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A Postweaning Reduction in Circulating Ghrelin Temporarily Alters Growth Hormone (GH) Responsiveness to GH-Releasing Hormone in Male Mice But Does Not Affect Somatic Growth

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Ghrelin was initially identified as an endogenous ligand for the GH secretagogue receptor. When administered exogenously, ghrelin stimulates GH release and food intake. Previous reports in ghrelin-null mice, which do not exhibit impaired growth nor appetite, question the physiologic role of ghrelin in the regulation of the GH/IGF-I axis. In this study, we generated a transgenic mouse that expresses human diphtheria toxin (DT) receptor (DTR) cDNA in ghrelin-secretion cells [ghrelin-promoter DTR-transgenic (GPDTR-Tg) mice]. Administration of DT to this mouse ablates ghrelin-secretion cells in a controlled manner. After injection of DT into GPDTR-Tg mice, ghrelin-secreting cells were ablated, and plasma levels of ghrelin were markedly decreased [nontransgenic littermates, 70.6 ± 10.2 fmol/ml vs. GPDTR-Tg, 5.3 ± 2.3 fmol/ml]. To elucidate the physiological roles of circulating ghrelin on GH secretion and somatic growth, 3-wk-old GPDTR-Tg mice were treated with DT twice a week for 5 wk. The GH responses to GHRH in male GPDTR-Tg mice were significantly lower than those in wild-type mice at 5 wk of age. However, those were normalized at 8 wk of age. In contrast, in female mice, there was no difference in GH response to GHRH between GPDTR-Tg mice and controls at 5 or 8 wk of age. The gender-dependent differences in response to GHRH were observed in ghrelin-ablated mice. However, GPDTR-Tg mice did not display any decreases in IGF-I levels or any growth retardation. Our results strongly suggest that circulating ghrelin does not play a crucial role in somatic growth. (*Endocrinology* 151: 1743–1750, 2010)

GH secretion is predominantly regulated by two hypothalamic peptides, one factor is GHRH and a second is somatostatin (SST). In 1999, Kojima *et al.* (1) discovered ghrelin as an endogenous ligand for the GH secretagogue receptor (GHS-R or ghrelin receptor) from rats' stomach. Ghrelin, an acylated peptide of 28 amino acids, is synthesized primarily in endocrine cells of the

stomach, named X/A-like or ghrelin cells (2). Peripheral administration of ghrelin strongly stimulates GH secretion (1, 3). Because coadministration of GHRH and ghrelin produces synergistic effects on pituitary GH release (4), circulating ghrelin may play a role in augmentation of GHRH-stimulated GH pulses. Therefore, circulating ghrelin was thought to be the third peptide which

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Abbreviations: BMD, Bone mineral density; CT, computed tomography; DT, diphtheria toxin; DTR, DT receptor; GHS-R, GH secretagogue receptor; GPDTR-Tg, ghrelin-promoter DTR-transgenic; HB-EGF, heparin-binding epidermal growth factor-like growth factor; SST, somatostatin; WT, nontransgenic littermates.

regulates GH secretion. Indeed, patients with a functional mutation in GHS-R, ghrelin receptor, display familial short stature (5). Okimura *et al.* (6), however, demonstrated that circulating ghrelin levels do not correlate with those of GH; also, administration of a GHS antagonist to freely moving rats did not reduce plasma GH levels. Ghrelin knockout mice also exhibit normal growth patterns (7). On the other hand, ghrelin receptor knockout mice exhibit modest, but significant, body weight reductions and decreased serum IGF-I levels (8). Together, these findings question the physiologic significance of ghrelin in the regulation of GH secretion. As always with such model mice, there may be confounding factors, such as developmental adaptation and other compensatory mechanisms. To avoid these factors, it may be necessary to ablate ghrelin after birth or before puberty. Moreover, during the prepubertal and pubertal period, GH-dependent proportional body growth is observed in many mammalian species. The fetal growth is GH-independent, and growth during the early postnatal is only partial dependent upon GH. Therefore, to evaluate whether an absence of circulating ghrelin can influence a somatic growth through GH/IGF-I axis modification, we think that it is appropriate to choose a postweaning model.

In this study, we adopted a diphtheria toxin (DT) receptor (DTR)-mediated conditional and targeted cell ablation strategy to ablate ghrelin secretion cells, X/A-like cell, in a specific and controlled manner (9). We generated a transgenic mouse expressing human DTR cDNA, which encodes human heparin-binding epidermal growth factor-like growth factor (HB-EGF), under the control of the transcriptional regulatory regions of ghrelin. In this mouse, ghrelin-secreting cells express the human DTR and can be ablated after the administration of a small amount of DT. By using this transgenic mouse, we ablated ghrelin-secretion cells after weaning, which allowed us to evaluate the physiologic significance of ghrelin in GH secretion and somatic growth.

Materials and Methods

All animal experiments were approved by the Kyoto University Graduate School of Medicine Committee on Animal Research. Procedures were performed in accordance with the principles and guidelines established by that committee.

Plasmid construction and generation of transgenic mice [ghrelin-promoter DTR-transgenic (GPDTR-Tg) mice]

The pGPDTR plasmid was constructed by replacement of the mouse albumin enhancer/promoter region of pMS7 (9) with a 4.1-kb *MulI-HindIII* fragment containing the 5'-flanking region of the human ghrelin gene (−4110/−33) derived from the

p-4110/−33GHRE plasmid (human ghrelin promoter in pGL3) (Fig. 1A) (10). The 6.4-kb *NotI-XhoI* fragment of pGPDTR was microinjected into the pronucleus of fertilized eggs obtained from C57/B6 mice (SLC, Shizuoka, Japan). The viable eggs were transferred into the oviducts of pseudopregnant female ICR mice (Japan CLEA, Osaka, Japan) by using standard techniques (11). Founder transgenic mice, identified by PCR analysis, were bred with C57BL/6 mice. Mice were housed in air-conditioned animal quarters, with light between 0800 and 2000 h. Except where noted, animals were fed standard rat chow (CE-2, 352 kcal/100 g; Japan CLEA) and water *ad libitum*.

Semiquantitative PCR

Total RNA was extracted using a Sepasol-RNA kit (Nacalai Tesque, Kyoto, Japan). RT used a high capacity cDNA RT kit (Applied Biosystems, Foster City, CA).

Semiquantitative PCR determined the distribution of the DTR in GPDTR-Tg mice, using the following primers: sense 5'-CCTCCTCTCGGTGCGGG-3' and antisense 5'-AGTCAC-CAGTGCCGAGAGAAGT-3'. Thirty-five cycles of thermal was performed with 94 C for 30 sec, 55 C for 30 sec, and 72 C for 30 sec. Human heart mRNA (purchased from Clontech, Palo Alto, CA) was used as a positive control.

DT injection

DT was purchased from Sigma-Aldrich Japan (Tokyo, Japan). According to the previous report using DTR-mediated cell ablation systems (9), DT was injected im.

Histological procedures

Formalin-fixed, paraffin-embedded tissue sections were immunostained using avidin-biotin peroxidase complex methods (Vectastain "ABC" Elite kit; Vector Laboratories, Burlingame, CA) as described (11). Sections were incubated overnight at 4 C with antighrelin-(1–11) antiserum that specifically recognizes acylated ghrelin (final dilution, 1:5000). Tissue sections were also stained with hematoxylin and eosine.

Measurement of plasma ghrelin levels

Measurement of plasma ghrelin levels was performed as reported previously (12). Blood samples drawn from the retro-orbital vein at 1000 h were immediately transferred to chilled siliconized glass tubes containing Na₂EDTA (1 mg/ml) and

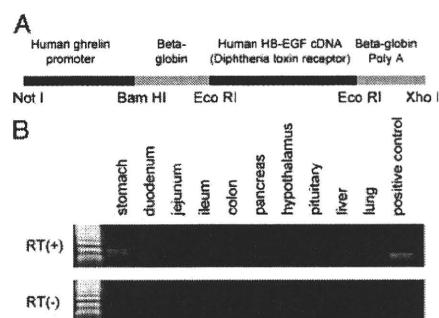


FIG. 1. Generation of GPDTR-Tg mice. A, The GPDTR-Tg construct contained a fusion gene comprised of the 5' flanking lesion of human ghrelin (4085 bp) and the DTR cDNA (human HB-EGF). B, Expression of DTR mRNA in various tissues of GPDTR-Tg mice at 8 wk of age. The human heart mRNA was used as a positive control.

TABLE 1. PCR primers and TaqMan probes

Ghrelin	Sense	5'-GCATGCTCTGGATGGACATG-3'
	Antisense	5'-TGGTGGCTTCTTGGATTCCCT-3'
	Probe	5'-AGCCAGAGCACCAGAAAGCCCA-3'
GH	Sense	5'-AAGAGTTCGAGCGTGCCTACA-3'
	Antisense	5'-GAAGCAATCCATGTTCGGTTC-3'
	Probe	5'-CCATTCAGAATGCCAGGCTGCTTTC-3'
GHRH	Sense	5'-AGGATGCAGCGACACGTAGA-3'
	Antisense	5'-TCTCCCTTGCTTGTTCATGA-3'
	Probe	5'-CCACCAACTACAGGAACTCCTGAGCCA-3'
SST	Sense	5'-AGCTGAGCAGGACGAGATGAG-3'
	Antisense	5'-ACAGGATGTGAATGTCTTCCAGTT-3'
	Probe	5'-CGAACCCAGCAATGGCACCCC-3'
IGF-1	Sense	5'-ACCCGACCTACCAAAATGAC-3'
	Antisense	5'-GGTGTGAAGACGACATGATGTGT-3'
	Probe	5'-CACCTGCAATAAAG-3'
GHS-R	Sense	5'-CACCAACCTCTATCCAGCAT-3'
	Antisense	5'-CTGACAACTGGAAGCGTTTGCA-3'
	Probe	5'-TCCGATCTGCTCATCTTCTGTGCATG-3'

aprotinin (1000 KIU/ml; Ohkura Pharmaceutical, Kyoto, Japan). After centrifugation at 4 C to separate out the plasma, hydrochloric acid was added to samples at a final concentration of 0.1 N. Plasma was immediately frozen and stored at -80 C until assayed. Plasma ghrelin concentrations were determined using a ghrelin ELISA kit (Mitsubishi Kagaku Iatron, Tokyo, Japan).

Real-time PCR analysis

Extraction of total RNA from various tissues and RT was performed as described above. Real-time quantitative PCR used an ABI PRISM 7500 Sequence Detection System (Applied Biosystems) using the primers and TaqMan probes described in Table 1. The mRNA expression levels of each gene were normalized to that of 18S rRNA. All samples were examined in triplicate in 96-well plates using an ABI Prism 7500 sequence detector according to the manufacturer's protocol.

GH provocative test

GH provocative test was carried out as previously described (12). These experiments were conducted in unanesthetized mice. Human GHRH was purchased from Sumitomo Pharmaceuticals Co., Ltd. (Osaka, Japan). Serum samples were collected at 15 and 30 min after sc injection of 180 mcg/kg of GHRH.

Ghrelin-rescue experiments

Osmotic infusion pumps (Alzet Micro-Osmotic pump, Model 1002; Durect Corp., Cupertino, CA) were implanted sc in 3-wk-old male GPDTR-Tg mice. Ghrelin (60 mcg/kg·d; Peptide Institute, Osaka, Japan) or saline was continuously infused through the osmotic infusion pumps. Then mice were started to treat with DT (50 ng/kg twice a week) a day after pump implantation. The average plasma ghrelin levels during continuous infusion of ghrelin were 31.6 ± 5.3 fmol/ml in the DT-treated GPDTR-Tg mice, whereas those without ghrelin infusion were 1.7 ± 0.2 fmol/ml. GH provocative test were carried out in these mice at the age of 5 wk.

