

Alterations of Pancreatic Beta-cell Mass and Islet Number due to *Ins2*-controlled Expression of Cre Recombinase: RIP-Cre Revisited; Part 2

Authors

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Key words

- diabetes
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- glucose metabolism
- insulin secretion
- islet of Langerhans
- pancreatic beta cell
- C57Bl/6

Abstract

Tissue-specific disruption of genes by targeted expression of Cre recombinase in insulin-producing cells has been widely used to explore pathways involved in regulation of pancreatic beta-cell mass. One particular line of transgenic mice [B6.Cg-Tg(*Ins2-cre*)25Mgn/J], commonly called RIP-Cre, in which the expression of Cre recombinase is controlled by a short fragment of the rat insulin II gene promoter has been used on at least 20 genes in at least 27 studies. In the majority of these studies (15 out of 27) inactivation of the gene of interest was associated with alterations in islet architecture, islet mass, or

pancreatic insulin content. We have tested the hypothesis that genomic integration or expression of Cre recombinase alone causes alterations of beta-cell mass by quantifying islet number and mass in RIP-Cre mice. We have observed a significant hypoplasia of beta-cells in young RIP-Cre mice, and a significant hyperplasia of islets in older RIP-Cre animals. These findings suggest that glucose intolerance and impaired insulin secretion previously described for younger RIP-Cre mice might be caused by transgene-associated islet hypoplasia, and that hyperplasia in older mice might reflect a compensatory response to transgene-related glucose intolerance.

Introduction

Cre recombinase-mediated excision of loxP-flanked genomic *loci* has become a widely used tool in studying glucose metabolism and insulin secretion in mice. In particular, numerous genes known to be expressed in the pancreatic beta-cell have been disrupted specifically in insulin-producing cells by transgenic expression of Cre recombinase under the control of various promoters. The most widely used line in this regard is commonly called RIP-Cre and is controlled by a short fragment of the rat insulin II gene promoter (*Ins II*) [1]. Since its first description in 1999, this line has been used to generate numerous beta-cell specific knock-out lines, including genes like insulin receptor, several insulin-receptor substrate genes (*IRSs*), numerous transcription factors, and others [2–28]. While most of these studies describe a pronounced phenotype concerning the glucose metabolism, a recent study summarizing observations from three independent laboratories suggests that integration or expression of Cre recombinase may alone be

responsible for some of the observations initially thought to be caused by gene targeting [29]. Specifically, at an age of 6 or 8 weeks of the mice, the different laboratories observed significant decreases in glucose tolerance in RIP-Cre mice, which was more pronounced in females than in males. Furthermore, alterations in insulin secretion similar to those found in human type 2 diabetes were observed. The authors came to the conclusion that integration or expression of Cre recombinase in pancreatic beta-cells *per se* might cause alterations in glucose tolerance and insulin secretion, while no mechanistic basis for these alterations was provided [29]. Based on these previous findings we have now tested whether alterations in islet architecture might be responsible for these phenotypical changes, especially due to the fact that the majority of studies have observed such changes in the respective knock-out models. We have now observed that integration or expression of Cre recombinase *per se* causes specific alterations in islet structure, and that changes previously shown to be caused by

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disruption of specific genes may be, at least in part, be related to undetected effects of transgene integration.

Material and Methods

RIP-Cre founders [B6. Cg-Tg(Ins2-cre)25Mgn/J] were reported to be 66% C57BL/6 initially and were backcrossed with C57BL/6 (Charles River, Munich, Germany) three times prior to breeding of the study group as previously described [8].

Mice were housed as previously described [8], briefly in a standard barrier facility according Federation of European Laboratory Animal Science Associations (FELASA) regulations including a 12-hour light, 12-hour dark cycle. Mice were fed standard rodent chow (Altromin GmbH, Lage, Germany). Breeding and genotyping was performed as previously described [8]. The RIP-Cre transgene was always kept in a hemizygous state in all animals evaluated.

