A mouse model of ghrelinoma exhibited activated growth hormone-insulin-like growth factor I axis and glucose intolerance

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Iwakura H, Ariyasu H, Li Y, Kanamoto N, Bando M, Yamada G, Hosoda H, Hosoda K, Shimatsu A, Nakao K, Kangawa K, Akamizu T. A mouse model of ghrelinoma exhibited activated growth hormone-insulin-like growth factor I axis and glucose intolerance. Am J Physiol Endocrinol Metab 297: E802–E811, 2009. First published July 14, 2009; doi:10.1152/ajpendo.00205.2009.—Ghrelin is a stomach-derived peptide that has growth hormone-stimulating and orexigenic activities. Although there have been several reports of ghrelinoma cases, only a few cases have elevated circulating ghrelin levels, hampering the investigation of pathophysiological features of ghrelinoma and chronic effects of ghrelin excess. Furthermore, standard transgenic technique has resulted in desacyl ghrelin production only because of the limited tissue expression of ghrelin O-acyltransferase, which mediates acylation of ghrelin. Accordingly, we attempted to create ghrelin promoter SV40 T-antigen transgenic (GP-Tag Tg) mice, in which ghrelin-producing cells continued to proliferate and finally developed into ghrelinoma. Adult GP-Tag Tg mice showed elevated plasma ghrelin levels with preserved physiological regulation. Adult GP-Tag Tg mice with increased plasma ghrelin levels exhibited elevated IGF-I levels despite poor nutrition. Although basal growth hormone levels were not changed, those after growth hormone-releasing hormone injection tended to be higher. These results indicate that chronic elevation of ghrelin activates GH-IGF-I axis. In addition, GP-Tag Tg mice demonstrated glucose intolerance. Insulin secretion by glucose tolerance tests was significantly attenuated in GP-Tag Tg, whereas insulin sensitivity determined by insulin tolerance tests was preserved, indicating that chronic elevation of ghrelin suppresses insulin secretion and leads to glucose intolerance. Thus, we successfully generated a Tg model of ghrelinoma, which is a good tool to investigate chronic effects of ghrelin excess. Moreover, their characteristic features could be a hint on ghrelinoma.

ghrelin; glucose metabolism

GHRELIN is a stomach-derived 28-amino acid (AA) peptide hormone with octanoyl modification of third Ser residue, which is essential for its binding to growth hormone (GH) secretagogue receptor (GHS-R) (20). There have been several reports regarding ghrelin-producing tumors (9, 17, 36, 37). As far as we know, only two cases have elevated plasma ghrelin level (9, 36). However, the ghrelin-producing cells in the stomach, known as X/A-like cells, account for about 20% of the endocrine cell population in the oxyntic glands (10). It may be reasonable to estimate that far more ghrelinoma cases have been overlooked and diagnosed as nonfunctioning tumors. Hormone-producing tumors demonstrate their characteristic symptoms by chronic effects of each hormone, which may be a key symptom to making a correct diagnosis. Conversely, the characteristic symptom often tells us the chronic effects of each responsible hormone. Acute effects of ghrelin have been studied extensively by many researchers, and a wide variety of acute effects of ghrelin have been discovered, such as the regulation of growth hormone (GH) release, food intake, gastric acid secretion, gastric motility, blood pressure, and cardiac output (23, 25, 26, 31, 33, 34). However, chronic effects of ghrelin have not been fully understood.

