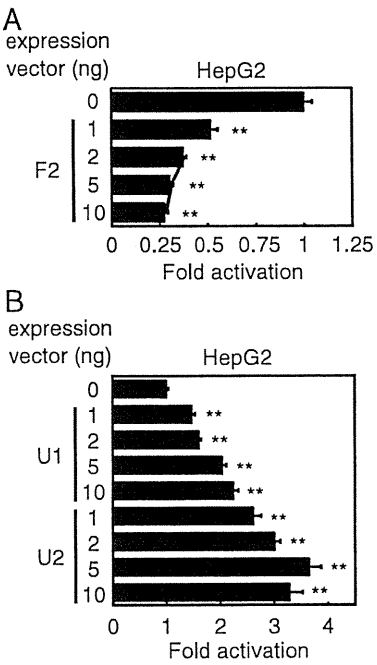

FIG. 4. Effect of overexpression of FOXA2 or USF on *hDIO1* promoter activity. −187/−4 hDIO1-Luc was transiently transfected into HepG2 cells in the presence of increasing amounts of vectors expressing FOXA2 (F2) (A) or USF1 or USF2 (U1 and U2, respectively) (B). Promoter activity was normalized to *Renilla* luciferase activity, then expressed as the relative activity to −187/−4 hDIO1-Luc cotransfected with pF4A without a cDNA insert. Statistical significance was determined by ANOVA followed by Dunnett's test. **, $P < 0.01$.

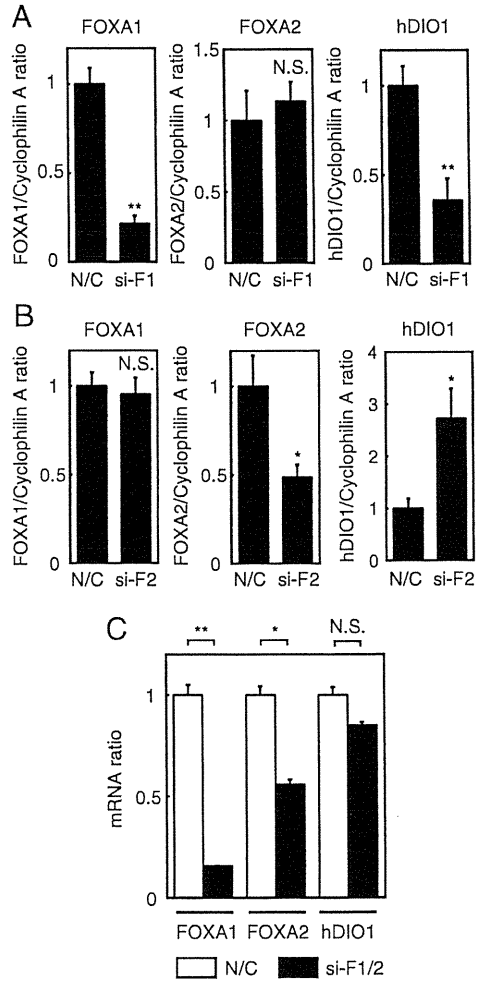

FIG. 5. Knockdown of FOXA1 and/or FOXA2. Effect of transfection of FOXA1 (A) and FOXA2 (B) siRNA on the mRNA expression level of FOXA1, FOXA2, and D1. Simultaneous knockdown of FOXA1 and FOXA2 (C) was performed using siRNA that has identical sequences in both FOXA1 and FOXA2. N/C, Negative control siRNA; si-F1, siRNA specific for FOXA1; si-F2, siRNA specific for FOXA2; si-F1/2, siRNA specific for both FOXA1 and FOXA2. mRNA expression level was normalized to that of cyclophilin A in each sample and expressed as the relative activity to the basal expression (N/C). Statistical significance was determined by unpaired *t* test. *, $P < 0.05$; **, $P < 0.01$. N.S., Not significant.

down of FOXA1 decreased the expression of *hDIO1* mRNA, and knockdown of FOXA2 increased the expression level of *hDIO1* mRNA. FOXA1 and FOXA2 did not affect each other's expression by knockdown of them (Fig. 5, A and B). In addition, when both FOXA1 and FOXA2 were knocked down simultaneously, no change in the expression of *hDIO1* mRNA was seen (Fig. 5C). Thus, *hDIO1* expression is positively regulated by FOXA1 and negatively regulated by FOXA2, and FOXA1 and FOXA2 interact with each other to regulate *hDIO1* expression.

Interaction between FOXA and USF in the activation of the *hDIO1* promoter

Transcription factors frequently interact to coordinately regulate gene expression, and we first wished to determine whether the FOXA binding site and the E-box present in the *hDIO1* promoter interact. We cotransfected a WT or mutated −187/−4 hDIO1-Luc construct and USF expression plasmids into HepG2 cells. When the FOXA binding site was mutated, the transcription activity of the *hDIO1* promoter in the presence of transfected USF was attenuated (Fig. 6A). Thus, activation of the *hDIO1* promoter by USF depends on the presence of a functional FOXA binding site. Next, we investigated the effects of FOXA on the response of the *hDIO1* promoter to USF. We knocked down the expression of FOXA and cotransfected a −187/−4 hDIO1-Luc construct along with USF expression plasmids into HepG2 cells. As shown in Fig. 6B, the transcription activity of the *hDIO1* promoter was attenuated by knockdown of FOXA1 and enhanced by knockdown of FOXA2. The transcription activity of the *hDIO1* promoter was also attenuated by simultaneous knockdown of FOXA1 and FOXA2 to an extent similar to that seen for the knockdown of FOXA1. The suppressed activity by knockdown of FOXA1 was not restored by overexpression of USF, and the enhanced activity by knockdown of FOXA2 was further enhanced by overexpression of USF. Thus, the response of the *hDIO1* pro-

