent comparison.

Overall, five sections per pancreas and animal were analysed and selected according to the criteria that have been discussed in the preceding chapter, in the same way pancreata were processed and sectioned following the method presented previously. The images of all islets, single beta-cells or beta-cell clusters within sections were captured using a confocal microscope.

In total two thousand one hundred and ninety eight images were analysed for the entire experiment, and all were examined anonymously.

4.3 Results

4.3.1 Response to pregnancy

As reported in Chapter 3, pregnancy is accompanied by multiple physiological and physical changes to accommodate to the new metabolic demand.

Similarly to a first pregnancy (Karnik et *al*., 2007), in a second pregnancy, we observed a 60% increase of the mean body weight in pregnant animals compared to that of non-pregnant controls, similarly the mean pancreas weight increase about 43% in pregnant animals in comparison to non-pregnant female animals (Figure 19 A and B). We calculated the beta-cell size in both groups by forming the ratio of total pixel number of cross-sectional area positive for insulin (detailed in chapter 3) over the number of beta cells counted, in the whole five sections per pancreas and per animal. We observed that the mean beta-cell area in pregnant animals (147±18.89 pixels) was slightly larger compared to that of non-pregnant controls

(131±12.45 pixels, figure 19C). The beta-cell area increased about 12% in pregnant animals, close to Avril et *al*. report (Avril et *al*. 2002).

Equally, during a first round of pregnancy (Scaglia et *al*., 1995, Aerts et *al*., 1997, Huang et *al*., 2009, Avril et *al*., 2002, Blondeau et *al*., 1999, Karnik et *al*., 2007, Parsons et *al*., 1995), we found that the beta-cell mass doubled in the pancreas of pregnant animals during the second pregnancy, compared to that of age-matched non-pregnant controls (Figure 19D).

Therefore, in line with other reports, in our animals, the beta-cell mass increased, observed in response to pregnancy must be due to an augmentation of number of the beta-cell population, with little contribution of increased beta-cell volume. Therefore, the next question was to investigate the origin of the new beta-cells.

4.3.2 Analysis of beta-cell origin during two cycles of pregnancy

The experimental design has been explained above and is also succinctly presented in Figure 20. The particularity of this experiment is to test the activation of progenitors from a lineage different from beta cells during two pregnancies, following beta-cell mass involution in the pancreas induced by apoptosis after a first pregnancy, in the post-partum period (Scaglia et *al*., 1995).

We have showed that a non-beta-cell source contributes to the beta-cell mass expansion during a first pregnancy. Based on the assumptions that the involution of the beta-cell mass occurs at random by beta-cell apoptosis and non-beta-cell progenitors contribute to the beta-cell mass increment during a second pregnancy, we would expect a further dilution of the HPAP labelling index during two cycles of pregnancy.

The first assumption does imply that unlabelled and labelled beta-cells are equally ablated by apoptosis, without any distinction between both populations.

In the same way, the second assumption does imply that the mechanism of the beta-cell mass expansion is similar to that in a first pregnancy (Figure 20).

Based on the assumptions above and inasmuch as Φ_1 defined as the ratio of the mean HPAP labelling index of non-pregnant animals over pregnant animals during a first pregnancy, is 1.37 (Chapter 3), Φ_0, the quotient of the mean HPAP labelling index of non-pregnant animals over pregnant animals at the end of the tamoxifen pulse, is 1, we estimated that the ratio Φ_3 of the mean HPAP labelling index of non-pregnant animals over the age-matched pregnant animals during two cycles of pregnancy, should be 1.88.

The HPAP labelling indices (Figure 21 A and B) in pancreata of non-pregnant and age-matched pregnant animals was calculated using data generated from both area measurement (Figure 21A) in pixel number, using ImageJ and manual cell counting (Figure 21B).

First, we confirmed that the HPAP labelling index values calculated from data produce by area measurement or generated by manual cell counting, for the same animal, are numerically similar.

Secondly, we observed that the HPAP labelling indices display large variations between animals of same group, namely between as well as the non-pregnant animals as compared all together that the animals in pregnant group.

This is likely due to an unanticipated problem with one batch of tamoxifen required to activate the labelling. Indeed, the animals from pair 1 received tamoxifen from the same batch used in studies in Chapter 3 and the proportions for beta-cells HPAP positive in animals from a first pregnancy were very similar (see Figure 15 in chapter 3). And, the rate of labelling, in animals injected with 25 and 20mg using this same batch of tamoxifen, showed that the marking was tamoxifen dose-dependent (see Figure 10 in chapter 3).

By contrast, the HPAP labelling indices in animals of pair 2 and 3 showed values clearly lower to that expected for the dose of tamoxifen injected. Pairs 2 and 3 were given tamoxifen from a new batch, as the original one had to be replaced.

This suggests that the tamoxifen quality can be variable from one batch to another or less likely that the animals from pair 2 and 3 might be different from the other animals used.

Nevertheless, these results demonstrate the importance of animal pairing to minimize variations other than those related to individual animals.

Statistical analysis of these data is limited, due to the great variation between pairs, but we can still use comparison between the paired designs, to examine the assumptions above.

To extract the information needed, we derived the ratio of the HPAP labelling indices of non-pregnant animal over pregnant animal, determined by cross-sectional area measurement in each pair for each experiment, namely, animals from experiment described in Chapter 3 (experiment 1, i.e. a single pregnancy) and in this Chapter (experiment 2, i.e. two pregnancies).

Figure 21C represents the mean ratio of the HPAP labelling indices in pancreata of non-pregnant controls over pregnant animals derived from the results of experiment 1 and 2 separately.

We observed that the mean ratio of the HPAP labelling index in pancreas of non-pregnant control over pregnant animals from experiment 1 (a single pregnancy, 1.37±0.209) is close to that of animals from experiment 2 (two pregnancies, 1.34±0.004). The difference between the two groups is not significant (P=0.53, two tailed, paired t-test).

According to the assumptions discussed above, namely, if ablation of unlabelled or labelled beta-cells occurs at random during the parturition period after a first pregnancy, and if the mechanism governing beta-cell mass expansion in a second pregnancy is similar to that of a first pregnancy, then the ratio expected for experiment 2, should be 1.88.

Given that the ratio obtained for experiment 2 (1.34±0.004) is different to that expected (1.88), accordingly, we cannot support both hypotheses.

We cannot conclude about the contribution of non-beta-cell progenitors during a second cycle of pregnancy.

The comparison of the proportion of labelled-free small islet sections between pregnant and non-pregnant animals shows that this ratio is systematically

greater in pregnant animals compared to that of the non-pregnant animals within the same pair (Figure 22D) despite the large variation between replicates of same group.

As shown during a first pregnancy, we observed, during a second pregnancy, small islet cross-sections positive or negative for HPAP immunoreativity, while intermediate or big islet cross-sections were always marked with the reporter (Figure 22 A, B and C).

In principle, one could have tested hypothesis 3, concerning the formation of whole new islets during two cycles of pregnancy, or alternatively tested the effect of a dilution in larger islets on the proportion of free-label small islet cross-sections, but because of the large variation between replicates, it was not possible to perform a robust statistical analysis to test the veracity of such propositions.

4.3.3 Beta-cell clusters associated with the pancreatic duct

Pancreatic duct epithelium in numerous reports is presented as a potential source for new beta-cells during normal growth in adult pancreas or after certain types of pancreatic injury. Consequently, our attention was directed specifically to this compartment. Unfortunately, the comparison of HPAP labelling indices in ductal and in the islets compartment in the pancreas of pregnant animals was not possible in this situation owing the large variation which was discussed above.

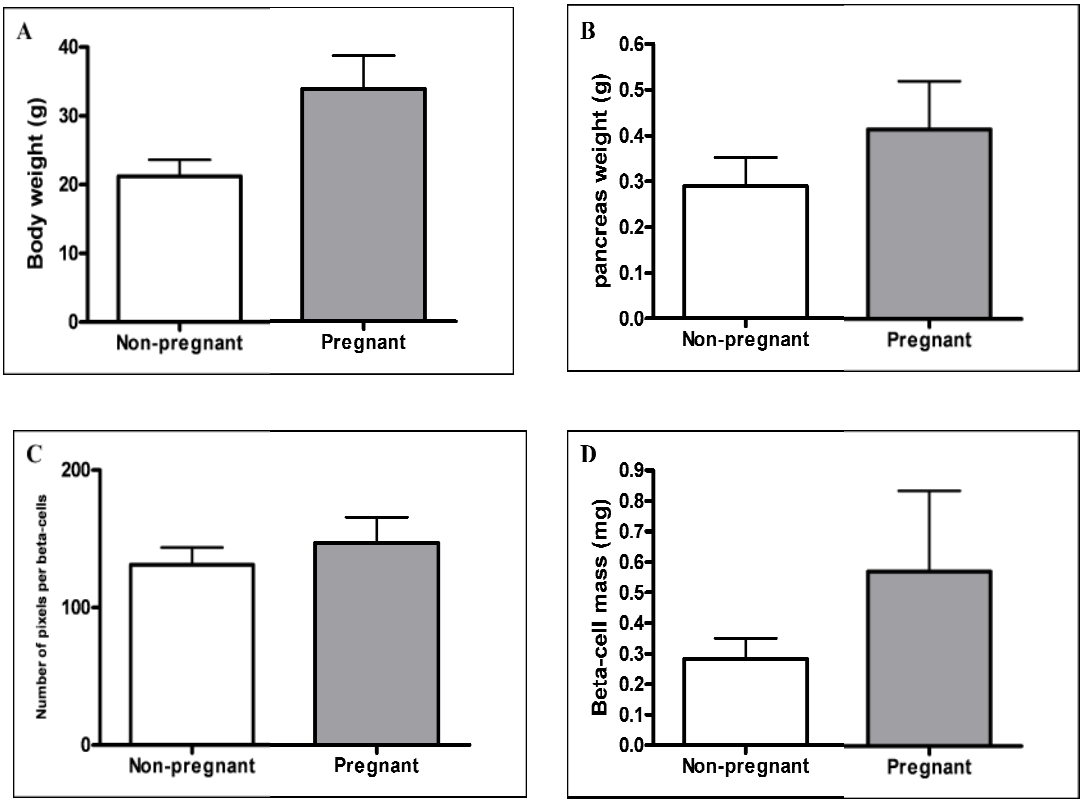


Figure 19 **Responses to pregnancy**. During pregnancy, the mean body weight increased by 60% (A), the mean pancreas weight by 43% (B), and the beta-cell area increased about 12% (C) as compared with their age-matched non-pregnant control counterparts. Mean beta-cell mass in pregnant animals doubled (D).

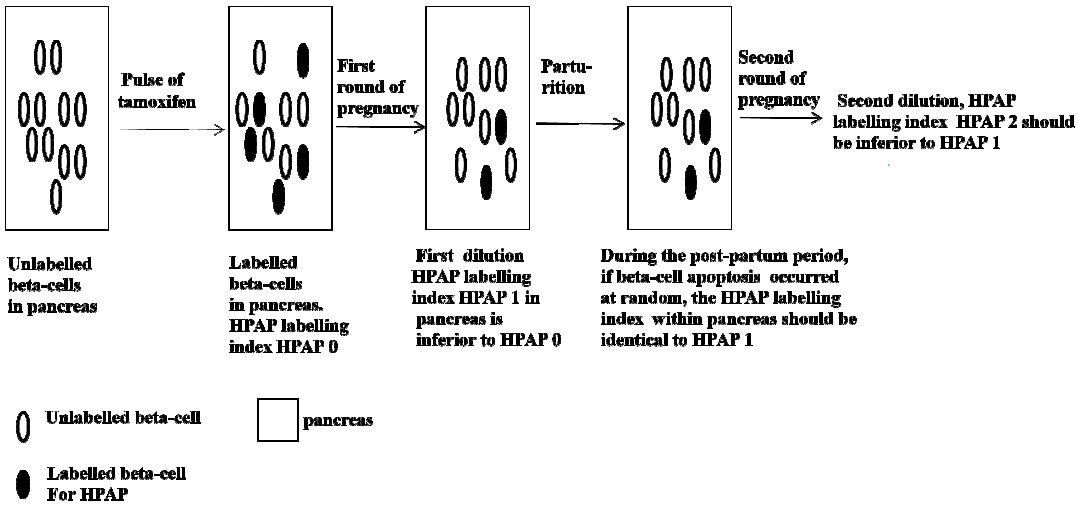


Figure 20 **Pulse-chase experiment and analysis of beta-cell origin.** Double transgenic female animals (Z/AP; RIP-CreER^{TAM}) were pulsed with tamoxifen to mark their beta-cells. Then during the chase period, pregnancy was induced in one group of female animals. Ten days after giving birth, pregnancy was induced again in the same group of animals. During a first pregnancy the HPAP labelling index decreases (HPAP1) when compared with the HPAP labelling index shortly after the pulse (HPAP 0). If during parturition, the beta-cell apoptosis occurs at random, the HPAP labelling index should be similar to that of animal pancreata during a first pregnancy. Consequently, during a second pregnancy, the HPAP labelling index is expected to be lower when compare to that of animal pancreata during a first pregnancy.

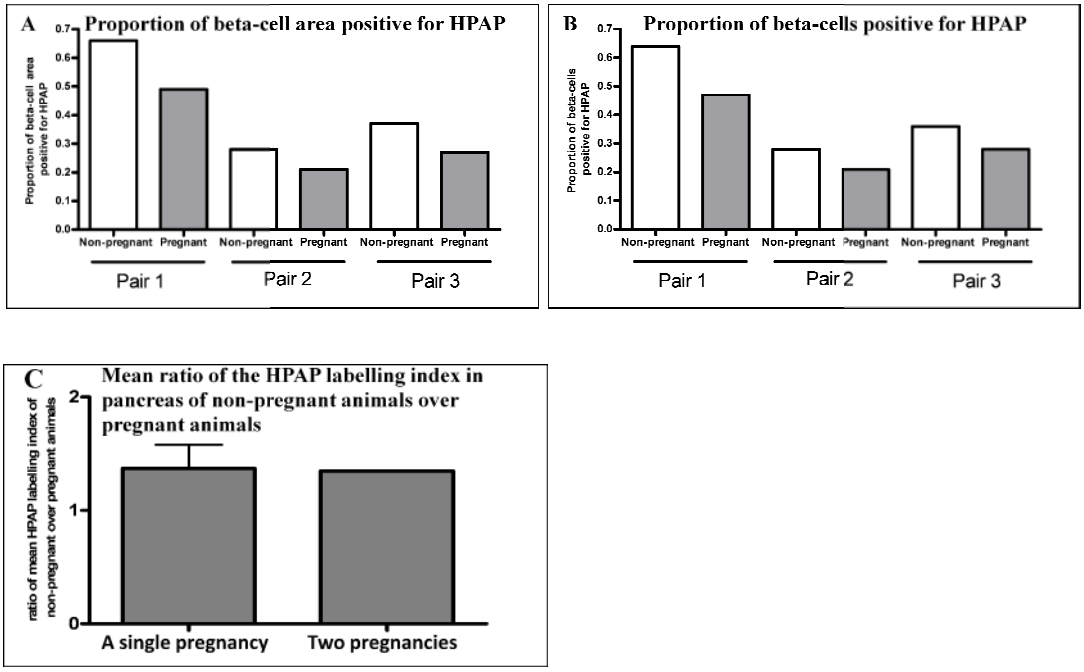


Figure 21 **Lineage tracing in pregnancy**. The HPAP labelling indices in pancreata of non-pregnant and age-matched pregnant animals were calculated using data generated from either an area measurement (A) in pixel number, using ImageJ or manual cell counting (B). The HPAP labelling index values calculated from data produced by area measurement or generated by manual cell counting, for the same animal, are numerically similar. (C) represents the mean ratio of the HPAP labelling indices in pancreata of non-pregnant controls over pregnant animals during a single (1.37± 0.209) and two (1.34 0±0.04) rounds of pregnancy.

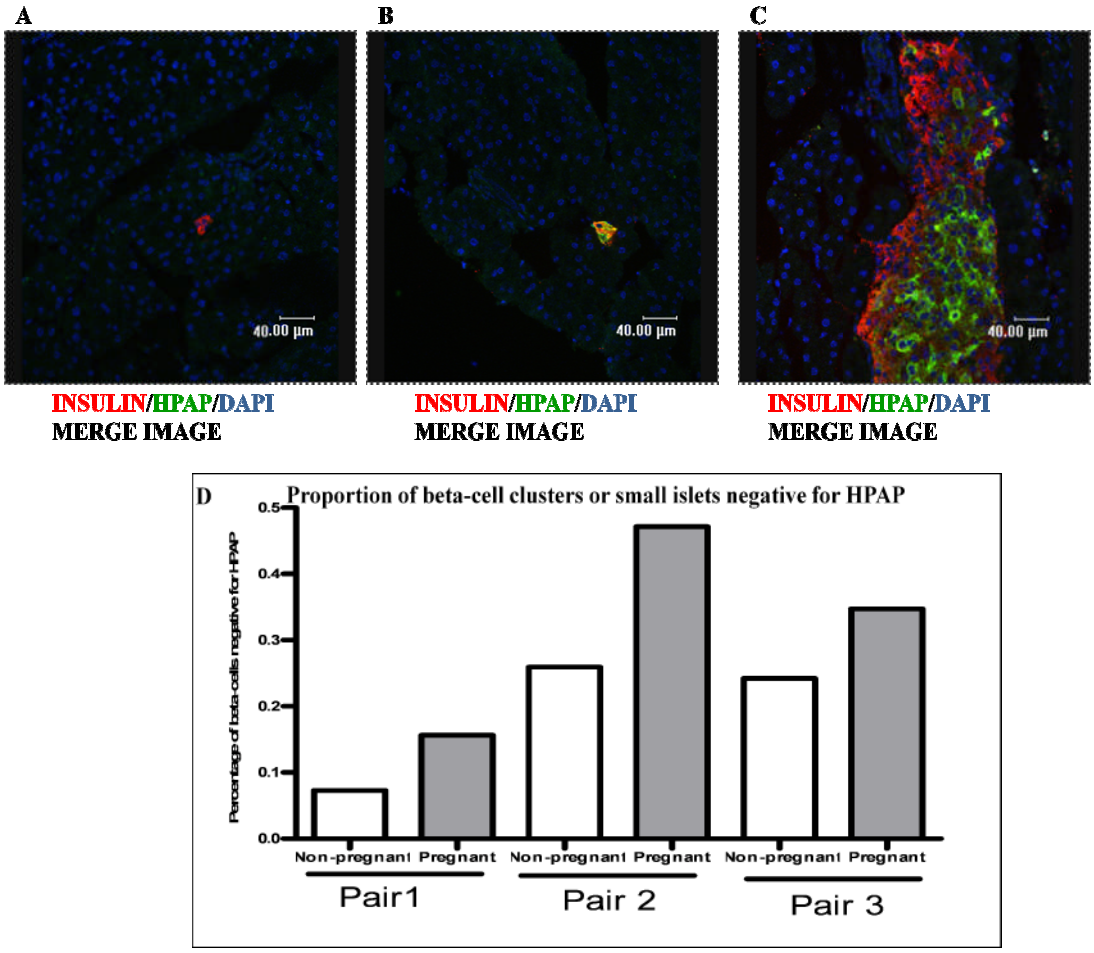


Figure 22 **Beta-cell clusters negative for HPAP during a second pregnancy**. Photomicrographs of pancreatic sections from pregnant animals stained for insulin (red), HPAP (green), and nuclei (blue, DAPI). Small islet sections are either completely HPAP-negative (A) or partly or wholly HPAP-positive (B), while intermediate or big islet cross-sections are always marked with the reporter (C). The comparison of the proportion of labelled-free small islet sections between pregnant and non-pregnant animals (D) shows that this ratio is systematically greater in pregnant animals compared to the non-pregnant animals within the same pair. Scale bar corresponds to 40µm.

Thus, instead of focusing on the HPAP labelling index as done in the preceding chapter, we examined the incidence of insulin-positive ductal cells in pregnant and non-pregnant animals during a second and first pregnancy (Figure 23A and B).

The data are presented in Figure 24. The scrutiny of insulin-containing cells in the ductal lining (Figure 24A), indicated an increase numbers of these cells, both during a first or two cycles of pregnancy. We also noticed that the numbers of ducts containing insulin-positive cell increased (Figure 24B). The analysis was carried out on five sections per pancreas, four and three replicates were viewed for the experiments conducted during a first and two cycles of pregnancy correspondingly.

The difference between the two groups, for both analyses was not significant. Combining the data from the experiment of one and two pregnancies (Figure 24A and B) did alter the P-value (paired, two-sided student t-test), but the difference is still not significant.

These tentative results suggest that during pregnancy, more ducts expressing insulin-positive cells may accommodate the new insulin load but the numerical analysis indicates that dramatic activation of this mechanism does not in fact occur.

Nonetheless, more experiments need to be performed to conclude about the extent of the contribution of the duct compartment.

Furthermore, we combined the results of the HPAP labelling indices obtained from paired animals in one and two pregnancies. We observed that the combined mean HPAP labelling index in the non-pregnant animals (0.44±0.12) is higher than that in pregnant animals (0.33±0.09), and the difference between the two groups is considered extremely significant (paired, two-sided student t-test, P-value 0.0007).

Similarly, the combined results of the proportion of small islets or beta-cell clusters negative for HPAP obtained from paired animals in one and two pregnancies, showed an increase in this proportion in pregnant animals (0.25±0.12) as compared with the non-pregnant group (0.14±0.08). The difference between the two groups is considered very significant (paired, two-sided student t-test, P-value 0.006). The latter indicates that either new islets are formed during pregnancy or alternatively, there is a dilution of the label in larger islets during this state.

4.3.4 Beta-cells do not transdifferentiate into other cell-lineages

In the previous chapter, we showed that beta-cells do not transdifferentiate into other pancreatic cell-lineages or do not fully loss differentiation two and half months after the last tamoxifen pulse. Here, we had a further opportunity to ask if this was still the case in animals three and half months following the last tamoxifen injection. We screened pancreatic sections for cells positive for HPAP and negative for insulin in non-pregnant and pregnant animals (Figure 25 A and B), we did not see any examples of cells remaining positive for HPAP without expressing insulin.