To understand the chronic effects of ghrelin, genetically engineered mouse models would be useful. Several groups, including ours, have developed transgenic animals in which ghrelin transgenes are driven by several different promoters (2, 4, 18, 29, 38, 41). All of these animals except for one line created by Reed et al. (29) using the neuron-specific enolase (NSE) promoter and another line recently reported by Bewick et al. (5) using the bacterial artificial chromosome produced only desacyl ghrelin rather than acylated ghrelin. Until the recent identification of ghrelin O-acyltransferase (GOAT), which mediates ghrelin octanoylation (40), it had been unclear how acylation of ghrelin takes place. GOAT is expressed mainly in stomach and intestine, and a small amount of GOAT is also present in pancreas (12). This limited expression area of GOAT made it impossible to create ghrelin-overproducing transgenic animals by standard procedures. When we started this study, GOAT had not yet been identified. Accordingly, we choose an approach in which an increase in the number of ghrelin-producing cells in mice would result in increased levels of circulating ghrelin. By taking this approach, we successfully obtained ghrelin promoter-SV40 T-antigen transgenic (GP-Tag Tg) mice. In these mice, ghrelin concentration elevates with age in concordance with the proliferation of ghrelin cells. The aim of this study was to elucidate the pathophysiological features of ghrelinoma and the chronic effects of ghrelin elevation.

MATERIALS AND METHODS

Animals. Two types of fusion genes comprising the 5'-flanking region of human ghrelin gene (4,085 or 1,479 bp) (19) and SV40 T-antigen were designed (Fig. 1A). The purified fragments (10 µg/ml) were microinjected into the pronucleus of fertilized C57/B6 mouse (SLC, Shizuoka, Japan) eggs. The viable eggs were transferred into the oviducts of pseudopregnant female ICR mice (SLC) by using standard techniques. Transgenic founder mice were identified by

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Southern blot analysis of tail DNAs. Transgenic mice were used as heterozygotes. Animals were maintained on standard rodent food (CE-2, 352 kcal/100 g; Japan CLEA, Tokyo, Japan) on a 12:12-h light-dark cycle unless otherwise indicated. All experimental procedures were approved by the Kyoto University Graduate School of Medicine Committee on Animal Research.

RT-PCR and real-time quantitative RT-PCR. Total RNA was extracted using a Sepasol RNA kit (Nacalai Tesque, Kyoto, Japan). Reverse transcription was performed with a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). RT-PCR was carried out with a GeneAmp 9700 using primers in Table 1 with AmpliTaq Gold PCR master mix (Applied Biosystems). Real-time quantitative PCR was performed using an ABI PRISM 7500 Sequence Detection System (Applied Biosystems) with primers and TaqMan probes or with Power SybrGreen (presented in Table 1). The mRNA expression in each gene was normalized to levels of 18S ribosomal RNA.

Immunohistochemistry. Formalin-fixed, paraffin-embedded tissue sections were immunostained using the avidin-biotin peroxidase complex method (Vectastain “ABC” Elite kit; Vector Laboratories, Burlingame, CA), as described previously (18). Sections were incubated with anti-COOH-terminal ghrelin (AA 13-28) (1:2,000 at final dilution), anti-NH2-terminal ghrelin (14) that recognizes the n-octanoylated portion of ghrelin (AA 1-11) (1:5,000), anti-glucagon (1:500; DAKO, Glostrup, Denmark), anti-somatostatin (1:500; DAKO), anti-gastrin (1:500; DAKO), and anti-GH (1:500; DAKO). The cell number of ghrelin-immunopositive cells was analyzed by WinRoof visual analysis software (Mitani, Fukui, Japan).

Measurements of plasma and tissue ghrelin concentrations. Collection of plasma samples was performed as reported previously (18). Plasma ghrelin and desacyl ghrelin concentrations were determined using two separate ELISA kits, an active ghrelin ELISA kit that recognizes the n-octanoylated portion of ghrelin (AA 1-11) (1:5,000), anti-glucagon (1:500; DAKO, Glostrup, Denmark), anti-somatostatin (1:500; DAKO), anti-gastrin (1:500; DAKO), and anti-GH (1:500; DAKO). The cell number of ghrelin-immunopositive cells was analyzed by WinRoof visual analysis software (Mitani, Fukui, Japan).

Western blot. Stomachs were boiled for 5 min in the 10-fold vol/wt of water. Acetic acid was added to each solution so that the final concentration was adjusted to 1 M, and the tissues were homogenized.
Fig. 2. Pathological findings and tissue ghrelin concentrations of stomachs in GP-Tag Tg mice.

