Diabetologia (2006) 49: 1222–1228
DOI 10.1007/s00125-006-0241-4

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A new model of insulin-deficient diabetes: male NOD mice with a single copy of *Ins1* and no *Ins2*

Received: 18 November 2005 / Accepted: 15 February 2006 / Published online: 13 April 2006
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Abstract *Aims/hypothesis:* We describe a novel model of insulin-deficient diabetes with a single copy of the gene encoding insulin 1 (*Ins1*) and no gene encoding insulin 2 (*Ins2*). *Materials and methods:* We constructed five lines of mice: mice with two copies of *Ins1* ($NOD^{Ins1+/+,Ins2-/-}$), mice with a single copy of *Ins1* ($NOD^{Ins1+/-,Ins2-/-}$), mice with two copies of *Ins2* ($NOD^{Ins1-/-,Ins2+/+}$), mice with a single copy of *Ins2* ($NOD^{Ins1-/-,Ins2+/-}$) and $NOD^{Ins1+/-,Ins2-/-}$ mice with a transgene encoding B16:Ala proinsulin. *Results:* By 10 weeks of age, all male $NOD^{Ins1+/-,Ins2-/-}$ mice were diabetic, whereas all female $NOD^{Ins1+/-,Ins2-/-}$ were not diabetic ($p<0.0001$). In contrast, neither male nor female $NOD^{Ins1-/-,Ins2+/-}$ with a single copy of *Ins2* (rather than single copy of *Ins1*) developed early diabetes and no mice with two copies of either gene developed early diabetes. Islets of the diabetic male $NOD^{Ins1+/-,Ins2-/-}$ at this early age had no lymphocyte infiltration. Instead there was heterogeneous (between islet cells) weak staining for insulin. Although only male $NOD^{Ins1+/-,Ins2-/-}$ mice developed diabetes, both male and female $NOD^{Ins1+/-,Ins2-/-}$ mice had markedly decreased insulin content. In $NOD^{Ins1+/+,Ins2-/-}$, there was also a significant decrease in insulin content, whereas $NOD^{Ins1-/-,Ins2+/+}$ mice, and even $NOD^{Ins1-/-,Ins2+/-}$ mice, were normal. Male $NOD^{Ins1+/-,Ins2-/-}$ mice were completely rescued from diabetes by introduction of a transgene encoding proinsulin. On i.p. insulin tolerance testing, male mice had insulin resistance compared with female mice. *Conclusions/interpretation:* These results suggest that *Ins1* is a 'defective gene' relative to *Ins2*, and that the mouse lines created provide a novel model of sex-dimorphic insulin-deficient diabetes.

Keywords Gender difference · Insulin biosynthesis/secretion · Insulin gene · Insulin resistance · Knock-out mouse · Metabolic diabetes · Nonobese diabetic mouse · Sex difference · Transgenic mouse

Abbreviations IAA: insulin autoantibody · *Ins1*: gene encoding insulin 1 · *Ins2*: gene encoding insulin 2 · ipGTT: intraperitoneal glucose tolerance test · ITT: insulin tolerance test · KO: knock-out · NOD: nonobese diabetic

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Introduction

Insulin is synthesised by pancreatic beta cells and plays a predominant role in glucose homeostasis. Mice have two genes encoding insulin, insulin 1 (*Ins1*) on chromosome 19, and insulin 2 (*Ins2*) on chromosome 7. *Ins1* differs from *Ins2* by two amino acids at positions B9 and B29 and there are additional differences in the leader sequence and the connecting peptide. In addition, *Ins1* lacks an intron present in *Ins2*. These structural features suggest that *Ins1* was generated by an RNA-mediated duplication–transposition event involving a transcript of *Ins2*, which was reinserted into the genome (retroposon) [1].

In a previous study, we created *Ins1* knock-out (*Ins1*-KO) and *Ins2*-KO mice on the nonobese diabetes (NOD) background. The *Ins1*-KO prevented the majority of progression to autoimmune type 1 diabetes, whereas the *Ins2*-KO accelerated the development of type 1 diabetes [2]. Similarly, Thebault-Baumont et al. have shown that *Ins2*-KO mice bred onto the NOD background develop accelerated insulitis and diabetes [3]. These studies have suggested that the role of each gene encoding insulin in autoimmune diabetes is different.

More recently, we have described double insulin KO (*Ins1*-KO and *Ins2*-KO) NOD mice with a mutated gene encoding preproinsulin (B16:Ala) that rescues the mice

from metabolic diabetes [4]. These mice lacking a native B:9–23 sequence do not develop autoimmune diabetes. During evaluation and creation of these mice, we unexpectedly found that male NOD mice with the genotype *Ins1*+/−, *Ins2*−/− (NOD$^{Ins1+/-,Ins2-/-}$) developed early onset of diabetes (<10 weeks of age). In this study, we describe the NOD$^{Ins1+/-,Ins2-/-}$ mice, a novel strain with non-autoimmune insulin-deficient diabetes.

