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Transgenic expression of human *INS* gene in *Ins1/Ins2* double knockout mice leads to insulin underproduction and diabetes in some male mice

Melis Karaca [1], Béatrice Durel [1], Laëtitia Languille [1], Luciane Lamotte [1], Cécile Tourrel-Cuzin [2], Loïc Leroux [1], Gretta Abou Sleymane [1], Susan Saint-Just [1], Danielle Bucchini [1], Alain Ktorza [2] and Rajiv L. Joshi [1]

[1] Institut Cochin, Department of Genetics and Development, INSERM U 567, CNRS UMR 8104, Université René Descartes Paris 5, Paris, France, [2] Laboratory of the Physiopathology of Nutrition, Université Paris 7, CNRS UMR 7059, Paris, France

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1. ABSTRACT

We have generated transgenic mouse lines expressing exclusively a human *INS* transgene on an *Ins1*/*Ins2* double knockout (mIKO) background. The transgene expression was driven by either a 4000 bp or a 353 bp promoter. These transgenic lines, designated mIKO:INS_{4000} and mIKO:INS_{353}, were viable and fertile. Determination of the amounts of insulin transcripts and total pancreatic insulin content revealed relative insulin underproduction in both lines, from birth to adulthood. Total pancreatic insulin stores in mIKO:INS_{4000} and mIKO:INS_{353} mice represented only about 50% and 27%, respectively, as compared to wild-type mice. Morphometric analysis of pancreas did not show any compensatory beta-cell hyperplasia. The majority of animals in both lines remained normoglycemic throughout their lives. Nevertheless, glucose tolerance tests revealed glucose intolerance in nearly half of mIKO:INS_{4000} male mice, likely due to impaired insulin secretion detected in those animals. In addition, a small fraction (2-4%) of male mice in both lines spontaneously developed diabetes with very distinct pathophysiological features. Diabetes was never seen in female animals. The diabetes developed by mIKO:INS_{353} mice was rapidly lethal, accompanied by a dramatic depletion of pancreatic insulin stores whereas the mIKO:INS_{4000} diabetic animals could live for several months. This suggests a possible link between the structure of the human *INS* gene promoter and the type of diabetes developed in these lines.

2. INTRODUCTION

Insulin is a peptide hormone synthesized, stored and secreted by the pancreatic beta-cells in a highly regulated manner and plays a vital role in the control of glucose homeostasis. The pancreatic insulin reserve is considered an important parameter of islet function, with tight coupling between insulin secretion and production being necessary for adequate beta-cell function. Insulin store size is determined by a balance between insulin secretion, insulin biosynthesis as well as total size of the beta-cell compartment.

In recent years, several genetically engineered mouse models have been developed to investigate the role of a number of genes in pancreatic beta-cell development and function, dysfunction or destruction and diabetes (1-3). Some of these models have revealed the key importance of beta-cell compensation to overcome insulin resistance in type 2 diabetes (4). Indeed, the capacity to adapt insulin secretion as well as insulin production and/or beta-cell mass to increased insulin demand under certain physiological or pathophysiological conditions appears mandatory for maintaining glucose homeostasis (5). A number of studies in both rodents and humans have suggested that progressive beta-cell failure in type 2 diabetes results from beta-cell dysfunction and/or reduction of beta-cell mass (6) due to adverse effects of genetic and/or environmental factors. The principal experimental animal model with reduced beta-cell mass which has been used so far is the rat made mildly diabetic by injection of streptozotocin in neonates or by partial pancreatectomy (7).

We became interested in examining the effects of manipulating the expression of the insulin gene itself on pancreatic insulin reserve, beta-cell mass and the control of glucose homeostasis. Rodents carry two non-allelic insulin genes (*Ins1/Ins2*) whereas there is a single insulin gene (*INS*) in humans, which is homologous to *Ins2*. It is also noteworthy that the human *INS* gene harbours a diabetes susceptibility *locus*, *IDDM2/VNTR* (Variable Number of Tandem Repeats), located in the 5'-regulatory region (8). We previously showed that mice carrying a null mutation in either *Ins1* or *Ins2* exhibited striking compensatory beta-cell hyperplasia and did not develop any metabolic alterations (9). Total pancreatic insulin stores in these mutant mice thus remained comparable to those found in wild-type (wt) animals. We report in this work the generation and characterization of two new transgenic mouse lines which express exclusively a human *INS* transgene on the *Ins1/Ins2* double knockout (mIKO) background (10). The transgene expression in these mouse lines was driven by its own promoter represented by either a 4000 bp or a 353 bp 5'-fragment (11, 12). The analyses of these lines revealed that they displayed relative insulin underproduction from birth to adulthood and did not exhibit any compensatory beta-cell hyperplasia. During the course of this study, we observed that a small fraction of male mice in both lines developed diabetes with very distinct pathophysiological features. We also report here the characterization of such diabetic animals.

