Studies on pathophysiological significance of intraislet ghrelin using transgenic animal model.

（遺伝子改変動物を用いた膵島由来グレリンの病態生理学的意義の検討）

坂東　美佳

Transgenic overexpression of intra-islet ghrelin does not affect insulin secretion or glucose metabolism *in vivo*

Running title: Transgenic overexpression of intra-islet ghrelin

Mika Bando[1, 3], Hiroshi Iwakura[1], Hiroyuki Ariyasu[1], Hiroshi Hosoda[4], Go Yamada[2], Kiminori Hosoda[2, 3], Souichi Adachi[3], Kazuwa Nakao[2], Kenji Kangawa[4], Takashi Akamizu[1, 5]

[1]Ghrelin Research Project, Translational Research Center, [2]Department of Medicine and Clinical Science, Endocrinology and Metabolism, [3]Department of Human Health Sciences, Kyoto University Hospital, Kyoto University Graduate School of Medicine, Kyoto 606-8507, Japan

[4]National Cerebral and Cardiovascular Center Research Institute, Osaka 565-8565, Japan, [5]The First Department of Medicine, Wakayama Medical University, Wakayama 641-8509, Japan

Address correspondence to and reprint request: Hiroshi Iwakura, M.D., Ph.D., 54 Shogoin Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan. TEL: +81-75-751-4735; FAX: +81-75-751-4731; Email:hiwaku@kuhp.kyoto-u.ac.jp

Abstract

While ghrelin is primarily produced in the stomach, but a small amount of ghrelin is also produced in pancreatic islets. Although exogenous administration of ghrelin suppresses insulin secretion *in vitro* or *in vivo*, the role of intra islet ghrelin in the regulation of insulin secretion *in vivo* remains unclear. To understand the physiological role of intra-islet ghrelin in insulin secretion and glucose metabolism, we developed a transgenic (Tg) mouse model, rat insulin II promoter ghrelin- internal ribosomal entry site –ghrelin O-acyl transferase (RIP-GG) Tg mice, in which mouse ghrelin cDNA and ghrelin O-acyltransferase are overexpressed under the control of the rat insulin II promoter.

Although pancreatic desacyl ghrelin levels were elevated in RIP-GG Tg mice, pancreatic ghrelin levels were not altered in animals on standard diet. When Tg mice were fed a medium chain triglyceride rich diet (MCTD), however, pancreatic ghrelin levels were elevated to approximately 16 times that seen in control animals. It seems likely that the gastric ghrelin cells possess specific machinery to provide the octanoyl acid necessary for ghrelin acylation, but that this machinery is absent from pancreatic β cells. Despite the overexpression of ghrelin, plasma ghrelin levels in the portal veins of RIP-GG Tg mice were unchanged from control levels. Glucose tolerance, insulin secretion and islet architecture in RIP-GG Tg mice were not significantly different even when the mice were fed a MCTD. These results indicate that

34 intra-islet ghrelin does not play a major role in the regulation of insulin secretion *in vivo*.

35 Key words: ghrelin, pancreas, insulin

36

Introduction

Ghrelin is a 28-amino acid peptide hormone with a unique modification of acylation at the third serine residue, first described by Kojima *et al.* in 1999 (17). The acyl-modification of ghrelin is mediated by the recently discovered enzyme ghrelin O-acyl transferase (29), and the modification is essential for ghrelin binding to its cognate receptor (12). Ghrelin is primarily produced in the stomach, but small amounts of ghrelin are also produced in pancreatic islets (1, 5, 8, 10, 12, 26, 27). Controversy remains about which type of islet cell produces ghrelin (5, 20, 26, 27). Date *et al.* reported that ghrelin is present in α cells in humans and rats (5), while Volante *et al.* reported that ghrelin is produced by β cells in humans (26). In contrast, Wierup *et al.* and Prado *et al.* reported that ghrelin-expressing cells comprise a new islet cell type distinct from α, β, δ and PP cells in human, rat, and mouse islets (20, 27, 28).

Exogenous ghrelin suppresses insulin secretion from pancreatic β cells *in vitro* (4, 9, 22) or *in vivo* (3, 22, 25). Although several studies have demonstrated contradictory results (1, 5, 11, 18, 24), data from genetically-engineered mice are consistent with this concept. Chronic elevation of plasma ghrelin levels suppresses insulin secretion, inducing glucose intolerance in transgenic mice (2, 13, 21), while ablation of ghrelin improves glucose tolerance by enhancing insulin secretion in diet-induced obesity (7) or ob/ob mouse models (23). Although *in vitro* studies demonstrate that intra-islet ghrelin can suppress insulin secretion from isolated islets (6),

55 the physiological role of intra-islet ghrelin on the regulation of insulin secretion *in vivo* is
56 unclear. As only minimal amounts of ghrelin are produced by the pancreas in comparison to that
57 made by the stomach (15), the effect of stomach-derived ghrelin may overpower the effects of
58 intra-islet ghrelin *in vivo*.

59 In this study, we developed a transgenic mouse model, in which the ghrelin and
60 ghrelin O-acyltransferase (GOAT) genes are overexpressed by pancreatic β cells under the
61 control of the rat insulin II promoter (RIP) to ascertain the physiological role of intra-islet
62 ghrelin on insulin secretion and glucose metabolism *in vivo*.
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Materials and Methods

Generation of RIP-ghrelin-GOAT Transgenic Mice

We designed a fusion gene comprised of RIP, mouse ghrelin cDNA, internal ribosomal entry site (IRES), and mouse GOAT cDNA coding sequences. The purified fragment (10 μg/ml) was microinjected into the pronuclei of fertilized C57/B6J mouse (SLC, Shizuoka, Japan) eggs. Viable eggs were transferred into the oviducts of pseudopregnant female ICR mice (SLC) using standard techniques. Transgenic founder mice were identified by Southern blot analyses of tail DNA using a mouse ghrelin cDNA fragment as a probe. For experimentation, we utilized heterozygous transgenic mice. Animals were maintained on a 12-h light/12-h dark cycle and fed with a standard diet (SD; CE-2, 352 kcal/100g, Japan CLEA, Tokyo, Japan) or an MCTD containing 45% Dermol M5 (C8:60%, C10:40%; Research Diet Inc., New Brunswick, NJ) as indicated. All experimental procedures were approved by the Kyoto University Graduate School of Medicine Committee on Animal Research.

Measurement of Plasma and Tissue Ghrelin Concentrations

Blood was drawn from the proximal end of the portal vein under ether anesthesia, transferred immediately to chilled siliconized glass tubes containing Na_2EDTA (1mg/ml) and aprotinin (1000 KIU/ml), and centrifuged at 4°C. Hydrogen

chloride was added to the samples at a final concentration of 0.1 N immediately after separation of plasma. Plasma was immediately frozen and stored at –80°C until assay. Plasma ghrelin concentration was determined by AIA-600 Ⅱ (Tosoh, Tokyo, Japan).

To measure tissue ghrelin concentrations, pancreata or stomachs were isolated from mice, then boiled for 5 min in the 10-fold v/w of water. Acetic acid was added to each solution to adjust the final concentration to 1 M before tissues homogenization. We determine the tissue ghrelin concentration in supernatants obtained after centrifugation by radioimmunoassay (RIA) using anti-ghrelin [13-28] (C-RIA) and anti-ghrelin [1-11] (N-RIA) antisera as described previously (12, 15).

Real-time Quantitative RT-PCR

Total RNA was extracted from pancreata using an RNeasy Protect mini kit (QIAGEN, Hilden, Germany). Reverse transcription (RT) was performed using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Real-time quantitative PCR was performed on an ABI PRISM 7500 Sequence Detection System (Applied Biosystems) using the following primers and TaqMan probes were used: mouse ghrelin (sense, 5'-GCATGCTCGGATGGACATG-3'; antisense, 5'-TGGTGGCTTCTTGGATTCCT-3'; TaqMan probe, 5'-AGCCCAGAGCACCAGAAAGCCCA-3'); mouse insulin (sense,

5'-CAGCTATAATCAGAGACCATCAGCAA-3'; antisense, 5'-GGGTAGGAAGTGCACCAACAG-3'; TaqMan probe, 5'-CAGGTCATTGTTTCAAC-3'); GOAT (sense, 5'-AGGGACTCTAGGAAGGACAG-3'; antisense, 5'-CCCATCTGAAAGAAGAAGGT-3', with Power SybrGreen). Data were normalized to the content of 18 S rRNA in each sample.

Glucose Tolerance Tests

For glucose tolerance testing, the *ad libitum*-fed mice were intraperitoneally injected with 1.5 g/kg glucose. Blood was sampled from the tail veins before and 30, 60, 90, and 120 min after the injection. Blood glucose levels were determined by the glucose oxidase method using a Glutest sensor (Sanwa Kagaku, Kyoto, Japan).

Insulin Release

Ad libitum-fed mice were injected with 3.0 g/kg glucose intravenously. Plasma was sampled from a retroorbital vein before and 2 or 30min after injections into heparin-coated tubes. Insulin concentrations were measured by a high-range speedy mouse insulin kit (Morinaga, Yokohama, Japan).

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue sections were immunostained using the avidin-biotin peroxidase complex method (Vectastain "ABC" Elite Kit, Vector Laboratories, Burlingame, CA, USA) as described previously (14). Serial sections of a 5-μm thickness were

incubated with anti-C-terminal ghrelin (1:1000) (17), and anti-N-terminal ghrelin (1:2000) (17), anti-glucagon (1:500), anti-insulin (1:500), anti-somatostatin (1:500), and anti-pancreatic polypeptide (PP, 1:500, DAKO, Glostrup, Denmark) antisera.

Statistical Analysis

All values were expressed as the means ± S.E. The statistical significance of the differences in mean values was assessed by ANOVA with a post-hoc test (Turkey's test) or Student's t-test as appropriate. Differences with $P<0.05$ were considered significant. Statistical analyses were performed using Statcel2 (OMS, Saitama, Japan).

Results

Generation of RIP-ghrelin-IRES-GOAT transgenic mice.

After injecting the RIP-ghrelin-IRES-GOAT transgene into 286 eggs, we obtained three lines (3-4, 9-3 and 11-5) confirmed to be insulin II promoter-ghrelin-IRES-GOAT transgenic (RIP-GG Tg) mice. For further analyses, we selected the 9-3 line, which had the highest expression of ghrelin and GOAT mRNA in the pancreas (data not shown). The expression levels of pancreatic ghrelin mRNA in 9-3 line of RIP-GG Tg mice were approximately 20-fold higher than those seen in controls (Figure 1B), while GOAT mRNA levels were approximately 80-fold higher than those in controls (Figure 1C). There was also increment in ghrelin and GOAT mRNA levels in the hypothalamus of RIP-GG Tg mice (non vs. Tg: ghrelin; 1.0 ± 0.28 vs. 25.6 ± 5.6, GOAT; 1.0 ± 0.26 vs. 5735.5 ± 1189.1, arbitrary unit, $n=8$, $P<0.01$).