Materials and methods

Mice

We constructed four lines of mice: (1) mice with two copies of *Ins1* (NOD$^{Ins1+/+,Ins2-/-}$; *Ins2*-KO); (2) mice with a single copy of *Ins1* (NOD$^{Ins1+/-,Ins2-/-}$); (3) mice with two copies of *Ins2* (NOD$^{Ins1-/-,Ins2+/+}$; *Ins1*-KO); and (4) mice with a single copy of *Ins2* (NOD$^{Ins1-/-,Ins2+/-}$). The mice were established by breeding the original insulin knock-outs kindly provided by J. Jami (Cochin and Saint Vincent de Paul Hospital, Paris, France) on to NOD/Bdc mice using marker-assisted congenic methods [2]. NOD$^{Ins1+/-,Ins2-/-}$ and NOD$^{Ins1-/-,Ins2+/-}$ were produced by mating (*Ins1*-KO×*Ins2*-KO)F1 with *Ins2*-KO and mating (*Ins1*-KO×*Ins2*-KO)F1 with *Ins1*-KO, respectively. NOD$^{Ins1+/-,Ins2-/-}$ with a mutated transgene encoding proinsulin (NOD$^{Ins1+/-,Ins2-/-,Tg(+)}$) were constructed by mating with NOD$^{Ins1+/+,Ins2-/-}$ and NOD$^{Ins1-/-,Ins2-/-}$ with the transgene encoding B16:Ala proinsulin [4, 5]. Mice were housed in a pathogen-free animal colony at Barbara Davis Center for Childhood Diabetes with an approved protocol from the University of Colorado Health Sciences Center Animal Care and Use Committee. All mice had free access to tap water in an air-conditioned room (22–25°C) with a 12-h light–darkness cycle (06.00–18.00 h). In addition, we produced control strains with the genetic region of 129S1/SvImj mice surrounding *Ins1* and *Ins2* bred onto the NOD mice and these mice did not develop 'early' diabetes but typical insulitis associated with later onset of diabetes as reported [4].

Genotype analysis

Genomic DNA was extracted from mouse tails. The genotyping for the *Ins1*-knock-out gene, the *Ins1*-wild-type gene, the *Ins2*-knock-out gene and the *Ins2*-wild-type gene was performed by using PCR [5]. The PCR products were electrophoresed on 2% agarose gels and visualised by ethidium bromide staining.

Diagnoses of diabetes

Glucose was measured weekly with the FreeStyle blood glucose monitoring system (TheraSense, Alameda, CA, USA), and the mice were considered diabetic after two consecutive blood glucose values >13.9 mmol/l. After development of diabetes, the mice were killed immediately and the pancreas was fixed in 10% formalin to perform histological analysis.

Insulin autoantibody (IAA) assay

IAA was measured with a 96-well filtration plate micro-IAA assay as previously described [6] and expressed as an index. A value of 0.01 or greater is considered positive and exceeds the 99th percentile of normal controls.

Insulin content in pancreas

Mice were analysed for insulin content at the age of 4–5 weeks, and blood glucose was measured before and after overnight fasting to confirm lack of diabetes. Insulin was extracted with 4 ml acid–ethanol with an overnight incubation at 4°C. Insulin concentration of the supernatant after centrifuging and diluting (×1,000) was measured with an ELISA-based insulin kit (Mercodia, Uppsala, Sweden). Calculated insulin content was corrected for pancreatic weight or body weight.

Response to exogenous insulin

To detect insulin resistance, an insulin tolerance test (ITT) was performed by injecting human insulin (0.75 IU/kg) i.p. into overnight-fasted mice at the age of 10 weeks and blood glucose levels were measured at 0, 15, 30, 45 and 60 min.

Intraperitoneal glucose tolerance test (ipGTT)

To assess glucose tolerance in NOD$^{Ins1-/-,Ins2+/+}$ and NOD$^{Ins1-/-,Ins2+/-}$, an ipGTT (2 g glucose/kg body weight) was performed in overnight-fasted mice, and blood glucose levels were measured at 0, 30, 60, 90 and 120 min. We calculated the glucose AUCs according to the trapezoidal rule from the glucose measurements at baseline (0 min), 30, 60, 90 and 120 min.