Quantification of islet mass and number was performed as previously described [8], briefly using pancreata from three female RIP-Cre animals and three wild-type female littermates at each time point (4 weeks and 36 weeks of age), i.e., 12 animals in total. Three independent sections of at least 200 micrometers apart of each animal were studied, i.e., 36 sections in total. Histology and immunohistochemistry were performed using standard procedures. The primary antibody used was anti-insulin (Camon, Wiesbaden, Germany). Islet morphometry was performed using a digital microscope (Eclipse E1000; Nikon Deutschland GmbH, Düsseldorf, Germany) and quantification software (Lucia G version 5.0; Nikon Deutschland GmbH) as previously described [8].

Statistical analyses were performed as previously described briefly by Mann-Whitney U-tests to determine the statistical significance of histological values. p -Values ≤ 0.05 were considered significant. Data are presented in box plots, showing the distribution of the values within groups by SPSS software, version 8.0.

Results

We performed a literature search employing the usual online tools to determine whether a putative alteration of islet mass due to integration or expression of Cre recombinase is of any relevance in regard to previously published knock-outs. We obtained 28 articles using the RIP-Cre line which was used to disrupt 20 different genes [2–28]. From those 28 articles, only 10 articles explicitly used animals expressing RIP-Cre only as a control concerning knock-out phenotype. Furthermore, amongst those 28 articles, at least 15 articles found morphometric differences pertaining to islet mass or size, or provided evidence for altered pancreatic insulin content. In all of those 15 cases, differences in islet morphology were explained by specific effects of disruption of the corresponding gene (data not shown, see corresponding references).

To determine whether integration or expression of Cre recombinase *per se* might, at least in part, be responsible for the alterations in beta-cell morphology and mass, we analyzed pancreatic sections from RIP-Cre mice and their genetically unaltered littermates (wild-type mice) in regard to number and size of insulin-positive areas by immunohistochemistry. As shown in the microphotographs in **Fig. 1**, young RIP-Cre mice at 4 weeks of age show a reduction in insulin-positive area per pancreatic section in comparison to wild-type littermates. At an age of 36 weeks, RIP-Cre mice show an apparent increase in islet size in comparison to wild-type littermates.

To obtain quantitative evidence for these suggestive observations, we secondly quantified the number of insulin-positive areas per whole pancreatic section (**Fig. 2**). We observed significant differences in young mice, where the number of insulin-positive areas was significantly increased in comparison to littermates (**Fig. 2**). Furthermore we observed a significant increase in the number of insulin-positive areas over time (4 weeks vs. 36 weeks) in RIP-Cre animals only (**Fig. 2**) while no significant differences between 36-week old transgenics and littermates were observed.

Since the results depicted in **Fig. 2** are dependent on the absolute size of the respective pancreatic section studied, we next aimed to normalize our findings by analyzing defined pancreatic areas of identical size ($1 \times 10^6 \mu\text{m}^2$). When applying this approach, no differences in islet number in young animals were observed