Pancreatic and plasma ghrelin levels in RIP-GG Tg mice

Total ghrelin levels measured by C-RIA were significantly elevated in the pancreata of RIP-GG Tg mice on a SD or MCTD (Figure 2A). The ghrelin levels measured by N-RIA, however, were elevated only when RIP-GG Tg mice were fed an MCTD (Figure 2B). Although ghrelin levels 16-fold higher than those seen in control littermates were observed in the pancreata of RIP-GG Tg mice fed MCTD, these absolute levels were low in comparison to

148 those isolated from stomach (Figure 1D, E). Further, the ratio of ghrelin to total ghrelin in the
149 pancreas of RIP-GG Tg mice was significantly low on SD, which was elevated on MCTD
150 (Figure 1C). Still, the level was significantly low in comparison to that of the stomach (Figure
151 1F).

152 Immunohistochemistry showed that the ghrelin-like immunoreactivities were
153 increased in the core of the islet of RIP-GG Tg mice on MCTD (Figure 3), indicating that
154 increased tissue levels of pancreatic ghrelin was originated from β cells.

155 We measured plasma ghrelin levels in the portal veins of RIP-GG Tg mice fed MCTD
156 to determine if this level of ghrelin overexpression in islets could affect plasma ghrelin levels.
157 No significant changes were observed either in ghrelin and desacyl ghrelin levels in the portal
158 veins of RIP-GG Tg mice (Figure 4A, B), indicating that ghrelin overexpression from the
159 transgene in islets produces minimal effect on plasma ghrelin levels.

160 **Glucose metabolism and insulin secretion in RIP-GG Tg mice**

161 No significant changes in blood glucose levels were seen by intraperitoneal glucose
162 tolerance tests between 10 week-old RIP-GG Tg mice and controls on MCTD (Figure 5A).
163 Plasma insulin levels before and after a glucose load were not significantly altered in
164 15-week-old RIP-GG Tg mice on MCTD (Figure 5B). There were also no significant changes in
165 blood glucose and plasma insulin levels after glucose load in old mice (around 84-weeks old) or

166 in female mice (Figure 5C, D, E, F).

167 **Islet Architecture**

168 There were no obvious abnormalities in intra-islet cytoarchitecture or in the cell
169 numbers of insulin-, glucagon-, somatostatin-, and PP-producing cells in the islets of RIP-GG
170 Tg mice on MCTD (Figure 6A–D). Staining intensities for these four islet hormones within
171 islets of RIP-GG Tg mice did not differ from those of nontransgenic littermates.

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Discussion

In previous studies, we developed transgenic mice in which mouse ghrelin cDNA is overexpressed in pancreatic β cells under the control of the rat insulin II promoter to identify the effect of ghrelin on pancreatic islets (15). These Tg mice, however, displayed elevated expression of desacyl ghrelin only within the pancreas. At that time, the mechanism by which ghrelin received an n-octanoyl modification was unknown. Recently, Yang *et al.* identified ghrelin O-acyltransferase as the enzyme mediating this modification (29). In this study, we developed a transgenic mouse in which ghrelin produced in the pancreas might be both overexpressed and modified, with the overexpression of both mouse ghrelin and GOAT cDNA in pancreatic β cells under the control of the rat insulin II promoter.

To our surprise, while pancreatic desacyl ghrelin levels were elevated in RIP-GG Tg mice, pancreatic levels of (active, modified) ghrelin were unchanged on a SD. Ghrelin levels were only elevated when mice were fed MCTD. Similar results were reported by Kirchner *et al* (16), who created a transgenic mouse in which ghrelin and GOAT cDNA were overexpressed in the liver under the control of the APOE promoter. These mice demonstrated elevated plasma ghrelin levels only when mice were fed a medium-chain fatty acids rich-diet. Considering that gastric ghrelin-producing cells can produce ghrelin regardless of diet, even in a fasting state, it is likely that these gastric cells possess a specific machinery to generate the octanoyl acid

192 necessary for acylation, which is lacking from pancreatic β cells or hepatocytes.

193 In previous studies, we demonstrated that the chronic elevation of plasma ghrelin
194 levels at approximately 10-fold higher than the normal range suppresses insulin secretion and
195 induces glucose intolerance in mice (13). In this study, RIP-GG Tg mice, which produce
196 16-fold higher ghrelin levels from the pancreas as normal mice, exhibited normal glucose
197 tolerance and insulin secretion. The pancreatic ghrelin levels in RIP-GG Tg mice, while
198 elevated, were still considerably lower than the gastric ghrelin level. We tried to compare the
199 ghrelin levels in pancreatic vein with those in artery as Dezaki *et al.* conducted using rats (7), it
200 was difficult to determine the ghrelin levels in pancreatic vein of mice due to the small body
201 size. We measured ghrelin levels in portal vein instead, which were not elevated in RIP-GG Tg
202 mice. We cannot determine the exact concentration of ghrelin in the microenvironment
203 surrounding β cells, but these levels still seem to be overpowered by the circulating ghrelin
204 produced by the stomach. While it is possible that additional overproducing of ghrelin in islets
205 could eventually suppress insulin secretion, further enhancement of ghrelin expression by islets
206 would not be in the realm of physiological relevance. *In vitro,* intra-islet ghrelin may suppress
207 insulin secretion in a paracrine (or autocrine) manner where the effect of circulating ghrelin is
208 eliminated (6). This study, however, indicates that intra-islet ghrelin does not play a major role
209 in controlling insulin secretion *in vivo,* where high levels of circulating ghrelin are generated

by the stomach.

One drawback of this study is that elevated pancreatic ghrelin levels in RIP-GG Tg mice could not be obtained without feeding mice MCTD. The MCTD consists of medium-chain fatty acids (C6-C10) that can enter mitochondria without the carnitine shuttle. Medium-chain triglycerides generally have favorable effects on obesity or diabetes (19), suppressing fat accumulation and improving insulin sensitivity. We cannot exclude the possibility that MCTD may have interfered with the effects of ghrelin within islets. In addition, ghrelin and GOAT mRNA levels were increased not only in the islet but also in the hypothalamus of RIP-GG Tg mice. There is a possibility that the over-expressed ghrelin in the hypothalamus may have influenced on the effects of overexpressed ghrelin in the islet.

In summary, we have developed RIP-GG Tg mice, in which intra-islet ghrelin levels were elevated to approximately 16 times control levels when mice were fed MCTD. The glucose tolerance and insulin secretion of RIP-GG Tg mice were unchanged, indicating that intra-islet ghrelin does not play a major role in regulating insulin secretion *in vivo*.

Acknowledgement

We thank Ms. Chieko Ishimoto and Ms. Chinami Shiraiwa for their excellent technical assistance.

Grants

This study was supported by funds from the Ministry of Education, Culture, Sports, Science and Technology of Japan and the Ministry of Health, Labour and Welfare of Japan.

Disclosures

All authors have nothing to declare.

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Figure Legends

Figure 1. Constructs of RIP-GG Tg mice and the expression levels of ghrelin and GOAT mRNA in the pancreas.

A. We designed a fusion gene containing rat insulin II promoter (RIP), mouse ghrelin cDNA, IRES and mouse GOAT cDNA. B, C. The resultant expression levels of ghrelin (B) and GOAT (C) mRNA in the pancreata of RIP-GG Tg mice. non: nontransgenic littermate, Tg: RIP-GG Tg mice, n=7–11, **: $P<0.01$ in comparison to nontransgenic littermates

Figure 2. Pancreatic and gastric ghrelin levels in RIP-GG Tg mice on STD or MCTD.

A, B. Pancreatic ghrelin levels in RIP-GG Tg mice (black bar) and nontransgenic controls (open bar) measured by C-RIA (A) and N-RIA (B). Although total ghrelin levels measured by C-RIA were elevated in RIP-GG Tg mice on both a standard diet (SD) and a medium-chain triglyceride-rich diet (MCTD), ghrelin levels measured by N-RIA were only elevated when RIP-GG Tg mice were fed MCTD. E, F Gastric ghrelin levels of RIP-GG Tg mice (black bar) and nontransgenic controls (open bar) measured by C-RIA (E) or N-RIA (F) were significantly higher than pancreatic levels, regardless of diet. C, G. The ratio of C-RIA/N-RIA. **: $P<0.01$, *: $P<0.05$ in comparison to controls, ##: $P<0.01$ in comparison to SD, n=5–7

Figure 3. Immunohistochemical analysis of the expression of ghrelin in the islet of RIP-GG Tg mice.

Ghrelin-like immunoreactivities were increased in the core of the islet of RIP-GG Tg mice on MCTD.

Figure 4. Portal ghrelin levels of RIP-GG Tg mice.

A, B. Portal ghrelin (A) and desacyl ghrelin (B) levels in male RIP-GG Tg mice (black bar) and nontransgenic littermates (open bar) fed MCTD. n=7–8,

Figure 5. Glucose metabolism in GP-Tag Tg mice.

A, C, E. Glucose tolerance tests in 10-week-old male (A), 11-week-old female (C) or 83-week-old male (E) RIP-GG Tg mice on MCTD (■) and nontransgenic littermates (◆). n=7–10

B, D, F. Serum insulin levels at baseline and at 2 min or 30 min after intravenous glucose injection in 15-week-old male (B), 10-week-old female (D) or 84-week-old male (F) RIP-GG Tg mice fed MCTD (black bars) and in nontransgenic littermates (open bars). n = 5–10.

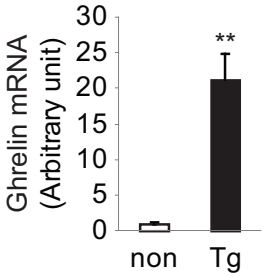
Figure 6. Islet morphology in RIP-G G Tg mice.

The pancreatic sections from RIP-GG Tg (Tg) mice and nontransgenic littermates (non) were stained with anti-insulin (A), anti-glucagon (B), anti-somatostatin (C), or anti-PP (D) antibodies. Representative images are presented.

A.


RIP
ghrelin
IRES
GOAT
poly A signal

B.


Ghrelin mRNA
(Arbitrary unit)
30
25
20
15
10
5
0
**
non
Tg

C.