Histology

The pancreata obtained from the mice were fixed in 10% formalin and paraffin-embedded. Paraffin-embedded tissue sections were stained with a monoclonal mouse anti-insulin antibody (Sigma, St Louis, MO, USA) followed by incubation with a peroxidase-labelled anti-mouse IgG antibody (DakoCytomation, Carpinteria, CA, USA), and also with a peroxidase-labelled broad-spectrum secondary antibody (Zymed/Invitrogen Corporation, Carlsbad, CA, USA) for peroxidase staining on adjacent sections. For immunofluorescence staining, the secondary antibody incubation took place with anti-guinea-pig AMCA (blue)-, anti-mouse Texas Red (red)-, and anti-rabbit Cy2

(green)-conjugated antibodies (Jackson ImmunoResearch, West Grove, PA, USA).

Statistics

Data are shown as means±SEM. Statistical analyses of insulin content, body weight, ITTs and ipGTTs were performed by Mann–Whitney's *U* test. Survival curves were analysed with the log-rank test. Statistical tests used PRISM software (Graphpad, San Diego, CA, USA). $p<0.05$ was regarded as significant.

Results

We produced four lines of mice varying their number of genes encoding insulin and monitored the mice for the development of diabetes (Fig. 1). As shown in Fig. 1a, all male mice with only a single *Ins1* gene (NOD$^{Ins1+/-,Ins2-/-}$) developed diabetes (12 out of 12) by 10 weeks of age, whereas no male mice with a single *Ins2* gene (NOD$^{Ins1-/-,Ins2+/-}$) (0 out of 12) developed diabetes by this age ($p<0.0001$). In addition, no male NOD$^{Ins1+/+,Ins2-/-}$ and NOD$^{Ins1-/-,Ins2+/+}$ had early-onset diabetes (0 out of 9 and 0 out of 18, respectively). No female mice developed early-onset diabetes, independently of the number of genes encoding insulin, even in female NOD$^{Ins1+/-,Ins2-/-}$ (0 out of 17, $p<0.0001$) (Fig. 1b).

After 10 weeks of age, female *Ins2*-KO (NOD$^{Ins1+/+,Ins2-/-}$ and NOD$^{Ins1+/-,Ins2-/-}$) mice had a high prevalence of diabetes (Fig. 1b) and severe insulitis. However, no *Ins1*-KO (NOD$^{Ins1-/-,Ins2+/+}$ and NOD$^{Ins1-/-,Ins2+/-}$) mice in either the males or females developed diabetes until 36 weeks of age (Fig. 1).

Since male NOD mice with a single copy of *Ins1* and lacking *Ins2* developed diabetes, we analysed the histology of the male NOD$^{Ins1+/-,Ins2-/-}$ mice with diabetes. Despite the presence of diabetes, islets on haematoxylin–eosin staining appeared normal and there was no lymphocytic infiltration (Fig. 2a). We also measured IAA (Fig. 2b). The level of IAA in male NOD$^{Ins1+/-,Ins2-/-}$ mice was the same as in wild-type male NOD and less than male NOD$^{Ins1+/+,Ins2-/-}$. A sex-related difference in IAA expression for mice <10 weeks of age was not detected in NOD$^{Ins1+/-,Ins2-/-}$ mice. These results suggest that diabetes in male NOD$^{Ins1+/-,Ins2-/-}$, which develops prior to 10 weeks of age, is not associated with enhanced autoimmunity, especially the lack of islet infiltrates.

We next measured pancreatic insulin content of mice at 4–5 weeks of age, before the development of diabetes (Fig. 3). The insulin content in male NOD$^{Ins1+/-,Ins2-/-}$ was significantly lower than that in mice with any other insulin genotype corrected for pancreatic weight (Fig. 3a) or body weight (Fig. 3b). In marked contrast, NOD$^{Ins1-/-,Ins2+/-}$ mice, with only one copy of the *Ins2* gene, had almost the same insulin content as wild-type NOD insulin mice or NOD$^{Ins1-/-,Ins2+/+}$ mice, suggesting NOD$^{Ins1-/-,Ins2+/-}$ mice compensate for loss of genes while NOD$^{Ins1+/-,Ins2-/-}$ mice cannot. NOD$^{Ins1+/+,Ins2-/-}$ mice had an intermediate insulin content compared with lower levels for NOD$^{Ins1+/-,Ins2-/-}$ and NOD$^{Ins1-/-,Ins2+/-}$ mice (Fig. 3c). Thus a single copy of *Ins2* results in greater insulin content than two copies of *Ins1*. In addition, as shown in Fig. 3, at this age, *Ins2*-KO mice (including NOD$^{Ins1+/-,Ins2-/-}$, NOD$^{Ins1+/+,Ins2-/-}$) weighed slightly less compared with *Ins1*-KO (including NOD$^{Ins1-/-,Ins2+/-}$, NOD$^{Ins1-/-,Ins2+/+}$) ($p<0.05$ in both male and female mice), and wild-type NOD ($p<0.05$ in male and $p<0.01$ in female mice). However, the body weight of *Ins1*-KO mice with *Ins2* was not significantly different from that of wild-type NOD mice, suggesting that low pancreatic insulin was associated with delayed growth and/or fat accumulation.