We can confirm that beta-cells do not transdifferentiate into other lineages or fully lose differentiation. It remains to be seen if this type of event can still take place in much longer time point that is six or twenty four months after the last tamoxifen pulse or in other circumstances.

Once again our examination corroborates results from studies on pancreatitis showing that beta-cell do not transdifferentiate into other cell types (Strobel et *al*., 2007)

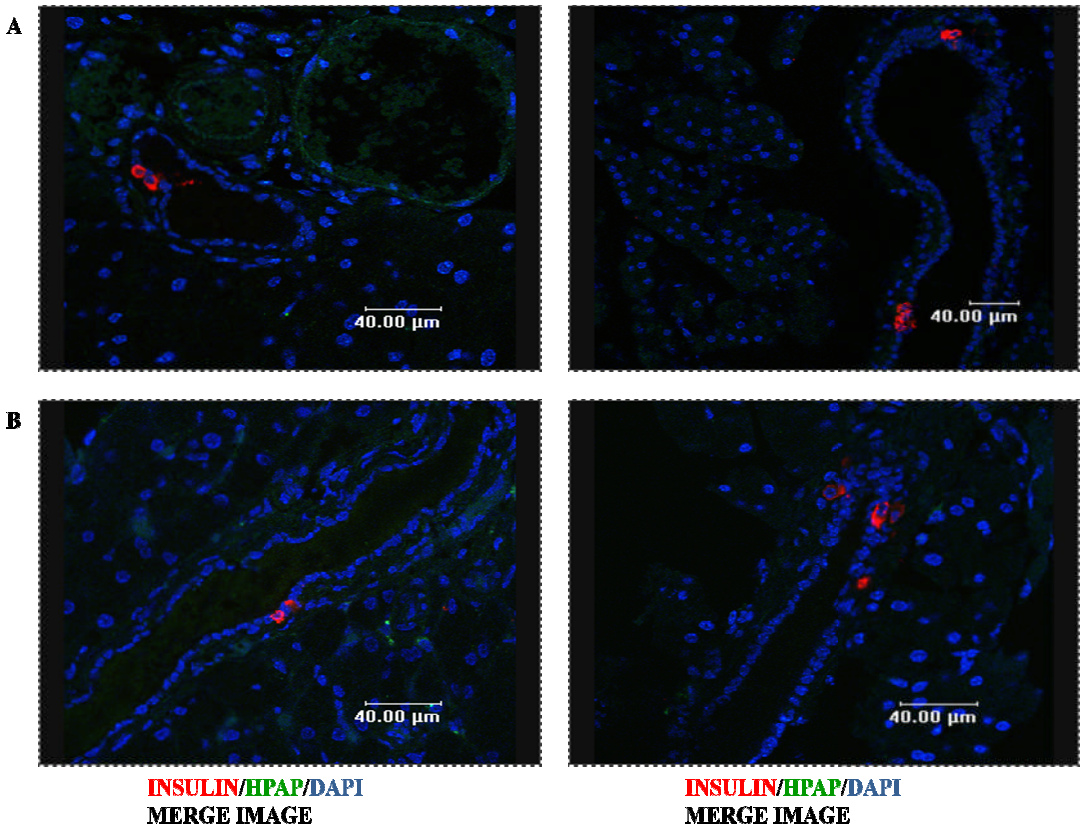


Figure 23 **Insulin-positive cells associated with the ductal epithelium in two pregnancies**. Pancreas sections from pregnant female animals (panel A) or non-pregnant female animals (panel B) stained for insulin (red), HPAP (green), and nuclei (blue, DAPI), show examples of beta-cells in the duct lining or associated with duct epithelium. Scales bar correspond to 40µm.

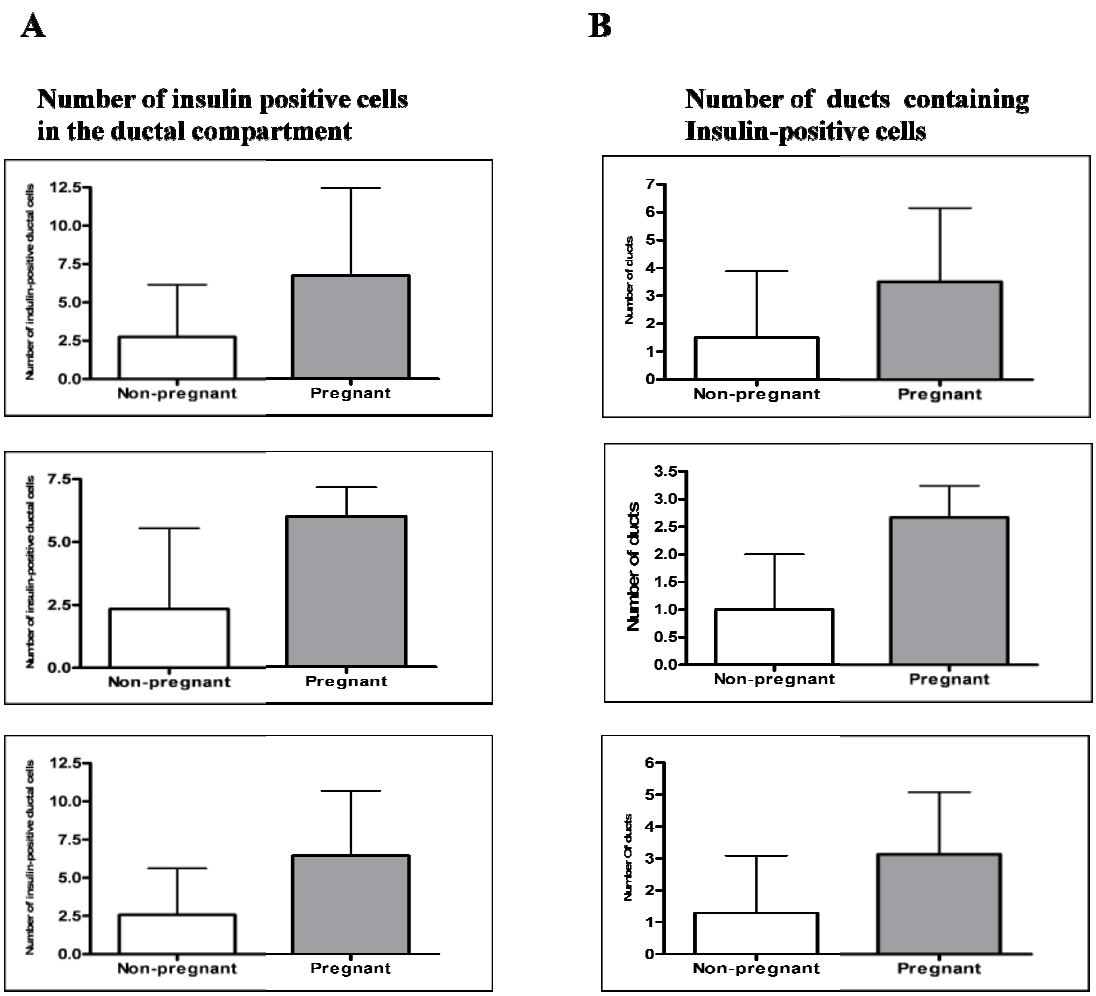


Figure 24 **Insulin-positive cells in the ductal lining and number of ducts containing insulin-positive cells**. (A) Graphs show the results of the number of insulin-positive cells in the ductal lining or associated with ducts for one pregnancy (upper panel), or two pregnancies (middle panel). Lower panel shows a graph representing the combined results for both one and two pregnancies. (B) Graphs show the results of the number of ducts containing insulin-positive cells for one pregnancy (upper panel), or two pregnancies (middle panel). Lower panel shows a graph representing the combined results for both one and two pregnancies

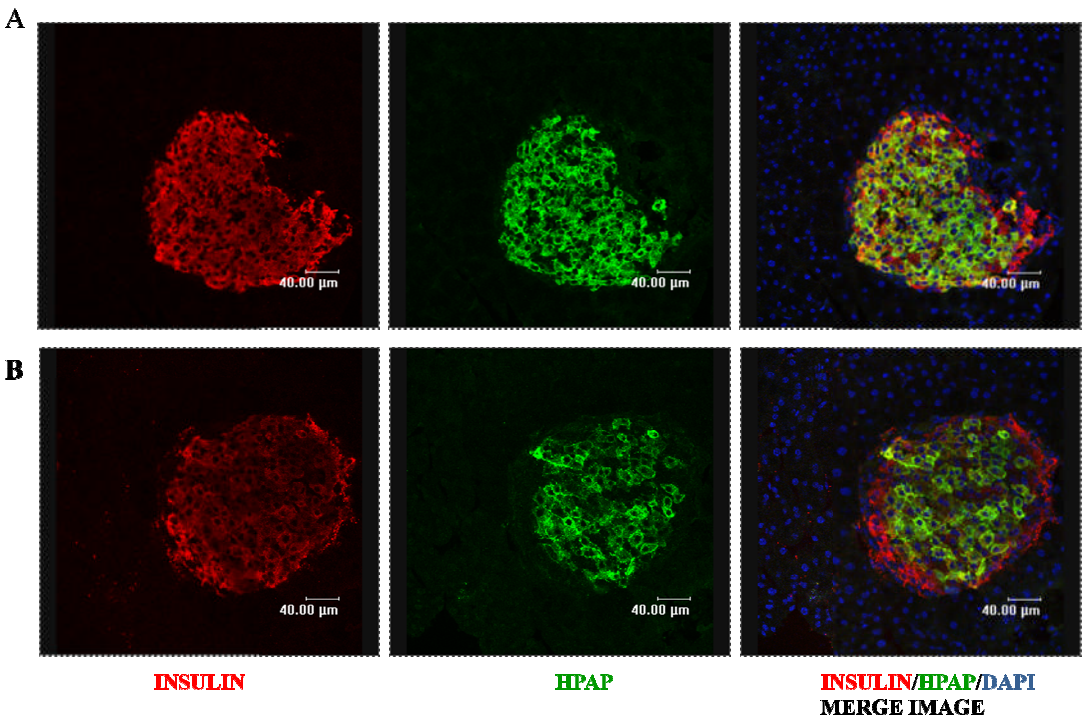


Figure 25 **Beta-cells do not transdifferentiate into other cell-lineages.** HPAP labelling remains restricted to beta-cells after a chase period. Pancreatic sections from non-pregnant control (panel A) and pregnant (panel B) females animals stained for insulin (red), HPAP (green), and nuclei (blue, DAPI), showing that the HPAP label is restricted to beta-cells at three and half months following the pulse of tamoxifen. Scale bar corresponds to 40µm.

4.4 Conclusion

In conclusion, using the same lineage tracing system employed to trace the fate of beta-cells during a first cycle of pregnancy, we have undertaken a similar experimentation to explore whether during two cycles of pregnancy, non-beta-cell progenitors contribute to the same extent to the formation of newly formed beta-cells.

We observed a 60% and 43% increase of the mean body weight and pancreas weight correspondingly in pregnant animals compared to non-pregnant controls.

Similarly, we confirmed an increment of two fold of the beta-cell mass in response to pregnancy to adapt to the new insulin load with little contribution of beta-cell hypertrophy.

Secondly, the comparison of the HPAP labelling index in non-pregnant controls over pregnant animals in one and two cycles of pregnancy led us to reject our hypothesis that, the HPAP labelling index would be markedly lower in the pregnant animals during two cycles of pregnancy compared to a first cycle.

The variation in the beta-cell labelling in the three replicates put a limitation to the analysis. Nonetheless, we observed both a small increase of the occurrence of insulin-positive cells in the ductal compartment and the number of ducts associated with these cells in a one and two pregnancies. These tentative results might suggest that duct progenitors could be activated to form de-novo beta-cells in pregnancy, but it was evident that this must be a minor mechanism.

However, the combined results of the HPAP labelling indices and the proportions of small islets or beta-cell clusters negative for HPAP from experiment 1 (a single pregnancy) and experiment 2 (two pregnancies), reinforce our conclusion regarding the contribution of non-beta-cell progenitors during the beta-cell mass expansion in response to pregnancy.

Lastly, we did not find any example of beta-cells that had transdifferentiated into other lineages, or fully lost differentiation in pregnant and non-pregnant animals even three and half months after the last pulse of tamoxifen.

As discussed at the beginning of this chapter, we expected that during two cycles of pregnancy, the dilution of beta-cells labelled for HPAP should be greater than in a first pregnancy, this if the beta-cell apoptosis process took place at random. And, the results obtained led us to reject that hypothesis. This unexpected outcome prompts more questions, which will be debated in the discussion chapter.

CHAPTER 5

ORIGIN OF NEW BETA-CELLS AFTER DIABETIC RECOVERY

Origin of new beta-cells after diabetic recovery

5.1 Introduction

The search for new therapies to cure diabetes and understanding tissue homeostasis has prompted researchers to investigate the origin of new beta-cells during postnatal growth, maintenance or beta-cell regeneration (Like and Chick, 1969). Studies from models of pancreatic injury in adult rodents, mooted for a long time the existence of progenitors within the adult pancreas playing an active role during beta-cell regeneration, and possibly during beta-cell renewal and maintenance in postnatal life (Nir and Dor, 2005, Bonner-Weir and Weir, 2005, Levine and Itkin-Ansari, 2008). In 2004, a first application of an inducible cell-lineage tracing for beta-cells performed by Dor et *al*. (Dor et *al*., 2004) has had an enormous impact in this field. For the first time they provided, with direct evidence, that pre-existing beta-cells were progenitors for new beta-cells and subsequently contributed essentially to beta-cell growth and maintenance during mouse postnatal development, and even after partial pancreatectomy.

The existence of putative pancreatic progenitors residing within adult pancreas or even the contribution of such progenitors to form de-novo beta-cells in adulthood has been seriously questioned since this publication.

However very recently, three reports (Inada et *al*., 2008, Xu et *al*., 2008, Collombat et *al*., 2009), have reinstated adult pancreatic progenitors on the map, by offering with direct proof ultimately that progenitors within adult pancreas do exist, are of ductal origin, and contribute significantly to the formation of new beta-cells shortly after birth or during beta-cell regeneration after duct ligation in mouse.

Undoubtedly there is a resurgence of the debate regarding the mechanism by which new beta-cell arise in normal and patho-physiological conditions with the advent of novel genetic cell-lineage techniques.

The dispute regarding whether stem/progenitor cells in the adult pancreas could be considered as legitimate cellular sources for newly formed beta cells under multiple situations is far from being resolved. It is justifiable to address the origin of

new beta-cell in different circumstances where other types of stimuli might be involved to better understand the mechanisms of these two complementary processes. For this reason, we aimed to use a direct approach in a specific model of pancreatic regeneration, to examine the origin of new beta-cells after toxic-genetic beta-cell ablation in transgenic mice over-expressing c-Myc (Pelengaris et *al*., 2002).

5.2 Experimental design

With the view to explore the cellular source giving rise to new beta-cells after diabetic recovery, we generated triple transgenic mice in which the inducible cell-lineage tracing system for beta-cells has been introduced in a mouse model of pancreatic regeneration (pIns-c-MycER^{TAM}).

First, the Z/AP mouse line was crossed with the RIP-CreER^{TAM} line to obtain double transgenic mice (Z/AP; RIP-CreER^{TAM}), the beta-cell lineage tracing system. Then, the latter were crossed with the toxic-genetic beta-cell ablation mouse line (pIns-cMycER^{TAM}) to generate the triple transgenic animals (Z/AP; RIP-CreER^{TAM}; pIns-c-MycER^{TAM}).

Using this strategy, one mouse out of eight was expected to be of the right genotype. The cell-lineage tracing in the bigenic mice (Z/AP; RIP-CreER^{TAM}) has been described in chapter 3, therefore we will describe only the transgenic line of beta-cell regeneration (pIns-c-MycER^{TAM}) in this section.

5.2.1 Toxic-genetic model of beta-cell ablation and regeneration in the single pINS-c-MycER^{TAM} mice

In the pIns-c-MycER^{TAM} monogenic mice, the switchable human basic helix-loop-helix leucine zipper (bHLH-LZ) transcription factor c-Myc, under the control of a preproinsulin promoter (pIns) is expressed restrictively in insulin-secreting beta-cells.

In this system the transcription factor c-Myc is in fact a chimerical protein which is fused with a truncated form of an oestrogen receptor (ER^{TAM}). In absence of 4-hydroxytamoxifen (4-OHT), the chimerical transcription factor domain is inactive owing to its binding to heat shock proteins (HSP) on its ER^{TAM} (Figure 26A).

The untreated animal presents a normal blood glucose level. However, upon tamoxifen or 4-hydroxytamoxifen (4-OHT) exposure, the c-$MycER^{TAM}$ is activated (see below) due to its binding to this synthetic drug in place of the HSP. As a result, the activated chimerical protein c-$MycER^{TAM}$ ablates specifically beta-cells by apoptosis. This apoptosis follows a short burst of proliferation (Pelengaris et *al*., 2002, Cano et *al*., 2008), leading to islet involution and the development of a diabetic phenotype in the transgenic animals.

By contrast, inactivation of c-Myc upon tamoxifen or 4-hydroxytamoxifen withdrawal, results in gradual beta-cell regeneration and diabetic recovery characterized by normalization of the glycaemia in the animals, as originally reported by Pelengaris et *al*. and Cano et *al*. (Pelengaris et *al*., 2002 and Cano et *a*l., 2008).

Figure 26, panel B, shows islet morphology in pancreatic sections in the pIns-c-$MycER^{TAM}$ transgenic animals. The microphotography (Figure 26Bi) shows a representative islet in an untreated animal (note the regular shape of the islet), but upon 4-OHT exposure, acute beta-cell apoptosis results in islet involution evident as islets of small mass with irregular contours (Figure 26Bii). Conversely, withdrawal of 4-OHT, leads to beta-cell regeneration (see discussion of this later) and islets grow back virtually to their original size (Figure 26Biii, Pelengaris et *al*., 2002, Cano et *al*., 2008).

In this model of beta-cell regeneration, we introduced the cell-lineage tracing system for beta-cells to study (in triple transgenic mice) the origin of new beta-cells following the toxic-genetic beta-cell ablation induced by c-Myc.

The active process of beta-cell regeneration after diabetic recovery, in the single pIns-c-$MycER^{TAM}$ mice, demonstrated by the authors above, might not occur in the triple transgenic (Z/AP; RIP-$CreER^{TAM}$; pIns-c-$MycER^{TAM}$) mice at the time point investigated.

5.2.2 Methodology applied to study beta-cell genesis after diabetic recovery

We generated multiple transgenic mice (Z/AP; RIP-CreER^{TAM}; pIns-c-MycER^{TAM}) to study in pulse-chase experiments the cellular source giving birth to de-novo beta-cells during beta-cell regeneration subsequent to beta-cell depletion induced by c-Myc.

The animals were split into three groups of three (Figure 27). The first group represents untreated control animals (group 1, 3 animals), in which beta-cells are not labelled for HPAP and the fed blood glucose level is normal.

The second one (group 2, 3 animals), animals were pulsed with the drug 4-OHT for ten consecutive days (the pulse period) and euthanized the following day after the last drug injection, at the time where the mice were overtly diabetic.

The last group (group 3, 3 animals) corresponds to animals also pulsed with 4-OHT for ten days but euthanized four weeks after the last day of the drug injection and after diabetic recovery (the chase period).

Thus, after daily injection of 1mg/0.2ml of 4-OHT for ten days each mouse about seven weeks of age, received after the end of the last pulse, 10mg of the drug in total. Then, animals from group1 (control untreated mice) and 3 (mice recovered from diabetes) were sacrificed contemporaneously, while animals from group 2 (diabetic mice) were sacrificed four weeks earlier before diabetic recovery. Pancreata of all animals were collected for analysis.

The fed blood glucose level was monitored in animals during the course of the whole experiment i.e. before, during, after 4-OHT injections, and at the time of sacrifice. Our animals were considered diabetic when their fed blood glucose level was above 12mM. After induction of diabetes in our mice, they did not require insulin treatment to recover for diabetes.

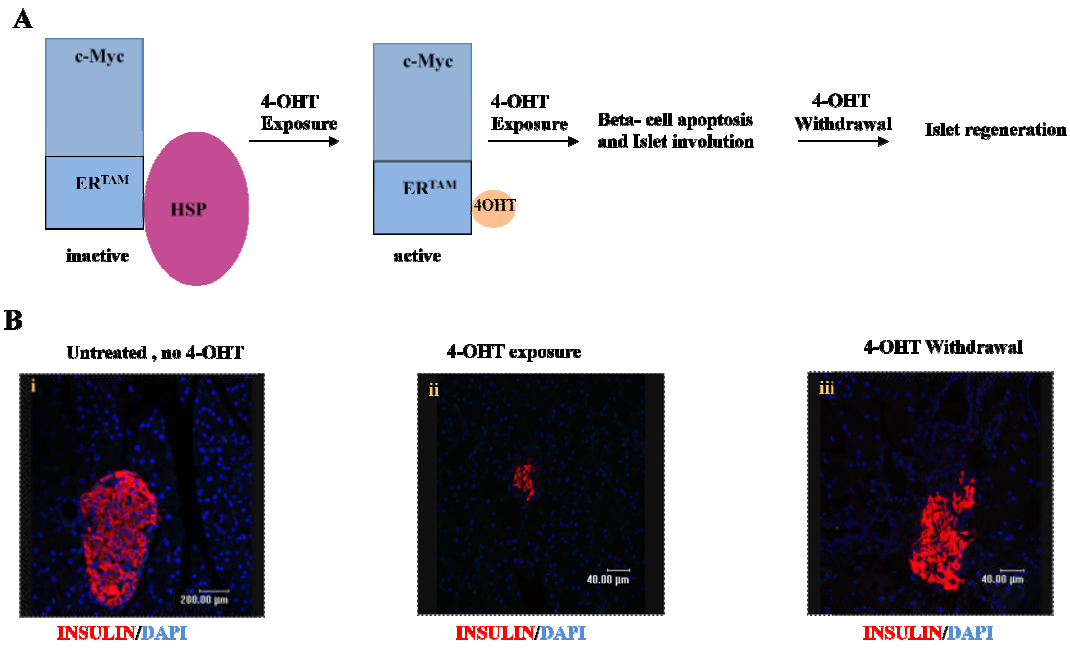


Figure 26 **Toxic-genetic model of beta-cell ablation and regeneration in the single pIns-c-MycER^{TAM} mice**. (A) In the absence of 4-hydroxytamoxifen (4-OHT), c-Myc is inactive due to its binding to heat shock proteins (HSP) on its ER^{TAM} domain. Upon 4-OHT exposure, the c-Myc is activated due to its binding to 4-OHT in place of the HSP. As a result, the c-Myc ablates specifically beta-cells by apoptosis, leading to islet involution. By contrast, inactivation of c-Myc upon 4-OHT withdrawal, results in gradual beta-cell regeneration. (B) Islet morphology in pancreatic sections in the pIns-c-MycER^{TAM} transgenic animals. The microphotography (i) shows a representative islet in an untreated animal, but upon 4-OHT exposure (ii), acute beta-cell apoptosis results in islet involution evident as islets of small mass. Conversely, withdrawal of 4-OHT (iii) leads to beta-cell regeneration.

