anti-ghrelin [1-11] antiserum might not be the exactly same that recognized by N-RIA or ELISA. Since the amount of n-octanoylated-ghrelin was so little that it could not be detected by RIA if any, we may as well consider that the phenotype of these transgenic mice is due to the effect of des-acyl ghrelin. Des-acyl ghrelin has been shown not to activate GHS-R (39). There have been several reports saying that des-acyl ghrelin has biological activities, such as promoting adipogenesis (41), inhibition of cell proliferation, inhibition of apoptosis (42) and counteracting the effect of n-octanoylated ghrelin (35).

We showed here that the ghrelin level in portal vein is significantly higher than that in retroorbital vein in wild type mouse. Ghrelin has been reported to be mainly synthesized in stomach and intestine. The step-up of plasma ghrelin level in gastric vein has been reported previously (43), but there has been no report showing the step-up of plasma ghrelin level in portal vein as compared to that in systemic circulation. The present study is the first report of the step-up of plasma ghrelin levels in portal vein. Moreover, the step-up of des-acyl ghrelin in RIP-G Tg was much higher than that in control littermates, indicating overproduction of des-acyl ghrelin by transgene in the pancreas.

The body weight, %body fat and food consumption of RIP-G Tg were not

significantly different from those of nontransgenic littermates. Recently, we and Asakawa et al. have reported the studies of β-actin promoter ghrelin transgenic mouse (44,45), in which plasma des-acyl ghrelin levels were 30 and 50 times higher than those of their nontransgenic littermates. These transgenic mice were reported to show small phenotype, although some discrepancy of interpretation regarding on etiology exists. Asakawa et al. reported that the triglyceride level of β-actin promoter ghrelin transgenic mouse was lower, but that cholesterol level and free fatty acid level were not changed compared to their nontransgenic littermates. The triglyceride levels of our RIP-G Tg only showed lower tendency compared to that of nontransgenic littermates. The lack of small phenotype and milder phenotype of lipid metabolism in RIP-G Tg may result from the fact that plasma des-acyl ghrelin level of RIP-G Tg was only 3.4 times higher than those of nontransgenic littermates.

The tissue sections of the pancreas of these transgenic mice showed no apparent disarrangement in the islet architecture and in β cell mass. There have been several reports on the transgenic mice overexpressing humoral factors in the β cells, such as parathyroid hormone-related peptide, hepatocyte growth factor, and insulin-like growth factor-I (46-49). Some of these transgenic mice showed islet hypertrophy or disarrangement of the endocrine cells in the islet (46-49). Our observation showed

that des-acyl ghrelin might have no apparent effects on the islet architecture and β cell mass.

In the present study plasma insulin levels after the 3.0 g/kg glucose injection were significantly lower in RIP-G Tg than those in nontransgenic littermates although there was no significant difference in plasma insulin levels between RIP-G Tg and nontransgenic littermates on the fasting state.

To rule out the decreased production of insulin caused by exogenous insulin promoter, we measured insulin mRNA level and content in the pancreata of our transgenic mice. The insulin mRNA level and content from the transgenic mice were not significantly different from those from nontransgenic littermates. Therefore, the insulin production might not be disturbed in these mice either in transcriptional or translational levels. The immunoreactivity of PDX-1, which is master regulator of the pancreas development and essential for insulin transcription, in RIP-G Tg β cell was not different from that in nontransgenic littermates' β cell. These results suggest that the suppression of glucose-stimulated insulin secretion in RIP-G Tg might not be due to the transcriptional disregulation of insulin caused by injection of exogenous insulin promoter.

RIP-G Tg did not show decreased-insulin secretion in response to arginine.

Arginine is known to stimulate insulin secretion by the mechanism that are different from those used by glucose, although the detail remains controversial (50,51). However, it seems certain that arginine somehow evoked Ca^{2+} influx into the β cell and that leads to the exocytosis of insulin-containing vesicles (52,53). So at least, the decreased insulin secretion in RIP-G Tg might not be due to disorders in exocytosis process. Egido reported that ghrelin inhibits insulin secretion from rat pancreas in response to arginine in vitro (28), however, there has been no report on the effect of des-acyl ghrelin on arginine-induced insulin secretion.

