Lille 2 University of health and law
Doctoral School "Biology and Health"
and
Aristotle University of Thessaloniki
Faculty of Medicine

This thesis is submitted as fulfillment of the requirements for the degree of Ph.D. in Medical Sciences

Speciality: 44 - 4403 – Cellular biology

Sofia GARGANI

Laboratory U859 «Biotherapy for Diabetes»

Adaptive changes of human islets to an obesogenic environment in the mouse

Thesis director: Julie KERR-CONTE
Laboratory director : Francois PATTOU

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Defense: 17 September 2013

Jury:

Pr Francois Pattou	**Chair**
Pr Katherine Maedler	**Reviewer**
Pr ZHANG Chang	**Reviewer**
Pr Amar Abderrahmani	**Examiner**
Pr Pantelis Zempekakis	**Examiner**
Pr Julie Kerr-Conte	**PhD supervisor**
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UNIVERSITE DU DROIT ET DE LA SANTE DE LILLE
et
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Ecole Doctorale Biologie Santé de Lille

Thèse présentée en vue de l'obtention du titre de Docteur en sciences de la vie et de la santé

Spécialité:44 - 4403 - Biologie Cellulaire

No : 3949

Sofia GARGANI

Adaptation des ilots humains à l'environnement de l'obésité

Thèse dirigée par le **Professeur Julie Kerr-Conte**

Date de soutenance : le 17 Septembre 2013

Jury :

Pr Francois Pattou	**President de Jury**
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ΠΑΝΕΠΙΣΤΗΜΙΟ ΔΙΚΑΙΟΥ ΚΑΙ ΥΓΕΙΑΣ ΤΗΣ ΛΙΛ
ΚΑΙ
ΑΡΙΣΤΟΤΕΛΕΙΟ ΠΑΝΕΠΙΣΤΗΜΙΟ ΘΕΣΣΣΑΛΟΝΙΚΗΣ

ΤΜΗΜΑ ΙΑΤΡΙΚΗΣ

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Σοφία ΓΑΡΓΑΝΗ

Προσαρμοστικές αλλαγές μεταμοσχευμένων ανθρώπινων παγκρεατικών νησιδίων σε περιβάλλον παχυσαρκίας

Επιβλέπουσα : Julie KERR-CONTE

Ημερομηνία υποστήριξης: 17 Σεπτεμβρίου 2013

Μέλη της επιτροπής :

Καθηγητής Francois Pattou	**Πρόεδρος επιτροπής**
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Καθηγητής John Yovos	**Συν-επιβλέπων**

Ἓν οἶδα ὅτι ***οὐδὲν οἶδα***

I know that I know nothing

Socrates

I dedicate this thesis in my family and my fiancé…

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CONTENTS

ABSTRACT

Introduction: Under normal healthy conditions, organisms maintain a dynamic endocrine cell mass throughout life. Pancreatic beta cell mass are able to maintain plasma glucose levels increasing insulin secretion in conditions as obesity. Beta cell inability to compensate in insulin demand provokes hyperglycemia and Type 2 Diabetes. Clinically, most obese individuals do not develop diabetes because islets compensate for insulin resistance. Direct evidence that human islet mass adapts longitudinally to obesity in vivo was lacking and, moreover, little information was available on the mechanisms and cell type(s) involved. Current evidence for increased beta cell mass in obese humans (vs lean) is based entirely on postmortem histology.

Aim: In this thesis, firstly, we explored the longitudinal adaptation of human islets to an obesogenic environment and showed direct evidence that non-diabetic human islets adapt both endocrine and beta cell mass, function and gene expression to obesity in vivo. Secondly, we investigated the mechanisms of human islet regeneration and we performed lineage tracing to determine which cell type alpha or beta give rise to the increase islet mass in obesity. Thirty, in this diet induced obesity model we developed, we looked at the differential gene expression with Affymetrix gene chips in a kinetic study on human islets which were laser capture microdissected at 6, 8 and 10 weeks on control or high fat diet.

Methods: Archived human pancreatic sections were immunostained for endocrine, beta, alpha, fat. In the obese/immunodeficient mouse model, non-diabetic Rag2–/– mice were transplanted under kidney capsule with human islets from human brain-deceased donors (non-diabetics donors and donors with overt metabolic dysfunction). Animals were fed for 12 weeks with a control or high-fat diet (HFD), and followed for weight, serum triacylglycerol, fasting blood glucose and human C-peptide. After the mice were killed, human grafts and the endogenous pancreas were analyzed for endocrine volume, distribution of beta and alpha cells, and mechanisms of regeneration.

Results: In the longitudinal study, concomitant with the increased weight gain, doubling of abdominal fat, increased serum triacylglycerol and reduced insulin sensitivity in 12 week HFD animals we reported that human islet grafts showed functional compensation, measured as a more than doubling of fasting human C-peptide in mouse serum, and histological adaptation of islet endocrine mass including increased beta cells.

Further analysis of the human grafts revealed proliferation and neogenesis as the responsible mechanisms for the doubling of the human endocrine mass.

Discussion: This novel model allows, for the first time, longitudinal studies of human islet adaptation to an obese murine environment and may be instrumental in deciphering pathways involved in human beta cell expansion, as well as in helping to identify factors predisposing human beta cells to undergo decompensation.

RESUME

Introduction: Dans les conditions normales, les organismes maintiennent une masse cellulaire endocrine stable tout au long de leur vie. En cas d'obésité, la masse de cellules b pancréatiques est capable de maintenir des taux de glucose plasmatique en augmentant la sécrétion en insuline. L'incapacité de ces cellules à fournir de l'insuline entraîne alors l'apparition d'une hyperglycémie et d'un diabète de type II. Cliniquement, la majorité des individus obèses ne développent pas de diabète car les îlots pallient à cette résistance à l'insuline. La preuve de l'adaptation de la masse d'îlots humains à l'obésité, in vivo, n'a pas été clairement décrite et, de plus, peu d'informations existent sur les mécanismes et les types cellulaires impliqués. Actuellement, la mise en évidence de l'augmentation de la masse des cellules b chez les humains obèses repose uniquement sur des études histologiques.

But : Au cours de cette thèse, nous avons étudié l'adaptation au cours du temps des îlots humains à un environnement obésogène. Nous avons montré ainsi que les îlots humains non diabétiques s'adaptent in vivo à l'obésité en modifiant la masse de cellules b, leur fonction et leur expression génique. En suite, on a cherché les mécanismes de régénération des ilots humaines (prolifération et néogèneses) et on a identifié le mécanisme de transdifférenciation des cellules alpha et beta en utilisant la méthode de lineage tracing. Finalement, on a déterminé la différence sur l'expression de gène des ilots humains greffé chez les souris sous régime control ou régime riche en graisse en utilisant les puces d'ARN (Illumina).

Methodes et Resultats: Au cours de l'étude longitudinale, des souris Rag2-/- non diabétiques ont été greffées sous la capsule rénale avec des îlots humains issus de donneurs en état de mort cérébrale (donneurs non diabétiques ou donneurs avec un dysfonctionnement métabolique déclaré). Les animaux ont été nourris pendant 2 semaines avec soit un régime contrôle soit un régime riche en graisse (high fat diet HFD). Un suivi du poids, du taux des triglycérides, de la glycémie et du C-peptide a été mis en place. Après sacrifice des souris, les greffons et les pancréas endogènes ont été analysés pour le volume endocrine, la distribution des cellules b et a et les mécanismes de régénération des cellules pancréatiques. Après 12 semaines sous régime gras, les souris montraient toutes les caractéristiques typiques de l'obésité, à savoir, une augmentation du poids, un doublement de la graisse abdominale, des triglycérides, de la glycémie et une sensibilité à l'insuline réduite. De plus, l'apparition sur ces animaux d'un

doublement rapide de la quantité de C-peptide humain dans le sérum murin nous indique la mise en place d'une compensation fonctionnelle. Une analyse histologique des greffons a permis de mettre en évidence une adaptation de la masse endocrine des îlots avec une augmentation des cellules b. D'autres analyses ont identifié la prolifération et la néogénèse comme les mécanismes responsables de ce doublement de la masse endocrine humaine.

Discussion: Ce nouveau modèle animal permet d'étudier, in vivo sur une longue période, l'adaptation des îlots humains à un environnement obésogène murin. Il peut être utilisé comme un outil dans le décryptage des voies de signalisation impliquées dans l'expansion des cellules b humaines et permettre également l'identification des facteurs prédisposant ces cellules à subir une décompensation.

Περίληψη

Εισαγωγή: Υπό φυσιολογικές συνθήκες, οι οργανισμοί διατηρούν μια δυναμική παγκρεατική ενδοκρινική μάζα. Τα β παγκρεατικά κύτταρα είναι υπεύθυνα για την διατήρηση των επιπέδων γλυκόζης στο αίμα αυξάνοντας την έκκριση της ινσουλίνης σε συνθήκες όπως στην παχυσαρκία. Ανικανότητα των β κυττάρων να απαντούν στη ζήτηση για ινσουλίνη προκαλεί υπεργλυκαιμία και Διαβήτη Τύπου 2. Στις περισσότερες, ωστόσο, κλινικές περιπτώσεις τα παχύσαρκα άτομα δεν αναπτύσσουν διαβήτη καθώς τα νησίδια του Langerhans απαντούν στην αντίσταση στην ινσουλίνη. Άμεσα δεδομένα για την προσαρμογή της ανθρώπινης παγκρεατικής μάζας μακροπρόθεσμα στην παχυσαρκία in vivo είναι ανεπαρκή και επίσης, πολύ λίγη είναι πληροφορία για τους μηχανισμούς που συμμετέχουν σε αυτή την προσαρμογή. Ισχύοντα δεδομένα για την αύξηση της μάζας των ανθρώπινων β κυττάρων στην παχυσαρκία προέρχονται από ιστολογικές μελέτες ατόμων που απεβίωσαν.

Υλικά και Μέθοδοι: Πρώτον, ιστολογικές τομές από ανθρώπινα παγκρέατα αναλύθηκαν για την ενδοκρινική μάζα, μάζα α και β κυττάρων και επίπεδα λίπους. Δεύτερον, ανθρώπινα παγκρεατικά νησίδια από μη διαβητικούς και διαβητικούς δότες οργάνων μεταμοσχεύτηκαν στην νεφρική κάψουλα ανοσοανεπαρκών ποντίκων Rag2–/– mice. Τα πειραματόζωα τρέφονταν για 12 εβδομάδες με τροφή χαμηλή ή υψηλή σε λιπαρά. Στην διάρκεια των 12 εβδομάδων, καταγράφηκε το βάρος των ποντικών, τα επίπεδα τριγλυκεριδίων, γλυκόζης και c πεπτιδίου. Μετά τις θυσίες των πειραματόζωων, το ενδογενές πάγκρεας και το μόσχευμα αναλύθηκαν για την ενδοκρινική μάζα, την αναλογία α και β κυττάρων και τους μηχανισμούς αναγέννησης.

Αποτελέσματα : Στην *in vivo* μελέτη, αύξηση του βάρους των ποντικών, διπλασιασμός των επιπέδων λίπους και τριγλυκεριδίων και αύξηση της αντίστασης σε ινσουλίνη την 12 εβδομάδα παρατηρήθηκε στα ποντίκια που τρέφονταν με τροφή υψηλής περιεκτικότητας σε λιπαρά. Στα ίδια πειραματόζωα, η μακροπρόθεσμη προσαρμογή των ανθρώπινων νησιδίων του Langerhans σε περιβάλλον παχυσαρκίας εξερευνήθηκε. Αρχικά, στοιχεία για την προσαρμογή, την λειτουργία και την έκφραση των γονιδίων της ενδοκρινής μάζας in vivo στην παχυσαρκία ανακαλύφθηκαν. Ανάλυση των ανθρώπινων μοσχευμάτων έδειξε λειτουργική προσαρμογή με αύξηση της έκκρισης του c-πεπτιδίου στο ορό του αίματος, διπλασιασμό της ανθρώπινης ενδοκρινικής μάζας και αύξηση της μάζας των β κυττάρων. Έπειτα, ερευνήθηκαν οι μηχανισμοί αναγέννησης της ενδοκρινικής μάζας και τεχνικές lineage tracing εφαρμόστηκαν για να προσδιορίσουν το

τύπο των κυττάρων (α ή β κύτταρα) που συμμετέχουν στην αύξηση της μάζα στην παχυσαρκία. Ανάλυση των μηχανισμών αναγέννησης, αποκάλυψε τον πολλαπλασιασμό, την νεογέννηση και την αποδιαφοροποίηση ως πιθανούς μηχανισμούς αύξησης της ενδοκρινικής μάζας. Τέλος, αλλαγές στην έκφραση των γονιδίων που συμμετέχουν στο κυτταρικό κύκλο και στο στρες του ενδοπλασματικού δικτύου με τη χρήση μικροσυστοιχιών προσδιορίστηκε για την $6^{η}$, $8^{η}$ και $10^{η}$ εβδομάδα παχυσαρκίας.

Συζήτηση: Το συγκεκριμένο πρωτότυπο μοντέλο, μας επιτρέπει για πρώτη φορά, την μακροπρόθεσμη μελέτη της προσαρμογής των ανθρώπινων παγκρεατικών νησιδίων σε περιβάλλον παχυσαρκίας *in vivo*. Μπορεί να χρησιμοποιηθεί για την διερεύνηση μονοπατιών που συμμετέχουν στην αύξηση της μάζας των ανθρώπινων β παγκρεατικών κυττάρων και στην ταυτοποίηση προδιαθεσικών παραγόντων για την δυσπροσαρμοστικότητα των παγκρεατικών νησιδίων του Langerhans.

RESUME DETAILLE

Le pancréas est un organe situé au niveau de l'abdomen, derrière l'estomac. Il est constitué de 3 parties distinctes : la tête, partie la plus large du pancréas qui s'insère au niveau du duodénum, le corps et la queue qui se prolonge jusqu'à la rate. Le pancréas est composé de 2 types de tissus : exocrine et endocrine. La partie exocrine renferme le système canalaire ou ductal et les cellules épithéliales acineuses. Ces dernières sécrètent des enzymes digestives (trypsine, chymotrypsine, lipase et amylase) qui se déversent dans le petit intestin via l'arbre canalaire. La partie endocrine est formée de 4 types cellulaires qui sont, historiquement, définis par l'hormone qu'ils expriment: 1) les cellules α (le glucagon), 2) les cellules β (l'insuline), 3) les cellules δ (la somatostatine) et 4) les cellules PP (le polypeptide pancréatique). Ces cellules sont regroupées sous forme de grappe et ne représentent que 1-2% de la masse pancréatique. Edouard Laguesse, professeur d'histologie à Lille, a été le premier à proposer d'appeler ces petites « grappes » pancréatiques 'ilots de Langerhans' d'après la description originelle de l'étudiant allemand Paul Langerhans en 1869. Récemment, un cinquième type cellulaire a été décrit : les cellules epsilon qui produisent la ghréline.

Il est généralement admis que l'organisation des cellules endocrines dans les ilots humains est différente de celle des ilots de rongeurs. Chez les rongeurs, les cellules β constituent le centre de l'ilot et les cellules non- β (cellules α, δ, PP) le manteau. Chez l'homme, une étude récente a décrit que les cellules des ilots de Langerhans sont agencées sous forme de plaque trilaminaire : une couche de cellules β intercalée entre deux couches de cellules α. Cette structure se présente sous forme d'un modèle replié permettant ainsi aux vaisseaux de se propager sur l'ensemble de ses cotés. Ils peuvent, également, s'amalgamer grâce à leurs contacts intracellulaires. Néanmoins, il n'y a aucun doute sur le fait que les ilots humains contiennent proportionnellement moins de cellules β et plus de cellules α que ceux des rongeurs.

Dans les conditions normales, les organismes maintiennent une masse de cellule endocrine dynamique tout au long de leur vie. Des études chez l'homme ont montré qu'une hémi-pancréatectomie provoque une intolérance au glucose peu de temps après la chirurgie, suggérant ainsi l'existence d'une relation entre le volume de cellules endocrines et les concentrations de glucose à jeun. D'anciennes études transversales réalisées chez l'homme et les primates ont montré une relation curviligne entre les deux. Les mécanismes de maintien du taux de cellules pancréatiques restent toujours inconnus

chez l'homme. Mais, des études sur le modèle animal ont mis en évidence trois mécanismes potentiels : (i) la réplication des cellules endocrines matures existantes, (ii) la différentiation (ou la néogenèse) des cellules ductales ou des cellules précurseurs pancréatiques acineuses et (iii) la mort programmée des cellules existantes. Récemment, des expériences induisant la suppression totale des cellules β (PDL avec alloxane et la toxine diphtérique) ont montré la transdifférentiation des cellules α en cellules β. Ceci apparait comme un nouveau mécanisme potentiel de la régénération des cellules β. La cellule endocrine présente une excellente plasticité à se régénérer. Le mode de régénération dépend du stimulus, qui peut être physiologique (grossesse, obésité), compensateur, ou après un dommage (ligature du canal, suppression des cellules β, pancréatectomie partielle).

Le glucose est la seule source d'énergie qui peut être utilisée par le cerveau et les globules rouges. Le glucose est stocké au niveau du foie sous forme de glycogène et ses taux circulants sont contrôlés par deux hormones pancréatiques : l'insuline (secrétée par les cellules β) et le glucagon (secrété par les cellules α). Banting et al ont dépeint pour la première fois des actions opposées de l'insuline et du glucagon. En effet, en réponse à des taux élevés de glucose, la pro-insuline est libérée par les cellules β. L'insuline stimule la capture du glucose et son stockage au niveau des tissus sous forme de glycogène (glycogenèse). Au contraire, des taux bas de glucose provoquent la sécrétion du glucagon par les cellules β et du PP par les cellules δ. Le glucagon permet la conversion du glycogène stocké dans le foie en glucose (glycogénolyse) et sa libération dans le sang. Au cours du jeûne ou d'un exercice intense, le glucose peut aussi être produit à partir des précurseurs des non-carbohydrates (pyruvate, acides aminés et glycérol), un processus appelé gluconéogenèse.

La perturbation du métabolisme glucidique peut causer un diabète, maladie endocrine, qui peut être divisée en 4 types.

Dans les conditions normales, la masse de cellules β est capable de maintenir des taux de glucose plasmatique en augmentant la sécrétion d'insuline. L'incapacité de la cellule à compenser la demande en insuline provoque l'hyperglycémie. La concentration en insuline requise pour une réponse semi-maximale est définie comme la 'sensibilité à l'insuline'. L'incapacité des cellules à utiliser l'insuline produite, entrainant la diminution de la sensibilité à l'insuline, est définie comme la 'résistance à l'insuline'. La relation entre la sensibilité à l'insuline et la libération d'insuline est non-linéaire. Pour atteindre une

condition physiologique, les changements de sensibilité à l'insuline (grossesse, prise de poids) doivent être accompagnés par des changements proportionnellement croissants à l'insuline libérée. La masse et la fonction des ilots jouent un rôle majeur au niveau de l'adaptation et du développement du diabète, causé par le manque d'acclimatation à ce changement. La fonction des ilots humains dans l'obésité est basée uniquement sur des études transversales d'histologie post-mortem.

L'activité sécrétrice des ilots pancréatiques est régulée par des hormones gastro-intestinales, des hormones tissulaires (adipocytes, ostéoblastes) et du système nerveux autonome (sympathique, parasympathique). Les principaux facteurs impliqués dans la régulation de la fonction des ilots ainsi que leurs taux plasmatiques dans l'obésité sont repris dans le tableau 2.

Objectif

L'objectif principal de cette étude a été d'explorer l'adaptation des ilots humains dans un environnement obésogène.

Les différentes étapes :

- Création d'un modèle de souris in vivo qui associe l'obésité et l'immunodéficience et qui soit compatible avec la transplantation d'ilots humains.
- Vérification de la capacité des ilots humains à s'adapter fonctionnellement à un environnement obésogène murin.
- Mise en évidence de la régénération in vivo des ilots humains (prolifération, néogenèse, transdifférentiation).
- Cinétique d'expression des gènes des ilots humains au cours de leur adaptation à l'obésité.

Matériels et Méthodes

Les pancréas ont été obtenus à partir de donneurs en état de mort cérébrale, en accord avec les règlements français et avec notre comité d'éthique institutionnel. Les ilots ont été isolés en utilisant une version modifiée de la méthode automatisée de Ricordi, comme déjà décrite. Au cours de ce projet de thèse, six pancréas de donneurs non-diabétiques et deux de donneurs possédant un dysfonctionnement du métabolisme déclaré (plus âgé, HbA1c élevé ou histoire de diabète) ont été utilisés. Des études chez les souris ont été réalisées selon l'accord du comité local d'expérimentation animale. 93 souris males C57BL6 RAG 2-/- immunodéficiences (A Bouloumié, INSERM U858 Toulouse France et Taconic USA RAGN12-M), âgées de 8 à 9 semaines ont été utilisées dans ce projet. Les animaux ont été nourris 4, 6, 8, 10 ou 12 semaines sous un régime contrôle ou HFD (Research Diets, New Brunswick, NJ – Ref D12450B). Toutes les souris ont été suivies au niveau du poids, du triacyglycerol sérique et du glucose sanguin à jeun à 6 heures.

Pour évaluer la prolifération, le Bromodeoxyuridine (BrdU) a été injecté 18 heures avant le sacrifice ou a été ajouté 7 jours avant dans l'eau potable. Le pancréas endogène de souris et les greffons humains ont été analysés.

Le lineage tracing est une technique qui permet l'identification de toute la progéniture d'une seule cellule. Elle a été utilisée pour déterminer la transdifférentiation des ilots greffés aux souris contrôles ou HFD.

La capture par microdissection laser, à partir de cryocoupes, a été réalisée pour isoler des ilots humains greffés chez les souris sous le régime contrôle ou HFD. Les souris sous régime contrôle ou HFD ont été sacrifiées à 6, 8 et 10 semaines (n=18). L'extraction d'ARN a été réalisée avec l'Arcturus Pico Pure RNA isolation kit (Applied Biosystems) et sa qualité a été déterminée par Bioanalyser et agilent 2100 expert_Eukaryote Total RNA Pico kit. L'expression des gènes a été estimée en utilisant le Affymetrix Human Gene 2.0 ST array (Affymetrix, Santa Clara, California) à l'institut Cochin (INSERM U1016, Paris).

Résultats

Manuscrit 1

Dans la première partie du mémoire, un nouveau modèle in vivo de souris obèse immunodéficiente a été mis au point. La compatibilité avec la transplantation des ilots humains a permis d'étudier l'adaptation des ilots et leur fonction dans un environnement obèsogène. L'analyse des pancréas endogènes a confirmé que ce modèle offre un environnement approprié à l'expansion des greffons humains et à l'amélioration de leur fonction. Les mécanismes de régénération des ilots humains (prolifération, néogenèse) ont été découverts grâce aux études de la voie par laquelle la masse des ilots est augmentée dans l'obésité. Dans la deuxième partie, des ilots humains dysfonctionnels, provenant de patients diabétiques de type 2, ont été greffés, mettant ainsi en évidence leur incapacité à s'adapter à un environnement obésogène.

Dans ce manuscrit, nous avons montré que :

1) Les souris immunodéficientes Rag2 ont développé des traits liés à l'obésité après un régime HFD de 2 à 4 semaines.
2) Les ilots humains greffés chez les souris ont montré une adaptation fonctionnelle à un environnement obésogène : augmentation de la sécrétion d'insuline et maintient de la normoglycémie (le 2ème mois).
3) Les ilots humains ont semblé perdre totalement leur fonction au 3ème mois de régime HFD.
4) Les ilots humains dysfonctionnels ne peuvent pas s'adapter à un environnement obésogène et les souris deviennent hyperglycémiques au début du deuxième mois.
5) L'analyse histologique a montré une augmentation progressive de la masse des cellules β sous régime HFD, causée par la régénération des ilots (prolifération et néogenèse).

Manuscrit 2

Très récemment, l'étude transversale de Saisho et Co a montré l'effet de l'obésité humaine sur la masse de cellules. Des échantillons d'autopsie d'individus non-diabétiques (53 minces et 61 obèses+surpoids) ont été utilisés pour quantifier la masse de cellules β pancréatiques. Il a été observé que : 1) chez les obèses, la masse de cellules β est 50% plus élevée à cause de l'augmentation du nombre de cellules β, aucune différence au niveau de la surface des cellule β n'a été trouvée, 2) l'accumulation de la graisse est plus élevée chez les obèses, 3) une corrélation positive entre la masse de cellules β et le BMI (r=0.5) a été mise en évidence, 4) le taux de prolifération reste inchangé et peu fréquent chez les obèses par rapport au groupe d'individus minces, et 5) l'apoptose est très rare (9 sur 236.711 cellules), aucune différence entre les 2 groupes. De plus, les sujets en surpoids ont été examinés dans le groupe des obèses pour déterminer le taux de réplication au début de l'obésité.

Les auteurs reconnaissent la nécessité d'études longitudinales pour comprendre comment la cellule beta s'adapte à un environnement obésogène. Les études transversales, comme souligné dans cet article, nous donnent peu d'information sur le mécanisme par lequel l'obésité conduit en même temps à une augmentation (50%) de la masse et du diamètre nucléaire moyen de la cellule beta ainsi qu'à l'expansion des cellules acineuses. Par conséquent, il apparait urgent de réaliser des études longitudinales chez l'homme afin de caractériser les mécanismes par lesquels l'obésité provoque des altérations de la masse et de la fonction des ilots. Mais, le manque d'un accès non-invasif direct à la glande pancréatique exclut l'étude de ce phénomène chez l'homme.

Notre lettre de commentaire s'est focalisée sur notre modèle publié de souris immunodéficiente-obèse transplantée avec des ilots humains, et son intérêt pour des études longitudinales futures.

Discussion

Ma recherche à Lille a débuté par la caractérisation de l'influence de l'obésité sur les ilots humains au niveau morphologique et fonctionnel (Master 2, 2009-2010). La question posée a été : est-ce que les ilots des personnes obèses sont les meilleurs ilots pour le traitement cellulaire du diabète ? Une étude rétrospective portant sur 283 donneurs de pancréas à l'université hospitalière de Lille a montré que le nombre total et la taille moyenne des ilots ainsi que le taux d'insuline intracellulaire moyen sont plus élevés chez les donneurs obèses que chez les donneurs normaux et en surpoids. L'analyse histologique de 37 donneurs a montré: une augmentation de la taille des ilots, du nombre total de cellules endocrines (cellules α, β, δ, pp) et de la graisse intra-pancréatique chez les donneurs obèses. De plus, les ilots des individus obèses possèdent plus de cellules □ et moins de cellules □ que les non-obèses. Par conséquent, au niveau morphologique, les ilots humains des obèses apparaissent sensiblement très différents de ceux des personnes normales et en surpoids. Cela étant dit, l'obésité n'est pas un critère d'exclusion pour le prélèvement de pancréas à but de transplantation. Certains centres d'isolement préfèrent isoler des pancréas provenant de personnes avec un BMI élevé car le rendement est meilleur.

Le but principal de ce programme de recherche a été de concevoir des modèles in vivo pour étudier la fonction et l'adaptation des ilots humains à leur environnement. Mon projet s'est premièrement concentré sur le développement d'un modèle murin : la caractérisation de l'adaptation fonctionnelle et morphologique des ilots humains transplantés chez des souris immunodéficientes soumises à un régime conduisant à l'obésité (régime HFD). L'adaptation des ilots humains à un environnement obésogène murin a été confirmée par une augmentation significative du C-peptide humain chez les souris HFD par rapport aux souris contrôles. Au cours de cette thèse, une adaptation fonctionnelle des ilots humains à l'environnement obèse a été démontrée et les mécanismes de régénération des ilots ont été étudiés. Des ilots pancréatiques de Langerhans provenant de 8 donneurs d'organes (non-diabétiques et diabétiques) ont été greffés chez des souris immunodéficientes Rag2 sous la capsule rénale comme déjà décrit.

Toutes les lignées de souris ne développent pas la même sensibilité à induire une obésité sous régime HFD. Des études antérieures menées au laboratoire ont permis d'explorer l'alimentation HFD chez des souris nude mâles C57Bl6. Ces souris ont gagné moins de

2% de leur poids (<8% prise de poids) 8 semaines après un régime HFD. D'autres études ont confirmé que les souris RAG1ko et RAG2ko sont sensibles au régime HFD ou au régime HFD + régime de sucre élevé. Dans notre étude, nous avons décidé d'utiliser les souris C57BL/6 Rag2-/-, car l'alimentation sous régime HFD (60% des calories proviennent de la graisse) pendant 12 semaines a provoqué une prise de poids supérieure à 60%. Au cours du premier mois sous régime HFD, les souris peuvent développer plusieurs caractéristiques associées à l'obésité. Des taux élevés de triglycérides sériques et des quantités importantes de graisse abdominale chez nos souris HFD ont confirmé le phénotype obèse. Le même degré de prise de poids, sur une courte durée, ne peut pas être observé chez l'homme.

L'analyse des pancréas endogènes des souris contrôles a montré une diminution progressive de la surface des cellules endocrines contrairement aux souris sous régime HFD, pour lesquelles une augmentation progressive a été observée, (différence significative à la douzième semaine (versus Contrôle)). Cependant, lorsque comparé aux souris HFD non greffées, la surface des cellules β apparait plus petite chez les souris HFD greffés. Ce résultat suggère un effet des ilots greffés sur le pancréas endogène des souris contrôles et HFD. De plus, un nombre plus faible d'ilots a été trouvé chez les souris contrôles à la douzième semaine. La quantification morphométrique a permis de mettre en évidence une augmentation progressive de la taille des ilots chez les souris HFD par rapport aux contrôles. Ajouté à ce résultat, lorsque la glycémie a été testée, la glycémie à jeun a été trouvée légèrement plus élevée après 6 semaines et significativement plus élevée après 10 et 12 semaines chez les souris HFD. Néanmoins, les taux de glucose sanguin n'ont jamais excédé 10mM aux semaines 6, 8, et 10 comme régulièrement observés chez les souris HFD non greffées de type C57BL/6 Rag2-/- ou chez les souris mâles C57BI6 WT. La prolifération des cellules β du pancréas endogène a été trouvée plus élevée chez les souris HFD à 6 et 12 semaines du, peut-être, à l'augmentation du glucose. En conséquence, une faible augmentation des quantités de glucose pourrait être à l'origine de la réplication des cellules β.

Le taux de C-peptide humain sécrété a été mesuré chez les souris greffées à jeun afin d'étudier la fonction des greffons humains. Celui-ci a progressivement augmenté chez les souris HFD par rapport aux souris contrôles. Au cours des premières semaines, les taux de C-peptide humain semblaient être indépendants du type de régime. Mais, au cours du temps, il a été affecté par la durée du régime et la prise de poids des souris. Très récemment, une étude a montré l'effet de la prise de poids sur la sécrétion d'insuline chez

les souris C57BL/6 sous régime HFD, les répondeurs-haut (prise de poids importante) ont 3 fois plus d'insuline que les répondeurs-bas (prise de poids faible). D'un autre côté, les taux élevés de C-peptide humain dans notre modèle d'obésité pourraient être expliqués par l'hyperglycémie. En effet, un jour après l'infusion de glucose aux souris, des études antérieures ont démontré une augmentation de 3 fois de la sécrétion d'insuline des ilots humains transplantés. Est-ce que les ilots des souris HFD sont des « supers ilots » ? Pour répondre à cette question, le modèle d'évaluation de l'homéostasie (HOMA) a été utilisé pour quantifier la résistance à l'insuline (HOMA%IR), la fonction des cellules β (HOMA%B) des greffons humains et le HOMA2%BS qui tient compte de la fonction beta à une sensibilité ou à une résistance à l'insuline donnée. Les HOMA B, IR et S sont calculés par rapport aux taux de base de l'insuline humaine, du C-peptide et de la glycémie à jeun. A cause de la prise de poids, une chute vertigineuse de la sensibilité à l'insuline (HOMA%S) a été observée (jusqu'à 70% tout au long des semaines) chez les souris HFD par rapport aux contrôles, comme décrit dans le manuscrit 1 (analyse longitudinale ; modèle mixte). Ce phénomène a été accompagné par une augmentation significative de la fonction β déterminée par le HOMA2%B, qui a été amélioré chez l'ensemble des souris HFD et contrôles. Cependant, à 12 semaines, la fonction totale (HOMA2%BS) des greffons humains chez les souris HFD a progressivement diminué par rapport aux contrôles. En particulier, le HOMA2%BS était 4-fois plus bas chez les souris HFD que chez les souris contrôles. Ce résultat suggère que les ilots humains ont vu leur fonction diminuée après 12 semaines de régime HFD. Dans cette étude, l'utilisation du HOMA pour le C-peptide est intéressante et spécifique, mais il peut faire l'objet d'un débat puisque la glycémie à jeun dans ce modèle peut être régulée à la fois par le greffon et le pancréas endogène encore en place.

Est-ce que notre modèle d'obésité provoqué par un régime HFD est capable de reproduire l'altération morphologique de la composition des cellules α et β comme observée au cours de mon Master dans les sections pancréatiques des obèses versus minces (8% de cellules α en moins chez les obèses versus les minces, exprimé par rapport au nombre total de cellules α+β). De ce fait, observerait-on ce phénomène si on greffait des ilots humains provenant d'un donneur mince chez des souris contrôles ou HFD ? Le ratio du volume α/α+β du greffon a été calculé pour pouvoir comparer la même valeur. Il a été observé que les ilots greffés aux souris HFD avaient 6% de cellules α en moins (18.08±1.49 chez les contrôles versus 12.45±1.69% chez les souris HFD). Si nous voulions aller encore plus loin, la question serait : si nous implantons des ilots provenant

de donneurs obèses, observerions nous ces altérations chez les souris contrôles ? Ce processus est-il réversible ? Pour le moment, nous n'avons pas la réponse à cette question.

Au cours d'une étude de cinétique, l'analyse du volume de cellules endocrines humaines a révélé une corrélation entre le volume de cellules α et β avec le glucose sanguin et la sécrétion de C-peptide humain. Des taux semblables de glucose sanguin et de C-peptide ont été mesurés chez les souris contrôles tout au long de l'étude. En parallèle, aucun changement important n'a été observé au niveau du volume endocrine et de cellules β. Une faible diminution de la sécrétion du C-peptide humain et du volume de cellules α a été trouvée au niveau des greffons contrôles à 12 semaines. Un résultat qui pourrait être expliqué par une intra-régulation des greffons humains. Par contre, des modifications du volume de cellules α et β ont été observées chez les souris HFD durant les 12 semaines. Pendant la période normo glycémique (4 semaines), le volume de cellules β a été augmenté en réponse aux besoins en insuline pour contrecarrer l'augmentation de la résistance à l'insuline au niveau des tissus périphériques, car les souris avaient déjà pris 28% de poids. Le volume de cellules α a été diminué (4 semaines). Rahier et co ont montré que le ration de cellules α: β ne diminuait pas avec l'augmentation du BMI chez l'homme. Ce résultat confirme notre observation sur 45 pancréas humains ($r=0.14$, $p=0,12$). Au cours d'une courte hyperglycémie (6 semaines), le volume de cellulеς β est faiblement diminué et le volume de cellules α est élevé (aucune différence comparé aux souris contrôles). Au cours d'une longue période d'hyperglycémie (>10 semaines), le volume de cellule β est augmenté et le volume de cellule □ apparait être plus élevé seulement à 12 semaines.

Dans nos 3 premières séries de préparations d'ilots humains, le BrdU a été ajouté 1 jour avant le sacrifice. Dans ces séries, une prolifération endocrine élevée avait tendance à apparaître, mais nous étions incapables d'identifier les cellules β en prolifération. Par conséquent, dans toutes les études suivantes, nous avons mis le BrdU dans l'eau potable 7 jours avant le sacrifice. L'étude cinétique nous a permis d'examiner la prolifération des cellules endocrines humaines, de manière longitudinale, chez les souris sous un régime contrôle ou HFD pendant 12 semaines. Durant la période normoglycémique (4 semaines), une prolifération élevée des cellules β a été observée chez les greffons HFD en comparaison aux greffons contrôles. Ce résultat est du à la demande en insuline causée par l'obésité (déterminé par l'analyse de la quantité de BrdU mis dans l'eau potable 7 jours avant le sacrifice). Des études transversales sur des pancréas humains

provenant de donneurs normoglycémiques ont mis en évidence des taux de prolifération de cellules β très bas. Cette prolifération a été légèrement augmentée chez les obèses. Aucun changement au niveau de la prolifération des cellules β chez les greffons HFD n'a été observé en comparaison aux contrôles.

Un résultat qui peut être partiellement expliqué par la diminution du volume de cellules α à ce moment là. Un pic à 6 semaines de la prolifération des cellules β humaines a été observé, tandis que l'hyperglycémie moyenne n'a pas entrainé de changements au niveau de la prolifération des cellules α. De manière inattendue, à la fin de cette période, lorsque les taux de glucose sanguin ont commencé à augmenter (8 semaines), une diminution de la prolifération de l'ensemble des cellules α et β est apparue. Finalement, la prolifération des cellules α et β s'est reproduite lorsque l'hyperglycémie a été établie. Une étude des ilots humains a montré que l'infusion de haut glucose a augmenté de 2-fois la réplication des cellules β chez les ilots greffés aux souris nudes. En particulier, la réplication croissante des cellules α a été attribuée à un taux élevé d'IL-6, une cytokine associée à la résistance à l'insuline dans l'obésité. Une rare réplication des cellules δ a été observée, mais de manière intéressante toutes les cellules ont été trouvées au niveau du greffon chez les souris HFD.

Le double marquage CK-19/chromogranine A, insuline ou glucagon a été effectué pour mettre en évidence la néogenèse chez les greffons humains. La néogenèse déterminée à 12 semaines chez 3 premiers donneurs avait des niveaux plutôt bas. L'étude cinétique a montré des taux de néogenèse plus élevé à 8 semaines comme décrit dans le manuscrit 1. Ce résultat doit être confirmé par des expériences ultérieures. A ce moment, l'hyperglycémie a commencé à augmenter et la prolifération des cellules endocrines à diminuer. Une analyse complémentaire a montré que 80% des cellules CK19+/ChromoA+ étaient des cellules α. Ce résultat confirme ceux d'autres études qui proposent que la néogenèse des cellules α survient en premier.

Dans notre modèle, la technique du test TUNEL n'a pas mis en évidence d'apoptose au niveau des cellules endocrines dans les greffons humains chez les souris contrôles et HFD comme attendu. Levitt et Co ont montré que la réactivité TUNEL des cellules β n'était pas mesurablement augmentée chez les greffons humains exposés au glucose sanguin élevé. De plus, dans d'autres études, le régime HFD et la prise de poids des souris n'avaient pas modifié le pourcentage de cellules caspase 3 positives dans le pancréas endogène des souris.

L'étude du lineage tracing a été réalisée pour expliquer l'altération du volume des cellules α et β, car aucune différence au niveau de l'apoptose n'avait été détectée chez les greffons HFD. Tout d'abord, une conversion des cellules β en cellules α a été observée. L'analyse des cellules GFP+ avant la transplantation des ilots a confirmé la spécificité du vecteur Rip-CRE comme décrit dans d'autres études utilisant les mêmes vecteurs (Pr Philippe Ravassard, BCBC/UMR-7225). Après le marquage des cellules β des ilots avec les vecteurs RIP-CRE et CMV-LOXP, l'analyse des cellules GFP-Ins+ et GFP+Gcg+ 12 semaines après la transplantation a montré que 24.71% et 35.62% de cellules GFP+ (ils expriment le promoteur à l'insuline) étaient des cellules α (gcg+) chez les souris contrôles et HFD1 respectivement. Cependant, le nombre de souris était trop petit, par exemple seulement 13.04% de conversion a été trouvé chez la souris HFD2. Ce résultat, pour la souris HFD2, a montré une très faible expression du C-peptide humain (~20pmol/l à 8 et 10 semaines sous HFD). Très récemment, la transition des cellules α matures en cellules β a été décrite dans les ilots humains greffés chez des souris NOD/SCID à 2 semaines. Des changements au niveau de l'expression des hormones endocrines ont été expliqués par la conversion de 10% des cellules β et non à cause de l'apoptose ou de la prolifération. Par ailleurs, la conversion des cellules α en cellules β a été testée en utilisant le vecteur Glu-CRE pour les ilots provenant d'un même donneur. L'analyse quantitative a montré que 41.53% des cellules GFP+ dans le contrôle 1 et 42.4% chez les souris HFD étaient des cellules β. De plus, 43.14% de conversion a été trouvé chez le contrôle 2. Une observation plus précise de ces souris a montré une sécrétion anormalement élevée du C-peptide, associé à une prise de poids de 15%. Dans des études antérieures, deux modèles ont été utilisés pour décrire la capacité de conversion des cellules α en cellules β ; l'expression transgénique de la toxine diphtérique chez la souris et le modèle de cellule β induit par alloxan plus PDL. Une approche plus consciencieuse de la transition des cellules humaines α en cellules β devra être réalisée.

Pour valider notre modèle, des ilots dysfonctionnels de deux donneurs diabétiques ont été greffés chez des souris sous régime contrôle et HFD (DD1 a été traité pendant 10 ans pour diabète de type 2). Aucune adaptation fonctionnelle associée à une sécrétion de C-peptide et à l'évaluation de HOMA2 n'a été observée dans ces greffes (données décrites dans le manuscrit 1). Le C-peptide humain à jeun a été moins élevé chez le DD2 au cours des 12 semaines par rapport aux donneurs contrôles. Il faut souligner que les ilots de DD1 sécrétaient moins de C-peptide que ceux de DD2. En plus, les taux de glucose sanguin ont augmenté au cours des 12 semaines chez ces souris (à partir de 2

semaines). Il a été observé que les souris greffées avec des ilots de DD1 avaient des taux de glucose sanguin plus élevés (>10mmol/l), mettant ainsi en évidence une mauvaise fonction et une mauvaise adaptation de ces ilots à l'obésité. Finalement, aucune adaptation au niveau histologique n'a été observée pour ces greffons. Le déficit de la masse de cellule β a été déjà décrit chez des patients de typeT2D. Il est connu que le vieillissement est corrélé à une diminution de la capacité de prolifération et à une masse de cellules β dysfonctionnelles chez les patients T2D. Le double marquage BrdU/Chromogranine A n'a pas détecté de prolifération des cellules endocrines comme attendu.