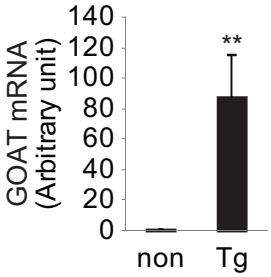

GOAT mRNA
(Arbitrary unit)
140
120
100
80
60
40
20
0
**
non
Tg

Figure 1

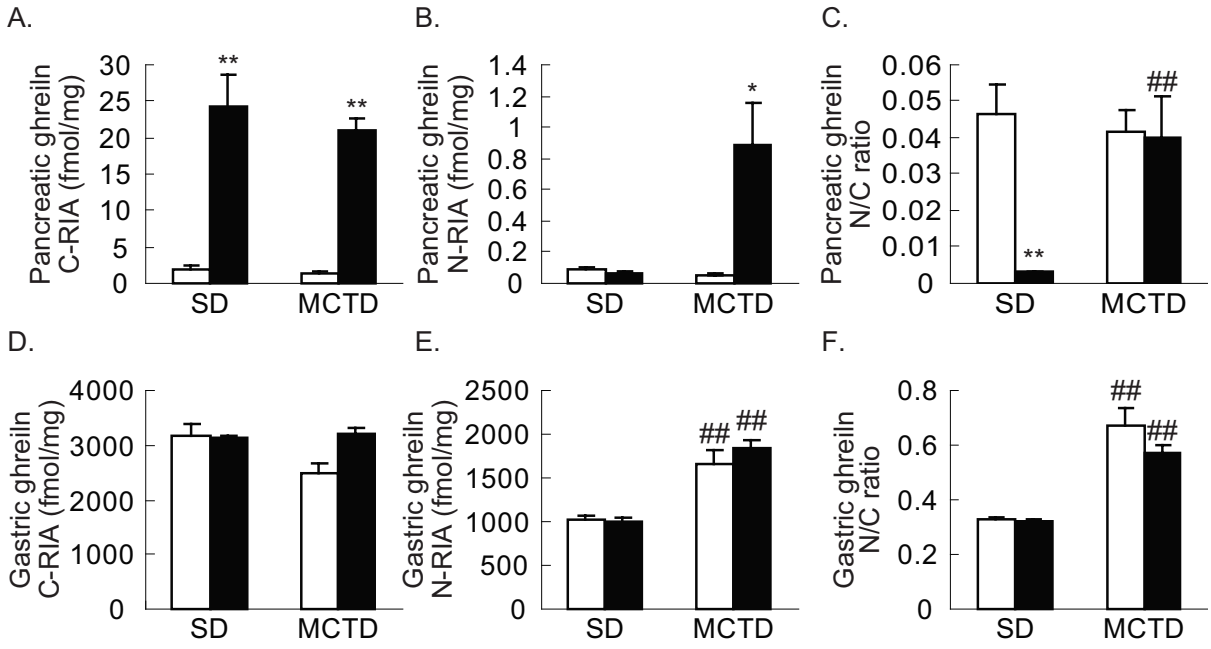

A.
Pancreatic ghreiln C-RIA (fmol/mg)
30
25
20
15
10
5
0
**
**
SD
MCTD
B.
Pancreatic ghreiln N-RIA (fmol/mg)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
SD
MCTD
C.
Pancreatic ghreiln N/C ratio
0.06
0.05
0.04
0.03
0.02
0.01
0
**
##
SD
MCTD
D.
Gastric ghreiln C-RIA (fmol/mg)
4000
3000
2000
1000
0
SD
MCTD
E.
Gastric ghreiln N-RIA (fmol/mg)
2500
2000
1500
1000
500
0
##
##
SD
MCTD
F.
Gastric ghreiln N/C ratio
0.8
0.6
0.4
0.2
0
##
##
SD
MCTD

Figure 2

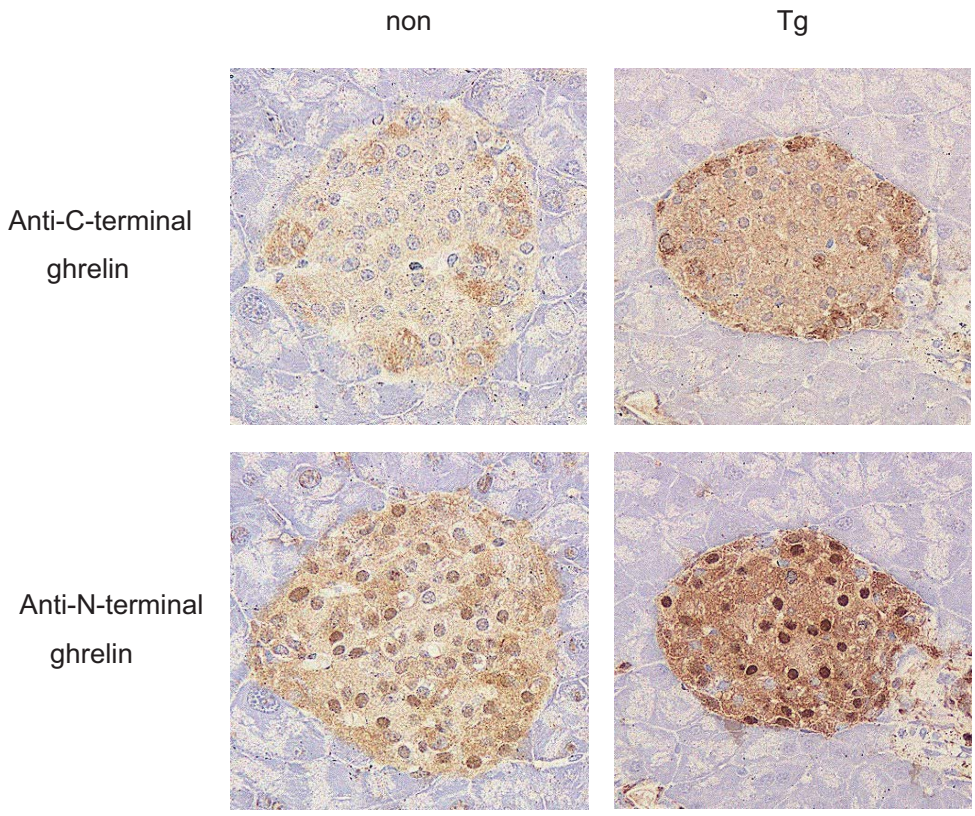


Figure 3

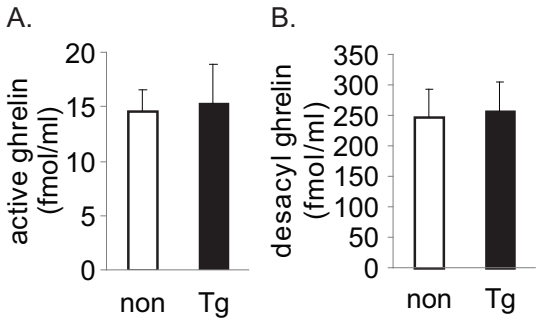

A.
active ghrelin (fmol/ml)
20
15
10
5
0
non
Tg
B.
desacyl ghrelin (fmol/ml)
350
300
250
200
150
100
50
0
non
Tg

Figure 4

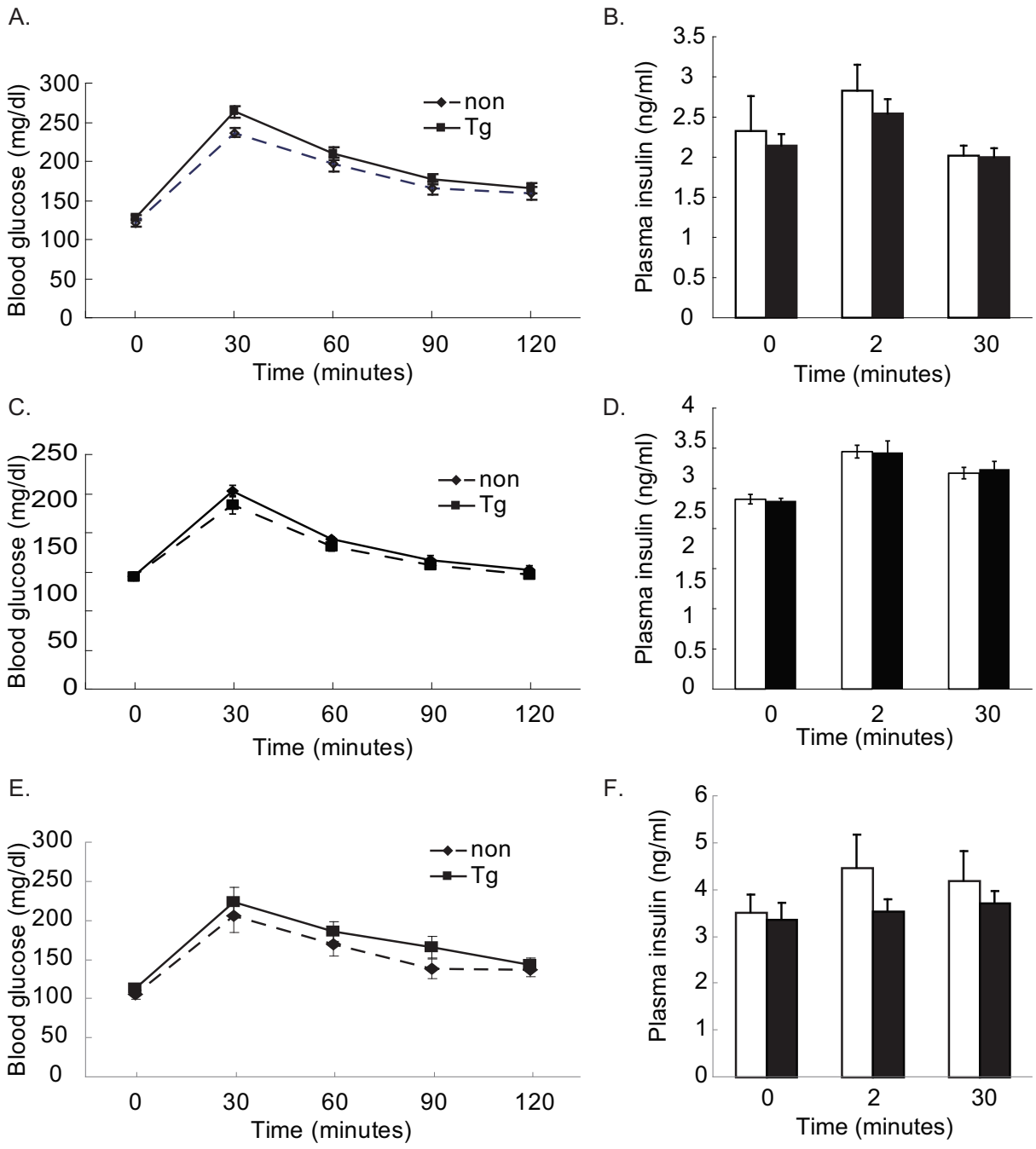

A.
Blood glucose (mg/dl)
300
250
200
150
100
50
0
non
Tg
0
30
60
90
120
Time (minutes)
B.
Plasma insulin (ng/ml)
3.5
3
2.5
2
1.5
1
0.5
0
0
2
30
Time (minutes)
C.
Blood glucose (mg/dl)
250
200
150
100
50
0
non
Tg
0
30
60
90
120
Time (minutes)
D.
Plasma insulin (ng/ml)
4
3.5
3
2.5
2
1.5
1
0.5
0
0
2
30
Time (minutes)
E.
Blood glucose (mg/dl)
300
250
200
150
100
50
0
non
Tg
0
30
60
90
120
Time (minutes)
F.
Plasma insulin (ng/ml)
6
5
4
3
2
1
0
0
2
30
Time (minutes)

Figure 5

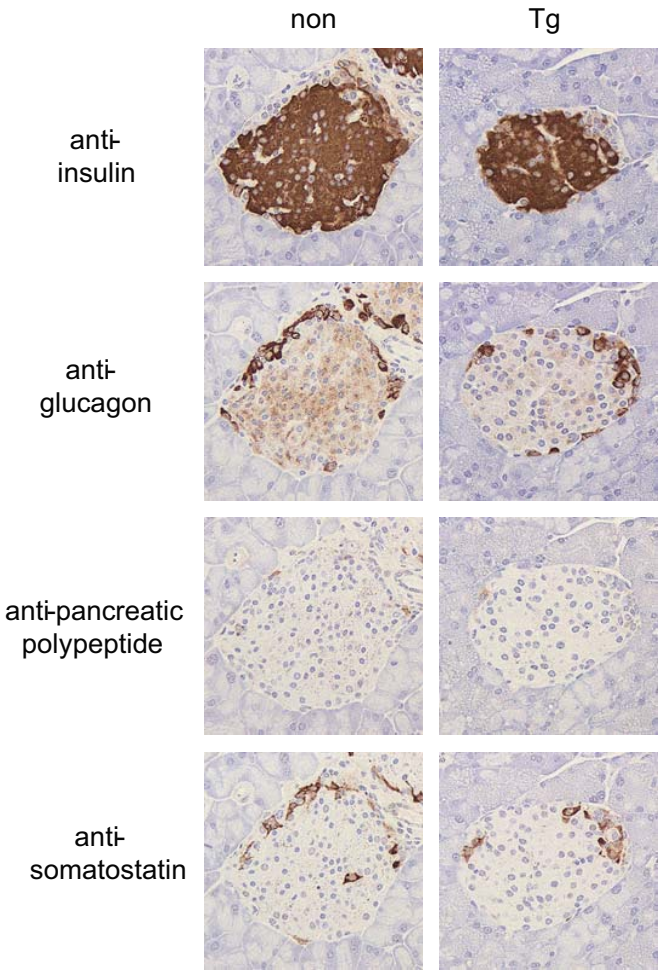

non
Tg
anti-
insulin
anti-
glucagon
anti-pancreatic
polypeptide
anti-
somatostatin