Given the low content of insulin at a young age before diabetes, we evaluated insulin expression by islets with immunocytochemistry (Fig. 4). Compared with wild-type NOD mice, mice with a single copy of *Ins1* had weak and heterogeneous (between beta cells) insulin expression. These results suggest that the diabetes of male NOD$^{Ins1+/-,Ins2-/-}$ results from deficient insulin expression. To confirm this, we

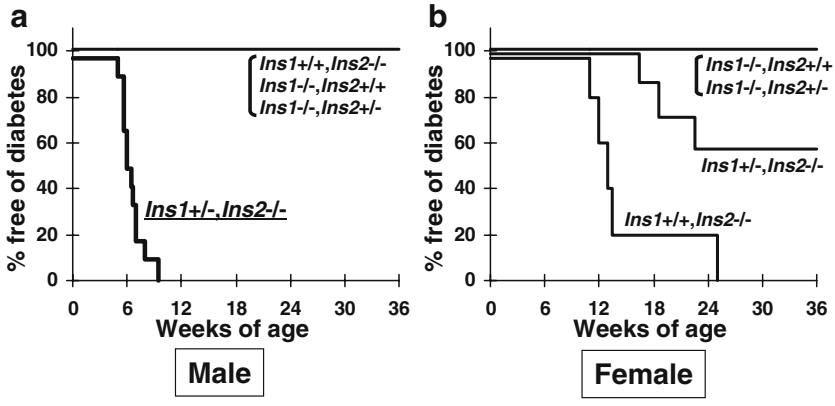

Fig. 1 Development of diabetes with differential *Ins* knock-outs. **a** Male NOD$^{Ins1+/-,Ins2-/-}$ (n=12), NOD$^{Ins1+/+,Ins2-/-}$ (n=9), NOD$^{Ins1-/-,Ins2+/-}$ (n=12), NOD$^{Ins1-/-,Ins2+/+}$ (n=18); **b** female NOD$^{Ins1+/-,Ins2-/-}$ (n=17), NOD$^{Ins1+/+,Ins2-/-}$ (n=8), NOD$^{Ins1-/-,Ins2+/-}$ (n=14), NOD$^{Ins1-/-,Ins2+/+}$ (n=15). By 10 weeks of age, only male NOD$^{Ins1+/-,Ins2-/-}$ developed diabetes (12 out of 12). $p<0.0001$ (log-rank test) for survival curves between male NOD$^{Ins1+/-,Ins2-/-}$ and female NOD$^{Ins1+/-,Ins2-/-}$, and between male NOD$^{Ins1+/-,Ins2-/-}$ and male NOD$^{Ins1-/-,Ins2+/-}$