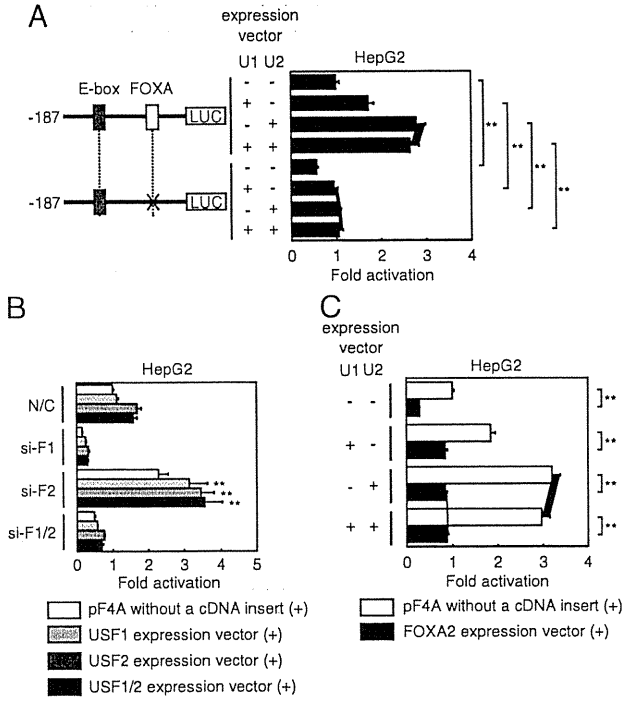

FIG. 6. Interaction between FOXA and USF in the activation of the *hDIO1* promoter. A, Dependence of the activation of *hDIO1* promoter by USF on the FOXA binding site. Schematic diagram in the *left* of figure representing WT and site-specific mutations of the *hDIO1* promoter, introduced into the upstream region of the luciferase gene. A *cross* represents the site-specific mutation of the putative FOXA binding site. Each construct was transiently cotransfected into HepG2 cells in the presence of 5 ng of vectors expressing USF1 (U1) and/or USF2 (U2). Promoter activity was normalized to *Renilla* luciferase activity, then expressed as the relative activity to −187/−4 hDIO1-Luc cotransfected with pF4A without a cDNA insert. Statistical significance was determined by ANOVA followed by Student-Newman-Keuls test. **, $P < 0.01$. B, Effect of knockdown of FOXA on the activation of the *hDIO1* promoter by USF. Short interfering RNA specific for FOXA1 and/or FOXA2 or a negative control siRNA were transfected into HepG2 cells as described in *Materials and Methods*. One day after siRNA transfection, −187/−4 hDIO1-Luc was transiently cotransfected in the presence of 5 ng of vectors expressing USF1 and/or USF2. N/C, Negative control siRNA; si-F1, siRNA specific for FOXA1; si-F2, siRNA specific for FOXA2; si-F1/2, siRNA specific for both FOXA1 and FOXA2. Promoter activity was normalized to *Renilla* luciferase activity, then expressed as the relative activity to −187/−4 hDIO1-Luc cotransfected with pF4A without a cDNA insert after knockdown by negative control siRNA. Statistical significance was determined by ANOVA followed by Student-Newman-Keuls test. **, $P < 0.01$ relative to −187/−4 hDIO1-Luc cotransfected with pF4A without a cDNA insert after knockdown by siRNA specific for FOXA2. C, Effect of overexpression of FOXA2 on the activation of the *hDIO1* promoter by USF. −187/−4 hDIO1-Luc was transiently transfected into HepG2 cells in the presence of 5 ng of vectors expressing USF1 (U1), USF2 (U2), and/or FOXA2. Promoter activity was normalized to *Renilla* luciferase activity, then expressed as the relative activity to −187/−4 hDIO1-Luc cotransfected with pF4A without a cDNA insert. Statistical significance was determined by ANOVA followed by Student-Newman-Keuls test. **, $P < 0.01$. LUC, Luciferase.

moter to USF was greatly attenuated by knockdown of FOXA1 and enhanced by knockdown of FOXA2. Furthermore, we cotransfected a −187/−4 hDIO1-Luc construct and USF expression plasmids with or without a FOXA2 expression plasmid into HepG2 cells. The transcription activity of the *hDIO1* promoter was enhanced in the presence of transfected USF (Fig. 6C, *white bar*), but the activity was greatly attenuated by cotransfection of the FOXA2 expression plasmid (Fig. 6C, *black bar*). Thus, the response of the *hDIO1* promoter to USF was attenuated by the coexpression of FOXA2. Collectively, these results indicate that FOXA1 is required for the activation of the *hDIO1* promoter by USF and that FOXA2 represses the transcription of *hDIO1* and disrupts the interaction of USF with FOXA1 by occupying the FOXA binding site.

Discussion

In this study, we analyzed the 5′-upstream region of *hDIO1* to identify protein-DNA interactions within the *hDIO1* promoter. Our experiments demonstrated that the region between nucleotides −187 and −132 is important for *hDIO1* promoter activity in HepG2 cells. We identified functional elements for FOXA and USF within this region, and we showed that these sites are important for the transcriptional regulation of *hDIO1*. Recently, Ohguchi *et al.* (9) identified a proximal hepatocyte nuclear factor (HNF)4α binding site in mice, and they demonstrated that the HNF4α binding site is essential for the activation of the mouse D1 gene by HNF4α. Deletion analyses of the 5′-flanking region of *hDIO1* were performed by Jakobs *et al.* (10) by transfecting 1.5- and 0.1-kb constructs into HepG2 cells, and they found that both constructs substantially increased luciferase activity compared with a promoterless vector. However, they did not perform a higher resolution promoter analysis, and we are the first to identify functional elements other than thyroid hormone responsive element in the *hDIO1* promoter.

The FOXA proteins were first identified as liver-enriched factors because of their ability to bind the transthyretin gene promoter, and they were originally termed HNF3 (11). There are three FOXA proteins, FOXA1 (HNF3α), FOXA2 (HNF3β), and FOXA3 (HNF3γ), which are encoded by different genes on different chromosomes (12). FOXA proteins play important roles in early embryonic development and organogenesis, and they are recognized as "pioneer factors" (13). In addition, the FOXA proteins control glucose metabolism through the regulation of multiple target genes in the liver, pancreas, and adipose tissue after birth (13). Our EMSA experiments demonstrated that FOXA1 and FOXA2 specifically bound the identical FOXA binding site of the *hDIO1* promoter. Although all three FOXA proteins exist relatively abundant in HepG2 cells (14) and recognize the same DNA sequences, slight differences in the binding affin-

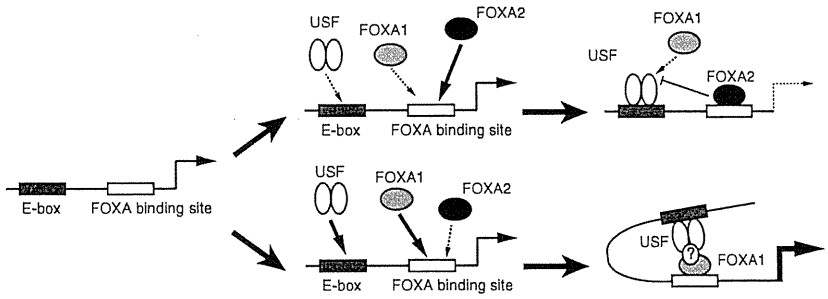

FIG. 7. A model for the regulation of liver-specific expression of *hDIO1* by FOXA1, FOXA2, and USF. FOXA1 and FOXA2 bind and share the identical FOXA binding site, and USF binds the E-box as a heterodimer. FOXA2 represses the transcription of *hDIO1* through the FOXA binding site (*upper panel*), and FOXA1 and USF work cooperatively to activate *hDIO1* transcription (*lower panel*).

ity and DNA binding capacity may account for their specificity (15). Indeed, there are very few reports that FOXA1 and FOXA2 share an identical binding site and coparticipate in the transcriptional regulation of a single gene (16). Our transfection assays and siRNA experiments demonstrated that *hDIO1* is positively regulated by FOXA1 and negatively regulated by FOXA2 and that FOXA1 and FOXA2 interact to coordinately regulate *hDIO1* expression. These results suggest that FOXA proteins are involved in thyroid hormone homeostasis.