Measurement of serum GH and IGF-I levels

Blood samples were collected from the tail veins of mice. Serum was isolated by centrifugation and stored at -20 C until

assayed. Serum GH levels and IGF-I levels were measured using the appropriate EIA kits from SPI-BIO (Bonde, France) and Diagnostic Systems Laboratories, Inc. (Webster, TX), respectively, according to the manufacturers' instructions.

Measurement of body lengths

Mouse body length was measured by manual immobilization and extension of mice to determine nose-to-anus length. All measurements were performed by the same individual in a blind fashion.

Measurement of fat mass and bone mineral density (BMD)

The fat mass (% fat) and BMD of mice were measured by computed tomography (CT) (Laboratory CT; Lacita, Aloka, Japan) under pentobarbital anesthesia.

Statistical analysis

Results are expressed as the means \pm SEM. Multiple comparisons between groups were made by Turkey-Krammer test, with α set at $P < 0.05$. The results on body weight and serum GH levels after GHRH injection were analyzed by a two-way ANOVA followed by Tukey's *post hoc* test, with α set at $P < 0.05$. Statistical analyses were carried out with STATVIE 4.0 software (Abacus Concepts, Inc., Berkeley, CA).

Results

Generation of transgenic mice in which ghrelin can be ablated in a controlled manner

Transgenic mice

To elucidate physiologic role of ghrelin in GH secretion and somatic growth, we developed transgenic mice in which ghrelin can be ablated in controlled manner. We adopted a DTR-mediated conditional and targeted cell ablation strategy. We created transgenic mice that expressed the gene for the human DTR, human HB-EGF,

under the control of the ghrelin promoter. By injecting transgenes into 184 eggs, we obtained three lines of transgenic mouse (Tg 1-2, Tg 5-1, and Tg 5-8). We continued with the Tg 5-1 transgenic line, because Tg 1-2 animals did not exhibit decreases in plasma ghrelin levels after injection of high-dose DT and Tg 5-8 required high doses of DT (50 mcg/kg) to ablate ghrelin-producing cells (data not shown). In Tg 5-1 transgenic animals, semiquantitative PCR analysis revealed high expression of DTR mRNA in the stomach and weak expression in the duodenum and jejunum. No expression, however, could be detected in the ileum, colon, pancreas, hypothalamus, pituitary, liver, or lung (Fig. 1B). In Tg 5-1 mice, the ghrelin-producing cells of the stomach were ablated by injection with low-dose DT (10 or 50 ng/kg) (Fig. 2, A, B, and D). We therefore designated the Tg 5-1 transgenic line and nontransgenic littermates as GPDTR-Tg mice and wild-type (WT) mice, respectively.

Ablation of ghrelin-producing cell

To determine the dose and timeframe of DT injection, preliminary studies were performed: GPDTR-Tg mice were injected with saline or DT twice a week at a dose of 10, 30, 50, 100, and 500 ng/kg (on d 0 and 2). Plasma ghrelin levels on d 4 were decreased to approximately 60, 30, 5, 5, and 5% of control mice (Tg mice treated with saline) after 10, 30, 50, 100, and 500 ng/kg of DT injection, respectively. Thus, we judged that 50 ng/kg of DT is the smallest effective dose to reduce plasma ghrelin. The final results using 10 and 50 ng/kg of DT were described below. Next, GPDTR-Tg mice were injected with 50 ng/kg of DT with four schedules: once a week (on d 0), twice a week (on d 0 and 2), three times a week (on d 0, 2, and 4), or daily (from d 0 to 6), and plasma ghrelin levels were measured on d 7. The once-a-week injection of DT was insufficient, but the twice-a-week injection of DT had enough effect on reduction in plasma ghrelin concentration.

To ablate ghrelin-producing cells, 8-wk-old male WT and GPDTR-Tg mice were injected im with 10 or 50 ng/kg DT daily on d 0 and 2 and analyzed on d 4. WT mice treated with saline or DT and GPDTR-Tg mice treated with saline were used as control mice.

To evaluate the effects of DT injection on ghrelin-producing cell, we analyzed stomach by immunohistochemical analysis with antighrelin antisera (Fig. 2A) and real-time PCR (Fig. 2, B and C). DT injection reduced in a dose-dependent manner both the number of ghrelin-positive cells and the expression of ghrelin mRNA in the stomach of GPDTR-Tg mice (Fig. 2, A and B). DT injection did not produce in any abnormalities in WT mice, because

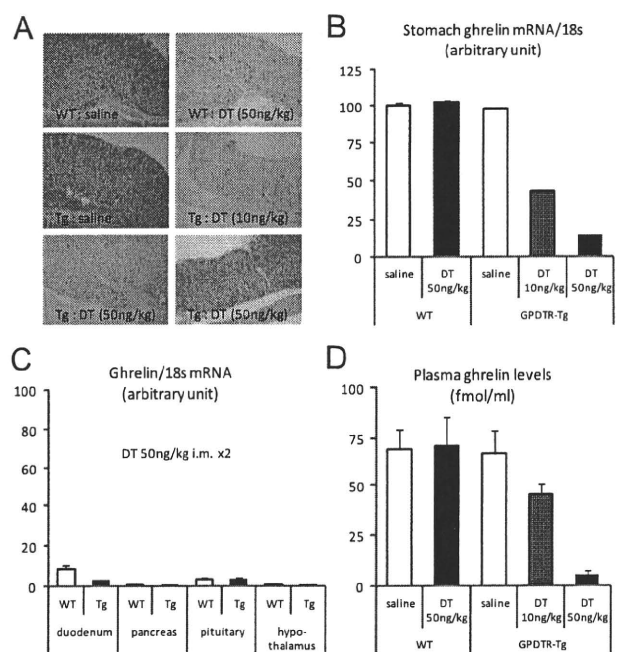


FIG. 2. Ablation of ghrelin-secretion cells. Eight-week-old male GPDTR-Tg mice (Tg) and nontransgenic littermates (WT) were injected with saline or 10 or 50 ng/kg of DT (im) on d 0 and 2, then analyzed on d 4. A, Histological analysis of stomach sections. Immunohistochemical analysis of ghrelin peptide expression and hematoxylin and eosin staining. Original magnification, $\times 100$. B, Ghrelin mRNA levels in the stomach. C, Ghrelin mRNA levels in the duodenum, pancreas, pituitary, and hypothalamus of GPDTR-Tg and WT mice injected with 50 ng/kg of DT. D, Plasma ghrelin levels in GPDTR-Tg and WT mice. For B–D, data represent the means \pm SEM ($n = 8$).

these mice do not possess the DTR, making them insensitive to DT. In transgenic animals, DT injection also reduced ghrelin mRNA expression in the duodenum, but not the pancreas, pituitary, or hypothalamus (Fig. 2C). Plasma ghrelin levels in GPDTR-Tg mice treated with 10 and 50 ng/kg of DT were decreased to approximately 60 and 5–7% of control mice, respectively (Fig. 2D). These results suggested that this transgenic mouse model is a useful tool for evaluating the physiologic role of circulating ghrelin.

Histological analysis with hematoxylin and eosin staining revealed that no inflammatory cell infiltration was seen in the stomach (Fig. 2A), small intestine, colon, pancreas, pituitary, and hypothalamus of the GPDTR-Tg mice with 50 ng/kg of DT injection. Other historical abnormalities were also not observed in these tissues (data not shown).

The effects of a reduction in circulating ghrelin after weaning on the GH/IGF-I axis and somatic growth

To study the effects of postweaning reductions in circulating ghrelin on the GH/IGF-I axis and somatic growth, 3-wk-old WT and GPDTR-Tg mice were treated with DT

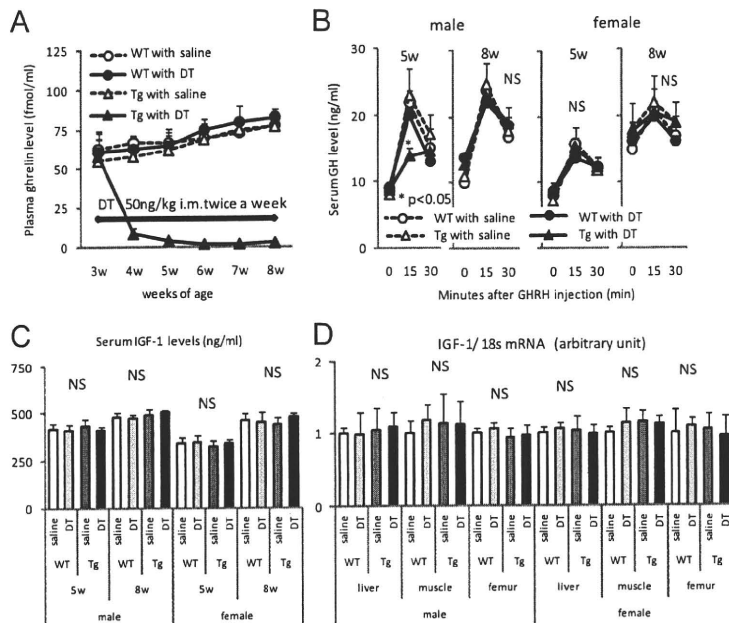


FIG. 3. The effects of a postweaning reduction in circulating ghrelin on the GH/IGF-1 axis. Three-week-old GPDTR-Tg and WT mice were injected saline or DT at a dose of 50 ng/kg twice a week for 5 wk (from 3 to 8 wk old). A, Plasma ghrelin levels before and after DT injection. B, GH response to GHRH administration (180 μ g/kg sc) in GPDTR-Tg and WT mice at 5 and 8 wk of age. C, Serum IGF-I levels of GPDTR-Tg and WT mice at 5 and 8 wk of age. D, IGF-I mRNA levels in liver, skeletal muscle, and femur in GPDTR-Tg and WT mice at 5 wk of age. Data represent the means \pm SEM (n = 12).

(50 ng/kg) or saline twice a week for 5 wk (from 3 to 8 wk old). After DT injection, plasma ghrelin levels of GPDTR-Tg mice decreased rapidly. In GPDTR-Tg mice, ghrelin levels were undetectable by 5 wk of age, remaining so thereafter (Fig. 3A). The data obtained from GPDTR-Tg mice were compared with those from three groups of control mice (WT with saline, WT with DT, and GPDTR-Tg with saline).

To elucidate whether a postweaning reduction in circulating ghrelin can influence GH secretion, we measured basal serum GH levels and performed GH provocative test with GHRH. There were no differences in basal serum GH levels between GPDTR-Tg mice treated with DT and control mice in either males or females at 5 or 8 wk of age. GH provocative test with GHRH showed some intriguing results (Fig. 3B). The GH responses to GHRH in male GPDTR-Tg mice treated DT were significantly lower than those in three controls at 5 wk of age. However, those responses were normalized at 8 wk of age. On the other hand, there were no differences in GH response to GHRH among four groups (WT with saline or DT, and Tg with saline or DT) in females at 5 or 8 wk of age.

To elucidate whether temporarily attenuation of GH responses to GHRH can affect IGF-I regulation, we investigated serum IGF-I levels and IGF-I mRNA expres-

sions in the liver, skeletal muscle, and distal femur. There were no differences in serum IGF-I levels among any animal groups in either males or females at 5 or 8 wk of age (Fig. 3C). There were also no differences in IGF-I mRNA expressions in the liver, skeletal muscle, or distal femur among any animal groups at 5 wk of age (Fig. 3D). We then investigated the effects of decreases in circulating ghrelin on the expression of mRNA encoding GHRH and SST within the hypothalamus and encoding GH and GHS-R in the pituitary. There were no differences in mRNA expression levels of these mediators among any animal groups in male and female at 5 wk of age (Fig. 4).