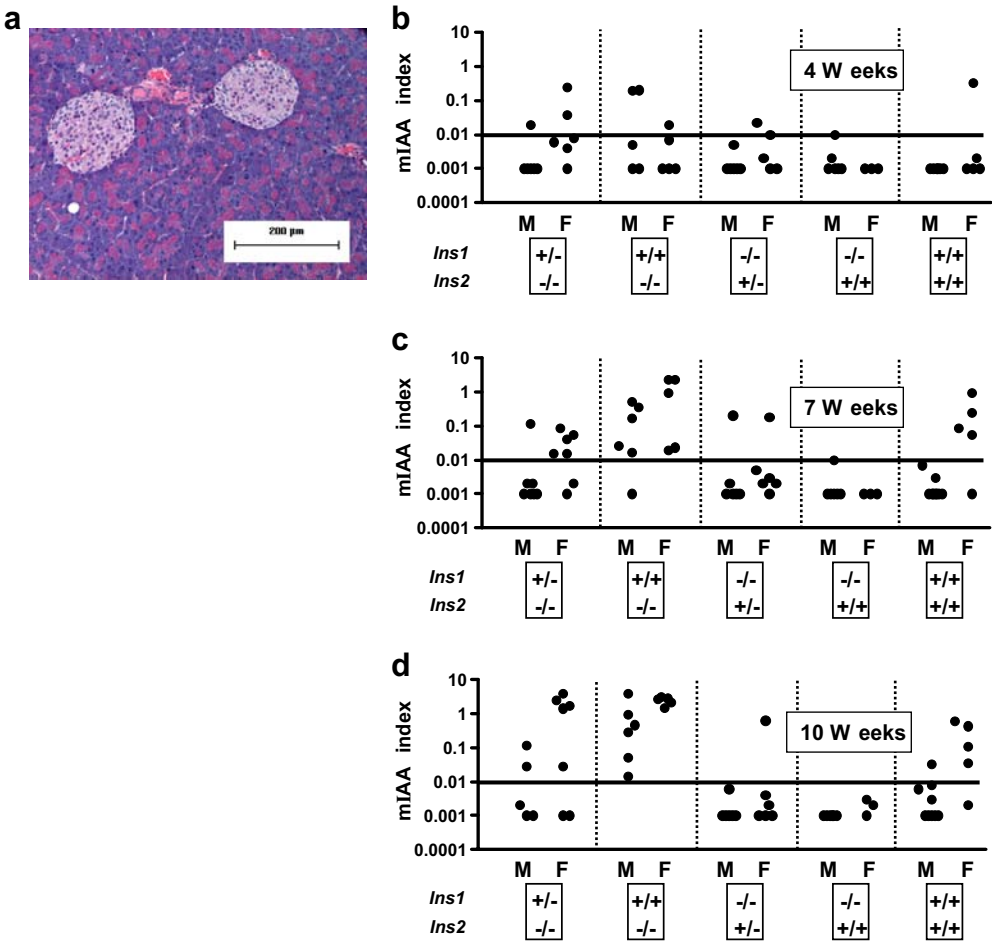

Fig. 2 **a** Histology of a diabetic NOD$^{Ins1+/-,Ins2-/-}$ mouse with diabetes that developed before 10 weeks of age (haematoxylin–eosin staining). **b–d** Longitudinal analysis of IAA. The level of IAA in male (M) NOD$^{Ins1+/-,Ins2-/-}$ mice was the same as in wild-type male NOD and less than male NOD$^{Ins1+/+,Ins2-/-}$. A sex-related difference in IAA before 10 weeks of age was not detected in NOD$^{Ins1+/-,Ins2-/-}$ mice. *F* Female. *Horizontal line*, cut-off for mIAA (0.01)

genetically rescued male NOD$^{Ins1+/-,Ins2-/-}$ mice from diabetes with a transgene encoding proinsulin that we had previously developed to study a specific epitope of insulin (B:9–23). This transgene, driven off the rat insulin promoter, produces biologically active insulin despite a single amino acid change (B16:Ala). Male NOD$^{Ins1+/-,Ins2-/-}$ mice with the transgene (n=17) did not develop early onset of diabetes (p<0.0001; log-rank test). In addition, mice with the