The cartridge was washed with 2.5 ml of 5% CH3CN-0.1% trifluoroacetic acid (Waters, Milford, MA) preequilibrated with 0.9% NaCl after centrifugation. The supernatant was loaded onto a Sep-Pak C18 cartridge (Waters, Milford, MA). Then, the cartridge was washed with 2.5 ml 60% CH3CN-0.1% trifluoroacetic acid and eluted with 2.5 ml 5% CH3CN-0.1% trifluoroacetic acid. The eluate was vaporized, lyophilized, and dissolved in Novex Tricine SDS Sample Buffer (Invitrogen, Carlsbad, CA). After being heated at 85°C for 2 min, 20 ng of samples of initial weight were subjected to tricine-SDS PAGE and electroblotted to polyvinylidene fluoride membranes (Invitrogen). Transferred membranes were blocked with Immunoblock (Dainippon Seiyaku, Osaka, Japan) and then incubated with anti-COOH-terminal ghrelin antibody (1:5,000). After being washed with PBS-0.1% Tween-20, membranes were reacted with secondary antibodies and developed with ECL plus (GE Healthcare, Buckinghamshire, UK) as instructed by the manufacturer. The signal on the blot was detected with Lumino-Image Analyzer LAS-3000 mini system (Fuji Photo Film, Tokyo, Japan).

Table 1. PCR primers and TaqMan probes

<table>
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<tr>
<th>Gene</th>
<th>Primer Sequence</th>
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<tr>
<td>Ghrelin</td>
<td>Sense 5′-GCATGCTCTGGATGACATG-3′</td>
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<tr>
<td></td>
<td>Antisense 5′-CTGCTGGTCTCGGATCCCT-3′</td>
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<td>TaqMan probe 5′-ACCCAGAGCCAGCCACCA-3′</td>
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<td></td>
<td>NPY Sense 5′-TCCGCTCTGGGACACACTAT-3′</td>
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<tr>
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<td>Antisense 5′-GAGGGCTTCAAGCCCTTG-3′</td>
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<td></td>
<td>TaqMan probe 5′-GAAGGCTTCTGGCATATCTCTG-3′</td>
</tr>
<tr>
<td>AgRP</td>
<td>Sense 5′-GTCCAAGAGGCGAGGCTATG-3′</td>
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<td></td>
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<td>GHRH Sense 5′-AGATGCAAGGAGCAGCTAGA-3′</td>
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<td></td>
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<td>Antisense 5′-TCCCTGGTTTACTGACATGGA-3′</td>
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NPY, neuropeptide Y; AgRP, agouti-related protein; GHRH, growth hormone(GH)-releasing hormone; GHS-R, GH secretagogue receptor; GHRH-R, GHRH receptor; PC1/3, prohormone convertase 1/3.

The supernatant was loaded onto a Sep-Pak C18 cartridge (Waters, Milford, MA) preequilibrated with 0.9% NaCl after centrifugation. The cartridge was washed with 2.5 ml of 5% CH3CN-0.1% trifluoroacetic acid and eluted with 2.5 ml 60% CH3CN-0.1% trifluoroacetic acid. The eluate was vaporized, lyophilized, and dissolved in Novex Tricine SDS Sample Buffer (Invitrogen, Carlsbad, CA). After being heated at 85°C for 2 min, 20 ng of samples of initial weight were subjected to tricine-SDS PAGE and electroblotted to polyvinylidene fluoride membranes (Invitrogen). Transferred membranes were blocked with Immunoblock (Dainippon Seiyaku, Osaka, Japan) and then incubated with anti-COOH-terminal ghrelin antibody (1:5,000). After being washed with PBS-0.1% Tween-20, membranes were reacted with secondary antibodies and developed with ECL plus (GE Healthcare, Buckinghamshire, UK) as instructed by the manufacturer. The signal on the blot was detected with Lumino-Image Analyzer LAS-3000 mini system (Fuji Photo Film, Tokyo, Japan).