USF proteins were first identified as regulators of adenovirus major late promoter transcription (17, 18). There are two USF proteins, 43 kDa (USF1) and 44 kDa (USF2), encoded by different genes on different chromosomes (19, 20). USF proteins primarily bind as dimers to consensus sequences containing the CACGTG motif termed an E-box (18, 19, 21). USF proteins are ubiquitously expressed, although different ratios of USF homo- and heterodimers are found in different cell types (22). The molecular details of USF binding and activity have been well characterized, but its biological role remains poorly understood. USF proteins regulate the expression of several genes related to glucose and lipid metabolism and peptide hormone synthesis, including liver-type pyruvate kinase (23) and glucokinase (24), fatty acid synthase (25), apolipoprotein A-II (26), calcitonin/calcitonin gene-related peptide (27), and ghrelin (8). In our study, we demonstrated that the putative E-box site in the *hDIO1* promoter specifically bound the USF1/USF2 heterodimer and that promoter activity increased in a dose-dependent manner with the cotransfection of the USF1/2 expression plasmid. These results suggest that USF positively regulate *hDIO1* expression. Additionally, promoter activity was almost completely abolished by mutation of the E-box motif, indicating that USF proteins are critical for the transcriptional regulation of *hDIO1* and thyroid hormone homeostasis in the liver and possibly kidney.

The response of the *hDIO1* promoter to USF was greatly attenuated by mutation of the FOXA binding site or knockdown of FOXA1, indicating that FOXA1 is necessary for the expression of *hDIO1* by USF. FOXA1 plays an essential role in the "pioneering" of gene regulatory elements, allowing for the recruitment of additional factors required for gene regulation (28), and our data suggest that USF cooperates with FOXA1 to regulate *hDIO1* promoter activity. Although we could not confirm the interaction between FOXA1 with USF by coimmunoprecipitation experiments in our experimental condition (data not shown), a direct physical interaction between FOXA1 and USF has been reported through the use of immunoprecipitation and glutathione *S*-transferase pull-down assays (29). Furthermore, the cooperation between FOXA1 and USF likely contributes to the liver-specific activation of *hDIO1*; although FOXA1 was expressed in both HepG2 and TSA201 cells in our preliminary experiments (data not shown), only HepG2 cells demonstrated substantial differences in promoter activity by transfection of −187/−4 hDIO1-Luc and mutation of the FOXA binding site. Interactions between cell-specific factors and other regulators are thought to contribute to the tissue-specific control of gene expression by the ubiquitous USF proteins (26, 30–34), and Fig. 7 shows our working model for the regulation of liver-specific expression of *hDIO1* by transcription factor binding to the −187 to −132 region of the *hDIO1* promoter. In this model, *hDIO1* promoter activity is modulated by FOXA1, FOXA2, and USF proteins, and these transcription factors interact with each other to fine-tune the *hDIO1* promoter activity.

In conclusion, we have shown that FOXA1, FOXA2, and USF regulate *hDIO1* expression in the liver. FOXA1 and FOXA2 both participate in the liver-specific regulation of *hDIO1* expression, and FOXA1 and USF act together to promote the liver-specific activation of *hDIO1*. FOXA1 and FOXA2 are likely involved in thyroid hormone homeostasis in the liver.

Acknowledgments

We thank Mami Yoshida and Yudai Takeuchi for technical assistance and Miyuki Ban for secretarial assistance.

Address all correspondence and requests for reprints to: Naotetsu Kanamoto, M.D., Ph.D., Department of Medicine and Clinical Science, Kyoto University Graduate School of Medicine, 54 Shogoin Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan. E-mail: kyotetsu@kuhp.kyoto-u.ac.jp.

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Health, the Labor and Welfare of Japan, Ministry of Education, Culture, Sports, Sciences, and Technology of Japan Grants 18790635, 19591075, and 21119013, and the Takeda Science Foundation.

Disclosure Summary: The authors have nothing to disclose.