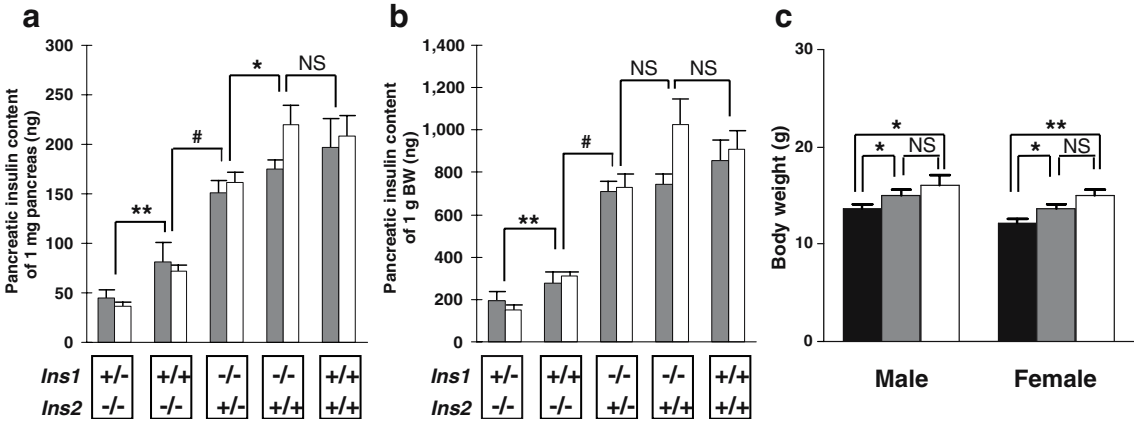

Fig. 3 **a**,**b** Pancreatic insulin content of NOD$^{Ins1+/-,Ins2-/-}$ (n=8, 6, male and female, respectively), NOD$^{Ins1+/+,Ins2-/-}$ (n=5, 6), NOD$^{Ins1-/-,Ins2+/-}$ (n=8, 9), NOD$^{Ins1-/-,Ins2+/+}$ (n=8, 8) and wild-type NOD mice (n=7, 7). *Filled bars*, male mice; *open bars*, female mice. Insulin content was per milligram of pancreatic weight (**a**) or gram of body weight (*BW*) (**b**). NOD$^{Ins1+/-,Ins2-/-}$ had extremely low pancreatic insulin content. No sex-related difference in pancreatic insulin content was detected. **c** Body weight of *Ins2*-KO (combining NOD$^{Ins1+/-,Ins2-/-}$ and NOD$^{Ins1+/+,Ins2-/-}$ [n=13, 12, male and female, respectively]), *Ins1*-KO (combining NOD$^{Ins1-/-,Ins2+/-}$ and NOD$^{Ins1-/-,Ins2+/+}$ [n=16, 17]) and wild-type NOD mice (n=7, 7). *Black bars*, *Ins2*-KO mice; *grey bars*, *Ins1*-KO mice; *open bars*, wild-type NOD mice. *p<0.05, **p<0.01, $^{\#}p$<0.0001 combined male and female

transgene recovered pancreatic insulin content at least to the same level as NOD$^{Ins1+/+,Ins2-/-}$ (Fig. 5). These results strongly suggest that rescue from diabetes was possible with the introduction of insulin message independently of the wild-type insulin loci and that male NOD$^{Ins1+/-,Ins2-/-}$ developed diabetes because of low insulin expression.

As shown in Fig. 3, insulin content in male and female NOD$^{Ins1+/-,Ins2-/-}$ mice was similar, although only male mice developed diabetes. We hypothesised that male NOD mice might be more insulin-resistant than female NOD mice. Therefore we evaluated the glucose response to i.p. insulin in male and female NOD mice (Fig. 6). Male NOD mice had significantly higher blood glucose than female mice (Fig. 6a), and the blood glucose at 15 min (%basal) after i.p. insulin was also significantly higher, and the slope of glucose decline was decreased compared with female mice (Fig. 6b). In addition, as shown in Fig. 7, even among male and female NOD$^{Ins1-/-,Ins2+/+}$ that did not develop any diabetes and had no differences in pancreatic insulin content, the male mice have higher glucose on an ipGTT than females. These results suggest that sex differences in insulin resistance may contribute to the metabolic differences between male and female knock-out mice.

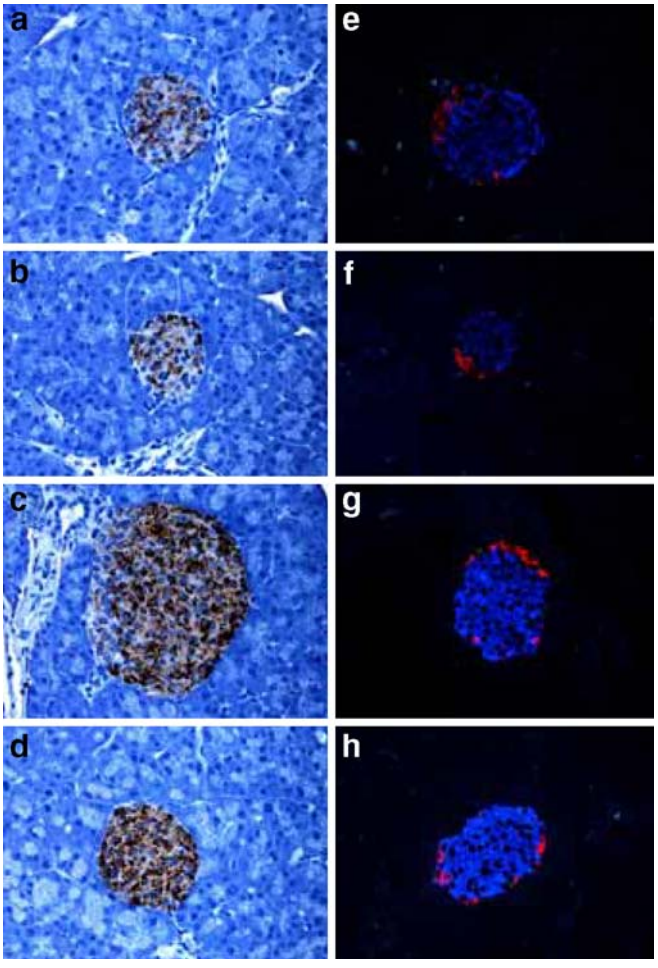

Fig. 4 No insulitis and weak and heterogeneous staining for insulin in male NOD$^{Ins1+/-,Ins2-/-}$ islets with insulin immunoperoxidase staining (**a**) and insulin immunofluorescence staining (**e**) despite the presence of normal islet architecture. Female NOD$^{Ins1+/-,Ins2-/-}$ (**b**,**f**) had similar signal levels to male NOD$^{Ins1+/-,Ins2-/-}$, whereas male NOD$^{Ins1-/-,Ins2+/-}$ (**c, g**), and wild-type NOD (**d, h**) had more uniform and brighter insulin staining compared with male NOD$^{Ins1+/-,Ins2-/-}$. All samples were collected at 5–6 weeks of age before development of diabetes. **a–d** Insulin immunoperoxidase staining (*brown:* insulin). **e–h** Insulin immunofluorescence staining (*blue*: insulin, *red*: glucagon, *green*: somatostatin)