Figure 6

Overexpression of intra-islet ghrelin enhances β-cell proliferation after streptozotocin-induced β-cell injury in mice

Running head: Ghrelin enhances β-cell proliferation

Mika Bando[1, 3], Hiroshi Iwakura[1], Hiroyuki Ariyasu[1], Hiroyuki Koyama[2], Kiminori Hosoda[2, 3], Souichi Adachi[3], Kazuwa Nakao[2], Kenji Kangawa[4], Takashi Akamizu[1, 5]

[1]Ghrelin Research Project, Translational Research Center, [2]Department of Medicine and Clinical Science, Endocrinology and Metabolism, [3]Department of Human Health Sciences, Kyoto University Hospital, Kyoto University Graduate School of Medicine, Kyoto 606-8507, Japan

[4]National Cerebral and Cardiovascular Center Research Institute, Osaka 565-8565, Japan, [5]The First Department of Medicine, Wakayama Medical University, Wakayama 641-8509, Japan

Address correspondence to and reprint request: Hiroshi Iwakura, M.D., Ph.D., 54 Shogoin Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan. TEL: +81-75-751-4735; FAX: +81-75-751-4731; Email:hiwaku@kuhp.kyoto-u.ac.jp

Abstract

Previously, we reported that exogenous administration of ghrelin ameliorates glucose metabolism in a neonate streptozotocin (STZ)-induced diabetic rat model through enhancement of β-cell proliferation. However, it was not clear whether the observed β-cell proliferation was a direct or indirect effect (*e.g.*, via orexigenic or growth hormone–stimulated pathways) of ghrelin activity. Here, we aim to investigate whether ghrelin directly impacts β-cell proliferation after STZ-induced injury in mice.

Seven-week-old male rat insulin II promoter-ghrelin internal ribosomal sequence ghrelin O-acyltransferase transgenic (RIP-GG Tg) mice, which have elevated pancreatic ghrelin levels, but only minor changes in plasma ghrelin levels, when fed a medium-chain triglyceride-rich diet, were treated with STZ. Then, serum insulin, pancreatic insulin mRNA expression, and islet histology were evaluated.

We found that the serum insulin levels, but not blood glucose levels, of RIP-GG Tg mice were significantly ameliorated 14 days post-STZ treatment. Pancreatic insulin mRNA expression was significantly elevated in RIP-GG Tg mice, and β-cell numbers in islets were increased. Furthermore, the number of phospho-histone $H3^+$ or $Ki67^+$ proliferating β cells was significantly elevated in RIP-GG Tg mice, while the apoptotic indices within the islets, as determined by the TUNEL assay, were not changed.

These results indicated that ghrelin can directly stimulate β-cell proliferation *in vivo* after β-cell injury even without its orexigenic or GH-stimulating activities.

Key words: ghrelin, beta cell, diabetes, streptozotocin

Introduction

Decreased insulin secretion is one of the major features of diabetes. Insulin is produced in pancreatic islets by β cells, whose numbers are reduced or eliminated during the pathology of the disease. Autoimmune-mediated destruction of β cells causes type I diabetes, and a decrease in β-cell mass is also noted in patients with type II diabetes (5). Accordingly, a substantial effort has been made towards preventing or reversing β-cell degradation. One approach has been to find hormones or growth factors that impact proliferation or survival after β-cell injury. Several hormones, including growth hormone (22), prolactin (11) and GLP-1(11), have been suggested to stimulate β-cell proliferation in cell lines or animal models. Although these hormones have not yet been tested in the clinic, this approach may lead to the development of a new class of anti-diabetic drugs.

Ghrelin is a 28 amino acid stomach-derived peptide hormone bearing a unique acyl modification on the third Ser residue, which is essential for binding to its receptor (18). We previously reported that exogenous ghrelin administration prevents the development of diabetes at the adult stage of a rat neonate streptozotocin (STZ) model (13). In that study, we observed increased numbers of phospho-histone $H3^{+}$/$insulin^{+}$ cells in the islets of ghrelin-treated rats, suggesting that ghrelin had enhanced β-cell proliferation. However, it was not clear whether that was a direct or indirect effect of ghrelin treatment. Because ghrelin strongly stimulates GH

secretion (18, 27) and food intake (20, 25), we could not rule out the possibilities that elevated GH or nutritional status may have affected β-cell proliferation (6).

Here, we directly examined the effects of ghrelin on β cells after STZ treatment by using a recently developed rat insulin II promoter-ghrelin internal ribosomal sequence ghrelin O-acyl transferase (GOAT) transgenic (RIP-GG Tg) mice, in which ghrelin and GOAT genes are overexpressed in pancreatic β cells under the control of the rat insulin II promoter (2). As compared to control mice, RIP-GG Tg mice display a ~16-fold increase in pancreatic ghrelin concentrations, but no change in plasma ghrelin levels, when fed a medium-chain triglyceride rich diet (MCTD) (2). The aim of this study was to determine whether ghrelin directly stimulated the proliferation of β cells after STZ-induced injury.

Materials and Methods

RIP-GG Tg Mice

RIP-GG Tg mice were generated as reported previously (2). In this study, we used male heterozygous transgenic mice along with their nontransgenic littermates as controls. Animals were maintained on a 12-h light/12-h dark cycle and fed with a standard diet (SD; CE-2, 352 kcal/100g; Japan CLEA, Tokyo, Japan) or an MCTD containing 45% Dermol M5 (C8:60%, C10:40%; Research Diet Inc., New Brunswick, NJ) as indicated. RIP-GG Tg mice show elevated pancreatic ghrelin only when they were on MCTD presumably due to the lack of machinery providing octanoyl acid for acylation in β cells (2). RIP-GG Tg mice have normal glucose tolerance and insulin secretion in the absence of STZ (2). All experimental procedures were approved by the Kyoto University Graduate School of Medicine Committee on Animal Research.

STZ treatment

Seven-week-old male mice were randomly assigned to vehicle or STZ groups. STZ (100 mg/kg body weight in 100 mM citrate buffer, pH 4.5; Sigma-Aldrich, St. Louis, MO) or vehicle alone was injected after overnight fasting.

Blood glucose levels were determined by the glucose oxidase method using a Glutest sensor (Sanwa Kagaku, Kyoto, Japan) and serum insulin levels were determined using an

85 Ultrasensitive Plus Mouse Insulin kit or a High-Range Speedy Mouse Insulin kit (Morinaga,
86 Yokohama, Japan).

87 **Real-time Quantitative RT-PCR**

88 Total RNA was extracted from pancreata using an RNeasy Protect mini kit (QIAGEN,
89 Hilden, Germany). Reverse transcription (RT) was performed using a high-capacity cDNA
90 reverse transcription kit (Applied Biosystems, Foster City, CA). Real-time quantitative PCR was
91 performed on an ABI PRISM 7500 Sequence Detection System (Applied Biosystems) using the
92 following primers and TaqMan probes: mouse ghrelin, sense,
93 5'-GCATGCTCGGATGGACATG-3', antisense, 5'-TGGTGGCTTCTTGGATTCCT-3';
94 TaqMan probe, 5'-AGCCCAGAGCACCAGAAAGCCCA-3'; mouse insulin 1, sense,
95 5'-CAGCTATAATCAGAGACCATCAGCAA-3', antisense,
96 5'-GGGTAGGAAGTGCACCAACAG-3'; TaqMan probe, 5'-CAGGTCATTGTTTCAAC-3';
97 mouse Pdx1, sense, 5'-CAAAGCTCACGCGTGGAA-3', antisense,
98 5'-TGTAGGCAGTACGGGTCCTCTT-3'; TaqMan probe, 5'-AGGAGGTGCTTACAC-3';
99 mouse GHS-R, sense, 5'-CTGCTCACCGTGATGGTATG-3', antisense,
100 5'-CAGCAGAGGATGAAAGCAAA-3', with Power SybrGreen. Data were normalized to the
101 18 S rRNA content in each sample.

102 **Pancreatic insulin concentration**

To measure of pancreatic insulin concentration, pancreata were obtained from the mice under the ether anesthesia and homogenized in acid-ethanol. The supernatants were used for assay after centrifugation.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue sections were immunostained using the avidin–biotin peroxidase complex method (Vectastain ABC Elite Kit; Vector Laboratories, Burlingame, CA, USA) as described previously (14). Serial sections (5 μm) were incubated with anti-insulin antibody (1:500; DAKO, Glostrup, Denmark). Counter staining was performed with Myer's hematoxylin.

Quantitative evaluations of insulin$^+$ areas were performed using WinROOF (Mitani, Fukui, Japan). For each pancreas, insulin$^+$ areas and islets were evaluated using five sections spaced more than 40 μm apart. The number of insulin$^+$ cells within an islet was counted in five sections spaced more than 40 μm apart. The relative volume of insulin$^+$ cells was determined by calculating the ratio between the area occupied by insulin$^+$ cells and the area encompassed by islet cells.

β-cell proliferation

To detect β-cell proliferation, pancreatic tissue sections were double-stained to detect both phospho-histone H3 (Ser10) or Ki67 and insulin. First, the immunoreactivity of the anti-phospho-histone H3 (Ser10) antibody (1:50; Cell Signaling Technology, Beverly, MA) or

anti-Ki67 antibody (1:25; BD Pharmingen, Franklin Lakes, NJ) was detected using a Vectastain ABC Elite Kit with a DAB (DAKO) substrate. Then, the sections were incubated with anti-insulin antibody (1:500, DAKO), which was visualized with VECTOR VIP (Vector Laboratories). Quantitation of β-cell proliferation was performed by counting phospho-histone $H3^+$ or $Ki67^+$/$insulin^+$ cells using five sections spaced more than 40 μm apart. The relative number of phospho-histone $H3^+$ or $Ki67^+$cells was determined by calculating the ratio between the numbers of phospho-histone $H3^+$ or $Ki67^+$cells and $insulin^+$ cells.

Apoptosis

Apoptotic cells were detected using the terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) assay (ApoMark apoptosis detection Kit; Exalpha Biologicals, Maynard, MA). Quantitation of apoptotic cells was performed by counting $TUNEL^+$ cells within islets using five sections spaced more than 40 μm apart. The number of $TUNEL^+$ cells was presented as the number of $TUNEL^+$ cells/area of necrotic β cells.