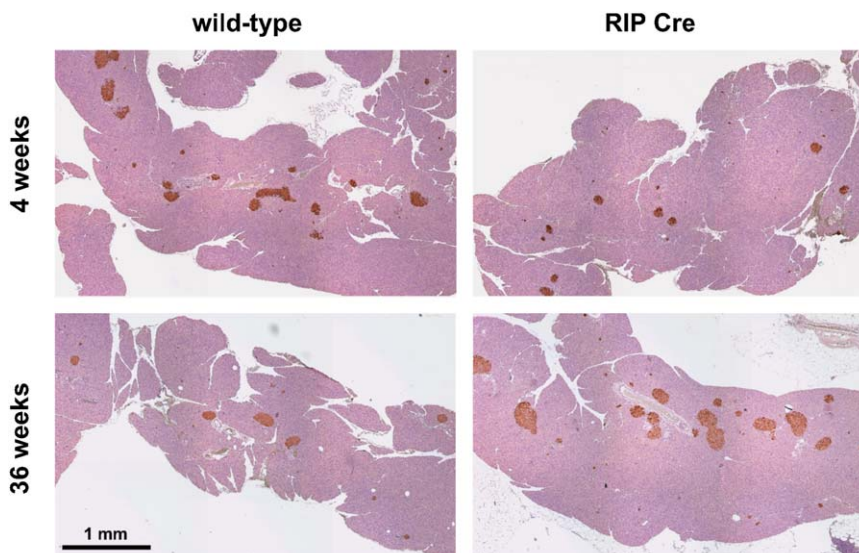


Fig. 1 Differential regulation of beta-cell mass. Representative photographs of pancreata evaluated, stained with hematoxylin-eosin and immunostained with an anti-insulin antibody.

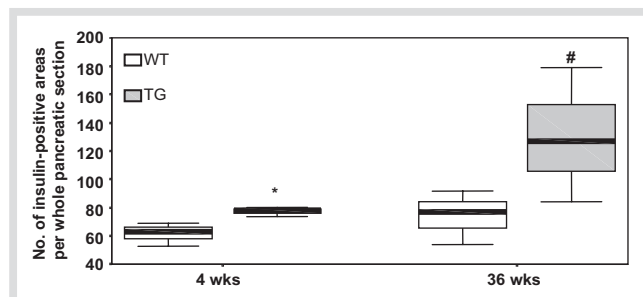


Fig. 2 Number of insulin-positive areas per whole pancreatic section. Evaluated were mice of different genotypes and at different ages. The middle line of the box plot indicates the median for each group, the box edges mark the distribution between the 25th and 75th percentile, and whiskers represent the maximum and the minimum values without being outliers or extreme values. * $p \leq 0.05$ (TG vs. WT respective time points), and # $p \leq 0.05$ (WT vs. WT or TG vs. TG).

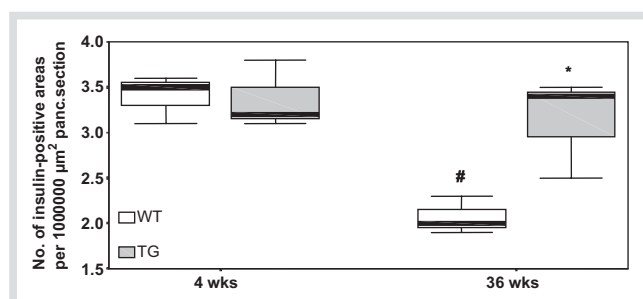


Fig. 3 Number of insulin-positive areas per normalized pancreatic area. Normalized pancreatic area was chosen as a $1 \times 10^6 \mu\text{m}^2$ fraction of whole pancreatic sections. For box-plot legend and p-value legends, see Legend to **Fig. 2**.

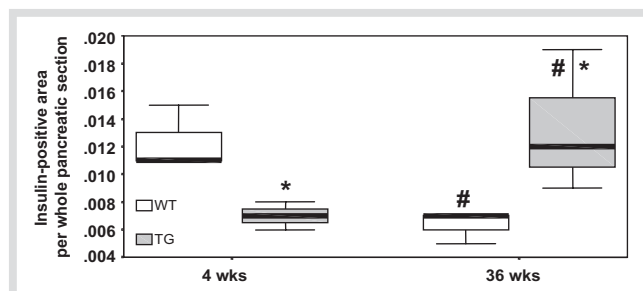


Fig. 4 Ratios of total insulin-positive area per whole pancreatic section. For box-plot legend and p-value legends, see Legend to **Fig. 2**.

(**Fig. 3**). Nevertheless a significant loss of islets over time (4 weeks vs. 36 weeks) was observed in wild-type mice only, while the number of islets per defined pancreatic area stayed constant in Cre-expressing transgenic animals (**Fig. 3**). This is also reflected in a significantly different number in islets per defined pancreatic section in 36 week-old animals when transgenics and wild-type animals are compared (**Fig. 3**).