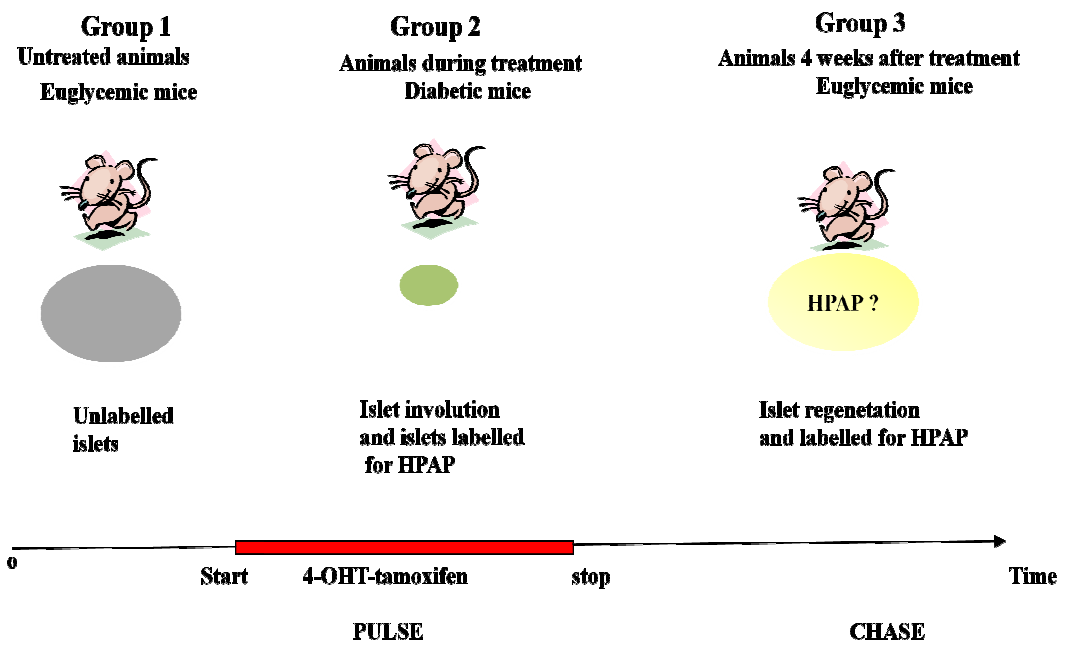


Figure 27 **Pulse-chase experiment in triple transgenic mice (Z/AP; RIP-CreER^{TAM}; pIns-c-MycER^{TAM}) mice**. In absence of 4-OHT the triple transgenic mice are euglycemic and no expression of HPAP in their beta-cells (group 1). Upon 4-OHT exposure (the pulse period), beta-cells are ablated by c-Myc activation and labelled for HPAP by removal of stop codon sequences upstream of HPAP gene by cre-recombinase. Beta-cell ablation leads to islet involution and the animals develop a diabetic phenotype (group 2). Conversely, upon 4-OHT withdrawal, 4 weeks after the last pulse of 4-OHT (the chase period), the animals recover from diabetes and become euglycemic due to beta-cell or islet regeneration (group 3).

It is important to emphasise that in the triple transgenic mice, upon 4-OHT injection, beta-cells are both labelled for HPAP resulting from removal of stop signal sequences upstream of the HPAP reporter gene catalyzed by the Cre-recombinase, and are also ablated by apoptosis following c-Myc activation.

Thus, during the 4-OHT treatment, the two events occur concurrently, leading to the marking of the surviving beta-cell population that escaped apoptosis induced by c-Myc activation. Thenceforth, at the end of the last pulse of 4-OHT, the mice develop a diabetic phenotype due to beta-cell depletion. Our initial expectation was that upon 4-OHT removal, the triple transgenic mice (Z/AP; RIP-CreER^{TAM}; pIns-c-MycER^{TAM}) recover from diabetes due to formation of new beta-cells by regeneration in the same way as the single transgenic pIns-c-MycER^{TAM} mice, after diabetic recovery to replenish the missing beta-cells in their pancreas.

By which mechanism are new beta-cells produced in the triple transgenic mice after diabetic recovery subsequent to selective beta-cell ablation and labelling?

To answer to this question, triple transgenic mice were generated and injected with 4-OHT for ten consecutive days to ablate and label their beta-cells, then upon 4-OHT withdrawal, they were allowed to recover from diabetes before excising their pancreata for analyses.

Pancreata were processed and analysed in a systematic way to avoid any forms of bias, as described in chapter 3 and Materials and Methods. ImageJ was used for performing pixel counts on captured images of sectioned islets, single and clusters of beta-cells stained for HPAP or insulin.

Overall, one thousand seven hundred and six images were analysed for the whole experiment comprised of nine mice. In total an average of seventy eight beta-cell clusters or islets were analysed per mouse in group 2 (diabetic animals), seventy four beta-cell clusters or islets were analysed per mouse in group 3 (animals after diabetic recovery) and one hundred and thirty two beta-cell clusters and islets were analysed per mouse in group 1 (untreated control mice).

The HPAP labelling index and beta-cell mass for each animal were calculated as detailed previously.

5.2.3 Interpretation of possible lineage-tracing outcomes

Using the triple transgenic mice bearing the ablation-tracing system in a pulse-chase experiment, we could examine the origin of new beta-cells after diabetic recovery and beta-cell regeneration.

The analysis of the lineage tracing results and the strategy described above are presented succinctly in Figure 28. In the absence of 4-OHT, the beta-cells in the multiple transgenic animals are unlabelled for beta-cells, and exhibit normal fed blood glucose level.

Upon 4-OHT addition, during the pulse period, beta-cells in the animals are ablated and labelled for HPAP simultaneously, resulting in the development of a diabetic phenotype.

Withdrawal of 4-OHT leads to diabetic recovery and beta-cell regeneration during the chase period.

Comparisons of the HPAP labelling indices in animals at the end of the pulse period (when the mice are diabetic) and in animals four weeks after the last 4-OHT pulse, after diabetic recovery during the chase period, should allow deductions about the mechanism by which newly formed beta-cells arise.

We must consider three hypotheses. First, the percentage of beta-cells labelled for HPAP in animals after diabetic recovery or beta-cell regeneration in the chase period is similar to that in diabetic animal shortly after the last pulse of 4-OHT. One concludes that new beta-cells are produced by mitogenesis (Hypothesis 1).

Secondly, if there is a diminution of the proportion of beta-cells positive for HPAP in animals after diabetic recovery compared to the proportion of labelled beta-cells in diabetic animals, one deduces that new beta-cells are the progeny of non-beta-cell progenitors in combination or not with pre-existing beta-cells (Hypothesis 2).

At last, if the percentage of islets devoid for the HPAP label is higher in animals after diabetic recovery than in the diabetic animals, one can infer that new whole islets are formed from non-beta-cell progenitors (Hypothesis 3).

5.3 Results

5.2.1 Induction of diabetic by activation of c-myc in the triple transgenic mice

In order to study the origin of new beta-cell after diabetic recovery in the triple transgenic mice, the mice were pulsed with 10mg of 4-OHT, to ablate and labelled their beta-cells, then euthanized four weeks following the pulse.

The fed blood glucose was monitored at the three time points presented in Figure 29A. Untreated animals and animals before treatment present a normal fed blood glucose level. The mean fed blood glucose in those animals was 4.7±1.5mM.

After the last pulse of 4-OHT, treated animal developed a diabetic phenotype, the mean fed blood glucose level was 21.6±6.0mM.

The difference between the two groups was significant (One-way Analysis of Variance (ANOVA), P-value <0.001).

Then four weeks after the last 4-OHT injection, the last time point on the graph, the mice partially recovered from diabetes, illustrated by a mean fed blood glucose level about 10.5±2.6mM.

The difference of mean fed blood glucose level between diabetic animals and animals after diabetic recovery was significant (One-way Analysis of Variance (ANOVA), P-value <0.01), whereas the difference between untreated animals and animals after diabetic recovery was not (One-way Analysis of Variance (ANOVA), P-value >0.05).

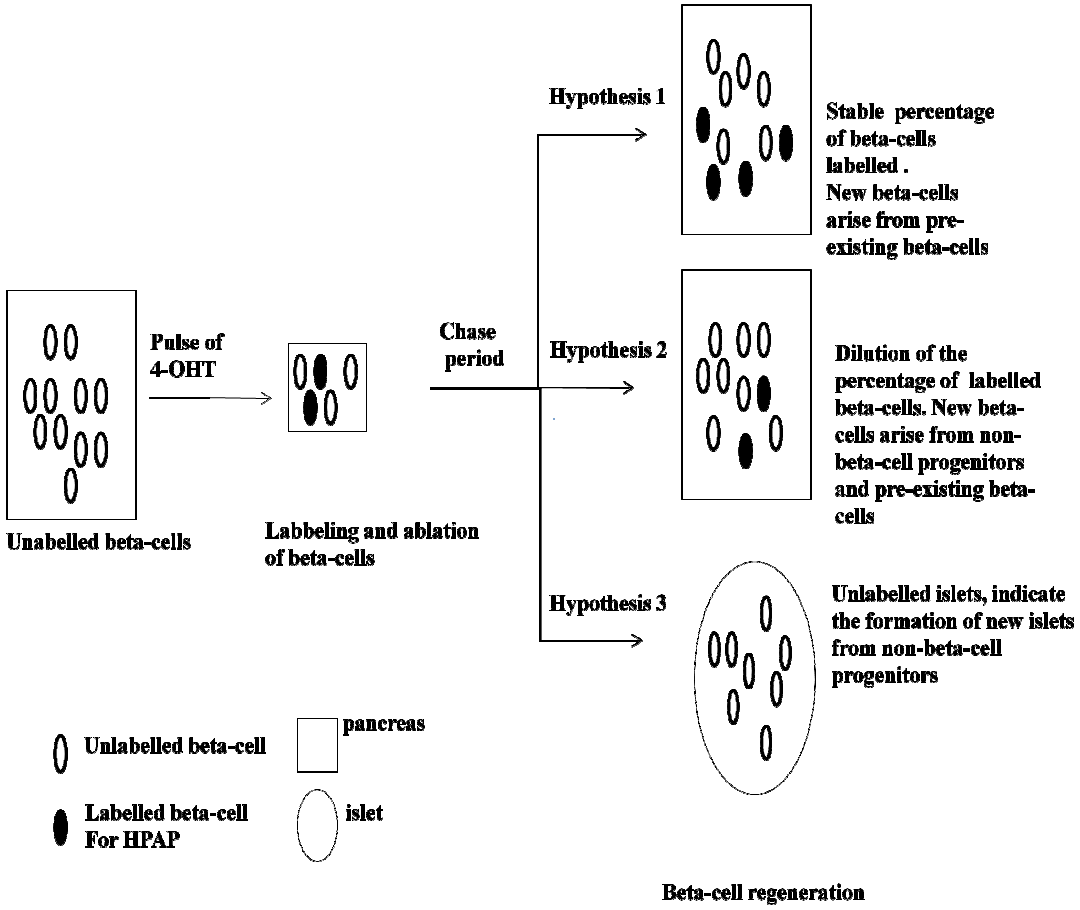


Figure 28 **Analysis of the lineage tracing results in triple transgenic mice (Z/AP; RIP-CreER^{TAM}**. In the absence of 4-OHT, the beta-cells in triple transgenic animals are unlabelled for beta-cells. Upon 4-OHT addition (the pulse period), beta-cells are ablated and labelled for HPAP concurrently. Withdrawal of 4-OHT leads to beta-cell regeneration during the chase period. Comparisons of the HPAP labelling indices between animals at the end of the pulse period and four weeks after the last 4-OHT pulse, allows deductions about the mechanism by which newly formed beta-cells arise. Three hypotheses must be considered. Hypothesis 1, if the percentage of beta-cells labelled for HPAP in animals after beta-cell regeneration during the chase period is similar to that in animal shortly after the last pulse of 4-OHT. One concludes that new beta-cells are produced by mitogenesis. Hypothesis 2, if there is a diminution of the proportion of beta-cells positive for HPAP in animals during the chase phase compared to that in animals during the pulse one deduces that new beta-cells are the progeny of non-beta-cell progenitors in combination or not with pre-existing beta-cells. Hypothesis 3, at last, if the percentage of islets devoid for the HPAP label is higher in animals during the chase period than in animals shortly after the pulse, one can infer that new whole islets are formed from non-beta-cell progenitors.

Following the 4-OHT treatment the mice developed a diabetic phenotype as expected, suggesting that the beta-cell mass in the pancreas of the treated mice was depleted by apoptosis induced by c-Myc activation. This treatment was also expected to mark the remaining beta-cells for HPAP.

Were the beta-cell mass ablated and the survival beta-cells marked for HPAP during the drug treatment?

In order to answer those questions, we collected pancreas from untreated animals and animals at the end of the pulse and also pancreata after diabetic recovery.

The HPAP labelling index and beta-cell mass were determined. We observed that the mean beta-cell mass of untreated animal is higher (0.83±0.20) than that of the diabetic animals after the end of the treatment (0.16±0.07mg). The difference between the two groups was statistically significant (One-way Analysis of Variance (ANOVA), P-value<0.01). We also compared the three groups altogether, see below.

Thus, we found that the diabetic phenotype observed in triple transgenic mice was due to beta-cell ablation induced by apoptosis subsequent to c-Myc activation by 4-OHT. These data corroborate Pelengaris et *al*. and Cano et *al*. results (Pelengaris et *al*. and Cano et *al*.).

Another important point to verify was the labelling of the remaining beta-cells after activation of the Cre-recombinase. Therefore, we immunostained for insulin and HPAP as described in Materials and Methods, pancreatic sections of triple transgenic animals treated or not for 4-OHT. The results are presented in Figure 30. In the absence of 4-OHT (Figure 30A), the overall islets were largely unlabelled for HPAP in all three untreated animals, even though, we observed limited labelling scattered in few islet sections. On the other hand, beta-cells of animals treated with 4-OHT (Figure 30B and D), expressed the HPAP reporter. The islets shown in Figure 30 are representative of the overall islet population observed in the pancreatic sections of animals for each time point.

We observe that the activation of the Cre-recombinase upon 4-OHT exposure induced expression of the label exclusively in the beta-cells of treated triple transgenic animals.

Also Figure 31 shows examples of paired post-mitotic cells positive for HPAP in treated animals which must be the progeny of pre-existing beta-cells as evidenced by the HPAP label. These examples are relatively rare but the overall labelling index in the triple transgenic mice is also low. It is not unreasonable to state that the dose of 4-OHT injected in the mice do not ablate all their beta-cells as evident in Figure 30B, and so one can expect in the same way, that a proportion of surviving beta-cells not to be labelled as well.

5.2.2 Analysis of origin of new beta-cell after diabetic recovery

With the view to determine the source for new beta-cells after diabetic recovery in the triple transgenic mice, we calculated the HPAP labelling index in animals of each group. The results are presented in Figure 29B.

The HPAP labelling index in the untreated mice (group1) is close to zero as compared with that of treated animals.

The mean HPAP labelling index of the diabetic mice (0.047±0.028, group 2) and mice after diabetic recovery (0.06±0.026, group 3) are not dissimilar (two-tailed, unpaired t-test, P-value=0.587).

This result might be taken to that new beta-cells after diabetic recovery arise mainly from pre-existing beta-cells (hypothesis 1). This is consistent with the examples of paired post-mitotic beta-cells positive for HPAP in pancreata of treated animals.

However to validate Hypothesis 1, one must prove that new beta-cells were produced during diabetic recovery. Therefore, we measured the beta-cells mass in diabetic animals in which beta-cells were ablated (group 2) and in animals after diabetic recovery (group 3), the results are presented in Figure 29C.

The mean beta-cell mass of diabetic mice (0.16±0.07mg) is not dissimilar to the mean beta-cell mass of mice after diabetic recovery (0.13±0.06mg). The

difference between the two groups was not statistically significant (One-way Analysis of Variance (ANOVA), P-value>0.05.

Surprisingly, from these data, after diabetic recovery, there is no evidence that new beta-cells were produced despite the fact that the fed blood glucose of the mice returned to normal values.

Large scale regeneration can be excluded by these data, but limited regeneration giving rise to new beta-cells cannot be ruled out in the context of the beta-cell mass remaining low.

5.4 Conclusion

In conclusion, in the triple transgenic mice we showed that c-Myc and the Cre-recombinase were activated upon 4-OHT exposure, leading to the development of a diabetic phenotype, and the labelling of remaining beta-cells for HPAP.

We also showed that the labelling was 4-OHT-dependent, heritable and specific for beta-cells.

Unfortunately, we were unable to demonstrate in the triple transgenic that diabetic recovery was associated with beta-cell regeneration, as was previously reported in the single transgenic mice model of beta-cell regeneration (pIns-c-MycER^{TAM}). Therefore we could not examine the cellular source giving rise to new beta-cells after diabetic recovery in this model. However we have seen examples of paired post-mitotic cells positive for HPAP which must be the progeny of pre-existing beta-cells in treated animals.

The similar labelling indices before and after recovery are specifically consistent with the observation that new beta-cells are not formed from progenitors.

What is the mechanism for glucose homeostasis in this model? This will be discussed in Chapter 7.

By contrast to the triple transgenic mice, in the pIns-c-MycER^{TAM} mice, we and others (Pelengaris et *al*., 2002 and Cano et *al*., 2008), have shown that the activation of c-Myc induced massive beta-cell loss by apoptosis, resulting in development of a diabetic phenotype in the animals. And more importantly, that after 4-OHT withdrawal, the mice recover from diabetes due to active beta-cell regeneration.

A pertinent question to address, after the beta-cell regenerative process occurred in these single (N.B. not triple transgenic) transgenic mice, relates to the properties of such regenerated beta-cells. Namely, if they still have the ability to completely match the functionality of true beta-cells such as the capability to maintain blood glucose level within a narrow physiological range particularly after a meal.

This is the subject of the next chapter.

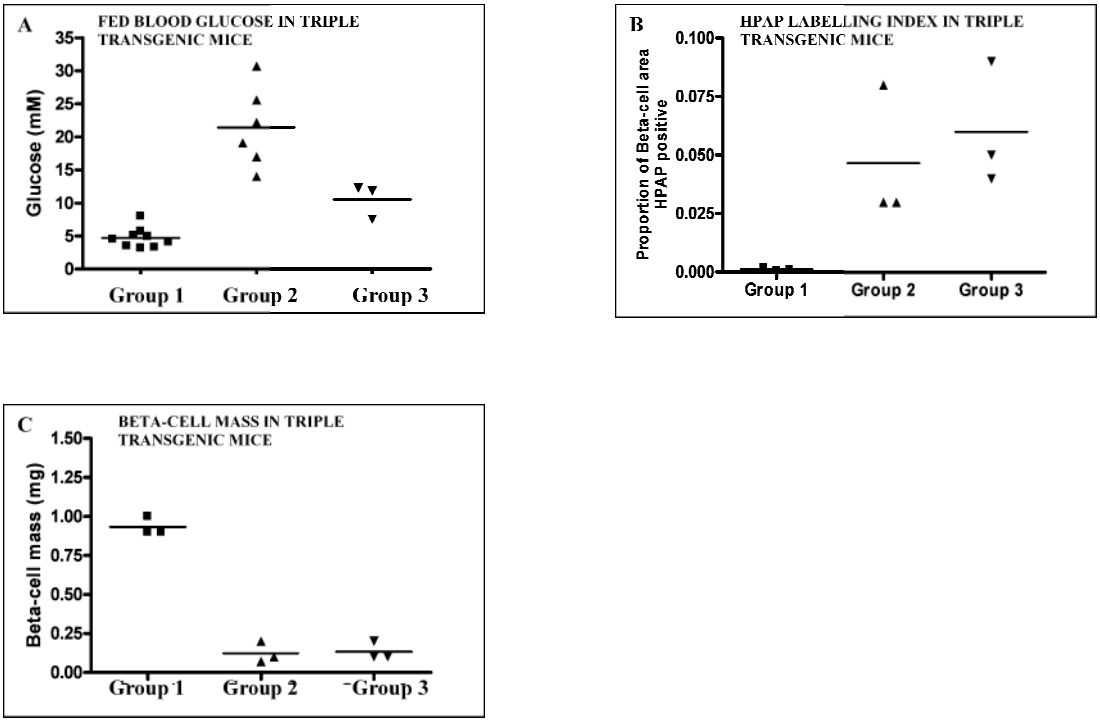


Figure 29 **Diabetes and beta-cell labelling induction in triple transgenic mice (Z/AP; RIP-CreER^{TAM}**. (A) Fed blood glucose level in animals non-treated or before treatment with 4-OHT (group 1), pulsed with 4-OHT (group 2), and 4 weeks after the last pulse of 4-OHT (group 3). Upon 4-OHT injection, the mice developed a diabetic phenotype due to beta-cell depletion, but withdrawal of 4-OHT, 4 weeks later allowed the mice to recover partially from diabetes. (B) and (C) display the HPAP labelling index and the beta-cell mass respectively, in non treated animals (group 1), animals euthanized shortly after the 4-OHT pulse (group 2), and 4 weeks following the last 4-OHT injection (group 3).