To detect apoptotic cells with DNA not yet fragmented, pancreatic tissue sections were stained with anti-cleaved caspase-3 (Asp175) antibody (1:300; Cell Signaling Technology, Beverly, MA). Quantitation of apoptotic cells was performed by counting cleaved caspase-3^+ cells within islets using five sections spaced more than 40 μm apart. The number of cleaved caspase-3^+ cells was presented as the number of cleaved caspase-3^+ cells/area of necrotic β cells.

Statistical Analyses

All values were expressed as the mean ± S.E. The statistical significance of differences in mean values was assessed by the Student's *t*-test. Differences where $p < 0.05$ were considered significant. Statistical analyses were performed using Statcel2 (OMS, Saitama, Japan).

Results

Glucose metabolism and insulin secretion in RIP-GG Tg mice treated with STZ

When RIP-GG Tg mice and their nontransgenic littermates were fed a diet of MCTD and treated with STZ, blood glucose levels were significantly elevated in both groups at 7 and 14 days post-treatment as compared to those in vehicle-treated mice (Figure 1A), and body weights were significantly decreased in both groups at 7 and 14 days post-treatment as compared to those in vehicle-treated mice (Figure 1B). At 14 days post-treatment, serum insulin levels were significantly decreased in STZ-treated mice, and when compared between genotypes, the insulin levels, but not blood glucose levels, were significantly higher in RIP-GG Tg mice than those in nontransgenic littermates (Figure 1D), although only the tendency was observed at 7 days post-treatment (Figure 1C).

Insulin mRNA expression and β-cell numbers in RIP-GG Tg mice treated with STZ

The pancreatic insulin 1 and PDX-1 mRNA levels were not changed in RIP-GG Tg mice 7 days after STZ treatment, but were significantly elevated in RIP-GG Tg mice 14 days after STZ treatment with increased tendency in pancreatic insulin contents (Figure 2A, B, E). Pancreatic ghrelin mRNA levels were increased by ~70-fold in RIP-GG Tg mice as compared to their nontransgenic littermates (Figure 2C). The pancreatic GHS-R mRNA levels were not changed with STZ treatment and not different between the genotype (Figure 2D). We assessed β

cell numbers in the islets of RIP-GG Tg mice 7 days and 14 days after STZ treatment. (Figure 3A-F). In accord with the insulin mRNA levels, the ratio of insulin$^+$ cell area per islet was significantly higher in RIP-GG Tg mice than in their nontransgenic littermates 14 days after STZ treatment (Figure 3D, E), although the restoration of β cell area was limited, considering the fact that the β cell area in vehicle-treated RIP-GG Tg mouse was 83.7±0.67% and their nontransgenic littermates was 82.9±0.74% (Figure 3G). And the difference was not observed without STZ treatment (β cell areas on day 0: RIP-GG Tg vs. non: 88.9±0.71% vs. 87.6±0.99%, P=0.29) as reported previously (2). The number of insulin$^+$ cells per islet was also significantly higher in RIP-GG Tg mice as compared to vehicle-treated control animals 14 days after STZ treatment (Figure 3F). These differences were not observed 7 days after STZ treatment (Figure 3A-C).

Phospho-histone H3$^+$/insulin$^+$ cells and Ki67$^+$/insulin$^+$ cells in RIP-GG Tg mice treated with STZ

To determine whether the increased number of insulin$^+$ cells in the islets of RIP-GG Tg mice was due to increased β-cell proliferation, we assessed phospho-histone H3 and Ki67 expression, which indicate proliferating cells, in the islets of RIP-GG Tg mice 7 days and 14 days after STZ treatment. The ratio of phospho-histone H3$^+$/insulin$^+$ cells or Ki67$^+$/insulin$^+$ cells to insulin$^+$ cells were not changed in RIP-GG Tg mice 7 days after STZ treatment (Figure4A-D),

but were significantly higher in the islets of RIP-GG Tg mice 14 days after STZ treatment (Figure 4E-H), indicating that β-cell proliferation had increased in these animals at 14 days post treatment.

Short-term effects of STZ-treatment: residual β-cell numbers and apoptotic index in islets of RIP-GG Tg mice

Finally, we attempted to elucidate whether overexpressed ghrelin had direct protective effects on β cells against STZ treatment. Since we could not detect any TUNEL positive cells or cleaved caspase-3 positive cells in the islets 14 days after STZ treatment (data not shown), we examined residual β cells and the apoptotic index in islets of RIP-GG Tg mice soon after STZ administration. One day post-administration of the drug, cell nuclei in the islet core were diminished, however strong immunoreactivity for insulin was still broadly observed, probably due to leakage of insulin from damaged β cells (Figure 5A). This artifact made it difficult to accurately determine the number of residual β cells. As an alternative, we assessed insulin mRNA levels in the pancreas of RIP-GG Tg mice before and 1 day post-treatment. The pancreatic insulin mRNA levels were significantly decreased in both groups 1 day post-treatment, and there was no difference in insulin mRNA levels between the genotypes, indicating that β-cell destruction by STZ was not affected by overexpressed ghrelin (Figure 5B). In addition, to determine whether the apoptotic cells were increased, we assessed TUNEL and

cleaved caspase-3 expression, in islets from RIP-GG Tg mice. The ratio of $TUNEL^+$ cell or cleaved caspase-3^+ cell number per islets area was not significantly different from that of their nontransgenic littermates (Figure 5C-F).

Discussion

In this study, we found that the overexpression of intra-islet ghrelin ameliorated insulin secretion in an STZ-induced diabetic mouse model by stimulating the proliferation of β cells in the islets. This finding is in accord with our previous reports that exogenous ghrelin administration stimulates β-cell proliferation in STZ-treated neonate rats (13). In the previous study, it was not clear whether the stimulatory effects of ghrelin on β cells were direct or indirect. We hypothesized that indirect mechanisms could be mediated through ghrelin's GH-stimulating and/or orexigenic properties. Here, by using RIP-GG Tg mice, in which intra-islet ghrelin levels are elevated without major changes in plasma ghrelin levels (2), we clearly demonstrated that ghrelin directly stimulated β-cell proliferation *in vivo* after STZ treatment.

Although serum insulin levels were elevated in STZ-treated RIP-GG Tg mice, glucose levels were not improved to the degree observed in ghrelin-treated neonate STZ rats (13). The relatively weak effect observed in this study may have been due to the differences in age and species as compared to the previous study. In rats, β-cell numbers continue to increase after birth, and reach a steady-state level at weaning (10). Accordingly, in the neonate STZ-treated rat model, β-cell numbers recover to some degree even without any therapeutic treatment and elevated glucose levels temporally return to normal for several weeks after STZ administration

223 (30). Here, we used adult mice with limited capacity for β-cell proliferation (10). Since RIP-GG
224 Tg mice must be fed with MCTD in order to increase islet ghrelin levels, we could not study the
225 mice before weaning. The age-related differences in β-cell proliferative capacities may explain
226 the disparities in the intensity of ghrelin activity between the current study and the previous
227 report. Another possibility is that the differences reflect species-specific variations. β-cell
228 sensitivity to STZ is known to be different among species (31). For example, rats are more
229 sensitive than mice to the effects of the drug (31). This difference in STZ sensitivity may have
230 affected the results of these studies. Age and species differences aside, we cannot completely
231 rule out the possibility that exogenously administered ghrelin may have exhibited both direct
232 and indirect effects on β cells in the neonate rat STZ model.

233 Ghrelin is reported to stimulate the proliferation of several cell lines, including the
234 pancreatic cancer cell line PANC1 (9), the somatotroph cell line GH3 (21), the prostate cancer
235 cell line PC3 (15) and osteoblasts (19). Conversely, the peptide has been observed to inhibit the
236 growth of tumors and tumor-derived cell lines including human breast carcinoma (6), and fetal
237 thyroid and thyroid follicular tumors (28). Thus, our results are in accord with previous reports
238 that ghrelin can stimulate cell proliferation. Given that β-cell proliferation is not increased at a
239 basal state in RIP-GG Tg mice (2), the proliferative effects of ghrelin on β cells seem to be
240 limited. β-cell proliferation is enhanced in STZ- (30) or alloxan-treated rodents (29), in a

241 partially pancreatectomized rat (4), and in a ductally ligated hamster (23). However, the
242 mechanisms underlying the stimulation of β-cell proliferation in these injury models have not
243 yet been completely elucidated. Ghrelin may synergize with these injury-derived proliferative
244 effects on β cells. Further studies will be needed to clarify the precise mechanisms by which
245 ghrelin stimulates β-cell proliferation.

246 Several lines of evidence suggest that ghrelin can exhibit anti-apoptotic effects on a
247 variety of cell types (1, 7, 12, 16, 17). With respect to β cells, Granata *et al.* reported that ghrelin
248 prevented apoptosis in the β-cell lines HIT-T15 and INS-1E, as well as in human islets (12). By
249 contrast, in this study, we could not detect differences in the apoptotic index of the islets
250 between RIP-GG Tg and control mice. The discrepancy between the previous results and this
251 study may be due to differences in experimental conditions. For example, Granata *et al.* used
252 β-cell lines and isolated islets *in vitro*, and induced apoptosis by serum starvation or the addition
253 of interferons (12), while we used an *in vivo* STZ-induced diabetic mouse model. Furthermore,
254 it has been reported that low doses of STZ induce β-cell apoptosis, whereas high doses cause
255 β-cell necrosis (24). In this study, we used 100 mg/kg, which is a relatively high dose. Therefore,
256 although we detected very few apoptotic cells in RIP-GG Tg islets, based upon these results we
257 cannot determine whether ghrelin directly protected β cells from apoptosis.

258 The results of this study indicate that introduction of ghrelin and GOAT to β cell may

have beneficial effects on diabetes in the sense that it may increase β cell mass. On the other hand, previous reports indicate that exogenous ghrelin administration suppresses insulin secretion and elevates blood glucose level and that inhibition of ghrelin or GOAT ameliorates glucose tolerance in mice by enhancing insulin secretion (3, 26, 32). Considering that RIP-GG Tg mice has normal glucose tolerance and insulin secretion, the level of ghrelin needed to stimulate β cell proliferation after STZ-induced β cell injury seems to be lower than the level to suppress insulin secretion. It would be necessary to keep in mind the deleterious side of ghrelin's effect on β cell when therapeutic application of ghrelin on β cell injury is considered.

One drawback of this study is that ghrelin may be produced in the tissues other than the β cell such as hypothalamus as is the case in the RIP-Cre mice (8), which may have affected β cell proliferation. Actually, the mRNA levels of ghrelin and GOAT was elevated in the hypothalamus of RIP-GG Tg mice (2). However, when we examined the expression of the peptide by immunohistochemistry, we found no apparent differences of the ghrelin-like immunoreactivities in the hypothalamus between Tg mice and controls (data not shown). Further, there were no differences in body weights between two groups. Therefore, we doubt that physiologically meaningful levels of ghrelin were produced in the hypothalamus of RIP-GG Tg mice. Nonetheless, we cannot completely eliminate the possibility that the leakage expression of ghrelin in other tissues may have also affected

277 the β cell proliferation indirectly.

278 In conclusion, we found that serum insulin levels, β-cell numbers and β-cell
279 proliferation were significantly elevated in RIP-GG Tg mice after STZ treatment. These results
280 indicated that ghrelin can directly stimulate β-cell proliferation *in vivo* after β-cell injury even
281 without its orexigenic or GH-stimulating activities.

282

Acknowledgement

We thank Ms. Chieko Ishimoto and Ms. Chinami Shiraiwa for their excellent technical assistance.