As expected from the results of the IGF-I studies, no evidence of growth retardation could be found in either male or female GPDTR-Tg mice treated with DT during the observation period. There were no difference in body weight or length in comparison with three groups of control mice at any point (Fig. 5, A and B, for male; and Fig. 5, D and E, for female animals). CT analysis of body composition demonstrated that there were no differences in percent fat or BMD among any animal groups at 5 and 8 wk of age (Fig. 5C for male, and Fig. 5F for female animals).

There were no differences in weekly food intake from 3 to 8 wk of age [WT *vs.* GPDTR-Tg (treated with DT); male, 18.4 \pm 0.5 *vs.* 18.9 \pm 0.7; female, 18.4 \pm 1.0 *vs.* 18.5 \pm 0.6 (g/wk)]. These results suggested that although GH responses to GHRH were temporarily reduced under conditions of decrease in circulating ghrelin, somatic growth was not impaired.

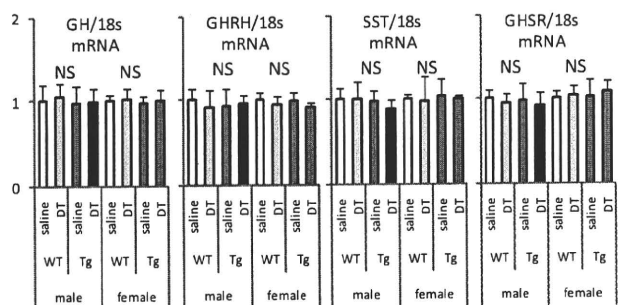


FIG. 4. The effects of a postweaning reduction in circulating ghrelin on the expression of mRNA encoding GHRH, SST, GH and GHS-R. Three-week-old GPDTR-Tg and WT mice were injected saline or DT at a dose of 50 ng/kg twice a week for 5 wk (from 3 to 8 wk old). Pituitary mRNA levels of GH and GHS-R and hypothalamic mRNA levels of GHRH and SST in GPDTR-Tg and WT mice at 5 wk of age. Data represent the means \pm SEM (n = 12).

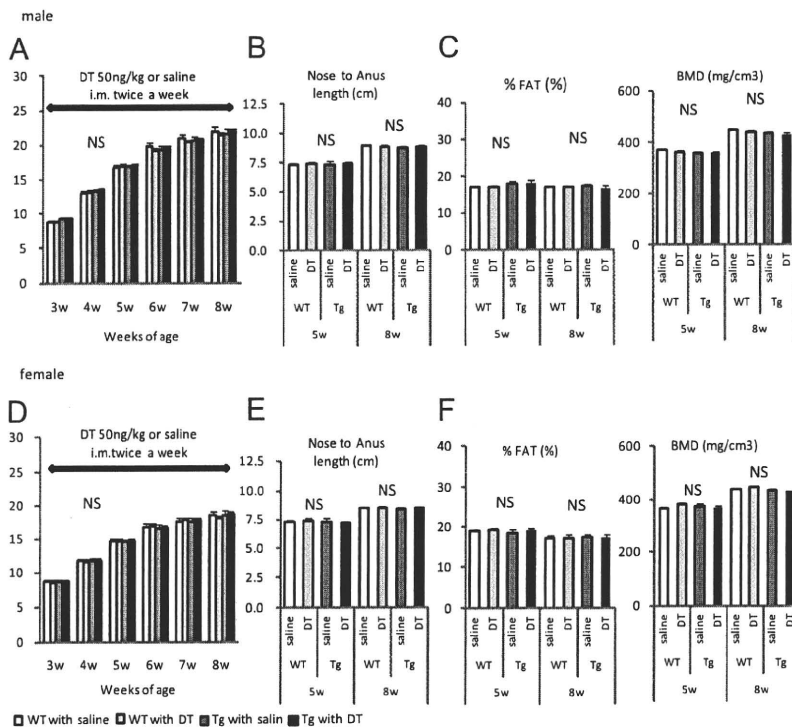


FIG. 5. The effects of a postweaning reduction in circulating ghrelin levels on somatic growth. Three-week-old GPDTR-Tg and WT mice were injected saline or DT at a dose of 50 ng/kg twice a week for 5 wk (from 3 to 8 wk old). A and D, Changes in body weight in male (A) and female mice (D). B and E, Nose to anus length in male (B) and in female mice (E) at 5 and 8 wk of age. C and F, Body composition (% Fat) and BMD as analyzed by CT in male (C) and in female mice (F) at 5 and 8 wk of age. Data represent the means \pm SEM ($n = 12$).

GH response to GHRH in the ghrelin-rescued GPDTR-Tg mice

To elucidate whether GH responsiveness to GHRH can be ameliorated by ghrelin replacement in the ghrelin-ablated mice, GH provocative test were carried out in the DT-treated GPDTR-Tg mice whose circulating ghrelin were rescued by continuously administration of ghrelin with osmotic pump. The average plasma ghrelin levels during continuous infusion of ghrelin were 31.6 ± 5.3 fmol/ml in the DT-treated GPDTR-Tg mice, whereas those without ghrelin infusion were 1.7 ± 0.2 fmol/ml. GH provocative test were carried out at the age of 5 wk.

GH responsiveness to GHRH was ameliorated by ghrelin replacement. Serum GH levels at 0, 15, and 30 min after GHRH administration in the ghrelin-rescued mice were 7.8 ± 1.6 , 26.2 ± 4.2 , and 12.3 ± 0.8 ng/ml, respectively, whereas those in mice without ghrelin replacement were 6.8 ± 1.5 , 10.9 ± 2.6 , and 11.3 ± 1.6 ng/ml, respectively. These results suggested that attenuated response to GHRH seen in ghrelin-ablated mice without ghrelin replacement was due to acute ghrelin deficiency.

Discussion

In this study, we generated transgenic mice expressing the DTR driven by the transcriptional regulatory machinery of ghrelin. Injection of DT into this mouse can ablate ghrelin-secreting cells. Approximately 70–80% of circulating ghrelin originates from the stomach (13). Ghrelin-producing cells are also found throughout the small intestine, with the duodenum producing approximately one-tenth that of the stomach (14). Semiquantitative PCR revealed that DTR was only expressed in stomach and not in pituitary, hypothalamus, and pancreas and the intensity of the band of DTR in stomach was very low. Three possibilities might be considered to explain this result. The first is the low efficiency of gene transfection. Three lines of GPDTR-Tg mice that we generated in this study were inserted with low copy numbers of transgene (DTR cDNA). Thus, the expression levels of DTR mRNA could be very low even in stomach. The second is the efficiency of gene expression. In this study, we designed a fusion gene comprising the 4085-bp fragment contained a partial sequence of the 5'-flanking region of the human

ghrelin gene and human DTR. The efficiency of gene expression driven by this fragment might be lower than those driven by the original ghrelin promoter region. The last, except gastrointestinal tract, transcription of ghrelin gene might be driven by a different size of fragment of the 5'-flanking region. Immunohistochemical and PCR analyses demonstrated that ghrelin-secreting cells in the stomach and duodenum were ablated after DT injection into GPDTR-Tg mice, resulting in marked reduction of plasma ghrelin levels. In contrast, ghrelin-producing cells of the pituitary and hypothalamus were unaffected. Thus, this transgenic mouse is a useful model to explore the role of circulating ghrelin, because plasma ghrelin levels can be abrogated in a controlled manner without altering pituitary and hypothalamic ghrelin mRNA expression levels.

The physiologic roles of ghrelin in the regulation of GH secretion remain unclear, because previous reports using rodents deficient or reduced in ghrelin signals have given conflicting results (7, 8, 15, 16). Sun *et al.* (7) reported that ghrelin-deficient mice did not exhibit any growth retardation or decreases in serum IGF-I levels. Wortley *et al.*

(15) also were unable to observe any significant differences between ghrelin-deficient mice and WT mice in body weight or basal serum GH levels, when fed a standard diet. Moreover, Zigman *et al.* (16) demonstrated there was no significant difference in serum IGF-I levels between ghrelin receptor knockout and WT mice. Sun *et al.* (8), however, showed that ghrelin receptor knockout mice exhibited only a small reduction in body weight and serum IGF-I levels. In addition, Pantel *et al.* (5) showed that two unrelated families with short stature have a missense mutation of GHS-R. This mutation impairs the constitutive activity of the GHS-R. They also reported a young patient with growth delay who has a recessive partial isolated GH deficiency due to GHS-R mutations (17). These results indicate importance of ghrelin/GHS-R signals in GH secretion and somatic growth.

The purpose of this study is to evaluate whether an absence of circulating ghrelin can influence GH secretion and somatic growth via GH/IGF-I axis in mammals. First, we investigated basal serum GH levels and the GH response to GHRH. Although basal serum GH levels in the ghrelin-abrogated mice did not differ from those seen in WT mice, the GH responses to GHRH in male GPDTR-Tg mice were significantly lower than those in WT mice at 5 wk of age. As coadministration of GHRH and ghrelin produces synergistic effects on pituitary GH release (4), circulating ghrelin may play a role in augmentation of GHRH-stimulated GH pulses. Indeed, GH responsiveness to GHRH was ameliorated by ghrelin replacement in the ghrelin-ablated mice. However, the attenuated response to GHRH in the ghrelin-ablated mice had persisted only for a short term. The GH responses to GHRH in male GPDTR-Tg mice were recovered and were not different from those in WT mice at 8 wk of age. It is possible that an adaptation to reduced circulating ghrelin occurred within a short term. Indeed, Popovic *et al.* (18) reported that 10 patients who underwent total-gastrectomy at least 2 yr ago, a state of acquired chronic hypoghrelinemia, exhibited normal GH response to GHRH compared with normal subjects. Meanwhile, in female mice, there were no differences in either basal serum GH levels or GH response to GHRH between WT and GPDTR-Tg mice at 5 or 8 wk of age. The secretory pattern of GH in rodents is sexually differentiated. In male rats, GH is secreted in episodic pattern with low levels between pulses, whereas in females, the pulses are lower and plasma GH levels between pulses are higher than males (19). The secretory pattern of GH differs between male and female by 30 d of age (20). Gonadal steroids are thought to produce the sexual differences in GH secretion. We assumed that the sexual differences in GH response to GHRH in ghrelin-ablated mice may depend on gonadal steroids.

As GH secretion is pulsatile in nature, a single measurement of GH concentration in blood would not adequately reflect endogenous GH secretion. To estimate the amplitude and frequency of GH pulses, short-interval blood sampling under a conscious state is required. Such studies are difficult to perform in mice. Instead, we investigated serum IGF-I levels, skeletal muscle IGF-I mRNA expression, and anthropometric parameters that reflect pulsatile GH release under similar nutritional conditions (21). Serum IGF-I levels and IGF-I mRNA expression in skeletal muscle did not decrease in the ghrelin-abrogated mice in comparison with WT mice. These results suggest that circulating ghrelin does not play a dominant role in the GH/IGF-I axis. Due to significant differences between species in the regulation of GH secretion (21), we have to give careful considerations to apply the results of animal experiments concerning GH secretion directly to humans; insulin-induced hypoglycemia is a potent stimulus of GH secretion in humans, whereas rats respond to the stress of hypoglycemia by decreasing GH secretion (22, 23). L-arginine is a potent GH secretagogue in humans, but does not (or less overtly) stimulate GH secretion in rats (21, 24).