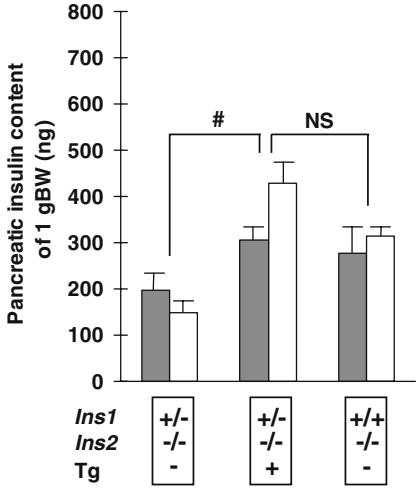

Fig. 5 Pancreatic insulin content of NOD$^{Ins1+/-,Ins2-/-,Tg(-)}$ (n=8, 6, male and female, respectively), NOD$^{Ins1+/-,Ins2-/-,Tg(+)}$ (n=11, 11), and NOD$^{Ins1+/+,Ins2-/-,Tg(-)}$ (n=5, 6). Pancreatic insulin content in the NOD$^{Ins1+/-,Ins2-/-,Tg(+)}$ was significantly higher than that in the NOD$^{Ins1+/-,Ins2-/-,Tg(-)}$ ($^{\#}p<0.0001$) and similar to that in the NOD$^{Ins1+/+,Ins2-/-,Tg(-)}$ (p=0.28), suggesting that insulin content in the pancreas was rescued by the transgene encoding B16:Ala mutated proinsulin. *Filled bars*, male mice; *open bars*, female mice

Discussion

In this study, we report that male NOD mice with only a single copy of *Ins1* (NOD$^{Ins1+/-,Ins2-/-}$) develop 'metabolic diabetes' at less than 10 weeks of age. The pathogenesis is probably a defect of insulin production with low and heterogeneous expression of insulin that can be corrected by additional transgenic expression of proinsulin. A single copy of *Ins2* gene is sufficient to prevent diabetes and is associated with normal insulin content. This suggests that *Ins1* gene is a functionally defective gene relative to *Ins2* gene and interestingly, there is no compensation sufficient to prevent diabetes with only a single *Ins1* gene.

It has been reported that *Ins1*-KO and *Ins2*-KO, when on a C57BL/6 strain background and with two copies of each gene encoding insulin, have insulin content in the pancreas similar to wild-type mice, in addition to normal glucose tolerance, because of the compensatory response of insulin transcription [7, 8]. In contrast to these studies, our NOD$^{Ins1+/+,Ins2-/-}$ mice have lower insulin content compared with wild-type NOD mice. A major difference between these studies may be related to the background genome, namely NOD background in our study vs C57BL/6 background. We are surprised that there is not

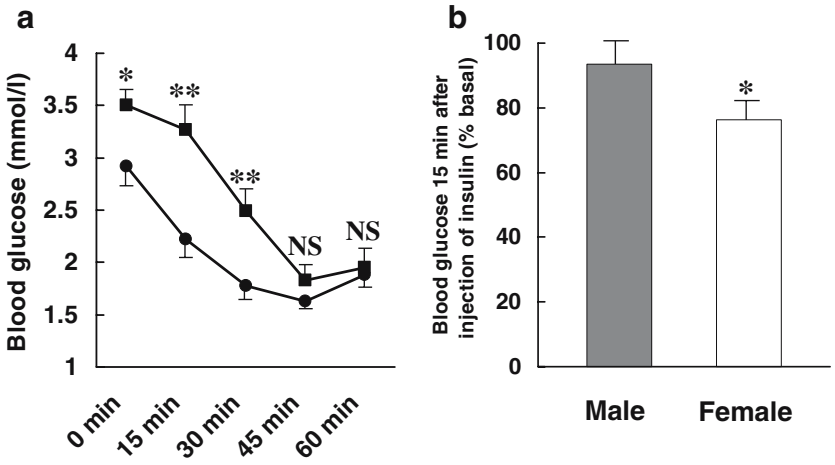

Fig. 6 ITTs in NOD mice at 10 weeks of age. *Filled squares*, male mice (n=10); *filled circles*, female NOD mice (n=12). Male NOD mice had significantly higher blood glucose than female (*p<0.05, **p<0.01) (**a**) and the blood glucose at 15 min (percentage of basal) was also significantly higher than that in the female (p<0.05) (**b**)