References

1. Bianco AC, Salvatore D, Gereben B, Berry MJ, Larsen PR 2002 Biochemistry, cellular and molecular biology, and physiological roles of the iodothyronine selenodeiodinases. Endocr Rev 23:38–89
2. Berry MJ, Banu L, Larsen PR 1991 Type I iodothyronine deiodinase is a selenocysteine-containing enzyme. Nature 349:438–440
3. Toyoda N, Zavacki AM, Maia AL, Harney JW, Larsen PR 1995 A novel retinoid X receptor-independent thyroid hormone response element is present in the human type 1 deiodinase gene. Mol Cell Biol 15:5100–5112
4. Nagaya T, Fujieda M, Otsuka G, Yang JP, Okamoto T, Seo H 2000 A potential role of activated NF-κB in the pathogenesis of euthyroid sick syndrome. J Clin Invest 106:393–402
5. Moriyama K, Tagami T, Akamizu T, Usui T, Saijo M, Kanamoto N, Hataya Y, Shimatsu A, Kuzuya H, Nakao K 2002 Thyroid hormone action is disrupted by bisphenol A as an antagonist. J Clin Endocrinol Metab 87:5185–5190
6. Heinemeyer T, Wingender E, Reuter I, Hermjakob H, Kel AE, Kel OV, Ignatieva EV, Ananko EA, Podkolodnaya OA, Kolpakov FA, Podkolodny NL, Kolchanov NA 1998 Databases on transcriptional regulation: TRANSFAC, TRRD and COMPEL. Nucleic Acids Res 26:362–367
7. Piekielko-Witkowska A, Master A, Wojcicka A, Boguslawska J, Brozda I, Tanski Z, Nauman A 2009 Disturbed expression of type 1 iodothyronine deiodinase splice variants in human renal cancer. Thyroid 19:1105–1113
8. Kanamoto N, Akamizu T, Tagami T, Hataya Y, Moriyama K, Takaya K, Hosoda H, Kojima M, Kangawa K, Nakao K 2004 Genomic structure and characterization of the 5′-flanking region of the human ghrelin gene. Endocrinology 145:4144–4153
9. Ohguchi H, Tanaka T, Uchida A, Magoori K, Kudo H, Kim I, Daigo K, Sakakibara I, Okamura M, Harigae H, Sasaki T, Osborne TF, Gonzalez FJ, Hamakubo T, Kodama T, Sakai J 2008 Hepatocyte nuclear factor 4α contributes to thyroid hormone homeostasis by cooperatively regulating the type 1 iodothyronine deiodinase gene with GATA4 and Kruppel-like transcription factor 9. Mol Cell Biol 28:3917–3931
10. Jakobs TC, Schmutzler C, Meissner J, Köhrle J 1997 The promoter of the human type I 5′-deiodinase gene—mapping of the transcription start site and identification of a DR+4 thyroid-hormone-responsive element. Eur J Biochem 247:288–297
11. Lai E, Prezioso VR, Smith E, Litvin O, Costa RH, Darnell JE Jr 1990 HNF-3A, a hepatocyte-enriched transcription factor of novel structure is regulated transcriptionally. Genes Dev 4:1427–1436
12. Kaestner KH, Hiemisch H, Luckow B, Schütz G 1994 The HNF-3 gene family of transcription factors in mice: gene structure, cDNA sequence, and mRNA distribution. Genomics 20:377–385
13. Friedman JR, Kaestner KH 2006 The Foxa family of transcription factors in development and metabolism. Cell Mol Life Sci 63:2317–2328
14. Motallebipour M, Ameur A, Reddy Bysani MS, Patra K, Wallerman O, Mangion J, Barker MA, McKernan KJ, Komorowski J, Wadelius C 2009 Differential binding and co-binding pattern of FOXA1 and FOXA3 and their relation to H3K4me3 in HepG2 cells revealed by ChIP-seq. Genome Biol 10:R129
15. Yu X, Gupta A, Wang Y, Suzuki K, Mirosevich J, Orgebin-Crist MC, Matusik RJ 2005 Foxa1 and Foxa2 interact with the androgen receptor to regulate prostate and epididymal genes differentially. Ann NY Acad Sci 1061:77–93
16. Shimizu S, Miyamoto Y, Hayashi M 2002 Cell-type dependency of two Foxa/HNF3 sites in the regulation of vitronectin promoter activity. Biochim Biophys Acta 1574:337–344
17. Miyamoto NG, Moncollin V, Egly JM, Chambon P 1985 Specific interaction between a transcription factor and the upstream element of the adenovirus-2 major late promoter. EMBO J 4:3563–3570
18. Sawadogo M, Roeder RG 1985 Interaction of a gene-specific transcription factor with the adenovirus major late promoter upstream of the TATA box region. Cell 43:165–175
19. Gregor PD, Sawadogo M, Roeder RG 1990 The adenovirus major late transcription factor USF is a member of the helix-loop-helix group of regulatory proteins and binds to DNA as a dimer. Genes Dev 4:1730–1740
20. Sawadogo M, Van Dyke MW, Gregor PD, Roeder RG 1988 Multiple forms of the human gene-specific transcription factor USF. I. Complete purification and identification of USF from HeLa cell nuclei. J Biol Chem 263:11985–11993
21. Bendall AJ, Molloy PL 1994 Base preferences for DNA binding by the bHLH-Zip protein USF: effects of MgCl2 on specificity and comparison with binding of Myc family members. Nucleic Acids Res 22:2801–2810
22. Sirito M, Lin Q, Maity T, Sawadogo M 1994 Ubiquitous expression of the 43- and 44-kDa forms of transcription factor USF in mammalian cells. Nucleic Acids Res 22:427–433
23. Vallet VS, Casado M, Henrion AA, Bucchini D, Raymondjean M, Kahn A, Vaulont S 1998 Differential roles of upstream stimulatory factors 1 and 2 in the transcriptional response of liver genes to glucose. J Biol Chem 273:20175–20179
24. Iynedjian PB 1998 Identification of upstream stimulatory factor as transcriptional activator of the liver promoter of the glucokinase gene. Biochem J 333(Pt 3):705–712
25. Casado M, Vallet VS, Kahn A, Vaulont S 1999 Essential role in vivo of upstream stimulatory factors for a normal dietary response of the fatty acid synthase gene in the liver. J Biol Chem 274:2009–2013
26. Ribeiro A, Pastier D, Kardassis D, Chambaz J, Cardot P 1999 Cooperative binding of upstream stimulatory factor and hepatic nuclear factor 4 drives the transcription of the human apolipoprotein A-II gene. J Biol Chem 274:1216–1225
27. Lanigan TM, Russo AF 1997 Binding of upstream stimulatory factor and a cell-specific activator to the calcitonin/calcitonin gene-related peptide enhancer. J Biol Chem 272:18316–18324
28. Cirillo LA, McPherson CE, Bossard P, Stevens K, Cherian S, Shim EY, Clark KL, Burley SK, Zaret KS 1998 Binding of the winged-helix transcription factor HNF3 to a linker histone site on the nucleosome. EMBO J 17:244–254
29. Sun Q, Yu X, Degraff DJ, Matusik RJ 2009 Upstream stimulatory factor 2, a novel FoxA1-interacting protein, is involved in prostate-specific gene expression. Mol Endocrinol 23:2038–2047
30. Greenall A, Willingham N, Cheung E, Boam DS, Sharrocks AD 2001 DNA binding by the ETS-domain transcription factor PEA3 is regulated by intramolecular and intermolecular protein.protein interactions. J Biol Chem 276:16207–16215
31. Heckert LL 2001 Activation of the rat follicle-stimulating hormone receptor promoter by steroidogenic factor 1 is blocked by protein kinase a and requires upstream stimulatory factor binding to a proximal E box element. Mol Endocrinol 15:704–715
32. Ismail PM, Lu T, Sawadogo M 1999 Loss of USF transcriptional activity in breast cancer cell lines. Oncogene 18:5582–5591
33. Martin CC, Svitek CA, Oeser JK, Henderson E, Stein R, O'Brien RM 2003 Upstream stimulatory factor (USF) and neurogenic differentiation/β-cell E box transactivator 2 (NeuroD/BETA2) contribute to islet-specific glucose-6-phosphatase catalytic-subunit-related protein (IGRP) gene expression. Biochem J 371:675–686
34. Qyang Y, Luo X, Lu T, Ismail PM, Krylov D, Vinson C, Sawadogo M 1999 Cell-type-dependent activity of the ubiquitous transcription factor USF in cellular proliferation and transcriptional activation. Mol Cell Biol 19:1508–1517

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

Hiroyuki Ariyasu, Hiroshi Iwakura, Go Yamada, Naotetsu Kanamoto, Mika Bando, Kenji Kohno, Takahiro Sato, Masayasu Kojima, Kazuwa Nakao, Kenji Kangawa, and Takashi Akamizu