ABBREVIATION LIST

BMI	Body Mass Index
BrdU	Bromodeoxyuridine
cAMP	Cyclic adenosine Monophosphate
CAMs	Cell Qdhesion Molecules
CDKs	Cyclin-Dependent Kinases
CKK	Cholecystokinin
CMV	Cytomegalovirus
DAB	Diaminobenzidine
DNA	Deoxyribonucleic acid
EGF	Epidermal Growth Factor
Eph	Ephrin
ER	Estrogen Receptors
GABA	Gamma-Aminobutyric Acid
GFP	Green Fluorescent Protein
GIP	Glucose-dependent Insulinotropic Polypeptide
GLP-1	Glucagon-like Peptide-1
GPR30	G Protein-Coupled Receptor 30
HbA1c	Hemoglobin A1c
HFD	High Fat Diet
HOMA	Homeostatic model assessment (HOMA)
IEQ	Islet Equivalents
IGT	Impaired Glucose Tolerance
IL-1β	Interleukin-1β
IL-6	Interleukin 6
IPGTT	Intraperitoneal Glucose Tolerance Test
IST	Insulin Suppression Test
IVGTT	Intravenous Glucose Tolerance Test
LADA	Latest Autoimmune Diabetes in Adults
LIF	Leukaemia Inhibitory Factor
MODY	Maturity-Onset Diabetes of the Young
MRI	Magnetic Resonance Imaging
NCAM	Neural Cell Adhesion Molecule

NF-kB	Nuclear Factor kappa-light-chain-enhancer of activated B c
OGTT	Oral Glucose Tolerance Test
PCD	Programmed Cell Death
PDL	Pancreatic Duct Digation
PTH	Parathyroid Hormone
PYY	Peptide YY
RIN	RNA Integrity Number
RNA	Ribonucleic acid
STZ	Streptozotocin
T1/2D	Type ½ Diabetes
T1D	Type 1 Diabetes
T2D	Type 2 Diabetes
TNF-a	Tumor Necrosis Factor-a
WHO	World Health Organization
Wnt	Drosophila melanogaster wingless
YFP	Yellow Fluorescent Protein

FIGURES LIST

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Chapter I

Introduction

1. Structure of human and mouse pancreas

The pancreas, an elongated organ located across the back of the abdomen, behind the stomach, is constituted of three distinct parts: the head, the widest part which lies in the curve of the duodenum, the body, and the tail that is located near the spleen (Fig 1a). The pancreas is composed of exocrine and endocrine tissue. The exocrine part contains the ductal system and the epithelial acinar cells which secrete digestive enzymes (trypsin, chymotrypsin, lipase and amylase) into the small intestine (Fig 1b, c) via the ductal tree. Edouard Laguesse a histology professor in Lille was the first to suggest that the small clusters in the pancreas should be called “islets of Langerhans” based on the original description by the German medical student Paul Langerhans in 1869; they comprise only 1-2% of the pancreatic mass. Islets are comprised of four endocrine cell types that have historically been defined by their hormone expression: 1) **α cells** secrete glucagon, 2) **β cells** secrete insulin, 3) **δ cells** secrete somatostatin and 4) **PP cells** secrete pancreatic polypeptide (Fig 1d). A few years ago, a fifth islet cell type, grhelin-producing epsilon cells, was described [1, 2].

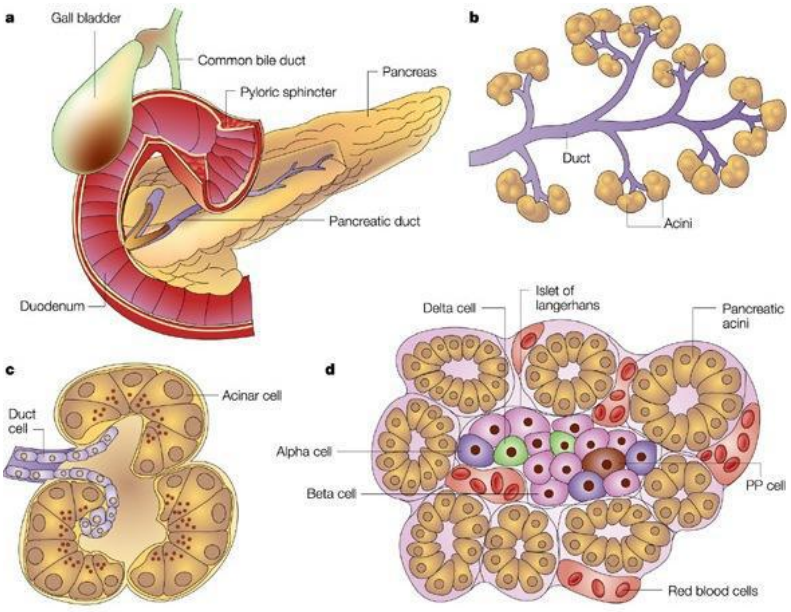


Fig. 1 Pancreas structure.a) Anatomy of the human pancreas b) The exocrine pancreas c) A single acinus d) An islet of Langerhans embedded in exocrine tissue *(Bardeesy, 2002)*

It is generally admitted that the endocrine cell organization in human islets is different from that of rodent islets, where β-cells compose the core and non β cells (α, δ, PP-cells) the mantle of the islets. A recent study describes that human islet cells are arranged in a trilaminar plate comprised of one layer of beta cells sandwiched between two layers of alpha cells. This structure has a folded pattern and vessels circulate along both of its sides. Moreover, they can be considered intermingled because of their intracellular contacts [3]. Thus, there is no doubt that the

human islets contain proportionally fewer β-cells and more α-cells than the mouse islets [4].

2. Overview of transcription factors in pancreatic development

Most of our knowledge of pancreatic development is based on studies in mouse, chicken, zebrafish and *Xenopus* models. Knowledge about human pancreas development is more limited and some differences exist between the mouse and the human pancreas. The development of the three primary layers called gastrulation (ectoderm, endoderm, mesoderm) starts at embryonic day 6.5 (2wks in humans). Canonical Wnt pathway contributes to the differentiation of the mesendoderm cells [5, 6]. Cells within the endoderm (E7.5-9.5/3 wks) are specified for several transcription factors and give genesis to gut tube. FGF2 and activin are also crucial for the gut tube development via inactivation of Shh gene [7].

First evidence for pancreas formation appears at 9 embryonic days (4wks) after the development of two pancreatic buds, dorsal endoderm and ventral endoderm. A network of transcription factors interact at this stage such as Pdx1, Hb9, Sox9, Sox17, Cpa1. Pdx1 is activated by retinoic acid-mediated pathway and has an important role for the pancreas development and the beta cell identity later. At stage of E9, immature glucagon producing cells are observed when immature insulin producing cells can be detected later at E10.5 (end of 4wks) [8]. Endocrine lineage is specified by the expression of transcription factor of Neurogenin 3 (Ngn3). Ngn3 inactivation by inhibitory factor Hes1 (Notch-Delta pathway) leads to exocrine precursors development (E10.5-12.5/5 wks [9]. Then, transcription activity of Hnf6 gives genesis to duct cells or expression of Ptf1a, Rpbj leads to acinar cell differentiation. On the other hand, active ngn3 promotes the expression of Arx, Pax4, NeuroD1, Nkx2.2, Nkx6.1, which allows endocrine precursors to express one of the endocrine hormones. Arx and Pax4 were found to be specific for α-/PP cells and β-/δ cells differentiation respectively [10, 11]. Nk2.2 and Nkx6.1 appears only in beta cells, when Pax6 and Isl1 are responsible to delta cell formation [8]. Additionally, expression of MafA and MafB, is crucial for β and α cells maturation respectively [12].

Studies in rodents revealed that epsilon cells can be detected in the mouse pancreas as early as embryonic day 10.5 and that they are the major source of ghrelin during fetal life [13]. Recent study demonstrated that epsilon cells represent a multi-potent

progenitor cell population that delineates a major subgrouping of the islet endocrine cell populations [14]. Contrarily, there is little information on epsilon cells during fetal development of the human pancreas. A study of Andralojc *et al*, showed that during fetal development epsilon cells show an ontogenetic and morphogenetic pattern that is distinct from that of alpha and beta cells [15]. The transcriptions factors which characterize the different type of endocrine cells are given in Fig 2.

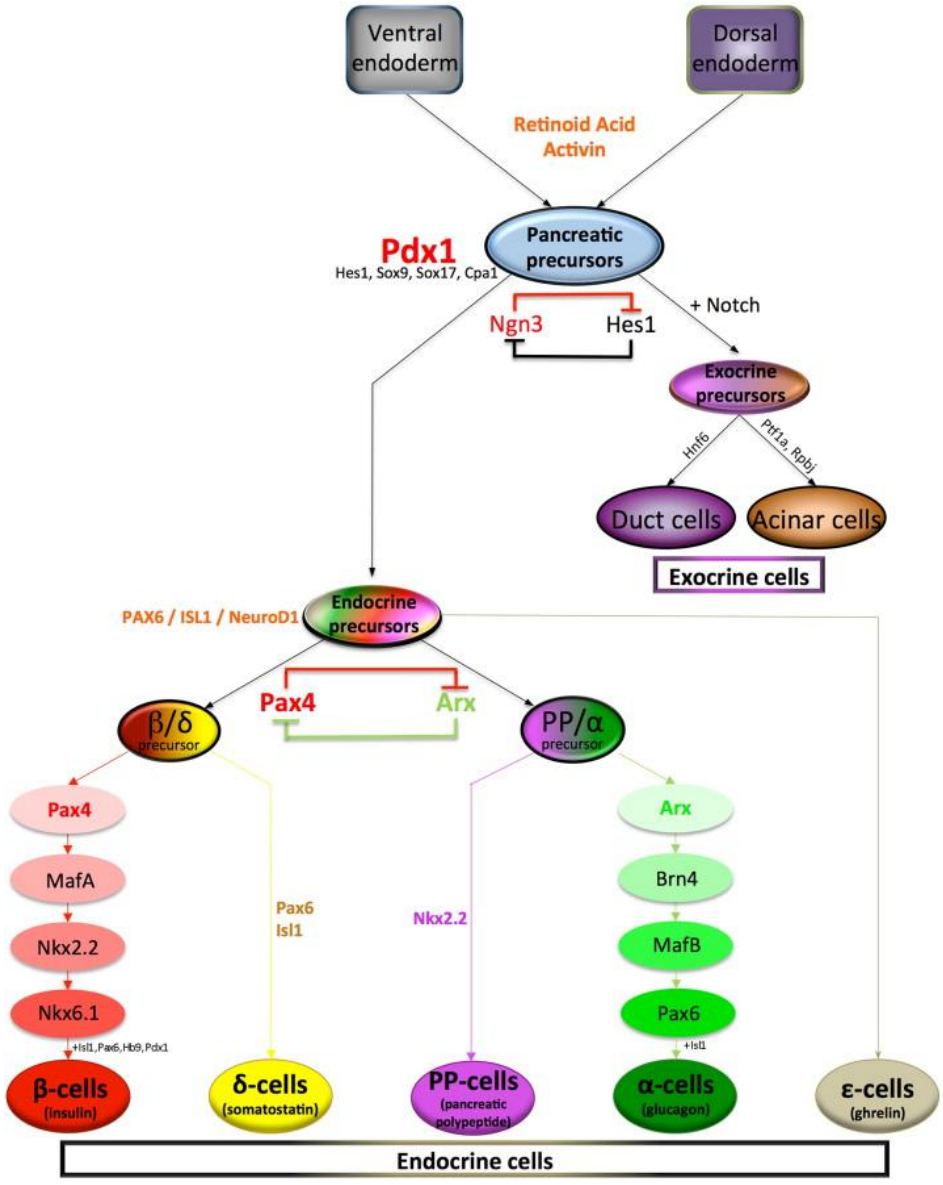


Fig 2: Transcriptions factors involved in endocrine cells development. Schematic overview of (Ben-Othman, 2013)

3. Mechanisms of pancreatic cell maintenance

Under normal healthy conditions, organisms maintain a dynamic endocrine cell mass throughout life. Studies in humans revealed that hemi-pancreatectomy causes glucose intolerance in short term after surgery proposing the relationship between endocrine cell volume and fasting blood glucose concentrations [16]. Further cross-sectional studies in humans and primates showed a curvilinear relationship between beta cell volume and fasting blood glucose concentrations [17, 18]. The mechanisms of pancreatic cell maintenance are still unknown in humans, but studies in animal model reveal three potential mechanisms: (i) replication of existing mature endocrine cells (ii) differentiation (or neogenesis) by ductal or acinar pancreatic precursor cells and (iii) programmed cell death of existing cells. Recently, experiments induced extreme β cell ablation (PDL with alloxan and diphtheria toxin) showed α to β cell transdifferentiation as novel potential mechanism of beta cell regeneration [19, 20]. The endocrine cell has great plasticity to regenerate [21, 22] (Fig 3). The mode of regeneration depends on the stimulus which can be physiological (pregnancy, obesity) or compensatory, following damage (duct ligation, beta cell ablation, partial pancreatectomy).

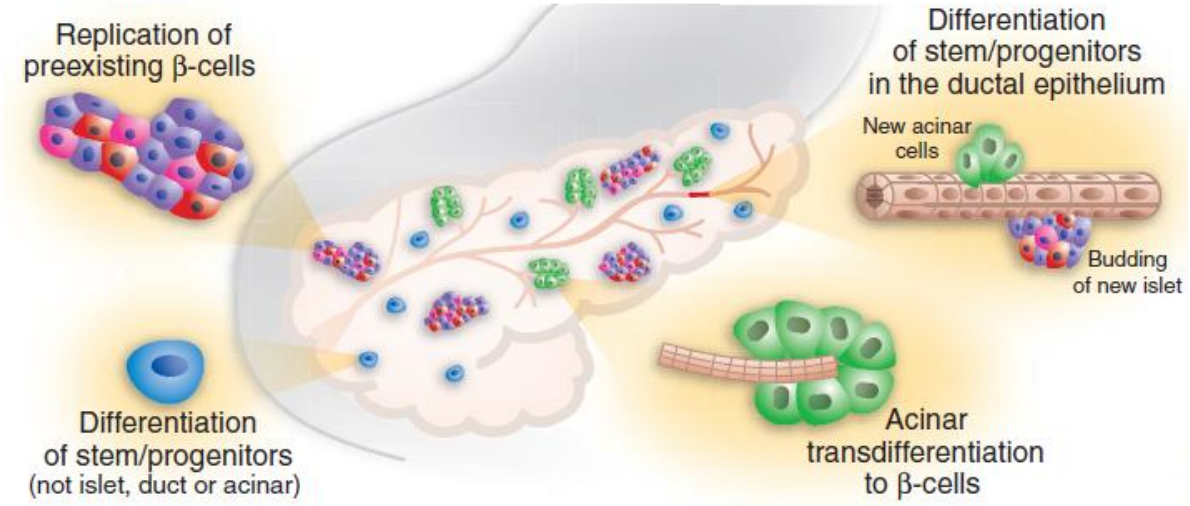


Fig 3: Approaches of insulin secreting cells regeneration. Replication of preexisting beta cells and (trans)differentiation of ductal or acinar precursor cells (Bonner-Weir S, 2005)

3.1 Pancreatic endocrine cell replication in vivo

The strongest evidence of *in vivo* beta cell proliferation was provided by animal studies. Dor *et al* demonstrated for the first time that preexisting beta cells were the major source of new beta cells during adult life in mice and following damage [23]. Studies in embryonic and neonatal rodent pancreases revealed the role of the cell cycle regulator, cyclin D2, in the massive proliferation of beta cells [24]. Rodent islet function and proliferation decreases with aging [25, 26]. Furthermore, endogenous beta cells replicates in response to insulin demand in physiological models of pregnancy and obesity [27-32]. Likewise, proliferation of surviving beta cell plays the major role in regeneration in rodents after duct ligation, partial pancreatectomy and extreme β cell ablation in transgenic mice by diphtheria toxin or after alloxan perfusion [33-40].

While significant progress has been made towards the understanding of b-cell regeneration in adults, very little is known about the regeneration of the non-β endocrine cells such as glucagon producing α-cells and somatostatin producing δ-cells. Studies in obese mice revealed the association between interleukin 6 (IL-6) and alpha cell proliferation [41]. Recently, it has been shown that alpha and delta cells can proliferate following STZ-induced beta cell destruction [42]. Previous studies showed endogenous alpha cell proliferation in PDL plus alloxan before their conversion in beta cells [19].

In humans, pancreatic beta cell mass may expand several fold from birth to adulthood, but human islet proliferation is very low in adults [43] as proliferation already decreases 3% six months after birth [44]. Human islet function and proliferation also decrease with aging [26, 45]. The cell cycle protein cyclin D1, D2 and D3 with the absence of p16 and p27 expression appears to promote beta cell replication in prenatal beta cells [46]. Very recently, the repertoire of G1/S regulatory proteins was delineated for the adult human beta pancreatic cells. The human beta cell G1/S atlas revealed that the only nuclear molecules are the cell cycle inhibitors pRb, p57, p21 and none of the cyclins or cdks are present in the nuclear compartment [47]. This may explain the refractoriness of human beta cell proliferation. Although the knowledge about cell cycle regulation is abundant, the mechanisms beyond human beta cell proliferation in physiological conditions are still unknown but are a subject of intense research in particular in vitro [48, 49].

The majority of studies on human islet proliferation and neogenesis in physiological conditions (pregnancy, obesity) are based on histological findings of autopsied pancreatic

tissue following accidental or pathology-induced death. Cross- sectional studies showed low levels of human beta cell replication with no change in pregnant individuals and no significant increase in obese subjects (0.06%) [50, 51]. Table 1 shows further details for the *in vivo* studies providing evidence for beta cell proliferation in animals and humans.

Table 1: Studies providing evidence of *in vivo* beta cell proliferation

Species	Condition	Observation	References
Rodent	Neonates	-B cell replication is the primary mechanism for maintaining postnatal b cell mass	Georgia,S. and BhushanA. *et al*[24]
	Adult	-Pre-existing beta-cells, are the major source of new beta-cells during adult life in mice -Decrease of beta cell proliferation rates in aged adult mice	Dor, Y. *et al*[23] Teta, M. *et al*[25]
	Pregnancy	-Lactogenic hormones, growth hormones and prolactine promote beta cell proliferation during pregnancy	Karnik, S.K. *et al*[27] Brelje, T.C. *et al*[28]
	Obese rodent model	-Long-term fat feeding is associated with an increase in the beta cell population but an inadequate functional adaptation. -Islet volume and beta cell proliferation increase in adult ob/ob mice -Beta cell proliferation increases with 4 days glucose infusion	Hull, R.L. *et al*[29] Davis, D.B. *et al*[30] Bock, T. *et al*[31] Alonso, L.C. *et al*[32]
	Partial Duct Ligation (PDL)	-10 fold higher proliferation (BrdU+Ins+) after PDL via Ngn3 gene activation	Xu, X. *et al*[34] Van de Casteele, M. *et al*[35]
	Partial Pancreatectomy	-FoxM1 is required for increased beta cell proliferation after 60% partial pancreatectomy -High level of beta cell replication 14 days after 70% partial pancreatectomy -90% partial pancreatectomy enhance beta cell replication	Ackermann Misfeldt, A. *et al*[36] Lee, S.H. *et al*[37] Liu, T. *et al*[38] Bonner-Weir, S. *et al*[40]
	Transgenic beta cells expressing diphtheria toxin upon treatment of mice with doxycycline	-Proliferation of surviving β cells played the major role in regeneration, after 80-90% of beta cell ablation	Nir, T. *et al*[33]
	Alloxan	-Beta cell replication was observed in nonperfused part of pancreas	Waguri, M. *et al*[39]
Pig	Islet graft in STZ mice	-Increase in beta cell mass due to proliferation of differentiated β-cells in porcine islet graft	Trivedi, N. *et al*[52]
Human/ Cross-sectional studies	Neonates	-High rate of beta cell proliferation is coincident with the major postnatal expansion of beta cell mass -3% decrease of beta cell proliferation after 6 months of age	Meier, J.J. *et al*[43] Kassem, S. A. et al [44]
	Adult	-The frequency of beta cell replication is very low in normal, obese, diabetic subjects (0.03-0.06%) -Islet function and replication decrease with aging	Butler, A.E. *et al*[50] Maedler, K. *et al*[26]
	Pregnancy	-Increase beta cell mass with no change in replication	Butler, A.E. *et al*[51]
	Obesity	-Very low beta cell proliferation level (0.06%) with no significant difference compared to normal individuals (0.04%)	Butler, A.E. *et al*[50]
	Gastric Bypass	-No evidence for beta cell replication after hyperinsulinemic hypoglycemia	Meier, J.J. *et al*[53]
	Intra-pancreatic gastrinomas	-Increase islet beta cell replication	Meier, J.J. *et al*[54]

3.2 Neogenesis of beta cells by ductal pancreatic precursor cells

The role of ductal tissue as a source for the formation of new islets remains a controversial hypothesis. Neogenesis of islets occurs during normal development and in response to physiological stress. Mathematical models proposing two phases of neogenesis (in birth and 3 weeks after birth) were confirmed by studies in newborn rats [55-57]. On the contrary, Solar *et al* proposed the lack of β cell neogenesis after birth [58]. Studies in classic rodent models of partial duct ligation also provided evidence for *in vivo* differentiation of exocrine duct cells. Islet neogenesis is characterized by increase of small islets and islet cell clusters after PDL [59]. Very recently, Van de casteele *et al* confirmed *in situ* their previous study that Ngn3+ cell contributes not only to beta cell proliferation but also to its neogenesis after PDL [34, 35]. However, islet neogenesis after partial pancreatectomy seems to be controversial. Some reports showed both neogenesis and replication [36, 40], while others reported only increased proliferation [23, 37]. Finally, extreme β-cell ablation with alloxan seems to increase insulin positive duct cells in perfused part of pancreas [39].

Two independent cross-sectional studies in human pancreases showed a low range of 0-2.2% insulin positive duct cells before 7 years old and a range of 0-1.2% after 7 years old proposing decline of neogenesis level with aging [43, 60]. Furthermore, study in pregnancy in the age of 18-42 years old showed three fold higher level of insulin positive duct cells in pregnant individuals (0.4% in control group vs 1.2% in pregnant group). In parallel, two fold higher insulin positive duct cells was observed in obese individuals in the age of 64-81 years old (0.4% in control group and 0.8%in obese group) [50, 51, 53].

3.3 (Trans or de)-differentiation of endocrine cells

Recent findings revealed new ideas for the potential mechanisms of endocrine cell regeneration. It has been demonstrated the possibility to directly or indirectly convert differentiated cells into other cell subtypes.

Acinar cell conversion into beta cells: Evidence for acinar cell transdifferentiation was showed in rat exocrine pancreatic cells treated *in vitro* with two growth factors, epidermal growth factor (EGF) and leukaemia inhibitory factor (LIF) [61]. Later, Zhou *et al* provided evidence that differentiated exocrine cells can be directly reprogrammed into single beta cells or small clusters within 3 days. Transfection of a combination of the transcription factors Ngn3, Pdx1 and MafA induced 20% direct conversion of exocrine cells into insulin positive cells. In this study, dedifferentiation via progenitors was excluded, because rapid division or expression of Sox9 and Hnf6 was not detected [62].

Alpha to beta cell transdifferentiation: Due to the fact that mature α-and β-cells share several transcription factors and a common ancestor and are functionally very close, the α-cell represents an appropriate candidate for reprogramming to β-cell phenotype. Firstly, Collombat *et al* showed that Pax4 regulates the balance between α-cells and β-cells by antagonizing Arx in endocrine progenitors [10]. Additionally, it was reported that Pax4 expression in embryonic α-cells provokes their conversion into β-cells [63]. *In vivo* lineage reprogramming was shown by Thorel *et al* using a transgenic model of diphtheria-toxin-induced β cell ablation [64]. In this study, it was reported that cells co-expressing glucagon and insulin are increased the days after β cell destruction. 90% of these cells were YFP-labeled under Cre-Lox proposing that pre-existing α-cells started expressing insulin. One month later, 65% of these cells expressed only insulin and YFP. Another model following pancreatic duct ligation and elimination of pre-existing β cell with alloxan, was used to examine the conversion of mature α-cells [19]. Expression of the transcription factors MafA (expressed only in rodent β-cells) and MafB (expressed only in rodent α-cells) was examined to determine the nature of b cells that appeared 2 weeks after PDL with alloxan. One week following 99% of β cell ablation, 64% of insulin positive cells expressed MafB when only 22% insulin+MafB+ were found after two weeks proposing a novel mechanism of beta cell regeneration.

Beta to alpha cell transdifferentiation: Study in Dnmt1 (an enzyme that participates in DNA methylation during cell division)- deficient beta cells, revealed the beta cell conversion into alpha cells [65]. Arx repression in beta cells was required via its

methylation and so, deletion of methylation in beta cells provokes activation of Arx and conversion into alpha cells. Recently, the role of Pax4 and MafA was examined in normal and dysfunctional human islets. Lower expression of MafA in dysfunctional human beta cells was observed in two independent studies. Interestingly, expression of Pax4 and MafA was found in 50% of human alpha cells which did not confirmed in rodent islets studies [66] (Bonnavion *et al*, accepted in Plos one).

Beta cell dedifferentiation: In *in vitro* experiments with mouse beta cells, increased vimentin expression and loss of the markers Pdx1, insulin, Glut2, and PC1/3 (80% in 21 days) proposing their dedifferentiation into mesenchymal cells [67]. This was confirmed with human beta cells in culture [68]. In addition, experiments in diabetic mice proposed that a decrease of beta cell mass associates with loosening of FoxO1 expression. Further studies in FoxO1 KO mice showed that beta cell didn't die but dedifferentiated decreasing the expression of beta cell specific markers (MafA, Pdx1). Moreover, FoxO1-deficient beta cells often gained expression of other islet hormone such as glucagon. This was a provocative explanation for beta cell failure and alpha cell hyperfunction in type 2 diabetes [69].

3.4 Programmed cell death in endocrine cells

Programmed cell death (PCD) is death of a cell mediated by an intracellular program. In physiological process, apoptosis and autophagy are both forms of PCD. During the neonatal period, the increasing level of β cell mass is followed by increases in β cell death [44]. In adults, massive β cell apoptosis and reduction of β cell mass were shown in type 2 diabetic patients [50, 70].The apoptotic caspases were found as the main participants in apoptosis. They are classified as initiators (caspase-2, 8, 9, 10) or executioners (caspase-3, 6, 7); the first activates the second [71]. There have been only a few studies investigating the role of caspases in the mechanism of β cell apoptosis *in vivo*. Studies of mice with βcell caspase-8 deficiency showed protection against multiple low doses of streptozotocin and high fat diet-inducedβ cell death and diabetes development. However, under basal conditions, loss of caspase-8 leads to an age-dependent decrease in β cell mass and glucose intolerance [72], providing evidence for a critical role in maintaining endocrine cell mass. Furthermore, deletion of caspase-3 in the

β cells was also shown to be protective against multiplelow doses of streptozotocin model without islet tumor promotion [73].

Autophagy is a process of removal of damaged organelles/proteins for recycling providing signals for removal of apoptotic cells and genome stability. Data proposed the protective role of autophagy during β cell stress when in other conditions autophagy had a detrimental role to cell survival. Autophagy deficient mice (Atg7) showed increased apoptosis and decreased proliferation of β cells promoting hypoinsulinemia and hyperglycemia [74]. Additionally, experiments in obesity-induced diabetic mice (db/db) revealed increasing numbersof autophagosomes in β cells [75]. Autophagy is necessary to maintain the structure, mass and function of pancreatic cells. Direct inter relationship between autophagy and endoplasmic reticulum stress-induced obesity suggests that autophagy deficiency may contribute to the progression from obesity to diabetes [76].

4. Role of pancreas in glucose homeostasis

Glucose is the only energy source that brain and red blood cells can use. Glucose is stored in the liver as glycogen and its circulating levels are controlled by two pancreatic hormones, insulin (secreted by beta pancreatic cells) and glucagon (secreted by alpha pancreatic cells). Banting *et al* showed for the first time the opposing actions of insulin and glucagon [77]. In response to high glucose levels, pro-insulin is released from pancreatic beta cells. Insulin stimulates the uptake of glucose and storage in the tissues as glycogen (glycogenesis). In contrast, low glucose levels cause secretion of pancreatic peptide hormone glucagon from alpha cells. Glucagon promotes the conversion of liver glycogen to glucose (glycogenolysis) and release of glucose back into the blood [78-80]. During starvation and intense exercise, glucose can also be generated from non-carbohydrate precursors (i.e. pyruvate, amino acids and glycerol), in a process called gluconeogenesis (Fig 4).

Disturbance of glucose metabolism may cause Diabetes Mellitus, one of the most common endocrine diseases, which can be divided into four types. Type 1 Diabetes (T1D) is an autoimmune disorder characterized by absolute loss of insulin-producing beta cells and Type 2 Diabetes (T2D) provoked by a progressive decline in beta cell function and

insulin secretion. Additionally, 10% of phenotypic type 2 diabetic patients are positive for at least one of islet autoantibodies and they are referred as latest autoimmune diabetes in adults (LADA) or Type ½ Diabetes (T1/2D) [81]. Finally, MODY (Maturity-Onset Diabetes of the Young) is a group of monogenic disorders characterized by autosomally dominantly inherited diabetes or hyperglycemia typically detected during adolescence or young adulthood [82]. Historically, three different concepts for the etiology of diabetes have been supported. For thirty years, after the discovery of insulin (1922), lack of insulin was considered as the unique cause of all diabetes abnormalities (insulinocentric trend). Although glucagon had been identified much earlier, its role on diabetes abnormalities was taken into account from 1975 (bihormonal trend). Nowadays, part of scientific community declaims that decline of insulin secretion provokes some diabetes abnormalities and excess glucagon causes all the others (glucagocentric trend) [83].

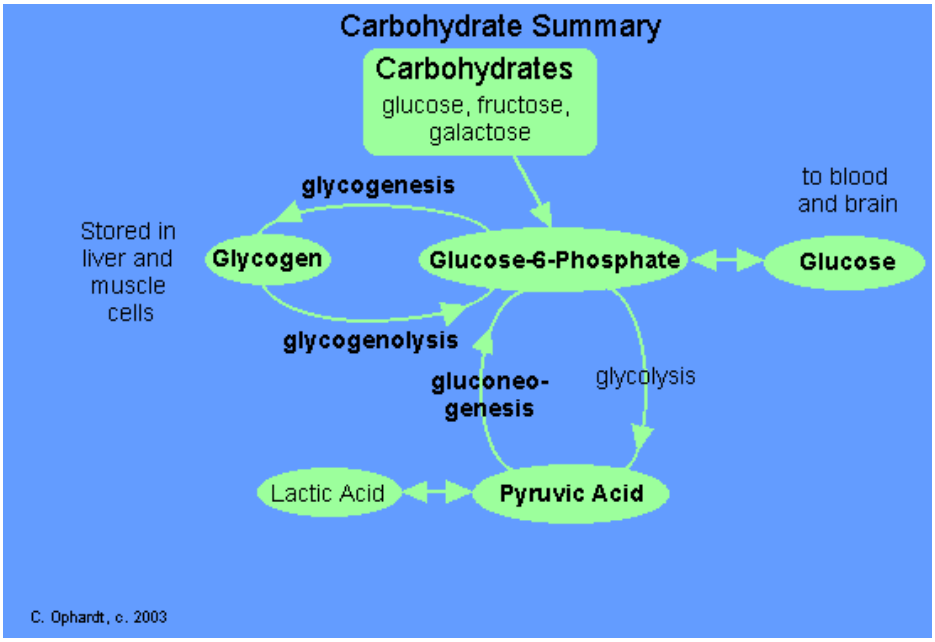


Fig 4: Mechanisms of glucose homeostasis regulation. The action of insulin and glucagon of pancreas regulates glucose homeostasis by the mechanisms of glycogenesis, glycogenolysis and glyconeogenesis in liver.

5. Pancreas in obesity

Overweight and obesity are defined as abnormal or excessive fat accumulation that presents a risk to health. Body mass index (BMI) is a simple index of weight-for-height that is commonly used to classify overweight and obesity in adults. BMI higher than 30Kg/m² characterizes obesity status, while overweight condition refers to BMI levels equal or more than 25Kg/m². According to WHO (World Health Organization) more than 1.4 billion adults were overweight in 2008. Among these, over 200 million men and nearly 300 million women were obese. Moreover, in 2011, more than 40 million children under the age of five were overweight (data of WHO). Overweight and obesity are major risk factors for a number of chronic diseases, including diabetes, cardiovascular diseases and cancer. The most devastating of these diseases seems to be Type 2 diabetes. Relationship between obesity and diabetes prevalence rates worldwide for 2012 is shown in Figure 5 [84]. However, as shown in the same figure, obesity does not fully explain diabetes prevalence rates, because some countries with high diabetes prevalence rates have low obesity rates. Both obesity and T2D are associated with insulin resistance but most obese, insulin-resistant peoples do not develop hyperglycemia.

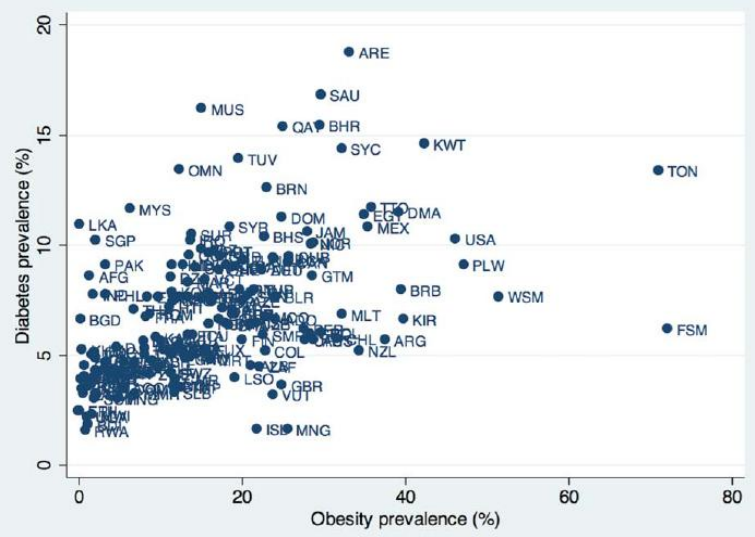


Fig 5: Relationship between obesity and diabetes prevalence rates worldwide in adults. Data from WHO 2012 and International Diabetes Federation, Atlas 2011 (Basu S, 2013)

5.1 Adaptation of islets in obesity

Under normal conditions, pancreatic beta cell mass is able to maintain plasma glucose levels increasing insulin secretion. Beta cell inability to compensate in insulin demand provokes hyperglycemia. Insulin concentration required for a half-maximal response defines as “insulin sensitivity“. Inability of cells to use insulin-produced by beta cells decreases the insulin sensitivity and is defined as “insulin resistance”. The relationship between insulin sensitivity and insulin release is nonlinear [85]. In order to reach a physiological condition, changes in insulin sensitivity for example in pregnancy or weight gain must be followed by a proportionate incremental changes of insulin release (Fig 6). Islet mass and function play a major role in adaptation and development of diabetes based on failure to adapt to this change. Evidence for human islet function in obesity is provided only by cross-sectional studies based on postmortem histology.

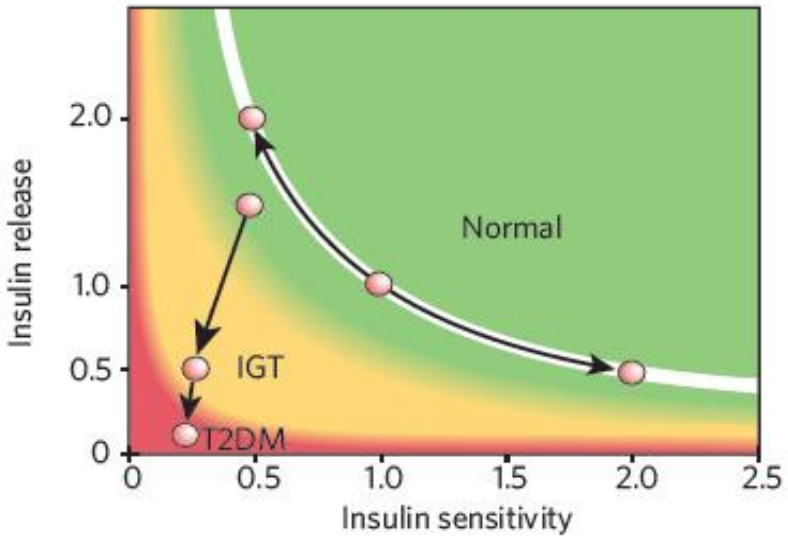


Fig 6: Hyperbolic relationship between insulin sensitivity and insulin release. If changes in insulin sensitivity cannot be followed by a proportionate opposite changes of insulin release, individuals go out the physiological range (green) and become pre-diabetics (yellow) or diabetics (red) (Kahn, 2006)

5.2 Distribution and communication of islet cells in obesity

Human islets tend to contain fewer β-cells and more α-cells compared to rodent islets. Cabrera *et al* reported little difference in the proportion of endocrine cells in islets from different regions of the pancreas where beta cells intermingled with alpha and delta cells throughout the human islet [4]. Islets seem to have three layers and all endocrine cells touch the blood vessels, the middle layer is enriched with beta cells and the two others are enriched with alpha cells [3]. In obese humans, β-cells increase in number to compensate for increased insulin demand. In obese patients with type 2 diabetes, there are reduced numbers of β-cells due to increased apoptosis. Study in type 2 diabetic individuals also showed the presence of amyloid deposits in the dysfunctional islets [86].

The interaction of endocrine cells plays an important role in islet function and insulin secretion. High heterologous intracellular contacts of beta cells has an effect on insulin secretion [87]. Cell adhesion molecules (CAMs), cadherins, gap junction, protein connexin-36, Eph receptors, ephrin ligands and insulin are some of the regulators of β cell-β cell interactions [88]. Neural cell adhesion molecule (NCAM) is essential for α cell-α cell interaction and glucagon secretion as alpha cell intermingled with β cells in NCAM deficient mice [89]. Somatostatin inhibits insulin and glucagon secretion and ghrelin inhibits only insulin secretion where its effect on glucagon secretion remains controversial [90, 91]. Little is known about the physiological alteration of cell-cell communication in obesity. Recently, studies on mice fed with high fat diet for 2 months showed changes in connexin-36 made GAP channels decreasing β cell-β cell coupling [92].

6. Regulation of islet function in obesity

The secretory activity of pancreatic islets is regulated by gastrointestinal hormones, hormones of tissues (adipocytes, osteoblasts) and autonomic nervous system (sympathetic, parasympathetic). Brief report of the most common factors that regulate islet function and their plasma levels in obesity is given in Table 2.

6.1 Gastrointestinal hormones

The gastrointestinal hormones (or gut hormones) constitute a group of hormones secreted by enteroendocrine cells in the stomach, pancreas, and small intestine. Hormones like Glucagon-like peptide-1 (GLP-1), Glucose-dependent insulinotropic polypeptide (GIP), Peptide YY (PYY), Cholecystokinin (CKK), gastrin and ghrelin have an important role on insulin secretion. Hormone level depend on glucose load and insulin demand in different situations such as pregnancy, obesity and type 2 diabetes. 70% of the secreted insulin after a meal in healthy individuals is induced by the GLP-1 and GIP, called incretin effect. They are released from the gut in response to food intake. GLP-1 has a dual action, as a satiety signal and as incretin hormone stimulating insulin release. Findings regarding GLP-1 levels in obesity have been inconsistent. Increased levels of GLP-1 are observed in metabolically healthy obese individuals [93]. In these cases, inhibition of glucagon secretion and decrease of hepatic gluconeogenesis via GLP-1 response in insulin demand increase insulin sensitivity. Some studies have reported reduced postprandial GLP-1 levels in obese compared with lean subjects, while others showed a correlation of GLP-1 to BMI [94]. In all cases, it is well established that individuals with T2D display an impaired incretin effect. Study in mouse and human islets revealed the interaction between TCF7L2 and GLP-1R/GIP-R expression in T2D while low levels of TCF7L2 protein was associated with downregulation of GLP-1 and GIP-receptors [95]. On the other hand, HFD mice seem to have higher levels of GIP and GIPKO mice are protected from obesity and insulin resistance [96, 97]. Furthermore, overexpression of GIP in transgenic mice increases beta cell function and improves insulin sensitivity, but mice were resistant to HF diet-induced glucose intolerance [98]. Finally, it has been shown that GIP has glucagonotropic effects in human subjects and the increased GIP levels in at-risk obese might contribute to the increased glucagon basal levels and to glucagon’s inappropriate suppression after glucose load [99].

Peptide YY (PYY) is a gut hormone whichis also expressed in the pancreas. Obese subjects seem to have significantly reduced circulating levels of PYY [100]. Selective ablation of PYY in pancreas provokes severe hyperglycemia decreasing insulin secretion by disruption of islet morphology in mice [101]. Cholecystokinin (CKK) is also an important regulator of insulin sensitivity. Microarray analysis revealed that CKK was the most upregulated gene in islets of ob/ob mouse model. Another study showed that CKK contributes to islets expansion by increasing beta cell survival [102]. On the other hand, cholecystokinin-deficient mice seem to have impaired insulin secretion [103].

Ghrelin is expressed in pancreatic islets and released into pancreatic microcirculations and its role is the inhibition of insulin release in mice, rats, and humans [91]. When the systemic demand for insulin exceeds the physiological range, including insulin resistance and obesity, antagonism of ghrelin function can promote insulin secretion and thereby prevent from glucose intolerance [104]. Epidemiological studies showed low ghrelin concentration in obese individuals, however there was no relevant data for diabetes state [105, 106].

Finally, gastrin is also released by the pancreas, but no changes in serum concentrations were detected in obese individuals. However, gastrin has an important role on human and mouse islet function. It stimulates beta cell neogenesis and increases islet mass in ligated part after PDL [107]. Furthermore, expansion of transplanted human islets was confirmed in mice treated by gastrin and GLP-1 [108].