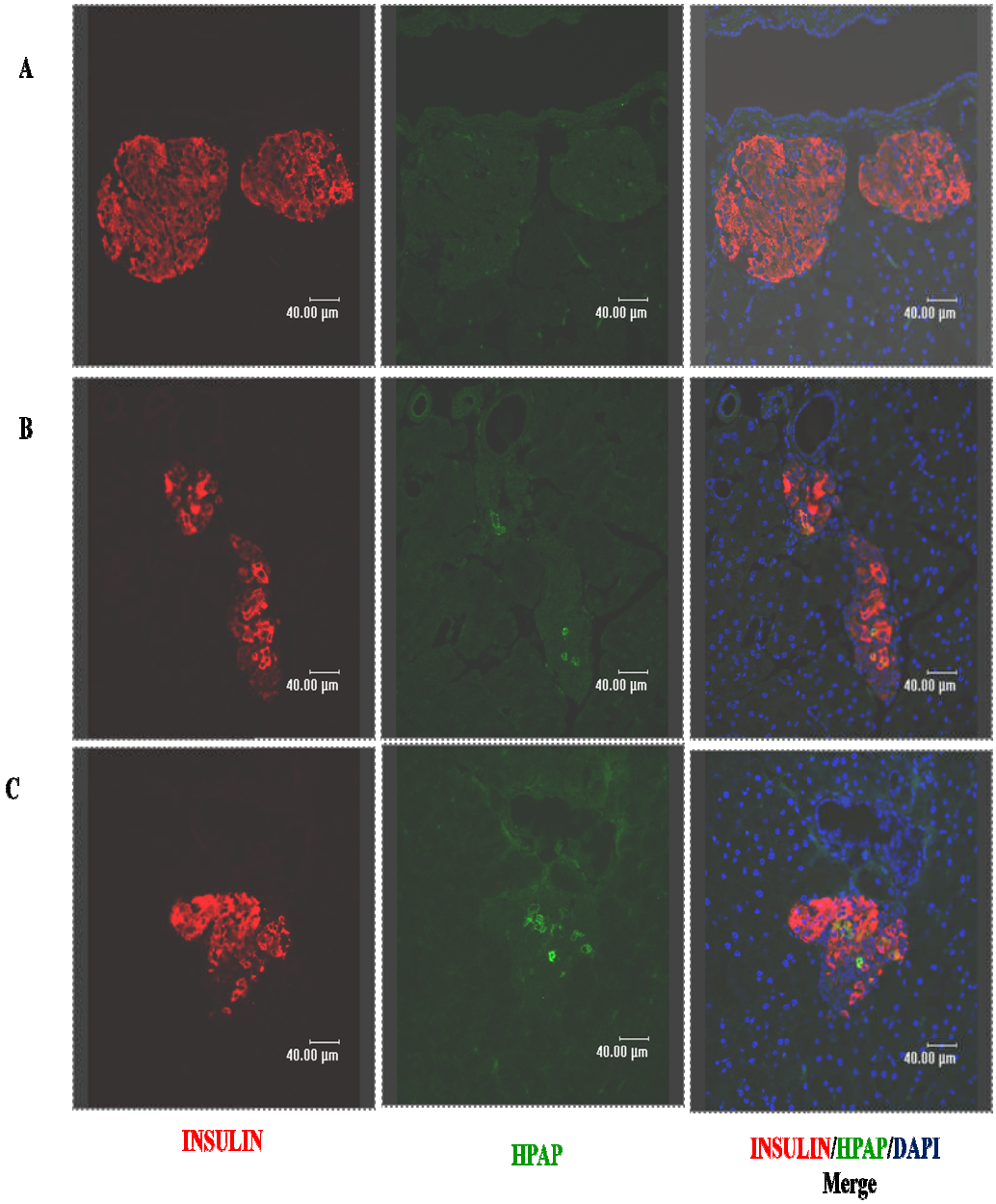


Figure 30 **Labelling for HPAP in triple transgenic mice**. (A) Pancreatic section of an untreated control animal. The two other panels represent pancreatic sections of animals treated with 10mg of 4-OHT and euthanized either at the end of the pulse (B), or 4 weeks later, (C). The sections were triple stained by immunohistohemistry for HPAP (green) and insulin (red). DAPI, the blue stain is a nuclear dye. Scale bar, 40µm.

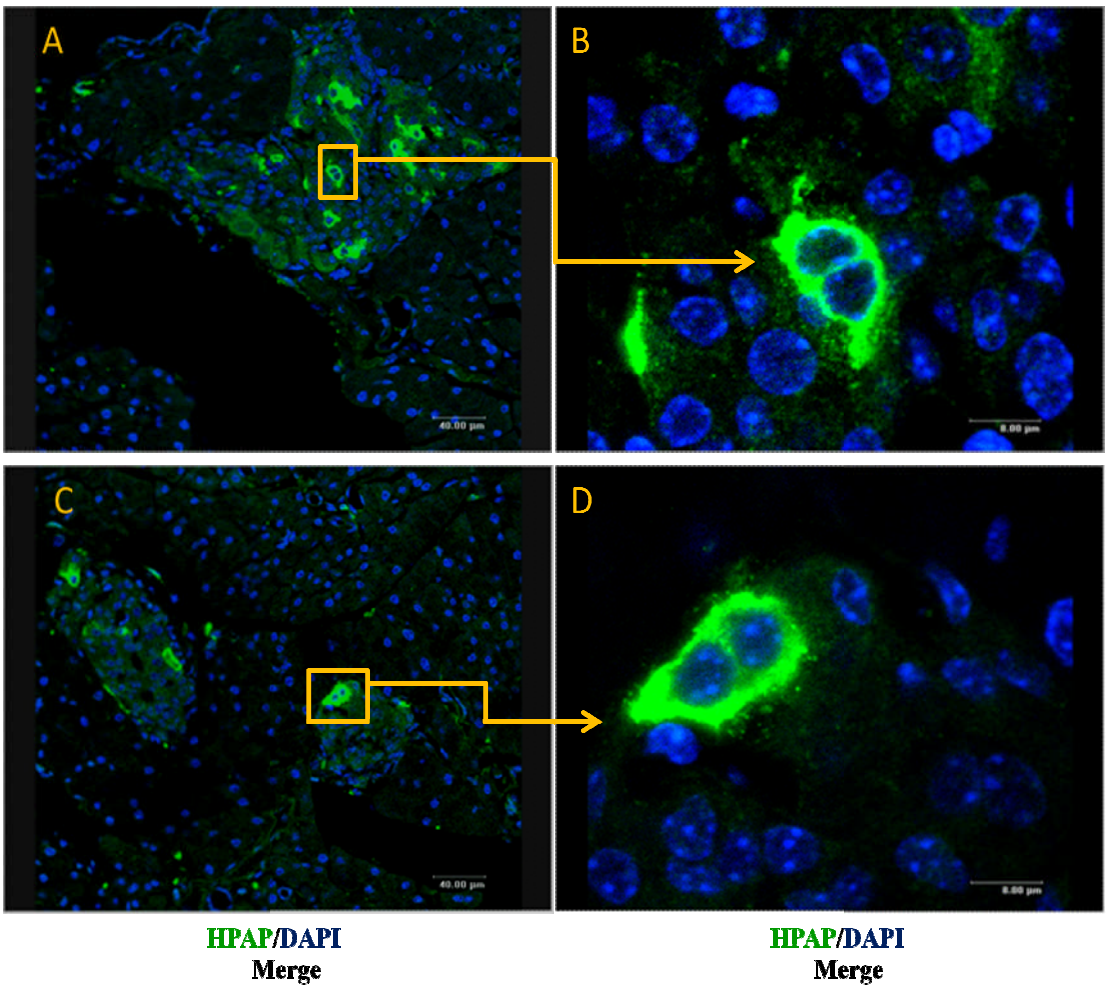


Figure 31 **HPAP labelling in the triple transgenic mice after 4OHT treatment**. (A) and (B) represent pancreatic sections from two different animals that were treated with 20mg of 4-OHT and euthanized either on the day of the last pulse or 4 weeks later respectively (scale bar, 40µm). (B) and (D) are an enlargement of micrographs A and C respectively (scale bar, 8µm).). The sections were double stained by immunohistohemistry for HPAP (green) and DAPI for the nucleus (blue).

CHAPTER 6

BETA-CELL FUNCTIONALITY AFTER DIABETIC RECOVERY

Beta-cell functionality after diabetic recovery

6.1 Introduction

In physiological situations such as pregnancy, beta-cell mass expansion is generally associated with beta-cell function enhancement to accommodate the new insulin requirement particular to the pregnant state (Sorenson and Brelje, 1997). Similarly during obesity, an augmentation of both beta-cell number and beta-cell responsiveness is also observed to compensate for the insulin resistance induced by this state, a key trait of this condition (Kargar and Ktorza, 2008).

However, in specific models of pancreatic injury associated with hyperglycaemia, such as 90% subtotal pancreatectomy (Laybutt et *al*., 2007) or selective beta-cell ablation induced by activation of caspase 8 (Wang et *al*., 2008), the regenerative process characterised by an increase beta-cell mass was not correlated with an improvement of the beta-cell functionality. Similar features were observed in the Kir6.2G123S transgenic mice, a spontaneous model of beta-cell regeneration (Oyama et *al*., 2006). In those models, the regenerated beta-cells are not fully functionally mature (Kargar and Ktorza, 2008).

Consequently, in our system in which beta-cells do regenerate after massive ablation by c-Myc (Pelengaris et *al*., 2002, Cano et *al*., 2008), it is also of interest to address the properties of new beta-cells and determine to which extent their functional capacity matches that of original beta-cells.

Using single cell microfluorimetry, we examined the beta-cell function after diabetic recovery in the pIns-c-MycER^{TAM} mice. We stress that the mice studied here are singly and not triply transgenic.

6.2 Experimental design

We analysed the beta-cell responsiveness in islets isolated from pIns-c-MycER^{TAM} mice after diabetic recovery using calcium microfluorimetry.

Single transgenic pIns-c-MycER^{TAM} and wild-type mice about three months of age were daily injected with a dose regime of 1mg/0.2ml of 4-hydroxytamoxifen (4-OHT) for six consecutive days, while control pIns-c-MycER^{TAM} mice were injected with peanut oil, the control vehicle.

At the end of the pulse, the pIns-c-MycER^{TAM} mice upon 4-OHT withdrawal were allowed to recover. Nine months after 4-OHT withdrawal, the islets from mice after diabetic recovery, from wild-type and control pIns-c-MycER^{TAM} mice were isolated for analysis.

It is important to point out that pIns-c-MycER^{TAM} mice, at around three months of age at the beginning of the experimentation, were injected contemporaneously with peanut oil with or without 4-OHT, and nine months after the last pulse of 4-OHT or peanut oil were euthanized concurrently at the end of the experiment to isolate their islets. The wild-type mice, on the contrary, were injected with 4-OHT at different times, at three months of age, but their islets were also isolated at the same time as those of the single transgenic mice for consistency.

Three to four replicates were used per group for the experiment and islets were isolated from each individual animal. Also, we isolated islets from three month old pIns-c-MycER^{TAM} control and wild-type mice that did not received any treatment, for additional control tests of functionality.

Islets were isolated from three mice per group. Fed blood glucose levels and glucose tolerance tests (see Materials and Methods for further details) were performed on the animals during the experiment before euthanizing the mice for islet isolation.

The islets were isolated using the collagenase digestion procedure, then the purified islets were cultured on 3-aminopropyltriethoxysilane-coated glass coverslips in a standard RPMI medium and incubated at 37°C in the presence of 5% CO_2 for two to three days until analysis (details of these procedures are described in Materials and Methods).

The calcium microfluorimetric measurements to study beta-cell functionality were performed by Dr Paul E. Squires according to the protocol described by Squires et *al*. (Squires et *al*., 2000).

What is the mechanism that allows beta-cells to tightly regulate the release of insulin in mammals and how can the single-cell microfluorimetric method be used to evaluate the efficiency of this process?

Beta-cells can be compared to a micro-factory that manufactures insulin that is stored in secretory vesicles or granules before its release into the medium by exocytosis. Insulin secretion is highly regulated and depends on intricate intracellular signalling in response to a variety of stimuli. The process involved in glucose-induced insulin release by beta-cells, is succinctly presenting in Figure 32 and it follows the following steps.

First, an increase of glucose concentration in the extracellular medium leads to glucose uptake by the beta-cells, catalysed by the glucose transporter-2 (Glut2). Then, the metabolic breakdown of the sugar that ensues results in the increase of the intracellular ATP concentration at the expense of ADP (or the increase of the ATP/ADP ratio) which induces the closure of an ATP-dependent potassium channel (K_{ATP}-channel). The closing of this channel causes a progressive depolarisation of the plasma membrane and the initiation firing of action potentials. These in turn open voltage-sensitive, L-type Ca^{2+} channels and provoke the influx of Ca^{2+} in the beta-cells. This results in an increase of the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) that can be considered as the key stimulus that triggers insulin-containing granules to fuse with the plasma membrane and release insulin into the medium by exocytosis (Rutter, 2004, Rorsman and Renstrom, 2003).

Glucose-stimulated insulin release by beta-cells, is in fact ultimately due to the Ca^{2+} influx. Accordingly, one can quantify this specific part of the process to appraise the efficiency of the beta-cell functionality by measuring a change in $[Ca^{2+}]_i$ in the cells after appropriate treatments known to induce insulin release

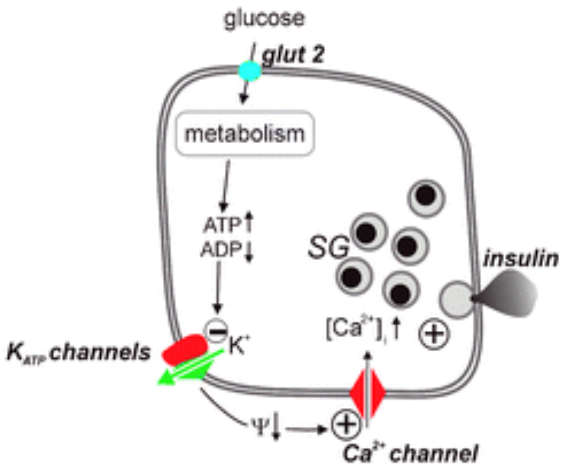


Figure 32 **Mechanism of glucose induced insulin release in beta cells**. The entry of glucose via the glucose transporter-2 (Glut2) in the beta-cells results in the glucose metabolic breakdown that leads to the generation of ATP at the expense of ADP. The change in the ratio of ATP/ADP results in the closure of the ATP-dependant potassium channel (K_{ATP} channel). Subsequently a membrane depolarization occurs; this opens voltage-gated Ca^{2+} channels initiating electrical activity and Ca^{2+} influx in the cells. The consequent rise in intracellular Ca^{2+} ($[Ca^{2+}]_i$) triggers the insulin release process. Secretory granule (SG), membrane potential (Ψ ↓), ion potassium (K^+), inhibition (-), stimulation (+), increase (↑) or decrease (↓).(Rorsman and Renstrom, 2003).

The single cell fluorimetric technique is based on the measurement of a change in $[Ca^{2+}]_i$ by using a Ca^{2+}-fluorophore fura-2A/M (Squires et *al*., 2000).

Upon binding calcium the maximum excitation wavelength of the fura-2A/M undergoes a blue shift from 380nm (when it is calcium free) to 340nm (when it is saturated with calcium). By detecting the intensity of the emitted light (wavelength of 510nm) coming from the fluorophore after illumination alternatively with the excitation wavelength of 340nm and that of 380nm, the increase of $[Ca^{2+}]_i$ due to the influx of calcium into the beta-cells will be characterized (Palmer and Moore, 2000).

Changes in $[Ca^{2+}]_i$ is represented by the ratiometric values of the intensity of fluorescence emitted by fura-2A/M after excitation with the 340nm wavelength over the intensity of fluorescent emitted by fura-2A/M after excitation with the 380 wavelength. This emission ratio can be written as 340/380nm.

To trigger insulin release from the beta-cells in vitro, the cells maybe incubated with high concentrations of glucose, about 20 to 30mM, but other components can be used to elicit the release of insulin. Potassium chloride at 20mM depolarises the membrane and forces the voltage-sensitive, L-type Ca^{2+} channel to open, which in turn leads to the influx of extracellular Ca^{2+} in the cells and insulin release by exocytosis. Tolbutamide (100µM), actives the sub-unit Sur1 of the K_{ATP}-channel, which also results in depolarisation of the membrane, which in turn induces the opening of the voltage-sensitive, L-type Ca^{2+} channel and so forth. Finally, ATP (100µM) which binds to P2-purinoreceptors, in turn leads to the formation of IP3 (inositol-3phosphates) resulting in the release of Ca^{2+} from the endoplasmic reticulum to the cytoplasm of the cell. In this situation the Ca^{2+} is released from inside the cells.

An important point regarding this technique is that the fluorescence measurement is carried out only in regions (called region of interest (RIO)) located at the periphery of the islets because only these areas are in focus under the binocular microscope, so that the fluorescence coming from fluorophore fura-2A/M can be recorded.

6.3 Results

6.3.1 Metabolic measurements

6.3.1.1 Fed blood glucose and glucose tolerance tests in pIns-c-MycER^{TAM} and wild-type animals.

Wild-type and transgenic pIns-c-MycER^{TAM} mice were injected with 4-OHT or a control vehicle for six consecutive days to induce diabetes by ablation of beta-cells upon c-Myc activation.

Figure 33 presents the fed blood glucose (a) in wild-type animals, (b) in pIns-c-MycER^{TAM} mice injected with control vehicle (c-MycER^{TAM} off), (c) in pIns-c-MycER^{TAM} mice on the last day of c-Myc activation by 4-OHT (c-MycER^{TAM} on), and (d) in pIns-c-MycER^{TAM} mice in which c-Myc was activated and then deactivated upon 4-OHT removal after nine months (c-MycER^{TAM} on-off). The values were, (a) 7.3±1mM, (b) 4±0.4mM, (c) 30±3mM and (d) 4.1±.5mM respectively.

The difference between all the groups was statistically significant (One-way Analysis of Variance (ANOVA) P-value <0.05), except for the mean fed blood glucose of pIns-c-MycER^{TAM} mice after diabetic recovery (4.1±.5mM, c-Myc on-off) and that of control pIns-c-MycER^{TAM} (4±0.4mM, c-Myc off).

These results indicate that upon activation of c-Myc by 4-OHT, the mice developed a diabetic phenotype (30±3mM) but following removal of 4-OHT, the transgenic mice recovered from diabetes (4.1±.5mM). The difference between the two groups is highly significant (One-way Analysis of Variance (ANOVA) P-value <0.0001). These results corroborate Pelengaris et *al*. (Pelengaris et *al*., 2002) and Cano et *al*. (Cano et *al*., 2008) reports.

Also, the data show that the mean fed blood glucose is higher in wild-type mice in general (7.3±1mM) than in pIns-cMycER^{TAM} mouse line (4±0.4mM), (One-way Analysis of Variance (ANOVA) P-value <0.05).

We carried out glucose tolerance tests by injecting intra-peritoneally, glucose (2g/kg) in animals after overnight fasting. The blood glucose level was recorded prior to and at 10, 30, 60 and 120 minutes after glucose administration.

Figure 34 presents the intra-peritoneal glucose tolerance test (IPGTT) results in non-treated wild-type (n=4) and pIns-c-MycER^{TAM} (n=3) mice aged three months, and also pIns-c-MycER^{TAM} mice (n=4) treated with control vehicle, aged twelve months.

Following the IPGTT, one can see that 120min after the glucose challenge, the blood glucose level of animals of each group returns to normal. Only at the time point prior to and 120 min after glucose administration, the wild-type animals show a glucose level different from the two other groups. The difference was significant (One-way Analysis of Variance (ANOVA) P-value <0.05).

Also the calculation of the area under the curve (AUC), (prism 4, University of Warwick) of the glucose tolerance test curve for each individual mouse in each group, showed that the difference of the mean AUC between non-treated wild-type (1541±276), pIns-c-MycER^{TAM} (1359±285) mice, both aged three months and pIns-c-MycER^{TAM} mice aged twelve months (1015±326) was not statistically significant (One-way Analysis of Variance (ANOVA) P-value >0.05).

First, these results indicate that the age of the transgenic pIns-c-MycER^{TAM} mice, does not affect the capacity of their beta-cells to respond properly to a glucose challenge. Second, the mice of each group were glucose tolerant, suggesting that their beta-cells were functional. Third, it is of interest to note that the fed blood glucose of wild-type animals is higher than that of our transgenic animals.

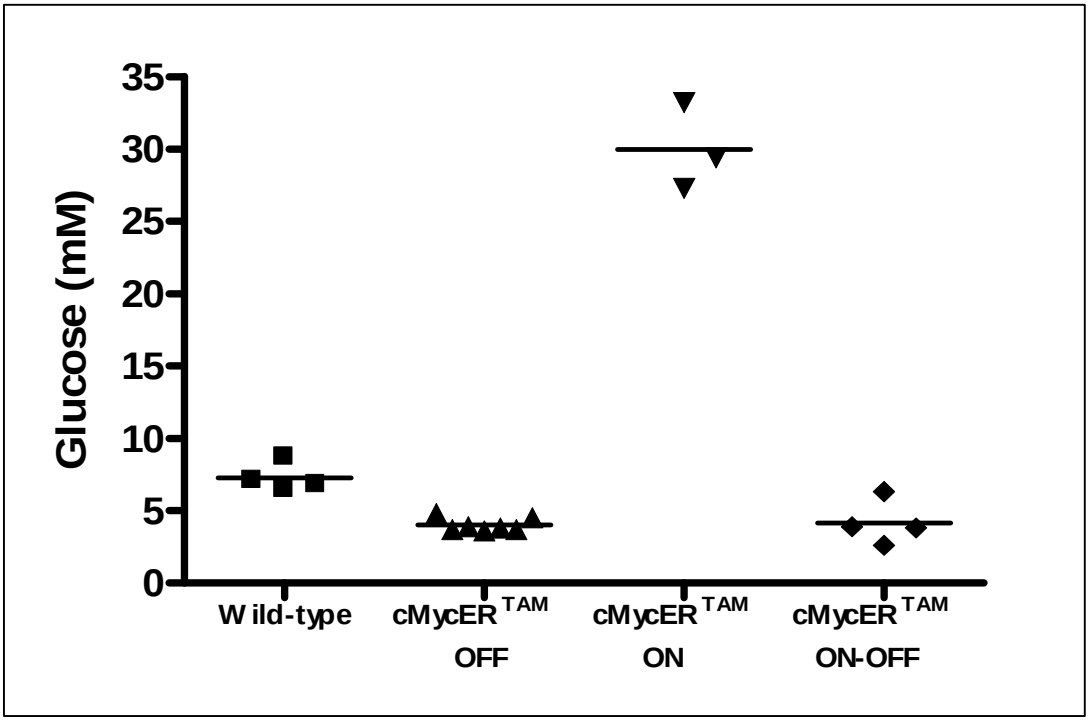


Figure 33 **Fed blood glucose**. Blood fed glucose of 3 month old wild-type animals treated with 4-OHT, 3 month old pIns-c-MycER^{TAM} mice before treatment (c-Myc off), 3 month old pIns-c-MycER^{TAM} mice shortly after the last pulse of 4-OHT (c-Myc on), and 12 month old pIns-c-MycER^{TAM} mice nine months after 4-OHT withdrawal (c-Myc on-off).