Ghrelin Research Project (H.A., H.I., M.B., K.Ka., T.A.), Translational Research Center, Kyoto University Hospital, Kyoto 606-8507, Japan; Department of Endocrinology and Metabolism (G.Y., N.K., K.N.), Kyoto University Graduate School of Medicine, Kyoto 606-8507, Japan; Laboratory of Molecular and Cell Genetics (K.Ko.), Graduate School of Biological Sciences, Nara Institute of Science and Technology, Nara 630-0192, Japan; Molecular Genetics (M.K., T.S.), Institute of Life Sciences, Kurume University, Kurume 839-0864, Japan; and Department of Biochemistry (K.Ka.), National Cardiovascular Center Research Institute, Osaka 565-8565, Japan

Ghrelin was initially identified as an endogenous ligand for the GH secretagogue receptor. When administrated 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

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

doi: 10.1210/en.2009-1040 Received September 1, 2009. Accepted January 12, 2010.
First Published Online February 25, 2010

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 correlated 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-Hin*dIII 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 *Not*I-*Xho*I 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-CAGTGCCGAGAGAACTG-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

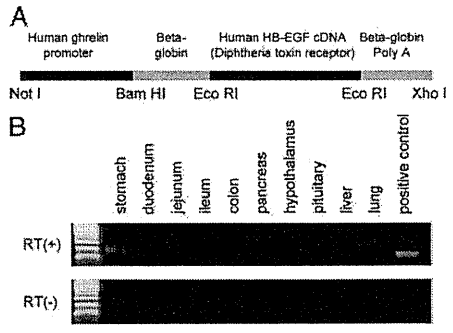

FIG. 1. Generation of GPDTR-Tg mice. A, The GPDTR-Tg construct contained a fusion gene comprised of the 5′flanking lesion of human ghrelin (4085 bp) and the DTR cDNA (human HB-EGF). B, Expression of DTR mRNA in various tissues of GPDTR-Tg mice at 8 wk of age. The human heart mRNA was used as a positive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

TABLE 1. PCR primers and TaqMan probes

Ghrelin	Sense	5′-GCATGCTCTGGATGGACATG-3′
	Antisense	5′-TGGTGGCTTCTTGGATTCCT-3′
	Probe	5′-AGCCCAGAGCACCAGAAAGCCCA-3′
GH	Sense	5′-AAGAGTTCGAGCGTGCCTACA-3′
	Antisense	5′-GAAGCAATTCCATGTCGGTTC-3′
	Probe	5′-CCATTCAGAATGCCCAGGCTGCTTTC-3′
GHRH	Sense	5′-AGGATGCAGCGACACGTAGA-3′
	Antisense	5′-TCTCCCCTTGCTTGTTCATGA-3′
	Probe	5′-CCACCAACTACAGGAAACTCCTGAGCCA-3′
SST	Sense	5′-AGCTGAGCAGGACGAGATGAG-3′
	Antisense	5′-ACAGGATGTGAATGTCTTCCAGTT-3′
	Probe	5′-CGAACCCAGCAATGGCACCCC-3′
IGF-1	Sense	5′-ACCCGGACCTACCAAAATGAC-3′
	Antisense	5′-GGTGTGAAGACGACATGATGTGT-3′
	Probe	5′-CACCTGCAATAAAG-3′
GHS-R	Sense	5′-CACCAACCTCTATCCAGCAT-3′
	Antisense	5′-CTGACAAACTGGAAGCGTTTGCA-3′
	Probe	5′-TCCGATCTGCTCATCTTCCTGTGCATG-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 unanesthelized 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 ± 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., Berkley, 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, preliminarily 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

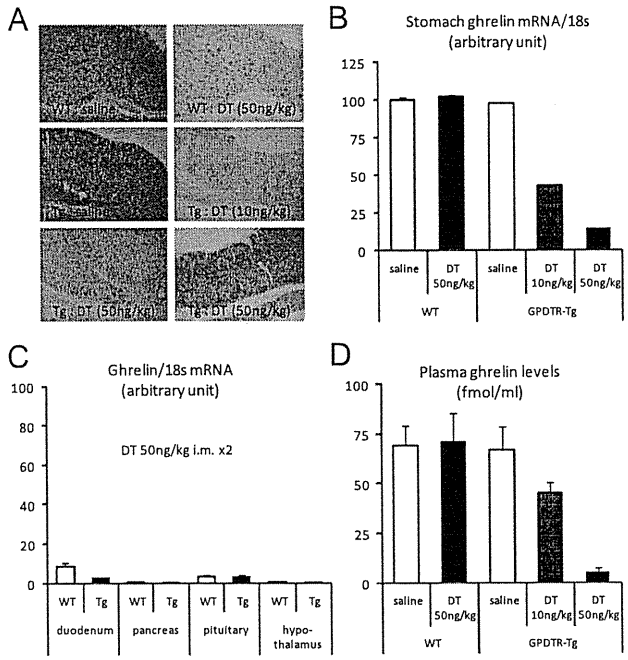

FIG. 2. Ablation of ghrelin-secretion cells. Eight-week-old male GPDTR-Tg mice (Tg) and nontransgenic littermates (WT) were injected with saline or 10 or 50 ng/kg of DT (im) on d 0 and 2, then analyzed on d 4. A, Histological analysis of stomach sections. Immunohistochemical analysis of ghrelin peptide expression and hematoxylin and eosin staining. Original magnification, ×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 ± 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

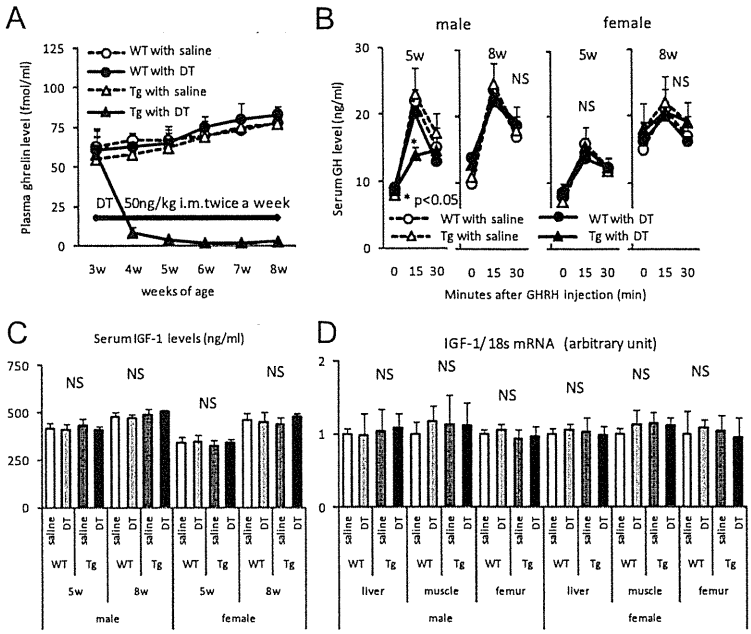

FIG. 3. The effects of a postweaning reduction in circulating ghrelin on the GH/IGF-I axis. Three-week-old GPDTR-Tg and WT mice were injected saline or DT at a dose of 50 ng/kg twice a week for 5 wk (from 3 to 8 wk old). A, Plasma ghrelin levels before and after DT injection. B, GH response to GHRH administration (180 μg/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 ± 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 expressions 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 ± 0.5 *vs.* 18.9 ± 0.7; female, 18.4 ± 1.0 *vs.* 18.5 ± 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.