6.1 Adipocyte-secreted factors

The interaction between adipocytes and pancreatic islets is not fully known, but it seems that some adipocyte-secreted factors such as leptin, adiponectin, resistin, interleukin-6, tumor necrosis factor-a and interleukin-1β have an important roleon islet cell survival and function. The change in cytokine profiles in islets and plasma is associated with pancreatic β-cell dysfunction and apoptosis. Cytokines have important roles in regulation of pancreatic β-cell function[109].

Leptin and adiponectin are two important peptide hormones secreted by adipocytes that are involved in the regulation of metabolism and energy homeostasis. Both of them have a crucial role in islet function. Leptin-to-adiponectin ratio is also proposed to be a better index of β-cell dysfunction than leptin or adiponectin alone.Leptin

receptors are expressed in pancreatic beta-cells.The direct effect of leptin on pancreatic insulin secretion has been examined in several studies with various leptin concentrations. In physiological concentrations, leptin significantly downregulates insulin secretion from β-cells in the presence of high glucose concentrations, increases beta cell proliferation and blocks lipid accumulation by reducing beta cell apoptosis [110, 111]. It has been reported that plasma levels of leptin are positively correlated with BMI and obese subjects have higher leptin levels than their lean counterparts. The observation that obese subjects have greater leptin but also high insulin concentrations indicates that there is a state of leptin resistance in obese subjects [112]. Contrary to the leptin concentration, adiponectin is the only cytokine downregulated in obesity and type 2 diabetes. In healthy adults, adiponectin circulates in serum at a high concentrations and stimulates insulin secretion by enhancing exocytosis of insulin granules [109].Studies on adiponectin knockout mice revealed the development of severe insulin resistance in response to high fat diet [113]. Adiponectin has been also shown to positively correlate with insulin sensitivity and inversely correlate with fasting proinsulin concentration and the proinsulin-to-insulin ratio, a marker of β-cell failure [114].

Resistin is another factor; its role on obesity remains controversial. Although, clinical studies showed a positive correlation of resistin with BMI, its controversial correlation to insulin sensitivity in some studies or insulin resistance in others proposes an important role of resistin in the link between obesity and type 2 diabetes [115-117]. In physiological concentrations, resistin increases beta cell viability while high concentration reduces insulin receptor activity and provokes beta cell apoptosis in *in vitro* experiments [118].

Interleukin-6 (IL-6), Interleukin-1β (IL-1β) and Tumor Necrosis Factor-a (TNF-□) are also elevated in obesity.IL-6 is considered as a predictive factor for T2D development in obese subjects with systematically increased levels.Interleukin-6 has a protective effect on beta cells. The elevation of IL-6 production in obese and type 2 diabetic individuals may be involved in the beta-cell compensation for insulin resistance in these conditions. It has been showed that IL-6 regulates beta cell function by increasing insulin secretion and viability and alpha cell expansion in obesity [41, 119]. On the other hand, increased circulating IL-1β concentrations are associated with a greater risk of developing T2D while that leads to beta cell death and dysfunction. Studies in high fat diet mice showed that decreased levels of IL-1β using IL-1R antagonist protects from hyperglycemia. Additionally, elevated secretion of IL-1β in human beta cell after high glucose exposure

provokes beta cell dysfunction and apoptosis via NF-kB activation [120, 121]. Finally, TNF-ais a cytokine implied in systemic inflammation. Overexpression of TNF-a was observed in ob/ob and db/db mouse models. In humans, it is associated with obesity, adipocyte cell volume, and inhibition of glucose uptake in adipocytes [109, 122].In the insulin resistant state, TNF-a has been reported to inhibit insulin secretion through activation of several serine kinases in insulin signaling pathway and induce cell apoptosisvia caspase activation[123].

6.2 Osteoblast-associated factors

The observation of vitamin D accumulation in mature beta cells urged the investigation on the role of vitamin D on insulin biosynthesis and secretion. Interestingly, global gene analysis of murine islets revealed huge effects of vitamin D on intercellular junction formation, cytoskeletal organization, exocytosis of insulin [124]. Several studies showed that low plasma vitamin D levels are positively associated with both β-cell function and insulin sensitivity in healthy obese and obese with impaired glucose tolerance [125, 126]. However, no correlation of vitamin D levels with insulin sensitivity and beta cell function was revealed in young healthy obese, prediabetic and diabetic obese subjects[127]. Parathyroid hormone (PTH) is another factor that participates in islets compensation in obesity. Increased levels of PTH may contribute oncompensatory mechanism in response to low circulating levels of vitamin D [126]. Parathyroid hormone-related protein overexpression in mouse islets leads to islet hyperplasia and increase insulin secretion[128].

6.3 Sex hormones

Estrogen and testosterone are two steroid hormones. The principal binding receptors for estrogen are the nuclear estrogen receptors (ER)-α and –β and G protein-coupled receptor 30 (GPR30). Human and animal islet studies suggest that 17-estradiol (E2), one of the most common types of estrogens, might promote insulin production in diabetic individuals and prolong cell survival. Additionally, the antiapoptotic effects of estrogen in female islets was confirmed via activation of GPR30[129]. More recently, it has been

found that estradiol improves the function of human islets transplanted in nude mice[130]. Finally, human cross-sectional studies showed the protective effect of testosterone and estradiol on the development of metabolic syndrome. High testosterone levels is associated with high insulin sensitivity and better beta cell function [131].

6.4 Autonomic nervous system

Neurotransmitters are endogenous chemicals that transmit signals froma neuron to a target cell across a synapse. Many neurotransmitters were identified as regulators of islet function in obesity such as glutamate, GABA, dopamine, epinephrine, norepinephrine, serotonin, somatostatin and acetylcholine.

High levels of glutamate and γ-aminobutyric acid (GABA), two amino acids classified as neurotransmitters, have been observed in obesity. Recently, two independent studies showed that the flux of glutamate modulated insulin release from insulin granules. Decrease of glutamate transport enhanced insulin secretion, but did not change glucagon secretion [132, 133]. Furthermore, GABA seems also to modulate insulin secretion and have an important role on islet mass maintenance [134].

High or low levels of the amines like dopamine, epinephrine, norepinephrine and serotonin modulate islet function in obesity. Obese individuals seem to have less dopamine receptors proposing low levels of dopamine [135, 136]. *In vivo* studies and studies in isolated human islets showed that dopamine inhibits insulin secretion and this inhibition correlates with a reduction in frequency of the intracellular [Ca2] oscillations[137]. Opposite levels have been identified for epinephrineand norepinephrineonobesity. A positive association has been shown between plasma epinephrine levels and insulin sensitivity in healthy obese individuals [138]. Additionally, it is known that epinephrine induces glucagon secretion through the activation of b-adrenoceptors and the generation of cAMP [133]. On the other hand,norepinephrine levels tended to decrease in obesity. It was showed that norepinephrine increases glucose uptake in brown adipose tissue in mouse [139]. Physiologically,it inhibits insulin secretion by three actions a) increasingCa^{+2}concentrations by activation of K^{+} channels b) inactivating of adenylylcyclases and inhibiting exocytosis and c) reducing the activity of the L-type Ca^{+2} channels [140]. Finally, human obesity was associated with chronic elevation of brain serotonin. Overweight individuals seem to have high levels of serotonin metabolites in one study, without data documenting their diabetes state [141]. Additionally, high levels of plasma serotonin were observed in T2D patients. Recently, a

study in db/db mice confirmed the elevated expression of serotonin in islets and revealed that inhibition of serotonin using antagonist improves insulin secretion [142].

Somatostatin and acetylcholine are two neurotransmitters for which plasma levels do not change in obese or obese patients with impaired glucose tolerance (IGT), and type 2 diabetes. Exogenous somatostatin infusion in mouse models (ob/ob, db/db) and in vitro experiments with human islets showed that somatostatin inhibits insulin and glucagon secretion [143]. Acetylcholine is crucial for pancreatic beta cell function as it stimulates insulin secretion by increasing the cytoplasmic free Ca^{2+} concentration and enhancing insulin exocytosis. Rodriguez-Diaz *et al* recently showed that acetylcholine secreted by human alpha cells sensitizes beta cells to secrete insulin [144].

Table2: Studies providing evidence of regulation on pancreatic cell function

	Type	Obesity	Regulation	References
Gastrointestinal Hormones	GLP-1	Controversial results but attenuated in obese with insulin resistance	-physiologicaly, it has insulinotropic effect, inhibits pancreatic glucagon secretion, decreases hepatic gluconeogenesis, and decreases insulin resistance -low level in obese type 2 diabetic patients	Muscelli, S. *et al*[93] Madsbad, S. *et al*[94]
	PYY	Low concentration predispose the development of obesity	-regulate beta cell function and survival via the receptor Y(1/2)	Batterham *et al*[100] Sam, A.H *et al*[101]
	Ghrelin	Decreased in human obesity	-inhibit insulin release in rodents and humans -obestatin (ghrelin gene-derived peptide) increase insulin secretion in high fat diet mice	Dezaki, K. *et al*[91], Granata, R. *et al*[104], Tschop, M. *et al*[105]Rosicka, J. *et al*[106]
	GIP	Unaffected levels in healthy obese but increased at risk obese subjects	-Gluganotropic effect in healthy individuals -Overexpression of GIP in trangenic mice increase beta cell function and improve insulin sensitivity but they were resistant to HF diet-induced glucose intolerance -GIPR ko mice do not develop obesity	Meier, J.J. *et al*[99] Calanna, S. *et al*[96] Kim, S.J. *et al*[98] Miyawaki, K. *et al*[97]
	Gastrin	No significant difference in obese subjects (serum concentration)	-stimulation of beta cell neogenesis in duct-ligated rats -expansion of human islets transplanted in NOD-SCID mice treated by gastrin+GLP-1	Rooman, I. *et al*[107] Sadry, S.A.*et al*[108]
	CCK	+++	- increase beta cell survival in ob/ob mice -impaired insulin secretion and increasing insulin sensitivity in CKKko mice	Lavine, J.A. *et al*[102] Lo, C.M *et al*[103]
Adipose tissue	Leptin	+++	-Inhibit insulin secretion in rodents and human islets by acting directly in beta cells and reduce glucose transport into beta cells -Regulator of beta cell mass and survival, antiapoptotic affect	Kulkarni, R.N. *et al*[111] Lee, Y.N. *et al*[110]
	Adiponectin	Downregulated	-Adiponectin knockout mice develop severe insulin resistance in response to a high-fat diet -is associated to insulin resistance in humans -antiapoptotic affect	Maeda, N. *et al*[113] Kern, P.A. *et al*[114] Lee, Y.N. *et al*[110]
	Resistin	+++ (positive correlation to BMI)	-controversial correlation to insulin sensitivity in humans -associated to insulin resistance in rodents and humans -increase beta cell viability in physiological concentration but high level provoke beta cell dysfunction (resistance state)	Silha, J.V. *et al*[115] Lee, J.H. *et al*[116] Wang, C. *et al*[117]
	IL-6	+++	-regulate pancreatic alpha cell expansion -stimulate insulin secretion via direct cell-based mechanisms	Ellingsgaard, H. *et al*[41] da Silva Krause, M. *et al*[119]

	IL-1β	+++	-beta cell itself produce IL-1β after high glucose exposure -IL-1β antagonists improve beta cell survival and function in HFD animals	Sauter. N.S. *et al*[120] Ardestani, A. *et al*[121]
	TNF-a	+++	-inhibitinsuilin secretion and induce beta cell apoptosis	Zhao, Y.F. *et al*[109]
Osteoid tissue	Vitamin D	Low level in obese adults and no changes in youth obese individuals	-Positive association with β-cell function and insulin sensitivity in humans -Effect on intercellular junction formation, cytoskeletal organization, exocytosis (murine islets)	de Las Heras, J. *et al* Wolden-Kirk, H. *et al* Karnchanasorn, R. *et al*[124, 125, 127]
	Parathyroid Hormone	+++ (serum concentration)	-PTHrP over expression increase islet mass and insulin secretion	Guasch, A. *et al* Porter, S.E. *et al*[126, 128]
Sex hormones	Estrogen	+++	-improve human islet survival, revascularization and function grafted in nude mice -antiapoptotic effect in human islets through activation of GPR30 when GPR30 expression in islets is related to BMI	Liu, S. *et al* ; Kumar, R. *et al*[129, 130]
	Testosterone	+++	-higher levels are associated with a higher insulin sensitivity and reduced the risk of the metabolic syndrome	Muller, M. *et al*[131]
Neuro-transmitters	Glutamate	+++	-Insulin secretion is modulated by the flux of glutamate through the secretory granule -The intra-cellular glutamate pool is likely of lesser importance in α-cells	Gammelsaeter, R. *et al* Feldmann, N. *et al*[132, 133]
	GABA	+++	-role in maintain beta cell mass	Taneera, J. [134]
	Dopamine	Lower D2 receptors availability	- Exhibits insulin secretion in isolated islets and in vivo	Wang, G.J. *et al*; Rubi, B. *et al* ; Ustione, A. *et al*[135-137]
	Norepinephrine	tended to decrease	-Increases glucose uptake in brown adipose tissue -Three effects on beta cell that exhibits insulin secretion	Dallner, O.S. *et al* Straub, S.G. *et al*[139, 140]
	Epinephrine	Increased in healthy obese individuals	-Epinephrine induces glucagon secretion through the activation of β-adrenoceptors and the generation of cAMP - positive association between plasma epinephrine level and insulin sensitivity in obese individuals	Feldmann, N. *et al* Dai, X.P. *et al*[133, 138]
	Serotonin	High plasma levels in obese T2D patients	-Inhibition of serotonin receptor improve insulin secretion	Zhang, Q.[142]; Markianos, M. [141]
	Somatostatin	No significant changes	-exogenous SST inhibit insulin and glucagon secretion in rodents and humans	Hauge-Evans, A.C. *et al*[143]
	Acetylcholine	No significant changes	-Secreted by alpha cells and sensitize beta cell to response in increased glucose concentration	Rodriguez-Diaz, R.*et al*[144]

7. Regulation of gene expression by microRNAs in obesity

MicroRNAs (miRNAs) are single-stranded non-protein coding gene products (20-22 nucleotides) that regulate negatively the expression of target genes at the post-transcription level through interactions with specific mRNAs. The first miRNAs were characterized in the early 1990s [145]. However, miRNAs were not recognized as a distinct class of biological regulators with conserved functions until the early 2000s. Different sets of expressed miRNAs are found in different cell types and tissues. The human genome may encode over 1000 miRNAs, which may target about 60% of mammalian genes and are abundant in many human cell types.

It have been shown that miRNAs regulate multiple diverse biological processes inhibiting translation or degrading the target mRNA such as development, insulin secretion and cell differentiation [146]. The role of many microRNAs was identified in regulation of insulin expression (miR-30d, miR-375, miR-124a2), insulin secretion (miR-9, miR-375, miR-124a2), glucose-stimulated insulin secretion (miR-369-5p, miR-130a, miR-27a miR-410, miR-200a, miR-337, miR-532, miR-320, miR-192, miR-379, miR-375, miR-124a2) and insulin sensitivity (miR-103, miR-107, miR-29, miR-320).

MiRNAs have been also found to be important for the proper development of the pancreas and beta cells physiology. Recently, different miRNAs were identified to express in human pancreatic alpha and beta cells, 134 were expressed more in beta cells and 7 in alpha cells [147]. Jacovetti *et al*, revealed the miRNAs contribution to beta cel expansion during pregnancy and obesity [148]. Decreased level of miR-338-3p activated the GPR30 (G protein-coupled estrogen receptor) and GLP1R (glucagon-like peptide 1) in obese mice. Thus, blockage of mi-338-3p increased proliferation and improved survival of beta cell *in vitro* and *in vivo*. Moreover, inhibition of miRNA-7a has been also showed to regulate proliferation in adult pancreatic beta cells via mTOR pathway [149]. Finally, very recently Nesca *et al,* proposed that the maintenance of blood glucose homeostasis or progression toward glucose intolerance and type 2 diabetes may be determined by the balance between expression changes of particular microRNAs [150]. Modifications in the levels of miR-34a, miR-146a, miR-199a-3p, miR-203, miR-210 and miR-383 primarily occur in diabetic mice and result in increased beta cell apoptosis.

8. Methods of quantification of insulin sensitivity/resistance

Different methods for the quantification of insulin sensitivity and insulin resistance are used in humans and animal models. The Hyperinsulinemic Euglycemic Glucose Clamp and Insulin Suppression Test (IST) are the two direct methods to measure insulin sensitivity. In the glucose clamp technique, after an overnight fast, insulin is infused intravenously at a constant state (hyperinsulynemic state). Under this condition, blood glucose is measured, while 20% dextrose is given intravenously at an appropriate rate to conserve glycemia in the normal range (euglycemic). In spite of technical difficulties, time and money consuming, it is considered one of the best methods to measure insulin sensitivity, because glycemia remains in the same levels, as the infusion rate of glucose and the glucose consuming depends only on serum insulin [151, 152]. IST is based on suppression of endogenous insulin and glucagon secretion following somatostatin orocteotide infusion. The ability of exogenous insulin to mediate disposal of the glucose is provided directly by IST [152, 153]. Dynamics or indirect tests were also developed to quantify insulin sensitivity such as Intravenous Glucose Tolerance Test (Minimal Model/IVGTT), Intraperitoneal Glucose Tolerance Test (IPGTT) and Oral Glucose /Meal Tolerance Test (OGTT). In these cases, glucose is administered intravenously, intraperitoneally and orally then, blood glucose and endogenous insulin is measured to determine the indexes of insulin sensitivity [152, 154].

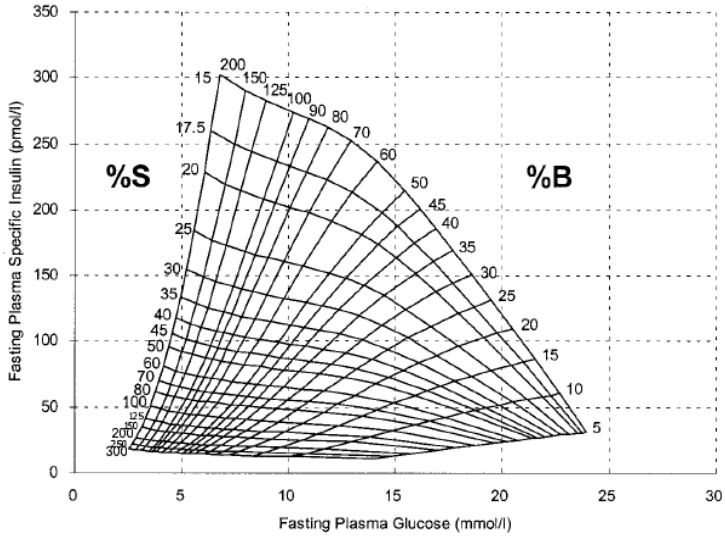


Fig 7: Nonlinear correlation of basal plasma insulin and glucose concentrations in the updated HOMA model. Higher insulin secretion in response to low glucose concentration leads to highest insulin sensitivity (S=200%) and highest beta cell function (B=200%) (Wallace, 2004)

Finally, simple surrogate indexes for insulin sensitivity/resistance are available. Homeostatic model assessment (HOMA), first described in 1985, is a method for assessing beta cell function and insulin resistance from basal (fasting) glucose and insulin or C-peptide concentrations.

Decreases in beta cell function were modeled by changing the beta cell response to plasma glucose concentrations. Insulin sensitivity was modeled by proportionally decreasing the effect of

plasma insulin concentrations at both the liver and the periphery. Figure 7 shows the nonlinear correlation of basal plasma insulin and glucose concentrations in the updated HOMA model (Homa2: computer model) [155]. Insulin secretion curve is modified by increasing insulin secretion in response to plasma glucose concentrations. Higher insulin secretion in response to low glucose concentrations leads to highest insulin sensitivity (S=200%) and highest beta cell function (B=200%). A computer model can calculate the %S and %B using insulin or C-peptide concentrations and gives a value of Homa %S and Homa %B, where 100% is considered as normal. In addition, Homa %BxS index is calculated from insulin secretion (Homa %B) and insulin sensitivity (Homa %S) to describe the overall function of beta cells [156, 157].

9. Non invasive imaging of islet mass

The improvement of non invasive imaging technologies of pancreatic islet mass is a crucial field for investigation. Progress has been hindered by many problems. The small size of beta cells dispersed throughout of pancreas put obstacles in development of clinical beta cell imaging. In addition, some of these techniques necessitate the *in vitro* manipulation of islets and are limited by the short half-life of available labeling molecules.

Nuclear imaging, Mn^{+2} MRI, Zn^{+2} MRI, optical imaging and functional imaging into eye anterior are some of these methods. Nuclear imaging technologies are based on labeled molecules that enter into beta cell modulating insulin secretion as fluorine 19 labeled mannoheptulose and glibenlamide. Mannoheptulose inhibits insulin secretion via inhibition of glucose phosphorylation acting on glucose transporters (GLUT2) [158]. Glibenlamide promotes insulin secretion via activation of Ca^{2+} channels in beta pancreatic cells [159]. Mn^{+2} MRI methods are accompanied with glucose injection as manganese enters into beta cells through Ca^{2+} channels in a glucose dependent manner. Additionally, zinc ions are released with insulin in response to glucose and Zn^{+2} MRI is used in beta cell mass quantification [160, 161]. Optical imaging technologies investigate endocrine pancreas using optical coherence, confocal or 2-photon microscopy [162, 163]. Functional clinical imaging modalities with very high sensitivity, such as PET or single photon emission computed tomography (SPECT) are hampered by the partial volume effect, leading to an underestimation of the signals derived from objects smaller than the spatial resolution of the

scanner [164]. Nowadays, none of these techniques has been associated with the overall beta cell function and none is used in clinical practice.

On the other hand, non invasive approaches, allowing transplanted-islet functional imaging, were developed. Speier *et al* proposed the anterior chamber of the eye as a novel non invasive imaging method to investigate pancreatic cell function and survival [165]. After transplantation, the morphology and cellular composition of the islets engrafting in the anterior chamber of the eye are preserved and are able to recover hyperglycemia. Recently, radioligand of exendin-4 (GLP-1 analog) was used to evaluate the function and survival of transplanted islets into human brachioradialis muscle [166].

10. Islet transplantation

The first successful use of exogenous insulin in the treatment of diabetes was at 1922. Patients with T1D depend on external insulin for their survival. Whole pancreas transplantation is associated with complications such as bleeding, infection and inflammation of the pancreas and it is performed in specific cases. Nowadays, islet transplantation is a more physiological method to recover hyperglycemia than exogenous insulin therapy and pancreas transplantation. Shapiro *et al* reported for the first time islet transplantation and insulin independence in all patients in Edmonton Center at 2000 [167]. In clinical practice, naked islets are transplanted into the liver via the portal vein. This allows delivery of insulin to the portal circulation as occurs with normal physiology. The main disadvantages of islet transplantation method are the graft rejection, poor vascularization, hypoxia and the large number of islets as at least two donors pancreases are needed for one recipient [168]. Historically, all age and BMI donors were frequently used. High donor BMI and large pancreas size are important for successful human islet isolation (250 000 islets) [169]. Obese donors tend to be avoided for pancreas transplantation because of fat content within their pancreas [170] but these donors often are accepted for islet cell isolation and subsequent transplantation because of the high yields [171]. Some islet isolation laboratories only accept high BMI pancreatic donors for islet isolation/transplant trials [172]. The capacity of obese islets for clinical islet transplantation should be investigated.

Immunoisolation devices are proposed to facilitate islets transplantation providing sufficient oxygen and separating islets from blood. Some materials used to encapsulate

islets are natural (arginate, agarose and collagen) or synthetic (PLL/poly-L-ornithine coating and polyethylene glycol) [173-176]. Animal models are used for technical improvement of islet transplantation and further investigation on human islets using these encapsulated methods is needed. Figure 8 shows different sites of islet transplantation. Islet transplantation into intraportal vein offers physiologically delivery of insulin to the portal circulation, but has limited space and increases the risk of complications including vein thrombosis and hemorrhage (Fig 8A) [177, 178]. In addition, transplantation into muscle is recently proposed as an alternative site which offers vascular network and unlimited space (Fig8B) [166]. Furthermore, animal studies use sites as kindey capsule and subcutaneous tissue offering quick revascularization of islets (Fig 8D, 8F) [179-181]. On the other hand, peritoneal cavity and omentum are two sites with unlimited space, but no vascular network and invasive procedure is required (Fig 8C) [182, 183]. Finally, islet transplantation in anterior chamber of the eye is proposed as the method with the easiest access for islet structure and function investigation but with no practical application in clinical procedure (large number of islets in humans transplantation) (Fig 8E) [165].

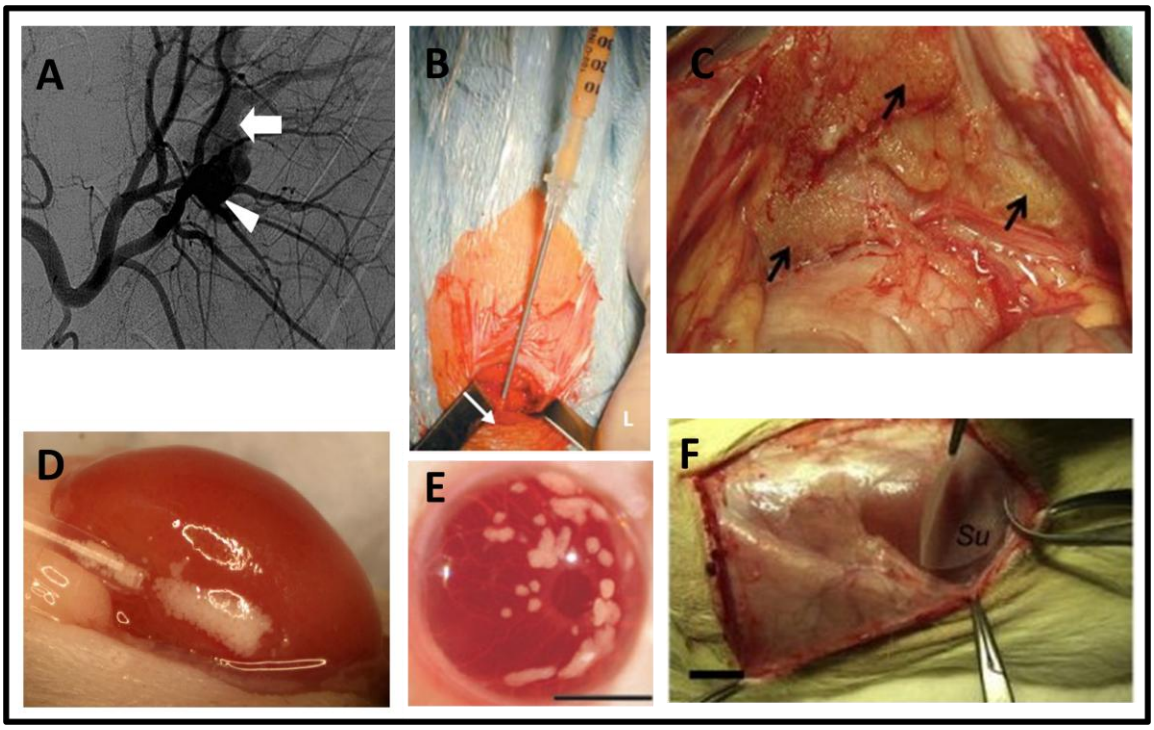


Fig 8: Different islet transplantation sites. A) Intraportal vein B) muscle C) Peritoneal cavity D) Under kindey capsule E) Anterior chamber of eye, and F) Subcutaneous tissue/ epididymal fat pad.

Chapter II

Background and Aim of study

1. Background

Human islets were isolated in Lille University Hospital since 1994. Routinely performed in our laboratory, human islet function is quantified after transplantation in immunodeficient mice by dosing human C-peptide in mouse blood [184]. In the context of organ donation for pancreas transplantation, obesity is an exclusion criteria [170]. This was initially the case for pancreases harvested for islet isolation and transplantation. However, lean (BMI<21Kg/m²) pancreatic donors are characterized by significantly lower islet yields as compared with normal (BMI<21-24 Kg/m²) and obese (BMI>24 Kg/m²) donors [185]. In Lille laboratory, likewise 62% of organ donors, that were clinically suitable for islet transplantation, were overweight or obese (BMI>25Kg/m²).

Cross-sectional study during my master project, exploiting archived paraffin sections of pancreases (U859 collection), confirmed morphological differences in human islets between obese and lean donors: pancreases of obese donors had four basic characteristics: 1) increased total endocrine mass (a,β,δ,pp cells), 2) individual islets were bigger in size 3) increased of intrapancreatic fat tissue and 4) increased beta cell mass and decreased alpha cell mass in islets expressed in addition of alpha and beta cells. Yet, direct evidence that human islet mass adapts longitudinally to obesity *in vivo* is lacking and, moreover, little information is available on the mechanisms and cell type(s) involved. Current evidence for increased beta cell mass in obese humans (vs. lean) is based entirely on postmortem histology.

2. Aim

Generally, the aim of this study was to explore the longitudinal adaptation of human islets to an obesogenic environment. The following steps were followed:

- Creation of a novel *in vivo* mouse model that associates obesity and immunodeficiency and is compatible with human islet transplantation.
- Verification of human islets capacity to functionally adapt to the murine obesity environment.
- Providing evidence for *in vivo* regeneration of human islets (proliferation, neogenesis, transdifferentiation).
- Kinetic Gene expression profiling of human islets during islet adaptation to obesity.

Chapter III

Materials and Methods

1. Human islets

Pancreases were harvested from human brain deceased donors in agreement with French regulations and with our institutional ethics committee. Islets are isolated from the pancreases using a modified version of the automated Ricordi's method as previously described [186, 187]. During the thesis project, six pancreases from non-diabetic donors and two from donors with overt metabolic dysfunction (older, high HbA1c or a history of diabetes) were used. The characteristics of non diabetic and diabetic human donors were given in Tables 3 and 4 respectively.

Table 3: Characteristics of non diabetic human donors

Donors	Sex M/F	Age years	BMI Kg/m^2	Hba1c %(mmol/mol)	Viability %
D1	M	16	19.5	5,5(34.1)	90.7
D2	F	41	31.6	6(37.2)	98
D3	M	22	20.5	5,3(32.9)	96.4
D4	M	16	21.2	5,3(32.9)	94.8
D5(H723)	M	41	26.2		95
D6(H742)	M	34	24.2	5,4(33.5)	98
Mean+SEM		28.33±4.82	23.87±1.85	5.5±0.16(34.2±1.02)	94.9±1.56

Table 4: Characteristics of dysfunctional human donors

Donors	Sex M/F	Age years	BMI Kg/m^2	Hba1c %(mmol/mol)	Viability %
DD1*	M	60	24.1	6.5	98
DD2	M	59	37.7	7	99.1

* treated for 10 years for type 2 diabetes

Human islets were cultured in CMRL medium (Sigma C0422), Stem Ease (Abc0103), 10% human serum AB (Sigma H4522), 5ml Penicillin-Streptomycin. Viability of islet cells was performed with dithizon-trypan blue method (Annex 1). All islets showed more than 90% viability after culture, but were quantitatively insufficient for clinical islet transplantation.

2. Animals and induction of obesity

Mouse studies were performed in accordance with the local Animal Experimentation Committee. Ninety-three male C57BL6 RAG $2^{-/-}$immunodeficient mice (A Bouloumié, INSERM U858 Toulouse France [188] and Taconic USA RAGN12-M) 8-9 week-old were used for this project. Animals were fed 4, 6, 8, 10 or 12 weeks with Control or HFD (Research Diets, New Brunswick, NJ ref D12450B is given in **Table 5**). All mice were followed for weight, serum triacylglycerol and 6 hour fasting blood glucose. Fat content was quantified by Magnetic Resonance Imaging (MRI) using 10 images/mouse before sacrifice as described [189].

Table 5: Composition of Research Diets (New Brunswick, NJ ref D12450B)

	Control	HFD
Protein (%)	20	20
Carbohydrate (%)	70	20
Fat (%)	10	60

3. Human islet transplantation

Four hundred human islet equivalents (IEQ) were transplanted under the kidney capsule as described [184]. To control for potential differences in islet quality between the donors, islets from each human donor were distributed symmetrically across experimental groups [190]. More than ten mice were transplanted per donor per condition.

3.1 Human C-peptide Measurements on mice

Human islet function is quantified after transplantation by dosing human C-peptide in mouse serum. Within the pancreatic beta cells, proinsulin is cleaved into one molecule of C-peptide and one molecule of insulin. C-peptide is subsequently released into circulation at concentrations equimolar to those of insulin. Specific C-peptide ELISA kit (Ultrasensitive human C-peptide kit, Mercodia, Uppsala, Sweden) allows the distinction

between human C-peptide and murineC- peptide allowing follow up of human graft function. It is based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the human C-peptide molecule. Blood was harvested from tail vein in heparinized tubes. After centrifugation, plasma was collected and frozen for human C-peptide dosage. Control mouse serum is negative for human C-peptide. The procedure of C-peptide measurement is given in Annex 2.

3.2 Quantification of insulin resistance/sensitivity with OGTT and Homeostatic model assessment

At the end of the study, mice were fasted for 6 hours before receiving an oral bolus of glucose (2g per Kg body weight) diluted in saline buffer (0.9% Sodium Chloride). Tail Blood was collected at 0, 30, 60 minutes after administration to evaluate human C-peptide levels. Blood glucose was measured (GlucometerAccu-chek® Go, Roche France) up to 180 minutes. Homeostatic model assessment, calculated using fasting human C-peptide and blood glucose in mice (http://www.dtu.ox.ac.uk/homacalculator/index.php) was used as an index of insulin sensitivity (HOMA2%S), beta cell function (HOMA2%B) and the hyperbolic product (HOMA2%S x HOMA2%B /100). Results of 12 clinical grade islet preparations (glycemia, human C-peptide) transplanted in 20 immunodeficient mice, as described were used to calculate theoretical HOMA2%B and S.

4. Histological analysis in endogenous mouse pancreas and human islet graft in mice

To assess proliferation, Bromodeoxyuridine (BrdU) was injected 18h before sacrifice or administered 7 days in the drinking water. Both the endogenous mouse pancreas and human grafts were analyzed.

4.1 Fixation of mouse and human tissues

Different types of tissue fixation were used adjusted to the needs of the analysis. Endogenous mouse pancreas and human grafts were fixed overnight in 4% PFA or formalin and embedded in paraffin (Annex 3a). Sections of 5μm thick were cut using microtome and were used for immunohistochemistry. For the study of lineage tracing, human grafts were fixed overnight in 4% PFA and embedded in gelatin. Isopentane (2-methylbutane) cooled by dry ice were used to freezing gelatin blocks containing the kidney with transplanted human islets (Annex 3b). Cryo-sections of 10μm thick were used for these analyses. Directly immersion of fresh human grafts into liquid nitrogen was performed for the microdissection process. Isopentane (2-methylbutane) cooled by liquid nitrogen were used to freezing OCT blocks. Cryo-sections of 10-12 thick were used for microdissection following RNA extraction. Finally inclusion in thrombin and fibrinogen was performed for single islets or cells (Annex 3c).

4.2 Immuno-histochemistry and fluorescence

Specific antibodies for islet cell, duct cell, cell nucleus and markers for proliferation were used to determine the distribution of islet cell and mechanisms of regeneration for mouse and human islets. Endocrine mass of the endogenous pancreas was determined with Papanicolaou staining of sections. Two, three or four color fluorescence or DAB staining was performed to reveal the reaction of these antibodies. The basic excitation wavelengths of fluorochromes are green, red, infrared and blue. Table 6 shows the primary and secondary antibodies used in this project. Immunostaining protocols are given in Annex 4.

Table 6: List of primary and secondary antibodies

Type	Species	Specificity	Provider	Reference	Dilution
Primary Antibody					
Insulin/Proinsulin	mouse	beta cells	AbDserotec	5330-3339	1/300
Insulin	guinea pig	beta cells	Dako	A0564	1/300
C-peptide	mouse	human Beta cells	MONOSAN	C-pep-01	1/300
Glucagon	mouse	alpha cells	Sigma	G2654	1/300
Glucagon	rabbit	alpha cells	Gentaur	20076	1/300
Chromogranin A	rabbit	endocrine cells	Dako	A0430	1/300
Somatostatin	rabbit	delta cells	Dako	A0566	1/100
CK-19	mouse	duct cells	Dako	RCK108	1/200
BrdU	rat	S phase of cell cycle	AbDserotec	013T0030	1/200
Ki67	mouse	Active phases of cell cycle	Dako	MIB-1	1/100
GFP	chicken	green fluorescent protein	GeneTex	GTX13970	1/1000
c-Myc	mouse	c-Mycepitope Tag	Abcam	Ab32	1/200
Secondary antibody					
Alexa 488	goat	chicken	Invitrogen	A11039	1/500
Alexa 488	donkey	rabbit	Invitrogen	A21206	1/500
Alexa 488	goat	mouse	Invitrogen	A11001	1/500
Alexa 594	donkey	mouse	Invitrogen	A21203	1/400
Alexa 594	goat	rabbit	Invitrogen	A11012	1/400
Alexa 594	goat	rat	Invitrogen	A11007	1/300
Alexa 647	donkey	rabbit	Invitrogen	A31573	1/400
Dylight 405	goat	guinea pig	Interchim	106-475-003	1/400

5. Microscopy and morphometric analysis

Three types of optical microscope (bright field, fluorescence and confocal) were used for sections analysis. To quantify graft size, volume of islet, volume of alpha and beta cell in human grafts, the sections were scanned with Nikon Eclipse Ti microscope (motorized stage). The fractional area of the pancreas was digitally quantified using a color based threshold using the Nikon software (Nis Elements AR 3.0). Both Leica DM-R and Nikon Eclipse Ti fluorescence microscope were used toquantify endogenous pancreas and islets, distribution of endocrine cells, endocrine cell proliferation and endocrine cell neogenesis.Co-localisation of cytoplasmic proteins was determined usingconfocal microscope Zeiss 710.The quantification of total number of nuclei and individual cells, cytoplasm and nuclei surface were identified by the ImageJ (NIH, USA) software using a color based threshold.

Planimetry, used for clinical tumour volume appreciation (surface in microns^2×distance in microns between paraffin sections), was used to determine endocrine, beta and alpha volumes (mm^3) of entire human islet grafts.Mouse kidneys were completely cut in 5µm sections. One in every ten section was stained by chromogranin A, C-peptide and glucagon and were used for volume determination (red arrows in **Figure 9**). Stained surface was measured and volume was determined as the addition ofsurface (µm^2) x distance in microns between two paraffin sections (s1-s2). Specifically, V(µm^3)=s1(µm^2)x50(µm) + s2(µm^2)x50(µm) + s3(µm^2)x50(µm) … + sn(µm^2)x50(µm) (**Fig 9**).

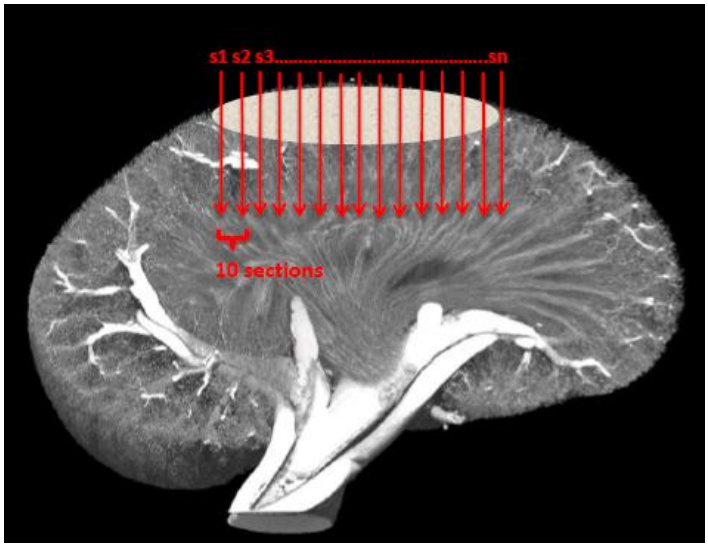


Fig 9: Method of determination of cell volume in islets transplanted under kidney capsule. One in every ten paraffin sections were stained and measured. Volume was determined as the addition of surface (µm2) x distance in microns between two paraffin sections (s1-s2).

6. Lineage Tracing

Lineage tracing is the identification of all progeny of a single cell. This technique was used to determine the transdifferentiation of islet cell transplanted in control and HFD mice. The principal vector, pTRIP-CMV-eGFP-ΔU3 [191], expresses the eGFP gene under the control of an internal cytomegalovirus (CMV) promoter. Two new lentiviral vectors, pTRIP-RIP-eGFP-ΔU3 and pTRIP-Gcg-eGFP-ΔU3, were constructedwith the Rat insulin II gene promoter (RIP) and glucagon promoter in order torestrict the expression of eGFP to insulin and glucagon-producing cells respectively. Two other vectors were constructed based on the two first excluding the expression of eGFP, pTRIP-RIP-nlsCRE-ΔU3 and pTRIP-Gcg-nlsCRE-ΔU3 as previously described [192]. The nlsCRE gene translates CRE recombinase, an enzyme derived from P1 bacterophage. Finally, one reporter vector pTRIP-CMV-loxP-cmycdsred2-loxP-eGFP-ΔU3 was constructed by insertion of loxP-cmycdsred2-loxP cassette into the principal vector. Cre-lox system is a site-specific recombinase technology which used to carry out deletions, insertions, translocations and inversions at specific sites in the DNA of cells. For our project, two types of vectors were used to tag beta and alpha cells; pTRIP-RIP-nlsCRE-ΔU3, pTRIP-Gcg-nlsCRE-ΔU3 and one reporter vector; pTRIP-CMV-loxP-cmycdsred2-loxP-eGFP-ΔU3 (provided by Professor Philippe Ravassard, BCBC/UMR-7225). Two independent experiments were performed, one for beta cell and the other for alpha cell differentiation. The pair of vectors used for beta and alpha cells was given in **Figure 10.** In the first case, beta cells which received the two vectors express eGFP and all the others express dsred2. On the other hand, alpha cells which received the two vectors express eGFP and all the others express dsred2.