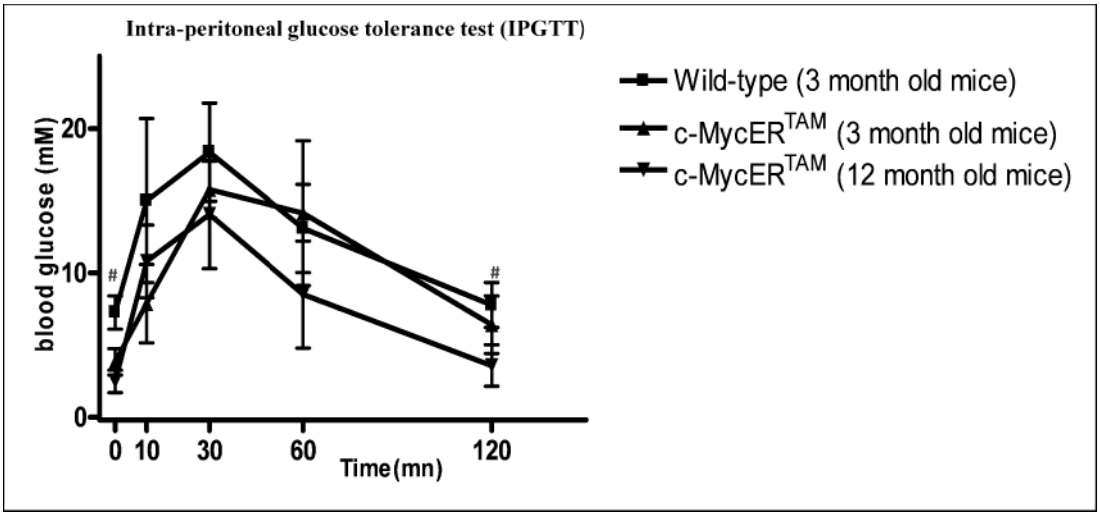


Figure 34 **Intra-peritoneal glucose tolerance test (IPGTT).** IPGTT results recorded prior to, 10, 30, 60 and 120min after glucose challenge, in non-treated wild-type (n=4) and pIns-c-MycER^{TAM} (n=3) mice aged three months, and also in pIns-c-MycER^{TAM} mice (n=4) treated with control vehicle, aged twelve months. Only at the time point prior to and 120min after glucose administration, the wild-type animal group displayed a glucose level different from the two other groups. The difference was significant (One-way Analysis of Variance (ANOVA) #: P-value <0.05).

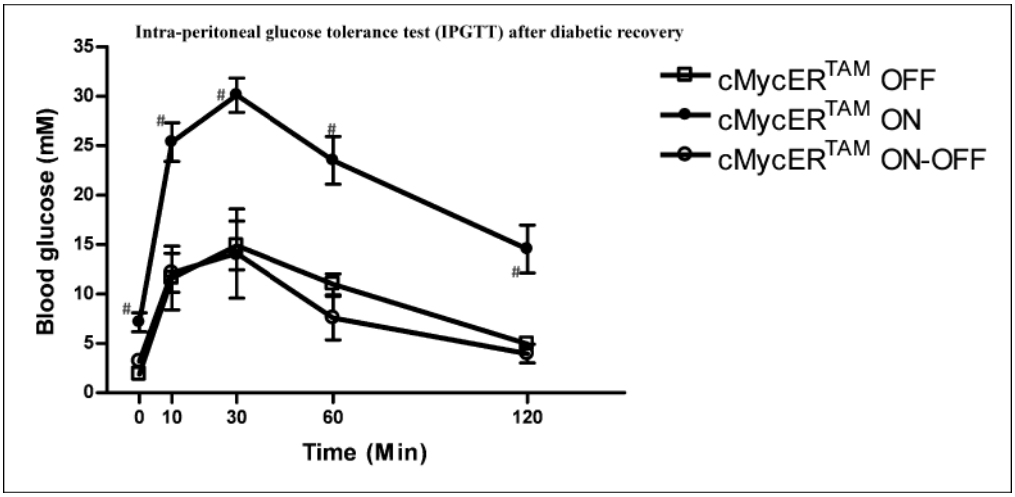


Figure 35 **Intra-peritoneal glucose tolerance test (IPGTT)**. IPGTT results from pIns-c-MycER^{TAM} animals (n=3) before 4-OHT treatment (c-Myc off), shortly after 4-OHT pulse (c-Myc on) and 9 months upon 4-OHTwithdrawal (c-Myc on-off). The IPGTT values of animals shortly after treatment compared with those of both the mice before treatment and after diabetic recovery, were significantly different at all the time points (One-way Analysis of Variance (ANOVA) #: P-value <0.05). In stark contrast, IPGTT curves of animals before treatment (c-MycER^{TAM} off) and after diabetic recovery (c-MycER^{TAM} on-off) were not significantly dissimilar from each other.

6.3.1.2 Glucose tolerance tests in pINS-c-MycER^{TAM} mice after diabetic recovery

Three pIns-c-MycER^{TAM} mice about three months of age, were treated with 4-OHT for six consecutive days, and then allowed to recover from diabetes upon 4-OHT removal.

IPGTT was carried out on the same mice during the course of the experiment, that is before 4-OHT treatment, shortly after the end of the treatment (that is when the mice were overtly diabetic) and nine months after the last 4-OHT treatment (that is when the mice recovered from diabetes). The results are recorded in Figure 35. The analysis of the IPGTT in the animals shortly after 4-OHT treatment (c-MycER^{TAM} on) indicates clearly that the mice were glucose intolerant and 120 min after the glucose challenge they were still not able to normalise their blood glucose level.

The IPGTT values of animals shortly after treatment compared with those of both the mice before treatment and after diabetic recovery, were significantly different at all the time points (One-way Analysis of Variance (ANOVA) P-value <0.05).

In stark contrast, IPGTT curves of animals before treatment (c- MycER^{TAM} off) and after diabetic recovery (c- MycER^{TAM} on-off) were not significantly dissimilar from each other. Thus, analysis of IPGGT curves in these two groups indicates that the animals were able to normalise their blood glucose level 120min after the glucose challenge.

Also, the calculation of the AUC of the glucose tolerance test curve of each individual mouse shows that the difference of the mean AUC of animal before treatment (1197± 273) and after diabetic recovery (1010±466) was not statistically significant (One-way Analysis of Variance (ANOVA) P-value >0.05).

By contrast, comparison of the mean AUC of the animals during treatment (2661±359) with that of animals before treatment or after diabetic recovery, indicates that the difference between the two groups was statistically significant (One-way Analysis of Variance (ANOVA) P-value <0.001).

It is noteworthy that the same animal was glucose tolerance tested prior to, at the end of and after the 4-OHT treatment, in this experiment.

Collectively, these results indicate that upon 4-OHT treatment, pIns-c-MycER^{TAM} mice develop a diabetic phenotype (become glucose intolerant), but after 4-OHT removal, namely nine months after the drug pulse, the animals recover from diabetes, as evidenced by normalisation of their fed blood glucose and by becoming glucose tolerant again.

In addition, the fact that the animals after diabetic recovery were glucose tolerant again, suggested that their regenerated beta-cells were functional.

6.3.2 Analysis of beta-cell functionality using single-cell microfluorimetry

Using single-cell microfluorimetry we studied islet beta-cell functionality of pIns-c-MycER^{TAM} mice before and after diabetic recovery. The study was performed in collaboration with P. Squires.

For this experiment, wild-type animals were treated with 4-OHT prior to islet isolation. pIns-c-MycER^{TAM} mice were treated with 4-OHT or control vehicle and islets were isolated nine months after 4-OHT or control vehicle administration. In each group, islets were isolated from three different animals. Figures 36, 37 and 42 represent graphs of the islet beta-cell functionality of islets from wild-type animals. Each figure represents the result of the beta-cell functionality test of an islet from an individual animal. The islets were incubated, first with glucose (20mM) then with different components not in a particular order, tolbutamide (100µm), ATP (100µm) or K^+Cl^- (20mM). The y-axis, corresponds to the changes in $[Ca^{2+}]_i$ symbolised by an increase of the ratio 340/380nm, whilst the x-axis to the time in seconds. Series or RIO (region of interest) represents a region in the islet that was examined. At least eight different regions of interest were concurrently monitored per islet, for stimulated changes in $[Ca^{2+}]_i$. Each oscillatory curve characterizes changes in $[Ca^{2+}]_i$ for each RIO or series tested. The three graphs show unequivocally an increase of the ratio 340/380nm, indicating an augmentation of the $[Ca^{2+}]_i$, due either to an influx of Ca^{2+} release into the cells or the cytoplasm, after the islets were stimulated

with nutrient and non-nutrient. These results indicate clearly that the islet beta-cells from wild-type animals were completely functional.

By contrast, the islet beta-cells from pIns-c-MycER^{TAM} mice after diabetic recovery (Figures 38 and 39) show very little increase of the ratio 340/380nm or change in $[Ca^{2+}]_i$ after stimulation by glucose or other components. This indicates a marked impairment of the beta-cell functionality of mice after diabetic recovery.

To verify that the impaired beta-cell functionality was a feature of the beta-cells after diabetic recovery, we checked the islet beta-cells functionality in pIns-c-MycER^{TAM} mice treated with control vehicle (Figures 40 and 41). Surprisingly, after stimulation of the islets with glucose, tolbutamide or K^+Cl^-, little increase in change in $[Ca^{2+}]_i$ was observed, similar to islets of animals after diabetic recovery.

To rule out the fact that the age of the pIns-c-MycER^{TAM} animals might affect their beta-cell responsiveness, we examined the islet beta-cell functionality of pIns-c-MycER^{TAM} (Figure 43 and 44) and wild-type (Figures 45 and 46) animals aged three months.

The graphs of the islets from wild-type animals, once again, show a higher amplitude of the increase of the $[Ca^{2+}]_i$ compared to those from pIns-c-MycER^{TAM} mice.

Collectively, the profound attenuation of the beta-cell responsiveness in pIns-c-MycER^{TAM} mice either in untreated controls or after diabetic recovery shows explicitly that beta-cell functionality is abnormal in this transgenic line.

Also, the finding that the fura-2M/A uptake was similar in wild-type and pIns-c-MycER^{TAM} islets, characterized by comparable dye base line, indicates that the beta-cells were viable at the start of the experiment.

The graphs of islets from pIns-c-MycER^{TAM} mice untreated controls and after diabetic recovery, show no evidence that the beta-cell functionality after diabetic recovery is different from that of untreated control pIns-c-MycER^{TAM} mice (albeit abnormal).

6.4 Conclusion

Based on the results of the glucose tolerance test, we concluded that the transgenic pIns-c-MycER^{TAM} mice prior to c-Myc activation were glucose tolerant. This indicates that their beta-cells are functional in maintaining glucose homeostasis, and the age of the transgenic mice did not affect this property.

By contrast, upon 4-OHT treatment, pIns-c-MycER^{TAM} mice developed a diabetic phenotype, becoming glucose intolerant, but upon 4-OHT removal, the animals recovered eventually from diabetes and became glucose tolerant again, suggesting that regenerated beta-cells are at least partially functional after diabetic recovery.

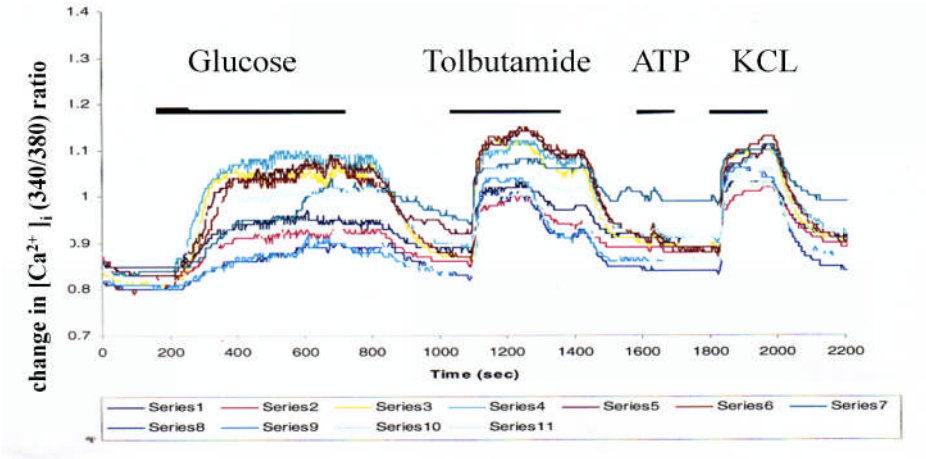
To explore whether beta-cell function was normal after diabetic recovery, we tested their functionality using single-cell microfluorimetry.

Based on the results of calcium microfluorimetry, we concluded that the islet beta-cells from control wild-type animals were completely functional.

In stark contrast, the profound attenuation of the beta-cell responsiveness in pIns-c-MycER^{TAM} mice untreated controls and after diabetic recovery, demonstrated that the mechanism of the beta-cell functionality in this line was altered.

In addition, the comparison of the profile in the change of intracellular Ca^{2+}concentration between islets from pIns-c-MycER^{TAM} mice untreated controls and after diabetic recovery, showed no evidence that beta-cell functionality after diabetic recovery was different from the (albeit abnormal) functionality of untreated control pIns-c-MycER^{TAM} mice.

The discrepancy observed between the physiological responsiveness of transgenic animals and the beta-cell responsiveness *in vitro* in the pIns-c-MycER^{TAM} line is quite intriguing and will be discussed in the next chapter.



Change in $[Ca^{2+}]_i$ in islet from 3 month old wild-type mouse treated with 4-OHT

Figure 36 **Islet beta-cell functionality of wild-type animals**. For this experiment, three month old wild-type animals were treated with 4-OHT prior to islet isolation. The islets were incubated, first with glucose (20mM) then with different components, tolbutamide (100µm), ATP (100µm) or K^+Cl^- (20mM). The y-axis, corresponds to the changes in $[Ca^{2+}]_i$ symbolised by an increase of the ratio of the fluorescence at 340/380nm, whilst the x-axis to the time in seconds. Each series represents a distinct region in the islet that was examined.

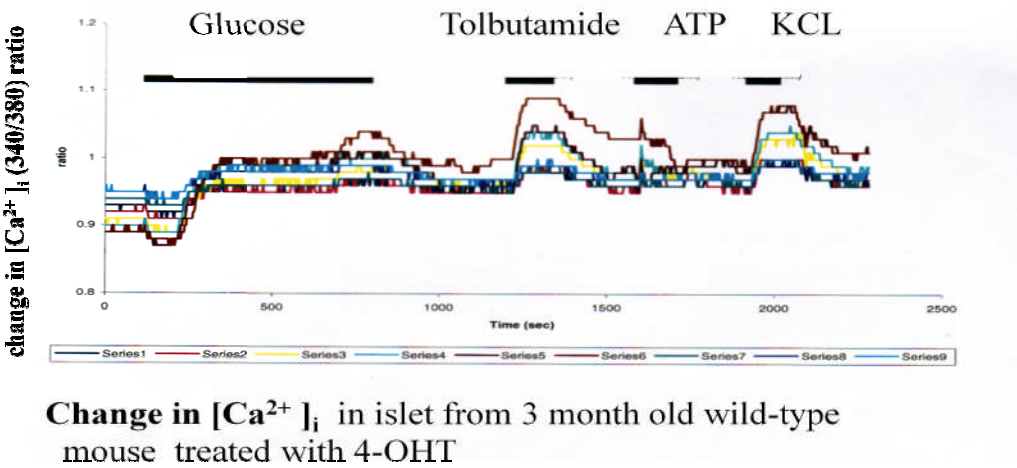
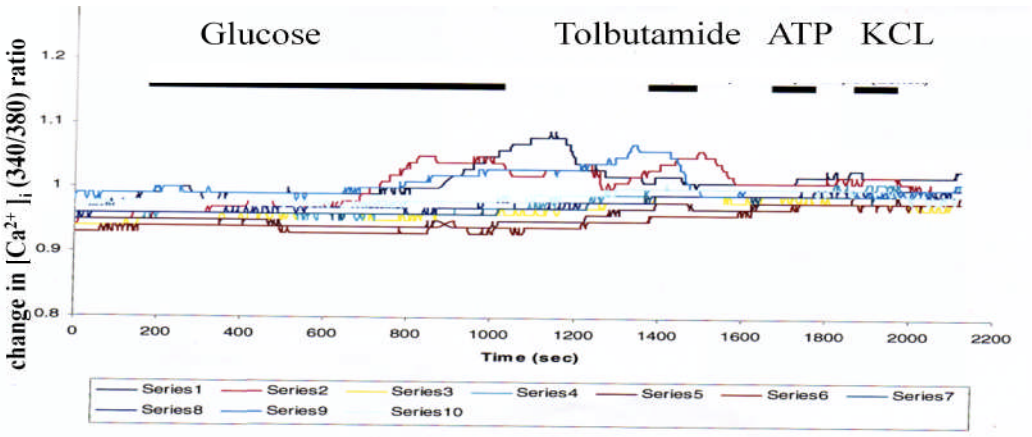


Figure 37 **Islet beta-cell functionality of wild-type animals**. For this experiment, three month old wild-type animals were treated with 4-OHT prior to islet isolation. The islets were incubated, first with glucose (20mM) then with different components, tolbutamide (100µm), ATP (100µm) or K^+Cl^- (20mM). The y-axis, corresponds to the changes in $[Ca^{2+}]_i$ symbolised by an increase of the ratio of the fluorescence at 340/380nm, whilst the x-axis to the time in seconds. Each series represents a distinct region in the islet that was examined.



Change in $[Ca^{2+}]_i$ in islet from 12 month old pIns-c-MycER^{TAM} mouse treated with 4-OHT and after diabetic recovery

Figure 38 **Islet beta-cell functionality of pIns-c-MycER^{TAM}mice after diabetic recovery**. For this experiment, pIns-c-MycER^{TAM} islets were isolated from twelve month old animals allowed to recover from diabetes for nine months after 4-OHT withdrawal. The islets were incubated, first with glucose (20mM) then with different components, tolbutamide (100µm), ATP (100µm) or K^+Cl^- (20mM). The y-axis, corresponds to the changes in $[Ca^{2+}]_i$ symbolised by an increase of the ratio of the fluorescence at 340/380nm, whilst the x-axis to the time in seconds. Each series represents a distinct region in the islet that was examined.

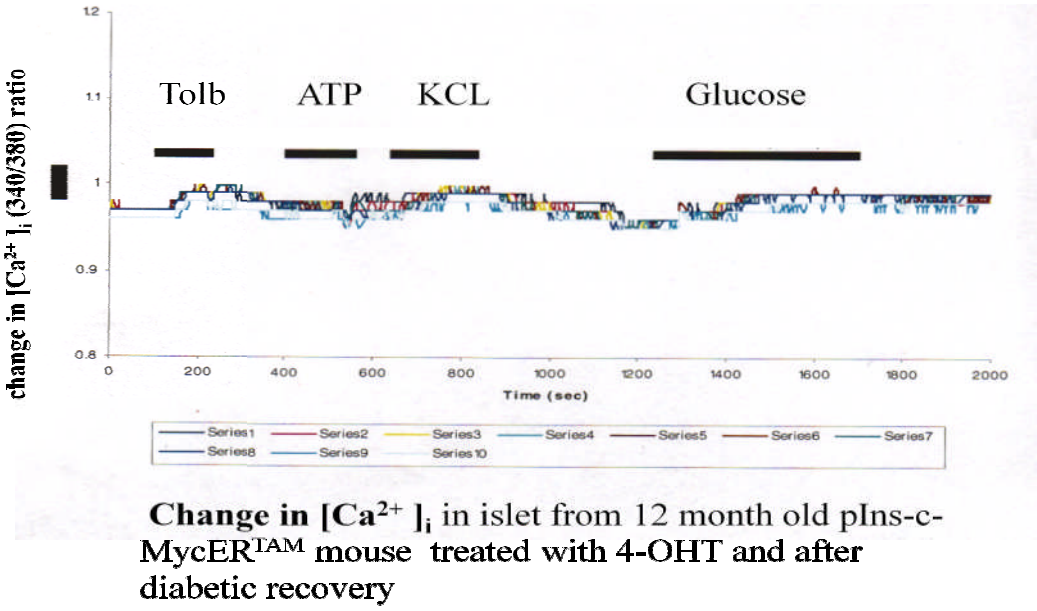


Figure 39 **Islet beta-cell functionality of pIns-c-MycER^{TAM} mice after diabetic recovery**. For this experiment, pIns-c-MycER^{TAM} islets were isolated from twelve month old animals allowed to recover from diabetes for nine months after 4-OHT withdrawal. The islets were incubated, first with glucose (20mM) then with different components, tolbutamide (100µm), ATP (100µm) or K^+Cl^- (20mM). The y-axis, corresponds to the changes in $[Ca^{2+}]_i$ symbolised by an increase of the ratio of the fluorescence at 340/380nm, whilst the x-axis to the time in seconds. Each series represents a distinct region in the islet that was examined.