3. MATERIALS AND METHODS

3.1. Animals and genotyping

Transgenic mice expressing the human *INS* gene under control of 4000 bp or 353 bp promoter were crossed with $Ins1^{-/-}Ins2^{+/-}$ mice to obtain $Ins1^{+/-}Ins2^{+/-}$ mice carrying either one of the human *INS* transgenes. These animals were then intercrossed to generate *Ins1/Ins2* double knockout mice expressing the long or the short version of the human *INS* gene. The progeny in these crosses were genotyped by PCR on tail DNA using Taq DNA polymerase and the following primer sets: 5'-CAGTAGTTCTCCAGCTGGTA-3' and 5'-GGCTTCTTCTACACACCCA-3' (to detect *Ins1*, *Ins2* or human *INS*); 5'-ACGGCACGCTGATTGAAGCA-3' and 5'-CCAGCGACCAGATGATCACA-3' (to identify $Ins2^{+/-}$ genotype by detecting *lacZ* previously inserted at the *Ins2 locus*). The reaction cycles used were: 95°C 5 min, 55°C 1 min and 72°C 1 min, followed by 30 cycles of 94°C 1 min, 55°C 1 min and 72°C 1 min and finally 72°C 7 min. The lengths of the PCR products were: 169 bp for *Ins1*, 675 bp for *Ins2*, 946 bp for human *INS,* and 301 bp for *lacZ*.

All transgenic as well as wt mice (C57Bl/6 x CBA) F1 were maintained in transgenic mouse facility with normal light-dark cycle of 14 h of light and fed on standard diet *ad libitum*. The study was performed mainly with 2 to 6 months old adult male mice. The genetic background of the transgenic mice obtained by breeding different transgenic lines was heterogeneous (129/sv, C57Bl/6, CBA, DBA/2). All animal manipulations were performed according to the French ethical rules for animal experimentation.

3.2. Analysis of insulin transcripts by RT-PCR

Total RNA was extracted using TRIzol (Invitrogen) from pancreas frozen in liquid N_2 and ground in a cooled mortar and pastel. After treatment with RQ1 DNase (Promega), the RNA samples (2 µg) were subjected to reverse transcription using random hexamer primers and Superscript II (Invitrogen). The resulting cDNAs (200 ng) were used to amplify transcripts for insulin and beta-actin by PCR. The primers used for insulin were as mentioned above and the oligonucleotide probe was: 5'-[^{32}P]-ACAATGCCACGCTTCTG-3'. The primers and the oligonucleotide probe for beta-actin were: 5'-CGTGGGCCGCCCTAGGCACCA-3' and 5'-TTGGCCTTAGGGTTCAGGGGGG-3' and 5'-[^{32}P]-AAGGACTCCTATGTGGGTGACG-3'. The PCR conditions were as described above. The products were analyzed on agarose gels, transferred onto Hybond-N+ membranes (Amersham), hybridized using ^{32}P-labeled oligonucleotide probes and exposed to X-ray films.

3.3. Extraction of pancreatic insulin

Pancreatic extracts were prepared by homogenizing entire pancreas using the ethanol/acid mixture and insulin was partially purified as described (13). The homogenates were left at 4°C overnight with gentle agitation. They were then centrifuged and the supernatants were collected. The amount of insulin in these extracts was quantified using a RIA kit (Insulin ^{125}I RIA; ICN).

3.4. Determination of metabolic parameters

Blood samples were collected from the orbital sinus. Blood glucose levels were measured using Glucotide strips and Glucometer 4 (Bayer-Diagnostics). Serum insulin levels were determined by RIA as described above. Urinary glucose was detected using Keto-Diastics strips (Bayer).

3.5. Glucose tolerance tests

Glucose tolerance tests were performed upon intraperitoneal injection of D-glucose (1.5 g/kg) after overnight fasting. Blood samples were collected from the orbital sinus at the indicated times and glucose levels were determined as described above.

3.6. Immunohistochemical and morphometric analyses of the pancreas

The pancreata were fixed overnight in 4% paraformaldehyde at 4°C, dehydrated and embedded in paraffin. Each pancreatic block was serially sectioned (5 μm) and then mounted on slides. For each pancreas 12 sections were randomly chosen at a fixed interval through the block (every 100 μm), a procedure previously shown to ensure that the selected sections are representative of the whole pancreas. Dewexed sections were first blocked for endogenous peroxydase activity by incubation in methanol/H_2O_2 mixture and then rehydrated and treated by proteinase K (1 μg/ml) at 37°C. After preincubation in goat serum, the sections were exposed to guinea pig anti-insulin antibodies (DAKO). Detection was by biotin-labeled goat anti-guinea pig IgG (Jackson ImmunoResearch Labs) followed by incubation with peroxydase-labeled streptavidin (DAKO) and development in diaminobenzidine (Sigma).