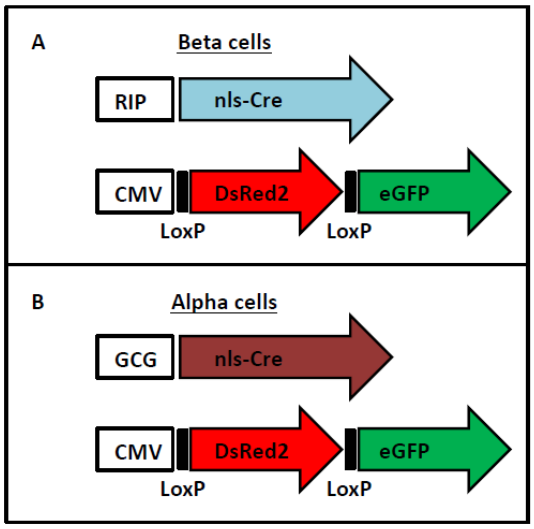


Fig 10: Pair of vectors of pancreatic cells infection.A) Specific for beta cells infection and B) specific for alpha cell infection.

6.1 Cell culture and infection

Human islets from normal donor (D5, see Table 3) were cultured in CMRL medium with 0.5% steam ease (Abc0103), 10% human serum AB (Sigma H4522), 5ml Penicillin-Streptomycin. After 2 days in culture, islets were washed with PBS and infected with a 1:1mixture of the two viruses at multiplicity of infection (MOI) 3:1 in same medium containing 1/1000 diethylaminoethyl-dextran (Sigma-Aldrich, Saint-Quentin Fallavier, France) for 1,5 hour. To validate the efficacy of vectors, a part of islets was infected only with reporter vector (pTRIP-CMV-loxP-cmycdsred2-loxP-eGFP-ΔU3). Viability of islet cells was performed with dithizon-trypan blue method (Annex 1). Five hundred islets from the same donor were transplanted in mice (n=14) fed with control or HFD for 10 weeks. Cryo-sections of human grafts were analyzed for several antibodies (**Table 6**).

7. Cryosections and Laser microdissection

Laser capture microdissection was performed to specifically isolate human islet grafts transplanted under kidney capsule of control and HFD mice. Human islets grafted under kidney capsule and mice, fed with control or HFD, were sacrificed at 6, 8 and 10 weeks (n=18). Cryo-sections of mouse kidney with transplanted human islets were collected withspecific Arctrurus PEN membrane slides (Applied Biosystem). Slides were incubated for 2 minutes in 100% ethanol and 2 minutes in xylene. ArcturusXT laser microdissection system (Applied Biosystems) was used to cut the selected region which was provided by the laboratory of Professor Bart Staels in institute Pasteur of Lille, technical responsible is Jonathan Vanhoutte (**Fig 11A**). Selected region of the tissue (islet graft) was collected in specific cap capsure (macro LSM caps of Applied Biosystem) whichinserted onto microcentrifuge tube with RNA extraction buffer **(Fig 11B).** RNA isolation was performed with Arcturus Pico Pure RNA isolation kit (Applied Biosystems) (Annex 5). RNA quality was determined with Bioanalyser and agilent 2100 expert_Eukaryote Total RNA Pico kit.

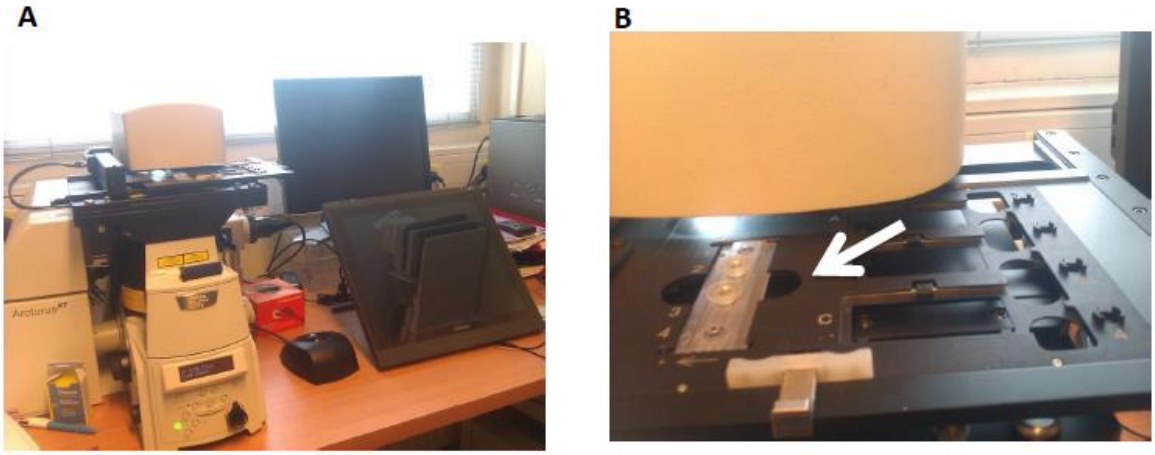


Fig 11: Laser Microdissection equipment.A) ArcturusXT laser microdissection of applied biosystems and Nikon microscopy system and B) Selection of tissue with macro LSM caps (white arrow).

8. Gene expression profile

Gene expression was measured using the Affymetrix Human Gene 2.0 ST array (Affymetrix, Santa Clara, California) at Cochin Institut (INSERM U1016, Paris). RNA was labeled using NuGEN Ovation Version 1. Labeled cDNA was hybridized to Affymetrix Human Gene 2.0 arrays and scanned with an Agilent G2500A GeneArray Scanner. In brief, RNA was reverse transcribed into cDNA and biotin-labelled cRNA was prepared by in vitro transcription (Enzo Diagnostics Inc, Farmingdale, NY). After hybridization, the arrays were scanned using the Affymetrix GeneArray GCS3000 scanner and visualized using GeneChip Operating Software (GCOS, Affymetrix). Gene expression

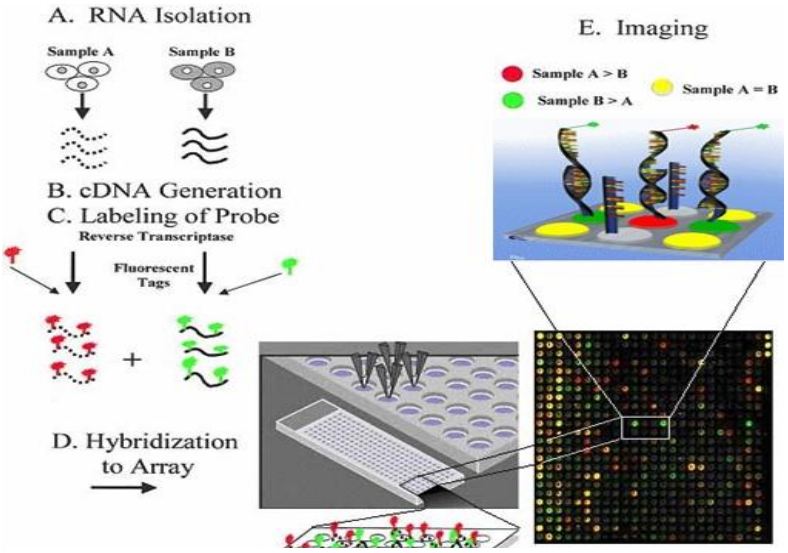


Fig 12: Gene expression microarray procedure. A) RNA isolation from human islets after laser microdissection B) Reverse transcription of RNA to cDNA, C) Probes labeling with fluorescence, D) Hybridization to array and E) Imaging

levels were normalized using the Robust Multiarray Average (RMA)(Fig 12). Microarrays analysis was performed with Ingenuity System provided by Institute Cochin in Paris.

9. Statistical analysis

Two different statistical approaches were applied. Differences between the two groups (control and HFD) in each time point were determined by non parametric Mann-Whitney test. More interestingly, changes of continuous traits overtime (period of 12 weeks) in the two groups were determined by a mixed model analysis. Diet was considered as a fixed effect, donors or mice (in the case of one donor) as a random effect and time as covariable.

Statistical analyses were performed using StatView (SAS Institute, Cary, NC, USA) and SAS (SAS Institute Inc., Cary, NC, USA). *P* values<0.05 were considered as statistically significant. Data are mean± SEM (standard error of mean).

Chapter IV

Results

1. Preview of manuscript 1: *Adaptive changes of human islets to an obesogenic environment in the mouse.*

In the first part of the manuscript, a novel *in vivo* immunodeficient obese mouse model was created. The compatibility with human islet transplantation permitted the study of human islets adaptation and function in obese environment. In parallel, analysis of endogenous pancreas confirmed that this model offers an appropriate environment for human graft expansion and increased function. Mechanisms of human islet regeneration (proliferation, neogenesis) were revealed explaining the way with which islet mass is increased in obesity. In the second part, dysfunctional human islets from type 2 diabetic patients were transplanted providing evidence for inability to adapt in obese environment.

In this manuscript, generally, we showed that:

6) Rag2 Immunodeficient mice developed obesity associated traits after 2-4 weeks on high fat diet.
7) Human islets transplanted to mice showed functional adaptation in obese environment, insulin secretion was increased and normoglycemia was maintained (2^{nd} month).
8) Human islets seemed to lose their overall function in the third month on high fat diet.
9) Dysfunctional human islets cannot adapt in obese environment and mice became hyperglycemic early in the second month.
10) Histological analysis showed progressive increase of beta cell mass during high fat diet due to islet regeneration (proliferation and neogenesis).

2. Manuscript 1

3. Unpublished data related to manuscript 1

As statistical analyses showed, there was no effect of donor in our results. In the article, the data of the four donors (D1, D2, D3, D4) were analyzed together. The following supplementary unpublished data will be presented with the same way. Data for the two diabetic donors will be given separately as in the article.

3.1 Human islet graft function during 12 weeks on high fat diet

For the mice grafted with normal human islets from non diabetic donors, fasting blood glucose was higher in HFD mice from 6 weeks with a statistical significant difference at 6 and 12 weeks, p=0.003 and p=0.002 respectively (**Fig 13A**) (2 wks: 5.5±0.2 control vs. 6.09±0.34 mmol/l HFD; 4 wks: 6.2±0.3 control vs. 6.8±0.75 mmol/l HFD; 6 wks: 5.9±0.3 control vs. 8.6±0.5 mmol/l HFD; 8 wks: 7.7±0.5 control vs. 9.1±0.5 mmol/l HFD; 12 wks: 4.6±0.48 control vs. 8.2±0.8 mmol/l HFD). Compared to mice grafted with dysfunctional islets (diabetic donors), fasting blood glucose was significantly higher over the entire time; p<0.05 (**Fig 13B**) (DD2/2 wks: 5.41±0.4 control vs. 9.7±0.47 mmol/l HFD; 4 wks: 7.3±0.75 control vs. 10.04±1.15 mmol/l HFD; 6 wks: 4.7±0.44 control vs. 8.67±0.63 mmol/l HFD; 8 wks: 3.39±0.08 control vs. 8.06±1.12 mmol/l HFD; 12 wks: 6.3±0.28 control vs. 8.89±0.56 mmol/l HFD). Values for blood glucose were higher for the mice grafted with islets provided by D1 donor treated 10 years for type 2 diabetes (**Fig 13C)** (DD1/2 wks: 5,951±0.16 control vs. 10.38±0.71 mmol/l HFD; 4 wks: 9.3±0.46 control vs. 13.42±0.72 mmol/l HFD; 6 wks: 7.8±0.85 control vs. 12.19±0.37 mmol/l HFD; 8 wks: 5.9±1.8 control vs. 11.24±0.46 mmol/l HFD; 12 wks: 7.5±0.67 control vs. 9.2±0.12 mmol/l HFD).

Fasting C-peptide was slightly higher from 8 weeks on high fat diet with p=0.04 (2 wks: 172.42±28.75 control vs. 479.54±148.84 pmol/l HFD; 4 wks: 388±113.4 control vs 523.546±136.96 pmol/l HFD; 6 wks: 386.17±147.445 control vs. 507.05±0.5 pmol/l HFD; 8 wks: 237.156±130.278 control vs. 956.83±130.27 pmol/l HFD; 12 wks: 370.89±97.91 control vs. 1081.41±216.32 pmol/l HFD) (**Fig 13D**). On the other hand, no difference in fasting c-peptide was observed between the two groups in mice grafted with dysfunctional islets with lower secretion for DD1 (**Fig 13E, F**). (DD2/2 wks: 332.5±12.31 control vs. 369.36±32.51 pmol/l HFD; 4 wks: 282.87±36.83 control vs. 372.01±22.65 pmol/l HFD; 6 wks: 312.35±83.98 control vs. 554.23±47.97 pmol/l HFD; 8 wks: 237.11±120.8 control vs. 425.87±21.33 pmol/l HFD; 12 wks: 362.65±58.58 control

vs. 412.23±19.19 pmol/l HFD) and (DD1/2 wks: 111.92±16.18 control vs. 79±4.7 pmol/l HFD; 4 wks: 120.94±24.69 control vs. 50.36±17.76 pmol/l HFD; 6 wks: 37.51±1.5 control vs. 29.5±12.52 pmol/l HFD; 8 wks: 461.74±47.43 control vs. 392.34±31.82 pmol/l HFD; 12 wks: 387.54±24.62 control vs. 256.01±15.45 pmol/l HFD).

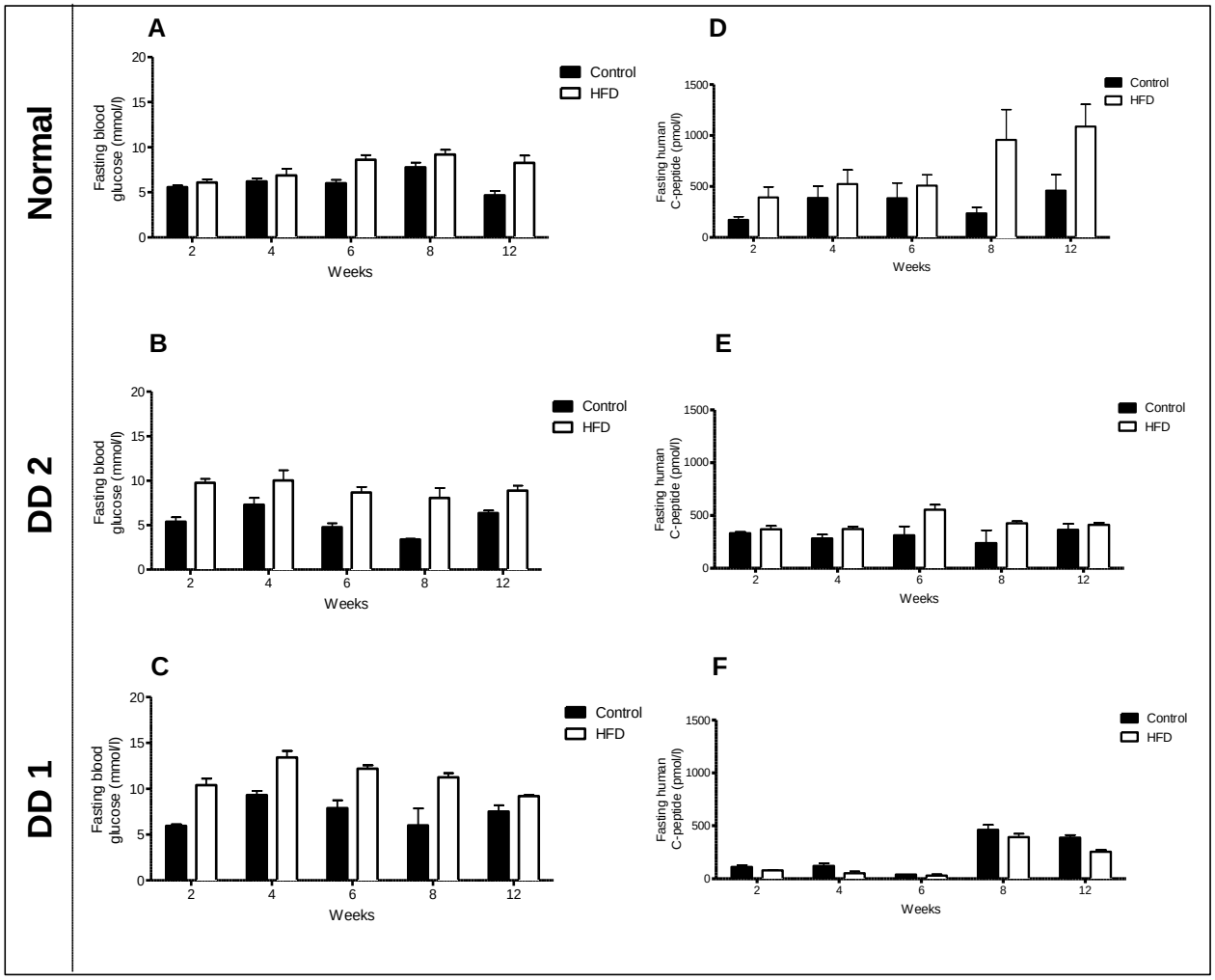


Fig 13: Fasting blood glucose and human C-peptide for mice grafted with islets (normal or diabetic donors). A) normal donors (D1, D2, D3, D4) B) Diabetic donor 2 (DD2) C) diabetic donor 1 treated for 10 years (DD1) and fasting C-peptide for the same groups D) normal donors (D1, D2, D3, D4) E) diabetic donor 2 (DD2) F) diabetic donor 1 (DD1).

Human C-peptide expressed as a function of glycemia was also higher at 8 and 12 weeks on high fat diet mice transplanted with islets from normal donors; no statistically significant difference was observed (**Figure 14**) (2 wks: 30.09±4.69 control vs. 55.5±10.25 pmol/mmol HFD; 4 wks: 61.57±19.05 control vs. 79.27±15.94 pmol/mmol HFD; 6 wks: 65.19±28.6 control vs. 59.14±12.74 pmol/mmol HFD; 8 wks: 32.77±5.09

control vs. 114.24±35.46 pmol/mmol HFD; 12 wks: 107.92±38.35 control vs. 193.24±48.3 pmol/mmol HFD).

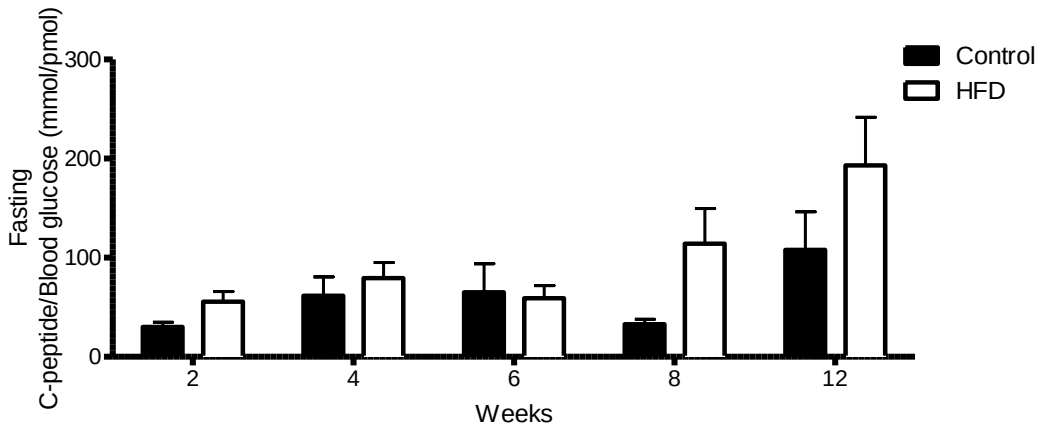


Fig 14: Fasting C-peptide expressed as a function of blood glucose for mice grafted with islets from normal donors (D1, D2, D3, D4) at 2, 4, 6, 8 and 12 weeks on high fat diet.

3.2 Kinetic study of normal human islet adaptation (results of n=1 donor)

Kinetic study was performed to evaluate normal human islet adaptation. BrdU was administrated in the drinking water for 7 days prior to killing at 4, 6, 8 and 12 weeks. Mice of this study group had higher blood glucose at 10-12 weeks (2 wks: 5.3±0.13 control vs. 6 ±0.25 mmol/l HFD; 4 wks: 5.9±0.49 control vs. 4.9±1.08 mmol/l HFD; 6 wks: 6.6±0.27 control vs. 8.37±0.7 mmol/l HFD; 8 wks: 8.62±0.41 control vs. 8.08±0.34 mmol/l HFD; 10 wks: 6.7±0.11 control vs. 12.51±0.52 mmol/l HFD; 12 wks: 6.57±0.3 control vs. 10.28±0.55 mmol/l HFD) (**Fig 15A**). However, mixed model analysis showed that neither diet nor diet duration had significantly effect on blood glucose levels ($p>0.05$). Moreover, fasting C-peptide was significantly higher from 6 to 12 weeks (2 wks: 186.78±19.57 control vs. 215.95±18.97 pmol/l HFD; 4 wks: 236±11.4 (control vs. 252.26±63.54 pmol/l HFD; 6 wks: 166.17±13.84 control vs. 291.96±42.35 pmol/l HFD; 8 wks: 282.195±47.71 control vs. 491.64±68.78 pmol/l HFD; 10 wks: 261.75±14.59 control vs. 479.32±107 pmol/l HFD; 12 wks: 120.47±1.17 control vs. 297.95±68.16 pmol/l HFD) (**Fig 15B**). In this case, C-peptide seemed to be unaffected by the type of diet (control or HFD), but it was significantly influenced by diet duration in HFD mice ($p=0.01$).

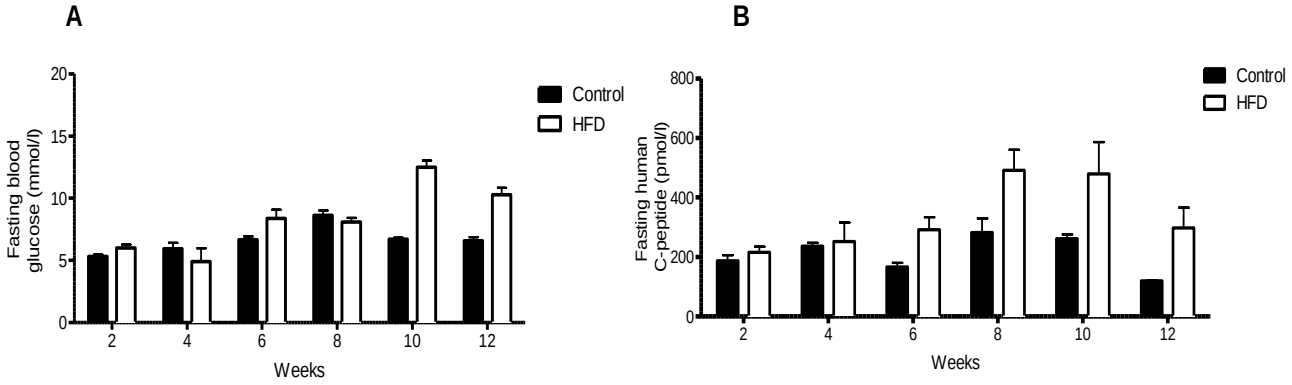


Fig 15: Fasting blood glucose and human C-peptide for mice grafted with normal islets (Kinetic study). A) Fasting blood glucose of mice transplanted with normal islets and B) fasting C-peptide of human islets

Human C-peptide expressed as a function of glycemia was also higher at 6, 8 and 12 weeks on high fat diet mice of kinetic study with significant difference at 8 weeks (p=0.02) **(Fig 16)** (2 wks: 35.15±3.66 control vs. 36.1±3.17 pmol/mmol HFD; 4 wks: 44.25±6.49 control vs. 48.48±6.24 pmol/mmol HFD; 6 wks: 25.12±2.12 control vs. 32.14±4.74 pmol/mmol HFD; 8 wks: 32.77±5.09 control vs. 60.33±7.21 pmol/mmol HFD; 10 wks: 39.05±2.81 control vs. 38.73±10.16 pmol/mmol HFD; 12 wks: 18.36±0.66 control vs. 28.69±5.09 pmol/mmol HFD). Longitudinal analysis (mixed model) did not reveal significant difference for C-peptide/glycemia ratio due to higher levels of blood glucose in HFD mice at 10 and 12 weeks.

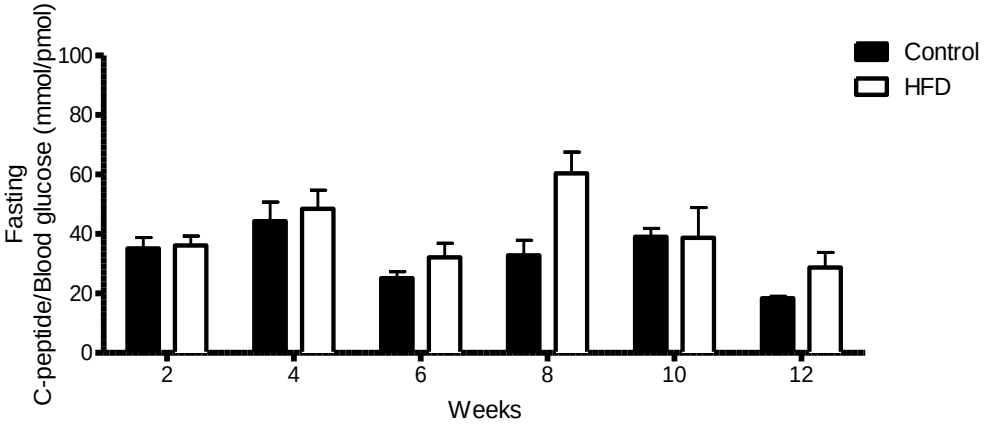


Fig 16: Fasting c-peptide expressed as a function of blood glucose for mice (kinetic study).

3.3 Morphometric analysis of endogenous mouse pancreas in kinetic study

Morphometric analysis in 633 (325 control/308 HFD) islets showed that total endocrine area increased progressively in HFD mice and was five times higher at 12 weeks ($p<0.05$) (**Fig 17A**). Lower endocrine cell area was observed at 4 weeks ($p>0.05$) (4 wks: 0.404±0.06 control vs. 0.26±0.04 % HFD; 6 wks: 0.29±0.07 control vs. 0.52±0.11 % HFD; 8 wks: 0.26±0.05 control vs. 0.4±0.12 % HFD; 12 wks: 0.1±0.01 control vs. 0.64±0.07 % HFD). Difference in islet number was observed only at 12 weeks ($p=0.01$; 12 wks: 3.56±0.54 control vs. 8.3±1.69 x 10^{-4} % HFD) (**Fig 17B**).

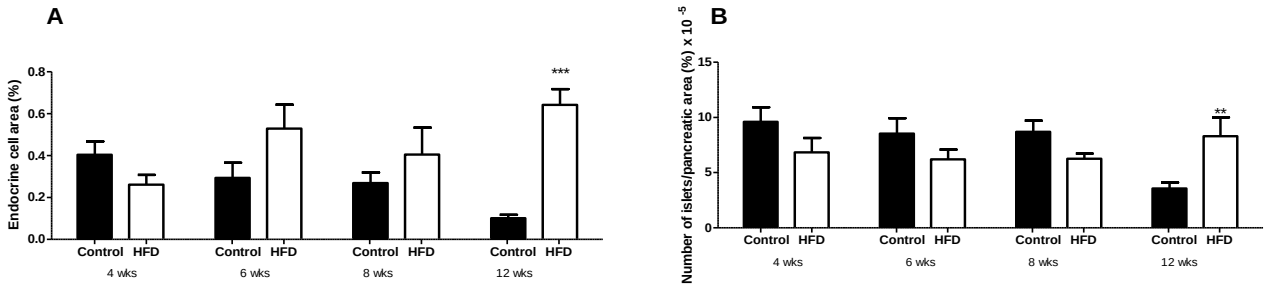


Fig 17: Morphometric analysis of endogenous mouse pancreas. A) Endocrine cell area expressed in total pancreatic area (%) and B) number of islets in pancreatic area (%)

Further analysis was performed for the islet size. Islets seemed to become larger in HFD mice as significantly more small islets (0-5000μm^2) were observed at 4 weeks ($p=0.024$) and significantly more big islets (>10000μm^2) were observed at 12 weeks in HFD mice ($p=0.01$). There was difference in islet with size 5000-10000μm^2 between two groups (**Fig 18).**

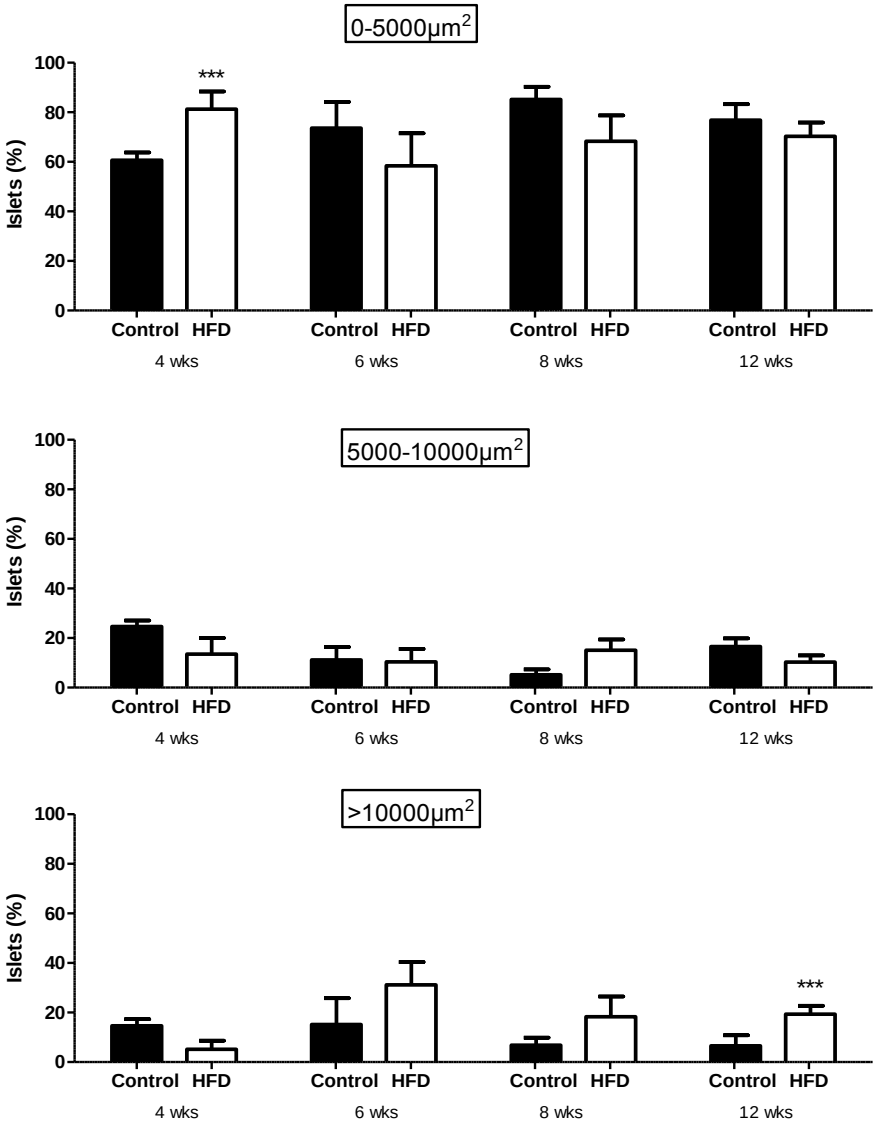


Fig 18: Classification of mouse islets according to their size. (0-5000µm^2; 5000-10000µm^2; >10000µm^2) in mice at 4, 6, 8 and 12 weeks on control and HFD

Finally, BrdU-insulin staining revealed the proliferation levels in endogenous pancreas (**Fig 19A**). Quantification of BrdU+ Ins+ cells in total islet cells showed that proliferation peaked at 6 and 12 weeks with significant difference at 12 weeks (p=0.02) (4 wks: 3.5±1.11 control vs. 2.66±1.9 % HFD; 6 wks: 9.72±1 control vs. 26.1±19.9 % HFD; 8 wks: 6.9±2.34 control vs. 9.38±2.9 % HFD; 12 wks: 30.01±1.64 control vs. 39.8±4.94 % HFD) (**Fig 19B**).

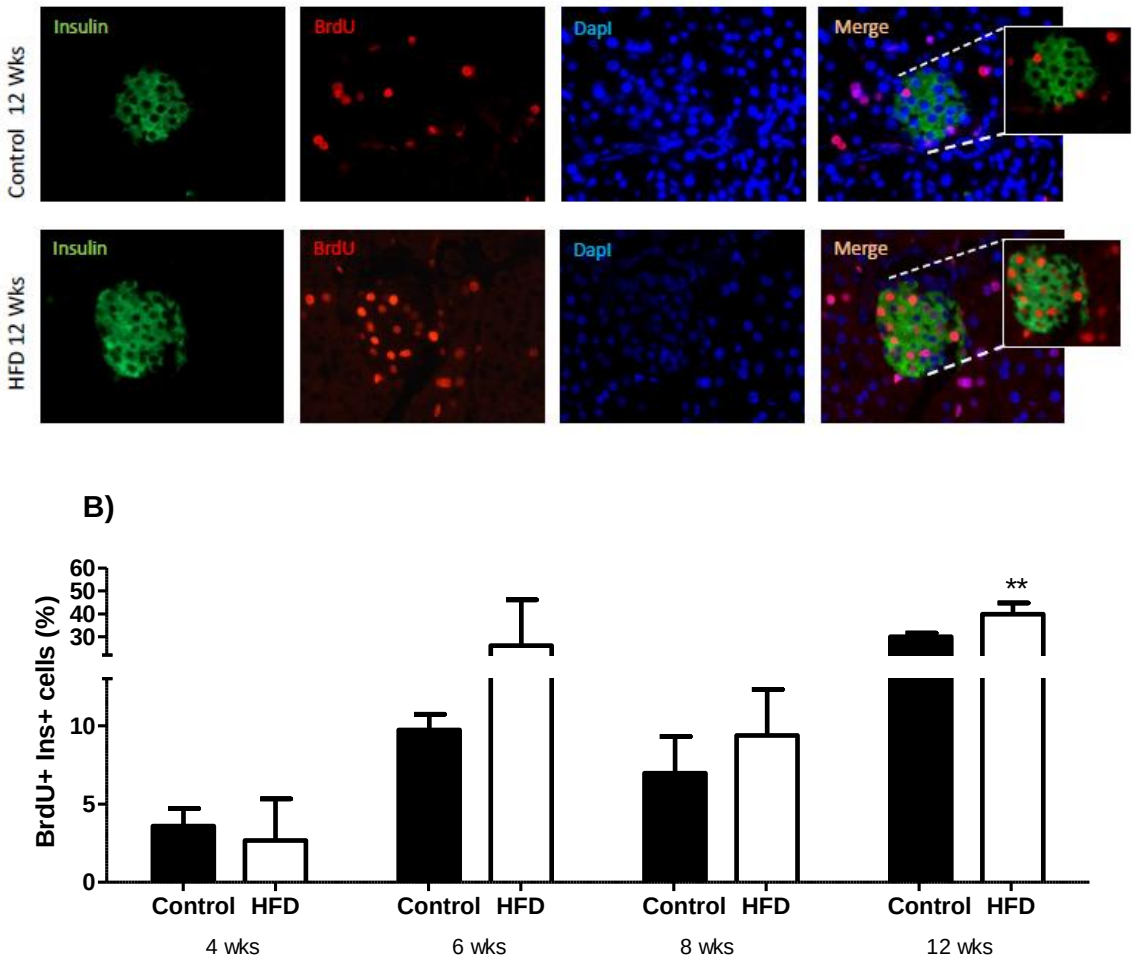


Fig 19: Proliferation of mouse beta cells. A) Representative sections of paraffin-embedded endogenous pancreas stained by Insulin (green), BrdU (red) and DAPI (blue) B) Quantification of BrdU+Ins+ expressed in total beta cells (Ins+) in mice at 4, 6, 8 and 12 weeks on control diet and HFD

3.4 Morphometric analysis of human grafts of kinetic study

Human graft volume, beta and alpha cell volume were measured for all mice of kinetic study as described in the article. Human endocrine volume increased progressively in mice after 8 weeks on high fat diet. Triple endocrine volume was observed at 12 weeks on HFD (0.021±0.02 control vs. 0.057±0.02 mm^3 HFD) (**Fig 20A**). Interestingly, human beta and alpha cell volume maintained in control mice over time with small increase of alpha cell volume at 12 weeks. Contrary, beta cell volume was higher at 4 weeks, decrease at 6 weeks and increase at 8 and 12 weeks on HFD mice compared to control group (4 wks: 0.013±0.0004 control vs. 0.02±0.001 mm^3 HFD; 6 wks: 0.0084±0.002 control vs. 0.008±0.0009 mm^3 HFD; 8 wks: 0.009±0.0005 control vs. 0.018±0.007 mm^3 HFD; 12 wks: 0.014±0.0007 control vs. 0.04±0.0008 mm^3 HFD)(**Fig 20B**). Alpha cell

volume was lower at 4 weeks (0.008±0.0004 control vs. 0.005±0.0017 mm^3 HFD) but remained higher after 6 weeks on HFD mice (vs. control) (**Fig 20C**).

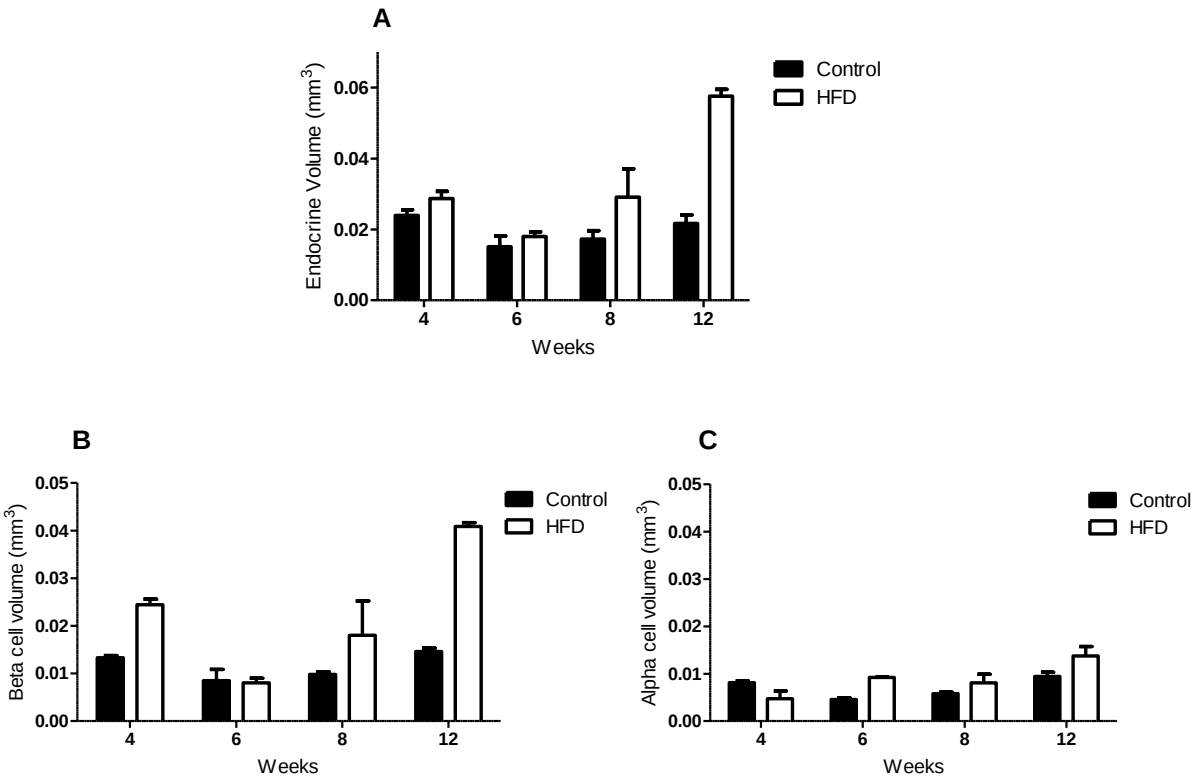


Fig 20: Morphometric analysis of human endocrine cell volume (grafts). A) total human endocrine volume (stained by chromogranin A) B) human beta cell volume (stained by c-peptide) and C) human cell volume (stained by glucagon) in mice fed with control and high fat diet 4, 6, 8 and 12 weeks

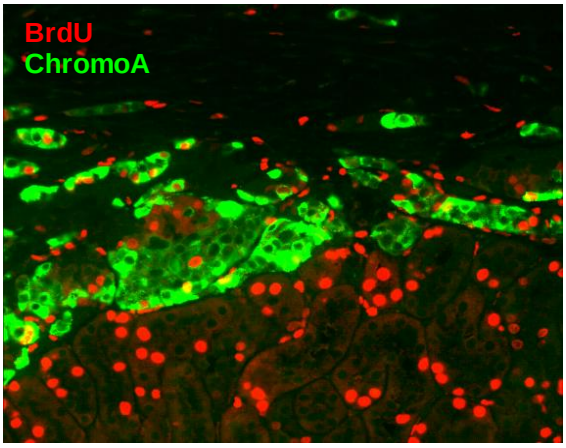
3.5 Evidence for human endocrine cell proliferation (n=1)

In our initial study design, BrdU was injected one day prior to sacrifice, however proliferation levels measured with BrdU or Ki67 were low. Therefore in subsequent studies after validation, BrdU was administered 7 days prior to sacrifice in drinking water. The following kinetic proliferation results were performed on one series of animals, in which BrdU was injected 7 days before sacrifice at 4, 6, 8 and 12 weeks. Double staining with anti BrdU- chromogranin A, C-peptide, glucagon or somatostatin was performed to determine proliferation level of total endocrine, beta, alpha or delta cells respectively (**Fig 21A, C, E, G**). More than 40000 cells were measured to determine the proliferation levels of the different types of human endocrine cells.