Measurement of food intake. Mice were housed individually with continuous access to chow and water. Food intakes were measured by subtracting the remaining weight of the chow from that originally presented. As for measuring the food intake by ghrelin, ad libitum-fed mice were injected with ghrelin (120 or 360 μg/kg) or saline subcutaneously. Food intakes were measured for 2 h after injection.

Measurements of lean body mass, fat mass, and bone mass. Mice were anesthetized with pentobarbital sodium. Lean body mass, fat mass, and bone mass of mice were measured by an animal computed tomography system (Latheta LTC-100; Aloka, Tokyo, Japan).

Measurements of hormones and blood glucose levels. Serum GH levels were determined by a rat GH EIA kit (SPI Bio, Massy Cedex, France). Serum insulin-like growth factor I (IGF-I) levels were measured using a mouse IGF-I immunosassay kit (R & D Systems, Minneapolis, MN). Blood glucose levels were determined by glucose oxidase method using Glutest Sensor Neo (Sanwa Kagaku, Kyoto, Japan). Measurement of serum insulin concentrations was performed by ELISA using an ultrasensitive rat insulin kit (Morinaga, Yokohama, Japan).

GH- provocative test. GH- provocative test was carried out as described previously (16). Serum samples were collected at 15 min after subcutaneous injection of 180 μg/kg of GH-releasing hormone (GHRH) or 120 μg/kg of ghrelin. We choose these doses according to the results of our previous study (16).

Glucose and insulin tolerance tests. For the glucose tolerance test, after overnight fast, the mice were injected with 1.5 g/kg glucose intraperitoneally. For the insulin tolerance test, after a 4-h fast, mice were injected with 1.0 mU/g human regular insulin (Novolin R; Novo Nordisk, Bagsvaerd, Denmark) intraperitoneally. Blood was sampled from the tail vein before and 30, 60, 90, and 120 min after the injection.

Insulin release. After overnight fast, the mice were injected with 3.0 g/kg glucose intraperitoneally. Blood was sampled from the retroorbital vein at 2, 30 min after the injection using a glass tube.

Statistical analysis. All values were expressed as means ± SE. The statistical significance of the differences in mean values was assessed by repeated-measures ANOVA or Student’s t-test. The statistical difference in the changes of ghrelin levels in plasma being assessed by paired t-test. Pearson’s correlation coefficient analysis and simple regression were used to assess the relations between plasma ghrelin level and body weight. Difference of correlation coefficients of the regression lines obtained from GP-Tag Tg mice and nontransgenic littermates was determined by testing the t value.

RESULTS

Generation of GP-Tag Tg mice. By injecting transgenes into 846 eggs, we obtained 11 lines of GP (4.85) Tag Tg mice. We succeeded in breeding three of these lines (1-5, 3-1, and 4-3). Among these three lines, mice of the 3-1 line developed gastric tumor and showed elevated plasma ghrelin levels, as described below. Mice of the 1-5 line showed very aggressive tumor development and died at ~13 wk of age because of thyroid, pancreatic, and gastric tumors. Mice of the 4-3 line showed very slow tumor development. The proliferation of ghrelin cells was...
modest even at 50 wk of age in the 4-3 line. Accordingly, we analyzed mainly GP-Tag Tg mice of the 3-1 line.

We could not get a transgene-positive mouse of GP (1479) Tag Tg mouse by injecting transgenes into 631 eggs.

The expression levels of SV40-Tag mRNA among various tissues. We first examined the expression levels of SV40-Tag mRNA in various tissues of GP-Tag Tg mice, including stomach, small intestine, colon, hypothalamus, pituitary, thyroid,
pancreas, liver, heart, kidney, and testis (Fig. 1B). The highest expression levels were observed in stomach, and the second-highest levels were observed in small intestine. The expression pattern of SV40-Tag mRNA was almost similar to that of ghrelin (Fig. 1C).