Somatic growth is affected not only by GH and IGF-I but also by thyroid hormones, sex steroids, and glucocorticoids. It also depends on genetic background and nutrition. Adequate nutrition is one of the most important factors affecting somatic growth. In present study, there were no differences in food intake between the ghrelin-abrogated mice and WT mice. Body weight, length, and body composition also were not influenced by plasma ghrelin levels. These results suggest that circulating ghrelin does not play a dominant role in somatic growth.

We cannot exclude the possibility that hypothalamic ghrelin may regulate GH secretion, as hypothalamic ghrelin-secreting cells were preserved in this animal model. Shuto *et al.* (25) demonstrated that transgenic rats expressing antisense GHS-R mRNA within the arcuate nucleus of the hypothalamus displayed growth retardation, suggesting that ghrelin/GHS-R systems in the hypothalamus function in the regulation of GH. Further studies will be needed to elucidate the role of hypothalamic ghrelin in GH secretion.

In summary, we have succeeded in generating transgenic mice in which circulating ghrelin can be abrogated in a controlled manner after birth. Our results suggest that circulating ghrelin does not play a crucial role in somatic growth.

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Establishment of a Novel Ghrelin-Producing Cell Line

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To establish a tool to study ghrelin production and secretion *in vitro*, we developed a novel ghrelin-producing cell line, MGN3-1 (mouse ghrelinoma 3-1) cells from a gastric ghrelin-producing cell tumor derived from ghrelin-promoter Simian virus 40-T-antigen transgenic mice. MGN3-1 cells preserve three essential characteristics required for the *in vitro* tool for ghrelin research. First, MGN3-1 cells produce a substantial amount of ghrelin at levels approximately 5000 times higher than that observed in TT cells. Second, MGN3-1 cell expressed two key enzymes for acyl modification and maturation of ghrelin, namely ghrelin O-acyltransferase for acylation and prohormone convertase 1/3 for maturation and the physiological acyl modification and maturation of ghrelin were confirmed. Third, MGN3-1 cells retain physiological regulation of ghrelin secretion, at least in regard to the suppression by somatostatin and insulin, which is well established in *in vivo* studies. Thus, MGN3-1 cells are the first cell line derived from a gastric ghrelin-producing cell preserving secretion of substantial amounts of ghrelin under physiological regulation. This cell line will be a useful tool for both studying the production and secretion of ghrelin and screening of ghrelin-modulating drugs. (*Endocrinology* 151: 2940–2945, 2010)

Ghrelin, a stomach-derived hormone, is composed of 28 amino acid residues with unique acyl-modification (1). Plasma ghrelin levels are regulated by acute and chronic energy status: serum levels are increased during a postprandial period and decreased after refeeding (2, 3). Ghrelin levels are low in obese individuals and high in lean people (4, 5). The direct factors regulating ghrelin secretion from ghrelin-producing cells (X/A-like cells), however, are not fully understood.

One method to investigate the direct effect of a specific factor on ghrelin secretion is to use a ghrelin-producing cell line. Ghrelin production has been reported by several cell lines, including TT (6), HL-60, THP-1, SupT1 (7), and HELL (8) cells. All of these cell lines differ completely from endogenous ghrelin-producing cells. TT cells originate from human thyroid medullary cancer, whereas HL-60, SupT1, and HELL cells are leukocyte in origin, with HELL

being erythroleukemic line. Although these cell lines may be used to study ghrelin production (9), they are not ideal tools to study the regulation of ghrelin secretion. Therefore, establishment of a cell line originating from ghrelin-producing cells of the stomach would be useful for studying ghrelin production and secretion. Furthermore, it is vital to establish such cell line for studying factors directly affecting ghrelin production and secretion.

In this study, we established a ghrelin-producing cell line from a gastric tumor derived from ghrelin-promoter-Simian virus 40 T-antigen transgenic (GP-Tag Tg) mice.

Materials and Methods

Animals

GP-Tag Tg mice were generated as described previously (10). KSN nude mice were purchased from Shimizu Laboratory Supplies (Kyoto, Japan). Animals were maintained on

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Abbreviations: AA, Amino acid; FBS, fetal bovine serum; GOAT, ghrelin O-acyltransferase; GP-Tag Tg, ghrelin-promoter-Simian virus 40 T-antigen transgenic; KRB, Krebs-Ringer buffer; PC, prohormone convertase; siRNA, small interfering RNA.

standard rodent food (CE-2, 352 kcal/100 g; Japan CLEA, Tokyo, Japan) on a 12-h light, 12-h dark cycle unless otherwise indicated. All experimental procedures were approved by the Kyoto University Graduate School of Medicine Committee on Animal Research.

Cell culture

A gastric tumor resected from a GP-Tag Tg mouse was minced and digested with the combination of 1.5 mg/ml collagenase type I (Sigma-Aldrich, St. Louis, MO) and 0.5 mg/ml dispase (Roche, Basel, Switzerland) in DMEM (11995-065; Life Technologies, Inc., Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) at 37 C for 90 min. After washing with PBS, tumor cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μ g/ml streptomycin at 37 C in 10% CO₂. Stromal cells were diminished by serial passage of tumor cells into new dishes 2–3 h after seeding the cells to the first dishes. After several passages of cells in 3-d intervals, the cells were cloned by dilution cloning onto a feeder layer of mitomycin-C-treated embryonic fibroblasts in 96-well microplates.

TT cells were cultured in Ham's F-12K supplemented with 10% FBS at 37 C in 5% CO₂ as described previously (6).

Immunocytochemistry

Cells were cultured in a chamber slide system (Nulge Nunc, Rochester, NY) and then fixed with 10% formalin for 15 min. Formalin-fixed slides were immunostained using the avidin-biotin peroxidase complex method (Vectastain ABC Elite kit; Vector Laboratories, Burlingame, CA) as described previously (11). Slides were incubated with anti-carboxy (C)-terminal ghrelin (amino acid, AA: 13–28) (12) (1:2000 at final dilution), which detects both ghrelin and desacyl-ghrelin, amino N-terminal ghrelin (12) that recognizes the *n*-octanoylated portion of ghrelin (AA: 1–11; 1:5000), antiglucagon (1:500) (Dako, Glostrup, Denmark), antisomatostatin (1:500) (Dako), and antigastrin (1:500) (Dako).

Electron microscope

Electron microscope study was performed as described previously (13). Cell pellets were fixed with 1% glutaraldehyde at 4 C for 2 h. After washing in phosphate buffer, samples were postfixed with 2% OsO₄ at 4 C for 2 h dehydrated with ethanol and embedded in Quetol 812 (Nisshin EM, Tokyo, Japan). Ultrathin sections of samples were cut, stained with uranyl acetate for 15 min followed by lead acetate for 5 min, and then viewed with an H-300 electron microscope (Hitachi, Tokyo, Japan).

Measurements of ghrelin concentrations in cells and culture medium

Cells were detached from dishes in enzyme-free cell dissociation buffer (Life Technologies). After centrifugation, cells were dissolved in PBS and boiled for 5 min. Acetic acid was added to each solution to a final concentration of 1 M. After needle shearing and centrifugation, the cell supernatants were applied to Sep-Pak C18 cartridges (Waters Corp., Milford, MA) preequilibrated with 0.9% saline. Cartridges were washed in saline and 5% CH₃CN/0.1% trifluoroacetic acid and eluted with 60% CH₃CN/0.1% trifluoroacetic acid. Elu-

ates were lyophilized and subjected to ghrelin RIA. To measure ghrelin concentrations in culture medium, the collected culture media were centrifuged, and the resulting supernatants were immediately applied to Sep-Pak C18 cartridges and processed as described above. RIAs were performed using anti-C-terminal ghrelin (AA: 13–28) antiserum (C-RIA), which detects both ghrelin and desacyl-ghrelin, and anti-N-terminal ghrelin (AA: 1–11) antiserum (N-RIA), which detects ghrelin only, as described previously (12, 14).

RT-PCR and 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 transcriptase kit (Applied Biosystems, Foster City, CA). RT-PCR was performed using a GeneAmp 9700 cyclor (Applied Biosystems) with AmpliTaqGold using appropriate primers (Supplemental Table 1 published on The Endocrine Society's Journals Online web site at <http://endo.endojournals.org>). Real-time quantitative PCR was performed using an ABI PRISM 7500 sequence detection system (Applied Biosystems) using appropriate primers and TaqMan probes or with Power SybrGreen (Supplemental Table 1). The mRNA expression of each gene was normalized to levels of 18S rRNA.

Western blotting

The molecular size of ghrelin in the medium was determined by tricine SDS-PAGE and Western blot analysis as described previously (10). MGN3-1 cells were seeded in 10-cm dishes (5.0 \times 10⁶ cells/dish). Culture media were collected after a 3-d incubation and subjected to HPLC purification and lyophilization as described above. Tricine SDS-PAGE and Western blot analysis were performed as described previously using anti-COOH-terminal ghrelin antibody (1:5000) (10).

Reverse-phase HPLC

MGN3-1 cells were seeded in a six-well dish (5 \times 10⁵ cells/well). After a washing in PBS, cells were incubated at 37 C for 4 h in DMEM supplemented with 0.5% BSA. Culture medium was collected and subjected to reverse-phase HPLC as described previously (6).

Small interfering RNA (siRNA)

Synthetic siRNAs and a negative control were purchased from Invitrogen (Carlsbad, CA). Two types of siRNAs specific for ghrelin *O*-acyltransferase (GOAT) were used: GCGCUUCUGUUAAUUAUCUCUGCA (si1) and AGGAAGUCCAUAGCUGACCUUCUU (si2). siRNAs were delivered into MGN3-1 cells using Lipofectamine RNAi Max (Invitrogen) according to the protocol provided by the manufacturer. The medium was changed after a 24-h incubation with siRNA. One mM of octanoic acid was added to the medium. Ghrelin levels in the media were measured after additional 6-d incubation.

Transplantation of MGN3-1 cells in nude mice

Eight-week-old male KSN nude mice were sc injected with 1.0 \times 10⁷ of MGN3-1 cells dissolved in PBS. Mice were housed individually with continuous access to chow and water. Food intake was measured by subtracting the remaining weight of the chow from that originally presented.

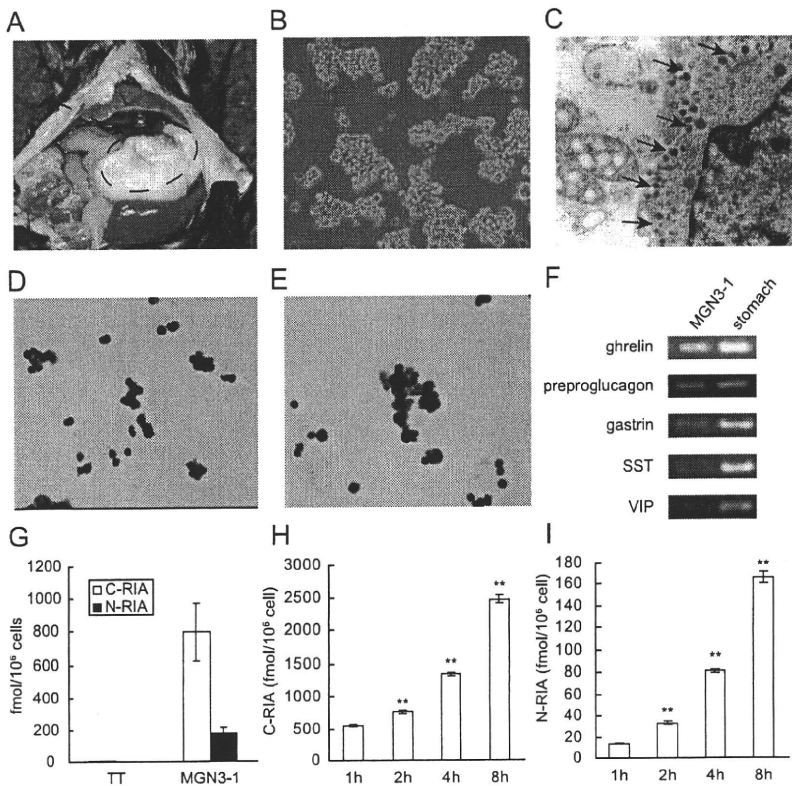


FIG. 1. Establishment of MGN3-1 cells. A, Macroscopic findings of a ghrelinoma in a GP-Tag Tg mouse. B and C, Morphology of MGN3-1 cells by optic (B) and electron (C) microscopy. Secretory granules were observed (arrow). D and E, MGN3-1 cells were immunostained with anti-C-terminal ghrelin (D) and anti-N-terminal (E) ghrelin antibodies. F, RT-PCR analysis of the expression of mRNAs encoding gastric hormones in MGN3-1 cells. SST, Somatostatin; VIP, vasoactive intestinal polypeptide. G, Ghrelin peptide content determined by C-RIA and N-RIA in TT and MGN3-1 cells. H and I, Time course changes of ghrelin levels in the medium in which MGN3-1 cells were incubated. **, $P < 0.01$ compared with 1 h ($n = 9$).