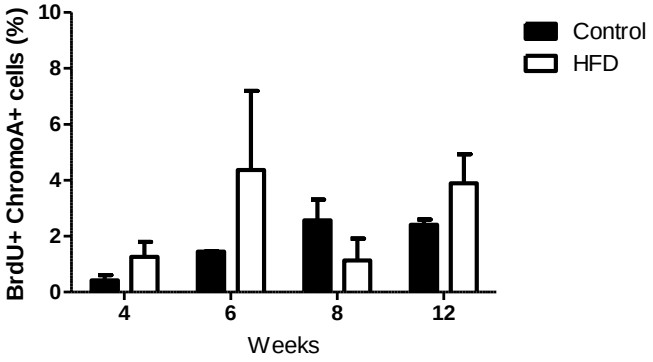
Morphometric analysis revealed that proliferation of human endocrine cells was higher at 4, 6 and 12 weeks and lower at 8 weeks on HFD mice (vs control) (Fig 21B) (4 wks: 0.4±0.18 control vs. 1.2±0.5% HFD; 6 wks: 1.4±0.007 control vs. 4.3±2.8% HFD; 8 wks: 2.56±0.74 control vs 1.14±0.7% HFD; 12 wks: 2.4±0.18 control vs. 3.8±1.044% HFD). The same proliferation profile was observed for the human beta cells which peaked at 6 and 12 weeks for the grafts in HFD mice vs controls (Fig 21D) (4 wks: 0.44±0.14 control vs. 1.49±0.04% HFD; 6 wks: 0.83±0.16 control vs. 4.1±2.8% HFD; 8 wks: 2.24±0.002 control vs. 0.83±0.16% HFD; 12 wks: 1.83±0.46 control vs. 4.99±0.33% HFD).

Interestingly, higher levels of alpha cell proliferation were observed both for control and HFD grafts at 6 weeks compared to 4 weeks (4 wks: 0.75±0.18 control vs. 0.59±0.15% HFD; 6 wks: 2.5±0.3 control vs. 2.53±0.36% HFD). However, alpha cell proliferation was significantly reduced at 8 weeks and re-peaked at 12 weeks in HFD mice (**Fig 21F**) (8 wks: 2.8±2.03 control vs. 0.38±0.2 % HFD; 12 wks: 1.96±1.7 control vs. 5.57±0.95% HFD). Finally, few proliferating delta cells were observed after BrdU staining, but surprisingly, all were detected in grafts in HFD mice (Fig 21H).

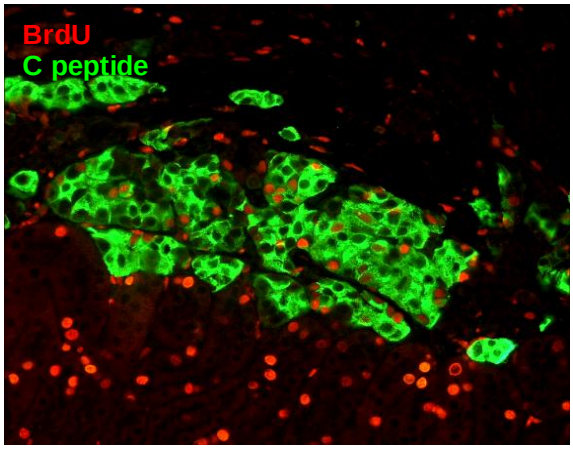
A


BrdU
ChromoA

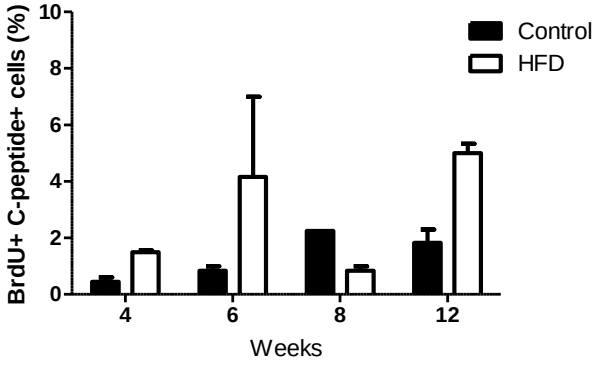
B


BrdU+ ChromoA+ cells (%)
10
8
6
4
2
0
4
6
8
12
Weeks
Control
HFD

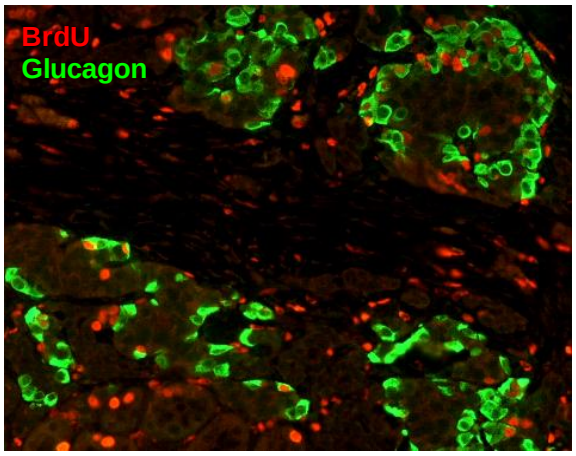
C


BrdU
C peptide

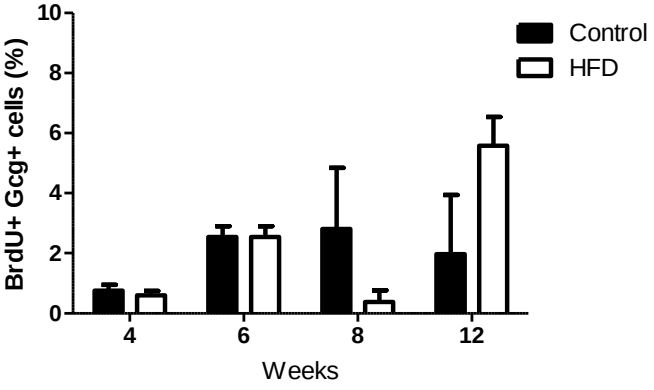
D


BrdU+ C-peptide+ cells (%)
10
8
6
4
2
0
4
6
8
12
Weeks
Control
HFD

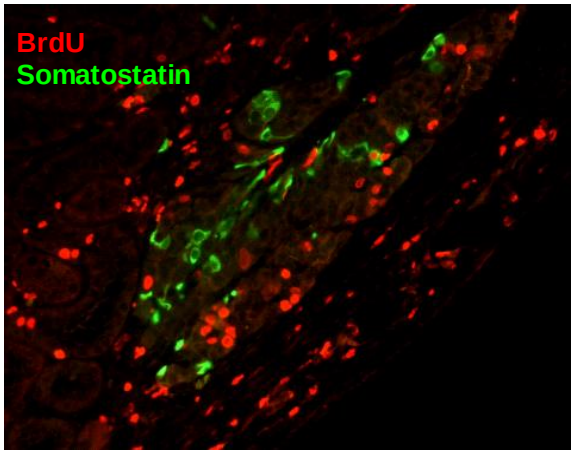
E


BrdU
Glucagon

F


BrdU+ Gcg+ cells (%)
10
8
6
4
2
0
4
6
8
12
Weeks
Control
HFD

G


BrdU
Somatostatin

H

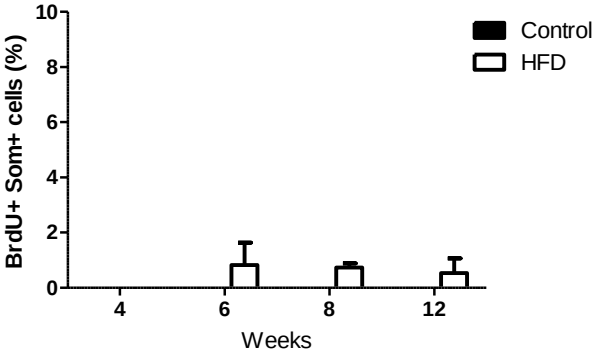

BrdU+ Som+ cells (%)
10
8
6
4
2
0
4
6
8
12
Weeks
Control
HFD

Fig 21: Determination of proliferation on human endocrine cells in human grafts. A) Double staining of anti-BrdU and chromogranin A in paraffin sections of human islets transplanted in mice fed with control or HFD B) Morphometric analysis for total human endocrine cell proliferation C) Double staining of anti-BrdU and c-peptide in paraffin sections of the same human grafts D) Morphometric analysis for human beta cell proliferation E) Double staining of anti-BrdU and glucagon in paraffin sections of the same human grafts F) Morphometric analysis for human alpha cell proliferation G) Double staining of anti-BrdU and somatostatin in paraffin sections of the same human grafts H) Morphometric analysis for human delta cell proliferation.

3.6 Evidence for human endocrine cell neogenesis

Neogenesis was determined by double staining anti CK19-chromogranin A a pan- endocrine marker as described in the manuscript 1. Furthermore, double staining of anti-CK19 with glucagon or insulin (**Fig 22**) revealed that 80% of CK19+ChromoA+ cells were CK19+Gcg+ cells.

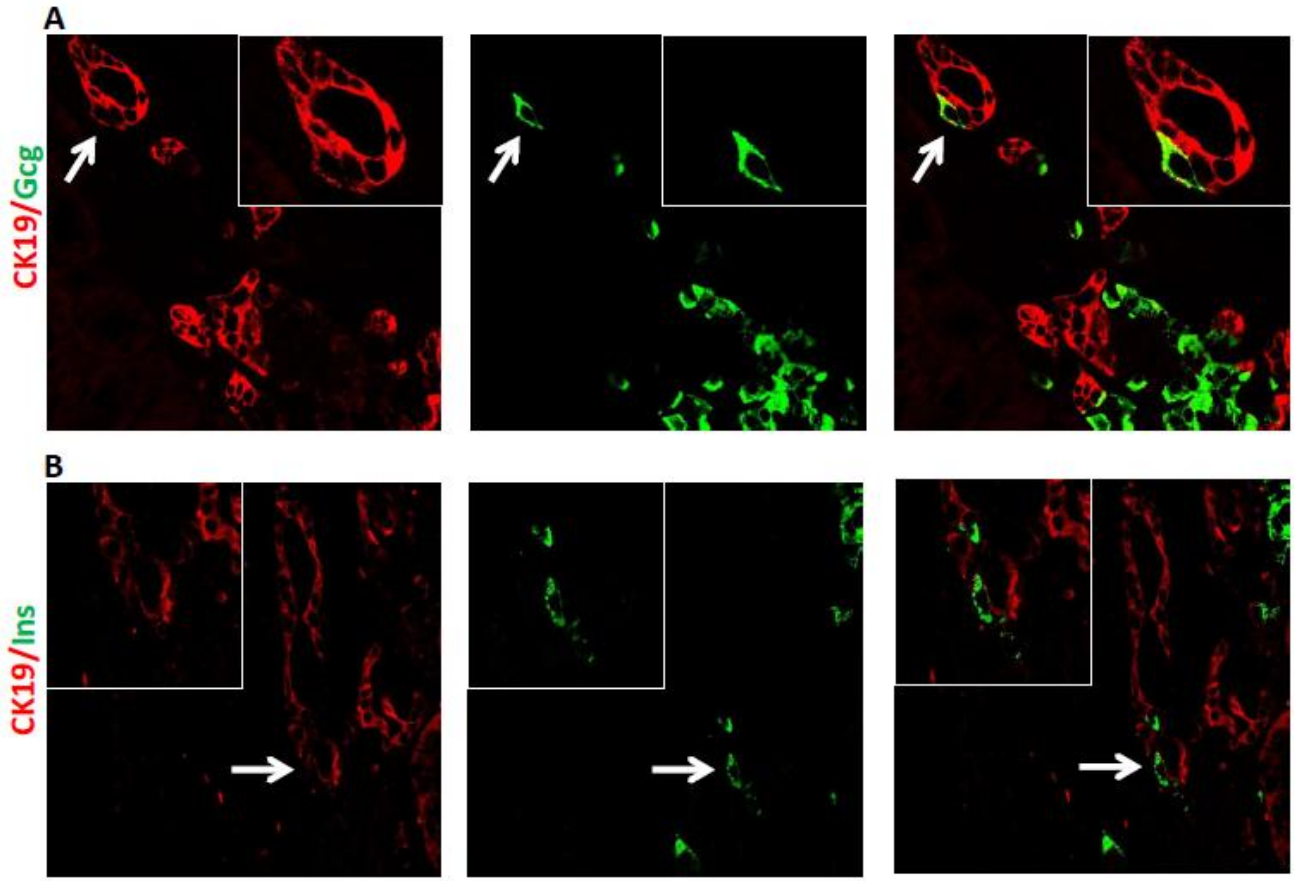


Fig 22: Determination of neogenesis in human endocrine cells in human grafts. A) Double staining of anti-CK19 (red) and glucagon (green) and B) Double staining of anti-CK19 (red) and insulin (green) in paraffin sections of human islets transplanted in mice fed with

control or HFD; white arrows show co-localization of duct cells with alpha or beta cell respectively.

4. Lineage Tracing: Preliminary results of a pilot study

4.1 Permanent Human islet tagging by transfection and function after transplantation

To determine which cells (beta or alpha) give rise to the doubling of the human islet mass 12 weeks after HFD (vs controls) we performed, in collaboration with P Ravassard, two experiments to permanently tag human beta cells and alpha cells within primary islets with the cre-lox technology, as previously published by their lab. One aliquot of human islets was transfected with the vectors RIP-CRE and CMV- LOX and another aliquot with GLU-CRE and CMV-LOX. Transfection with only CMV-LOX vector was also performed as a control. Viability of islets was reduced after transfection determined by trypan blue (Table 7)

Table 7: Viability of human islets after transfection

Viability (%)	Without vectors	CMV-LOX	RIP-CRE+CMV-LOX	GLU-CRE+CMV-LOX
Before transfection	95			
After transfection	87.35	69.77	76.88	72.38

One and a half hours after transfection, human islets were washed and transplanted under kidney capsule of mice. A preliminary viral safety study was performed to quantify the viral count in each wash after transfection in collaboration with Morvane Colin (INSERM U837 Lille) in order to ensure that there was no manipulator risk during transplantation. Following transplantation (Figure 23 shows study design), mice were fed either with control or high fat diet for 10 weeks. Islets without vectors were transplanted in mice to validate the self-expression of eGFP and c-myc. Figure 24A confirms the weight gain of mice fed with control of HFD over 10 weeks, with increased body weight in HFD mice after 2 weeks ($p<0.001$ vs controls). One control mouse died just after transplantation and one HFD died at 2 weeks due to infection.

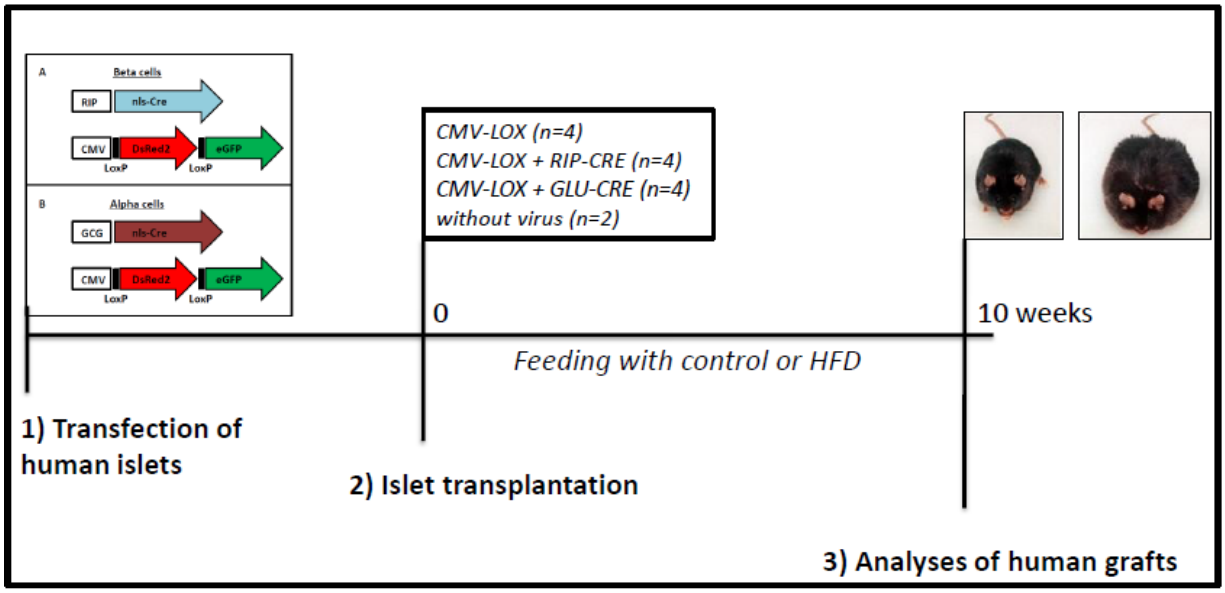


Fig 23: Lineage Tracing Study design: 1) Human islets transfected independently with the two pairs of vectors (CMV-LOX+RIP-CRE or CMV-LOX+GLU-CRE) or only with CMV-LOX ; 2) Islet transplantation was performed in day 0 and mice were fed 10 weeks with control aqnd HFD; 3) Human grafts were analyzed at 10 weeks

Fasting blood glucose remained in the same levels for control mice over 10 weeks. HFD mice had higher levels after 2 weeks (vs. control) corresponding to weight gain difference with significant difference only at 6, 8 and 10 weeks (Fig 24A) (6 wks: 7.09±0.49 control vs. 9.47±0.56 mmol/l HFD, p=0.009; 8 wks: 6.66±0.15 control vs. 8.11±0.29 mmol/l HFD, p=0.001; 10 wks: 6.5±0.29 control vs. 9.34±0.67mmol/l HFD, p=0.003). Fasting human C-peptide was also measured for transplanted islets after transfection. C-peptide levels were determined according to baseline level (feeding start) as islet cell viability was different after transfection. For control mice, C-peptide secretion was maintained over time with minor increase at 10 weeks. However, C-peptide levels were increased in HFD mice peaked at 4 weeks and remaining higher compared to control mice (**Fig 24B**) (2 wks: 83.09±24.76 control vs. 171.7±29.08% HFD, p=0.0042; 4 wks: 89.61±21.27 control vs. 232.61±31.05% HFD, p=0.003; 6 wks: 78.55±10.81 control vs. 139.98±22.98% HFD, p=0.03; 8 wks: 69.3±13.24 control vs. 128.47±43.49% HFD, p=0.4; 10 wks: 180.238±55.56 control vs. 132.72±37.82% HFD, p=0.4).

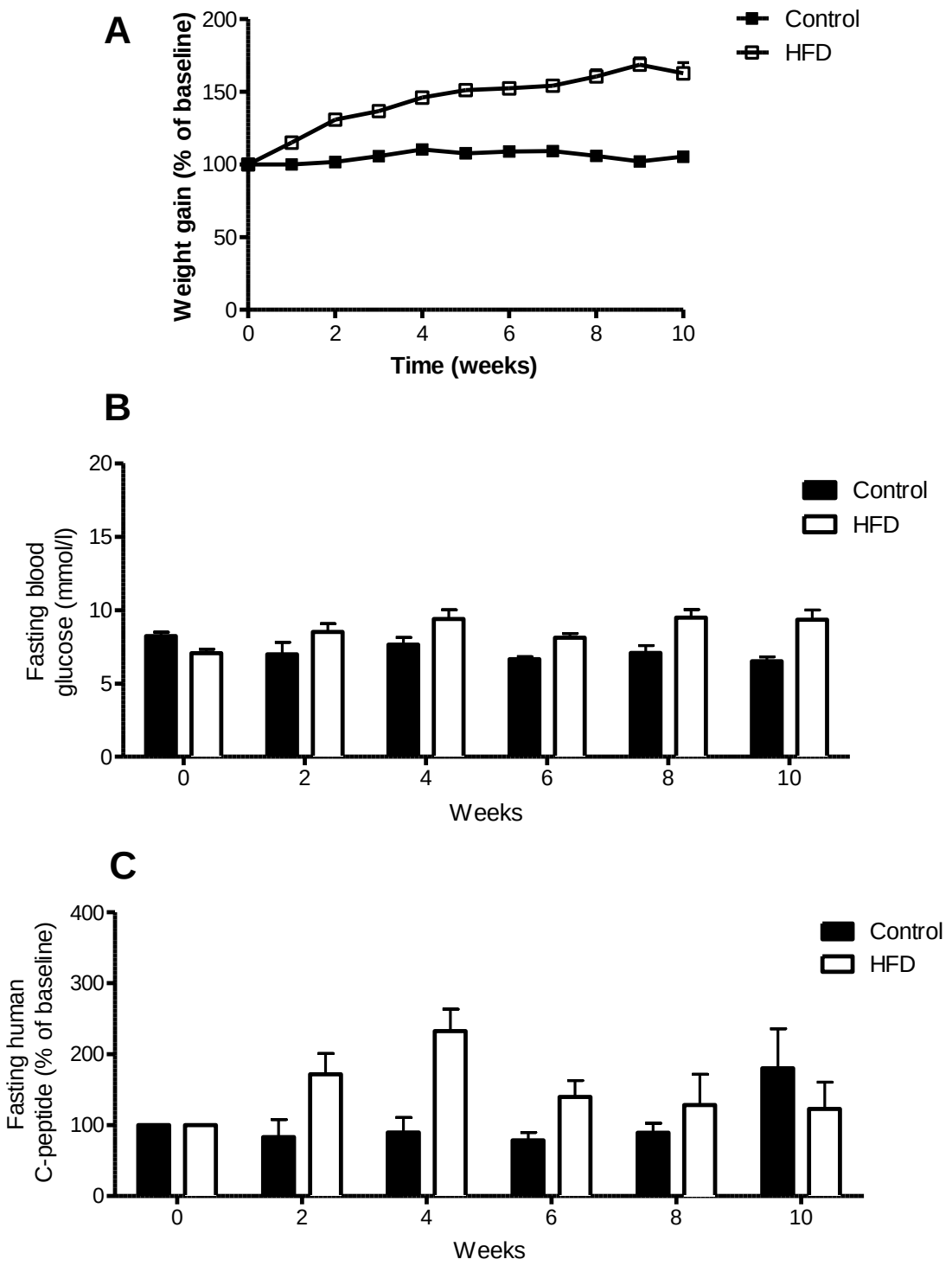


Fig 24: Weigh gain, fasting blood glucose and human C-peptide for mice grafted with islets (Lineage tracing study). A) Weight gain (% of baseline) over 10 weeks in animals grafted with human islets and fed with control or high fat diet (Lineage Tracing Study) B) Fasting blood glucose of mice transplanted with normal islets fed with control and HFD and C) fasting c-peptide of human islets transfected with viral vectors before transplantation

4.2 Validation of viral vector labeling of human islets

Islets without vectors were transplanted in mice to validate the self-expression of eGFP and cmyc. Quadruple immunostainings (anti-GFP, anti-cmyc, anti-ins and anti-gcg) were used to determine the GFP and cmyc expression in alpha and beta cells of grafts in cryo sections. Confocal microscopy revealed that non-transfected human islets were completely negative for GFP and cmyc expression.

Validation of CMV-LOX vector

Transfection with only CMV-LOX vector was performed in human islets before transplantation in control and HFD mice. Staining with four antibodies for insulin, glucagon, GFP and c-Myc showed co-expression of c-Myc and insulin or glucagon (**Fig 25**). Few cells positive for c-myc and negative for insulin or glucagon were also identified. Measurements revealed that 20% of grafted cells were positive for c-Myc after transfection with only CMV-Lox. Contrary, GFP staining was completely negative as expected in islets transplanted both in control and HFD mice.

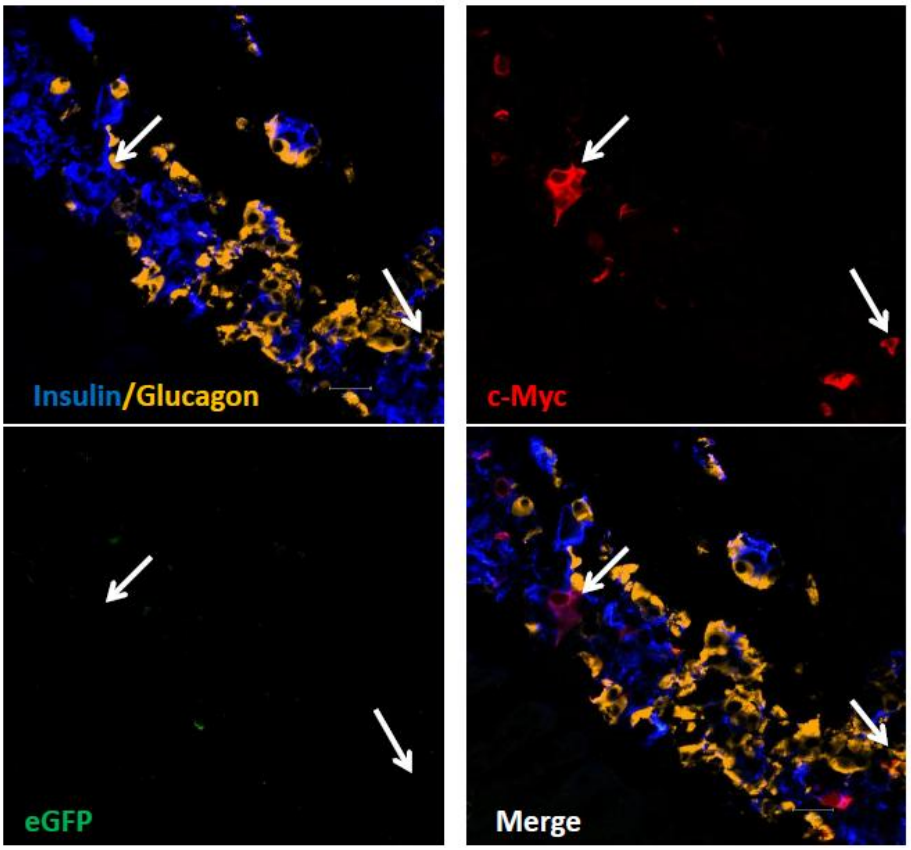


Fig 25: Staining of c-myc and GFP after transfection only by CMV-Lox vector. Transfection of human islets with CMV-Lox vector: staining with anti- insulin (blue), glucagon (orange), c-Myc (red) and eGFP (green) in human grafts; co-localization of c-Myc and insulin or glucagon was determined by confocal microscope (white arrows).

Tagging human beta cells by transfection with RIP-CRE and CMV-LOX vectors

Two hundred human islets were cultured one week after transfection with RIP-CRE and CMV-LOX vectors for the validation of the specificity of the tagging method with viral vectors. Islets were stained for insulin, glucagon, c-Myc and GFP. Only one glucagon positive cell was found to express GFP after one week in culture confirming the specificity of beta cells for RIP-Cre vector.

Human islet grafts were also analyzed 10 weeks post transplantation with the same antibodies. GFP expression was observed only in ins+ or gcg+ cells (alpha or beta). More GFP+ cells were found in grafts on HFD mice (~12.5% vs 10% control). Confocal microscope revealed co-localisation of GFP-insulin and GFP-glucagon staining proposing conversion of beta cells to alpha cells (**Fig 26**).

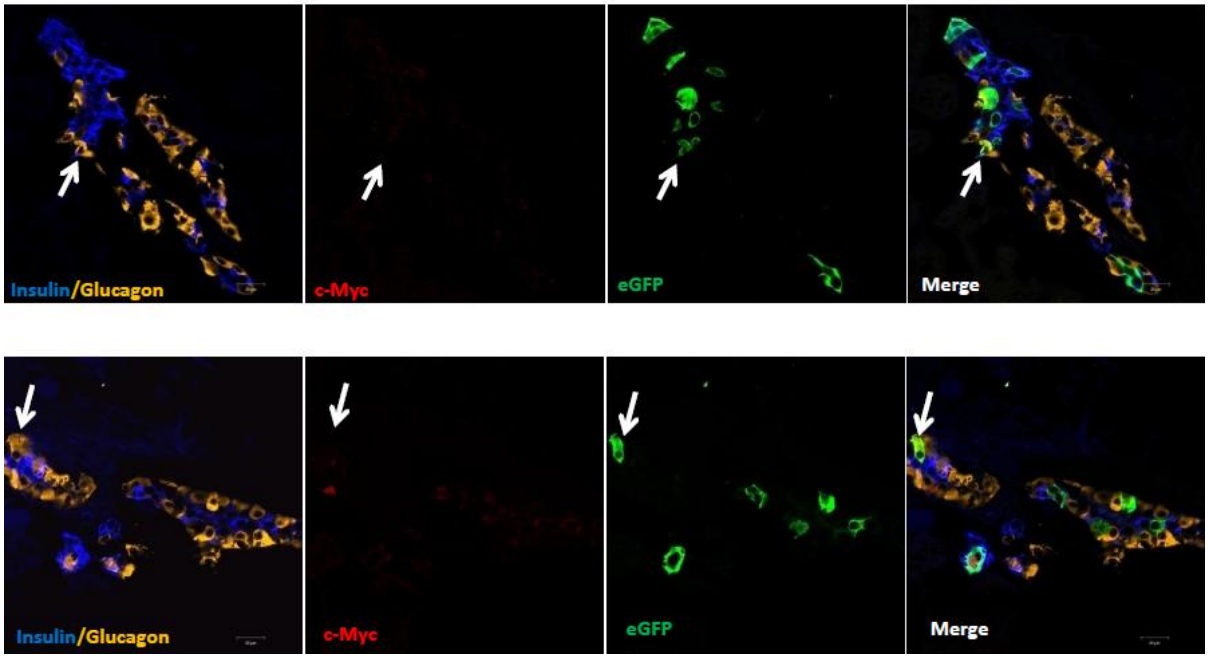


Fig 26: Staining of c-myc and GFP after transfection only by CMV-Lox vector and RIP-cre vectors. Transfection of human islets with CMV-Lox and RIP-cre vectors: staining with anti-insulin (blue), glucagon (orange), c-Myc (red) and eGFP (green) in human grafts; co-localization of GFP with insulin or glucagon was determined by confocal microscope; white arrows show GFP+GCG+ cells.

Quantification of double staining cells showed more GFP+Ins+ cells than GFP+Gcg+ in both control and HFD mice as expected but without a difference in conversion between control and HFD mice (**Table 8**).

Table 8: Analysis of cell conversion after transfection of human islets (CMV-Lox and RIP-cre vectors). Transfection of human islets with CMV-Lox and RIP-cre vectors: quantification of double staining GFP+Ins+ and GFP+Gcg+ cells in human islets grafted in control or HFD mice; percent(%) was determined by total GFP+ or by total ins+ and gcg+ cells.

	GFP+Ins+/GFP+(%)	GFP+Gcg+/GFP+(%	GFP+Ins+/Total(%	GFP+Gcg+/Total(%
Control	75.28	24.71	7.47	2.45
HFD1	63.72	35.62	7.66	4.28
HFD2	84.05	13.04	10.39	1.61

Tagging human alpha cells by transfection with GLU-CRE and CMV-LOX vectors

Islets were also cultured one week after transfection for the validation of the method. In this case, very few insulin positive cells were found to express GFP after one week in culture confirming the specificity of Glu-cre vector.

Human islet grafts were also analyzed with insulin, glucagon, GFP and c-Myc antibodies. GFP expression was observed in ins+ or gcg+ and also in ins-gcg- cells (non alpha or beta). More GFP+ cells were found in grafts on HFD mice (~17,3% vs 13% and 16% in controls). Confocal microscope revealed co-localization of GFP-insulin and GFP-glucagon staining proposing conversion of alpha cells to beta cells (**Fig 27**).

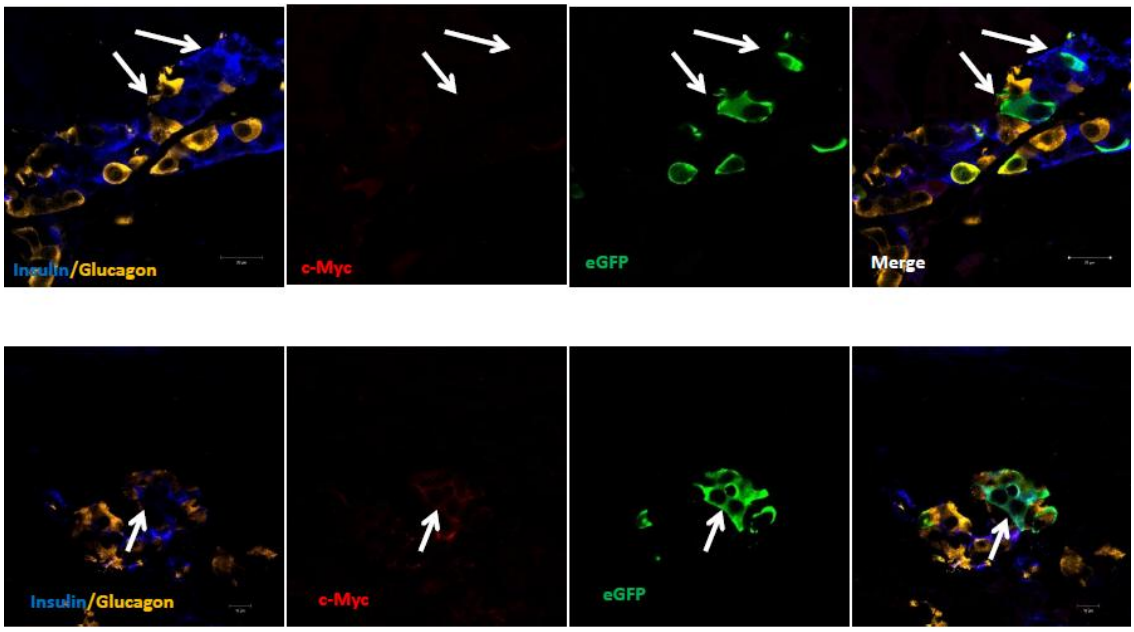


Fig 27: Staining of c-myc and GFP after transfection only by CMV-Lox vector and Glu-cre vectors. Transfection of human islets with CMV-Lox and Glu-cre vectors: staining with anti-insulin (blue), glucagon (orange), c-Myc (red) and eGFP (green) in human grafts; co-localization of GFP with insulin or glucagon was determined by confocal microscope; white arrows show GFP+Ins+ cells

Quantification of double staining cells showed slightly more GFP+Gcg+ cells than GFP+Ins+ in both control and HFD mice as expected but without a difference in the percent of conversion between control and HFD mice (**Table 9**).

Table 9: Analysis of cell conversion after transfection of human islets (CMV-Lox and Glu-cre vectors. Transfection of human islets with CMV-Lox and Glu-cre vectors: quantification of double staining GFP+Ins+ and GFP+Gcg+ cells in human islets grafted in control or HFD mice; percent(%) was determined by total GFP+ or by total ins+ and gcg+ cells

	GFP+Ins+/GFP+(%	GFP+Gcg+/GFP+(%	GFP+Ins+/Total(%	GFP+Gcg+/Total(%
Control1	41,53	56,54	5,53	7,53
Control2	43,14	54,67	7,04	8,92
HFD	42,42	52,56	7,34	9,10

5. Kinetic study of gene expression profiling in human islet grafts during adaptation to obesity by high fat diet

The goal of this experiment was to follow the gene expression profiles during the adaptation of human islet grafts after 6, 8, 10 weeks HFD in comparison to control fed animals transplanted with the same human islet donor. For this study we purposely transplanted human islets from a young donor. Following transplantation, animals were sacrificed at 6, 8 and 10 weeks, and human grafts were snap frozen in isopentane in preparation for subsequent laser capture microdissection which aimed to minimize the contaminating murine tissue. After extraction, quantification, and quality controls, RNA was shipped to Hopital Cochin (Inserm U1016) for Affymetrix chip analysis. Remaining RNA and frozen sections will be subsequently used respectively to confirm chip data on an RNA and protein level.

5.1 Confirmation of the adaptation of human islets to obesity induced by high fat diet

In this series of animals, mice were fed with control or high fat diet for 10 weeks. **Figure 28** confirms the weight gain of mice fed with control of HFD over 10 weeks. HFD mice significantly increased body weight after 2 weeks ($p<0.001$ vs. controls).

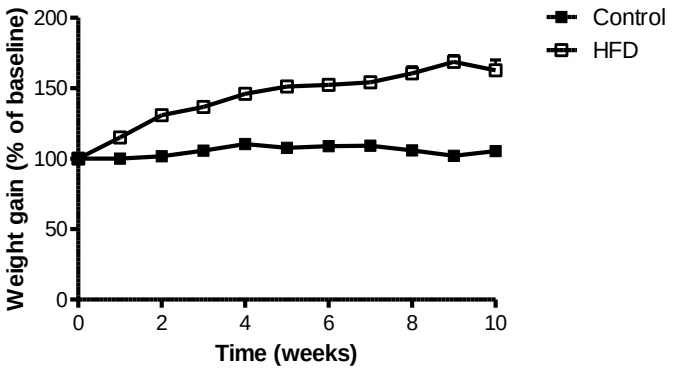


Fig 28: Weight change and diet in mice. Weight gain (% of baseline) over 10 weeks in animals grafted with human islets and fed with control or high fat diet (Gene expression profile study).

Compared to our other series, mice in this group had slightly higher blood glucose at 8-10 weeks (0 wks: 7.6±0.32 control vs. 6.5±0.33 mmol/l HFD; 2 wks: 5.7±0.52 control vs. 7.84±0.32 mmol/l HFD; 4 wks: 6.2±0.29 control vs. 7.5±0.25 mmol/l HFD; 6 wks: 7.14±0.53 control vs. 8.4±0.6 mmol/l HFD; 8 wks: 7.4±0.31 control vs. 9.87±0.96 mmol/l HFD; 10 wks: 8.19±0.98 control vs. 10.47±0.68 mmol/l HFD) **(Fig 29A**). However, mixed model analysis showed that neither diet nor diet duration had significantly effect on blood glucose levels ($p>0.05$). We were able to confirm also that fasting C-peptide was significantly higher from 2 to 10 weeks (0 wks: 633.11±63.19 control vs. 760.77±53.09 pmol/l HFD; 2 wks: 561±134.8 control vs. 1101.91±44.72 pmol/l HFD; 4 wks: 560.82±64.50 control vs. 776.74±29.46 pmol/l HFD; 6 wks: 624.63±133.4 control vs. 1023.499±70.06 pmol/l HFD; 8 wks: 730.01±84.1 control vs. 991.33±112.47 pmol/l HFD; 10 wks: 520.25±94.46 control vs. 834.29±51.52 pmol/l HFD) **(Fig 29B**).

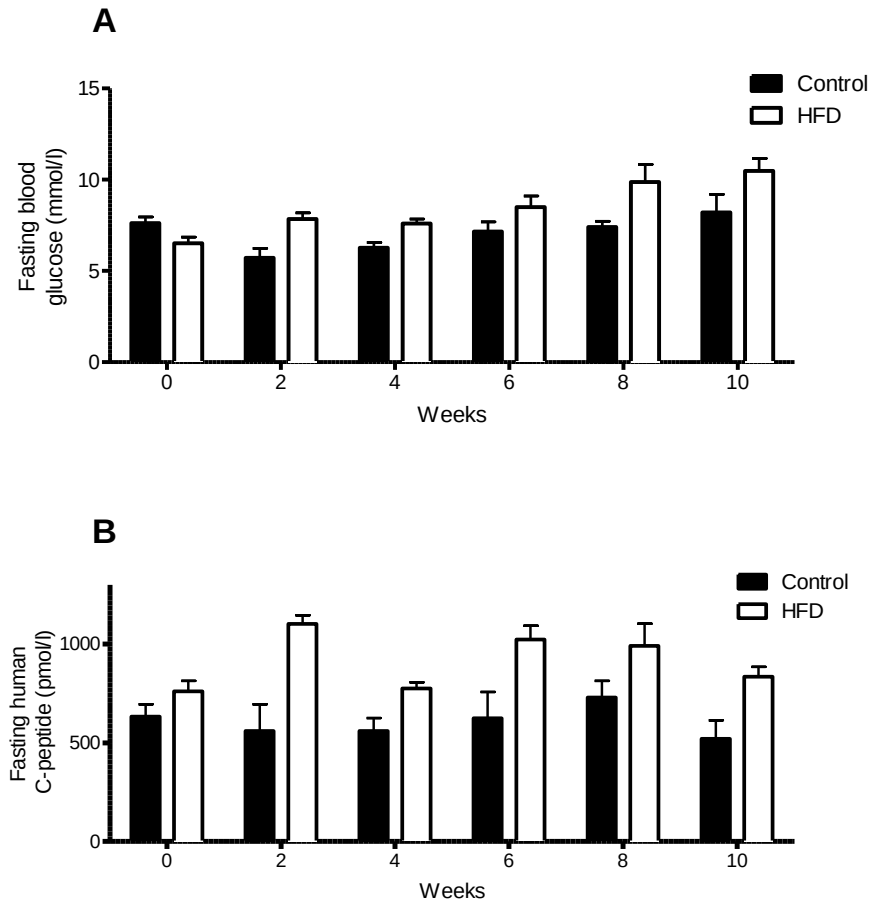


Fig 29: Fasting blood glucose and human C-peptide for mice grafted with islets (Gene expression profile study). A) Fasting blood glucose of mice transplanted with normal islets fed with control and HFD and B) fasting c-peptide of human islets

5.2 Laser capture microdissection procedure to recover human islet enriched grafts

The different structure of human islet graft compared to mouse kidney tissue allowed us to separate the two tissues under a light microscope (Fig 30A). In collaboration with Jonathan Vanhoutte (INSERM U 1011, professor B Staels) the human graft region was outlined with a pen on the screen (Fig 30B) and laser microdissection system cut the selected region using two lasers (**Fig 30C**). Finally, the selected microdissected region adhered to the cap (Fig 30D) which was subsequently inserted onto microcentrifuge tube with RNA extraction buffer. Samples were transferred to –80°C for storage and subsequent RNA extraction.