For morphometric analysis, the areas of pancreatic sections and insulin-positive cells were measured using a microscope connected through a video camera to a computer using the software Biocom VisioL@b 2000 (Explora Novo). The beta-cell mass was estimated by multiplying the percentage of the area occupied by insulin-positive cells by the weight of the pancreas. To determine the mean beta-cell surface, some sections stained for insulin were counterstained with hematoxylin (Shandon); the area of groups of cells was determined and divided by the number of nuclei counted.

For histopathological analyses, certain pancreatic sections were also subjected to trichrome staining.

3.7. Islet isolation and *in vitro* analysis of insulin secretion

Mice were sacrificed and pancreata were perfused with 3 ml of collagenase (1.6 mg/ml; Sigma) prepared in HHG (Hanks-Hepes 1X, Glucose 30%), excised and further digested in collagenase for 15 min at 37°C with gentle shaking. The homogenates were diluted with HHG/B (HHG containing 5 mg/ml BSA) and passed in a syringe through a 14 G needle. The material was washed 3 times in HHG/B by decantation and put into Petri dishes containing HHG/B. The islets were hand-picked with a pipette using a binocular and transferred in Petri dishes containing basal glucose (5.5 mM) in KRBH/B (Hepes Bicarbonate 1X, Mixed Salts 1X, BSA-fatty acid free 0.5 mg/ml). The perifusion of islets was performed at 37°C at pH 7.4 as described (14). Briefly, the islets (150) were placed between two layers of Bio-Gel P-2 fine (Biorad) in a flow column and perifused with basal (5.5 mM) or stimulating (16.7 mM) solutions of D-glucose in KRBH/B buffer. The eluates were collected at the rate of 1 ml/min and insulin concentration was determined by RIA using a kit (INSULIN-CT, CIS-Bio International).

3.8. Statistical analyses

The results are presented as mean values ± SEM. Statistical analyses were performed using unpaired Student's t test. The differences were considered significant for values of $p < 0.05$.

4. RESULTS

4.1. Generation of *Ins1/Ins2* double knockout mice carrying a human *INS* transgene

Transgenic mice expressing the human *INS* gene under control of either a 4000 bp or a 353 bp native promoter were previously generated and characterized (11, 12). By breeding these transgenic lines with heterozygous mutant mice carrying null allele(s) for *Ins1/Ins2*, we could obtain mice expressing exclusively the long or the short version of the human *INS* transgene on a *Ins1/Ins2* null background. Such mice producing only human insulin were viable and fertile, and were designated mIKO:INS_{4000} and mIKO:INS_{353}, respectively. Thus, expression of the human *INS* transgene completely rescued *Ins1/Ins2* double knockout mice, which otherwise would have developed severe diabetic ketoacidosis at birth and died within 48 hours (10).

4.2. Analysis of human *INS* gene expression in mIKO:INS_{4000} and mIKO:INS_{353} mice

The expression of the human *INS* transgene in mIKO:INS_{4000} and mIKO:INS_{353} mice was first examined by RT-PCR using total pancreatic RNAs. Our results indicate that the amounts of human *INS* transcripts present in these mice were significantly lower, compared to total insulin transcripts (*Ins1/Ins2*) found in wt controls (figure 1A). In accordance with these results, total pancreatic insulin contents in mIKO:INS_{4000} and mIKO:INS_{353} mice were also found to be reduced and represented only about 50% and 27%, respectively, as compared to wt controls (figure 1B). The relative insulin underproduction in mIKO:INS_{4000} and mIKO:INS_{353} mice was present at birth and persisted throughout adult life.