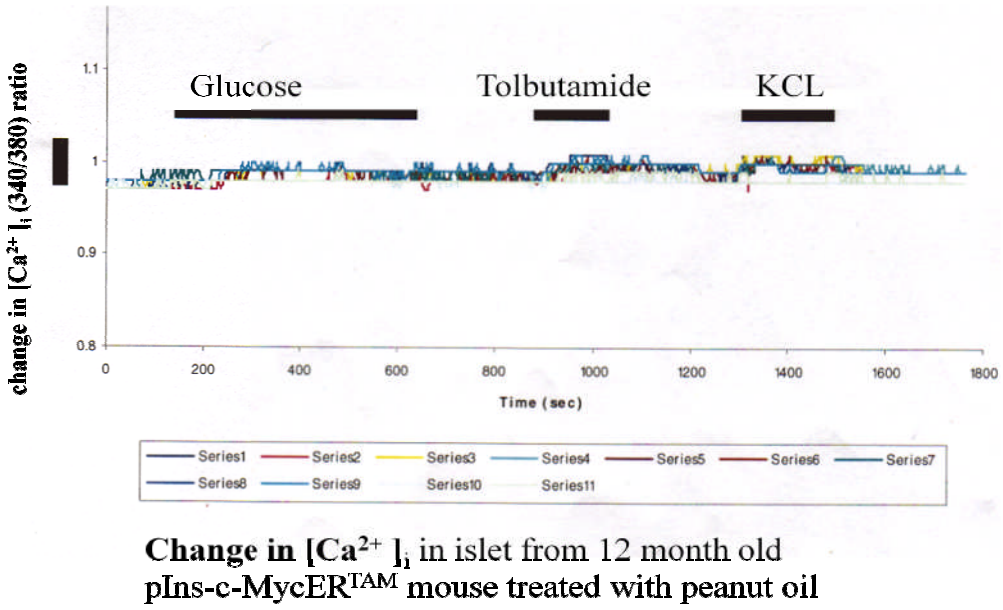


Figure 40 **Islet beta-cell functionality of control pIns-c-MycER^{TAM} mice**. For this experiment, twelve month old pIns-c-MycER^{TAM} islets were isolated from animals treated with peanut oil. The islets were incubated, first with glucose (20mM) then with different components, tolbutamide (100µm), ATP (100µm) or K^+Cl^- (20mM). The y-axis, corresponds to the changes in $[Ca^{2+}]_i$ symbolised by an increase of the ratio of the fluorescence at 340/380nm, whilst the x-axis to the time in seconds. Each series represents a distinct region in the islet that was examined.

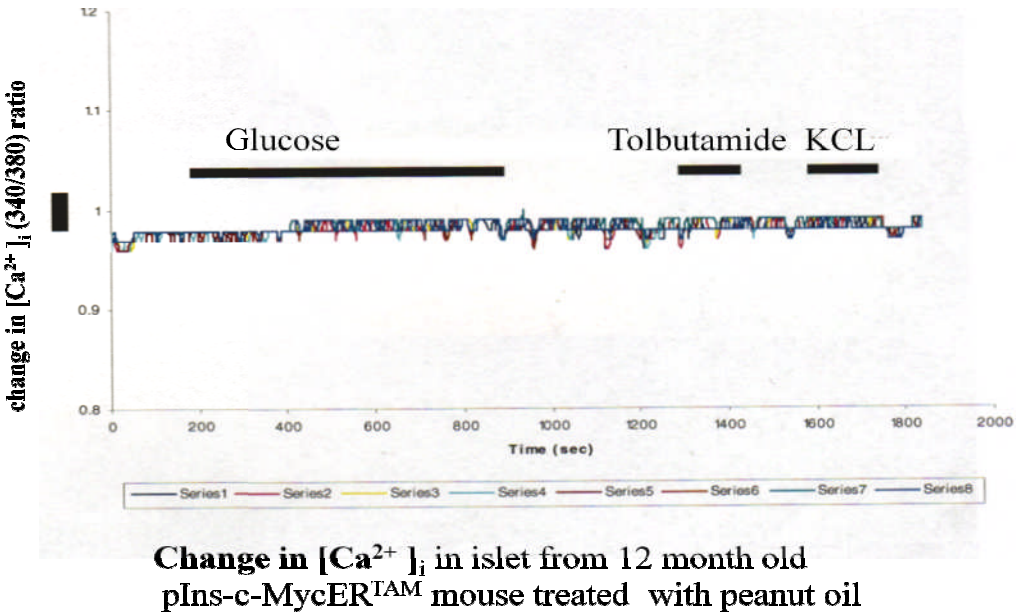


Figure 41 **Islet beta-cell functionality of control pIns-c-MycER^{TAM} mice**. For this experiment, twelve month old pIns-c-MycER^{TAM} islets were isolated from animals treated with peanut oil. The islets were incubated, first with glucose (20mM) then with different components, tolbutamide (100µm), ATP (100µm) or K^+Cl^- (20mM). The y-axis, corresponds to the changes in $[Ca^{2+}]_i$ symbolised by an increase of the ratio of the fluorescence at 340/380nm, whilst the x-axis to the time in seconds. Each series represents a distinct region in the islet that was examined.

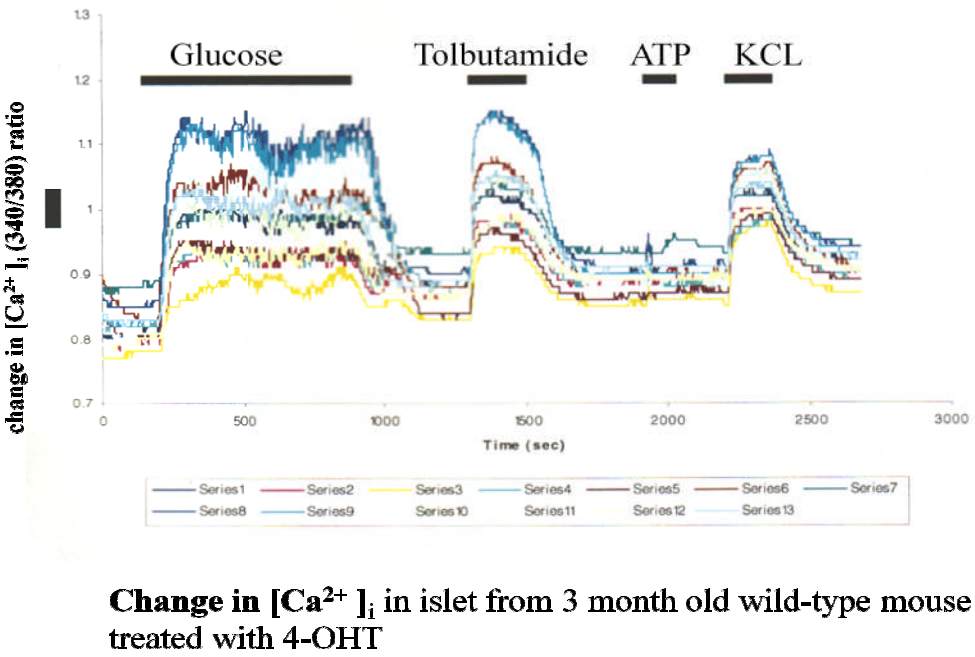
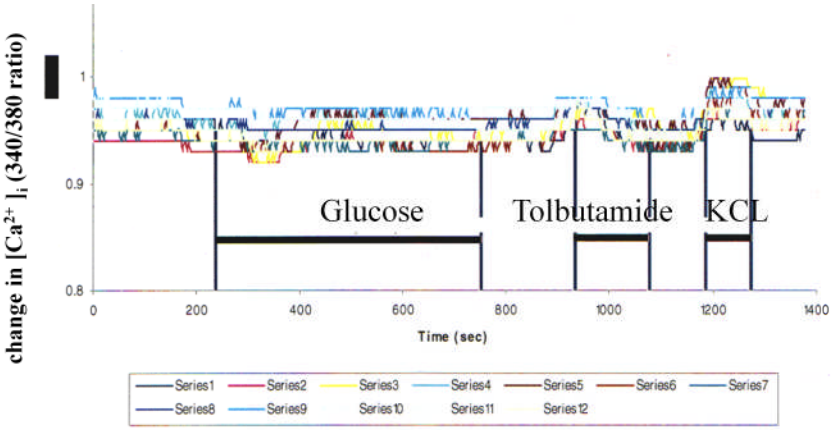
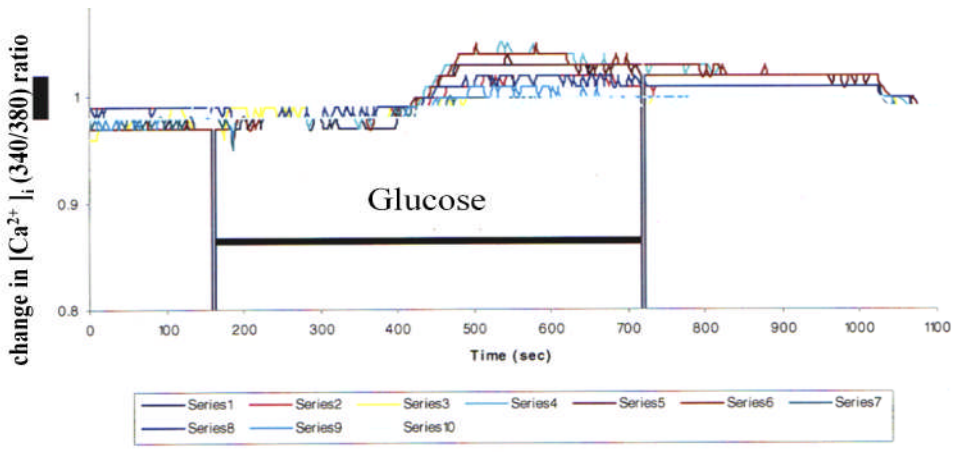


Figure 42 **Islet beta-cell functionality of wild-type mice**. For this experiment, three month old wild-type animals were treated with 4-OHT prior to islet isolation. The islets were incubated, first with glucose (20mM) then with different components, tolbutamide (100µm), ATP (100µm) or K^+Cl^- (20mM). The y-axis, corresponds to the changes in $[Ca^{2+}]_i$ symbolised by an increase of the ratio of the fluorescence at 340/380nm, whilst the x-axis to the time in seconds. Each series represents a distinct region in the islet that was examined.



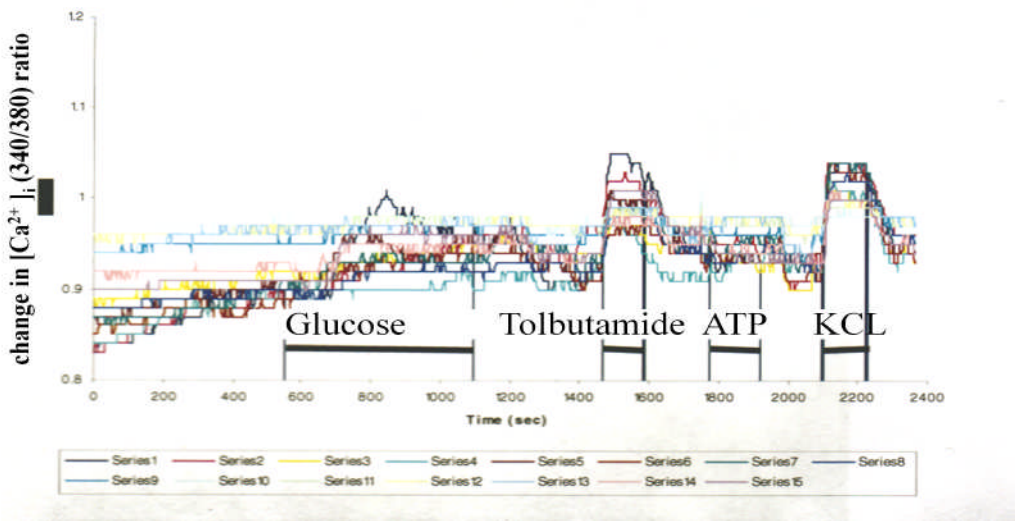
Change in $[Ca^{2+}]_i$ in islet from 3 month old untreated pIns-c-MycER^{TAM} mouse

Figure 43 **Islet beta-cell functionality of pIns-c-MycER^{TAM} mice**. For this experiment, islets from three month old untreated pIns-c-MycER^{TAM} animals were isolated. The islets were incubated, first with glucose (20mM) then with different components, tolbutamide (100µm), ATP (100µm) or K^+Cl^- (20mM). The y-axis, corresponds to the changes in $[Ca^{2+}]_i$ symbolised by an increase of the ratio of the fluorescence at 340/380nm, whilst the x-axis to the time in seconds. Each series represents a distinct region in the islet that was examined.



Change in $[Ca^{2+}]_i$ in islet from 3 month old untreated pIns-c-MycER^{TAM} mouse

Figure 44 **Islet beta-cell functionality of pIns-c-MycER^{TAM} mice**. For this experiment, islets from three month old untreated pIns-c-MycER^{TAM} animals were isolated. The islets were incubated, first with glucose (20mM) then with different components, tolbutamide (100µm), ATP (100µm) or K^+Cl^- (20mM). The y-axis, corresponds to the changes in $[Ca^{2+}]_i$ symbolised by an increase of the ratio of the fluorescence at 340/380nm, whilst the x-axis to the time in seconds. Each series represents a distinct region in the islet that was examined.



Change in $[Ca^{2+}]_i$ in islet from 3 month old untreated wild-type mouse

Figure 45 **Islet beta-cell functionality of wild-type mice**. For this experiment, islets from three month old untreated wild-type animals were isolated. The islets were incubated, first with glucose (20mM) then with different components, tolbutamide (100µm), ATP (100µm) or K^+Cl^- (20mM). The y-axis, corresponds to the changes in $[Ca^{2+}]_i$ symbolised by an increase of the ratio of the fluorescence at 340/380nm, whilst the x-axis to the time in seconds. Each series represents a distinct region in the islet that was examined.

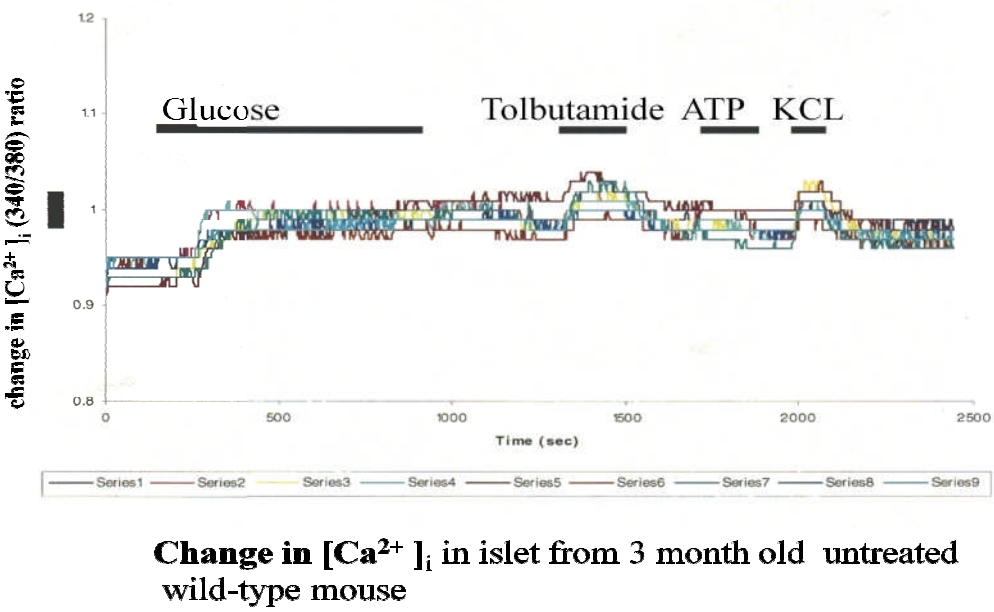


Figure 46 **Islet beta-cell functionality of wild-type mice**. For this experiment, islets from three month old untreated wild-type animals were isolated. The islets were incubated, first with glucose (20mM) then with different components, tolbutamide (100µm), ATP (100µm) or K^+Cl^- (20mM). The y-axis, corresponds to the changes in $[Ca^{2+}]_i$ symbolised by an increase of the ratio of the fluorescence at 340/380nm, whilst the x-axis to the time in seconds. Each series represents a distinct region in the islet that was examined.

CHAPTER 7

DISCUSSION

Discussion

7.1 Origin of new beta-cells under normal physiological condition

We investigated the origin of new beta-cells in response to one and two cycles of pregnancy using a cell-lineage tracing for beta-cells in pulse-chase experiments. We first showed that the labelling system in the double transgenic mice (Z/AP; RIP-CreER^{TAM}) was 1) specific to beta-cells, 2) irreversible and heritable and 3) tamoxifen dose-dependant.

7.1.1 Origin of new beta-cells during a first round of pregnancy

Secondly, we showed that in response to a first cycle of pregnancy, 1) the mean body and pancreatic weight increased about 45 and 51% respectively in pregnant animals compared to the aged-matched non-pregnant control animals, 2) the mean beta-cell mass increased around 91% in pregnant mice compared with the age-matched non-pregnant control mice.

The analysis of the proportion of beta-cells labelled for HPAP, showed that the labelling index of the non-pregnant animals (0.44±0.05) was greater that in the pregnant group (0.33±0.06). The difference between the two groups was statistically significant (paired two-tailed t-test, P-value 0.021).

Furthermore the combined results obtained from paired animals during one and two pregnancies reinforce this result, by indicating that the difference between the two groups was extremely significant (paired, two-sided student t-test, P-value 0.0007).

Taken together, Theses results are consistent with hypothesis 2 and indicate that non-beta-cell progenitor contribute significantly to the increase of the beta-cell mass in response to pregnancy.

The analysis of the proportion of the beta-cell clusters or small sized-islets devoid of HPAP, showed that the means of the beta-cell clusters or small islets

negative for HPAP was greater in the pregnant group (0.19±0.06) than in the non-pregnant controls (0.11±0.04). The difference between the two groups was not statistically significant (P=0.093, paired two tailed t-test). There is a trend indicating that new islets are formed during pregnancy or alternatively, these results might reflect the dilution of the label in expanded islets.

However, the combined results of these proportions in paired animals in one and two pregnancies strongly indicate that new islets are formed during pregnancy or there is a dilution of the label in larger islets during the pregnant state. Inasmuch, the difference between the pregnant and non-pregnant groups is considered very significant (paired, two-sided student t-test, P-value 0.006).

The analysis of the HPAP labelling indices in the beta-cells in the lining of the ductal component (0.21, n=57 cells, representing 4 replicates) and in the islet compartment in the same sections (0.31, n=7053 cells, representing 4 replicates) showed that the two indices are not dissimilar (2x2 contingency table, the chi-square approximation, two-sided, P= 0.15).

On the assumption that the number of insulin-positive cells in the ductal component increases substantially during pregnancy, the similar HPAP labelling indices in the ductal component and in the islets are not consistent with the duct being a “ preferential” source of new beta-cells during pregnancy.

Finally, we did not observed any example of cells positive for HPAP and negative for insulin in the pancreatic sections of non-pregnant and pregnant animals, indicating that during this time window beta-cells do not fully lose differentiation or transdifferentiate into other lineages of either endocrine or exocrine compartment.

In conclusion, using a cell-lineage tracing system for beta-cells we demonstrated, for the first time, that the beta-cell mass increase in response to a first cycle of pregnancy is associated not only with pre-existing beta-cells duplication but more importantly with the activation of non-beta-cell progenitor sources.

We need to exclude alternative explanations, most critically, whether the rate of proliferation of pre-existing beta-cells labelled for HPAP is slowed down with regard to beta-cells devoid of the label, i.e. the label may alter the beta-cell replication process. This is unlikely to happen, since Nir et *al*. (Nir et *al*., 2007),

previously demonstrated, using the same cell lineage tracing system, that the proliferation rate of beta-cells labelled for HPAP was similar to that of the non marked beta-cells. What is more, the authors did not observe any dilution of the label during the beta-cell mass regeneration after diabetic recovery. In line with this result, Dor et *al*. (Dor et *al*., 2004) also reported no dilution of the label during both normal growth and after pancreatectomy, strongly suggesting that it is unlikely that the label affects the replication rate of beta-cells containing the reporter.

We cannot give an exact number of the non-beta-cell progenitor contribution during the beta cell mass expansion. Yet, on the other hand, we can give an estimate of this precursor involvement. In principle, if the beta-cell mass doubles in mice during pregnancy, as reported by us and others (Huang et *al*., 2009, Karnik et *al*., 2007), and if the non-beta-cell progenitors are exclusively accountable for this beta-cell mass population increment, then, the HPAP labelling index in pregnant animals should be half of that of the non-pregnant animals. In non-pregnant controls, we observed a HPAP labelling index about 0.44, consequently, we would expect a proportion of the labelled beta-cells for HPAP around 0.22 in pregnant animals. The observed number is 0.33, suggesting that approximately, 50% of new beta-cells arise from pre-existing beta-cells by replication, while the other half must come from the activation of a non-beta-cell progenitor. Our finding validates results from previous authors suggesting that beta-cell mitogenesis is responsible for the beta-cell mass increment during pregnancy (Karnik et *al*., 2007, Teta et *al*., 2007, Scaglia et *al*., 1995, Avril et *al*., 2002, Parsons et *al*., 1992). But using a cell-lineage tracing for beta-cells, we were able to demonstrate that pre-existing beta-cells are not the only lineage to contribute to this process, and non-beta-cell precursor source plays an active role in this beta-cell mass increase as well.

Our finding does not contradict Teta et *al*. conclusions (Teta et *al*., 2007) regarding the absence of contribution of non-beta-cell specialized progenitors during pregnancy. Those authors used DNA analogue-based lineage tracing technique to detect multiple cycles of cell division in vivo to assess if progenitors that divided rapidly give rise to new beta-cells. We can estimate that if there are 3% of progenitors residing in the pancreas of animals, the progenitors will need to undergo only three to four rounds of division to increase the beta-cell mass about 50%, in response to pregnancy. This suggests that the progenitors do not need to go through

many rounds of replication, consistent with Teta's finding. Additionally, the technique used by those authors to co-label the cells with the two different thymidine analogs, can easily underestimate the number of cycles of replication that the precursors can actually undergo.

Karnik et *al*. and Huang et *al*. (Karnik et *al*., 2007, Huang et *al*., 2009) reported that the over-expression of Menin specifically in beta-cells, or the loss of expression of prolactin receptor in the whole organism, both abolished the beta cell mass increment during pregnancy in mice. These results led the authors to conclude that pre-existing beta-cells were the major lineage contributing to the beta cell mass increase. If the over-expression of Menin or the lack of prolactin receptors affect profoundly the duplication of beta-cells, they could also affect the replication of potential precursors of beta cells, and this would account for their observation.