The immunoreactivity of GLUT2, glucose transporter in the pancreatic β cell, in RIP-G Tg β cell was indistinguishable from that of in nontransgenic littermates' β cell. Although immunohistochemistry is not so suitable for quantitative analysis, at least no apparent decreased expression or disposition of GLUT2 in RIP-G Tg β cell exists. Chronic exposure to the high level of des-acyl ghrelin may not influence on GLUT2 expression.

We performed batch incubation study of RIP-G Tg islet. The insulin secretion from isolated islet of RIP-G Tg was indistinguishable from that of nontransgenic littermates. This finding indicates that insulin secretion was not affected by over expression of ghrelin transgene in vitro but was affected in vivo. The

difference of observations between in vitro and in vivo may be explained by dilution of ghrelin produced by transgene with incubation buffer. Alternatively, suppression of insulin secretion of RIP-G Tg was not due to the effect of des-acyl ghrelin on insulin secretion from β cell but on insulin sensitivity. Recently Gauna et al. reported that co-administration of des-acyl ghrelin and active ghrelin improves insulin sensitivity in humans (54), and that des-acyl ghrelin suppress glucose output from liver(55). Although insulin tolerance test did not show statistically significant difference in blood glucose levels between RIP-G Tg and their nontransgenic littermates, there were tendency that blood glucose levels of RIP-G Tg were lower. Moreover, plasma triglyceride levels of RIP-G Tg showed lower tendency. Taken together, these results may indicate that des-acyl ghrelin may improve insulin sensitivity of RIP-G Tg. The suppression of insulin secretion of RIP-G Tg is likely due to the effect of des-acyl ghrelin on insulin sensitivity.

To explore if chronic exposure to high-level des-acyl ghrelin may influence on the expression level of GHS-R, we investigated the mRNA level of GHS-R in the pancreas and pituitary of RIP-G Tg. No significant differences were found in GHS-R mRNA levels in pancreas or in pituitary between RIP-G Tg and their nontransgenic littermates. These finding indicates that chronic exposure to high-level des-acyl

ghrelin might not influence on GHS-R mRNA expression level.

We also developed RGP-G Tg. The pancreatic tissue ghrelin concentrations determined by C-RIA of RGP-G Tg were about 50 times higher than those of their nontransgenic littermates, indicating that ghrelin was overexpressed in RGP-G Tg. However, there was no obvious phenotype regarding insulin secretion and pancreatic morphology. Considering the observation that portal ghrelin levels were not elevated in RGP-G Tg compared to those in their nontransgenic littermates, the amount of secreted ghrelin from α cell may not outstrip the amount from stomach.

In summary we developed RIP-G Tg, in which pancreatic des-acyl ghrelin content was approximately 1,000 times higher than that in control littermates. We detected n-octanoylated-ghrelin-like immunoreactivity in pancreatic β cells by immunohistochemistry, indicating that the mechanism of acylation may exist not only in pancreatic α cells but also in β cells. The glucose-stimulated insulin secretion of RIP-G Tg was decreased. There was no abnormality about arginine-induced insulin secretion, pancreatic histology, pancreatic insulin mRNA levels and insulin content in the RIP-G Tg. Absence of insulin suppression in islet batch incubation study, lower tendency of blood glucose levels in ITT and lower tendency of plasma triglyceride level may indicate that the suppression of insulin secretion of RIP-G Tg is likely due to the

effect of des-acyl ghrelin on insulin sensitivity. Although we also developed RGP-G Tg with 50-fold increase of pancreatic des-acyl ghrelin content, we did not find obvious phenotype regarding insulin secretion and pancreatic morphology. The present study raises the possibility that des-acyl ghrelin may have influence on glucose metabolism.

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Figure legends

Fig.1 A. C-terminal ghrelin-like immunoreactivity in adult mouse islet. The staining was observed in the peripheral region of the islet. B. Glucagon-like immunoreactivity in serial section.