Pathological feature and tissue ghrelin concentration of stomach of GP-Tag Tg mice. Stomach walls of GP-Tag Tg mice became hypertrophic with age (Fig. 2A and B). Immunohistochemical analysis by both anti-COOH-terminal and anti-NH2-terminal ghrelin antibodies revealed hyperplasia of ghrelin-immunopositive cells (Fig. 2C and D), although the staining in GP-Tag Tg mice was paler than that in nontransgenic littermates (Fig. 2C). These hyperproliferating cells were not immunostained with anti-glucagon, somatostatin, or gastrin antibodies (data not shown).

The mRNA levels of ghrelin in the stomachs of 12-wk-old male GP-Tag Tg mice were significantly lower than those of nontransgenic littermates (P < 0.01, n = 6; Fig. 2E). Consistent with this observation, tissue concentrations of ghrelin (N-RIA; fmol/mg tissue) and total ghrelin (desacyl ghrelin plus ghrelin) (C-RIA) of 12-wk-old male GP-Tag Tg mice were significantly lower than those of nontransgenic littermates (P < 0.01, n = 6; Fig. 2F). However, since the weights of the stomach of GP-Tag Tg mice were significantly higher than controls (non-Tg vs. Tg, 83.4 vs. 362.0 mg, P < 0.01) due to the hypertrophy of the stomach wall, the tissue ghrelin concentration per whole stomach tended to be higher in GP-Tag Tg mice (not significant [NS], n = 6; Fig. 2G). The size of ghrelin content of GP-Tag Tg mice was similar to that of nontransgenic littermates when analyzed by tricine-SDS PAGE and Western blot analysis (Fig. 2H), indicating that processing of preproghrelin to ghrelin occurred in hyperproliferating ghrelin cells in GP-Tag Tg mice. The mRNA of prohormone convertase 1/3, which processes preproghrelin to ghrelin, was detected in the stomachs of GP-Tag Tg mice (Fig. 2I).

Plasma ghrelin levels of GP-Tag Tg mice. Plasma ghrelin and desacyl ghrelin levels of GP-Tag Tg mice were almost equal to those of nontransgenic littermates at 9 wk of age and then increased with age (n = 3–17, Fig. 3A and B), with some variations in the levels among animals. We next examined whether physiological regulation of ghrelin secretion is preserved in GP-Tag Tg mice. Plasma ghrelin and desacyl ghrelin levels of GP-Tag Tg mice were increased by fasting and decreased by refeeding (P < 0.01, (n = 7–13; Fig. 3C and D). Plasma ghrelin and desacyl ghrelin levels of female GP-Tag Tg mice were significantly higher than those of nontransgenic littermates at 9 wk of age and then increased with age (n = 3–17, Fig. 3A and B), with some variations in the levels among animals. We next examined whether physiological regulation of ghrelin secretion is preserved in GP-Tag Tg mice. Plasma ghrelin and desacyl ghrelin levels of GP-Tag Tg mice were increased by fasting and decreased by refeeding (P < 0.01, (n = 7–13; Fig. 3C and D). Plasma ghrelin and desacyl ghrelin levels of female GP-Tag Tg mice were significantly higher than those of male GP-Tag Tg mice (P < 0.01, (n = 7–13; Fig. 3C and D). Plasma ghrelin and desacyl ghrelin levels of female GP-Tag Tg mice were significantly higher than those of male GP-Tag Tg mice (P < 0.01, (n = 7–13; Fig. 3C and D).
male GP-Tag Tg mice at 12 wk of age (Fig. 3E). Plasma ghrelin levels of 12-wk-old male GP-Tag Tg mice correlated to body weight ($r = 0.574, P < 0.05, n = 13$; Fig. 3F). The regression coefficient of the regression line of GP-Tag Tg mice was bigger than that of nontransgenic littermates ($t = 2.08, P < 0.05, n = 13$; Fig. 3F). These results indicate that regulation of plasma ghrelin and desacyl ghrelin levels of GP-Tag Tg mice were preserved, at least with regard to feeding status, body weight, and sex difference.