4.3. Morphometric analysis of the pancreas in mIKO:INS_{4000} and mIKO:INS_{353} mice

Immunostaining for pancreatic hormones did not show any abnormality in islet morphology in mIKO:INS_{4000} and mIKO:INS_{353} mice (data not shown). Immunohistochemical staining of pancreatic sections with anti-insulin antibodies suggested a relative decrease in insulin content in the islets of mIKO:INS_{4000} and mIKO:INS_{353} mice (figure 2). Quantitative morphometric analysis of pancreatic sections stained for insulin did not

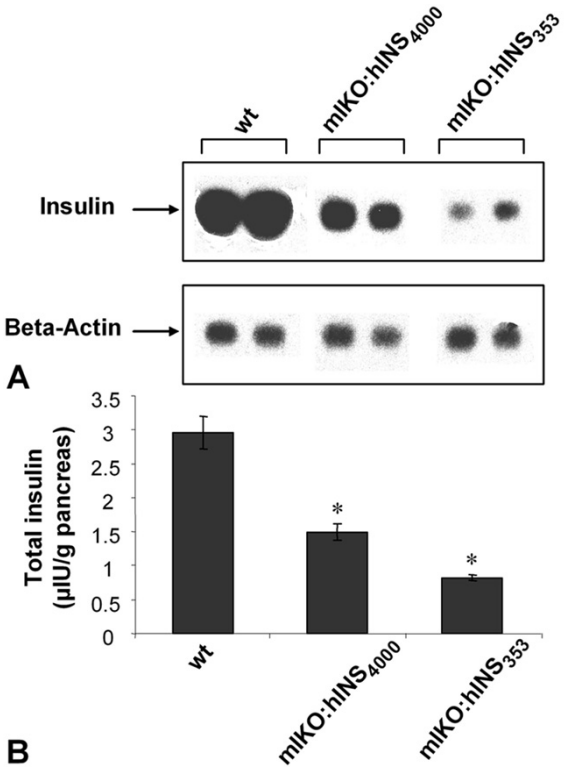

Figure 1. Analysis of insulin gene expression in wt, mIKO:INS_{4000} and mIKO:INS_{353} mice. (A) Insulin transcripts (*Ins1*/*Ins2* in wt and human *INS* in mIKO:INS_{4000} and mIKO:INS_{353} mice) were analyzed by RT-PCR using total pancreatic RNA. RT-PCR products were analyzed by Southern blot and the autoradiogramme is presented. Beta-Actin mRNA was amplified as an internal control. The sizes of RT-PCR products were 180 bp for insulin and 250 bp for beta-actin mRNA. (B) Total pancreatic insulin content as determined by RIA performed using insulin extracts from whole pancreas of adult mice. The mean values ± SEM are presented (n = 5-8). *p < 0,05 vs. wt .

reveal any statistically significant difference in the relative beta-cell area, beta-cell mass or islet density in mIKO:INS_{4000}, mIKO:INS_{353} and wt mice (table 1). The number of beta-cells per mean islet surface were also similar. Thus, insulin underproduction in mIKO:INS_{4000} and mIKO:INS_{353} mice was not accompanied by any compensatory increase in the size of the beta-cell compartment.

4.4. Glycemic control in mIKO:INS_{4000} and mIKO:INS_{353} mouse lines

Notwithstanding the markedly reduced pancreatic insulin content, the majority of animals in both mIKO:INS_{4000} and mIKO:INS_{353} lines remained capable of maintaining normal glucose homeostasis throughout their lives. The blood glucose levels in mIKO:INS_{4000} and mIKO:INS_{353} mice were comparable to those in wt controls under both fed and starved conditions (figure 3A). Serum insulin levels in mIKO:INS_{353} and wt mice were comparable and significantly decreased when starved (fed: wt, 16.84±1.92 µIU/ml; mIKO:INS_{353}, 16.02±1.81 µIU/ml; starved: wt, 9.56±0.74 µIU/ml; mIKO:INS_{353}, 9.48±0.50 µIU/ml). However, in the case of mIKO:INS_{4000} mice, the mean value for serum insulin under fed conditions was relatively low (11.89±0.7 µIU/ml) and was rather close to that in starved animals (9.43±0.8 µIU/ml). This suggested a possible insulin secretory defect specifically in the mIKO:INS_{4000} mouse line. We therefore examined the ability of mIKO:INS_{4000} and mIKO:INS_{353} mice to regulate provoked hyperglycemia by performing intraperitoneal glucose tolerance tests. As shown in figure 3B, all mIKO:INS_{353} and wt mice tested were able to normalize blood glucose levels with similar efficiency. However, in the mIKO:INS_{4000} line, about 50% of the animals analyzed were able to correct induced hyperglycemia with comparable efficiency whereas the remaining 50% exhibited marked glucose intolerance. To assess whether this effect could be accounted for by an insulin secretory defect in the mIKO:INS_{4000} mice, we analyzed insulin secretion using islets prepared from mIKO:INS_{4000}, mIKO:INS_{353} and wt mice, under basal and stimulating conditions by perifusion of D-glucose solutions (5.5 mM or 16.7 mM). As shown in figure 3C, insulin secretion was indeed impaired in islets prepared from mIKO:INS_{4000} mice, which were glucose intolerant. The insulin secretory defect in the mIKO:INS_{4000} mice appears also to exist *in vivo* since numerous attempts to detect the first peak of insulin secretion upon injection of D-glucose in these mice were unsuccessful whereas this peak was readily detected upon injection of L-arginine (data not shown). Consistent with these results, we observed that when fed a high glucose diet, about half of the mIKO:INS_{4000} mice developed glucosuria with blood glucose levels up to 350 mg/dl within one to two months (data not shown). These animals were presumably those exhibiting glucose intolerance. In contrast, all mIKO:INS_{353} and wt mice remained normoglycemic on such a diet.