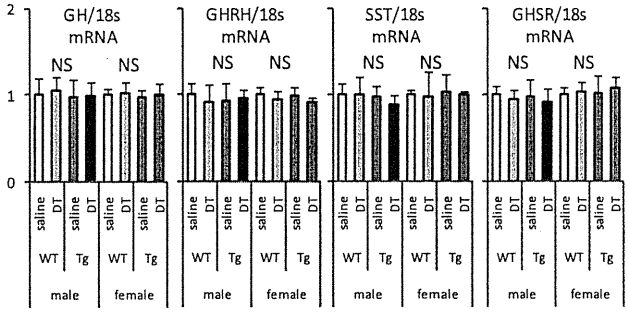

FIG. 4. The effects of a postweaning reduction in circulating ghrelin on the expression of mRNA encoding GHRH, SST,GH and GHS-R. Three-week-old GPDTR-Tg and WT mice were injected saline or DT at a dose of 50 ng/kg twice a week for 5 wk (from 3 to 8 wk old). Pituitary mRNA levels of GH and GHS-R and hypothalamic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

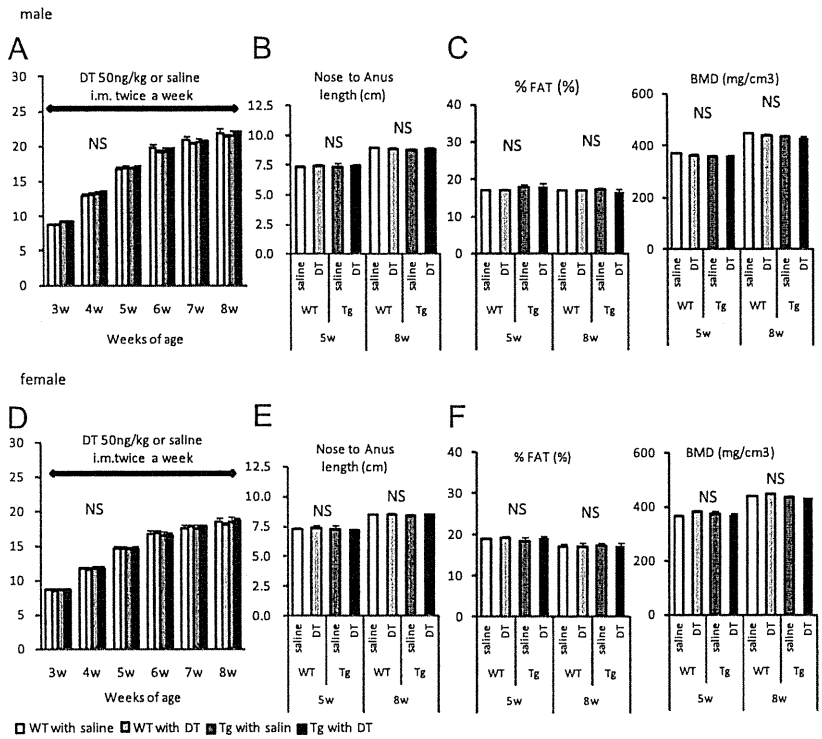

FIG. 5. The effects of a postweaning reduction in circulating ghrelin levels on somatic growth. Three-week-old GPDTR-Tg and WT mice were injected saline or DT at a dose of 50 ng/kg twice a week for 5 wk (from 3 to 8 wk old). A and D, Changes in body weight in male (A) and female mice (D). B and E, Nose 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 ± 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′-franking 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 statue 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 pluses 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.

Acknowledgments

We thank Dr. Shimatsu for fruitful discussions and Ms. Chieko Ishimoto and Ms. Chinami Shiraiwa for their excellent technical assistance.

Address all correspondence and requests for reprints to: Hiroyuki Ariyasu, M.D., Ph.D., Ghrelin Research Project, Translational Research Center, Kyoto University Hospital, 54 Shogin Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan. E-mail: ariyasu@kuhp.kyoto-u.ac.jp.

This work was supported by funds from the Japan Ministry of Education, Culture, Sports, Science, and Technology and the Ministry of Health, Labor, and Welfare of Japan, and by research grants from the Program for the Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation and from the Takeda Foundation in Japan.

Disclosure Summary: The authors have nothing to disclose.