compensation for insulin deficiency with the single copy of *Ins1* and with two copies of *Ins1*. However, a strain derived from the same colony as the NOD mouse, the Nagoya–Shibata–Yasuda mouse [9, 10], a model of type 2 diabetes, which may share background genome with NOD [11, 12], has no compensational hypertrophy of pancreatic islets despite increasing insulin resistance with ageing [9]. In addition, Kulkarni et al. reported the importance of background genome in the induction of type 2 diabetes [13].

Male and female NOD$^{Ins1+/-,Ins2-/-}$ mice had similarly decreased levels of pancreatic insulin content in young non-diabetic mice. However, diabetes incidence between male and female NOD$^{Ins1+/-,Ins2-/-}$ mice was significantly different. We hypothesise that small but statistically significant differences in response to insulin as evidenced by our i.p. ITT may relate to sex differences in development of overt diabetes of these insulin-deficient NOD$^{Ins1+/-,Ins2-/-}$ mice. We believe that greater insulin resistance for male mice with marginal insulin production leads to hyperglycaemia. In fact, most animal models of type 2 diabetes (including Nagoya–Shibata–Yasuda mice) show a high prevalence of type 2 diabetes in male mice [9, 10].

In humans, a polymorphism upstream of the *INS* promoter correlates with insulin expression in the thymus, but has little effect on pancreatic insulin expression [14, 15]. Therefore, loci other than the insulin locus may contribute to pancreatic insulin expression. Further analysis of crosses of NOD and B6 mice with insulin gene knock-outs may help clarify such genetic loci that possibly relate to regulation of insulin production. Of note, humans have only the insulin 2 homologous gene.

Analysing structural features of the gene encoding insulin, Soares et al. reported that *Ins1* was generated by an RNA-mediated duplication–transposition event involving a transcript of *Ins2* [1]. In addition, only *Ins2* is expressed in the NOD thymus with both genes expressed in islet beta cells [3, 16, 17]. This study clearly indicates that *Ins1* (on the NOD genetic background) is a functionally defective gene compared with *Ins2*, and provides a novel model of sex-dimorphic insulin-deficient diabetes.

Acknowledgements This work was supported by grants from the National Institutes of Health (DK32083, DK55969, DK62718), Diabetes Endocrine Research Center (P30 DK57516), the American Diabetes Association, the Juvenile Diabetes Foundation, and the Children's Diabetes Foundation. N. Babaya was supported by the Osaka Medical Research Foundation for Incurable Diseases. M. Nakayama was supported by a fellowship from the Juvenile Diabetes Foundation.

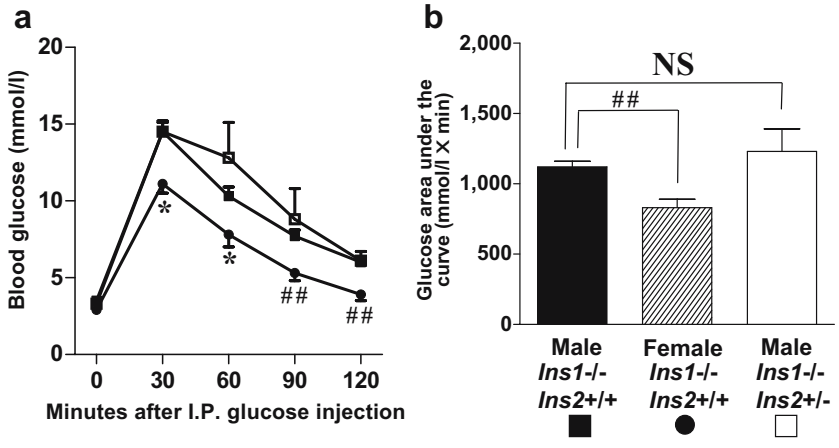

Fig. 7 **a**,**b** Intraperitoneal GTTs in male NOD$^{Ins1-/-,Ins2+/+}$ (n=10, *filled squares*) mice, female NOD$^{Ins1-/-,Ins2+/+}$ (n=6, *filled circles*) mice, and male NOD$^{Ins1-/-,Ins2+/-}$ (n=3, *open squares*) mice at 10 weeks of age. *p<0.05, $^{\#}p$<0.005 compared with male NOD$^{Ins1-/-,Ins2+/+}$

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