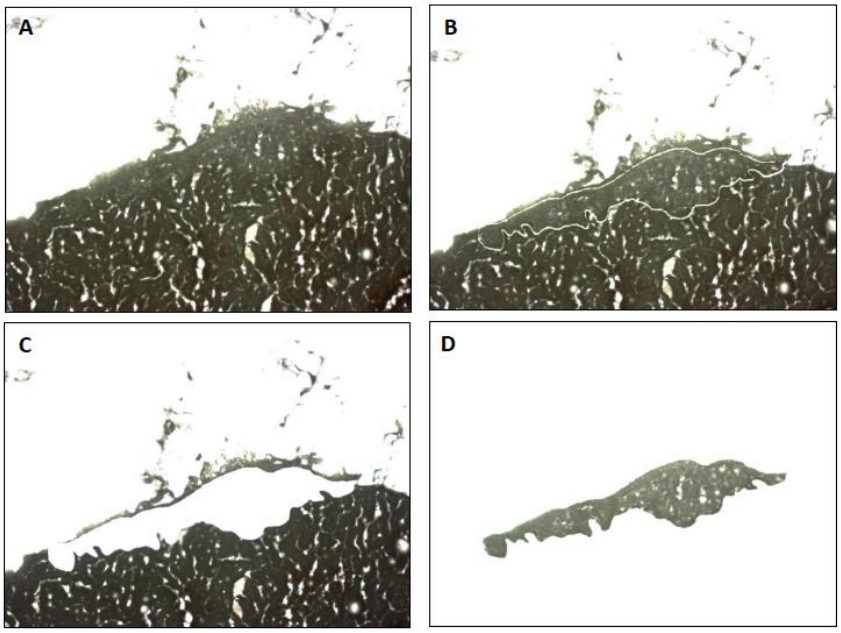


Fig 30: Laser microdissection procedure. A) View in light microscopy of A) Cryosection of a mouse kidney section with a human islet graft under the kidney capsule B) Selection of human graft region with specific pen on the screen C) Two lasers cut the selected region and D) Piece of human graft adhered to the cap

5.3 RNA quality

RNA isolation was performed with Arcturus Pico Pure RNA isolation kit as described in the manual. Integrity of RNA (RIN) was 6.5 to 7.5 and the concentration was between 2-15ng/µl for all samples. The same profile for RNA electrophoresis was taken for all samples (**Fig 31)**. Ribosomal RNA peaked at 28S/18S and the ratio indicated good quality to perform microarrays analysis for all samples. If RNA quality or quantity were insufficient, kidney sections were recut and microdissected.

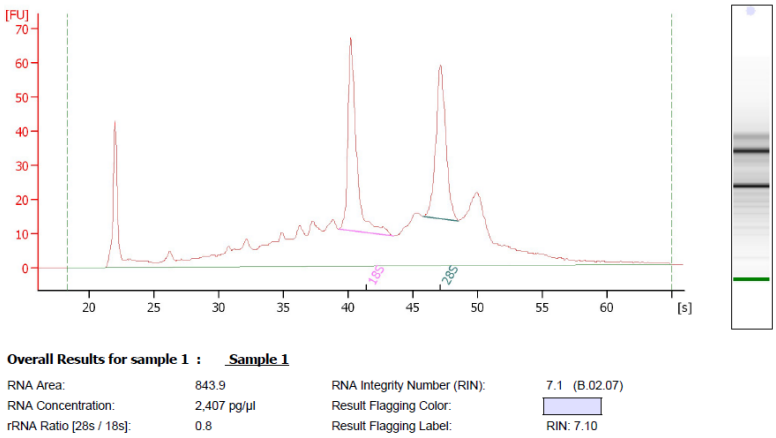


Fig 31: Representative electrophoregram of total RNA extracted from human grafts. The ratio of 28S to 18S of ribosomal RNA indicates good quality of total RNA.

5.4 Gene expression profiling of human islets grafted in control and HFD mice/ preliminary analysis (Kinetic study)

Expression of ~24,000 genes was measured using the Affymetrix Human Gene 2.0 ST array (Affymetrix, Santa Clara, California) at Cochin Institut (INSERM U1016, Paris). The fold change of a given gene measured in two samples is calculated by dividing the two measured intensities and is, therefore, referred to as a ratio. These raw ratios are generally log-transformed (usually log2). This is expected to give a mean log-ratio of

zero and improve the symmetry of the data distribution. This means that a two-fold up- or down-regulation in gene expression is equivalent to log-ratios of +1 or −1 respectively.

Classification of the genes into groups based on their expression values was performed. Firstly, general estimation for the 18 samples was given by unsupervised clustering. In figure 32, unsupervised clustering is performed hierarchically with gene clusters within clusters for all samples.

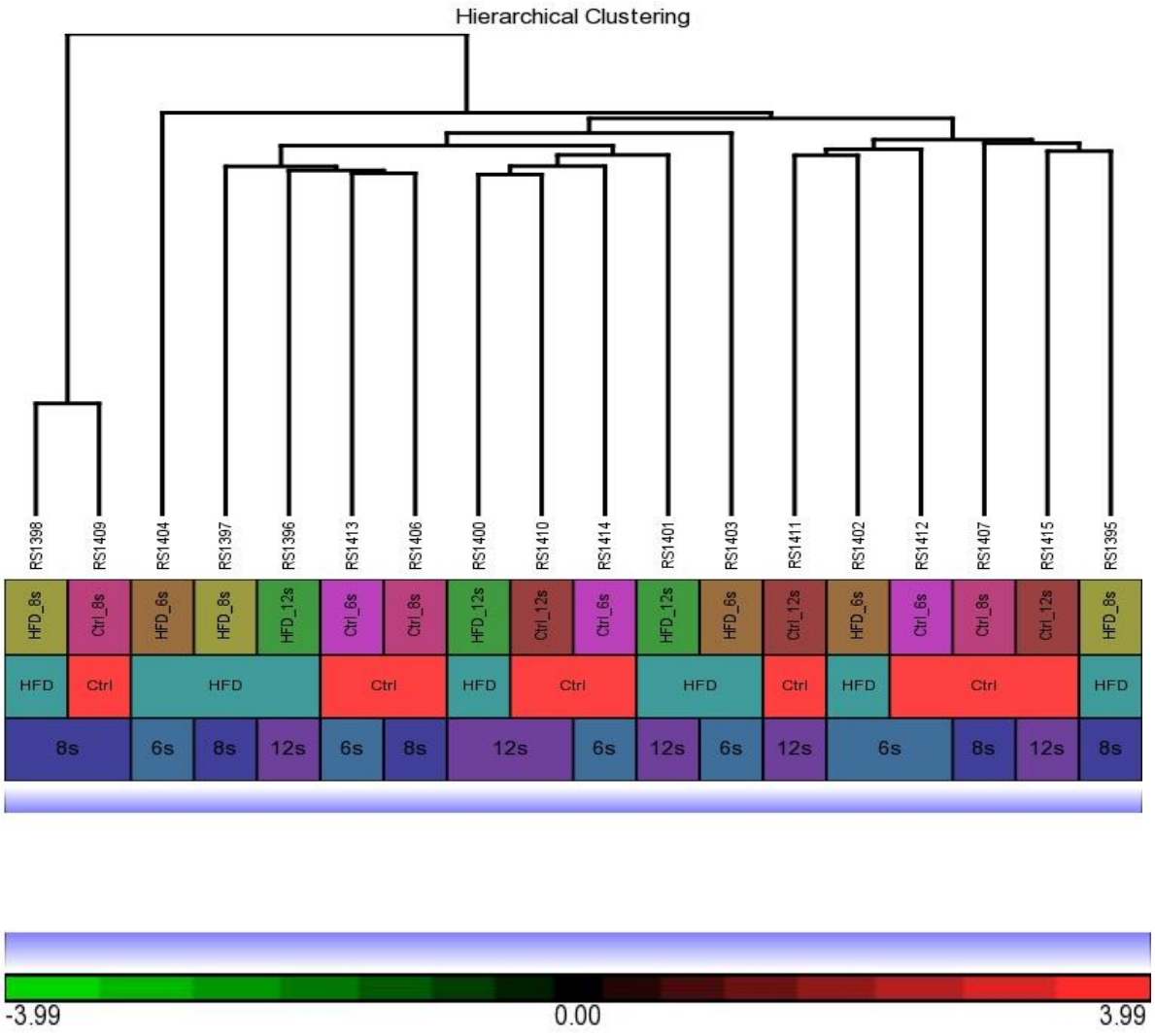


Fig 32: Hierarchically Clustering of microarrays data from 18 human islet grafts in kinetic study (6, 8 and 10 weeks on control or HFD diet)

Moreover, two unsupervised clustering was created based on the type of diet (Control or HFD) and or duration of feeding (6, 8 and 10 weeks) (Fig 33A, B). Principal Components Analysis (PCA) is a method that reduces data dimensionality by

performing a covariance analysis between factors. In our analysis, PCA analysis gave 26.1% variance.

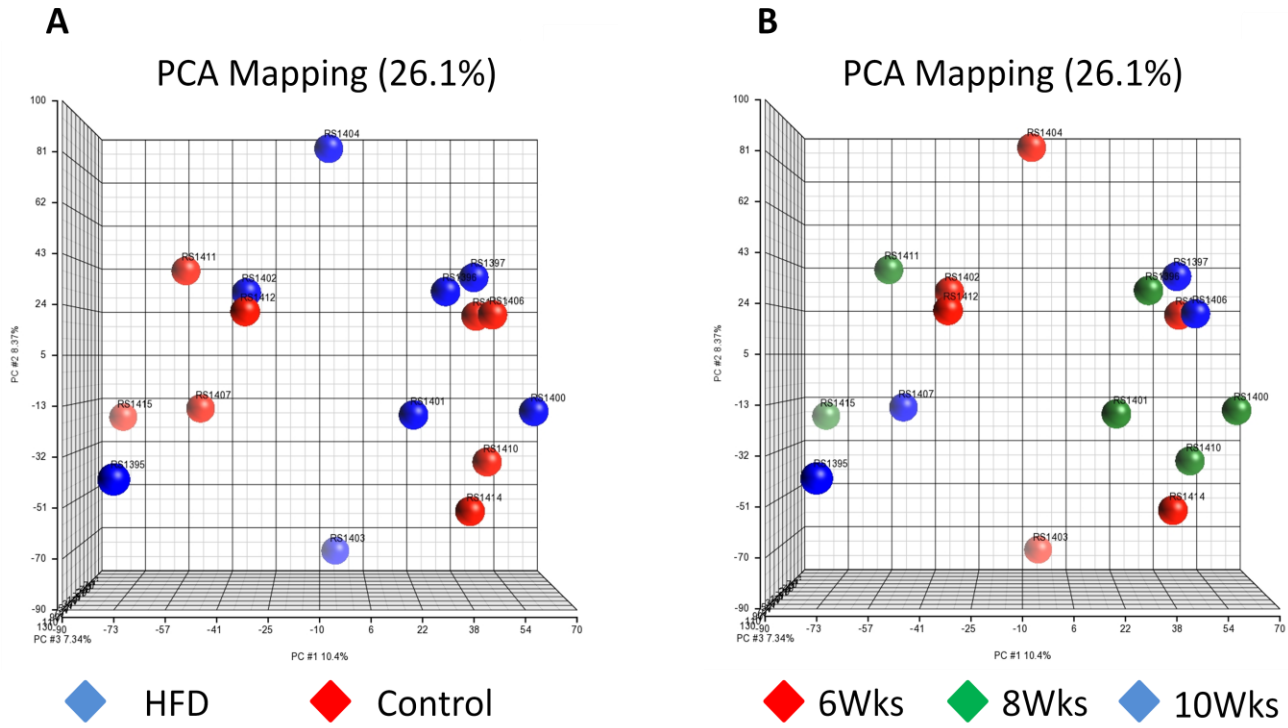


Fig 33: 3D Condition Scatter Plot and Principal Components Analysis (PCA) based on A) type of diet and B) duration of diet

In addition, supervised analysis was used to determine changes of gene expression between samples. In the first preliminary analysis, strict filters were used (fold>2). Figure 34 shows the number of genes which changed at 6, 8 and 10 weeks between islets grafted in control and HFD mice. Different genes were observed at 6 and 8 and 10 weeks.

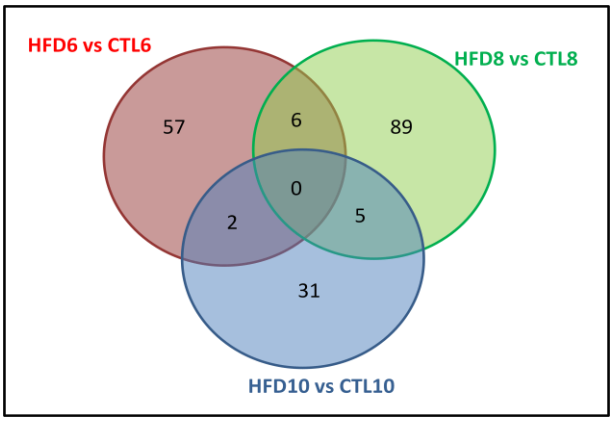


Fig 34: Cycles plot giving the number of genes in human grafts that changed their expression at 6, 8 and 10 weeks on HFD compared to control.

Gene specific for pancreatic endocrine mass in human islet grafts

Analysis of the expression of genes which are specific for the human islets showed higher expression of insulin (fold>6), glucagon (fold>4), somatostatin (fold>3) and islet amyloid polypeptide gene (fold>5) at 6 weeks compared to control. Moreover, there were no significant changes of genes expression at 8 and 10 weeks compared to control except from insulin expression gene that remained higher over time compared to control. However, longitudinal reduction of genes expression was observed over time in the group of HFD mice (Fig 35A).

Gene expression of transcription factors involved in endocrine cell development was also determined. Significantly higher expression of the transcription factors PDX1, NKX6.1, PAX6 and Isl1 and no changes for PAX4 and ARX were observed at 6 weeks in HFD grafts compared to control. At 8 weeks, significant higher level of ISL1 expression and significant lower level of ARX were observed in HFD mice compared to control. Finally, at 12 weeks, lower level of PDX1 and NKX6.1 expression was determined in HFD mice compared to control mice. Interestingly, significant longitudinal reduction of the expression was revealed for PDX1, NKX6.1, PAX6 and ISL1 genes in the group of HFD mice (Fig 35B).

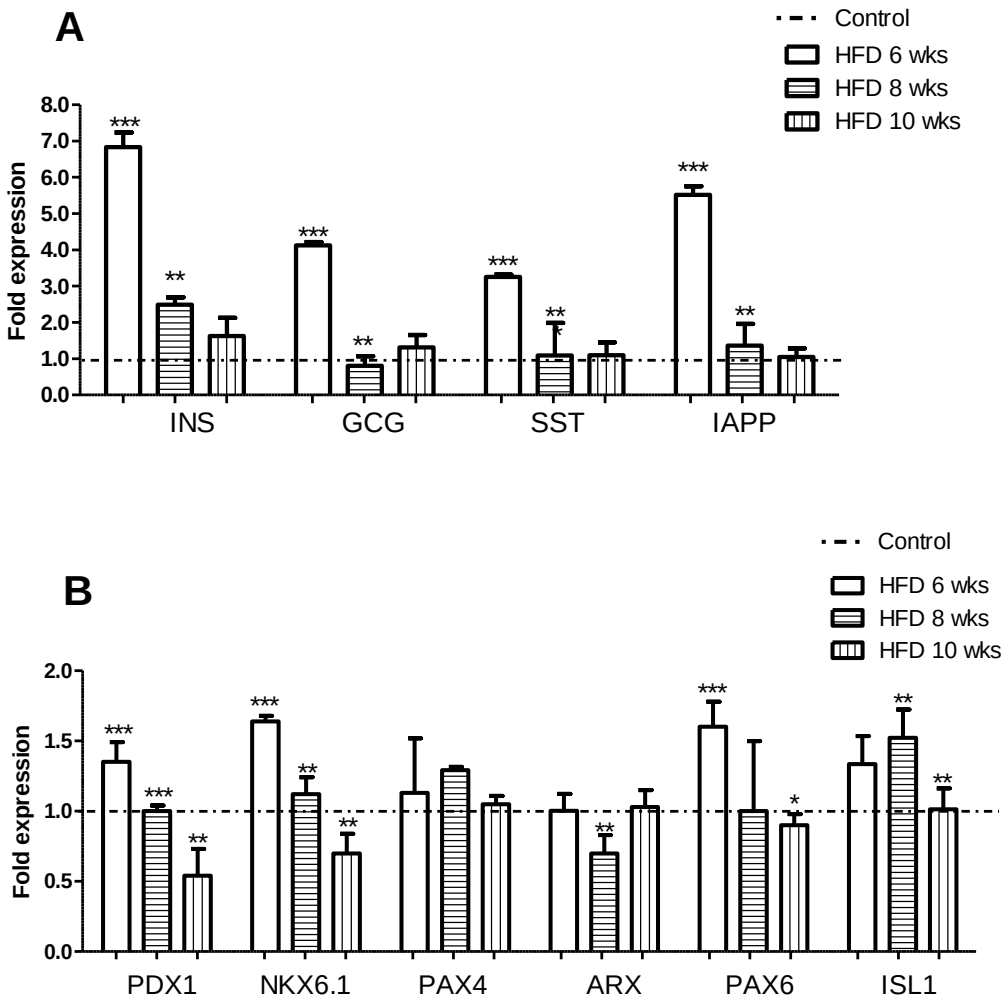


Fig 35: Relative expression of A) endocrine cell specific genes (INS, GCG, SST, IAPP) **and B) transcription factors** (PDX1, NKX6.1, PAX4, ARX, PAX6 and ISL1) (***p<0.001, **p<0.01, *p<0.05)

Cell cycle genes in human islet grafts

Very recently, Fiaschi-Taesch et al provided the immunocytochemical atlas of G1/S cell cycle control molecules in the human beta cell. Expression of cell cycle genes in human islet grafts was determined longitudinally according to this atlas. Firstly, no significant changes of the expression of the three pocket proteins were observed. These proteins play crucial roles in the cell cycle through interaction with members of

the E2F transcription factors family (Fig 36A). In addition, expression of the most E2F transcription factors did not change at 6 weeks on HFD (vs control). However, E2F4 was significantly downregulated at 6 weeks. At 8 weeks, only the regulators of S phase of cell cycle, E2F7 and E2F8, were significantly upregulated (Fig 36B).

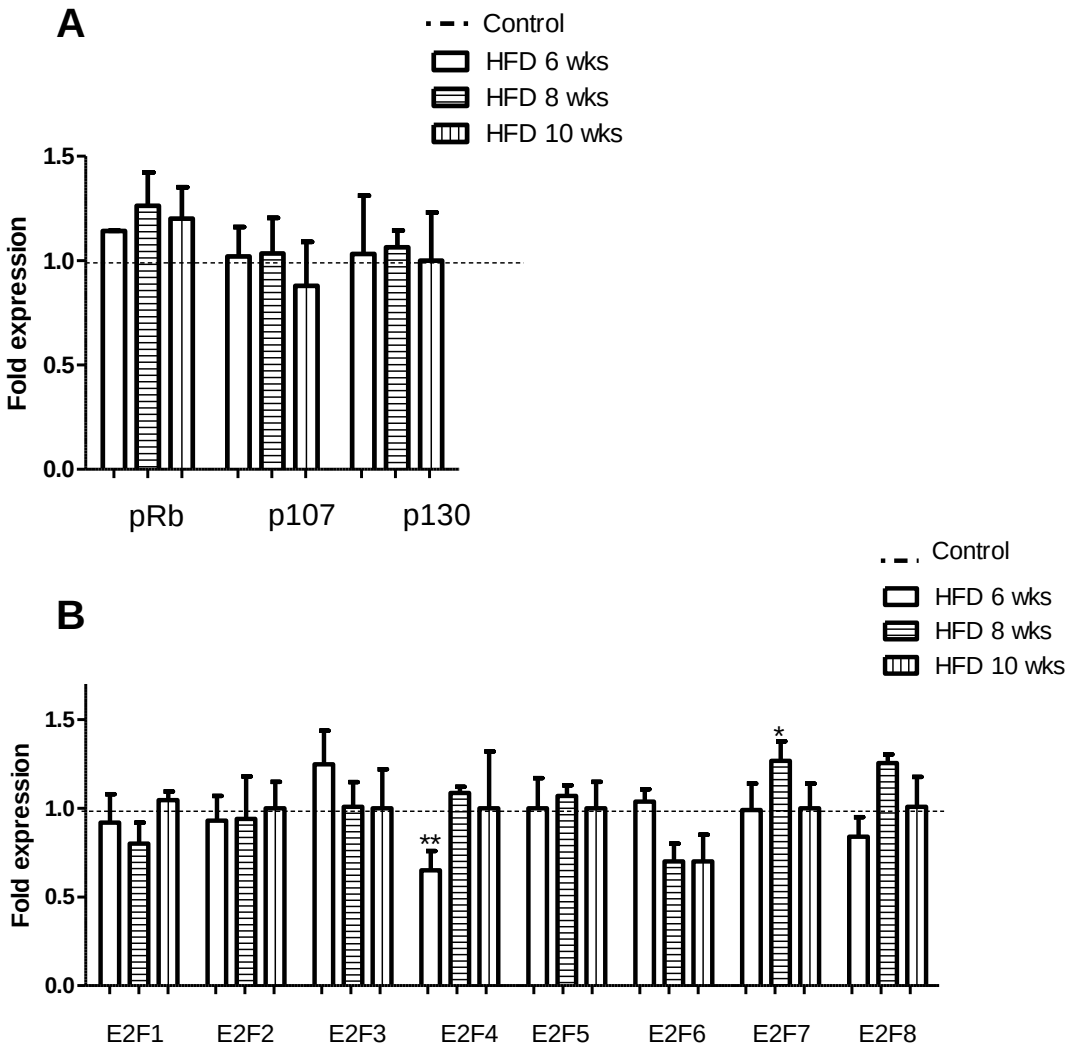


Fig 36: Relative expression of A) protein pocket (pRb, p107, p130) **and B) E2F transcription factors family** (E2F1-8) (**p<0.01, *p<0.05)

Moreover, the expression of cyclins and cdks, which are necessary to drive human cell proliferation, were determined. Here, only cdk4 was upregulated at 8 weeks in HFD mice. Cdk4 is a Ser/Thr-kinase that phosphorylate and inhibit members of

the retinoblastoma (RB) protein family including RB1 (pRb) (Fig 37A). Finally, the expression of the cyclin-dependent kinase inhibitors: INK4 family members (p15, p16, p18, p19) and CIP/KIP members (p21, p27, p57) were observed (Fig 37B). Only p18 seemed to be induced but not significantly at 8 weeks. It is known that p18 interacts strongly with CDK6, weakly with CDK4 inhibiting cell growth and proliferation with a correlated dependence on endogenous retinoblastoma protein RB. In addition, p21, p27, p57 were also upregulated at 8 weeks with significant difference only for p57. They are strong inhibitor of several G1 cyclin/Cdk complexes and a negative regulator of cell proliferation which may explain why most of the cyclin / Cdk complex is inhibited.

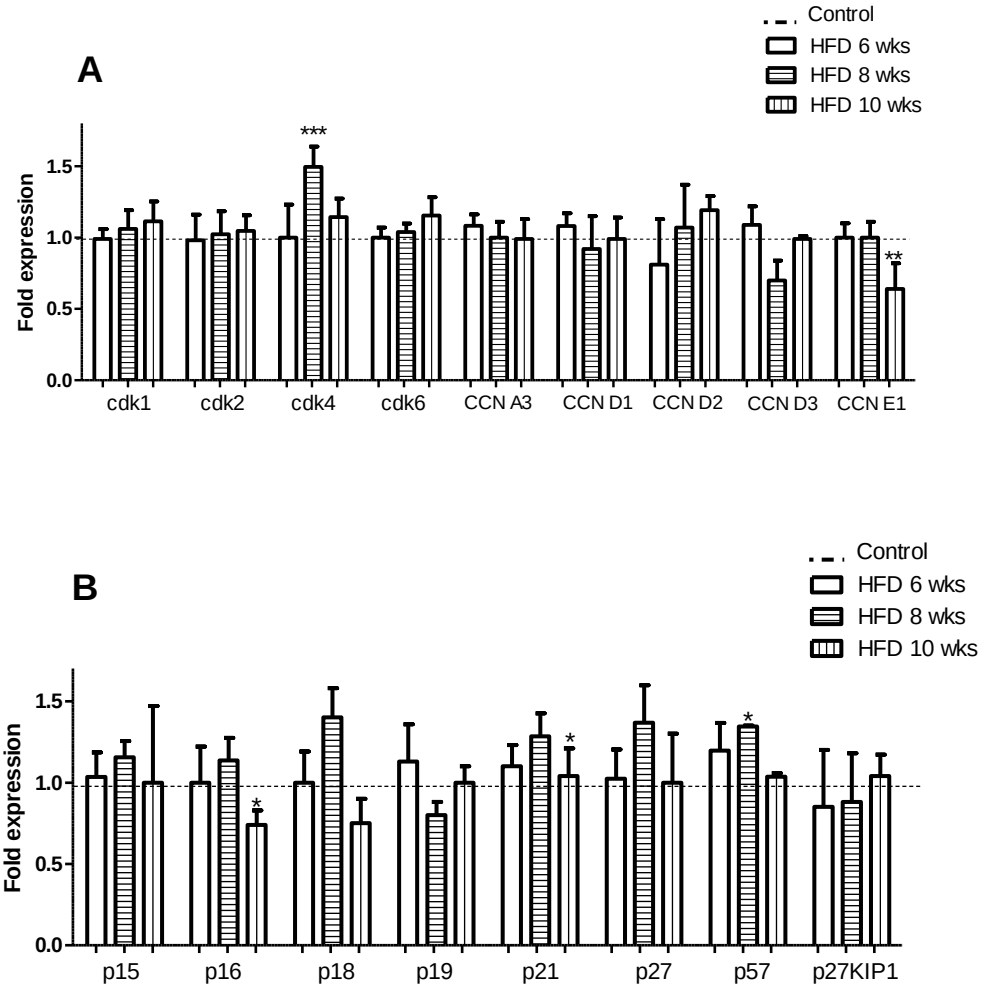


Fig 37: Relative expression of A) cyclins and cdks (cdk1, cdk2, cdk4, cdk6, cyclin A3, D1, D2, D3, E1) **and B) INK4 and CIP/KIP family members** (p15, p16, p18, p19 and p21, p27, p57) (***p<0.001, *p<0.05)

ER stress genes in human islet grafts

Endoplasmic reticulum (ER) stress has been proposed as a mechanism for b-cell dysfunction and death in type 2 diabetes. The expression of the adaptive ER stress gene was determined in our model (BiP, p58, Erp72, Fkbp11, Grp94, XBP1). Bip, Grp94 and XBP1 were upregulated at 6 weeks promoting the adaptation of human islets in obesity. However, the expression of these genes decreased progressively at 8 and 10 weeks in HFD mice proposing failure of ER adaptation long term in obesity (Fig 38)

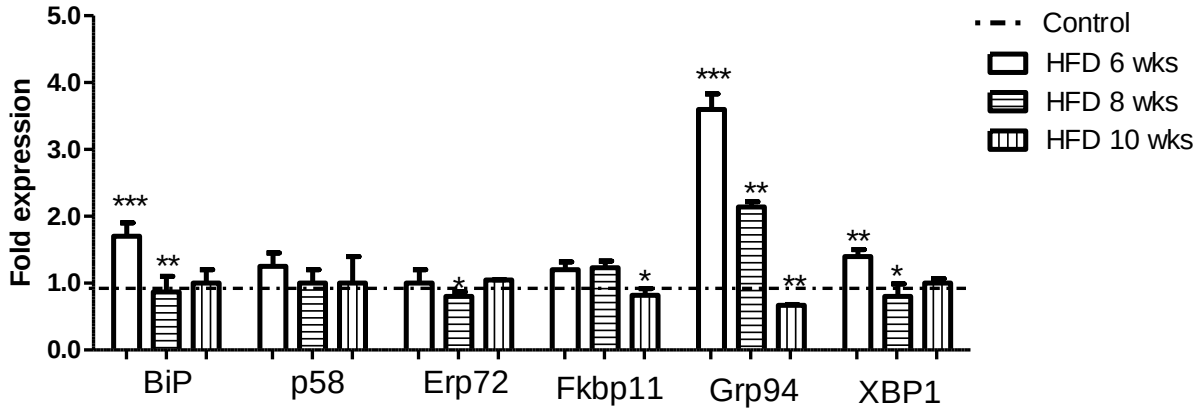


Fig 38: Relative expression of adaptive ER stress genes: BiP, p58, Erp72, Fkbp11, Grp94, XBP1 at 6, 8 and 10 weeks on HFD mice compared to control (***p<0.001, **p<0.01, *p<0.05)

6. Manuscript 2: Comment on: Saisho et al. β-Cell Mass and Turnover in Humans: Effects of Obesity and Aging.

6.1 Preview of manuscript 2: comment letter

Recently, cross-sectional study of Saisho *et al* showed the effect of human obesity on beta cell mass[193]. Autopsy samples (53 lean and 61 obese +overweight) from non diabetic individuals were examined for pancreatic beta cell mass. They observed 1) 50% more beta cell mass in obesity by the increase of beta cell number as no difference in individual beta cell area was determined, 2) higher fat accumulation in obese individuals, 3) positive correlation between beta cell mass and BMI (r=0.5), 4) infrequent and unchanged replication levels in obese vs. lean group and 5) rare apoptotic evident in beta cells (9 to 236.771 cells) without difference between the two groups. Additionally, overweight subjects were also examined in obese group to determine replication level earlier in obesity.

Authors admitted the necessity of longitudinal studies to investigate beta cell adaptation in obesity. Cross-sectional studies, as underlined in this article, give little information on the mechanism underlying how obesity leads to both increased (50%) beta cell mass, mean beta cell nuclear diameter, and ductal cell expansion. Thus, there is an urgent need for longitudinal studies, to characterize the mechanism(s) by which obesity leads to alterations in islet mass and function in humans. The current lack of a direct noninvasive access to the pancreatic gland precludes the direct study of this phenomenon in man.

Our comment letter strived to call attention to our published immunodeficient–obesity model with transplanted human islets, and its interest for future longitudinal studies.

6.2 Manuscript 2 : comment letter

6.3 Unpublished data related to comment letter

Regarding exhaustively the study of Saisho *et al*, great variation of beta cell mass among obese individuals was identified. Descriptive cross-sectional study in human pancreas was performed in our laboratory. Morphometric analysis of pancreatic sections stained by chromogranin A (Fig 39A), confirmed the 30% augmentation of endocrine cell mass in obese individuals compared to lean and overweight without achieving statistical significance due to samples variation (p=0.2; n=45) (Fig 39C). Furthermore, no change in endocrine cell mass was observed between lean and overweight subjects. On the other hand, significant differences were observed in beta cell mass between the three groups, 20% and 25% increase in overweight and obese individuals respectively compared to leans (p<0.01, n=18) [194]. Analysis of the nuclear size showed a positive correlation of the nuclear size with BMI (r=0.72, n=8) as also described in the article of Saisho *et al* [193] (Fig 39D).

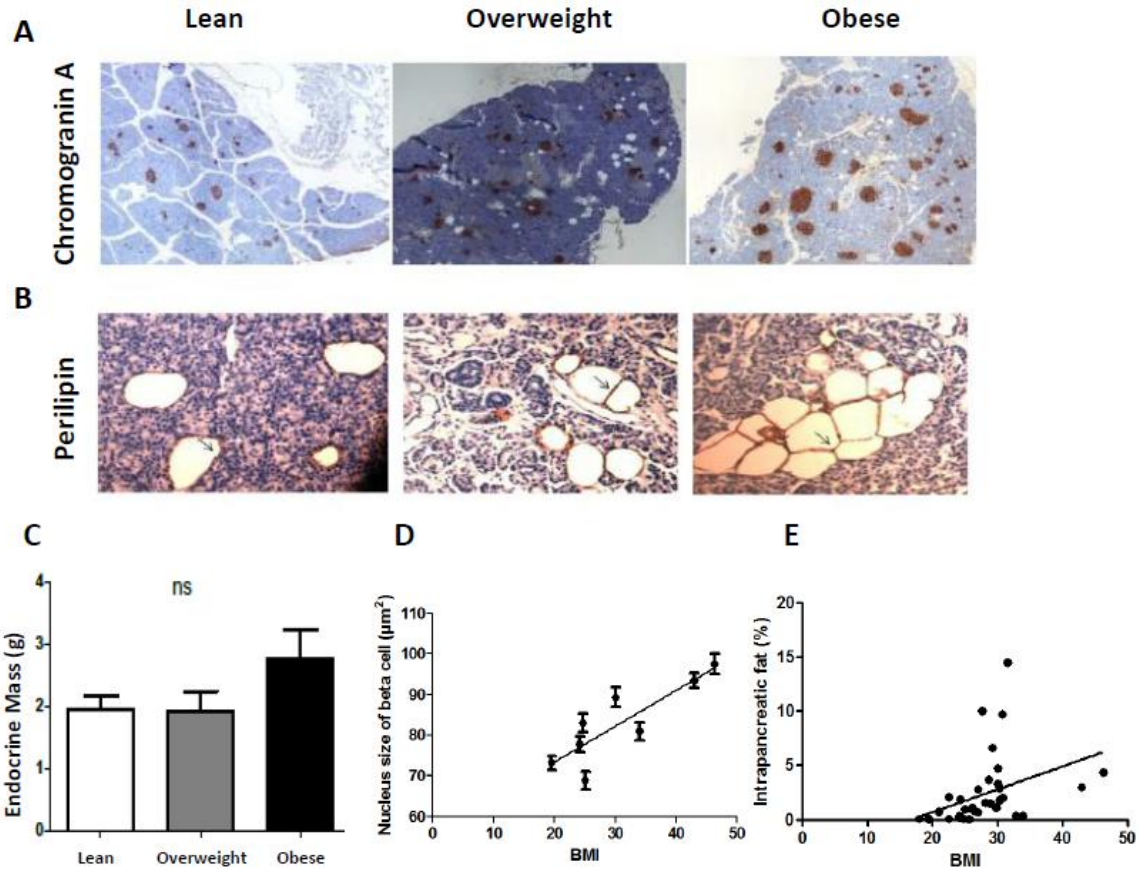


Fig 39: Study of the characteristics of human pancreas (n=40) A) Sections of human pancreas provided by lean, overweight and obese donors, stained by chromogranin A B) Sections of human pancreas stained by perilipin C) Morhometric analysis of pancreatic

endocrine mass from lean, overweight and obese donors D) Correlation between nucleus size of beta cell and BMI OF 8 individual (r=0.72, p=0.007) E) Correlation between intrapancreatic fat (%) with BMI in non diabetic individuals. r=0.13, p=0.02

Staining with perilipin (Fig 39B), an adipocyte marker on paraffin sections of pancreas revealed intrapancreatic fat accumulation in three groups. Analysis showed progressively more fat surface in overweight and obese individuals compared to leans (leans: 0.49±0.19%; overweight: 2.67±0.9%; obese: 3.98±1.19%). However, no correlation between intrapancreatic fat and BMI was observed due to samples variation as we observed in Fig 32 (r=0.13, p=0.02) (Fig 39E).

In our model, a doubling of beta cell mass was determined in grafts in mice which gained 50% of their weight (12 weeks). We were interested in exploring if indeed the diameter or area of each human beta cell increased in HDF vs controls (ie hypertrophy). Further analysis of beta cell size determined as surface area was also performed for the islet grafts in control and HFD mice. No difference in individual beta cell size/area of the grafts was observed suggesting that the increase of total beta cell mass can be attributed to increased numbers and not individual beta hypertrophy. However, nucleus size measurements showed modest increases of beta cell size in grafts on HFD mice vs. control (333.75±19.73 vs. 363±8.99 μm^2 HFD). Longitudinal study of our model revealed higher levels of proliferation in grafts on HFD mice proposing that this model may be used as tool for investigation of islet adaptation to obesity.

6.4 Response to Comment on: Saisho et al. β-Cell Mass and Turnover in Humans: Effects of Obesity and Aging.

COMMENTS AND RESPONSES

Response to Comment on: Saisho et al. β-Cell Mass and Turnover in Humans: Effects of Obesity and Aging. Diabetes Care 2013;36:111–117

We thank Gargani et al. (1) for their interest in our article reporting β-cell mass in age-matched obese (n = 61) and lean (n = 53) human pancreas samples. In that study, we reported an ~50% greater β-cell mass (0.8 to 1.2 g) in obese humans compared with lean humans (2). We acknowledged that our study was cross-sectional and so we could provide no time course for the difference we reported. In the interesting report that Gargani et al. direct us to, we note their approach to the question of human islet adaptation to dietary insulin resistance. They implanted 400 human islet equivalents under the renal capsule in immunodeficient mice and then performed a cross-sectional study of the implants 12 weeks later after high fat (n = 6) versus a regular diet (n = 7). It is well known that the first week after implantation of human islets there is a substantial and variable β-cell loss, presumed to be in part due to anoxia after loss of islet vasculature and formation of islet amyloid (3).

Since there was no reported measure of β-cell apoptosis or necrosis in the report by Gargani et al., it is not clear to what extent the changes in graft β-cell volume 12 weeks later were due to differences in β-cell loss. The insulin and GLP-1 signaling pathways inhibit apoptosis, and levels of both would be predicted to be increased in the high-fat diet–fed mice, so it is perhaps plausible that engrafted β-cell loss may have benefited from the high-fat diet environment. Gargani et al. found no difference in β-cell replication at the 12-week evaluation by Ki67, although they concluded after repeated BRDU labeling early after transplantation that there was an increase in β-cell replication by this measure. However, BRDU labeling also occurs during DNA repair that might predictably be increased shortly following islet transplantation (4). It would be of interest if the β-cell Ki67 was increased at the same early time points, as this measure is less vulnerable to this problem.

In general, it seems at present that it might be premature to interpret the islet adaptation in the studies of Gargani et al. purely in terms of new β-cell formation or indeed the origins of such formation. If after decades of obesity β-cell mass has increased by ~0.5 fold, it would indeed be intriguing if human islets adaptively increase by 5 times that in just 12 weeks in the devascularized implant under the renal capsule.

Finally, we are less surprised than Gargani et al. that we did not see an increase in β-cell replication in human obesity (5,6). Expansion of β-cell mass occurs in the early postnatal phase by β-cell replication, and then the capacity for β-cell replication is silenced by epigenetic repression of cell-cycle regulators.

We congratulate Gargani et al. for their interesting studies and pursuit of the important but difficult question of β-cell turnover in human islets. We wish them well in their ongoing research.

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No potential conflicts of interest relevant to this article were reported.

References

1. Gargani SA, Pattou F, Kerr-Conte J. Comment on: β-Cell mass and turnover in humans: effects of obesity and aging. Diabetes Care 2013;36:111–117 (Letter). Diabetes Care 2013;36:e111. DOI: 10.2337/dc13-0220
2. Saisho Y, Butler AE, Manesso E, Elashoff D, Rizza RA, Butler PC. β-Cell mass and turnover in humans: effects of obesity and aging. Diabetes Care 2013;36:111–117
3. Westermark GT, Westermark P, Berne C, Korsgren O; Nordic Network for Clinical Islet Transplantation. Widespread amyloid deposition in transplanted human pancreatic islets. N Engl J Med 2008;359:977–979
4. Butler PC, Meier JJ, Butler AE, Bhushan A. The replication of beta cells in normal physiology, in disease and for therapy. Nat Clin Pract Endocrinol Metab 2007;3:758–768
5. Meier JJ, Butler AE, Saisho Y, et al. Beta-cell replication is the primary mechanism subserving the postnatal expansion of beta-cell mass in humans. Diabetes 2008;57:1584–1594
6. Dhawan S, Tschen SI, Bhushan A. Bmi-1 regulates the Ink4a/Arf locus to control pancreatic beta-cell proliferation. Genes Dev 2009;23:906–911

Chapter V

Discussion

My research in Lille initially focused on characterizing of the influence of obesity on morphological and functional characteristics of human islets (Master's 2 thesis 2009-2010). The underlying question was: are obese human islets indeed the best islets for cell therapy of diabetes? A retrospective study in 283 pancreatic organ donors at the University Hospital in Lille showed that in obese donors compared to normal weight and overweight donors, the total number of islets increased, the average islet size increased and the mean intracellular insulin was higher. Histological analysis in 37 donor pancreases confirmed: obese donors were characterized by increased in total endocrine cells (a,β,δ,pp cells), bigger individual islets size, and increased intrapancreatic fat. Interestingly, we observed increased beta cells and decreased alpha cells in islets of obese vs non obese pancreata. Thus morphologically human islets in obese individuals are distinctly different from overweight and lean individuals.

That being said, obesity is an exclusion criterion for pancreas harvesting in the context of pancreas transplantation, yet these pancreases are not only routinely used for islet isolation and transplantation, they are preferred in certain isolation centers due to the improved technical success of islet isolation from high BMI pancreases [172, 195]. The general object of my PI's research program is to devise *in vivo* models to study the function and adaptation of human islets to their environment. My project focused first on developing the model and characterizing the functional and morphological adaptation of human islets to diet induced obesity in the immunodeficient mouse. The adaptation of human islets to the obese murine environment was confirmed by the significant increases in human c peptide in high fat diet mice compared with control diet mice. In this PhD thesis, a functional adaptation of human endocrine cells to the obese environment was determined and mechanisms of islets regeneration were investigated. Human pancreatic islets of Langerhans provided by eight organ donors (non diabetic and diabetic) were transplanted in Rag2 immunodeficient mice under kidney capsule as previously described [184, 196, 197].

Mouse strains differ in the susceptibility to diet-induced obesity. Previous studies in our lab explored HFD feeding of C57Bl6 male nude mice C57BL/6 nude mice gained less than 2gr of weight (<8% weight gain) after 8 weeks feeding with high fat diet [198]. Previous studies confirmed that RAG1ko and RAG2ko were sensitive to HFD or HFD and high sugar diet [199]. In our study, C57BL/6 Rag2$^{-/-}$ mice were selected, as feeding with HFD diet (60% calories from fat) for 12 weeks resulted in >60% weight gain. The

same degree of short term weight gain cannot be observed in humans as no more than 4% weight gain was observed after one month high fat feeding [200]. Within the first month on HFD, mice could develop several obesity associated traits [201]. High levels of serum triglycerides and abdominal fat content in our HFD mice confirmed the obese phenotype.

Analysis of endogenous pancreas showed progressive decrease of endocrine cell area in control mice and progressive increase in HFD mice with significant difference at 12 weeks (vs. control). However, when compared to HFD mice without grafts [202, 203], lower percent of endocrine cell area was detected in our HFD grafted mice. The latter proposes the effect of islets grafts on endogenous pancreas both in HFD and control mice [204]. In addition, lower number of islets was observed in control mice at 12 weeks. Further morphometric quantification showed progressive increase of islet size in HFD mice compared to control (Fig 18). Moreover, when glycemia tested, fasting blood glucose was found to be slightly increased after 6 weeks with significantly higher levels at 10 and 12 weeks in mice on high fat diet. However, blood glucose levels had never exceeded 10 mmol/l at 6, 8 and 10 weeks as routinely observed in HFD C57BL/6 Rag2$^{-/-}$ mice without islet graft [201, 205, 206] or in male C57Bl6 WT mice. Beta cell proliferation of endogenous pancreas was higher in HFD mice at 6 and 12 weeks probably due to glucose increase (Fig 19B). Even small increase on glucose levels could provoke beta cell replication [32, 207].