References

1. Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K 1999 Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature 402:656–660
2. Date Y, Kojima M, Hosoda H, Sawaguchi A, Mondal MS, Suganuma T, Matsukura S, Kangawa K, Nakazato M 2000 Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans. Endocrinology 141:4255–4261
3. Takaya K, Ariyasu H, Kanamoto N, Iwakura H, Yoshimoto A, Harada M, Mori K, Komatsu Y, Usui T, Shimatsu A, Ogawa Y, Hosoda K, Akamizu T, Kojima M, Kangawa K, Nakao K 2000 Ghrelin strongly stimulates growth hormone release in humans. J Clin Endocrinol Metab 85:4908–4911
4. Hataya Y, Akamizu T, Takaya K, Kanamoto N, Ariyasu H, Saijo M, Moriyama K, Shimatsu A, Kojima M, Kangawa K, Nakao K 2001 A low dose of ghrelin stimulates growth hormone (GH) release synergistically with GH-releasing hormone in humans. J Clin Endocrinol Metab 86:4552
5. Pantel J, Legendre M, Cabrol S, Hilal L, Hajaji Y, Morisset S, Nivot S, Vie-Luton MP, Grouselle D, de Kerdanet M, Kadiri A, Epelbaum J, Le Bouc Y, Amselem S 2006 Loss of constitutive activity of the growth hormone secretagogue receptor in familial short stature. J Clin Invest 116:760–768
6. Okimura Y, Ukai K, Hosoda H, Murata M, Iguchi G, Iida K, Kaji H, Kojima M, Kangawa K, Chihara K 2003 The role of circulating ghrelin in growth hormone (GH) secretion in freely moving male rats. Life Sci 72:2517–2524
7. Sun Y, Ahmed S, Smith RG 2003 Deletion of ghrelin impairs neither growth nor appetite. Mol Cell Biol 23:7973–7981
8. Sun Y, Wang P, Zheng H, Smith RG 2004 Ghrelin stimulation of growth hormone release and appetite is mediated through the growth hormone secretagogue receptor. Proc Natl Acad Sci USA 101:4679–4684
9. Saito M, Iwawaki T, Taya C, Yonekawa H, Noda M, Inui Y, Mekada E, Kimata Y, Tsuru A, Kohno K 2001 Diphtheria toxin receptor-mediated conditional and targeted cell ablation in transgenic mice. Nat Biotechnol 19:746–750
10. Kanamoto N, Akamizu T, Tagami T, Hataya Y, Moriyama K, Takaya K, Hosoda H, Kojima M, Kangawa K, Nakao K 2004 Genomic structure and characterization of the 5′-flanking region of the human ghrelin gene. Endocrinology 145:4144–4153
11. Ariyasu H, Takaya K, Iwakura H, Hosoda H, Akamizu T, Arai Y, Kangawa K, Nakao K 2005 Transgenic mice overexpressing desacyl ghrelin show small phenotype. Endocrinology 146:355–364
12. Iwakura H, Akamizu T, Ariyasu H, Irako T, Hosoda K, Nakao K, Kangawa K 2007 Effects of ghrelin administration on decreased growth hormone status in obese animals. Am J Physiol Endocrinol Metab 293:E819–E825
13. Ariyasu H, Takaya K, Tagami T, Ogawa Y, Hosoda K, Akamizu T, Suda M, Koh T, Natsui K, Toyooka S, Shirakami G, Usui T, Shimatsu A, Doi K, Hosoda H, Kojima M, Kangawa K, Nakao K 2001 Stomach is a major source of circulating ghrelin, and feeding state determines plasma ghrelin-like immunoreactivity levels in humans. J Clin Endocrinol Metab 86:4753–4758
14. Hosoda H, Kojima M, Matsuo H, Kangawa K 2000 Ghrelin and des-acyl ghrelin: two major forms of rat ghrelin peptide in gastrointestinal tissue. Biochem Biophys Res Commun 279:909–913
15. Wortley KE, Anderson KD, Garcia K, Murray JD, Malinova L, Liu R, Moncrieffe M, Thabet K, Cox HJ, Yancopoulos GD, Wiegand SJ, Sleeman MW 2004 Genetic deletion of ghrelin does not decrease food intake but influences metabolic fuel preference. Proc Natl Acad Sci USA 101:8227–8232
16. Zigman JM, Nakano Y, Coppari R, Balthasar N, Marcus JN, Lee CE, Jones JE, Deysher AE, Waxman AR, White RD, Williams TD, Lachey JL, Seeley RJ, Lowell BB, Elmquist JK 2005 Mice lacking ghrelin receptors resist the development of diet-induced obesity. J Clin Invest 115:3564–3572
17. Pantel J, Legendre M, Nivot S, Morisset S, Vie-Luton MP, le Bouc Y, Epelbaum J, Amselem S 2009 Recessive isolated growth hormone deficiency and mutations in the ghrelin receptor. J Clin Endocrinol Metab 94:4334–4341
18. Popovic V, Miljic D, Pekic S, Pesko P, Djurovic M, Doknic M, Damjanovic S, Micic D, Cvijovic G, Glodic J, Dieguez C, Casanueva FF 2005 Low plasma ghrelin level in gastrectomized patients is accompanied by enhanced sensitivity to the ghrelin-induced growth hormone release. J Clin Endocrinol Metab 90:2187–2191
19. Jansson JO, Edén S, Isaksson O 1985 Sexual dimorphism in the control of growth hormone secretion. Endocr Rev 6:128–150
20. Edén S 1979 Age- and sex-related differences in episodic growth hormone secretion in the rat. Endocrinology 105:555–560
21. Giustina A, Veldhuis JD 1998 Pathophysiology of the neuroregulation of growth hormone secretion in experimental animals and the human. Endocr Rev 19:717–797
22. Roth J, Glick SM, Yalow RS, Bersons A 1963 Hypoglycemia: a potent stimulus to secretion of growth hormone. Science 140: 987–988
23. Katz SH, Dhariwal AP, McCann SM 1967 Effect of hypoglycemia on the content of pituitary growth hormone (GH) and hypothalamic growth hormone-releasing factor (GHRF) in the rat. Endocrinology 81:333–339
24. Parker ML, Hammond JM, Daughaday WH 1967 The arginine provocative test: an aid in the diagnosis of hyposomatotropism. J Clin Endocrinol Metab 27:1129–1136
25. Shuto Y, Shibasaki T, Otagiri A, Kuriyama H, Ohata H, Tamura H, Kamegai J, Sugihara H, Oikawa S, Wakabayashi I 2002 Hypothalamic growth hormone secretagogue receptor regulates growth hormone secretion, feeding, and adiposity. J Clin Invest 109:1429–1436

Establishment of a Novel Ghrelin-Producing Cell Line

Hiroshi Iwakura, Yushu Li, Hiroyuki Ariyasu, Hiroshi Hosoda, Naotetsu Kanamoto, Mika Bando, Go Yamada, Kiminori Hosoda, Kazuwa Nakao, Kenji Kangawa, and Takashi Akamizu

Ghrelin Research Project (H.I., Y.L., H.A., M.B., T.A.), Translational Research Center, and Department of Medicine and Clinical Science, Endocrinology, and Metabolism (N.K., G.Y., K.H., K.N.), Kyoto University Hospital, Kyoto University Graduate School of Medicine, Kyoto 606-8507, Japan; and National Cardiovascular Center Research Institute (H.H., K.K.), Osaka 565-8565, Japan

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

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

doi: 10.1210/en.2010-0090 Received January 22, 2010. Accepted March 12, 2010.
First Published Online April 7, 2010

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 μg/ml streptomycin at 37 C in 10% CO_2. 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% CO_2 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% OsO_4 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% CH_3CN/0.1% trifluoroacetic acid and eluted with 60% CH_3CN/0.1% trifluoroacetic acid. Eluates 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 cycler (Applied Biosystems) with Ampli-taqgold 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 (5.0×10^6 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 (5×10^5 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: GCGCUUCUGUUUAAUUAUCUCUGCA (si1) and AGGAAGUCCAUAGGCUGACCUUCUU (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 1.0×10^7 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.