Measurements of plasma ghrelin concentrations

Plasma samples were collected as reported previously (10). Plasma ghrelin and desacyl ghrelin concentrations were determined using an active ghrelin ELISA kit that recognizes *n*-octanoylated ghrelin and a desacyl ghrelin ELISA kit (both from Mitsubishi Kagaku Iatron, Tokyo, Japan), respectively (15).

Batch incubation study

MGN3-1 cells were seeded and cultured overnight in 12-well plates (7.5×10^5 cells/well). After a washing in PBS, cells were incubated at 37°C for 4 h in DMEM or Krebs-Ringer buffer (KRB) supplemented with 0.5% BSA and the indicated additional reagents (octanoic acid, somatostatin, or insulin) before collecting supernatants. Ghrelin concentrations in the supernatant were measured by RIA as described above. To determine the expression levels of ghrelin and GOAT mRNA, cells were incubated at 37°C for 24 h in DMEM supplemented with 0.5% BSA and the indicated additional reagents.

Statistical analysis

All values were expressed as the means \pm SE. The statistical significance of the differences in mean values was assessed by ANOVA with a *post hoc* test (Tukey's test) or Student's *t* test as

appropriate. Difference with $P < 0.05$ was considered significant.

Results

Establishment of MGN3-1 cell line

GP-Tag Tg mice (10) develop gastric tumors (Fig. 1A), which produce and secrete ghrelin and preserving the physiological regulation, at least by feeding status, sex difference, and body weights *in vivo*. We established a cell line, MGN3-1 cell, from a gastric tumor derived from a GP-Tag Tg mouse (Fig. 1A). MGN3-1 cells formed round-shaped aggregates that stuck together with moderate adhesion to culture dishes (Fig. 1B). These cells contained secretory granules when observed by electron microscopy (Fig. 1C). MGN3-1 cells exhibited ghrelin-like immunoreactivity by immunocytochemistry using anti-N-terminal, which recognizes ghrelin only, and anti-C-terminal ghrelin, which recognizes both ghrelin and desacyl ghrelin, antibodies (Fig. 1, D and E). We found the production of ghrelin mRNA by MGN3-1 cells using RT-PCR (Fig. 1F). This method also detected low levels of preproglucagon and gastrin mRNA (Fig. 1F) in MGN3-1 cells, whereas immunostaining with antiglu-

cagon and antigastrin antibodies could not detect any expression of these proteins (data not shown). No expression of somatostatin and vasoactive intestinal polypeptide mRNA was observed in MGN3-1 cell (Fig. 1F). MGN3-1 cells contained approximately 140-fold higher levels of total ghrelin (*n*-octanoylated ghrelin plus desacyl ghrelin) measured by C-RIA and approximately 5000-fold higher levels of ghrelin measured by N-RIA than those observed in TT cells (Fig. 1G). The ghrelin levels in the medium were increased time dependently when MGN3-1 cells were incubated in DMEM (Fig. 1, H and I).

Acyl modification and maturation of ghrelin in MGN3-1 cells

MGN3-1 cells expressed GOAT, prohormone convertase (PC) 1/3 and PC2 mRNA (Fig. 2A). The molecular size of ghrelin examined by tricine SDS-PAGE and Western blot analysis in culture medium was consistent with that of mature ghrelin (Fig. 2B). In addition, when the culture

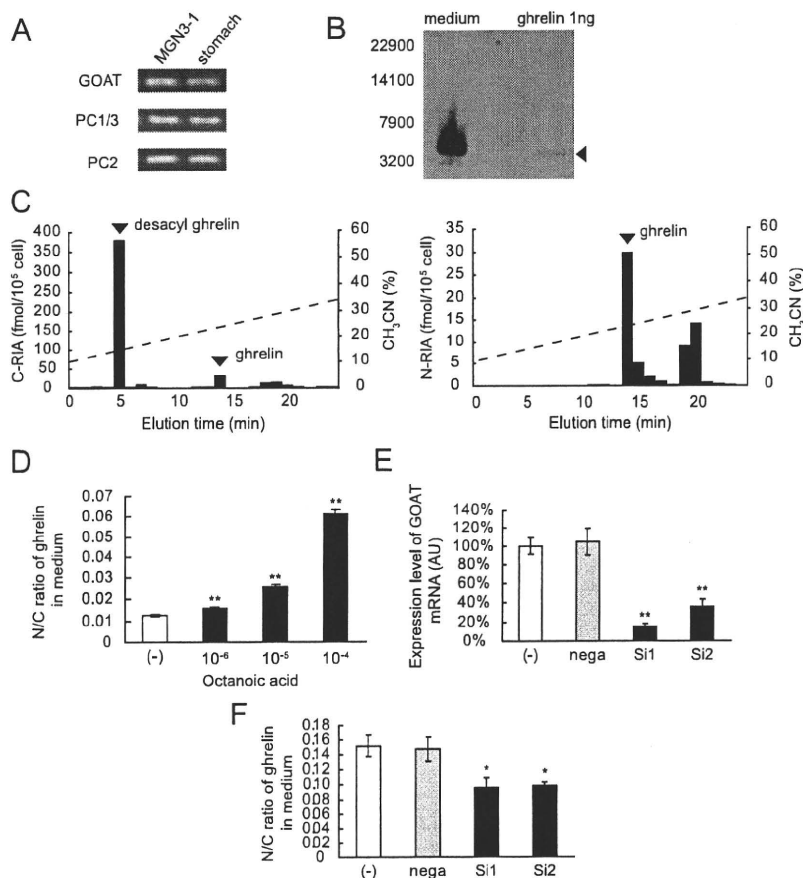


FIG. 2. Acylation and processing of ghrelin in MGN3-1 cells. **A**, RT-PCR analysis of GOAT, PC1/3, and PC2 mRNA expressions in MGN3-1 cells. **B**, Western blot analysis of culture medium in which MGN3-1 cells were cultured for 3 d. Rat ghrelin peptide was used as a positive control. **C**, Representative reverse-phase HPLC profiles of ghrelin immunoreactivity in the medium in which MGN3-1 cells were cultured for 4 h. **D**, The ratio of N-RIA to C-RIA of ghrelin secreted by MGN3-1 cells incubated for 4 h in KRB supplemented with 0.5% BSA and various concentrations of octanoic acid. **, $P < 0.01$ in comparison with (-) ($n = 9$). **E**, GOAT mRNA levels after siRNA treatment of MGN3-1 cells. Two types of siRNA specific for GOAT (Si1 and Si2) and negative control (nega) were introduced into MGN3-1 cell. *, $P < 0.05$, **, $P < 0.01$ in comparison with (-) ($n = 7$). AU, Arbitrary unit. **F**, The ratio of N-RIA to C-RIA of ghrelin secreted by MGN3-1 cells after siRNA treatment. *, $P < 0.05$, **, $P < 0.01$ in comparison with (-) ($n = 7$).

media were subjected to reverse-phase HPLC, the immunoreactive peaks detected by C-RIA and N-RIA were eluted at the positions identical with those of desacyl ghrelin and ghrelin (Fig. 2C). When MGN3-1 cells were incubated in KRB supplemented with 0.5% BSA, addition of octanoic acid to KRB significantly increased the ratio of *n*-octanoylated ghrelin measured by N-RIA to total ghrelin measured by C-RIA (N to C ratio) (Fig. 2D). Specific siRNA1 and siRNA2 treatment decreased GOAT mRNA levels in MGN3-1 cells to 85 and 65% of normal levels, respectively (Fig. 2E). The N to C ratio was significantly decreased by GOAT knockdown (Fig. 2F), indicating that GOAT mediates the acylation of ghrelin in MGN3-1 cells.

Transplantation of MGN3-1 cell to nude mouse

When MGN3-1 cells were injected sc into nude mice, they developed solid tumors (Fig. 3A). Plasma ghrelin and desacyl ghrelin levels were significantly elevated in nude mice 4 wk after MGN3-1 cell injection (Fig. 3, B and C). Mice injected with MGN3-1 cells exhibited significantly higher food intake in comparison with controls (PBS *vs.* cell: 21.4 ± 0.5 *vs.* 23.3 ± 0.7 g/wk, $P < 0.05$) 4 wk after injection (Fig. 3C).

The effect of somatostatin and insulin on ghrelin secretion and expression *in vitro*

MGN3-1 cells expressed the mRNAs encoding multiple somatostatin receptors, primarily type 2 and 5 with lower levels of type 3 and 4 (Fig. 4A). When somatostatin was added to culture media, ghrelin secretion was suppressed in a dose-dependent manner (Fig. 4, B and C). MGN3-1 cells also expressed mRNA of insulin receptor (Fig. 4A). Addition of insulin to culture media also suppressed ghrelin secretion from MGN3-1 cell (Fig. 4, D and E). Somatostatin treatment did not affect mRNA expression of either ghrelin or GOAT, even for 24 h incubation (Fig. 4, F and G), whereas insulin significantly suppressed them (Fig. 4, H and I).

Discussion

In this study, we successfully established the first ghrelinoma cell line, MGN3-1 cell. When establishing a cell line for a research tool, the most important thing is how the established cell line keeps its original characteristics. We evaluated the value of MGN3-1 cell by three points: the quantity of ghrelin production, the maintenance of machineries involved in acyl modification and maturation of ghrelin, and the preservation of known *in vivo* regulation of ghrelin secretion.

With regard to the amount of ghrelin production, MGN3-1 cells produced substantial quantities of ghrelin, approximately 5000-fold greater than that produced by

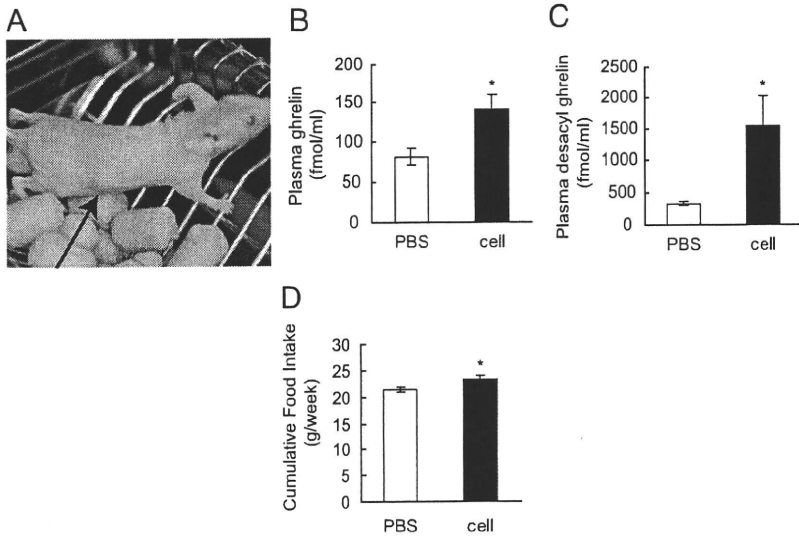


FIG. 3. Transplantation of MGN3-1 cell to nude mouse. A, Macroscopic findings of nude mice injected with MGN3-1 cells. B and C, Plasma ghrelin (B) and desacyl ghrelin (C) levels in nude mice at 4 wk after injection of saline or MGN3-1 cells. D, Cumulative food intake over a week (between 4 and 5 wk after injection) by mice injected with MGN3-1 cells (cell) or PBS. *, $P < 0.05$ in comparison with PBS ($n = 5$).