**Body weights, body composition, and food intake of GP-Tag Tg mice.** There was no difference in body weights between male GP-Tag Tg mice and controls until 12 wk of age ($n = 22–34$; Fig. 4A). After 13 wk of age, the body weights of the male GP-Tag Tg mice were significantly lower than those of nontransgenic littermates concomitantly with the decrease in the food intakes of male GP-Tag Tg mice after 11 wk of age (Fig. 4A and B). When the body compositions were examined by computed tomography scan, fat masses were significantly reduced in 15-wk-old male GP-Tag Tg mice ($P < 0.05, n = 7–9$; Fig. 4C), whereas lean body masses and body lengths were not changed (NS, $n = 7–9$; Fig. 4, D and E). We also examined hypothalamic mRNA levels of neuropeptide Y (NPY), agouti-related protein (AgRP), and GHS-R in 12-wk-old male GP-Tag Tg mice. No significant changes were observed in these mRNA levels (NS, $n = 7$; Fig. 4F). When 15-wk-old male GP-Tag Tg mice were injected with ghrelin, the food intake was stimulated to the same extent as in controls (NS, $n = 10–18$; Fig. 4G). Plasma leptin levels of 15-wk-old male GP-Tag Tg mice were significantly lower than controls ($P < 0.05, n = 6$; Fig. 4H).

**GH-IGF-I axis in GP-Tag Tg mice.** Serum IGF-I levels of 12- and 15-wk-old male GP-Tag Tg mice were significantly higher than those of nontransgenic littermates ($P < 0.05, n = 7–8$, and $P < 0.05, n = 6–7$, respectively; Fig. 5A). Although basal serum GH levels of 15-wk-old male GP-Tag Tg mice were not significantly different from controls, serum GH levels after GHRH injection tended to be high ($P = 0.077, n = 8–13$), which was not observed after ghrelin injection (Fig. 5B). We then investigated the effects of chronic ghrelin elevation on hypothalamic and pituitary mRNA levels of components involved in GH regulation. There were no differences in hypothalamic mRNA levels of GHRH and somatostatin or in pituitary mRNA levels of GH and GHRH receptor (GHRH-R) between 15-wk-old male GP-Tag Tg mice and their littermates.

![Fig. 4](http://ajpendo.physiology.org/)

A body weight, body composition, and food intake of GP-Tag Tg mice. A: body weights of male GP-Tag Tg mice (black bars) and nontransgenic littermates (open bars); $n = 22–34$. B: daily food intakes of male GP-Tag Tg mice (black bars) and nontransgenic littermates (open bars); $n = 19–26$. C and D: fat mass (C) and lean body mass (D) determined by animal computed tomography scan of 15-wk-old male GP-Tag Tg mice (black bars) and nontransgenic littermates (open bars). E: body length of 15-wk-old male GP-Tag Tg mice (black bars) and nontransgenic littermates (open bars); $n = 7–9$. F: hypothalamic mRNA levels of neuropeptide Y (NPY), agouti-related protein (AgRP), and growth hormone secretagogue receptor (GHS-R) in 12-wk-old male GP-Tag Tg mice (black bars) and nontransgenic littermates (open bars); $n = 7–9$. G: food intake for 2 h after injection of ghrelin (120 or 360 $\mu$g/kg or saline; $n = 10–18$). H: plasma leptin levels in 15-wk-old male Tg mice (black bars) and nontransgenic littermates (open bars); $n = 6–7$. *$P < 0.05$, **$P < 0.01$ compared with nontransgenic littermates.
Although plasma ghrelin level was elevated, pituitary GHS-R mRNA level was upregulated in gp-tag Tg mice (P < 0.05, n = 7; Fig. 5C). We also examined pituitaries of 15-wk-old male gp-tag Tg mice by immunohistochemical analysis. There were no obvious differences in somatotroph cell number or staining intensity of GH between gp-tag Tg mice and nontransgenic littermates (Fig. 5E).