4.5. Spontaneous diabetes development in some mIKO:INS_{4000} and mIKO:INS_{353} male mice

We observed that a small fraction (2 to 4%) of male mice in both mIKO:INS_{4000} and mIKO:INS_{353} lines spontaneously developed diabetes. The low occurrence of diabetes in these lines could be due to their heterogeneous genetic background. Interestingly, the pathophysiological features of diabetes developed by mIKO:INS_{4000} and mIKO:INS_{353} mice were very different. In the mIKO:INS_{4000} line, the age at which glucosuria could be detected was variable (1-5 months) and the diabetic animals could subsequently live for several months with no body-weight loss. In contrast, in the mIKO:INS_{353} line, mice developed diabetes at the age of 1-2 months, lost about 30-35% of body weight in 3-4 weeks and died within 1 month after the onset of glucosuria. The blood glucose levels in the diabetic mIKO:INS_{4000} or mIKO:INS_{353} mice reached values ranging from 300 to 400 mg/dl. The mIKO:INS_{4000} diabetic mice did not present hyperinsulinemia at any stage and their serum insulin levels remained comparable to those of non-diabetic fed animals. In the mIKO:INS_{353} diabetic mice, the serum insulin levels decreased and were found to be relatively low (10.1±0.4 µIU/ml) after two

Table 1. Morphometric analysis of pancreas

	Pancreas weight (mg)	Beta-cell area (%)	Beta-cell mass (mg)	Islet density (number/µm^2)	Mean islet surface (µm^2)	Beta-cells/mean islet surface (number/µm^2)
wt	130.4 ± 8.7	0.66 ± 0.02	0.86 ± 0.07	85.27 ± 2.25	7438 ± 438	56.14 ± 3.07
mIKO:hINS$_{4000}$	143.9 ± 9.7	0.54 ± 0.05	0.77 ± 0.09	65.10 ± 0.06	7710 ± 460	61.07 ± 2.85
mIKO:hINS$_{353}$	144.9 ± 10.4	0.59 ± 0.04	0.85 ± 0.06	88.09 ± 4.16	7479 ± 410	59.85 ± 2.62

The mean values ± SEM are presented (n = 6, 9, 7). All mice examined had no glucosuria.

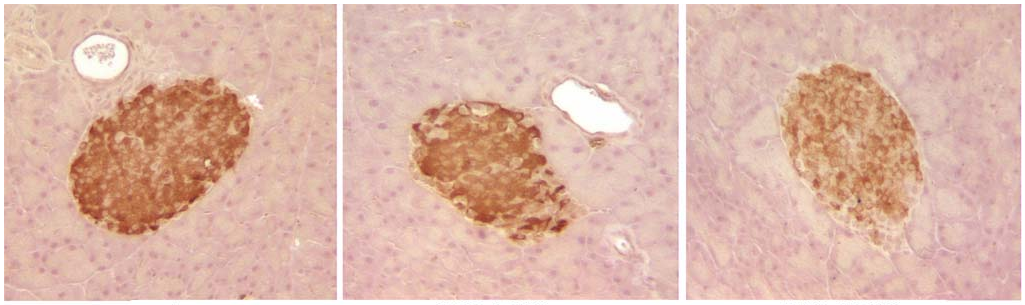

Figure 2. Immunohistochemical analysis of the pancreas from wt, mIKO:INS$_{4000}$ and mIKO:INS$_{353}$ mice. Pancreatic sections were stained for insulin using anti-insulin antibodies and counterstained with hematoxylin. Magnification was x160 for image acquisition using the microscope.

weeks of glucosuria. Diabetes was never observed in female animals.