TT cells (6). It may be reasonable because TT cell is originated from thyroid medullary carcinoma, not from gastric ghrelin-producing cells. As for the machinery of gh-

MGN3-1 cells, we performed a transplantation of MGN3-1 cells to nude mice, which resulted in the increase in plasma ghrelin levels and the enhancement of food intake in the nude mice. These results indicate that MGN3-1 cell keeps the ability to produce a substantial amount of bioactive ghrelin with normal acyl modification and maturation.

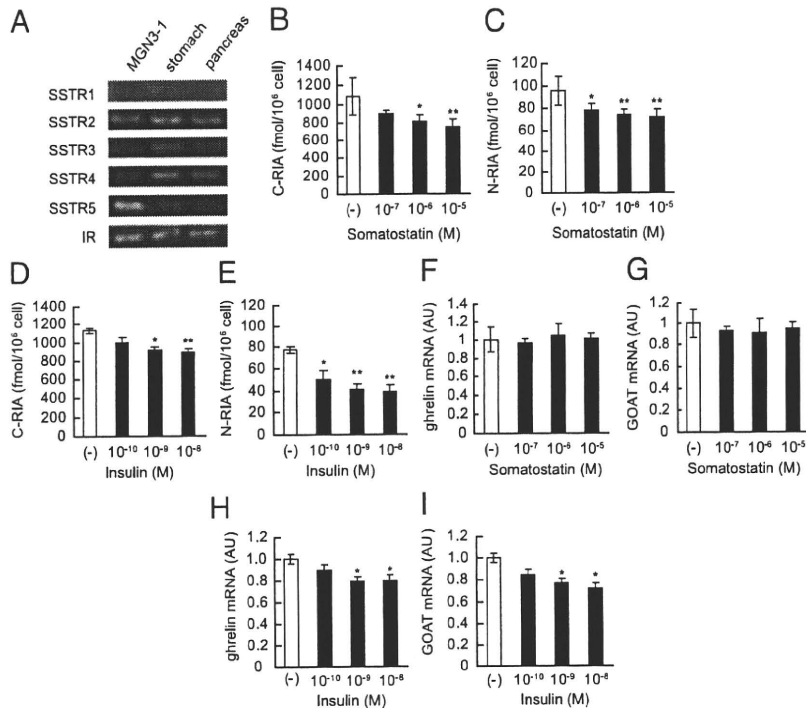


FIG. 4. The effect of somatostatin and insulin on ghrelin secretion and expression in MGN3-1 cells. A, RT-PCR analysis of somatostatin receptor (SSTR) types 1–5 and insulin receptor (IR) mRNA expressions in MGN3-1 cells. B–F, Ghrelin secretion was suppressed when MGN3-1 cells were incubated in DMEM supplemented with somatostatin (B and C) or insulin (D and E) for 4 h ($n = 6$). F–I, Somatostatin did not affect the expression levels of ghrelin (F) and GOAT (G) mRNA in MGN3-1 cell after 24 h incubation, whereas insulin significantly suppressed the expression levels of ghrelin (H) and GOAT (I) mRNA ($n = 6$). AU, Arbitrary unit. *, $P < 0.05$, **, $P < 0.01$ in comparison with (–).

relin acyl modification and maturation, the MGN3-1 cell expressed two key enzymes: GOAT for acylation (16) and PC1/3 for processing (17). We confirmed the activity of GOAT in the MGN3-1 cell by the experiment in which the ratio of ghrelin to total ghrelin levels was decreased by knocking down the GOAT in MGN3-1 cells. Furthermore, addition of octanoic acid significantly increased the N to C ratio of ghrelin in the medium, which is consistent with the *in vivo* finding by Nishi *et al.* (18). We consider these findings important because acyl modification of ghrelin is one of the targets of drug discovery.

On the other hand, tricine-SDS PAGE followed by Western blot analysis and reverse-phase HPLC confirmed the maturation of ghrelin in MGN3-1 cells. To provide a concrete evidence for the ability of producing bioactive ghrelin of

Lastly, we examined the effect of a factor known to effect ghrelin secretion *in vivo*. Numerous reports exist examining the factors that regulate ghrelin secretion *in vivo* (19–27). Among those, the suppression of ghrelin secretion by somatostatin (19–22) or insulin (28, 29) is well established. We confirmed that ghrelin secretion is suppressed by somatostatin and insulin in MGN3-1 cells, indicating that MGN3-1 cells preserves the intrinsic characteristics of ghrelin-producing cells, at least with regard to its regulation by somatostatin and insulin.

In summary, we have established the first ghrelinoma cell line MGN3-1. The MGN3-1 cell line produces high amounts of bioactive ghrelin with normal acyl modification and maturation and retains physiological regulation by somatostatin. MGN3-1 will be a useful tool for

studying ghrelin production and secretion as well as for screening of ghrelin-modulating drugs.

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Generation of Transgenic Mice Overexpressing a Ghrelin Analog

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After the discovery of ghrelin, we attempted to generate ghrelin gene transgenic (Tg) mice. These animals, however, produced only des-acyl ghrelin, which lacked the n-octanoyl modification at Ser³ necessary to manifest ghrelin activity. Because the mechanism for acyl-modification of ghrelin had been unclear until the recent identification of GOAT (ghrelin O-acyltransferase), it had been difficult to generate Tg mice overexpressing ghrelin using standard procedures. Therefore, we planned to generate Tg mice overexpressing a ghrelin analog, which possessed ghrelin-like activity in the absence of acylation at Ser³ and could be synthesized *in vivo*. As the replacement of Ser³ of ghrelin with Trp³ (Trp³-ghrelin) preserves a low level of ghrelin activity and Trp³-ghrelin can be synthesized *in vivo*, we generated mice overexpressing Trp³-ghrelin by using the hSAP (human serum-amyloid-P) promoter. Plasma Trp³-ghrelin concentrations in the Tg mice were approximately 85-fold higher than plasma ghrelin concentrations in non-Tg littermates. Because Trp³-ghrelin is approximately 1/10–1/20 less potent than ghrelin *in vivo*, plasma Trp³-ghrelin concentrations in Tg mice were calculated to have an activity approximately 6-fold greater than that of acylated ghrelin seen in non-Tg mice (85-fold x 1/10–1/20). Tg mice exhibited a normal growth and glucose metabolism in their early life stage. However, 1-yr-old Tg mice demonstrated impaired glucose tolerance and reduced insulin sensitivity. This model will be useful to evaluate the long-term effects of ghrelin or ghrelin analogs. In addition, this technique may be a useful method to generate gain-of-activity models for hormones that require posttranscriptional modifications. (*Endocrinology* 151: 5935–5940, 2010)

Ghrelin, an endogenous ligand for the GH secretagogue receptor (GHS-R) (or ghrelin receptor), is a stomach-derived 28-amino acid peptide hormone modified by n-octanoylic acid at the third Ser residue (Ser³) (1). This modification is essential for ghrelin activity (1).

Since ghrelin was discovered, several groups, including us, have been trying to generate transgenic (Tg) mice overexpressing ghrelin under the control of different promoters (2–7). All of these animals, with the exception of two lines created by Reed *et al.* (5) and Bewick *et al.* (8), produced des-acyl ghrelin only. This form lacks the n-octanoyl modification at Ser³ and is devoid of ghrelin activ-

ity. The mechanism for ghrelin acylation had been unclear until the recent identification of ghrelin O-acyltransferase (GOAT) (9). Because GOAT had not yet been identified when we initiated this study and it had proved to be difficult to generate the Tg mice overexpressing ghrelin by standard procedures, we planned to generate Tg mice overexpressing a ghrelin analog possessing ghrelin-like activity without Ser³ acylation that could be synthesized *in vivo*.

Matsumoto *et al.* (10) investigated the effect on ghrelin bioactivities of replacement of the octanoylated Ser at the third position with other amino acids, such as tryptophan

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Abbreviations: C-RIA, RIA recognizing the C-terminal region of ghrelin; GOAT, ghrelin O-acyltransferase; GHS-R, GH secretagogue receptor; hSAP, human serum-amyloid-P; N-RIA, RIA recognizing the N-terminal region of ghrelin; Tg, transgenic; Trp, tryptophan; Trp³-ghrelin, ghrelin analog with the third amino-acid residue (Ser³) replaced by Trp.

(Trp), Val, Leu, or Ile. The ghrelin-like activity of these synthetic peptides was evaluated by EC_{50} values, determined by an increase in intracellular calcium concentrations $[Ca^{2+}]_i$ in GHS-R-expressing cells. Replacement of Ser³ with Trp³ (Trp³-ghrelin) preserved ghrelin activity with an EC_{50} of 31 nM in comparison with 1.3 nM for intact ghrelin. Replacement of Ser³ with Val³, Leu³, or Ile³ led to complete loss of ghrelin potency. Although ghrelin analog, in which the Ser³ residue was replaced by Trp (Trp³-ghrelin) is approximately 24-fold less active than native ghrelin *in vitro*, it can be synthesized *in vivo*. Thus, we selected Trp³-ghrelin as a candidate ghrelin analog.

In this study, we examined whether Trp³-ghrelin exerts ghrelin-like activity *in vivo*. After confirming this activity, we generated Tg mice overexpressing Trp³-ghrelin.

Materials and Methods

All animal protocols were approved by the Kyoto University Graduate School of Medicine Committee on Animal Research. Animals, housed in air-conditioned animal quarters with light between 0800 and 2000 h, were maintained on standard rat chow (CE-2, 352 kcal/100 g; Japan CLEA, Osaka, Japan).

Experiment 1, the *in vivo* effects of Trp³-ghrelin

Eight-week-old male C57BL/6 mice were purchased from Japan CLEA. Ghrelin was obtained from Peptide Research Institute (Osaka, Japan). Trp³-ghrelin, in which the Ser³ residue was replaced by Trp, was synthesized as previously described (10).

Food intake

Mice ($n = 8$, each group) were injected sc with saline, ghrelin (120 or 360 mcg/kg), or Trp³-ghrelin (360, 1200, or 3600 mcg/kg) before measuring a 2-h food intake.

GH secretion

Mice ($n = 8$, each group) were injected with iv saline, ghrelin (4, 12, 40, or 120 mcg/kg), or Trp³-ghrelin (12, 40, 120, or 360 mcg/kg). Blood samples were collected from the retro-orbital vein 10 min after injection and stored at -20 C until assessed.

Inhibition of glucose stimulated insulin secretion

After a 12-h fast, mice ($n = 8$, each group) were injected iv with 1.0 g/kg glucose, together with saline, ghrelin (120 or 360 mcg/kg), or Trp³-ghrelin (1200 or 3600 mcg/kg). Blood samples were collected 1 and 10 min after injection and stored at -20 C until assessed.