**Glucose metabolism in gp-tag Tg mice.** Blood glucose levels of 15-wk-old male gp-tag Tg mice were significantly higher than controls (P < 0.05, n = 10; Fig. 6A), although those of 9-wk-old male gp-tag Tg mice were comparable with the controls (non-Tg vs. Tg: 96.0 ± 4.7 vs. 100.6 ± 4.7, P = 0.51, n = 9). Intraperitoneal glucose tolerance tests showed significantly higher blood glucose levels in 15-wk-old male gp-tag Tg mice (P < 0.05, n = 6–11; Fig. 6B). To estimate the insulin sensitivity of gp-tag Tg mice, we performed an insulin tolerance test. The blood glucose levels after insulin injection in 15-wk-old male gp-tag Tg mice were suppressed to the same level of those in controls (NS, n = 5–8; Fig. 6C). Although basal insulin levels of 15-wk-old male gp-tag Tg mice were not significantly different from those of control mice, those after glucose injection were significantly suppressed in gp-tag Tg mice (P < 0.05, n = 7–8; Fig. 6D). Pancreatic mRNA and protein levels of insulin in gp-tag Tg were comparable with those of nontransgenic littermates (NS, n = 6–8; Fig. 6, E and F).

**DISCUSSION**

In this study, we successfully established a mouse model of ghrelinoma, gp-tag Tg mouse. Gp-tag Tg mice exhibited chronic elevation of circulating ghrelin with physiological regulation. The elevation of circulating ghrelin in gp-tag Tg mice (~10-fold elevation) was much higher than that in bacterial artificial chromosome transgenic mice created by Bewick et al. (5) (only ~1.5-fold elevation). Nevertheless, the levels of circulating ghrelin in gp-tag Tg mice can be considered to be within the physiological range since the highest level of plasma ghrelin observed in the anorexia patients is about seven times higher than those of normal controls (3). One may be confused by low ghrelin mRNA levels and low ghrelin production per milligram of tissue in the stomachs of gp-tag Tg mice. In general, when the cell cycle progresses, endocrine cell produces far less amounts of hormone since the hormone production occurs mainly at the G0/G1 phase of the cell cycle. Since the hyperproliferating ghrelin-producing cells in gp-tag Tg mice were forced to proliferate by SV40 T-antigen, which suppresses RB protein and p53, promoting cell cycle progres-
sion, the amount of ghrelin production per cell was low. However, since the cell number was extremely increased, the net product by stomach was eventually elevated.

Several lines of evidence suggest that the GH-IGF-I axis is suppressed in the decreased GHS-R signaling state (28, 32). It has not yet been clear, however, whether chronic elevation of ghrelin within the physiological range could stimulate the GH-IGF-I axis. In this study, we found that adult GP-Tag Tg mice with elevated circulating ghrelin level showed elevated serum IGF-I level. Serum IGF-I level is regulated not only by GH but also by nutritional status. Malnutrition suppresses serum IGF-I level, whereas overnutrition elevates it (16). Since the nutritional state of GP-Tag Tg mice was poor because of decreased food intake, the elevated serum IGF-I levels in adult GP-Tag Tg mice were considered not to be due to overnutrition but to be due to activation of GH-IGF-I axis. Our findings indicate that desensitization of GH secretion to ghrelin or downregulation of GHS-R did not occur by chronic elevation of circulating ghrelin in GP-Tag Tg mice.