In order to investigate human graft function, fasting human C-peptide secreted by islet grafts was measured. Fasting human C-peptide was progressively increased in HFD mice compared to control. Human C-peptide level seemed to be independent of the type of diet in the first weeks, but it was affected by the duration of diet and the weight gain of mice. Very recently, a study showed the effect of weight gain on C57BL/6 mice fed with HFD in insulin secretion as high-HFD responders (more weight gain) had 3 fold higher insulin secretion than low-HFD responders (less weight gain) [205]. On the other hand, elevated human C-peptide levels in our obese model may be simply explained by hyperglycemia. Previous studies showed that one day after glucose infusion in mice insulin secretion of transplanted human islets was increased three times [208]. Are these islets in HFD mice “super islets”? To determine this, the homeostatic model assessment (HOMA) was used to quantify insulin resistance (HOMA%IR) and beta-cell function (HOMA%B) of human grafts and HOMA2%BS,

which takes into account beta cell function at a given insulin sensitivity or resistance. HOMA B, IR and S are calculated based on fasting human insulin, c-peptide and glycemia. A expected, upon weight gain a major incremental drop in insulin sensitivity (HOMA2%S) was observed -up to 70% over time in HFD mice compared to control as described in manuscript 1 (longitudinal analysis; Mixed model), was accompanied by an significant increase in beta cell function determined by HOMA2%B, which was improved in both control and HFD mice. However, the overall function (HOMA2%BS) of human grafts on HFD mice progressively decreased as compared to controls at 12 weeks. Particularly, at 12 wks, HOMA%BS was 4-fold lower in HFD mice than in control mice. This would on the contrary suggest that human islets on 12 weeks of HFD have impaired function. Use of HOMA in this study is interesting and specific (human C-peptide), but may be the subject of debate since fasting glycaemia in this model can be regulated by both the human graft and the endogenous pancreas still in place, which also adapts [155].

One question we asked ourselves is will our HFD induced obesity model be able to reproduce the morphological alteration in beta and alpha cell composition that we observed during my Masters degree in obese pancreatic vs lean sections (8% less alpha cell in obese individuals vs leans expressed in total alpha+beta cells). That is say if we implant human islets from a lean donor in control or HFD mice do we see an alteration? The ratio of alpha/ alpha+beta volume of the graft was calculated in order to compare the same value and it was observed that islets grafted in HFD mice had 6% less alpha cell (18.08±1.49 in control vs 12.45±1.69% in HFD mice). Taking things one step further if we implant islets from obese donors do we see alterations in the control mice- is the process reversible? We do not have the answer for the latter.

Analysis of human endocrine cell volume in kinetic study revealed an association of beta and alpha cell volume with weight gain of mice, blood glucose and human c-peptide secretion. Same levels of blood glucose and c-peptide were measured in control mice over time and in parallel, no important changes were observed in endocrine and beta cell volume of control mice. A slight decrease of human c-peptide secretion and a slight increase of alpha cell volume was observed in control grafts at 12 weeks (Fig 15B) which may be explained by intra islet regulation in human grafts [209, 210]. On the other hand, changes in beta and alpha cell volume were determined on HFD mice over 12 weeks. During the normoglycemic period (4 weeks), beta cell volume

was increased responding to insulin needs to overcome increasing insulin resistance of peripheral tissue as mice had already gained 28% of weight [50, 194]. Interestingly, alpha cell volume was decreased (4 weeks). Rahier *et al* showed that ratio alpha:beta cell did not decrease with the increase in BMI in humans confirming our observation in 45 human pancreas (r=0.14, p=0.12) [211]. In mild hyperglycemia (6 weeks), beta cell volume was slightly decreased and alpha cell volume was elevated (with no difference compared to control mice). In the hyperglycemic period (>10weeks) beta cell volume was increased and alpha cell volume seemed to be elevated only at 12 weeks (Fig 15 and 20) probably responding in hyperglycemia [17, 41, 212-214].

General discussion on proliferation (in our initial series of 3 control human islet prepartions BrdU was added 1 day prior to sacrifice. In these series there was a tendency for increased endocrine proliferation however we were unable to identify human beta cells undergong proliferation. All subsequent studies used 7 day BrdU in drinking water prior to sacifice.The kinetic study allowed us to examine proliferation of human endocrine cells longitudinally in mice fed with control or HFD over the 12 week period. During the normoglycemic period (4 weeks), higher beta cell proliferation was observed in HFD grafts compared to control grafts provoked by insulin demand in obesity (determined by BrdU into drinking water for 7 days before killing). Cross-sectional studies in human pancreas of normoglycemic donors showed very low levels of beta cell proliferation which was slightly increased in obese individuals [215]. No change in alpha cell proliferation was determined in HFD grafts compared to control which may partially be explained by the decrease of alpha cell volume at this time point. Human beta cell proliferation peaked at 6 weeks whereas mild hyperglycemia occurred without changes in alpha cell proliferation. Unexpectedly, at the end of this period when blood glucose levels started to increase (8 weeks), a decrease of proliferation in both beta and alpha cells was observed. Finally, beta and alpha cell proliferation reoccurred when hyperglycemia was established (Fig 21D and F). A study with human islets showed that high glucose infusion increased 2-fold beta cell replication in islets transplanted in nude mice [208]. Particularly, increased alpha cell replication has been attributed to high level of IL-6, a cytokine associated with insulin resistance in obesity [41]. Rare delta cell replication was observed but interestingly all cells were found in grafts on HFD mice (Fig 21H).

Staining by CK-19/chromogranin A and insulin or glucagon was performed to reveal neogenesis in human grafts. Neogenesis was determined at 12 weeks in the first 3 donors- yet levels were rather low. The kinetic study (HFD 11) showed that higher levels of neogenesis were observed at 8 weeks as described in manuscript 1. This will requires confirmation in subsequent experiments. At this time point hyperglycemia started to increase and proliferation of endocrine cells was decreased. Further analysis showed that 80% of CK19+/ChromoA+ cells were alpha cells confirming other studies which proposed that alpha cell neogenesis comes first [216-218].

Tunel test was also performed in our model and no measurable apoptosis of endocrine cell was observed in human grafts on control and HFD mice as expected. Levitt *et al* showed that beta cell TUNEL reactivity was not measurably increased in human grafts exposed to elevated blood glucose [208]. Additionally, high fat diet and weight gain of mice did not change the percent of caspase 3 positive cells in endogenous pancreas of mice in other study [29].

The lineage tracing study was performed to explain the alteration of alpha and beta cell volume as no difference in apoptosis was detected in HFD grafts. Firstly, conversion of beta cells to alpha cells was observed. Analysis of GFP+ cells before islet transplantation confirmed the specificity of Rip-CRE vector as described in other study using the same vectors (provided by Pr Philippe Ravassard, BCBC/UMR-7225) [192]. After labeling beta cells in islets with RIP-CRE and CMV-LOXP analysis of GFP+Ins+ and GFP+Gcg+ cells at 12 weeks post transplantation revealed that 24.71% and 35.62% of GFP positive cells (ie originally expressing the insulin promoter) were alpha cells (gcg+) in control and HFD1 mouse respectively. However, mouse numbers were small and for example only 13.04% conversion was found in HFD2 mouse. The result was unclear for HFD2 mouse as individual observation showed very low human c-peptide expression (~20pmol/l at 8 and 10 weeks on HFD). Very recently, transition of mature beta cells into alpha cells was described in human islets grafted in NOD/SCID mice for 2 weeks [219]. Changes in endocrine hormone expression of this recent study were explained by ~10% of beta cell conversion and not due to apoptosis or proliferation. On the other hand, conversion of alpha to beta cell was tested using a Glu-CRE vector for the islets provided from the same donor. Quantification analysis showed that 41.53% GFP positive cells in control1 and 42.42% in HFD mouse were beta cells. Furthermore, 43.14% conversion was found in control 2. Observation of this

mice revealed abnormally high c-peptide secretion associated with 15% of weight gain. Two models were used in previous studies to describe the capacity of alpha cell conversion to beta cell; transgenic expression of diphtheria toxin mouse mice [64] and PDL plus alloxan-induced beta cell model [19]. A more thorough study in transition of human alpha cells to beta cells should be performed.

Kinetic microarrays study was performed to reveal the gene expression profiling of human islets longitudinally in obese environment. In preliminary analysis, the expression of endocrine specific gene (insulin, glucagon, somatostatin, amylin) as well as the expression of specific transcription factors confirmed functional adaptation early in obesity at 6 weeks. However, the expression of cell cycle genes did not give strong evidence for whole islet proliferation. This may be explained by different proliferation levels each time individually for beta, alpha, delta and PP cells. Furthermore, the specific localization of these cell cycle proteins has a major role in proliferation as described [220]. Presence of these molecules in nuclear compartment drives human pancreatic cells to proliferation. Nuclear or cytoplasmic localization of cell cycle molecules should be determined in our model. Finally, the expression of ER stress genes was observed in our model. ER stress activates a signaling cascade known as the unfolded protein response (UPR), which has roles alleviating the ER stress through the upregulation of ER chaperones and folding enzymes and, paradoxically activating apoptosis via deleterious UPR signaling if the stress is too severe or prolonged. Very recently, study in ob/ob and db/db mice showed that early in obesity ER genes were upregulated in both models but only in db/db mice their expression decreased progressively proposing failure of islets adaptation [221]. The same profile was detected in our model confirming the dysfunction of human islets long term in obesity. Further studies should be performed to confirm these results.

To validate our model, dysfunctional islets from two diabetic donors were transplanted in mice fed with control and HFD (DD1 was treated for 10 years for time 2 diabetes). No functional adaptation associated with c-peptide secretion and HOMA2 assessment was observed in these grafts (data described in manuscript 1). Fasting human c-peptide was lower in DD2 over time (12 weeks) compared to the normal donors. It should be noticed that that islets of DD1 secreted much lower c-peptide levels compared to DD2. Additionally, blood glucose levels were increased over time in these mice (onset from 2 weeks). It was also observed that mice grafted with islets of DD1

had higher levels of blood glucose (>10mmol/l) providing more evidence for the bad function and maladaptation of these islets in obesity. Finally, no histological adaptation was observed for these grafts (ESM Fig 3 in manuscript 1). Deficit of beta cell mass has already described in T2D patients and it is known that aging correlates with decreased proliferation capacity and dysfunctional beta cell mass in T2D patients [26, 50, 60, 222]. Double staining BrdU/ Chromogranin A didn't reveal proliferation of endocrine cells as expected [50, 215].

Model description and potential mechanisms of human islets adaptation in obese environment are shown in **Fig 40**.

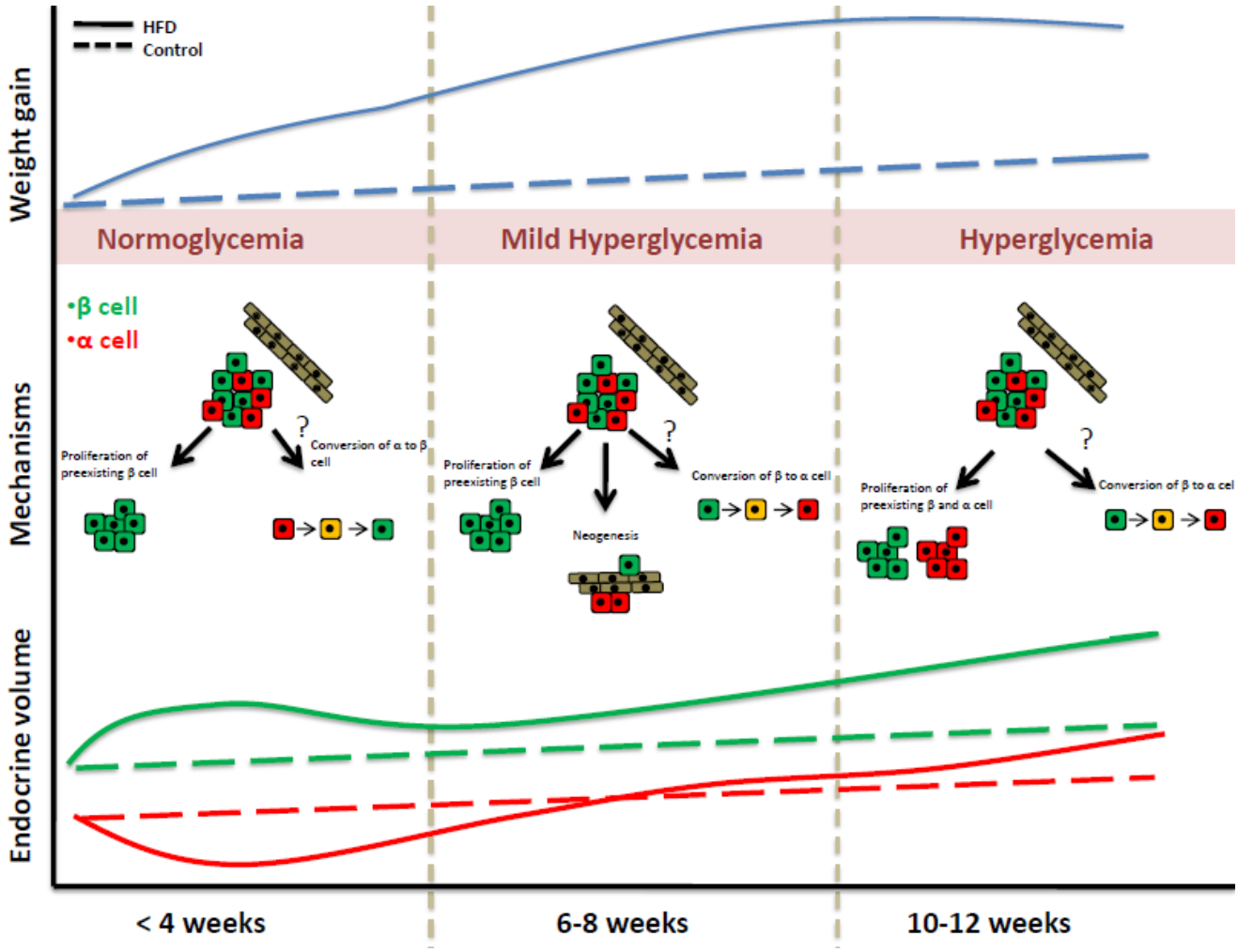

Fig 40: Model description and potential mechanisms of human islets adaptation to obese environment.

Chapter VI

Perspectives

An immunodeficient obese model was created to study the human islet adaptation to obesity. Longitudinal adaptation of human islets to the obese murine environment was confirmed by the significant increases in human c peptide in high fat diet mouse, and concomitantly morphometric analysis also revealed human endocrine cell expansion. Changes in endocrine cell contribution during 12 weeks on HFD were determined and mechanisms of regeneration were investigated. This novel model allows further investigation of molecular pathways involved in human beta cell expansion, as well as identification of factors predisposing human beta cells to undergo decompensation.

Progress would include imaging of the islets (and determination of graft volume in vivo) without sacrifice. This will require transplanting islets elsewhere besides the kidney capsule, as the kidney shows great background in diverse imaging techniques due to its role in eliminating tracers from the body. Our group [166, 223] as well as more recent work with human fetal pancreatic tissue [224] suggest the muscle may be the ideal spot (speed and accessibility) for transplantation and potential imaging for graft volume assessment.

The secretory activity of human islets in obesity is regulated by several circulating factors. Very recently, a new hormone expressed in mouse liver, betatrophin, was found to regulate the proliferation of mouse beta cells promising a novel therapy for type 2 diabetes [225]. However, the mechanisms by which betatrophin activates human beta cell proliferation remains unknown and this novel model may be used to respond to this question. Studies are underway in our laboratory to determine if betatrophin is increased in HFD and in addition if administration of an insulin receptor antagonist like S961 which leads to insulin resistance and islet compensation in mice, can lead to the adaptation of human Islets transplanted in immunodeficient mice. This model would be advantageous over the diet induced obesity model as it would require 7-14 days vs 3 months in the HFD model developed herein.

The mechanisms responsible for the inability of diabetic islets to adapt in obesity should be identified. We hope that in collaboration with P Froguel's group that this model may contribute to stratifying the multiple polymorphisms in polygenic forms of diabetes. In this context, the 2 labs received funding to develop a project DiaStem : Diabetic modeling in vitro using induced Pluripotent Stem Cell technology.

It is known that human beta cells do not proliferate *in vitro* and this immunodeficient mouse model developed in this project will be exploited for further experiences in islet proliferation. Knowledge of cell cycle regulation of beta cell *in vitro* is abundant but the mechanisms of human beta cell proliferation and adaptation to physiological conditions are still unknown. Menin has been reported as a regulator of beta cell proliferation and islet adaptation in pregnancy and obesity but the pathways in which menin provokes the expansion of endocrine cells remain unknown [27, 226].

Not long ago the transdifferenciation of mature endocrine cells was identified as potential mechanism of regeneration. Pax4 and Arx have been reported as the key transcription factors for beta and alpha cell conversion. It remains unclear when, why and how deletion of these factors provokes human endocrine cell conversion in physiological conditions. Kinetic study using our model should be firstly performed to determine longitudinally the rates of human endocrine cell conversion associated with mouse weight gain, duration of high fat diet and hyperglycemia and, secondly the molecular mechanisms implicated in islet transdifferentiation could be revealed.

The lineage tracing results performed in this study, are only preliminary and require confirmation. Recent evidence shows a differential and less alpha/beta specific expression of Pax 4 and Maf A and Maf B in human beta and alpha cells as compared to mouse beta cells and alpha cells. Expression of Pax4 and MafA was found in 50% of human alpha cells [227]. Further Lineage tracing experiments may prove to show that a degree of transdifferentiation in human pancreatic islet cells may occur in physiological processes like obesity.

Chapter VII

References

1. Wierup N, Svensson H, Mulder H, Sundler F: **The ghrelin cell: a novel developmentally regulated islet cell in the human pancreas**. *Regul Pept* 2002, **107**(1-3):63-69.
2. Prado CL, Pugh-Bernard AE, Elghazi L, Sosa-Pineda B, Sussel L: **Ghrelin cells replace insulin-producing beta cells in two mouse models of pancreas development**. *Proc Natl Acad Sci U S A* 2004, **101**(9):2924-2929.
3. Bosco D, Armanet M, Morel P, Niclauss N, Sgroi A, Muller YD, Giovannoni L, Parnaud G, Berney T: **Unique arrangement of alpha- and beta-cells in human islets of Langerhans**. *Diabetes* 2010, **59**(5):1202-1210.
4. Cabrera O, Berman DM, Kenyon NS, Ricordi C, Berggren PO, Caicedo A: **The unique cytoarchitecture of human pancreatic islets has implications for islet cell function**. *Proc Natl Acad Sci U S A* 2006, **103**(7):2334-2339.
5. Liu P, Wakamiya M, Shea MJ, Albrecht U, Behringer RR, Bradley A: **Requirement for Wnt3 in vertebrate axis formation**. *Nature genetics* 1999, **22**(4):361-365.
6. Van Hoof D, D'Amour KA, German MS: **Derivation of insulin-producing cells from human embryonic stem cells**. *Stem cell research* 2009, **3**(2-3):73-87.
7. Jorgensen MC, Ahnfelt-Ronne J, Hald J, Madsen OD, Serup P, Hecksher-Sorensen J: **An illustrated review of early pancreas development in the mouse**. *Endocrine reviews* 2007, **28**(6):685-705.
8. Ben-Othman N, Courtney M, Vieira A, Pfeifer A, Druelle N, Gjernes E, Faurite B, Avolio F, Collombat P: **From pancreatic islet formation to beta-cell regeneration**. *Diabetes research and clinical practice* 2013.
9. Oliver-Krasinski JM, Stoffers DA: **On the origin of the beta cell**. *Genes & development* 2008, **22**(15):1998-2021.
10. Collombat P, Mansouri A, Hecksher-Sorensen J, Serup P, Krull J, Gradwohl G, Gruss P: **Opposing actions of Arx and Pax4 in endocrine pancreas development**. *Genes & development* 2003, **17**(20):2591-2603.
11. Collombat P, Hecksher-Sorensen J, Broccoli V, Krull J, Ponte I, Mundiger T, Smith J, Gruss P, Serup P, Mansouri A: **The simultaneous loss of Arx and Pax4 genes promotes a somatostatin-producing cell fate specification at the expense of the alpha- and beta-cell lineages in the mouse endocrine pancreas**. *Development* 2005, **132**(13):2969-2980.

12. Artner I, Hang Y, Mazur M, Yamamoto T, Guo M, Lindner J, Magnuson MA, Stein R: **MafA and MafB regulate genes critical to beta-cells in a unique temporal manner**. *Diabetes* 2010, **59**(10):2530-2539.
13. Chanoine JP, Wong AC: **Ghrelin gene expression is markedly higher in fetal pancreas compared with fetal stomach: effect of maternal fasting**. *Endocrinology* 2004, **145**(8):3813-3820.
14. Arnes L, Hill JT, Gross S, Magnuson MA, Sussel L: **Ghrelin expression in the mouse pancreas defines a unique multipotent progenitor population**. *PLoS One* 2012, **7**(12):e52026.
15. Andralojc KM, Mercalli A, Nowak KW, Albarello L, Calcagno R, Luzi L, Bonifacio E, Doglioni C, Piemonti L: **Ghrelin-producing epsilon cells in the developing and adult human pancreas**. *Diabetologia* 2009, **52**(3):486-493.
16. Kumar AF, Gruessner RW, Seaquist ER: **Risk of glucose intolerance and diabetes in hemipancreatectomized donors selected for normal preoperative glucose metabolism**. *Diabetes Care* 2008, **31**(8):1639-1643.
17. Ritzel RA, Butler AE, Rizza RA, Veldhuis JD, Butler PC: **Relationship between beta-cell mass and fasting blood glucose concentration in humans**. *Diabetes Care* 2006, **29**(3):717-718.
18. Saisho Y, Butler AE, Manesso E, Galasso R, Zhang L, Gurlo T, Toffolo GM, Cobelli C, Kavanagh K, Wagner JD *et al*: **Relationship between fractional pancreatic beta cell area and fasting plasma glucose concentration in monkeys**. *Diabetologia* 2010, **53**(1):111-114.
19. Chung CH, Hao E, Piran R, Keinan E, Levine F: **Pancreatic beta-cell neogenesis by direct conversion from mature alpha-cells**. *Stem Cells* 2010, **28**(9):1630-1638.
20. Thorel F, Herrera PL: **[Conversion of adult pancreatic alpha-cells to beta-cells in diabetic mice]**. *Med Sci (Paris)* 2010, **26**(11):906-909.
21. Levine F, Itkin-Ansari P: **beta-cell Regeneration: neogenesis, replication or both?** *J Mol Med (Berl)* 2008, **86**(3):247-258.
22. Bonner-Weir S, Weir GC: **New sources of pancreatic beta-cells**. *Nat Biotechnol* 2005, **23**(7):857-861.
23. Dor Y, Brown J, Martinez OI, Melton DA: **Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation**. *Nature* 2004, **429**(6987):41-46.

24. Georgia S, Bhushan A: **Beta cell replication is the primary mechanism for maintaining postnatal beta cell mass**. *J Clin Invest* 2004, **114**(7):963-968.
25. Teta M, Long SY, Wartschow LM, Rankin MM, Kushner JA: **Very slow turnover of beta-cells in aged adult mice**. *Diabetes* 2005, **54**(9):2557-2567.
26. Maedler K, Schumann DM, Schulthess F, Oberholzer J, Bosco D, Berney T, Donath MY: **Aging correlates with decreased beta-cell proliferative capacity and enhanced sensitivity to apoptosis: a potential role for Fas and pancreatic duodenal homeobox-1**. *Diabetes* 2006, **55**(9):2455-2462.
27. Karnik SK, Chen H, McLean GW, Heit JJ, Gu X, Zhang AY, Fontaine M, Yen MH, Kim SK: **Menin controls growth of pancreatic beta-cells in pregnant mice and promotes gestational diabetes mellitus**. *Science* 2007, **318**(5851):806-809.
28. Brelje TC, Bhagroo NV, Stout LE, Sorenson RL: **Beneficial effects of lipids and prolactin on insulin secretion and beta-cell proliferation: a role for lipids in the adaptation of islets to pregnancy**. *J Endocrinol* 2008, **197**(2):265-276.
29. Hull RL, Kodama K, Utzschneider KM, Carr DB, Prigeon RL, Kahn SE: **Dietary-fat-induced obesity in mice results in beta cell hyperplasia but not increased insulin release: evidence for specificity of impaired beta cell adaptation**. *Diabetologia* 2005, **48**(7):1350-1358.
30. Davis DB, Lavine JA, Suhonen JI, Krautkramer KA, Rabaglia ME, Sperger JM, Fernandez LA, Yandell BS, Keller MP, Wang IM *et al*: **FoxM1 is up-regulated by obesity and stimulates beta-cell proliferation**. *Mol Endocrinol* 2010, **24**(9):1822-1834.
31. Bock T, Pakkenberg B, Buschard K: **Increased islet volume but unchanged islet number in ob/ob mice**. *Diabetes* 2003, **52**(7):1716-1722.
32. Alonso LC, Yokoe T, Zhang P, Scott DK, Kim SK, O'Donnell CP, Garcia-Ocana A: **Glucose infusion in mice: a new model to induce beta-cell replication**. *Diabetes* 2007, **56**(7):1792-1801.
33. Nir T, Melton DA, Dor Y: **Recovery from diabetes in mice by beta cell regeneration**. *J Clin Invest* 2007, **117**(9):2553-2561.
34. Xu X, D'Hoker J, Stange G, Bonne S, De Leu N, Xiao X, Van de Casteele M, Mellitzer G, Ling Z, Pipeleers D *et al*: **Beta cells can be generated from endogenous progenitors in injured adult mouse pancreas**. *Cell* 2008, **132**(2):197-207.

35. Van de Casteele M, Leuckx G, Baeyens L, Cai Y, Yuchi Y, Coppens V, De Groef S, Eriksson M, Svensson C, Ahlgren U *et al*: **Neurogenin 3(+) cells contribute to beta-cell neogenesis and proliferation in injured adult mouse pancreas**. *Cell Death Dis* 2013, **4**:e523.
36. Ackermann Misfeldt A, Costa RH, Gannon M: **Beta-cell proliferation, but not neogenesis, following 60% partial pancreatectomy is impaired in the absence of FoxM1**. *Diabetes* 2008, **57**(11):3069-3077.
37. Lee SH, Hao E, Levine F: **beta-Cell replication and islet neogenesis following partial pancreatectomy**. *Islets* 2011, **3**(4):188-195.
38. Liu T, Wang C, Wan C, Xiong J, Zhou F: **Proliferation and differentiation of duct epithelial cells after partial pancreatectomy in rats**. *J Huazhong Univ Sci Technolog Med Sci* 2006, **26**(5):567-569.
39. Waguri M, Yamamoto K, Miyagawa JI, Tochino Y, Yamamori K, Kajimoto Y, Nakajima H, Watada H, Yoshiuchi I, Itoh N *et al*: **Demonstration of two different processes of beta-cell regeneration in a new diabetic mouse model induced by selective perfusion of alloxan**. *Diabetes* 1997, **46**(8):1281-1290.
40. Bonner-Weir S, Baxter LA, Schuppin GT, Smith FE: **A second pathway for regeneration of adult exocrine and endocrine pancreas. A possible recapitulation of embryonic development**. *Diabetes* 1993, **42**(12):1715-1720.
41. Ellingsgaard H, Ehses JA, Hammar EB, Van Lommel L, Quintens R, Martens G, Kerr-Conte J, Pattou F, Berney T, Pipeleers D *et al*: **Interleukin-6 regulates pancreatic alpha-cell mass expansion**. *Proc Natl Acad Sci U S A* 2008, **105**(35):13163-13168.
42. Zhang Y, Zhang Y, Bone RN, Cui W, Peng JB, Siegal GP, Wang H, Wu H: **Regeneration of pancreatic non-beta endocrine cells in adult mice following a single diabetes-inducing dose of streptozotocin**. *PloS one* 2012, **7**(5):e36675.
43. Meier JJ, Butler AE, Saisho Y, Monchamp T, Galasso R, Bhushan A, Rizza RA, Butler PC: **Beta-cell replication is the primary mechanism subserving the postnatal expansion of beta-cell mass in humans**. *Diabetes* 2008, **57**(6):1584-1594.

44. Kassem SA, Ariel I, Thornton PS, Scheimberg I, Glaser B: **Beta-cell proliferation and apoptosis in the developing normal human pancreas and in hyperinsulinism of infancy**. *Diabetes* 2000, **49**(8):1325-1333.
45. Basu R, Breda E, Oberg AL, Powell CC, Dalla Man C, Basu A, Vittone JL, Klee GG, Arora P, Jensen MD *et al*: **Mechanisms of the age-associated deterioration in glucose tolerance: contribution of alterations in insulin secretion, action, and clearance**. *Diabetes* 2003, **52**(7):1738-1748.
46. Kohler CU, Olewinski M, Tannapfel A, Schmidt WE, Fritsch H, Meier JJ: **Cell cycle control of beta-cell replication in the prenatal and postnatal human pancreas**. *Am J Physiol Endocrinol Metab* 2011, **300**(1):E221-230.
47. Fiaschi-Taesch NM, Kleinberger JW, Salim F, Troxell R, Wills R, Tanwir M, Casinelli G, Cox AE, Takane KK, Scott DK *et al*: **A Human Pancreatic Beta Cell G1/S Molecule Cell Cycle Atlas**. *Diabetes* 2013.
48. Cozar-Castellano I, Fiaschi-Taesch N, Bigatel TA, Takane KK, Garcia-Ocana A, Vasavada R, Stewart AF: **Molecular control of cell cycle progression in the pancreatic beta-cell**. *Endocr Rev* 2006, **27**(4):356-370.
49. Cozar-Castellano I, Harb G, Selk K, Takane K, Vasavada R, Sicari B, Law B, Zhang P, Scott DK, Fiaschi-Taesch N *et al*: **Lessons from the first comprehensive molecular characterization of cell cycle control in rodent insulinoma cell lines**. *Diabetes* 2008, **57**(11):3056-3068.
50. Butler AE, Janson J, Bonner-Weir S, Ritzel R, Rizza RA, Butler PC: **Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes**. *Diabetes* 2003, **52**(1):102-110.
51. Butler AE, Cao-Minh L, Galasso R, Rizza RA, Corradin A, Cobelli C, Butler PC: **Adaptive changes in pancreatic beta cell fractional area and beta cell turnover in human pregnancy**. *Diabetologia* 2010, **53**(10):2167-2176.
52. Trivedi N, Hollister-Lock J, Lopez-Avalos MD, O'Neil JJ, Keegan M, Bonner-Weir S, Weir GC: **Increase in beta-cell mass in transplanted porcine neonatal pancreatic cell clusters is due to proliferation of beta-cells and differentiation of duct cells**. *Endocrinology* 2001, **142**(5):2115-2122.
53. Meier JJ, Butler AE, Galasso R, Butler PC: **Hyperinsulinemic hypoglycemia after gastric bypass surgery is not accompanied by islet hyperplasia or increased beta-cell turnover**. *Diabetes Care* 2006, **29**(7):1554-1559.

54. Meier JJ, Butler AE, Galasso R, Rizza RA, Butler PC: **Increased islet beta cell replication adjacent to intrapancreatic gastrinomas in humans**. *Diabetologia* 2006, **49**(11):2689-2696.
55. Bouwens L, Wang RN, De Blay E, Pipeleers DG, Kloppel G: **Cytokeratins as markers of ductal cell differentiation and islet neogenesis in the neonatal rat pancreas**. *Diabetes* 1994, **43**(11):1279-1283.
56. Finegood DT, Scaglia L, Bonner-Weir S: **Dynamics of beta-cell mass in the growing rat pancreas. Estimation with a simple mathematical model**. *Diabetes* 1995, **44**(3):249-256.
57. Bonner-Weir S, Toschi E, Inada A, Reitz P, Fonseca SY, Aye T, Sharma A: **The pancreatic ductal epithelium serves as a potential pool of progenitor cells**. *Pediatric diabetes* 2004, **5 Suppl 2**:16-22.
58. Solar M, Cardalda C, Houbracken I, Martin M, Maestro MA, De Medts N, Xu X, Grau V, Heimberg H, Bouwens L *et al*: **Pancreatic exocrine duct cells give rise to insulin-producing beta cells during embryogenesis but not after birth**. *Developmental cell* 2009, **17**(6):849-860.
59. Wang RN, Kloppel G, Bouwens L: **Duct- to islet-cell differentiation and islet growth in the pancreas of duct-ligated adult rats**. *Diabetologia* 1995, **38**(12):1405-1411.
60. Reers C, Erbel S, Esposito I, Schmied B, Buchler MW, Nawroth PP, Ritzel RA: **Impaired islet turnover in human donor pancreata with aging**. *European journal of endocrinology / European Federation of Endocrine Societies* 2009, **160**(2):185-191.
61. Baeyens L, De Breuck S, Lardon J, Mfopou JK, Rooman I, Bouwens L: **In vitro generation of insulin-producing beta cells from adult exocrine pancreatic cells**. *Diabetologia* 2005, **48**(1):49-57.
62. Zhou Q, Brown J, Kanarek A, Rajagopal J, Melton DA: **In vivo reprogramming of adult pancreatic exocrine cells to beta-cells**. *Nature* 2008, **455**(7213):627-632.
63. Collombat P, Xu X, Ravassard P, Sosa-Pineda B, Dussaud S, Billestrup N, Madsen OD, Serup P, Heimberg H, Mansouri A: **The ectopic expression of Pax4 in the mouse pancreas converts progenitor cells into alpha and subsequently beta cells**. *Cell* 2009, **138**(3):449-462.

64. Thorel F, Nepote V, Avril I, Kohno K, Desgraz R, Chera S, Herrera PL: **Conversion of adult pancreatic alpha-cells to beta-cells after extreme beta-cell loss**. *Nature* 2010, **464**(7292):1149-1154.
65. Dhawan S, Georgia S, Tschen SI, Fan G, Bhushan A: **Pancreatic beta cell identity is maintained by DNA methylation-mediated repression of Arx**. *Developmental cell* 2011, **20**(4):419-429.
66. Butler AE, Robertson RP, Hernandez R, Matveyenko AV, Gurlo T, Butler PC: **Beta cell nuclear musculoaponeurotic fibrosarcoma oncogene family A (MafA) is deficient in type 2 diabetes**. *Diabetologia* 2012, **55**(11):2985-2988.
67. Weinberg N, Ouziel-Yahalom L, Knoller S, Efrat S, Dor Y: **Lineage tracing evidence for in vitro dedifferentiation but rare proliferation of mouse pancreatic beta-cells**. *Diabetes* 2007, **56**(5):1299-1304.
68. Russ HA, Sintov E, Anker-Kitai L, Friedman O, Lenz A, Toren G, Farhy C, Pasmanik-Chor M, Oron-Karni V, Ravassard P *et al*: **Insulin-producing cells generated from dedifferentiated human pancreatic beta cells expanded in vitro**. *PLoS One* 2011, **6**(9):e25566.
69. Talchai C, Xuan S, Lin HV, Sussel L, Accili D: **Pancreatic beta cell dedifferentiation as a mechanism of diabetic beta cell failure**. *Cell* 2012, **150**(6):1223-1234.
70. Leonardi O, Mints G, Hussain MA: **Beta-cell apoptosis in the pathogenesis of human type 2 diabetes mellitus**. *European journal of endocrinology / European Federation of Endocrine Societies* 2003, **149**(2):99-102.
71. Kumar S: **Caspase function in programmed cell death**. *Cell death and differentiation* 2007, **14**(1):32-43.
72. Liadis N, Salmena L, Kwan E, Tajmir P, Schroer SA, Radziszewska A, Li X, Sheu L, Eweida M, Xu S *et al*: **Distinct in vivo roles of caspase-8 in beta-cells in physiological and diabetes models**. *Diabetes* 2007, **56**(9):2302-2311.
73. Liadis N, Murakami K, Eweida M, Elford AR, Sheu L, Gaisano HY, Hakem R, Ohashi PS, Woo M: **Caspase-3-dependent beta-cell apoptosis in the initiation of autoimmune diabetes mellitus**. *Molecular and cellular biology* 2005, **25**(9):3620-3629.
74. Jung HS, Chung KW, Won Kim J, Kim J, Komatsu M, Tanaka K, Nguyen YH, Kang TM, Yoon KH, Kim JW *et al*: **Loss of autophagy diminishes**

pancreatic beta cell mass and function with resultant hyperglycemia. *Cell metabolism* 2008, **8**(4):318-324.

75. Ebato C, Uchida T, Arakawa M, Komatsu M, Ueno T, Komiya K, Azuma K, Hirose T, Tanaka K, Kominami E *et al*: **Autophagy is important in islet homeostasis and compensatory increase of beta cell mass in response to high-fat diet**. *Cell metabolism* 2008, **8**(4):325-332.
76. Quan W, Hur KY, Lim Y, Oh SH, Lee JC, Kim KH, Kim GH, Kim SW, Kim HL, Lee MK *et al*: **Autophagy deficiency in beta cells leads to compromised unfolded protein response and progression from obesity to diabetes in mice**. *Diabetologia* 2012, **55**(2):392-403.
77. Banting FG, Best CH, Collip JB, Campbell WR, Fletcher AA: **Pancreatic Extracts in the Treatment of Diabetes Mellitus**. *Can Med Assoc J* 1922, **12**(3):141-146.
78. Lins PE, Wajngot A, Adamson U, Vranic M, Efendic S: **Minimal increases in glucagon levels enhance glucose production in man with partial hypoinsulinemia**. *Diabetes* 1983, **32**(7):633-636.
79. Freychet L, Rizkalla SW, Desplanque N, Basdevant A, Zirinis P, Tchobroutsky G, Slama G: **Effect of intranasal glucagon on blood glucose levels in healthy subjects and hypoglycaemic patients with insulin-dependent diabetes**. *Lancet* 1988, **1**(8599):1364-1366.
80. Myers SR, Diamond MP, Adkins-Marshall BA, Williams PE, Stinsen R, Cherrington AD: **Effects of small changes in glucagon on glucose production during a euglycemic, hyperinsulinemic clamp**. *Metabolism* 1991, **40**(1):66-71.
81. Naik RG, Brooks-Worrell BM, Palmer JP: **Latent autoimmune diabetes in adults**. *J Clin Endocrinol Metab* 2009, **94**(12):4635-4644.
82. Tattersall RB: **Mild familial diabetes with dominant inheritance**. *Q J Med* 1974, **43**(170):339-357.
83. Unger RH, Cherrington AD: **Glucagonocentric restructuring of diabetes: a pathophysiologic and therapeutic makeover**. *J Clin Invest* 2012, **122**(1):4-12.
84. Basu S, Yoffe P, Hills N, Lustig RH: **The relationship of sugar to population-level diabetes prevalence: an econometric analysis of repeated cross-sectional data**. *PloS one* 2013, **8**(2):e57873.

85. Kahn SE, Hull RL, Utzschneider KM: **Mechanisms linking obesity to insulin resistance and type 2 diabetes**. *Nature* 2006, **444**(7121):840-846.
86. Steiner DJ, Kim A, Miller K, Hara M: **Pancreatic islet plasticity: interspecies comparison of islet architecture and composition**. *Islets* 2010, **2**(3):135-145.
87. Wojtusciszyn A, Armanet M, Morel P, Berney T, Bosco D: **Insulin secretion from human beta cells is heterogeneous and dependent on cell-to-cell contacts**. *Diabetologia* 2008, **51**(10):1843-1852.
88. Jain R, Lammert E: **Cell-cell interactions in the endocrine pancreas**. *Diabetes, obesity & metabolism* 2009, **11 Suppl 4**:159-167.
89. Esni F, Taljedal IB, Perl AK, Cremer H, Christofori G, Semb H: **Neural cell adhesion molecule (N-CAM) is required for cell type segregation and normal ultrastructure in pancreatic islets**. *The Journal of cell biology* 1999, **144**(2):325-337.
90. Kanno T, Gopel SO, Rorsman P, Wakui M: **Cellular function in multicellular system for hormone-secretion: electrophysiological aspect of studies on alpha-, beta- and delta-cells of the pancreatic islet**. *Neuroscience research* 2002, **42**(2):79-90.
91. Dezaki K, Yada T: **Islet beta-cell ghrelin signaling for inhibition of insulin secretion**. *Methods in enzymology* 2012, **514**:317-331.
92. Carvalho CP, Oliveira RB, Britan A, Santos-Silva JC, Boschero AC, Meda P, Collares-Buzato CB: **Impaired beta-cell-beta-cell coupling mediated by Cx36 gap junctions in prediabetic mice**. *American journal of physiology Endocrinology and metabolism* 2012, **303**(1):E144-151.
93. Muscelli E, Mari A, Casolaro A, Camastra S, Seghieri G, Gastaldelli A, Holst JJ, Ferrannini E: **Separate impact of obesity and glucose tolerance on the incretin effect in normal subjects and type 2 diabetic patients**. *Diabetes* 2008, **57**(5):1340-1348.
94. Madsbad S: **The role of GLP-1 impairment in obesity and potential therapeutic implications**. *Diabetes, obesity & metabolism* 2013.
95. Shu L, Matveyenko AV, Kerr-Conte J, Cho JH, McIntosh CH, Maedler K: **Decreased TCF7L2 protein levels in type 2 diabetes mellitus correlate with downregulation of GIP- and GLP-1 receptors and impaired beta-cell function**. *Human molecular genetics* 2009, **18**(13):2388-2399.

96. Calanna S, Piro S, Di Pino A, Maria Zagami R, Urbano F, Purrello F, Maria Rabuazzo A: **Beta and alpha cell function in metabolically healthy but obese subjects: relationship with entero-insular axis**. *Obesity* 2013, **21**(2):320-325.