Experiment 2, generation of Tg mice overexpressing a ghrelin analog, Trp³-ghrelin

Plasmid construction and generation of Tg mice

We generated a fusion gene of the human serum-amyloid-P (hSAP) promoter and full-length mouse preproghrelin cDNA (1, 11). Plasmid hSAP-ghrelin was constructed by inserting mouse preproghrelin cDNA into the unique *EcoRI* site between the

hSAP promoter and the 3'-flanking sequence of the rabbit β -globin gene. Mutations were created using a QuikChange Site-Directed Mutagenesis kit, according to the manufacturer's instruction. The hSAP-ghrelin plasmid was used as the template for PCR amplification. To replace the AGC codon encoding Ser to a TGG codon encoding Trp, we used two oligonucleotide primers: 5'-GGACATGGCCATGGCAGGCTCCTGGTTCCTGAGCCCAGAGC-3' and 5'-GCTCTGGGCTCAGGAA~~CC~~AGGAGCC-TGCCATGGCCATGTCC-3'. The mutated construct was verified by sequencing (please see figure 2A). The DNA fragment encoding mutant ghrelin was excised from the plasmid by digestion with *SalI* and *HindIII* (see figure 2B), then purified and microinjected into the pronuclei of fertilized eggs as reported (11). Founder Tg mice were identified by PCR analysis and bred against C57BL/6 mice.

Please refer to Supplemental Methods, published on The Endocrine Society's Journals Online web site at <http://endo.endojournals.org>, for real-time quantitative RT-PCR, semiquantitative PCR, glucose and insulin tolerance tests, measurements of insulin-releasing ability, body weights, body length, body composition, daily food intake, hormonal parameters, and statistical analyses.

Results

Experiment 1, the *in vivo* effects of Trp³-ghrelin on food intake and GH secretion

To elucidate whether Trp³-ghrelin has ghrelin-like potency *in vivo*, 8-wk-old male C57BL/6 mice were administered vehicle, ghrelin, or Trp³-ghrelin before determining food intake over a period of 2 h. Injection of ghrelin or Trp³-ghrelin stimulated food intake in a dose-dependent manner (Fig. 1A). The 2-h food intake after injection of 3600 mcg/kg of Trp³-ghrelin was 0.47 ± 0.04 g, which was 2.2-fold higher than that seen in vehicle-injected mice (0.21 ± 0.02 g/2 h). This level of stimulation was similar to that seen in mice injected with ghrelin at a dose of 360 mcg/kg. Serum GH levels increased after injection of 360 mcg/kg Trp³-ghrelin to 133.9 ± 46.1 ng/ml, which was 21-fold higher than that seen after vehicle injection (6.4 ± 1.1 ng/ml) and similar to those seen after ghrelin injection at 40 mcg/kg (138.8 ± 26.5 ng/ml, respectively) (Fig. 1B). Injection of ghrelin or Trp³-ghrelin inhibited glucose-stimulated insulin release in a dose-dependent manner (Fig. 1C). The 10-min insulin response was significantly inhibited by 3600 mcg/kg Trp³-ghrelin and 360 mcg/kg to the same extent (0.78 ± 0.09 and 0.63 ± 0.06 ng/ml), compared with saline (1.10 ± 0.01 ng/ml). These results indicated that Trp³-ghrelin stimulates food intake and GH secretion and inhibits glucose-stimulated insulin secretion in a manner similar to ghrelin with a potency approximately 1/10–1/20 (Trp³-ghrelin needs about 20-fold amount for stimulation of food intake, about 10-fold amount for GH secretion, and about 10-fold amount for

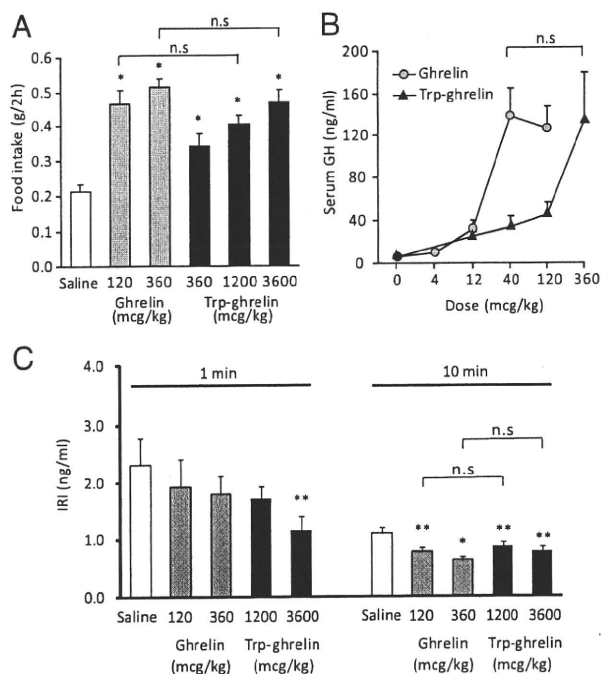


FIG. 1. Effects of the exogenous administration of Trp³-ghrelin on food intake, GH secretion, and inhibition of glucose stimulated insulin release. **A**, Two-hour food intake after saline (open bar), ghrelin at 120 or 360 mcg/kg (shaded bars), or Trp³-ghrelin at 360, 1200, or 3600 mcg/kg (closed bars) ip injection ($n = 8-10$). **B**, Serum GH levels were measured 10 min after 4, 12, 40, or 120 mcg/kg ghrelin (shaded circles) or 12, 40, 120, or 360 mcg/kg Trp³-ghrelin (closed triangles) iv injection ($n = 8-10$). **C**, Serum insulin levels were measured 1 and 10 min after iv injection of 1.0 g/kg glucose, together with saline (open bars), ghrelin (120 and 360 mcg) (shaded bars), or Trp³-ghrelin (1200 and 3600 mcg/kg) (closed bars) ($n = 8$). *, $P < 0.01$; **, $P < 0.05$; n.s., not significant compared with saline group; IRI, immunoreactive insulin. Data are presented as the means \pm SEM.

inhibition of glucose stimulated insulin release) that of ghrelin.

Experiment 2, generation of Tg mice overexpressing Trp³-ghrelin

Two Tg mouse lines, Tg6-2 and Tg6-5, were obtained. Hepatic transgene expression in Tg6-2 and Tg6-5 mice was 3.02 ± 1.15 and 0.07 ± 0.01 in arbitrary units, respectively, after normalization to preproghrelin mRNA expression levels seen in the stomachs of non-Tg littermates (non-Tg mice) (1.00 ± 0.18). No expression of preproghrelin mRNA was seen in the livers of non-Tg mice (Fig. 2C).

Two RIA methods [RIA recognizing the N-terminal region of ghrelin (N-RIA) and RIA recognizing the C-terminal region of ghrelin (C-RIA)] were performed to measure plasma ghrelin, des-acyl ghrelin, and Trp³-ghrelin concentrations. In a previous study, N-RIA has been demonstrated to recognize only the acylated N-terminal region of ghrelin, whereas C-RIA recognizes the C-terminal region of ghrelin, making it possible to detect both acylated

ghrelin and des-acyl ghrelin (12). We determined whether Trp³-ghrelin could be detected by one or both of these RIA systems. When synthetic Trp³-ghrelin was added to plasma samples from wild-type mice, Trp³-ghrelin could only be detected by C-RIA, not N-RIA (data not shown). At 8 wk of age, plasma ghrelin concentrations measured by N-RIA did not differ among genotypes. Total plasma ghrelin concentrations, including ghrelin, des-acyl ghrelin, and Trp³-ghrelin, measured by C-RIA were significantly elevated in Tg mice (Fig. 2, D and E). To determine precise plasma Trp³-ghrelin concentration, we also performed HPLC on Tg6-2 samples (Fig. 2F). Plasma ghrelin (40.5 ± 10.2 vs. 36.6 ± 4.4 fmol/ml) and des-acyl ghrelin (167.5 ± 51.8 vs. 235.7 ± 44.8 fmol/ml) concentrations did not differ among genotypes.

Plasma Trp³-ghrelin concentrations in Tg6-2 was 3437.8 ± 571.6 (2546.4–5101.7) fmol/ml, which was approximately 85-fold ($3437.8/40.5 = 84.9$ -fold) higher than plasma ghrelin (acylated ghrelin) concentrations seen in non-Tg mice. Because Trp³-ghrelin is approximately 1/10–1/20 less potent than ghrelin *in vivo* (experiment 1), plasma Trp³-ghrelin concentrations in Tg6-2 were calculated to have an activity approximately 6-fold greater than that of ghrelin (acylated ghrelin) seen in non-Tg mice ($84.9\text{-fold} \times 1/10\text{--}1/20 = 4.2\text{--}8.5\text{-fold}$). Total ghrelin concentrations measured by C-RIA in the Tg mice were roughly constant throughout the day.

We then analyzed the phenotype of the Tg6-2 line. Tg mice overexpressing Trp³-ghrelin (Tg6-2 line) were abbreviated as Trp³-ghrelin-Tg mice.

The analysis of the phenotypes of Trp³-ghrelin-Tg mice

During postnatal development, there were no significant differences in somatic growth between Trp³-ghrelin-Tg and non-Tg mice (Supplemental Fig. 1, A and B). Consistent with these results, no changes in serum GH and IGF-I concentrations were observed in Trp³-ghrelin-Tg mice (Supplemental Fig. 1, C and D). The average food intake of Trp³-ghrelin-Tg mice did not differ from that of non-Tg mice (Supplemental Fig. 1E). Trp³-ghrelin-Tg mice consumed the largest food portions during the dark phase ($75.4 \pm 2.7\%$), similar to the behavior seen in non-Tg mice ($75.9 \pm 1.6\%$). There were no differences between 10-wk-old Trp³-ghrelin-Tg and non-Tg mice in pituitary and hypothalamic mRNA levels of factors involved in GH secretion and food intake (Supplemental Fig. 2, A and B). In addition, glucose metabolism in Trp³-ghrelin-Tg mice did not differ from that seen in non-Tg mice in early life (Supplemental Fig. 1, F and G).