Immunohistochemical analysis of pancreatic sections using anti-insulin antibodies showed clearly detectable insulin staining in the islets in mIKO:INS$_{4000}$ diabetic mice whereas very few insulin-positive cells were present in the islets in mIKO:INS$_{353}$ diabetic mice (figure 4). In addition, the islets in some of the mIKO:INS$_{353}$ mice appeared to be infiltrated with immune cells, which was not the case for the islets in the pancreas of mIKO:INS$_{4000}$ diabetic mice. In addition, trichrome staining showed the presence of fibrosis within and around the islets of mIKO:INS$_{353}$ diabetic mice. In line with all these observations, the pancreatic insulin content was relatively preserved in mIKO:INS$_{4000}$ diabetic mice even 2-3 months after the onset of diabetes whereas insulin stores were completely depleted in mIKO:INS$_{353}$ diabetic mice after 2-3 weeks of persistent glucosuria (figure 5).

5. DISCUSSION

We have generated and characterized in this study two mouse lines expressing a human *INS* transgene, under control of its own 4000 bp or 353 bp promoter, on the *Ins1/Ins2* null background.

5.1. Mice expressing human *INS* transgenes as models of relative insulin underproduction

The mIKO:INS$_{4000}$ and mIKO:INS$_{353}$ mice presented relative insulin underproduction, which existed at birth and persisted throughout adult life. The total pancreatic insulin content in mIKO:INS$_{4000}$ and mIKO:INS$_{353}$ lines represented only about 50% and 27%, respectively, as compared to wt animals. Nevertheless, the vast majority of the mice in the two lines maintained normal blood glucose homeostasis throughout their lives.

Morphometric analysis of pancreas did not reveal any increase in the size of the beta-cell compartment. The relative area occupied by beta-cells and the apparent number of beta-cells in the pancreas were not augmented in mIKO:INS$_{4000}$ and mIKO:INS$_{353}$ mice as compared to wt controls (table 1). These results were quite unexpected since single knockout mice for *Ins1* or *Ins2* did not present insulin underproduction, as a result of compensatory beta-cell hyperplasia (9). Whether these differences are due to differences in the human and mouse transcription factors, which regulate the activity of insulin promoters, is not known.

The pancreatic insulin reserve and/or insulin secretory ability are considered to be important parameters which can determine the ability to control glucose homeostasis under certain physiological or pathophysiological situations of insulin resistance. The occurrence of only mild insulin resistance without diabetes in mutant mice lacking insulin receptor substrate (IRS)-1, an intracellular mediator of insulin signaling widely distributed in different cell types, was attributed to compensatory beta-cell hyperplasia and increased insulin secretion (15, 16). In agreement with this view, IRS-2-deficient mice developed overt diabetes and failed to increase their beta-cell mass (17, 18). Interestingly, the phenotype of mice lacking IRS-2 could be rescued by transgenic overexpression of the pancreatic transcription factor Pdx-1 in beta-cells, which increased the beta-cell mass in these mutants (19).

The study of the *Psammomys obesus* model of nutritionally induced insulin resistance and diabetes has also supported this view (20, 21). These animals remained normoglycemic when fed a regular diet but rapidly developed diabetes on hyperenergetic diets. It was shown that these animals failed to increase insulin production and

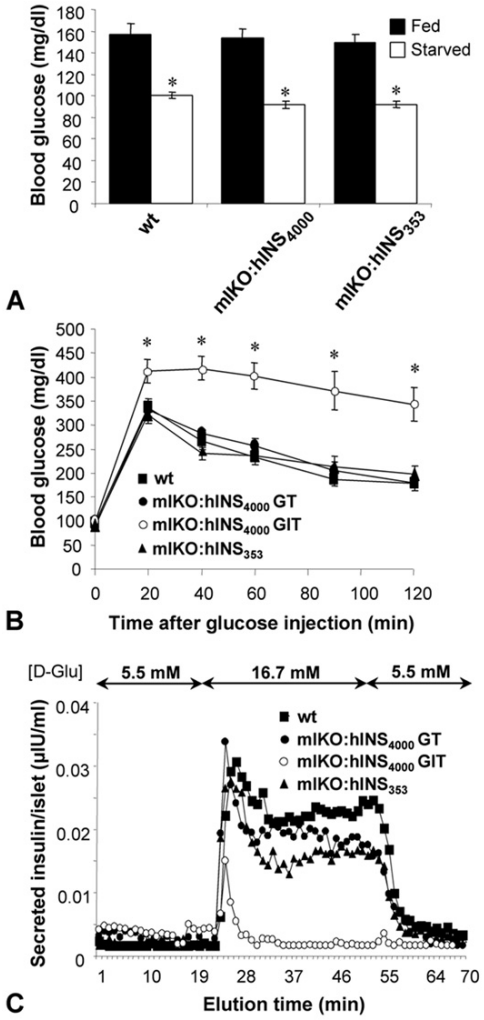

Figure 3. Glycemic control in wt, mIKO:INS_{4000} and mIKO:INS_{353} mice. (A) Blood glucose levels in fed and starved conditions in adult mice. The mean values ± SEM are presented (n = 3-7). *p < 0,05 vs. starved values. (B) Intraperitoneal glucose tolerance tests performed with adult mice. The mean values ± SEM are presented (n = 7). *p < 0,05 vs. wt . (C) Insulin secretion profiles obtained upon perifusion of D-glucose solutions (5.5 mM or 16.7 mM) using islets prepared from wt, mIKO:INS_{4000} and mIKO:INS_{353} adult mice. Insulin secretion was analyzed using 150 islets for each experiment. GT: glucose tolerant; GIT: glucose intolerant.