What might be the identity of the non-beta-cell progenitor lineages during a first cycle of pregnancy? Strong candidates must be ductal progenitors. In this area, three groundbreaking recent reports demonstrated, using a direct lineage tracing system that the ductal compartment indeed contains islet cell progenitors. The first report used the ductal marker carbonic anhydrase to follow the fate of duct cells during neonatal period, and after pancreatic injury induced by duct ligation (Inada et *al*., 2008). The second report concerns isolated ductal lining cells expressing a master switch gene *Ngn3*, involved in embryonic islet cell differentiation, in the ligated part of adult pancreas (Xu et *al*., 2008). And in the third report, the authors, using a lineage tracing system, showed that forced expression of Pax4 in glucagon-deficient mice, results in the continual conversion of glucagon-expressing alpha-cells into insulin-expressing beta-cells from Ngn3 precursors associated with the duct (Collombat et *al*., 2009). The authors showed that these ductal cells, given that they were able to differentiate into beta-cells, held progenitor properties. Even though, the progenitors identified by the authors might represent two different subsets of duct cells.

To investigate if similar events could take place during pregnancy, we compared the HPAP labelling indices of insulin-containing cells in contact or within the duct lining with visible lumen with the islets in the same sections. The labelling indices of both compartments were not significantly dissimilar, and led one to

conclude that the ductal component was not a "preferential source" for new beta-cell during pregnancy. An interesting question will be if these insulin-positive cells or progenitors produced in the duct compartment could be detached and migrate away from the ductal component to populate the islet compartment, as it has been already suggested by Xu et *al*. (Xu et *al*., 2008). This is an open question. Should insulin producing cells adjacent to the duct or part of the lumen lining have to be also considered as genuine beta-cells or the result of endocrine differentiation as reported by Bertelli and Bendayan (Bertelli and Bendayan, 2005). This is also another open question.

In addition Bertelli et *al*. (Bertelli et *al*., 2001) reported that, contrary to the commonly held view, there was an extensive connection between islets and the ductal tree. In the rat they showed that at least 73% of the islets were in contact with the duct, about 93% of those were associated with centroacinar cells or small sized duct. Unfortunately, the latter are easily overlooked by conventional histology staining, as mentioned by Bertelli et *al*. and Bouwens (Bertelli et *al*., 2001, Bouwens, 1998). This suggests that only a minority of insulin-positive cells can be detected using conventional histology staining, because they appear connected to medium and large sized-ducts with an observable lumen. Since it was not feasible to score insulin secreting cells in contact with duct without evident lumen and the counting of such cells was done at the end of pregnancy when the beta-cell mass expansion process had already occurred, our analysis must have underestimated the correct number of insulin secreting cells associated with the ductal tree. Therefore, we should qualify our previous statement and leave open the possibility that duct progenitors might be in a certain extent a source of new beta-cells during pregnancy.

Studies in rodents which aimed to isolate putative adult pancreatic precursors *in vitro* or to understand the mechanism by which newly formed beta-cell arise *in vivo*, during normal growth just after birth, beta-cell renewal and after pancreatic injury, compellingly suggest the existence of progenitors within islets which can differentiate into insulin-containing cells (Seaberg et *al*., 2004, Kodama et *al*., 2005, Guz et *al*., 2001, Li et *al*., 2004, Gu and Sarvetnick, 1993, Fernandes et *al*., 1997, Gu et *al*., 2002). Indeed, Kodama et *al*. (Kodama et *al*., 2005) reported the induction of *Ngn3* mRNA in islet-like cell clusters after streptozotocin-induced diabetes in mice. Likewise, Gu et *al*. (Gu et *al*., 2002) has provided evidence using a direct cell

lineage tracing system that the Ngn3 positive cells are islet progenitors in adult mice. In addition, Guz et *al*. (Guz et *al*., 2001) reported the occurrence of two presumptive intra-islet precursors in the regenerating islets following massive beta cell death induced by streptozotocin, one expressing the glucose transporter-2(Glut2) and the other co-expressing insulin and somatostatin. This result corroborates the conclusion of Pang et *al*. (Pang et *al*., 1994) postulating that beta-cells arise from Glut2-positive cells in the epithelium during rat development. Likewise Wang et *al*. (Wang et *al*., 2008) also reported an increased population of Glut2-positive, insulin-negative, cells during beta-cell regeneration, which might serve as beta-cell precursors in a transgenic mouse model called "PANIC-ATTAC". In this system beta-cells are ablated specifically and in an inducible fashion by apoptosis after activation of caspase 8 protein. The authors observed a novel population of Glut2 cells that might either represent a curiosity of this model or a bona fide beta-cell precursor, but more investigations have to be done to confirm its precursor status. However, this result is in line with another model of beta-cell regeneration, the pancreatic duct ligation system, in which an increased proliferation of these cells was also reported during the regenerative process (Wang et *al*., 1995). Another group reported in the Kir6.2G132S transgenic mouse model of spontaneously recovery from diabetes (Oyama et *al*., 2006), the occurrence of DBA-labelled cells expressing insulin in regenerated islets, which may represent potential ductal cell progenitors in this system. DBA (the lectin *Dolichos biflorus agglutinin*) is used for staining ductal cells. They also observed in the regenerated islets, at a lower degree, the incidence of PGP9.5 cells expressing insulin that might also contribute in part to beta-cell regeneration in this transgenic strain. PGP9.5 has been reported to be potential progenitor marker during beta-cell regeneration. In the light of these studies, we could also hypothesize that this potential intra-islet progenitor might also be activated during a first cycle of pregnancy and differentiate into insulin-producing cells to accommodate the new insulin demand, particularly, the Ngn3-positive progenitors described in adult mice during normal growth.

In conclusion, in the light of previous reports, we can postulate that non-beta-cell progenitors of ductal or/and intra-islet origin might be activated during a first cycle of pregnancy. Further experiments using positive cell-lineage tracing system for duct, exocrine or embryonic islet cell markers, such as carbonic anhydrase,

amylase, or Ngn3, respectively, should be employed to further explore our findings. Our data are compatible with the common view that beta-cell self-duplication plays an active role in the beta-cell mass expansion to adapt the new insulin load during a first cycle of pregnancy, but we also showed for the first time that this process occurred in combination with non-beta-cell progenitors.

7.1.2 Origin of new beta-cells during two rounds of pregnancy

Thirdly, to extend the results obtained in a first pregnancy, we wished to address the same question as to where new beta-cells arise during two pregnancies using the same cell-lineage tracing system and experimental design.

In response to two cycles of pregnancy, we observed a 60% and 43% increase of the mean body weight and pancreas weight compared to age-matched non-pregnant controls.

Similarly, we observed a doubling of the beta-cell mass induced by pregnancy to adapt the new insulin load, with little contribution of beta-cell hypertrophy.

The comparison of the ratio of the HPAP labelling index in pregnant controls over pregnant animals after one and two cycles of pregnancy showed that the two ratios were not dissimilar. This led us to reject our hypothesis that the HPAP labelling index was expected to be markedly lower in the pregnant animals during a second round of pregnancy compared to a first pregnancy.

The hypothesis assumed that the beta-cell apoptosis occurred at random during the parturition period.

The variability of the beta-cell marking at a similar rate in the three replicate pairs, precluded conclusion about both the formation of new whole islets, and whether duct progenitors are a preferential source for new beta-cells in a second pregnancy.

Nonetheless, we observed both an increase of the occurrence of insulin-positive cells in the ductal compartment, and increase of the number of ducts associated with these cells in a first and second pregnancy. These results suggest that duct progenitors could be activated to form de-novo beta-cells in pregnancy. To be conclusive, further analysis is required.

Lastly we did not observe any example of beta-cells that had transdifferentiated into other lineages, or fully lost differentiation, in pregnant and non-pregnant animals three and half months after the last pulse of tamoxifen. But we cannot rule out that this phenomenon could take place at a longer time point.

The rejection of our hypothesis because the ratio of the HPAP labelling index in pancreata of non-pregnant over pregnant animals in a second pregnancy was similar to that in a first pregnancy, is interesting per se.

To recapitulate, we posited that if during pregnancy non-beta-cell progenitors contributed substantially to the beta-cell mass increment, and if the beta-cell apoptosis process was indifferent with respect to labelled versus unlabelled cells following parturition, then during a second cycle of pregnancy, the proportion of beta-cell labelled for HPAP should be lower than in a first pregnancy.

We estimated that the ratio of the HPAP labelling index in pancreata of non-pregnant over pregnant animals in a second pregnancy should be 1.88, but 1.37 was the value observed. This led us to reject our hypothesis.

What assumption(s) was incorrect? Our first proposition to explain our results, would be that indiscriminate beta-cell apoptosis following parturition might be not true.

Actually, during the parturition period various events could take place. One could be a selective ablation of unlabelled beta-cells during the post-partum period. Furthermore, this ablated beta-cell population might be the progeny of the non-beta-cell progenitors.

This hypothesis will fit well with our results and is a very tantalizing explanation.

A second proposition would accept that beta-cell apoptosis process occurs at random, but the mechanism by which new beta-cells arise during a second pregnancy is different from that in a first pregnancy.

In this situation, the model of duplication of pre-existing beta-cells as the only process for producing new beta-cells fits our results.

Additional experimentation needs to be done to assess these two models. To test these assumptions, one could evaluate the proportion of beta-cells labelled for a marker of DNA synthesis to reflect the duplication of beta-cells, during pregnancy and the post-partum period.

Thus, one could verify if the percentage for this marker is similar, or not, at both time points. Or one could verify at both time points if the HPAP labelling index stays stable or not.

To confirm our previous results regarding the involvement of non-beta-cell progenitors in the formation of new beta-cells, we have conjectured that multiple rounds (i.e. more than two) of pregnancy could be examined. Our data show that the outcomes of these types of experiments are more complex than expected, and suggest that during the parturition period other intricate events might also take place.

To date no report has addressed the question of beta-cell expansion during several rounds of pregnancy. This is the first time that this was done.

Additionally our results prompt investigations of events in the immediate post-partum period when physiology of the non-pregnant state is re-established.

Finally, pregnancy is a normal physiological condition in which origins of new beta-cells can be studied because the adaptation is natural, rapid, substantial, reversible and repeatable over several rounds of pregnancy in the same animal. Also, the finding that non-beta-cell progenitors are involved in pregnancy makes this condition an appealing one for studying beta-cell mass expansion and reversal, the cells and the pathways involved, and hence the mechanisms by which these cells are generated.

7.2 Origin of new beta-cells under patho-physiological condition

Pelengaris et *al*. (Pelengaris et *al*., 2002) developed a mouse model of pancreatic regeneration (pIns-c-MycER^{TAM}) in which beta-cells can be ablated by apoptosis following c-Myc activation induced by 4-OHT. This results in the development of a diabetic phenotype in the animals. Upon 4-OHT removal, the single transgenic pIns-c-MycER^{TAM} mice recover from diabetes due to substantial beta-cell regeneration (Cano et *al*., 2008, Pelengaris et *al.*, 2002).

To investigate the nature of the cells responsible of the formation of the new beta-cells after diabetic recovery in this regenerative system, we generated triple transgenic mice (Z/AP; RIP-CreER^{TAM}; pIns-c-MycER^{TAM}) in which the inducible cell-lineage tracing system for beta-cells was introduced in the mouse model of pancreatic regeneration.

In the triple transgenic mice bearing the tracing-regenerative system, we observed that upon 4-OHT exposure, beta-cells are both labelled for HPAP and ablated. Indeed, the mean beta-cell mass in the untreated and diabetic triple transgenic mice was 0.83±0.20 and 0.1±0.06mg respectively, indicating that 88% of beta-cells were depleted upon activation of c-Myc, leading to the development of a diabetic phenotype observed in animals.

Inactivation of c-Myc allowed the mice to recover from diabetes. In this respect the triple transgenic animals follow the example of the single transgenic pIns-c-MycER^{TAM} mice. Therefore, we investigated in the triple transgenic mice the origin of new beta-cell after diabetic recovery.

We found that the mean HPAP labelling index in the diabetic mice and mice after diabetic recovery was 0.047±0.028 and 0.06±0.026 respectively. This let us to contemplate the possibility that we should accept hypothesis 1, namely mitogenesis might be the full mechanism giving birth to new beta-cells after diabetic recovery, as reported by Nir et *al*. (Nir et *al*., 2007) after toxi-genetic beta-cell ablation induced by expression of the diphtheria toxin specifically in beta-cells.

Surprisingly, the difference of the mean beta-cell mass between the diabetic triple transgenic mice (0.1±0.06mg) and in the triple transgenic mice after diabetic recovery (0.1±0.03mg) was not dissimilar.

There was no evidence of beta-cell regeneration and so we could not conclude as to where new beta-cells come from in this model of pancreatic regeneration induced by c-Myc, after diabetic recovery.

However, we observed examples of post-mitotic paired cells labelled for HPAP in treated multiple transgenic mice which must be the progeny of pre-existing beta-cells.

Could we take this observation as a revealing attempt to produce new beta-cells by beta-cell self-duplication to replenish the missing beta-cells?

It is known that activation of c-Myc in the single transgenic (pIns-c-MycER^{TAM}) mice, induced beta-cell apoptosis following a short burst of beta-cell proliferation (Pelengaris et *al*., 2002). Consequently the post-mitotic paired cells labelled for HPAP observed in the triple transgenic animals after diabetic recovery, might also be the progeny of residual beta-cells that underwent mitosis during the short wave of proliferation activated by c-Myc.

Unfortunately, we could not let the animals recover for longer time points and wait to see significant beta-cell mass recovery, because they developed tumours in their pancreata when they got older. This was also reported by Pascal et *al*. (Pascal et *al*., 2008) in old single transgenic mice (pIns-c-MycER^{TAM}).

We also observed that the treated triple transgenic mice did recover from diabetes without significant beta-cell mass augmentation, and that is intriguing.

What is the mechanism for glucose homeostasis in the triple transgenic mice? One plausible explanation can be related to the effect of c-Myc on the expression of markers important for beta-cell functionality. In this context, Laybutt et *al*. (Laybutt et *al*., 2002), reported that over-expression of c-Myc in transgenic mice results in down regulation of insulin and glucose transporter 2 (Glut2) gene expression. Cano et *al*. (Cano et *al*., 2008) reported similar features in the residual beta-cells of the single transgenic (pIns-c-MycER^{TAM}) mice upon activation of c-Myc. Yet, the author

also observed that this phenotype was reversible upon c-Myc inactivation in beta-cells of the single transgenic (pIns-c-MycER^{TAM}) mice. Namely, sixty days after tamoxifen removal, when the mice recovered from diabetes and after significant beta-cell mass increment, beta-cell markers cited above, displayed normal expression.

So, it is reasonable to state that similar to what occurs in the single transgenic (pIns-cMycER^{TAM}) mice upon c-Myc activation, beta-cell gene markers might also be down regulated, in addition of the beta-cell loss, in the residual beta-cells of the triple diabetic transgenic mice following 4-OHT exposure.

At the time point examined, twenty eight days after inactivation of c-Myc, the triple transgenic mice recovered form diabetes in absence of a large scale beta-cell mass recovery. To explain the normalisation of the blood glucose level, it is sensible to postulate that similar to the single transgenic mice, beta-cell marker expression might also reverse to normal level in the residual beta-cells of the triple transgenic mice after diabetic recovery. This might in part, contribute to the glycaemia normalisation, in the triple transgenics, without excluding the possible contribution of limited beta-cell regeneration.

In the end, the result observed in the triple transgenic mice after diabetic recovery suggests that the mechanism of diabetic recovery might be complex in this system. The anatomical beta-cell mass recovery on its own cannot be considered as an exclusive determinant parameter in this process.

7.3 Beta-cell functionality after hyperglycaemia recovery in single transgenic (pINS-c-MycER^{TAM}) mice

To study beta-cell functionality after beta-cell regeneration and diabetic recovery in single transgenic (pIns-c-MycER^{TAM}) mice, we analysed the beta-cell responsiveness in islets isolated from the transgenic animals using calcium microfluorimetry.

Based on the results of the glucose tolerance test, we concluded that the transgenic pIns-c-MycER^{TAM} mice prior to c-Myc activation were glucose tolerant

indicating that their beta-cells are functional and that the age of the transgenic mice did not affect this property.

By contrast, upon 4-OHT treatment, pIns-c-MycER^{TAM} mice developed a diabetic phenotype and became glucose intolerant. Upon 4-OHT removal the animals recovered from diabetes and became glucose tolerant again, indicating that regenerated beta-cells might be functional after diabetic recovery.

Second, to formally demonstrate that regenerated beta-cells were functional after diabetic recovery, we tested their functionality using single-cell microfluorimetry.

We concluded that the islet beta-cells from control wild-type animals were completely functional. In stark contrast, the profound attenuation of the beta-cell responsiveness in pIns-c-MycER^{TAM} controls and mice after diabetic recovery, demonstrated that beta-cell functionality in this line is altered.

In addition, comparison of the profiles of intracellular Ca^{2+}concentration between islets from pIns-c-MycER^{TAM} controls and mice after diabetic recovery shows no evidence that beta-cell functionality after diabetic recovery (albeit abnormal) was different from that of untreated control pIns-c-MycER^{TAM} mice. The impaired beta-cell functionality that we report here, in the pIns-c-MycER^{TAM} line by calcium fluorimetry has also been observed, recently by Pascal et *al*. (Pascal et *al*., 2008).

This result is interesting because our transgenic mice display a normal fed blood glucose level and respond properly to a glucose challenge *in vivo*.

What could explain this discrepancy?

A first possibility would be that the islet beta-cells isolated from the pIns-c-MycER^{TAM} mice might be more susceptible to damage during the isolation procedure. This can be ruled out since the fura-2A/M basal line for the transgenic and the wild-type animals, was similar indicating that the cells were viable.

If in the transgenic line the mechanism of the beta-cell responsiveness is altered, then how do the mice exhibit a normal glucose tolerance test?

One can postulate that the impaired functionality displayed by the transgenic mice can be compensated by a group effect of all the beta-cells present in the animal pancreas. Thus, if all beta-cells in the pancreas of the transgenic animals secrete a small quantity of insulin in concert, this may be enough to allow the mice to maintain a normal glucose homeostasis. Put another way, there is over-capacity of beta-cell function, or redundancy, in wild-type animals.

Another possibility would be that the population of beta-cells in this line is heterogeneous meaning that functionality within the beta-cell population is not uniform.

In either case, the altered beta-cell functionality in this line may due to the slight leakiness of c-Myc expression in absence of 4-OHT or tamoxifen, resulting in beta-cell impairment. Or the altered function may be related to the introduction of the transgene which might have altered other genes in the mouse genome related to beta-cell functionality.

7.4 General conclusion

We examined the origin and the functionality of beta-cells after diabetic recovery in a toxic-genetic beta-cell ablation mouse model (pIns-c-MycER^{TAM}). We showed that the beta-cell functionality in this line was abnormal. Our initial aim was to determine the origin of new beta-cells after diabetic recovery in this model in which a cell lineage tracing system for beta-cells was introduced (the triple transgenic mice). However, there was no evidence of large scale beta-cell regeneration at the time point investigated in the triple transgenic mice despite their evident recovery from diabetes.

We examined the origin of new beta-cells during the beta-cell mass expansion occurring in pregnancy to accommodate the increase metabolic demand, using the cell-lineage tracing system for beta-cells. We showed that new beta-cells were the progeny of pre-existing beta-cells, but more importantly, for the first time, we also demonstrated that non-beta-cell progenitors contribute significantly to the formation of new beta-cells during pregnancy. We also found that during two

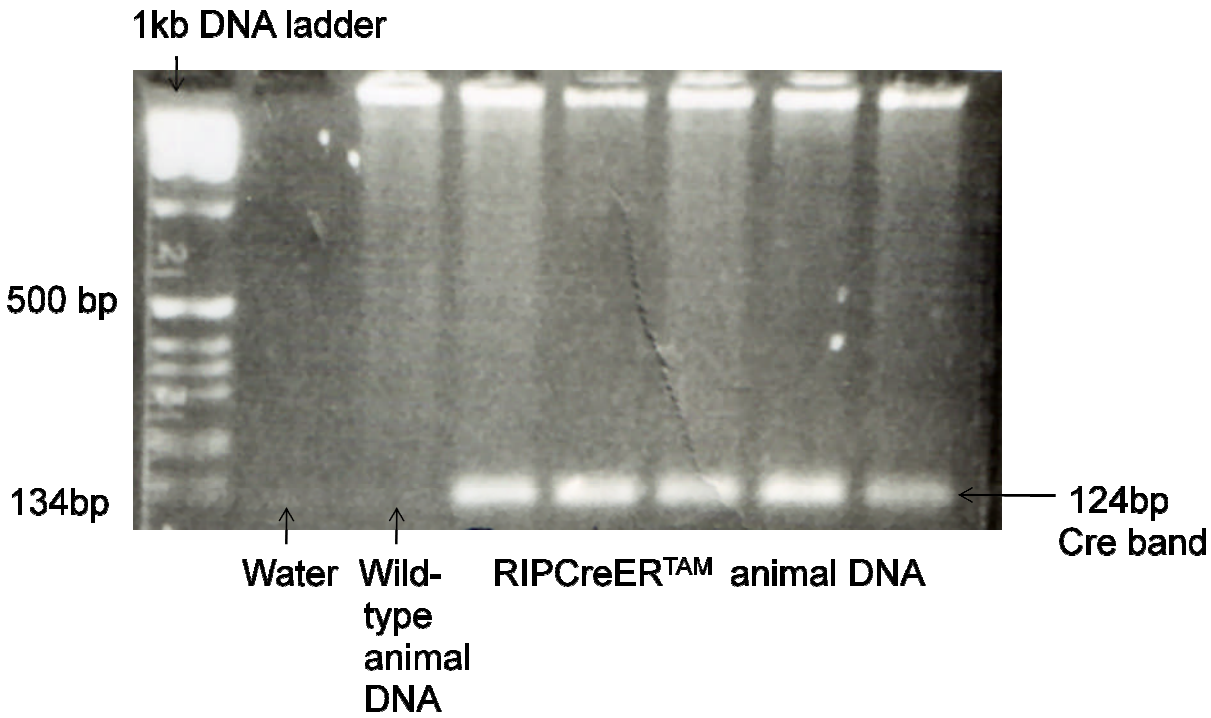
successive pregnancies, the beta-cell dynamics were unexpected. We conclude that either the mechanism for beta-cell loss following the first parturition is not random, and/or the subsequent mass increase in response to a second pregnancy occurs differently from a first pregnancy.