Next, we determined beta-cell mass by quantifying insulin-positive areas per whole pancreatic section (**Fig. 4**). We observed a significant reduction of beta-cell mass in young animals due to expression of Cre recombinase in comparison to wild-type animals. These differences were inverted in older animals, where transgenics showed a significantly greater beta-cell mass than

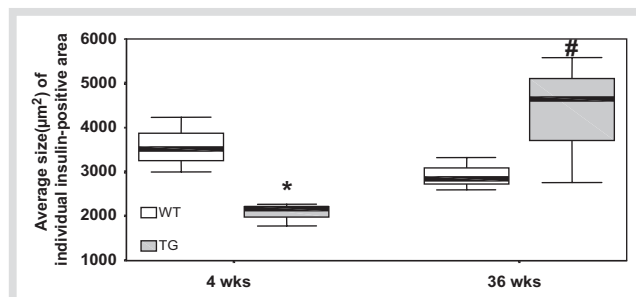


Fig. 5 Average size per insulin-positive area. For box-plot legend and p-value legends, see Legend to **Fig. 2**.

wild-type control animals did (**Fig. 4**), while control animals showed a significant reduction in islet number over time (**Fig. 4**).

Lastly, in regard to the individual size of insulin-positive portion of pancreatic islets, we observed a significant reduction in individual size in young RIP-Cre transgenics (**Fig. 5**). Furthermore, we observed a significant increase in the individual size of insulin-positive portion of pancreatic islets in RIP-Cre positive animals only (**Fig. 5**).

Taken together, these data suggest a significant hypoplasia of insulin-positive areas in young mice due to integration or expression of Cre recombinase *per se*, which is possibly compensated for in size and number over time leading to significant islet hyperplasia in older Cre-expressing animals.

Discussion

We have shown here that integration or expression of Cre recombinase under the control of a short fragment of the *rat insulin promoter II* leads to significant alterations of islet mass and number in C57/Bl6 mice. These findings potentially impact previous publications where the corresponding Cre-expressing line, commonly termed RIP-Cre, was used to obtain beta-cell specific disruptions of various targeted genes [2–28], and are most likely in those cases where no RIP-Cre controls were included into the corresponding study.

While previous reports from three different laboratories independently have shown that beta-cell specific Cre expression causes glucose intolerance, and impaired insulin secretion for unknown reasons [29], our current findings suggest that RIP-Cre animals primarily have a reduction in the size of insulin-positive pancreatic areas, i.e., beta-cell mass, while no reduction in islet number was observed. This reduction in beta-cell mass might be considered relevant for the development of glucose intolerance [30]. Since hyperglycemia, as typically observed in states of glucose intolerance, has been shown to induce a hyperplastic response in regard to islet mass [31–33], we tentatively interpret our findings as indicative for a secondary response of islet mass to glucose intolerance at early age, cumulating in islet hyperplasia in older mice.

Since a hyperplastic response in pancreatic islets is observed specifically in C57/Bl6 mice, but not necessarily in other inbred strains, these data also suggest that strain differences might play a role in the degree of hyperplasia observed. In particular, a number of researchers have used strains of mixed background, containing genetic material from 129Sv, DBA2, CD1, FVB and lastly C57/Bl6. This fact might also contribute to phenotypical

differences, as previously discussed [29], putatively based on different effects of Cre recombinase on islet mass. It may also explain why some researchers find no effect of Cre recombinase on islet mass, although mice expressing Cre recombinase only have been used as appropriate controls. In addition, experiments with mice from different laboratories [34,35] expressing Cre recombinase under the control of either the *Ins2* or other promoters should help to elucidate, whether our observations are strain-specific, or rather reflect an effect of cre expression in pancreatic islets.

Taken together, RIP-Cre mice show differences in islet mass presumably depending on the genetic background. While young mice on a >92% C57/BL6 background have hypoplastic islets, older mice on the same background exhibit islet hyperplasia possibly due to a morphologic response to impaired glucose tolerance. These findings should be taken into account when interpreting phenotypical data obtained from beta-cell specific knock-out models.

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