beta-cell mass under these conditions and their pancreatic insulin stores were rapidly depleted. It is however noteworthy that these animals naturally lack Pdx-1, which plays a crucial role in both the regulation of insulin gene transcription and insulin secretion.

The mIKO:INS_{4000} and mIKO:INS_{353} mouse models show that the pancreatic insulin stores can be reduced up to one third of what are found in wt animals, without affecting their ability to maintain glucose homeostasis under normal diet conditions. It is interesting to point out that about half of mIKO:INS_{4000} mice rapidly developed diabetes when fed a high glucose diet although their pancreatic insulin content was almost two-fold higher as compared to mIKO:INS_{353} mice. This could be due to impaired glucose-stimulated insulin secretion detected in the mIKO:INS_{4000} line. It would be interesting to further examine the dynamics of pancreatic insulin stores and/or changes in beta-cell mass when insulin demand is increased.

5.2. Possible mechanisms leading to diabetes in mIKO:INS_{4000} and mIKO:INS_{353} mice

The observation that a small fraction of male animals in both mIKO:INS_{4000} and mIKO:INS_{353} lines spontaneously developed diabetes is interesting. The low frequency of diabetes occurrence in these lines indicates that the genetic background must play an important role in the development of the disease and this model could represent an interesting tool for further examining this issue. It is tempting to consider whether the very different pathophysiological features of diabetes developed by mIKO:INS_{4000} and mIKO:INS_{353} mice could be linked to the structure of promoters driving expression of the human *INS* transgene. The reason why diabetes was seen only in males and not in females is not known.

It can be recalled that several genetic studies have now shown that the human *INS* gene itself harbours a diabetes susceptibility *locus*, *IDDM2/VNTR*, located in the 5'-regulatory region of the promoter (8). Although the correlation between class I *VNTR* alleles and the predisposition to type 1 diabetes as well as the underlying mechanisms based on the level of insulin gene expression in the thymus depending on *VNTR* alleles are now being well documented (22, 23), little data is available on the possible correlation between class III *VNTR* and the susceptibility to type 2 diabetes. Such correlation has been observed in some studies but the underlying mechanisms are not yet clearly understood (24). Interestingly, the 4000 bp promoter used in our model contains a class III *VNTR* (25) whereas the 353 bp promoter is devoid of *VNTR*.

The lethal diabetes developed by mIKO:INS_{353} mice is reminiscent of type 1 diabetes. These mice developed glucosuria at the age of 1-2 months, lost 30-35 % of body weight and died within the following month. These diabetic animals presented relative hypoinsulinemia after 2-3 weeks of persistent glucosuria. This was correlated with dramatic depletion of pancreatic insulin stores and markedly reduced insulin staining in the islets (figures 4, 5). In addition, the islets in some of the mIKO:INS_{353} mice appeared to be infiltrated with immune cells. This suggests that the mechanism leading to diabetes in mIKO:INS_{353} mice might involve auto-immune beta-cell destruction. However, the preliminary analysis of human *INS* transgene expression in the thymus did not reveal any difference between mIKO:INS_{353} and mIKO:INS_{4000} mice (results not shown), although this issue would need to be examined more extensively due to the heterogeneous genetic background of these mice. It is unlikely that insufficient insulin production leading to lethal diabetes in mIKO:INS_{353} line results from silencing of the transgene in