Identification of the non-beta-cell progenitors (of ductal, intra-islet or other origin) of beta-cells and the pathway giving birth to new beta-cells in pregnancy will be of great interest in our desire to better understand tissue homeostasis, and factors that command beta-cell regulation. Knowledge gained from these studies ultimately it may help design new strategies to generate unlimited sources for new beta-cells for cell-based therapy to treat diabetes or stimulate an endogenous beta-cell mass in diabetic patients.

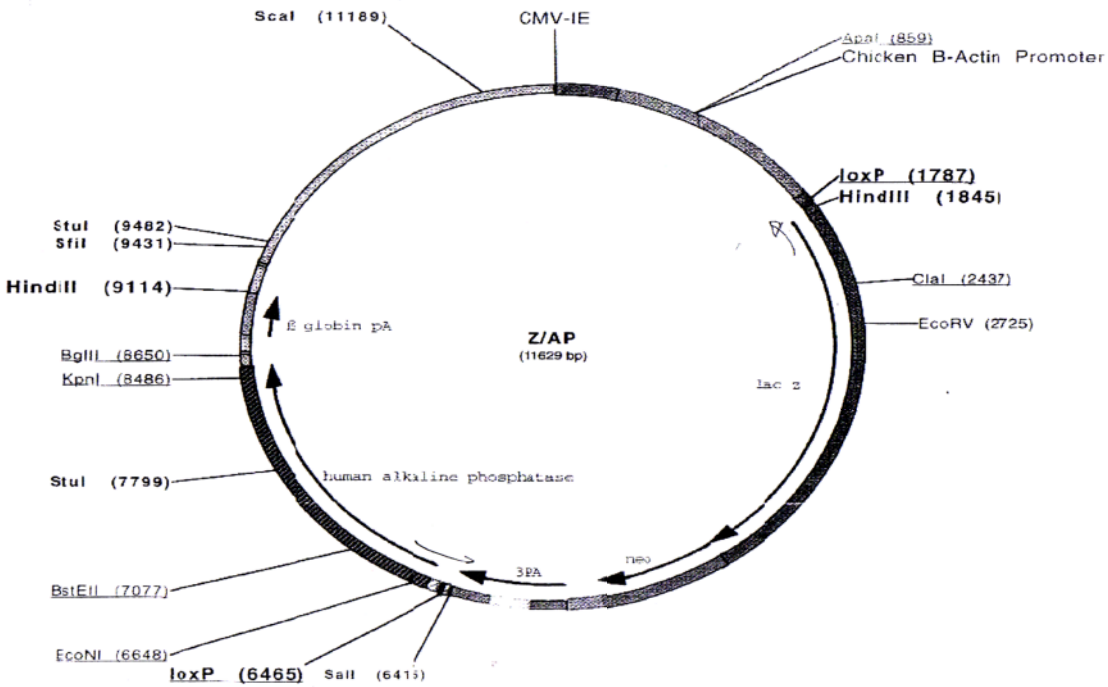
APPENDIX

APPENDIX 1

Gel electrophoresis of PCR products for genotyping Z/AP; RIP-CreER^{TAM} mice for the identification of the Cre-recombinase transgene using the Cre primers.



APPENDIX 2


ScaI (11189)
CMV-IE
ApaI (859)
Chicken B-Actin Promoter
loxP (1787)
HindIII (1845)
ClaI (2437)
EcoRV (2725)
StuI (9482)
SfiI (9431)
HindIII (9114)
β globin pA
Z/AP
(11629 bp)
BglII (8650)
KpnI (8486)
lac z
StuI (7799)
human alkaline phosphatase
3PA
neo
BstEII (7077)
EcoNI (6648)
loxP (6465)
SalI (6415)

APPENDIX 2

CHAPTER 3

ORIGIN OF NEW BETA-CELLS IN THE DOUBLE TRANSGENIC (Z/AP; RIP-CreER^{TAM}) MICE IN ONE PREGNANCY

HPAP and Insulin area in pregnant and non-pregnant animals in five coded sections per animal

Pair 1

Pregnant mouse

3940-1	HPAP area	Insulin area
PQx=L51-3940-1	34285.33333	90296.33333
Kyx=L113-3940-1	50772.33333	125315.6667
SSx=L231-3940-1	69830.33333	208869.6667
VVx=L271-3940-1	68016.33333	234907.6667
LGx=L315-3940-1	59451	184937.3333

Non-pregnant mouse

3940-2	HPAP area	Insulin area
TSx=L36-3937-1	53625.33333	97525.16667
HJx=L81-3937-1	78306.33333	138059.6667
NBx=L141-3937-1	139420	286030
DJx=L221-3937-1	92839.66667	195960
JMx=L293-3937-1	128100.3333	272590.6667

Pair 2

Pregnant mouse

3900-1	HPAP area	Insulin area
AAX=L61-3900-1	30139.33333	96970.66667
RWx=L130-3900-1	44919.33333	132727.6667
KMx=L211-3900-1	26604.66667	163459.6667
MRx=L283=3900-1	81869.33333	346472.1667
FCx=L310-3900-1	30106	119787.3333

Non-pregnant mouse

3900-2	HPAP area	Insulin area
BZx=L23-3900-2	41391.66667	85278
NGx=L83-3900-2	88581	210243
ZPx=L180-3900-2	107820	240334.6667
Pox=L243-3900-2	119978.6667	381051.6667
WPx=L304-3900-2	30933	70040.66667

Pair 3

Pregnant mouse

3974-1	HPAP area	Insulin area
Dax=L51-3974-1	41492	121731.6667
DSx=L111-3974-1	170808.3333	364063.1667
XDx=L163-3974-1	95998.33333	325545
Ojx=L234-3974-1	123781	434645.3333
HRx=L334-3974-1	36672.66667	116785.6667

Non-pregnant mouse

3974-2	HPAP area	Insulin area
Ycx=L31-3974-2	118341.3333	274959.3333
BTx=L81-3974-2	88366.33333	209352.6667
CVx=L144-3974-2	48160.33333	131770.6667
Wax=L211-3974-2	64325	163922
WSx=L264-3974-2	37543	102509

Pair 4

Pregnant mouse		
3866-1	HPAP area	Insulin area
Abx/L221-3866-1	100834.3333	268309.3333
FXx=L54-3866-1	62334.66667	168298
RDx=L105-3866-1	141908	370420.6667
Aqx=L172-3866-1	300651.6667	712194.6667
CRx=L272-3866-1	54808.66667	140986.6667

Non-pregnant mouse		
3866-2	HPAP area	Insulin area
BHx=L111-3866-2	50829.33333	92658.33333
SWx=L165-3866-2	68449.66667	167785
Rox=L222-3866-2	108338.3333	229977
GGx=L312-3866-2	105145.3333	202245
Acx/L3-3866-2	17270.66667	79556

HPAP labelling index

HPAP labelling index in pregnant mice

REPLICATE 1	3866-1	0.40
REPLICATE 2	3940-1	0.33
REPLICATE 3	3900-1	0.25
REPLICATE 4	3974-1	0.34

HPAP labelling index in non-pregnant mice

REPLICATE 1	3866-2	0.48
REPLICATE 2	3940-2	0.50
REPLICATE 3	3900-2	0.39
REPLICATE 4	3974-2	0.40

Proportion of small islets and beta-cell clusters negative for HPAP in pregnant and non-pregnant animals

Proportion of small islets or beta cell clusters negative for HPAP

REPLICATE 1	3866-2	0.215152
REPLICATE 2	3940-2	0.154696
REPLICATE 3	3900-2	0.140187
REPLICATE 4	3974-2	0.261589

HPAP labelling index in islets over ductal compartment in pregnant animals

Pregnant mice	beta-cell number positive for HPAP in ducts	beta-cell number negative for HPAP in duct
L51-3974-1	0	3
L201-3974-1 Test 2	0	3
L234-3974-1	0	4
L95-3866-1 Test2	0	4
L105-3866-1	1	3
L172-3866-1	3	1
L191-3866-1 extra	1	3
L330-3866-1 Test 2	0	1
L214-3866-1 extra	0	6
L231-3940-1	1	2
L251-3940-1extra	3	2
l351-3940-1 extra	2	0
L361-3940-1	0	12
L113-3900-1 Test 2	0	1
L250-3900-1 test 2	1	0
sum	**12**	**45**

Pregnant mice	beta-cell number positive for HPAP in islets	beta-cell number positive or not for HPAP in islets
L51-3974-1	74	284
L201-3974-1 Test 2	90	359
L234-3974-1	264	1035
L95-3866-1 Test2	117	334
L105-3866-1	458	1310
L172-3866-1	197	605
L191-3866-1 extra	90	229
L330-3866-1 Test 2	102	249
L214-3866-1 extra	118	350
L231-3940-1	57	175
L251-3940-1extra	54	202
l351-3940-1 extra	219	721
L361-3940-1	183	548
L113-3900-1 Test 2	129	548
L250-3900-1 test 2	16	104
sum	**2168**	**7053**

Ratio of HPAP labelling index of non-pregnant animals over that of pregnant animals in same pair

Pair 1	1.21
Pair 2	1.51
Pair 3	1.58
Pair 3	1.18

Beta-cell mass in pregnant and non-pregnant animals

		Beta-cell mass (g)	
		Non-pregnant mice	pregnant mice
REPLICATE 1	3866	0.0005	0.00139
REPLICATE 2	3900	0.0004221	0.00052
REPLICATE 3	3940	0.00046	0.00059
REPLICATE 4	3974	0.000453	0.001

Body weight in pregnant and non-pregnant animals

		Body weight (g)	
		Non-pregnant mice	pregnant mice
REPLICATE 1	3866	20.8	34.2
REPLICATE 2	3900	21.6	33.4
REPLICATE 3	3940	23	32.7
REPLICATE 4	3974	25.8	32.1

Pancreas weight in pregnant and non-pregnant animals

		pancreas weight (g)	
		Non-pregnant mice	pregnant mice
REPLICATE 1	3866	0.2394	0.387
REPLICATE 2	3900	0.2076	0.3088
REPLICATE 3	3940	0.1958	0.3372
REPLICATE 4	3974	0.2629	0.3344

CHAPTER 4

ORIGIN OF NEW BETA-CELLS IN THE DOUBLE TRANSGENIC (Z/AP; RIP-CreER^{TAM}) MICE IN TWO PREGNANCIES

HPAP and Insulin area in pregnant and non-pregnant animals in five coded sections per animal

Pair 1				
Pregnant mouse	HPAP area	Insulin area	cell count for HPAP	cell count for insulin
de=314L4083-3132	15265	22448	123	228
hg=41L4083-3132	25734	53513	177	371
mx=171L4083-3132	85064	200348	653	1526
nb=94L4083-3132	47896	89660	372	767
yr=232L4083-3132	90106	171969	532	1080

Non-pregnant mouse	HPAP area	Insulin area	cell count for HPAP	cell count for insulin
fd=302L4083-3135	4061	5824	48	73
lf=181L4083-3135	86931	124130	664	977
ob=105L4083-3135	57378	97551	535	877
vc=241L4083-3135	34644	55309	275	451
vw=34L4083-3135	81476	116084	638	1022

Pair 2

Pregnant mouse	HPAP area	Insulin area	cell count for HPAP	cell count for insulin
ah=275L4124-3312	48361	290520	258	1656
as=25L4124-3312	28596	56602	184	402
gw=298L4124-3312	39177	178159	242	1115
hk=405L4124-3312	16427	78607	108	638
ty=104L4124-3312	84637	420310	472	2254

Non-pregnant mouse	HPAP area	Insulin area	cell count for HPAP	cell count for insulin
kl=25L4124-3414	9989	53550	70	408
pb=294L4124-3414	26012	105676	209	786
qw=204L4124-3414	53462	179136	378	1309
rf=394L4124-3414	15173	43787	100	232
rt=101L4124-3414	45960	146692	290	1008

Pair 3				
Pregnant mouse	HPAP area	Insulin area	cell count for HPAP	cell count for insulin
dg=181L4173-3434	70063	244820.6667	542	1810
bn=31L4173-3434	15339	37768.66667	124	322
hb=321L4173-3434	32725	112133	241	837
mu=401L4173-3434	12402	90465	104	721
wx=94L4173-3434	22758.3333	72602.66667	146	384

Non-pregnant mice	HPAP area	Insulin area	cell count for HPAP	cell count for insulin
br=314L4173-3621	54809.6667	129142.3333	353	984
ce=401L4173-3621	12342.6667	32973.33333	100	242
cv=81L4173-3621	37273.6667	107751.3333	277	833
kd=21L4173-3621	22062.6667	65754.33333	172	541
az=171L4173-3621	37744	107616.3333	268	680

HPAP labelling index

		PREGNANT ANIMALS	
		total pixel number of area positive for HPAP/ total pixel number of area positive for insulin	total beta-cell number positive for HPAP/ total beta-cell number positive for insulin
REPLICATE 1	4083	0.49	0.47
REPLICATE 2	4124	0.21	0.21
REPLICATE 3	4173	0.27	0.28

		NON-PREGNANT ANIMALS	
		total pixel number of area positive for HPAP/ total pixel number of area positive for insulin	total beta-cell number positive for HPAP/ total beta-cell number positive for insulin
REPLICATE 1	4083	0.66	0.64
REPLICATE 2	4124	0.28	0.28
REPLICATE 3	4173	0.37	0.36

Ratio of HPAP labelling index of non-pregnant animals over that of pregnant animals in same pair

HPAP labelling index in non-pregnant mouse / HPAP labelling index in pregnant mouse	
count	**area**
1.36	1.35
1.34	1.34
1.35	1.26

Beta-cell mass in pregnant and non-pregnant animals

		Pregnant mice beta cell mass (mg)		Non-pregnant mice beta cell mass (mg)
REPLICATE 1	4083	0.38		0.21
REPLICATE 2	4124	0.87		0.30
REPLICATE 3	4173	0.46		0.34

Proportion of small islets and beta-cell clusters negative for HPAP in pregnant and non-pregnant animals

		proportion of small islets or beta-cell clusters HPAP negative	
		Pregnant mice	Non-pregnant mice
REPLICATE 1	4083	0.16	0.07
REPLICATE 2	4124	0.47	0.26
REPLICATE 3	4173	0.35	0.24

Body weight in pregnant and non-pregnant animals

		Pregnant mouse body weight (g)	Non-pregnant mouse body weight (g)
REPLICATE 1	4083	28.7	18.4
REPLICATE 2	4124	34.8	22.5
REPLICATE 3	4173	38.2	22.7

Pancreas weight in pregnant and non-pregnant animals

		Pregnant mouse pancreas weight (g)	**Non-pregnant mouse pancreas weight (g)**
REPLICATE 1	4083	0.3035	0.2194
REPLICATE 2	4124	0.4254	0.3054
REPLICATE 3	4173	0.5124	0.3426

Beta-cell area in pregnant and in non-pregnant animals

		total pixel number of cross-sectional beta-cell area	
		Pregnant mice	Non-pregnant mice
REPLICATE 1	4083	135.4326116	117.3228
REPLICATE 2	4124	168.8701292	141.2879
REPLICATE 3	4173	136.9145803	135.1334

Insulin-positive cell number in ductal compartment and number of ducts containing insulin-positive-cells in one pregnancy

Insulin-positive cell number in ductal compartment		
One pregnancy	Pregnant mouse	Non-pregnant mouse
replicate 1	6	7
replicate 2	7	0
replicate 3	14	0
replicate4	0	4
Number of ducts associated with insulin-positive cells		
One pregnancy	Pregnant mouse	Non-pregnant mouse
replicate 1	3	5
replicate 2	5	0
replicate 3	6	0
replicate 4	0	1

Insulin-positive cell number in ductal compartment and number of ducts containing insulin-positive-cells in two pregnancies

Insulin-positive cell number in ductal compartment		
Two pregnancies	Pregnant mouse	Non-pregnant mouse
replicate 1	8	0
replicate 2	6	6
replicate 3	4	1
Number of ducts associated with insulin-positive cells		
Two pregnancies	Pregnant mouse	Non-pregnant mouse
replicate 1	3	0
replicate 2	3	2
replicate 3	2	1

Combined results of insulin-positive cell number in ductal compartment and number of ducts containing insulin-positive-cells in one and two pregnancies

Insulin-positive cell number in ductal compartment

Pregnant mice	Non-pregnant mice
6	7
7	0
14	0
0	4
8	0
6	6
4	1

Number of ducts associated with insulin-positive cells

Pregnant mice	Non-pregnant mice
3	5
5	0
6	0
0	1
3	0
3	2
2	1

Combined results of HPAP labelling indices in one and two pregnancies

COMBINED RESULTS OF HPAP LABELLING INDICES OF ONE AND TWO PREGNANCIES

Group	Pregnant	non-pregnant
two pregnancies		
Pair 1	0.49	0.66
Pair 2	0.21	0.28
Pair 3	0.27	0.37
one pregnancy		
Pair 1	0.40	0.48
Pair 2	0.33	0.50
Pair 3	0.25	0.39
Pair 4	0.34	0.40

Combined results of proportion of small islets and beta-cell clusters in one and two pregnancies

Proportion of small islets or beta-cell clusters negative for HPAP

One pregnancy

	Non-pregnant mice	Pregnant mice
PAIR 1	0.15	0.22
PAIR 2	0.14	0.15
PAIR 3	0.07	0.14
PAIR 4	0.08	0.26

Two pregnancies

	Non-pregnant mice	Pregnant mice
PAIR 1	0.07	0.16
PAIR 2	0.26	0.47
PAIR 3	0.24	0.35

CHAPTER 5

ORIGIN OF NEW BETA-CELLS AFTER DIABETIC RECOVERY IN TRIPLE TRANSGENIC MICE (Z/AP; RIP-CreER^{TAM}; pIns-c-MycER^{TAM}) MICE

HPAP labelling index and beta-cell mass results

Mice after Diabetic recovery							
Replicate	HPAP area	Insulin area	**HPAP area/Insulin area**	tissue area	Insulin area/tissue area	pancreas weight (g)	**beta-cell mass (g)**
3899-2	8967.6667	196960.6667	**0.04**	279807552	0.000703915	0.1901	**0.00013**
3662-7	16174.8	170826.4	**0.09**	428332480	0.000398817	0.276	**0.00011**
3662-8	13258.2	262798.6	**0.05**	274762368	0.000956458	0.2251	**0.00022**

Diabetic mice							
Replicate	HPAP area	Insulin area	**HPAP area/Insulin area**	tissue area	Insulin area/tissue area	Pancreas weight (g)	**beta-cell mass (g)**
3899-1	9451	114378.6667	**0.08**	320789760	0.000356553	0.1991	**7.09898E-05**
3662-2	7500	283237.4	**0.03**	374769536	0.000755764	0.2622	**0.00020**
3662-3	8602.8	257035.4	**0.03**	352900480	0.000728351	0.159	**0.00012**

Untreated control mice							
Replicate	HPAP area	Insulin area	**HPAP area/Insulin area**	tissue area	Insulin area/tissue area	pancreas weight (g)	**beta-cell mass (g)**
D02	2190.3333	1357328	**0.002**	238185536	0.005698616	0.1717	**0.0010**
D05	516	1274149.667	**0.0004**	144463296	0.008819885	0.0994	**0.00088**
3662-4	790	729482.6667	**0.001**	189581312	0.003847862	0.2361	**0.00091**

Fed blood glucose results

Mice after Diabetic recovery	**Fed glucose (mM) before 4OHT pulse**	**Fed glucose (mM) after last pulse of 4OHT**	**Fed glucose (mM) 4weeks after last pulse of 4OHT**
Mouse number 3899-2	5.1	17	11.8
Mouse number3662-7	4.9	22.2	12.2
Mouse number3662-8	3.5	25.6	7.5
Diabetic mice			
Mouse number3899-1	4.5	19.1	
Mouse number3662-2	5.7	30.7	
3662-3	3.2	14	
Untreated control mice			
Mouse numberD02	4.1		
Mouse number D05	3.3		
Mouse number 3662-4	8		

CHAPTER 6

BETA-CELL FUNCTIONALITY IN THE SINGLE TRANSGENIC (pIns-c-MycER^{TAM}) MICE AFTER DIABETIC RECOVERY

Fed blood glucose results

Fed blood glucose (mM)			
3 month old wild type mice treated with 4-OHT	3 month old pIns-cMycERTAM mice before treatment	3 month old pIns-cMycERTAM mice shortly after 4-OHT pulse	12 month old pIns-cMycERTAM mice 9 months after the last 4-OHT pulse
8.7	3.6	29.4	3.9
6.8	3.7	27.3	3.8
6.5	4.8	33.2	2.6
7.1	3.7		6.3
	4.5		
	3.9		
	3.8		

Intraperitoneal glucose tolerance test (IPGTT) results

IPGTT in 3 month old untreated wild-type mice

replicate	1	2	3	4
Time (min)	blood Glucose (mM)	blood Glucose (mM)	blood Glucose (mM)	blood Glucose (mM)
0	6.9	8.9	6.2	7.0
10	9.8	10.4	20.4	19.4
30	16.8	18	15.4	23.2
60	12.4	14.3	9.2	16.4
120	7.8	7.7	5.9	9.7

IPGTT in 3 month old untreated pIns-cMycERTAM mice

replicate	1	2	3
Time (min)	blood Glucose (mM)	blood Glucose (mM)	blood Glucose (mM)
0	2.8	4.5	4.2
10	6.5	6.1	11.0
30	16.9	16.9	13.5
60	13.6	19.4	9.4
120	5.1	8.7	5.4

IPGTT in 12 month old pIns-c-MycERTAM mice treated with peanut oil

replicate	1	2	3	4
Time (min)	blood Glucose (mM)	blood Glucose (mM)	blood Glucose (mM)	blood Glucose (mM)
0	3.4	2.4	2.6	1.5
10	13.8	10.4	11.3	7.7
30	17.7	11.5	16.8	10.2
60	13.8	6.9	8.0	5.3
120	5.7	2.6	2.8	3.2

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