97. Miyawaki K, Yamada Y, Ban N, Ihara Y, Tsukiyama K, Zhou H, Fujimoto S, Oku A, Tsuda K, Toyokuni S *et al*: **Inhibition of gastric inhibitory polypeptide signaling prevents obesity**. *Nature medicine* 2002, **8**(7):738-742.

98. Kim SJ, Nian C, Karunakaran S, Clee SM, Isales CM, McIntosh CH: **GIP-overexpressing mice demonstrate reduced diet-induced obesity and steatosis, and improved glucose homeostasis**. *PloS one* 2012, **7**(7):e40156.

99. Meier JJ, Gallwitz B, Siepmann N, Holst JJ, Deacon CF, Schmidt WE, Nauck MA: **Gastric inhibitory polypeptide (GIP) dose-dependently stimulates glucagon secretion in healthy human subjects at euglycaemia**. *Diabetologia* 2003, **46**(6):798-801.

100. le Roux CW, Batterham RL, Aylwin SJ, Patterson M, Borg CM, Wynne KJ, Kent A, Vincent RP, Gardiner J, Ghatei MA *et al*: **Attenuated peptide YY release in obese subjects is associated with reduced satiety**. *Endocrinology* 2006, **147**(1):3-8.

101. Sam AH, Gunner DJ, King A, Persaud SJ, Brooks L, Hostomska K, Ford HE, Liu B, Ghatei MA, Bloom SR *et al*: **Selective ablation of peptide YY cells in adult mice reveals their role in beta cell survival**. *Gastroenterology* 2012, **143**(2):459-468.

102. Lavine JA, Raess PW, Stapleton DS, Rabaglia ME, Suhonen JI, Schueler KL, Koltes JE, Dawson JA, Yandell BS, Samuelson LC *et al*: **Cholecystokinin is up-regulated in obese mouse islets and expands beta-cell mass by increasing beta-cell survival**. *Endocrinology* 2010, **151**(8):3577-3588.

103. Lo CM, Obici S, Dong HH, Haas M, Lou D, Kim DH, Liu M, D'Alessio D, Woods SC, Tso P: **Impaired insulin secretion and enhanced insulin sensitivity in cholecystokinin-deficient mice**. *Diabetes* 2011, **60**(7):2000-2007.

104. Granata R, Gallo D, Luque RM, Baragli A, Scarlatti F, Grande C, Gesmundo I, Cordoba-Chacon J, Bergandi L, Settanni F *et al*: **Obestatin regulates adipocyte function and protects against diet-induced insulin resistance**

and inflammation. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 2012, **26**(8):3393-3411.

105. Tschop M, Weyer C, Tataranni PA, Devanarayan V, Ravussin E, Heiman ML: **Circulating ghrelin levels are decreased in human obesity**. *Diabetes* 2001, **50**(4):707-709.
106. Rosicka M, Krsek M, Matoulek M, Jarkovska Z, Marek J, Justova V, Lacinova Z: **Serum ghrelin levels in obese patients: the relationship to serum leptin levels and soluble leptin receptors levels**. *Physiological research / Academia Scientiarum Bohemoslovaca* 2003, **52**(1):61-66.
107. Rooman I, Lardon J, Bouwens L: **Gastrin stimulates beta-cell neogenesis and increases islet mass from transdifferentiated but not from normal exocrine pancreas tissue**. *Diabetes* 2002, **51**(3):686-690.
108. Sadry SA, Drucker DJ: **Emerging combinatorial hormone therapies for the treatment of obesity and T2DM**. *Nature reviews Endocrinology* 2013.
109. Zhao YF, Feng DD, Chen C: **Contribution of adipocyte-derived factors to beta-cell dysfunction in diabetes**. *The international journal of biochemistry & cell biology* 2006, **38**(5-6):804-819.
110. Lee YH, Magkos F, Mantzoros CS, Kang ES: **Effects of leptin and adiponectin on pancreatic beta-cell function**. *Metabolism: clinical and experimental* 2011, **60**(12):1664-1672.
111. Kulkarni RN, Wang ZL, Wang RM, Hurley JD, Smith DM, Ghatei MA, Withers DJ, Gardiner JV, Bailey CJ, Bloom SR: **Leptin rapidly suppresses insulin release from insulinoma cells, rat and human islets and, in vivo, in mice**. *The Journal of clinical investigation* 1997, **100**(11):2729-2736.
112. Koebnick C, Roberts CK, Shaibi GQ, Kelly LA, Lane CJ, Toledo-Corral CM, Davis JN, Ventura EE, Alexander K, Weigensberg MJ *et al*: **Adiponectin and leptin are independently associated with insulin sensitivity, but not with insulin secretion or beta-cell function in overweight Hispanic adolescents**. *Hormone and metabolic research = Hormon- und Stoffwechselforschung = Hormones et metabolisme* 2008, **40**(10):708-712.
113. Maeda N, Shimomura I, Kishida K, Nishizawa H, Matsuda M, Nagaretani H, Furuyama N, Kondo H, Takahashi M, Arita Y *et al*: **Diet-induced insulin resistance in mice lacking adiponectin/ACRP30**. *Nature medicine* 2002, **8**(7):731-737.

114. Kern PA, Di Gregorio GB, Lu T, Rassouli N, Ranganathan G: **Adiponectin expression from human adipose tissue: relation to obesity, insulin resistance, and tumor necrosis factor-alpha expression**. *Diabetes* 2003, **52**(7):1779-1785.
115. Silha JV, Krsek M, Skrha JV, Sucharda P, Nyomba BL, Murphy LJ: **Plasma resistin, adiponectin and leptin levels in lean and obese subjects: correlations with insulin resistance**. *European journal of endocrinology / European Federation of Endocrine Societies* 2003, **149**(4):331-335.
116. Lee JH, Chan JL, Yiannakouris N, Kontogianni M, Estrada E, Seip R, Orlova C, Mantzoros CS: **Circulating resistin levels are not associated with obesity or insulin resistance in humans and are not regulated by fasting or leptin administration: cross-sectional and interventional studies in normal, insulin-resistant, and diabetic subjects**. *The Journal of clinical endocrinology and metabolism* 2003, **88**(10):4848-4856.
117. Wang C, Guan Y, Yang J: **Cytokines in the Progression of Pancreatic beta-Cell Dysfunction**. *International journal of endocrinology* 2010, **2010**:515136.
118. Gao CL, Zhao DY, Qiu J, Zhang CM, Ji CB, Chen XH, Liu F, Guo XR: **Resistin induces rat insulinoma cell RINm5F apoptosis**. *Molecular biology reports* 2009, **36**(7):1703-1708.
119. da Silva Krause M, Bittencourt A, Homem de Bittencourt PI, Jr., McClenaghan NH, Flatt PR, Murphy C, Newsholme P: **Physiological concentrations of interleukin-6 directly promote insulin secretion, signal transduction, nitric oxide release, and redox status in a clonal pancreatic beta-cell line and mouse islets**. *The Journal of endocrinology* 2012, **214**(3):301-311.
120. Sauter NS, Schulthess FT, Galasso R, Castellani LW, Maedler K: **The antiinflammatory cytokine interleukin-1 receptor antagonist protects from high-fat diet-induced hyperglycemia**. *Endocrinology* 2008, **149**(5):2208-2218.
121. Ardestani A, Sauter NS, Paroni F, Dharmadhikari G, Cho JH, Lupi R, Marchetti P, Oberholzer J, Conte JK, Maedler K: **Neutralizing interleukin-1beta (IL-1beta) induces beta-cell survival by maintaining PDX1 protein nuclear localization**. *The Journal of biological chemistry* 2011, **286**(19):17144-17155.
122. Hotamisligil GS, Arner P, Caro JF, Atkinson RL, Spiegelman BM: **Increased adipose tissue expression of tumor necrosis factor-alpha in human**

obesity and insulin resistance. *The Journal of clinical investigation* 1995, **95**(5):2409-2415.

123. McArdle MA, Finucane OM, Connaughton RM, McMorrow AM, Roche HM: **Mechanisms of obesity-induced inflammation and insulin resistance: insights into the emerging role of nutritional strategies**. *Frontiers in endocrinology* 2013, **4**:52.
124. Wolden-Kirk H, Overbergh L, Gysemans C, Brusgaard K, Naamane N, Van Lommel L, Schuit F, Eizirik DL, Christesen H, Mathieu C: **Unraveling the effects of 1,25(OH)(2)D(3) on global gene expression in pancreatic islets**. *J Steroid Biochem Mol Biol* 2012.
125. Karnchanasorn R, Ou HY, Chiu KC: **Plasma 25-hydroxyvitamin D levels are favorably associated with beta-cell function**. *Pancreas* 2012, **41**(6):863-868.
126. Guasch A, Bullo M, Rabassa A, Bonada A, Del Castillo D, Sabench F, Salas-Salvado J: **Plasma vitamin D and parathormone are associated with obesity and atherogenic dyslipidemia: a cross-sectional study**. *Cardiovasc Diabetol* 2012, **11**:149.
127. de Las Heras J, Rajakumar K, Lee S, Bacha F, Holick MF, Arslanian SA: **25-Hydroxyvitamin D in Obese Youth Across the Spectrum of Glucose Tolerance From Normal to Prediabetes to Type 2 Diabetes**. *Diabetes Care* 2013.
128. Porter SE, Sorenson RL, Dann P, Garcia-Ocana A, Stewart AF, Vasavada RC: **Progressive pancreatic islet hyperplasia in the islet-targeted, parathyroid hormone-related protein-overexpressing mouse**. *Endocrinology* 1998, **139**(9):3743-3751.
129. Kumar R, Balhuizen A, Amisten S, Lundquist I, Salehi A: **Insulinotropic and antidiabetic effects of 17beta-estradiol and the GPR30 agonist G-1 on human pancreatic islets**. *Endocrinology* 2011, **152**(7):2568-2579.
130. Liu S, Kilic G, Meyers MS, Navarro G, Wang Y, Oberholzer J, Mauvais-Jarvis F: **Oestrogens improve human pancreatic islet transplantation in a mouse model of insulin deficient diabetes**. *Diabetologia* 2013, **56**(2):370-381.
131. Muller M, Grobbee DE, den Tonkelaar I, Lamberts SW, van der Schouw YT: **Endogenous sex hormones and metabolic syndrome in aging men**. *J Clin Endocrinol Metab* 2005, **90**(5):2618-2623.

132. Gammelsaeter R, Coppola T, Marcaggi P, Storm-Mathisen J, Chaudhry FA, Attwell D, Regazzi R, Gundersen V: **A role for glutamate transporters in the regulation of insulin secretion**. *PLoS One* 2011, **6**(8):e22960.
133. Feldmann N, del Rio RM, Gjinovci A, Tamarit-Rodriguez J, Wollheim CB, Wiederkehr A: **Reduction of plasma membrane glutamate transport potentiates insulin but not glucagon secretion in pancreatic islet cells**. *Mol Cell Endocrinol* 2011, **338**(1-2):46-57.
134. Taneera J, Jin Z, Jin Y, Muhammed SJ, Zhang E, Lang S, Salehi A, Korsgren O, Renstrom E, Groop L *et al*: **gamma-Aminobutyric acid (GABA) signalling in human pancreatic islets is altered in type 2 diabetes**. *Diabetologia* 2012, **55**(7):1985-1994.
135. Wang GJ, Volkow ND, Logan J, Pappas NR, Wong CT, Zhu W, Netusil N, Fowler JS: **Brain dopamine and obesity**. *Lancet* 2001, **357**(9253):354-357.
136. Rubi B, Ljubicic S, Pournourmohammadi S, Carobbio S, Armanet M, Bartley C, Maechler P: **Dopamine D2-like receptors are expressed in pancreatic beta cells and mediate inhibition of insulin secretion**. *J Biol Chem* 2005, **280**(44):36824-36832.
137. Ustione A, Piston DW: **Dopamine synthesis and D3 receptor activation in pancreatic beta-cells regulates insulin secretion and intracellular [Ca(2+)] oscillations**. *Mol Endocrinol* 2012, **26**(11):1928-1940.
138. Dai XP, Liu ZQ, Xu LY, Gong ZC, Huang Q, Dong M, Huang X: **Association of plasma epinephrine level with insulin sensitivity in metabolically healthy but obese individuals**. *Auton Neurosci* 2012, **167**(1-2):66-69.
139. Dallner OS, Chernogubova E, Brolinson KA, Bengtsson T: **Beta3-adrenergic receptors stimulate glucose uptake in brown adipocytes by two mechanisms independently of glucose transporter 4 translocation**. *Endocrinology* 2006, **147**(12):5730-5739.
140. Straub SG, Sharp GW: **Evolving insights regarding mechanisms for the inhibition of insulin release by norepinephrine and heterotrimeric G proteins**. *Am J Physiol Cell Physiol* 2012, **302**(12):C1687-1698.
141. Markianos M, Evangelopoulos ME, Koutsis G, Sfagos C: **Elevated CSF serotonin and dopamine metabolite levels in overweight subjects**. *Obesity* 2012.
142. Zhang Q, Zhu Y, Zhou W, Gao L, Yuan L, Han X: **Serotonin Receptor 2C and Insulin Secretion**. *PLoS One* 2013, **8**(1):e54250.

143. Hauge-Evans AC, King AJ, Carmignac D, Richardson CC, Robinson IC, Low MJ, Christie MR, Persaud SJ, Jones PM: **Somatostatin secreted by islet delta-cells fulfills multiple roles as a paracrine regulator of islet function**. *Diabetes* 2009, **58**(2):403-411.
144. Rodriguez-Diaz R, Dando R, Jacques-Silva MC, Fachado A, Molina J, Abdulreda MH, Ricordi C, Roper SD, Berggren PO, Caicedo A: **Alpha cells secrete acetylcholine as a non-neuronal paracrine signal priming beta cell function in humans**. *Nat Med* 2011, **17**(7):888-892.
145. Lee RC, Feinbaum RL, Ambros V: **The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14**. *Cell* 1993, **75**(5):843-854.
146. Williams MD, Mitchell GM: **MicroRNAs in insulin resistance and obesity**. *Experimental diabetes research* 2012, **2012**:484696.
147. Klein D, Misawa R, Bravo-Egana V, Vargas N, Rosero S, Piroso J, Ichii H, Umland O, Zhijie J, Tsinoremas N *et al*: **MicroRNA expression in alpha and beta cells of human pancreatic islets**. *PloS one* 2013, **8**(1):e55064.
148. Jacovetti C, Abderrahmani A, Parnaud G, Jonas JC, Peyot ML, Cornu M, Laybutt R, Meugnier E, Rome S, Thorens B *et al*: **MicroRNAs contribute to compensatory beta cell expansion during pregnancy and obesity**. *The Journal of clinical investigation* 2012, **122**(10):3541-3551.
149. Wang Y, Liu J, Liu C, Naji A, Stoffers DA: **MicroRNA-7 regulates the mTOR pathway and proliferation in adult pancreatic beta-cells**. *Diabetes* 2013, **62**(3):887-895.
150. Nesca V, Guay C, Jacovetti C, Menoud V, Peyot ML, Laybutt DR, Prentki M, Regazzi R: **Identification of particular groups of microRNAs that positively or negatively impact on beta cell function in obese models of type 2 diabetes**. *Diabetologia* 2013, **56**(10):2203-2212.
151. DeFronzo RA, Tobin JD, Andres R: **Glucose clamp technique: a method for quantifying insulin secretion and resistance**. *The American journal of physiology* 1979, **237**(3):E214-223.
152. Muniyappa R, Lee S, Chen H, Quon MJ: **Current approaches for assessing insulin sensitivity and resistance in vivo: advantages, limitations, and appropriate usage**. *American journal of physiology Endocrinology and metabolism* 2008, **294**(1):E15-26.

153. Harano Y, Hidaka H, Takatsuki K, Ohgaku S, Haneda M, Motoi S, Kawagoe K, Shigeta Y, Abe H: **Glucose, insulin, and somatostatin infusion for the determination of insulin sensitivity in vivo**. *Metabolism: clinical and experimental* 1978, **27**(9 Suppl 1):1449-1452.
154. Bergman RN, Ider YZ, Bowden CR, Cobelli C: **Quantitative estimation of insulin sensitivity**. *The American journal of physiology* 1979, **236**(6):E667-677.
155. Wallace TM, Levy JC, Matthews DR: **Use and abuse of HOMA modeling**. *Diabetes Care* 2004, **27**(6):1487-1495.
156. Bergman RN, Ader M, Huecking K, Van Citters G: **Accurate assessment of beta-cell function: the hyperbolic correction**. *Diabetes* 2002, **51 Suppl 1**:S212-220.
157. Utzschneider KM, Prigeon RL, Faulenbach MV, Tong J, Carr DB, Boyko EJ, Leonetti DL, McNeely MJ, Fujimoto WY, Kahn SE: **Oral disposition index predicts the development of future diabetes above and beyond fasting and 2-h glucose levels**. *Diabetes Care* 2009, **32**(2):335-341.
158. Malaisse WJ, Zhang Y, Louchami K, Sharma S, Dresselaers T, Himmelreich U, Novotny GW, Mandrup-Poulsen T, Waschke D, Leshch Y *et al*: **(19)F-heptuloses as tools for the non-invasive imaging of GLUT2-expressing cells**. *Archives of biochemistry and biophysics* 2012, **517**(2):138-143.
159. Schneider S, Feilen PJ, Schreckenberger M, Schwanstecher M, Schwanstecher C, Buchholz HG, Thews O, Oberholzer K, Korobeynikov A, Bauman A *et al*: **In vitro and in vivo evaluation of novel glibenclamide derivatives as imaging agents for the non-invasive assessment of the pancreatic islet cell mass in animals and humans**. *Experimental and clinical endocrinology & diabetes : official journal, German Society of Endocrinology [and] German Diabetes Association* 2005, **113**(7):388-395.
160. Malaisse WJ, Maedler K: **Imaging of the beta-cells of the islets of Langerhans**. *Diabetes research and clinical practice* 2012, **98**(1):11-18.
161. Lubag AJ, De Leon-Rodriguez LM, Burgess SC, Sherry AD: **Noninvasive MRI of beta-cell function using a Zn2+-responsive contrast agent**. *Proceedings of the National Academy of Sciences of the United States of America* 2011, **108**(45):18400-18405.
162. Villiger M, Goulley J, Friedrich M, Grapin-Botton A, Meda P, Lasser T, Leitgeb RA: **In vivo imaging of murine endocrine islets of Langerhans with**

extended-focus optical coherence microscopy. *Diabetologia* 2009, **52**(8):1599-1607.

163. Leibiger IB, Caicedo A, Berggren PO: **Non-invasive in vivo imaging of pancreatic beta-cell function and survival - a perspective**. *Acta physiologica* 2012, **204**(2):178-185.
164. Andralojc K, Srinivas M, Brom M, Joosten L, de Vries IJ, Eizirik DL, Boerman OC, Meda P, Gotthardt M: **Obstacles on the way to the clinical visualisation of beta cells: looking for the Aeneas of molecular imaging to navigate between Scylla and Charybdis**. *Diabetologia* 2012, **55**(5):1247-1257.
165. Speier S, Nyqvist D, Cabrera O, Yu J, Molano RD, Pileggi A, Moede T, Kohler M, Wilbertz J, Leibiger B *et al*: **Noninvasive in vivo imaging of pancreatic islet cell biology**. *Nature medicine* 2008, **14**(5):574-578.
166. Pattou F, Kerr-Conte J, Wild D: **GLP-1-receptor scanning for imaging of human beta cells transplanted in muscle**. *The New England journal of medicine* 2010, **363**(13):1289-1290.
167. Shapiro AM, Lakey JR, Ryan EA, Korbutt GS, Toth E, Warnock GL, Kneteman NM, Rajotte RV: **Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen**. *The New England journal of medicine* 2000, **343**(4):230-238.
168. O'Sullivan ES, Vegas A, Anderson DG, Weir GC: **Islets transplanted in immunoisolation devices: a review of the progress and the challenges that remain**. *Endocrine reviews* 2011, **32**(6):827-844.
169. Sakuma Y, Ricordi C, Miki A, Yamamoto T, Pileggi A, Khan A, Alejandro R, Inverardi L, Ichii H: **Factors that affect human islet isolation**. *Transplant Proc* 2008, **40**(2):343-345.
170. Bartlett ST, Kuo PC, Johnson LB, Lim JW, Schweitzer EJ: **Pancreas transplantation at the University of Maryland**. *Clin Transpl* 1996:271-280.
171. Ridgway D, Manas D, Shaw J, White S: **Preservation of the donor pancreas for whole pancreas and islet transplantation**. *Clin Transplant* 2010, **24**(1):1-19.
172. Matsumoto I, Sawada T, Nakano M, Sakai T, Liu B, Ansite JD, Zhang HJ, Kandaswamy R, Sutherland DE, Hering BJ: **Improvement in islet yield from obese donors for human islet transplants**. *Transplantation* 2004, **78**(6):880-885.

173. Duvivier-Kali VF, Omer A, Parent RJ, O'Neil JJ, Weir GC: **Complete protection of islets against allorejection and autoimmunity by a simple barium-alginate membrane**. *Diabetes* 2001, **50**(8):1698-1705.
174. Tuch BE, Keogh GW, Williams LJ, Wu W, Foster JL, Vaithilingam V, Philips R: **Safety and viability of microencapsulated human islets transplanted into diabetic humans**. *Diabetes care* 2009, **32**(10):1887-1889.
175. Dufour JM, Rajotte RV, Zimmerman M, Rezania A, Kin T, Dixon DE, Korbutt GS: **Development of an ectopic site for islet transplantation, using biodegradable scaffolds**. *Tissue engineering* 2005, **11**(9-10):1323-1331.
176. Lin CC, Anseth KS: **Glucagon-like peptide-1 functionalized PEG hydrogels promote survival and function of encapsulated pancreatic beta-cells**. *Biomacromolecules* 2009, **10**(9):2460-2467.
177. Low G, Hussein N, Owen RJ, Toso C, Patel VH, Bhargava R, Shapiro AM: **Role of imaging in clinical islet transplantation**. *Radiographics : a review publication of the Radiological Society of North America, Inc* 2010, **30**(2):353-366.
178. Lakey JR, Kin T, Warnock GL, Shapiro AM, Tsapogas P, Imes S, Korbutt GS, Kneteman NM, Rajotte RV, Ryan EA: **Long-term graft function after allogeneic islet transplantation**. *Cell transplantation* 2007, **16**(4):441-446.
179. Bertera S, Balamurugan AN, Bottino R, He J, Trucco M: **Increased yield and improved transplantation outcome of mouse islets with bovine serum albumin**. *Journal of transplantation* 2012, **2012**:856386.
180. Loganathan G, Graham ML, Radosevich DM, Soltani SM, Tiwari M, Anazawa T, Papas KK, Sutherland DE, Hering BJ, Balamurugan AN: **Factors Affecting Transplant Outcomes in Diabetic Nude Mice Receiving Human, Porcine, and Nonhuman Primate Islets: Analysis of 335 Transplantations**. *Transplantation* 2013.
181. Shimizu H, Ohashi K, Utoh R, Ise K, Gotoh M, Yamato M, Okano T: **Bioengineering of a functional sheet of islet cells for the treatment of diabetes mellitus**. *Biomaterials* 2009, **30**(30):5943-5949.
182. Qi M, Lacik I, Kollarikova G, Strand BL, Formo K, Wang Y, Marchese E, Mendoza-Elias JE, Kinzer KP, Gatti F *et al*: **A recommended laparoscopic procedure for implantation of microcapsules in the peritoneal cavity of non-human primates**. *The Journal of surgical research* 2011, **168**(1):e117-123.

183. Medarova Z, Evgenov NV, Dai G, Bonner-Weir S, Moore A: **In vivo multimodal imaging of transplanted pancreatic islets**. *Nature protocols* 2006, **1**(1):429-435.
184. Caiazzo R, Gmyr V, Kremer B, Hubert T, Soudan B, Lukowiak B, Vandewalle B, Vantyghem MC, Pattou F, Kerr-Conte J: **Quantitative in vivo islet potency assay in normoglycemic nude mice correlates with primary graft function after clinical transplantation**. *Transplantation* 2008, **86**(2):360-363.
185. Brandhorst H, Brandhorst D, Hering BJ, Federlin K, Bretzel RG: **Body mass index of pancreatic donors: a decisive factor for human islet isolation**. *Experimental and clinical endocrinology & diabetes : official journal, German Society of Endocrinology [and] German Diabetes Association* 1995, **103 Suppl 2**:23-26.
186. Hubert T, Gmyr V, Arnalsteen L, Jany T, Triponez F, Caiazzo R, Vandewalle B, Vantyghem MC, Kerr-Conte J, Pattou F: **Influence of preservation solution on human islet isolation outcome**. *Transplantation* 2007, **83**(3):270-276.
187. Vantyghem MC, Kerr-Conte J, Arnalsteen L, Sergent G, Defrance F, Gmyr V, Declerck N, Raverdy V, Vandewalle B, Pigny P *et al*: **Primary graft function, metabolic control, and graft survival after islet transplantation**. *Diabetes care* 2009, **32**(8):1473-1478.
188. Duffaut C, Galitzky J, Lafontan M, Bouloumie A: **Unexpected trafficking of immune cells within the adipose tissue during the onset of obesity**. *Biochem Biophys Res Commun* 2009, **384**(4):482-485.
189. Papademetris X, Shkarin P, Staib LH, Behar KL: **Regional whole body fat quantification in mice**. *Inf Process Med Imaging* 2005, **19**:369-380.
190. McCall M, Pawlick R, Kin T, Shapiro AM: **Anakinra potentiates the protective effects of etanercept in transplantation of marginal mass human islets in immunodeficient mice**. *Am J Transplant* 2012, **12**(2):322-329.
191. Castaing M, Guerci A, Mallet J, Czernichow P, Ravassard P, Scharfmann R: **Efficient restricted gene expression in beta cells by lentivirus-mediated gene transfer into pancreatic stem/progenitor cells**. *Diabetologia* 2005, **48**(4):709-719.

192. Russ HA, Bar Y, Ravassard P, Efrat S: **In vitro proliferation of cells derived from adult human beta-cells revealed by cell-lineage tracing**. *Diabetes* 2008, **57**(6):1575-1583.

193. Saisho Y, Butler AE, Manesso E, Elashoff D, Rizza RA, Butler PC: **beta-Cell Mass and Turnover in Humans: Effects of obesity and aging**. *Diabetes Care* 2013, **36**(1):111-117.

194. Rahier J, Guiot Y, Goebbels RM, Sempoux C, Henquin JC: **Pancreatic beta-cell mass in European subjects with type 2 diabetes**. *Diabetes Obes Metab* 2008, **10 Suppl 4**:32-42.

195. Hubert T, Strecker G, Gmyr V, Arnalsteen L, Garrigue D, Ezzouaoui R, Caiazzo R, Dezfoulian G, Averland B, Vandewalle B *et al*: **Acute insulin response to arginine in deceased donors predicts the outcome of human islet isolation**. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 2008, **8**(4):872-876.

196. Ricordi C, Scharp DW, Lacy PE: **Reversal of diabetes in nude mice after transplantation of fresh and 7-day-cultured (24 degrees C) human pancreatic islets**. *Transplantation* 1988, **45**(5):994-996.

197. Gaber AO, Fraga D, Kotb M, Lo A, Sabek O, Latif K: **Human islet graft function in NOD-SCID mice predicts clinical response in islet transplant recipients**. *Transplantation proceedings* 2004, **36**(4):1108-1110.

198. Luo J, Nguyen K, Chen M, Tran T, Hao J, Tian B, Rulifson IC, Zhang Y, Tian L, Zhang Y *et al*: **Evaluating insulin secretagogues in a humanized mouse model with functional human islets**. *Metabolism: clinical and experimental* 2013, **62**(1):90-99.

199. Winer S, Chan Y, Paltser G, Truong D, Tsui H, Bahrami J, Dorfman R, Wang Y, Zielenski J, Mastronardi F *et al*: **Normalization of obesity-associated insulin resistance through immunotherapy**. *Nat Med* 2009, **15**(8):921-929.

200. Heilbronn LK, Coster AC, Campbell LV, Greenfield JR, Lange K, Christopher MJ, Meikle PJ, Samocha-Bonet D: **The effect of short term overfeeding on serum lipids in healthy humans**. *Obesity* 2013.

201. Turner N, Kowalski GM, Leslie SJ, Risis S, Yang C, Lee-Young RS, Babb JR, Meikle PJ, Lancaster GI, Henstridge DC *et al*: **Distinct patterns of tissue-specific lipid accumulation during the induction of insulin resistance in mice by high-fat feeding**. *Diabetologia* 2013, **56**(7):1638-1648.

202. Ng SF, Lin RC, Laybutt DR, Barres R, Owens JA, Morris MJ: **Chronic high-fat diet in fathers programs beta-cell dysfunction in female rat offspring**. *Nature* 2010, **467**(7318):963-966.
203. Ellenbroek JH, Tons HA, de Graaf N, Loomans CJ, Engelse MA, Vrolijk H, Voshol PJ, Rabelink TJ, Carlotti F, de Koning EJ: **Topologically heterogeneous beta cell adaptation in response to high-fat diet in mice**. *PloS one* 2013, **8**(2):e56922.
204. Blume N, Skouv J, Larsson LI, Holst JJ, Madsen OD: **Potent inhibitory effects of transplantable rat glucagonomas and insulinomas on the respective endogenous islet cells are associated with pancreatic apoptosis**. *The Journal of clinical investigation* 1995, **96**(5):2227-2235.
205. Peyot ML, Pepin E, Lamontagne J, Latour MG, Zarrouki B, Lussier R, Pineda M, Jetton TL, Madiraju SR, Joly E *et al*: **Beta-cell failure in diet-induced obese mice stratified according to body weight gain: secretory dysfunction and altered islet lipid metabolism without steatosis or reduced beta-cell mass**. *Diabetes* 2010, **59**(9):2178-2187.
206. Kroon E, Martinson LA, Kadoya K, Bang AG, Kelly OG, Eliazer S, Young H, Richardson M, Smart NG, Cunningham J *et al*: **Pancreatic endoderm derived from human embryonic stem cells generates glucose-responsive insulin-secreting cells in vivo**. *Nature biotechnology* 2008, **26**(4):443-452.
207. Stamateris RE, Sharma RB, Hollern DA, Alonso LC: **Adaptive beta-cell proliferation increases early in high-fat feeding in mice, concurrent with metabolic changes, with induction of islet cyclin D2 expression**. *American journal of physiology Endocrinology and metabolism* 2013, **305**(1):E149-159.
208. Levitt HE, Cyphert TJ, Pascoe JL, Hollern DA, Abraham N, Lundell RJ, Rosa T, Romano LC, Zou B, O'Donnell CP *et al*: **Glucose stimulates human beta cell replication in vivo in islets transplanted into NOD-severe combined immunodeficiency (SCID) mice**. *Diabetologia* 2011, **54**(3):572-582.
209. Kawamori D, Kurpad AJ, Hu J, Liew CW, Shih JL, Ford EL, Herrera PL, Polonsky KS, McGuinness OP, Kulkarni RN: **Insulin signaling in alpha cells modulates glucagon secretion in vivo**. *Cell metabolism* 2009, **9**(4):350-361.
210. Gaisano HY, Macdonald PE, Vranic M: **Glucagon secretion and signaling in the development of diabetes**. *Frontiers in physiology* 2012, **3**:349.

211. Henquin JC, Rahier J: **Pancreatic alpha cell mass in European subjects with type 2 diabetes**. *Diabetologia* 2011, **54**(7):1720-1725.
212. Guardado-Mendoza R, Jimenez-Ceja L, Majluf-Cruz A, Kamath S, Fiorentino TV, Casiraghi F, Velazquez AO, Defronzo RA, Dick E, Davalli A *et al*: **Impact of obesity severity and duration on pancreatic beta- and alpha-cell dynamics in normoglycemic non-human primates**. *International journal of obesity* 2012.
213. Kou K, Saisho Y, Satoh S, Yamada T, Itoh H: **Change in beta cell mass in Japanese non-diabetic obese individuals**. *The Journal of clinical endocrinology and metabolism* 2013.
214. Matveyenko AV, Butler PC: **Relationship between beta-cell mass and diabetes onset**. *Diabetes, obesity & metabolism* 2008, **10 Suppl 4**:23-31.
215. Butler PC, Meier JJ, Butler AE, Bhushan A: **The replication of beta cells in normal physiology, in disease and for therapy**. *Nature clinical practice Endocrinology & metabolism* 2007, **3**(11):758-768.
216. Gmyr V, Kerr-Conte J, Belaich S, Vandewalle B, Leteurtre E, Vantyghem MC, Lecomte-Houcke M, Proye C, Lefebvre J, Pattou F: **Adult human cytokeratin 19-positive cells reexpress insulin promoter factor 1 in vitro: further evidence for pluripotent pancreatic stem cells in humans**. *Diabetes* 2000, **49**(10):1671-1680.
217. Bouwens L: **Cytokeratins and cell differentiation in the pancreas**. *The Journal of pathology* 1998, **184**(3):234-239.
218. Lima MJ, Muir KR, Docherty HM, Drummond R, McGowan NW, Forbes S, Heremans Y, Houbracken I, Ross JA, Forbes SJ *et al*: **Suppression of Epithelial to Mesenchymal Transitioning (EMT) Enhances Ex Vivo Reprogramming of Human Exocrine Pancreatic Tissue towards Functional Insulin Producing beta-Like Cells**. *Diabetes* 2013.
219. Spijker HS, Ravelli RB, Mommaas-Kienhuis AM, van Apeldoorn AA, Engelse MA, Zaldumbide A, Bonner-Weir S, Rabelink TJ, Hoeben RC, Clevers H *et al*: **Conversion of Mature Human beta-Cells Into Glucagon-Producing alpha-Cells**. *Diabetes* 2013, **62**(7):2471-2480.
220. Fiaschi-Taesch NM, Kleinberger JW, Salim FG, Troxell R, Wills R, Tanwir M, Casinelli G, Cox AE, Takane KK, Scott DK *et al*: **Human pancreatic beta-cell G1/S molecule cell cycle atlas**. *Diabetes* 2013, **62**(7):2450-2459.

221. Chan JY, Luzuriaga J, Bensellam M, Biden TJ, Laybutt DR: **Failure of the adaptive unfolded protein response in islets of obese mice is linked with abnormalities in beta-cell gene expression and progression to diabetes**. *Diabetes* 2013, **62**(5):1557-1568.
222. Goldfine AB, Kulkarni RN: **Modulation of beta-cell function: a translational journey from the bench to the bedside**. *Diabetes, obesity & metabolism* 2012, **14 Suppl 3**:152-160.
223. Sterkers A, Hubert T, Gmyr V, Torres F, Baud G, Delalleau N, Vantyghem MC, Kerr-Conte J, Caiazzo R, Pattou F: **Islet survival and function following intramuscular autotransplantation in the minipig**. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 2013, **13**(4):891-898.
224. Capito C, Simon MT, Aiello V, Clark A, Aigrain Y, Ravassard P, Scharfmann R: **The mouse muscle as an ectopic permissive site for human pancreatic development**. *Diabetes* 2013.
225. Yi P, Park JS, Melton DA: **Betatrophin: a hormone that controls pancreatic beta cell proliferation**. *Cell* 2013, **153**(4):747-758.
226. Lu J, Herrera PL, Carreira C, Bonnavion R, Seigne C, Calender A, Bertolino P, Zhang CX: **Alpha cell-specific Men1 ablation triggers the transdifferentiation of glucagon-expressing cells and insulinoma development**. *Gastroenterology* 2010, **138**(5):1954-1965.
227. Riedel MJ, Asadi A, Wang R, Ao Z, Warnock GL, Kieffer TJ: **Immunohistochemical characterisation of cells co-producing insulin and glucagon in the developing human pancreas**. *Diabetologia* 2012, **55**(2):372-381.

Chapter VIII

Annexes

Annex 1

Viability of islet cells

- Centrifuge 200 islets for 2 min and remove the medium
- Add cocktail of Dithizon (300µl),Trypan blue (300µl) and PBS (300µl) and mix gently
- Centrifuge for 2 min and remove ~850µl of the supernatant
- Add 1ml PBS
- Remove maximum of supernatant
- Add 300-400µl PBS
- Measure the islets of three droplets
- Islets are determined with red color (dithizon) and dead cell into islets with dark blue color (trypan blue)
- Islets classified in group with10%; 30%; 50%; 75%, 90% and 100% live cell and total viability is measured taking into account the number of islets in each group

Annex 2

Steps of procedure of human c-peptide measurement

Steps	Contents	Incubation
1	25µl Serum of mice	
2	50µl Assay Buffer	1hour at room temperature
3	350µl Wash Buffer (6times)	
4	100µl Enzyme	1hour at room temperature
5	350µl Wash Buffer (6times)	
6	200µl Substrate TMB	30 min at room temperature
7	50µl Stop Solution	
8	Read optical density at 450nm	

The concentration of c-peptide is obtained from the calibration curve. In each plate, the optical density at 450nm and the known concentration of calibrators (pmol/l) create a curve in which the concentration of unknown samples may be calculated according to their optical density.

Annex 3

a) Automatic inclusion in paraffin

- Immersion in 70% EtOH→ 1h
- Immersion in 95% EtOH→ 1h30
- Immersion in 95% EtOH→ 1h30
- Immersion in 100% EtOH→ 1h30
- Immersion in 100% EtOH→ 2h
- Immersion in 100% EtOH→ 2h
- Immersion in toluene → 2h
- Immersion in toluene → 2h
- Immersion in paraffin → 2h30
- Immersion in paraffin → 2h30

b) Inclusion in gelatin

- Immersion of tissue in 15% sucrose overnight at 4°C
- Inclusion in 7% gelatin/15% sucrose
- Gelatin block freezing into isopentane cooled by dry ice

c) Inclusion in thrombin/ fibrinogen

- Centrifugation of islets or cells
- Discard the supernatant
- Add V1 of thrombin
- Add V2=V1 fibrinogen
- Fixation overnight in 4% PFA

Annex 4

Immunohistochemistry -fluoresce protocol for paraffin section

1) Deparaffinizing and rehydrating the section
   - Xylene 1 for 5 min
   - Xylene 2 for 10 min
   - 100% EtOH for 5 min
   - 90% EtOH for 5 min
   - 70% EtOH for 5 min
   - 50% EtOH for 5 min
   - Distilled water for 5 min
   - PBS for 5 min
2) Antigen retrieval is depended by primary antibody
   - Heat-induced by microwave (9 min; 650W): Sodium citrate 10 mM or unmasking solution (Vector H 3300), pH 6.0
   - Heat-induced by microwave (9 min; 650W): Tris/EDTA pH 9.0
   - Enzymatic: Protease K
3) Leave slides to cool down for 30 min in the same solution
4) PBS washing 3 x 5 min
5) Blocking with Protein block (Dako) for 15-60 min
6) Incubation with primary antibody diluted for 3hours (room temperature) or overnight at 4°C (dilution with PBS or antibody diluant-DAKO)
7) PBS washing 3 x 5 min
8) Incubation with secondary antibody diluted
   a) Immunofluorescence: for 45min (PBS or antibody diluant-DAKO)
      - For double staining: PBS washing 3 x 5 min; incubation with 2nd primary antibody for 2-3 hours; PBS washing 3 x 5 min; incubation with 2nd secondary antibody for 45min
   b) Visible in light microscopy: Peroxide/DAB reaction
      - Endogenous peroxidases quenching: slides in H2O2 bath diluted in H20 for 30min (3%) or enzyme block solution (DAKO)
      - Biotinylated secondary antibody for 45min
      - DAB for 10-20 min

9) PBS washing 3 x 5 min
10) Nuclear staining Vectashield Mounting Medium with DAPI (immunofluorescence) or Hematoxyline (Peroxide/DAB reaction)

Immunofluoresce protocol for cryo-sections

1) PBS washing for 10 min at 40°C
2) PBS washing for 5 min at room temperature
3) PBTriton 0.2% washing for 10 min
4) Protein blocking with PBT0.2%+10% goat serum for 1 hour
5) Incubation with all the primary antibodies in PBT0.2%+2% goat serum overnight at 4°C
6) PBT0.2% washing 3 x 5 min
7) Incubation with all the secondary antibodies in PBT0.2%+2% goat serum for 1hour
8) PBT0.2% washing 3 x 5 min
9) Mounting slides with Vectashield mounting medium

Publications

- Related to PhD project

1) **Gargani S**, Thévenet J, Yuan JE, Lefebvre B, Delalleau N, Gmyr V, Hubert T, Duhamel A, Pattou F, Kerr-Conte J. Adaptive changes of human islets to an obesogenic environment in the mouse. ***Diabetologia.*** **2013** *Feb;56(2):350-358*

2) **Gargani S,** Pattou F, Kerr-Conte J. Comment on: β-cell mass and turnover in humans. Effects of obesity and aging. ***Diabetes Care***. **2013** Jul;36(7):e111. doi: 10.2337/dc13-0220;

- Others

1) Le Bacquer O, Kerr-Conte J, **Gargani S**, Delalleau N, Huyvaert M, Gmyr V, Froguel P, Neve B, Pattou F. TCF7L2 rs7903146 impairs islet function and morphology in non-diabetic individuals. ***Diabetologia.*** **2012** *Oct;55(10):2677-2681*

2) Prevost G, Arabo A, Jian L, Quelennec E, Cartier D, Hassan S, Falluel-Morel A, Tanguy Y, **Gargani S**, Lihrmann I, Kerr-Conte J, Lefebvre H, Pattou F, Anouar Y. The PACAP-Regulated Gene Selenoprotein T Is Abundantly Expressed in Mouse and Human β-Cells and Its Targeted Inactivation Impairs Glucose Tolerance. ***Endocrinology***. **2013** Oct;154(10):3796-806.

3) Bonnavion R, Jaafar R, Kerr-Conte J, Assade F, van Stralen E, Leteurtre E, Pouponnot C, **Gargani S,** Pattou F, Bertolino P, Cordier-Bussat M, Lu J, Zhang CX. Both PAX4 and MAFA Are Expressed in a Substantial Proportion of Normal Human Pancreatic Alpha Cells and Deregulated in Patients with Type 2 Diabetes. ***PLoS One.*** **2013** Aug 27;8(8):e72194