Grants

This study was supported by funds from the Ministry of Education, Culture, Sports, Science and Technology of Japan and the Ministry of Health, Labour and Welfare of Japan.

Disclosures

All authors have nothing to declare.

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402

403

Figure Legends

Figure 1. Serum insulin levels are increased in STZ-treated RIP-GG Tg mice as compared to control animals

A, B. Blood glucose levels (A) and body weight (B) in RIP-GG Tg mice (Tg) and their nontransgenic littermates (non-Tg) treated with STZ or vehicle alone. n = 8–10. **, ##. $p <$ 0.01 in comparison to vehicle alone. C, D. Serum insulin levels in RIP-GG Tg mice and their nontransgenic littermates treated with STZ or vehicle 7 days post treatment (C) or 14 days post treatment (D). n = 8–10. *$p < 0.05$.

Figure 2. Pancreatic insulin and PDX-1 mRNA levels are increased in STZ-treated RIP-GG Tg mice as compared to controls.

A, B, C, D. Pancreatic insulin1 (A), PDX-1 (B), ghrelin (C), GHS-R (D) mRNA levels in RIP-GG Tg mice (Tg) and their nontransgenic littermates (non-Tg) 0, 7, 14 days post-STZ treatment. n=7-10. At day14, pancreatic insulin 1, PDX-1, and ghrelin mRNA levels were significantly higher in RIP-GG Tg mice (Tg) as compared to their nontransgenic littermates (non-Tg). **$p < 0.01$. E. Pancreatic insulin concentration in RIP-GG mice (Tg) and their nontransgenic littermates (non-Tg) 14 days post-STZ treatment. n=7.

Figure 3. Compared to control animals, RIP-GG Tg mice have more insulin$^+$ islet cells after STZ treatment.

The area occupied by insulin$^+$ cells and the absolute number of these cells in islets 14 days post-STZ treatment were significantly higher in RIP-GG Tg mice as compared to their nontransgenic littermates. A, D. Representative images of tissue sections from RIP-GG Tg (Tg) and nontransgenic (non-Tg) islets 7 days post-STZ treatment (A) and 14 days post-STZ treatment (D) reacted with an anti-insulin antibody. B, E. Ratio of the area occupied by insulin$^+$ cells to the area of the entire islet 7 days post-STZ treatment (B) and 14 days post-STZ treatment (E). C, F. The number of insulin$^+$ cells in islets of RIP-GG Tg mice 7 days post-STZ treatment (C) and 14 days post-STZ treatment (F). n = 7–8. $^{**}p < 0.01$. G. Representative images of tissue sections from RIP-GG Tg (Tg) and nontransgenic (non-Tg) islets 14 days post-vehicle treatment. H. Ratio of the area occupied by insulin$^+$ cells to the area of the entire islet 14 days post-vehicle treatment. n = 7.

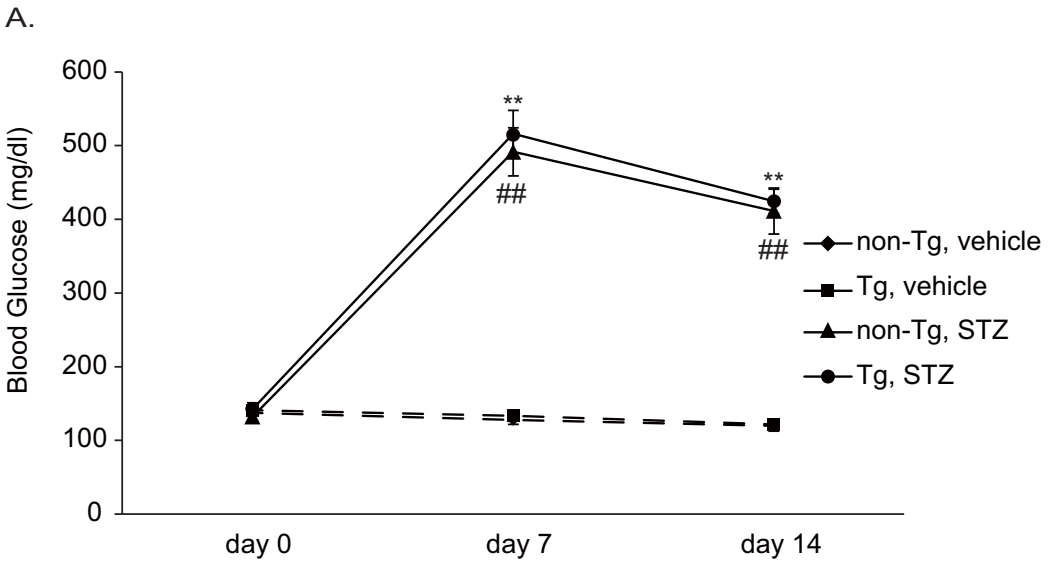
Figure 4. Phospho-histone H3$^+$ cells are more abundant in islets of RIP-GG Tg mice as compared to controls.

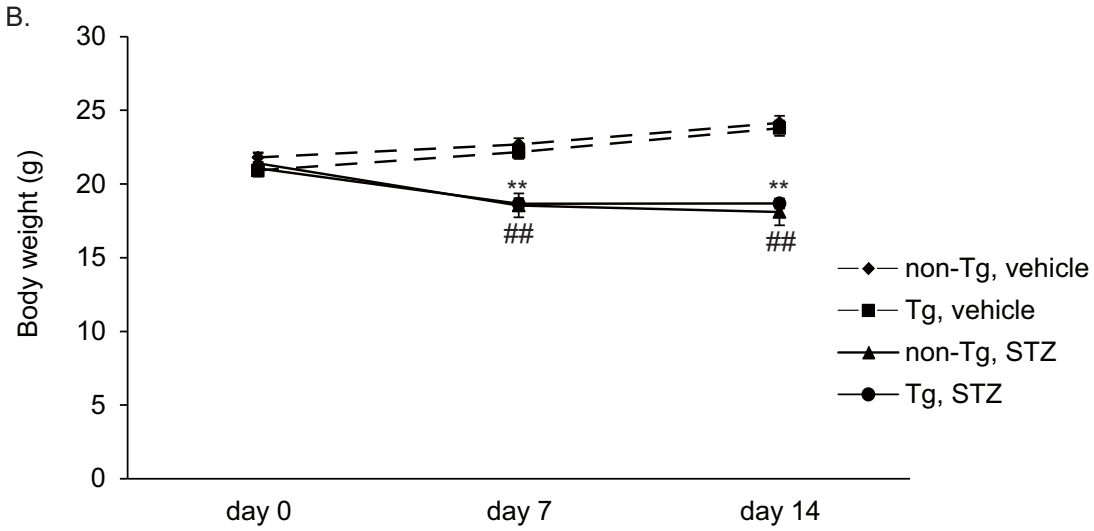
A, C. Representative images of islet tissue sections from RIP-GG Tg mice (Tg) and their nontransgenic littermates (non-Tg) 7 days post-STZ treatment. Sections were immunostained with an anti-phospho-histone H3 antibody (A) or an anti-Ki67 antibody (C) (brown, arrow) and an anti-insulin antibody (purple). B, D. Ratio of phospho-histone H3$^+$ cells (B) or Ki67$^+$ cells (D) to insulin$^+$ cells in islets of RIP-GG Tg mice (Tg) and their nontransgenic littermates

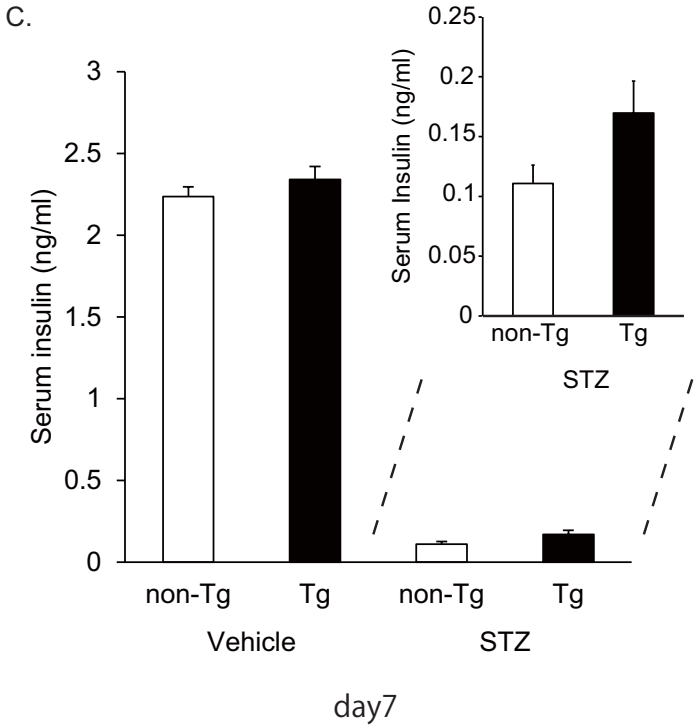
(non-Tg) 7 days post-STZ treatment. n = 5. E, G. Representative images of islet tissue sections from RIP-GG Tg mice (Tg) and their nontransgenic littermates (non-Tg) 14 days post-STZ treatment. Sections were immunostained with an anti-phospho-histone H3 antibody (E) or an anti-Ki67 antibody (G) (brown, arrow) and an anti-insulin antibody (purple). F, H. Ratio of phospho-histone $H3^+$ cells (F) or $Ki67^+$ cells (H) to $insulin^+$ cells in islets of RIP-GG Tg mice (Tg) and their nontransgenic littermates (non-Tg) 14 days post-STZ treatment. n = 7-8. $**p < 0.01$.

Figure 5. No differences were observed between the residual β-cell populations and the apoptotic indices in islets of STZ-treated RIP-GG Tg and control mice.

A. Representative images of islet tissue sections from RIP-GG Tg mice (Tg) and their nontransgenic littermates (non-Tg). One day post-STZ treatment, sections were stained with an anti-insulin antibody (brown). B. Pancreatic insulin 1 mRNA levels observed in RIP-GG Tg mice and their nontransgenic littermates before or one day post-STZ treatment. n = 11–12. **, ##. $p < 0.01$ in comparison to before. C, E. Representative images of tissue sections from RIP-GG Tg (Tg) and nontransgenic (non-Tg) islets one day post-STZ treatment reacted with TUNEL reagents (C) or an anti-cleaved caspase-3 antibody (E). D, F. The number of $TUNEL^+$ cells (D) or cleaved $caspase\text{-}3^+$ cells (F) in islet cores did not differ between RIP-GG Tg mice and controls (non-Tg). n = 5.