We conducted a precise evaluation of glucose metabolism using more aged mice. Thus we continued rearing

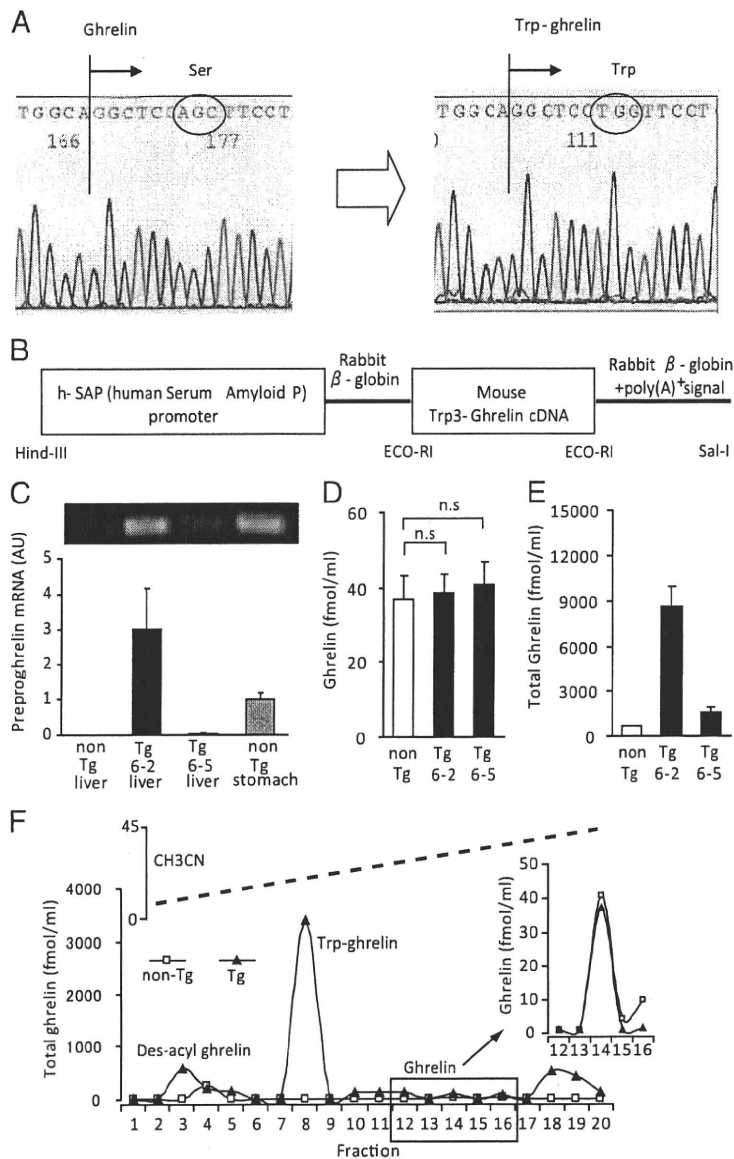


FIG. 2. Generation of Trp³-ghrelin overexpressing Tg mice. **A**, A mutant construct in which the AGC codon encoding Ser, the third amino acid of ghrelin that is modified by *n*-octanoic acid, was replaced by a TGG codon encoding Trp. **B**, The construct encoding Trp³-ghrelin used to generate Tg mice was a fusion gene of the hSAP promoter combined with the mutated cDNA of mouse ghrelin. **C**, The expression levels of preproghrelin mRNA or mutated preproghrelin mRNA. **D**, Plasma concentrations of ghrelin (acylated form) were measured by N-RIA ($n = 8-10$). **E**, Plasma concentrations of total ghrelin, which included ghrelin, des-acyl ghrelin, and Trp³-ghrelin, were measured by C-RIA ($n = 8-10$). **F**, Representative results of HPLC analysis (non-Tg, open square; Tg6-2, closed triangle). n.s., Not significant. Data are presented as the means \pm SEM (C–E).

these mice to 1 yr of age. Some intriguing results on glucose metabolism were obtained from 1-yr-old Trp³-ghrelin-Tg mice. Although there were no differences between Trp³-ghrelin-Tg and non-Tg mice in anthropometric parameters, including body weight, total body fat percentage, and lean body mass, Trp³-ghrelin-Tg mice exhibited impaired glucose tolerance and reduced insulin sensitivity (Fig. 3, A–F); blood glucose levels after glucose injection were significantly higher than those in non-Tg mice. The acute

phase of insulin secretion typically seen in response to glucose tended to be suppressed in Trp³-ghrelin-Tg mice ($P = 0.11$) (Fig. 3, C and D). In addition, the hypoglycemic response after the injection with insulin was blunted in Trp³-ghrelin-Tg mice (Fig. 3E). There were no differences, however, in pancreatic insulin mRNA levels between 1-yr-old Trp³-ghrelin-Tg and non-Tg mice (Fig. 3F). Because glucose tolerance and insulin sensitivity are influenced by GH, we examined whether GH secretion was augmented in 1-yr-old Trp³-ghrelin-Tg mice. Serum GH and IGF-I levels were unchanged in Trp³-ghrelin-Tg mice in comparison with those seen in non-Tg mice at 1 yr of age (Fig. 4, A and B). There was no difference between 1-yr-old Trp³-ghrelin-Tg and non-Tg mice in ghrelin or GOAT mRNA within the stomach or in plasma acylated ghrelin concentrations, which reflects the intrinsic secretion of ghrelin (Fig. 4, C and D). Because ghrelin can also affect the lipid metabolism, we measured serum nonesterified fatty acid, total cholesterol, and triglyceride levels. However, there was no significant difference in them (non-Tg vs. Tg: nonesterified fatty acid, 0.74 ± 0.03 vs. 0.82 ± 0.04 mEq/liter, $P = 0.12$; total cholesterol, 122.5 ± 9.8 vs. 143.4 ± 7.4 mg/dl, $P = 0.10$; triglyceride, 162.6 ± 12.8 vs. 159.5 ± 10.8 mg/dl, $P = 0.85$).

Discussion

It is challenging to generate ghrelin gain-of-activity models, because ghrelin requires posttranscriptional modification, an octanoylation of Ser³. GOAT

is responsible for this octanoylation of ghrelin, which confers its biological activity (10, 13). In this study, we succeeded in generating Tg mice overexpressing Trp³-ghrelin, a ghrelin analog that does not require posttranscriptional modification with GOAT for activity. Because expression of the mutated-ghrelin transgene was driven by the hSAP promoter, Trp³-ghrelin was continuously secreted from the liver after birth. Plasma concentrations of Trp³-ghrelin of Tg mice were calculated to have an equivalent activity

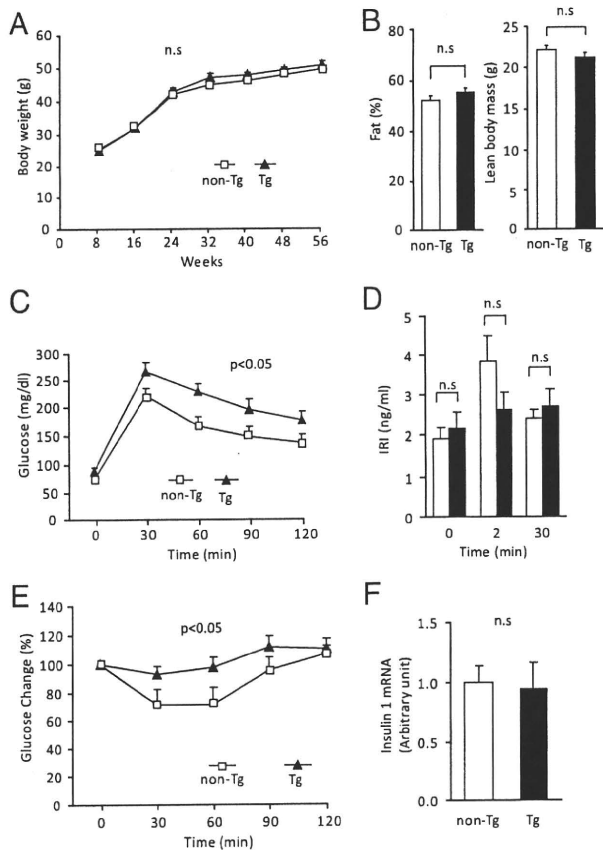


FIG. 3. Analysis of Trp³-ghrelin Tg mice. A, Changes of body weight in Trp³-ghrelin-Tg mice (closed triangles) and non-Tg littermates (open squares) (n = 20–25). B, Body fat percentage and lean body mass, as determined by computer tomography, in 52-wk-old Trp³-ghrelin-Tg mice (closed bars) and non-Tg littermates (open bars) (n = 14–16). C, Glucose tolerance test (0.75 g/kg) was performed in 52-wk-old Trp³-ghrelin-Tg mice (closed triangles) and non-Tg littermates (open squares) (n = 14–16; *, P < 0.05 in comparison with non-Tg littermates). D, Serum insulin levels at baseline, 2 min, and 30 min after ip glucose injection of 52-wk-old Trp³-ghrelin-Tg mice (closed bars) and non-Tg littermates (open bars) (n = 14–16, P = 0.11 in comparison with non-Tg littermates). E, Insulin tolerance test after treatment with 1.5 U/kg regular insulin in 52-wk-old Trp³-ghrelin-Tg mice (closed triangles) and non-Tg littermates (open squares) (n = 14–16; *, P < 0.05 in comparison with non-Tg littermates). F, Insulin 1 mRNA levels in the pancreases of 52-wk-old Trp³-ghrelin-Tg mice (closed bars) and non-Tg littermates (open bars) (n = 14). IRI, Immunoreactive insulin; n.s., not significant. Data are presented as the means ± SEM.

as 4.2- to 8.5-fold higher levels of acylated ghrelin in non-Tg mice. We think that this unique mouse model is a useful tool to evaluate the long-term pathophysiological and/or pharmacological effects of ghrelin or ghrelin analogs and provides insight into the physiological roles of ghrelin/GHS-R systems.

Bewick *et al.* (8) developed ghrelin-overexpressing mice using the endogenous ghrelin promoter. Although this mouse model was suitable to investigate the physiological role of ghrelin, it is not suitable to explore the pathophysiological or pharmacological effects of ghrelin, because the

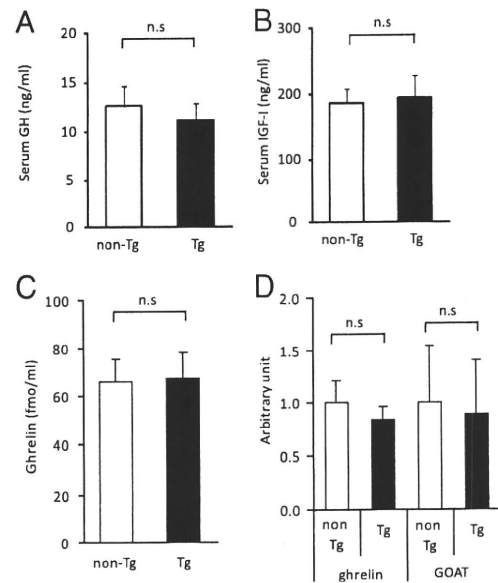


FIG. 4. We examined the levels of GH/IGF-I axis factors, plasma ghrelin levels, and ghrelin and GOAT mRNA levels in the stomachs of 52-wk-old Trp³-ghrelin-Tg mice. A and B, We measured serum GH (A) and IGF-I (B) levels in 52-wk-old Trp³-ghrelin-Tg mice (closed bars) and non-Tg littermates (open bars) (n = 10). C, Plasma ghrelin levels in 52-wk-old Trp³-ghrelin-Tg mice (closed bars) and non-Tg littermates (open bars) (n = 10). D, The mRNA levels of ghrelin and GOAT in the stomachs of 52-wk-old Trp³-ghrelin-Tg mice (closed bars) and non-Tg littermates (open bars) (n = 14). n.s., Not significant. Data are presented as the means ± SEM.

plasma ghrelin levels achieved in these mice were only 1.5-fold greater than that seen in non-Tg mice at the highest. Reed *et al.* (5) also developed ghrelin-overexpressing mice using the neuron-specific enolase promoter, reaching circulating ghrelin levels approximately 5-fold higher than those seen in non-Tg mice. Because these mice primarily produced ghrelin in the brain, it remains unclear whether the phenotype of these mice resulted from elevations in peripheral ghrelin and/or central ghrelin. Kirchner *et al.* (13) generated Tg mice simultaneously expressing human ghrelin and GOAT in the liver under the control of the human apolipoprotein E promoter. When fed a standard diet, these mice lack the circulating fatty-acid-modified forms of ghrelin, demonstrating high circulating concentrations of des-acyl ghrelin only. These mice exhibited elevated concentrations of fatty-acid-modified forms of ghrelin only when given a diet rich in medium-chain triglycerides. It may be difficult to characterize the phenotype of the mice precisely, especially the metabolic phenotype, under such a diet.

Trp³-ghrelin-Tg mice exhibited normal growth patterns and feeding behaviors. These results are consistent with previous results; ghrelin loss-of-function mice, ghrelin-deficient mice, or ghrelin-receptor-null mice all have normal growth rates, food intake, and body compositions (14–17). One-year-old Trp³-ghrelin-Tg mice demon-