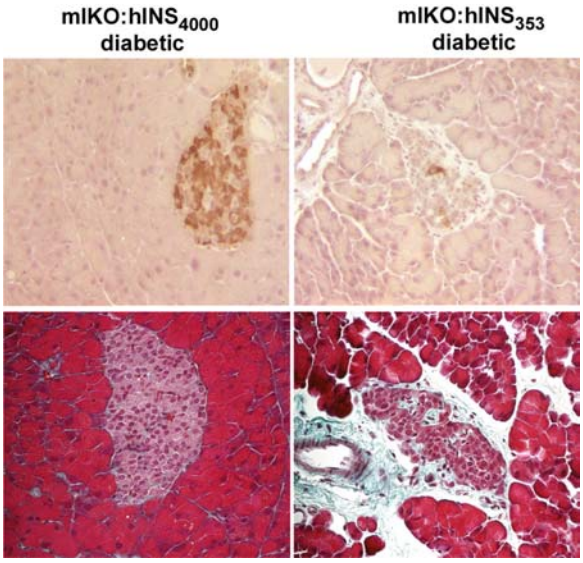

Figure 4. Histopathological analysis of the pancreas from mIKO:INS_{4000} and mIKO:INS_{353} diabetic mice. Pancreatic sections were stained for insulin using anti-insulin antibodies and counterstained with hematoxylin (top) or subjected to trichrome staining (bottom). The animals analyzed were diabetic for 3-4 months (mIKO:INS_{4000}) or 3-4 weeks (mIKO:INS_{353}). Magnification was x160.

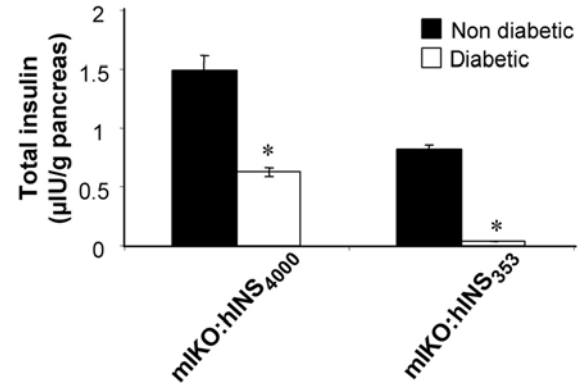

Figure 5. Total pancreatic insulin content in mIKO:INS_{4000} and mIKO:INS_{353} diabetic mice as compared to non-diabetic controls. The mean values ± SEM are presented (n = 4-8). *p < 0,05 vs. non-diabetic. The animals analyzed were diabetic for 3-4 months (mIKO:INS_{4000}) or 3-4 weeks (mIKO:INS_{353}).

certain animals through some epigenetic process. To rule out that the diabetic phenotype in mIKO:INS_{353} line might depend on the site of transgene integration, it should be mentioned that we also generated another mouse line expressing exclusively the human *INS* gene under the control of a shorter version (258 bp) of its own promoter. A small fraction of animals in this line (designated mIKO:INS_{258}) also developed diabetes with pathophysiological features identical to mIKO:INS_{353} diabetic mice (data not shown).

In contrast to mIKO:INS_{353} mice, the diabetes developed by mIKO:INS_{4000} animals was not lethal. The pancreatic insulin stores in mIKO:INS_{4000} diabetic mice were relatively preserved (figures 4, 5). Interestingly, when non-glucosuric mice of the mIKO:INS_{4000} line were examined for their tolerance to intraperitoneal injection of glucose, it appeared that half of the animals were markedly unable to restore normal blood sugar levels in time (figure 3B). The analysis of insulin secretion profiles upon perifusion of glucose solutions to purified islets revealed impaired glucose-stimulated insulin secretion in mIKO:INS_{4000} mice, which exhibited glucose intolerance (figure 3C). These defects were not detected in mice of the mIKO:INS_{353} line despite much lower pancreatic insulin stores present in these animals.

The glucose intolerance and impaired glucose-stimulated insulin secretion observed in mIKO:INS_{4000} line are reminiscent of pathologic features of type 2 diabetes (26). These defects might be more pronounced in mice which develop overt diabetes depending on background genes. Glucose intolerance was also detected in the two independent transgenic mouse lines (Tg 171, Tg 174) expressing the human *INS* gene under control of the 4000 bp promoter which were initially reported (10, 12, P. Lorès, unpublished observations) as well as in other transgenic lines expressing the human *INS* gene (27). Thus, the long human *INS* transgene version could have deleterious effects on the ability to control glucose homeostasis in the mouse even when both *Ins1/Ins2* genes were functional. In view of all these observations, it is likely that there could be a correlation between class III *VNTR* and impaired insulin secretion, as suggested in some studies (8, 28). In that case, the insulin secretory defect in type 2 diabetes could well be a primary defect and not necessarily be a secondary consequence of insulin resistance (29). However, to fully ascertain these conclusions, the contribution of the genetic background in the observed phenotype of mIKO:INS_{4000} mice would need to be dissected in future studies.

6. ACKNOWLEDGEMENTS

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Send correspondence to: Dr Rajiv L. Joshi, Institut Cochin, GD Department, 24 rue du Faubourg Saint-Jacques, 75014 Paris, France, Tel : 33-1-44-41-24-52, Fax: 33-1-44-41-24-48, E-mail: rajiv.joshi@wanadoo.fr

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