A.
Blood Glucose (mg/dl)
600
500
400
300
200
100
0
day 0
day 7
day 14
**
##
non-Tg, vehicle
Tg, vehicle
non-Tg, STZ
Tg, STZ


B.
Body weight (g)
30
25
20
15
10
5
0
day 0
day 7
day 14
**
##
non-Tg, vehicle
Tg, vehicle
non-Tg, STZ
Tg, STZ


C.
Serum insulin (ng/ml)
3
2.5
2
1.5
1
0.5
0
non-Tg
Tg
Vehicle
STZ
Serum Insulin (ng/ml)
0.25
0.2
0.15
0.1
0.05
0
day7

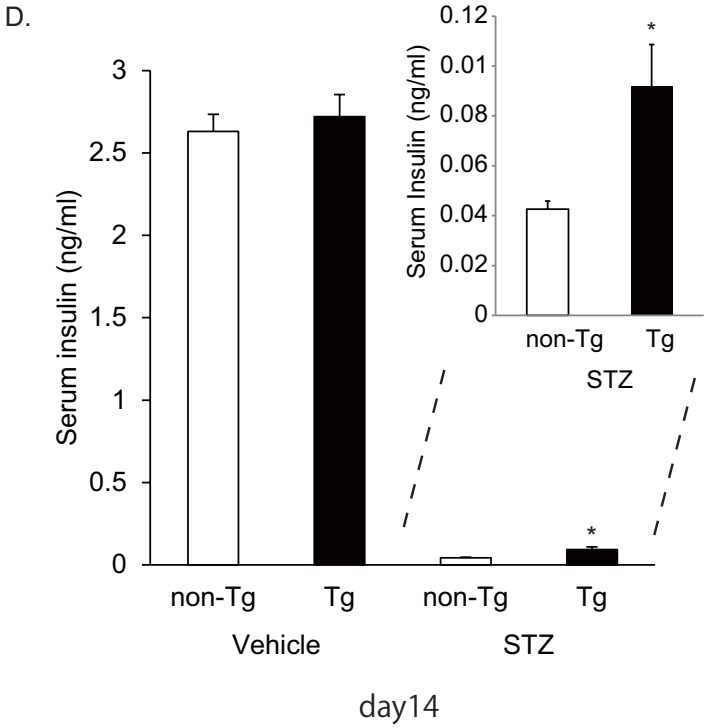

D.
Serum insulin (ng/ml)
3
2.5
2
1.5
1
0.5
0
non-Tg
Tg
Vehicle
STZ
Serum Insulin (ng/ml)
0.12
0.01
0.08
0.06
0.04
0.02
0
*
day14

Figure 1

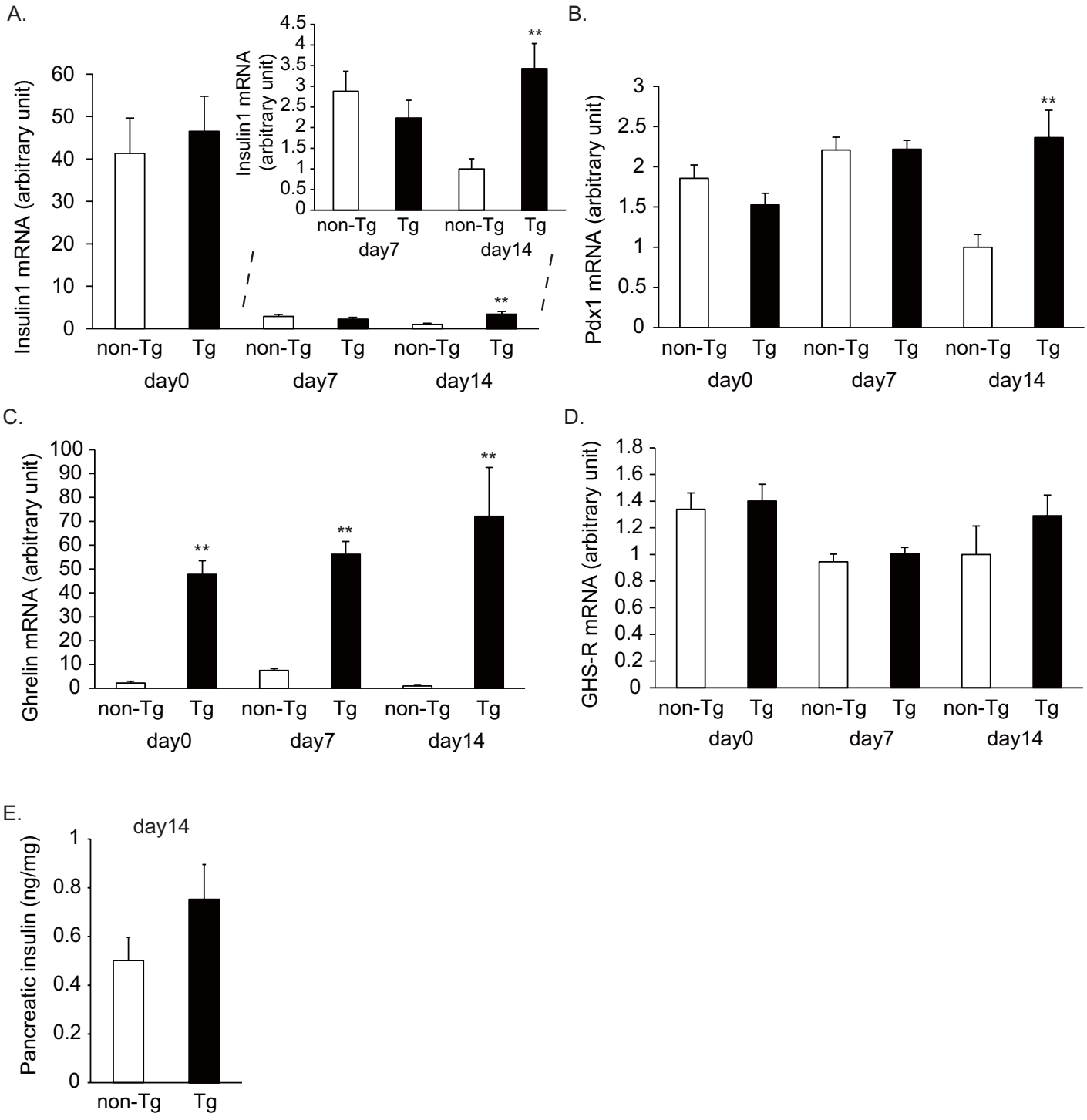

A.
Insulin1 mRNA (arbitrary unit)
60
50
40
30
20
10
0
non-Tg
Tg
day0
day7
day14
Insulin1 mRNA (arbitrary unit)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
**
B.
Pdx1 mRNA (arbitrary unit)
3
2.5
2
1.5
1
0.5
0
C.
Ghrelin mRNA (arbitrary unit)
100
90
80
70
60
50
40
30
20
10
0
D.
GHS-R mRNA (arbitrary unit)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
E.
Pancreatic insulin (ng/mg)
1
0.8
0.6
0.4
0.2
0

Figure 2

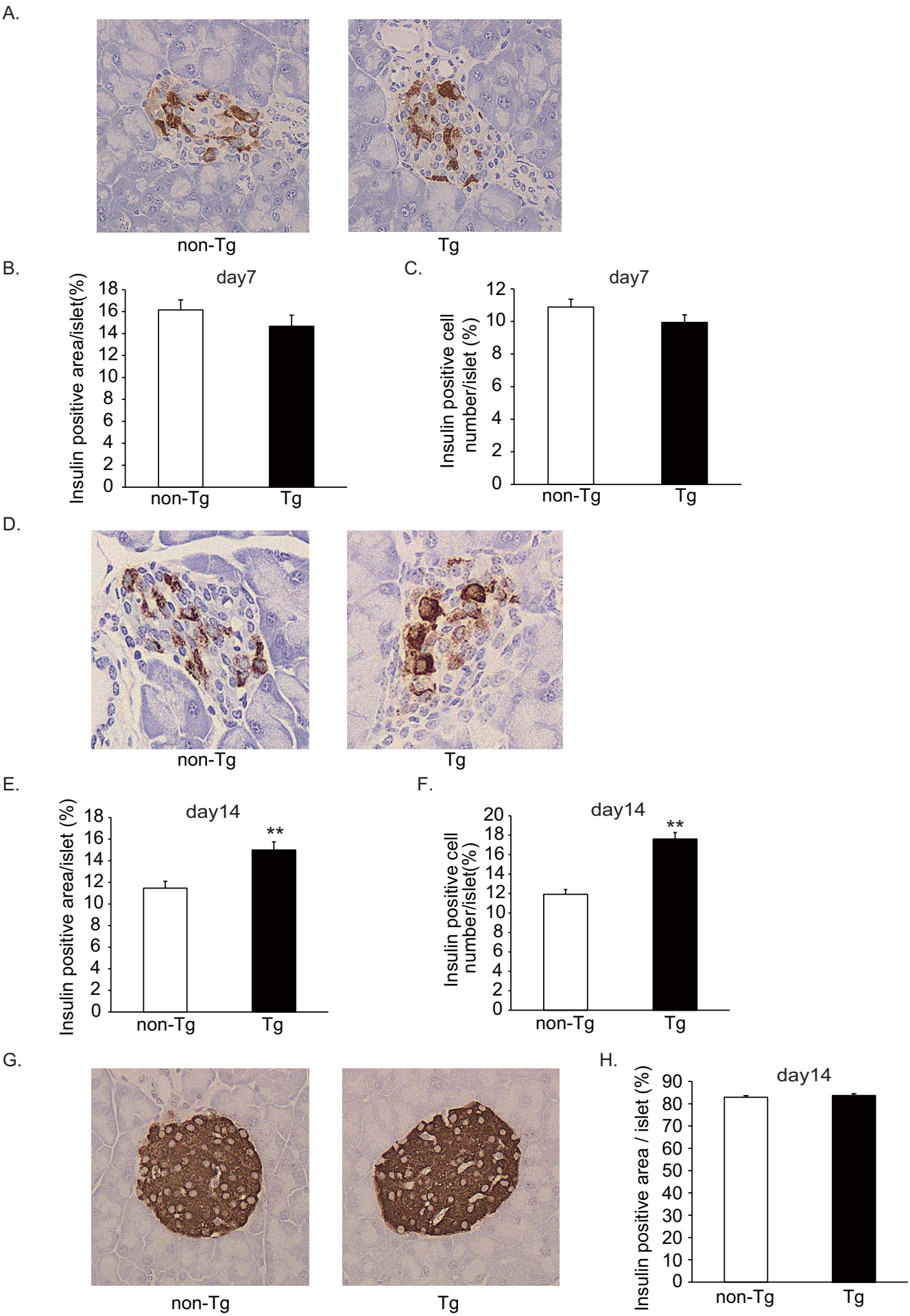
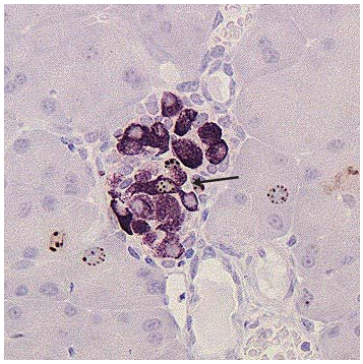
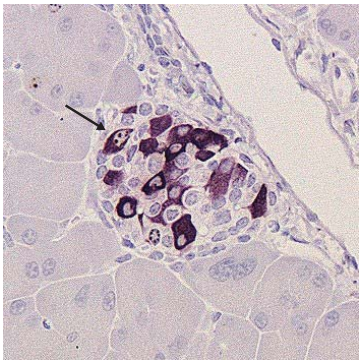

A.
non-Tg
Tg
B.
day7
Insulin positive area/islet(%)
18
16
14
12
10
8
6
4
2
0
non-Tg
Tg
C.
day7
Insulin positive cell number/islet (%)
12
10
8
6
4
2
0
non-Tg
Tg
D.
non-Tg
Tg
E.
day14
Insulin positive area/islet (%)
**
18
16
14
12
10
8
6
4
2
0
non-Tg
Tg
F.
day14
Insulin positive cell number/islet(%)
**
20
18
16
14
12
10
8
6
4
2
0
non-Tg
Tg
G.
non-Tg
Tg
H.
day14
Insulin positive area / islet (%)
90
80
70
60
50
40
30
20
10
0
non-Tg
Tg

Figure 3

A.