Fig.2 A: Structure of RIP-ghrelin transgene. B: Structure of RGP-ghrelin transgene. C, D: Pancreatic islet of RIP-ghrelin transgenic mouse stained with anti-ghrelin [13-28] (C) and anti-ghrelin [1-11] antisera (D). E, F: Pancreatic islet of RGP-ghrelin transgenic mouse stained with anti-ghrelin [13-28] (E) and anti-ghrelin [1-11] antisera (F). G: Plasma ghrelin levels collected from retroorbital and portal veins in RIP-G Tg. *: $P<0.05$ compared to retroorbital vein. #: $P<0.01$ compared to nontransgenic littermates. H: The step-up of ghrelin concentration from retroorbital vein to portal vein in RIP-G Tg. #: $P<0.01$ compared to their nontransgenic littermates.

Fig.3 A: Body weight of RIP-G Tg (Tg) and their nontransgenic littermates (non). B: Food intake of RIP-Tg (Tg) and their nontransgenic littermates (non).

Fig. 4 A, B. Blood glucose levels after overnight fast in RIP-G Tg (A) and RGP-G Tg (B) (Tg) and their nontransgenic littermates (non). C, E. Intraperitoneal (IP) glucose tolerance test (1.5 g/kg) in RIP-G Tg (C) and RGP-G Tg (E) (Tg) and their nontransgenic littermates (non). D, F: Plasma insulin concentration after IP glucose

(3g/kg) injection in RIP-G Tg (D) and RGP-G Tg (F) (Tg) and their nontransgenic littermates (non). G: Plasma insulin concentration after IP arginine (0.25g/kg) injection in RIP-G Tg (Tg) and their nontransgenic littermates (non). (H) Insulin (2.0 U/ kg) tolerance test in RIP-G Tg (Tg) and their nontransgenic littermates (non). Values are represented as mean ± SE. *: $P<0.05$ compared to nontransgenic littermates.

Fig.5 Islet morphology and β cell area in RIP-G Tg (A, B, C, D) and RGP-G Tg (E, F, G, H). The sections were stained with anti-insulin (A, E), anti-glucagon (B, F), anti-somatostatin (C, G) and anti-PP antiserum (D, H). I, J: The ratio of β cell area to that of whole section in RIP-G Tg (I) and RGP-G Tg (J). non, nontransgenic littermates; Tg, RIP-G Tg; NS, not significant.

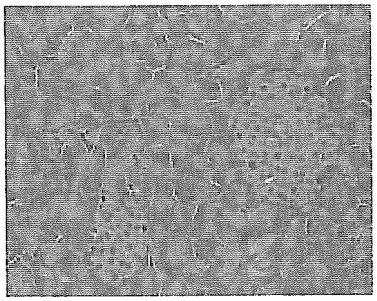
Fig. 6 mRNA level and peptide content of insulin in RIP-G Tg (Tg) and their nontransgenic littermates (non) pancreas. A. Representative blot of Northern blot analysis of insulin. B. Insulin mRNA levels C. Insulin peptide contents. NS, not significant.

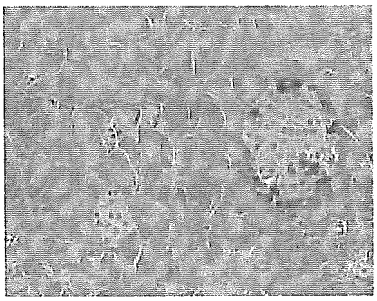
Fig. 7 A, B: Immunoreactivity of Glut-2 in the islet of RIP-G Tg (A) and nontransgenic littermates (B). C, D: Immunoreactivity of PDX-1 in the islet of RIP-G Tg (C) and nontransgenic littermates (D).

Fig. 8 mRNA level of GHS-R determined by quantitative RT-PCR in pancreas (A) and pituitary (B) of RIP-G Tg (Tg) and their nontransgenic littermates (non). NS, not significant.

Fig. 9 Batch incubation study of isolated islets of RIP-G Tg (Tg) and their nontransgenic littermates (non).

Figure 1

A 

B 

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