Adult GP-Tag Tg mice exhibited high glucose level in the basal state and by the glucose tolerance test. Although insulin production was not decreased in the pancreata of GP-Tag Tg mice, insulin secretion after glucose load was significantly attenuated. Since the insulin sensitivity of GP-Tag Tg mice was not reduced, the glucose intolerance in GP-Tag Tg mice was due mainly to the decreased insulin secretion. Given that GP-Tag Tg mice have gastric tumors, there is a possibility that the glucose intolerance is due to the tumors. However, the glucose intolerance observed in malignancy is due mainly to insulin resistance (8, 15), which may be evoked by cytokines (22, 24, 27). Since the glucose intolerance of GP-Tag Tg mice was caused mainly by decreased insulin secretion, it seems not to be the case. It has been reported that acute injection of ghrelin induces suppression of insulin secretion in rodents and humans (6, 30). Our findings suggest that chronic elevation of circulating ghrelin within the physiological range leads to glucose intolerance by suppressing insulin secretion.

There have been several reports regarding ghrelin-producing tumors (9, 17, 36, 37). Most of the cases did not present...
elevated plasma ghrelin levels except for a few cases. A malignant ghrelinoma case reported by Tsolakis et al. (36) showed elevated plasma ghrelin level. This patient maintained his weight despite progression of the tumor, a symptom that might be linked to the elevated ghrelin level. During the clinical course, he developed severe diabetes mellitus, which is consistent with the phenotype of GP-Tag Tg mice. GH and IGF-I levels were normal in this case. A pancreatic ghrelinoma case reported by Corbetta et al. (9) also showed normal GH and IGF-I levels despite elevated plasma ghrelin level. In contrast to these human ghrelinoma cases, GP-Tag Tg mice showed elevated IGF-I levels. The cause of the difference in the GH-IGF-I levels between our mice and these human ghrelinoma cases is unclear. Since the first case mentioned above was a malignant gastric ghrelinoma with liver metastasis, and the second case was of pancreatic origin, plasma ghrelin level might be elevated without any physiological regulation in these cases, although detailed plasma ghrelin level changes were not documented. Considering that the physiological regulation of ghrelin secretion was kept in GP-Tag Tg mice, the circadian rhythm may be needed for ghrelin to keep stimulating the GH-IGF-I axis. Indeed, several reports have shown that chronic treatment of ghrelin attenuates GH response both in vivo and in vitro (35, 39) and that in vitro treatment of pituitary with ghrelin results in decreased GHS-R mRNA levels (21). Further case studies will be required to reveal the relationship between plasma ghrelin levels and the GH-IGF-I axis in human ghrelinoma patients.

The limitation of this study is that the assessment of orexigenic action of ghrelin is difficult in this mouse model since stomach walls of GP-Tag Tg mice gradually become hypertrophic after 9 wk of age, which might affect the feeding behavior. Indeed, GP-Tag Tg mice exhibited decreased food intake and weight reduction despite the elevated plasma ghrelin levels. The hypothalamic mRNA levels of NPY and AgRP, which mediate the orexigenic action of ghrelin (7, 31), were not upregulated in GP-Tag Tg mice. There is a possibility that desensitization of GHS-R to chronic elevated ghrelin may be a cause of the lack of activation of these neurons besides the hypertrophy of the stomach wall. However, hypothalamic mRNA level of GHS-R was not changed. Furthermore, the food intake induced by acute ghrelin administration in GP-Tag Tg mice was comparable with control. These results may not support the idea of desensitization. Leptin and ghrelin have opposing effects on food intake. We examined whether plasma leptin levels of GP-Tag Tg mice were elevated as a compensation for the chronically elevated plasma ghrelin levels, which may cause anorexia. However, the leptin levels were decreased, provably reflecting the decreased fat mass of GP-Tag Tg mice.

In summary, we developed a mouse model of ghrelinoma, GP-Tag Tg mice, in which ghrelin concentrations were significantly elevated in adulthood. These GP-Tag Tg mice exhibited elevated IGF-I levels despite poor nutrition and glucose intolerance due to decreased insulin secretion. These characteristic features of this ghrelinoma mouse could be a guide to diagnose ghrelinoma.

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