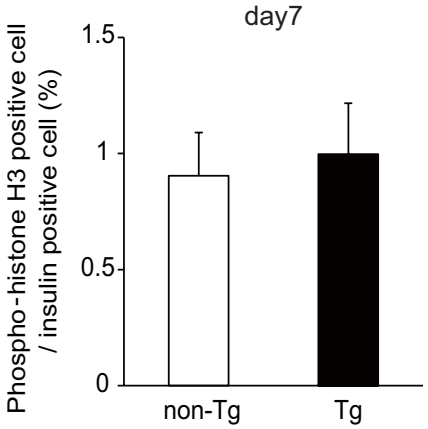


non-Tg

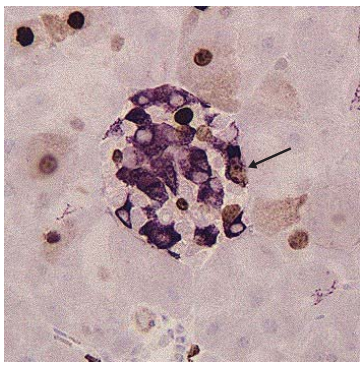


Tg

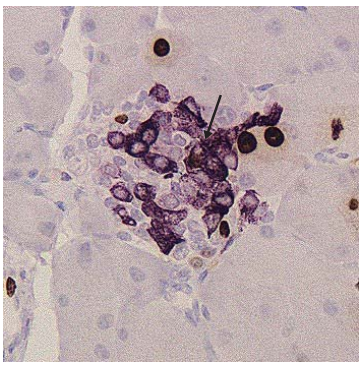
B.



C.

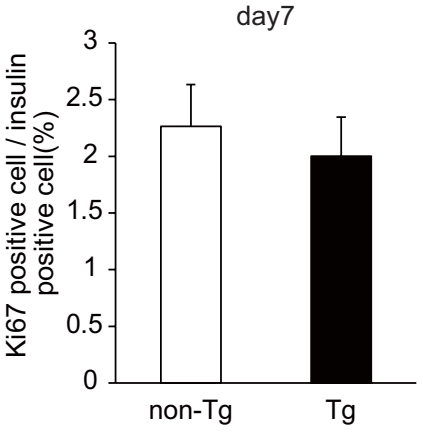


non-Tg

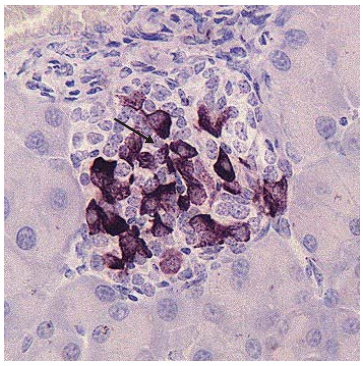


Tg

D.



E.



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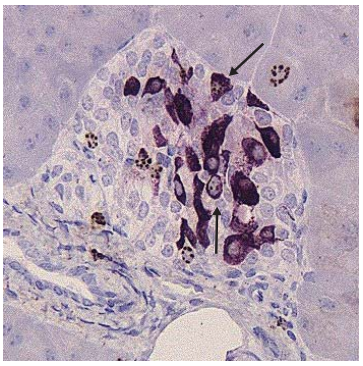
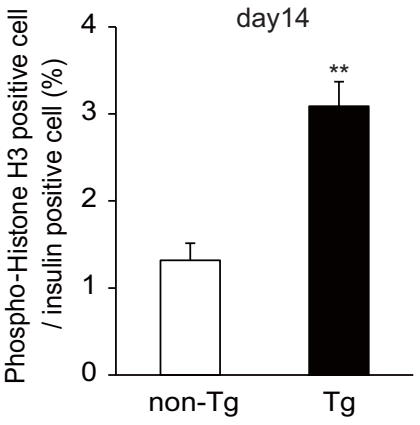
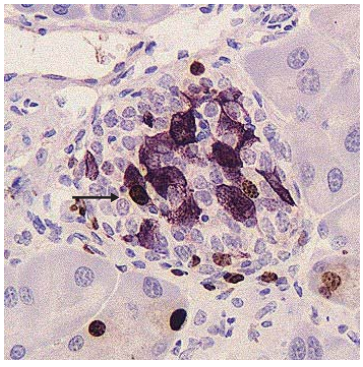


Tg

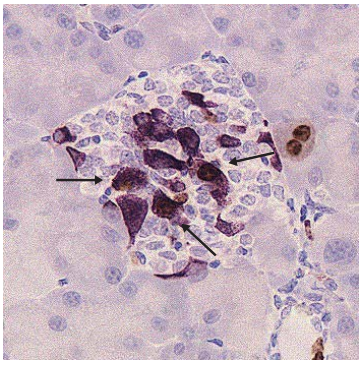
F.



G.



non-Tg



Tg

H.

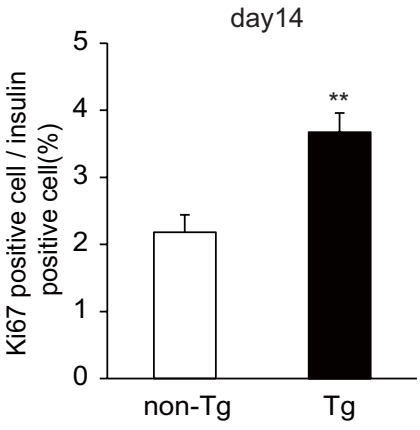
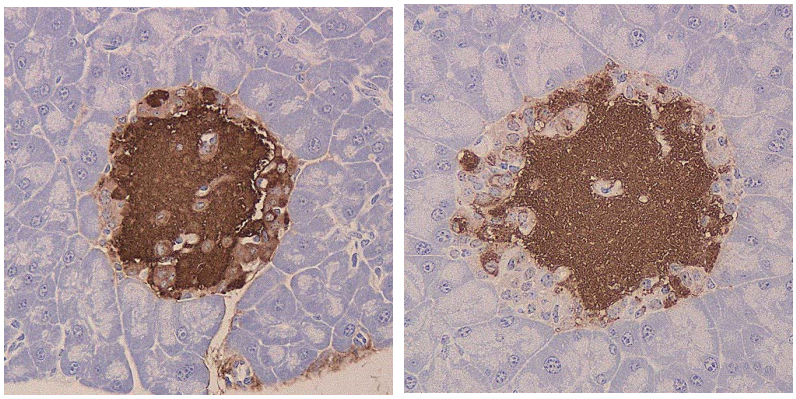


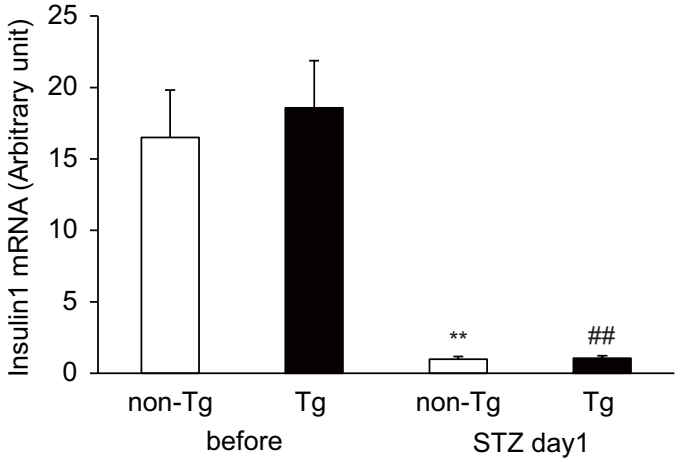
Figure 4

A.



non-Tg Tg

B.



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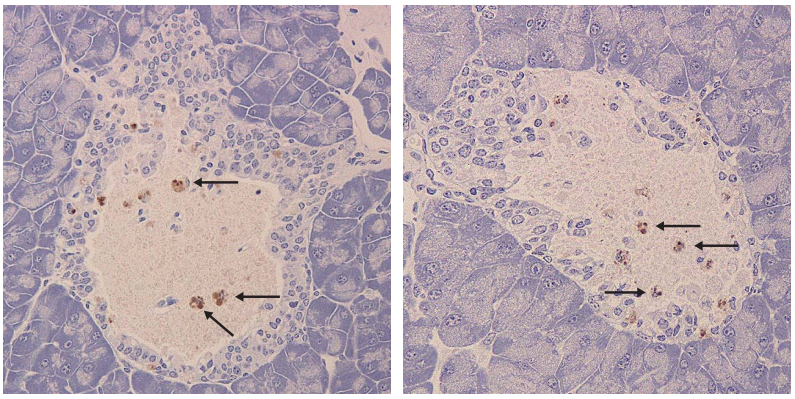
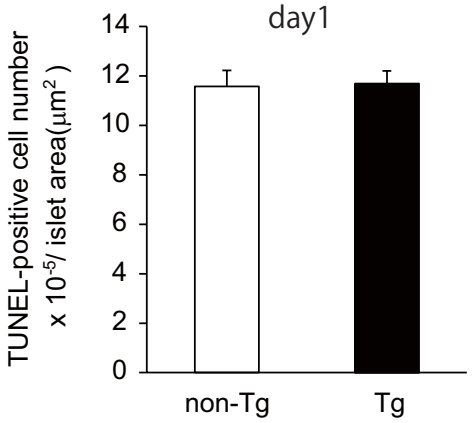
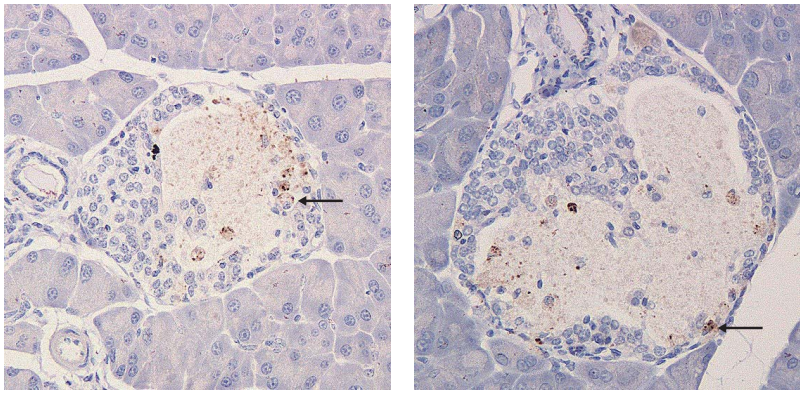


non-Tg Tg

D.



E.



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F.

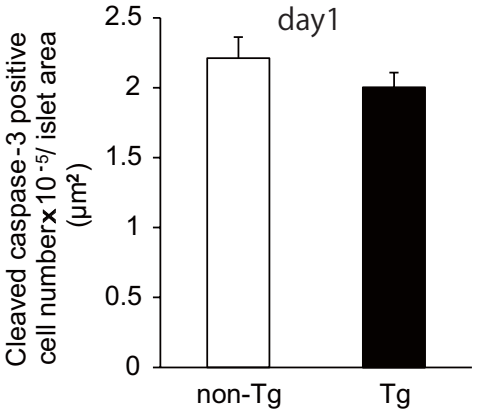

Figure 5