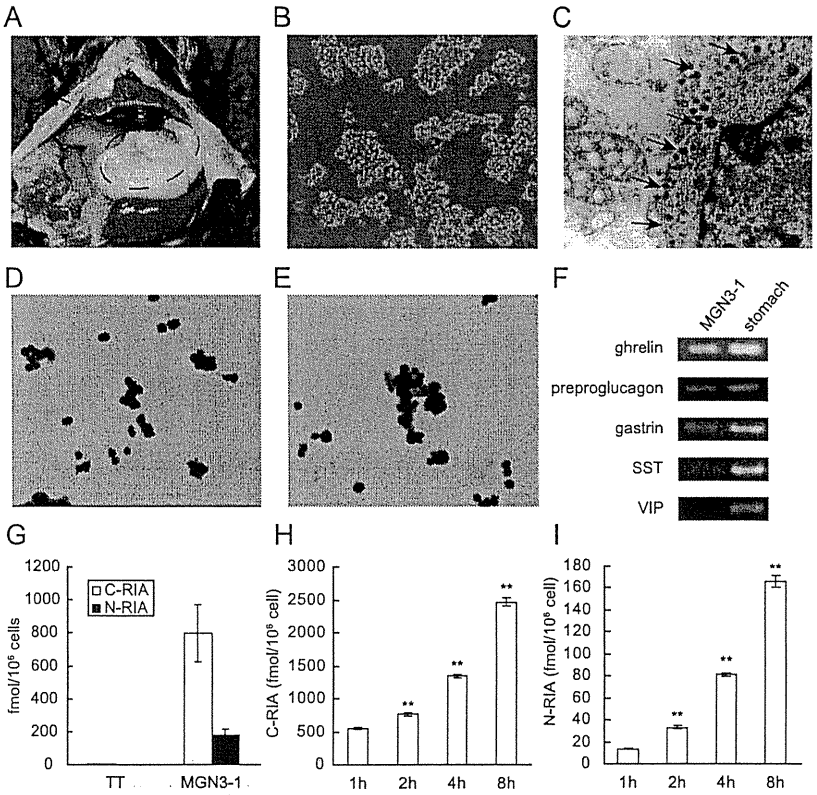

FIG. 1. Establishment of MGN3-1 cells A, Macroscopic findings of a ghrelinoma in a GP-Tag Tg mouse. B and C, Morphology of MGN3-1 cells by optic (B) and electron (C) microscopy. Secretory granules were observed (*arrow*). D and E, MGN3-1 cells were immunostained with anti-C-terminal ghrelin (D) and anti-N-terminal (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 ± 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 antiglucagon 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

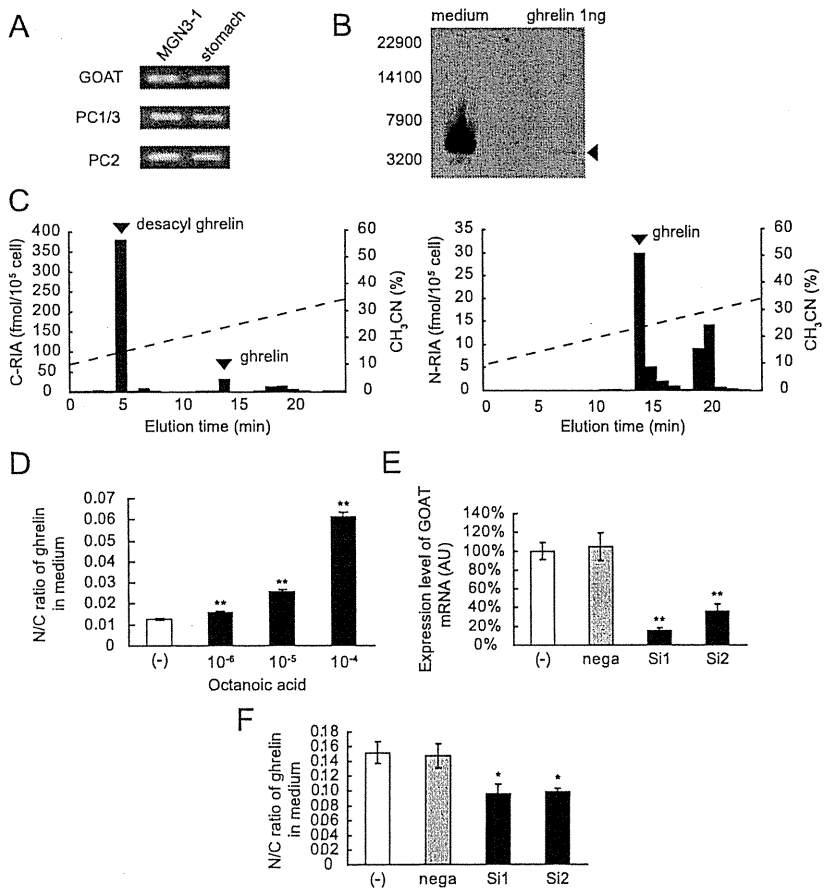

FIG. 2. Acylation and processing of ghrelin in MGN3-1 cells. A, RT-PCR analysis of GOAT, PC1/3, and PC2 mRNA expressions in MGN3-1 cells. B, Western blot analysis of culture medium in which MGN3-1 cells were cultured for 3 d. Rat ghrelin peptide was used as a positive control. C, Representative reverse-phase 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

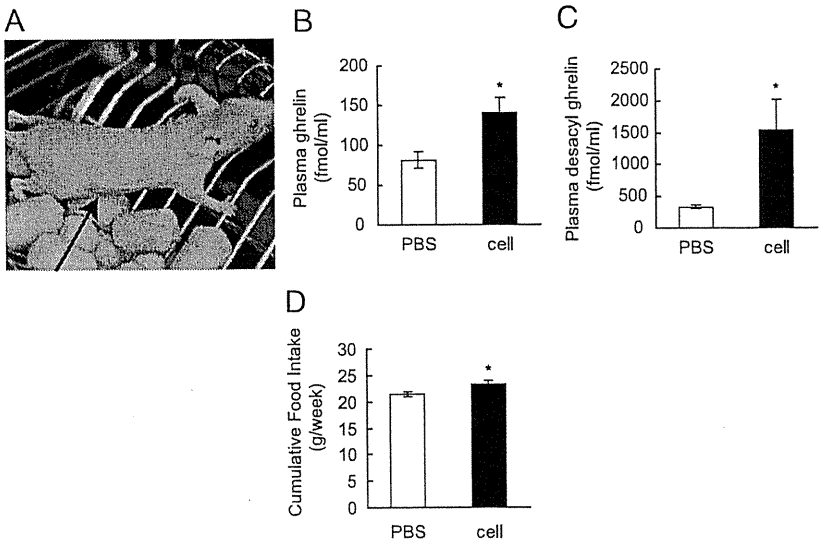

FIG. 3. Transplantation of MGN3-1 cell to nude mouse. A, Macroscopic findings of nude mice injected with MGN3-1 cells. B and C, Plasma ghrelin (B) and desacyl ghrelin (C) levels in nude mice at 4 wk after injection of saline or MGN3-1 cells. D, Cumulative food intake over a week (between 4 and 5 wk after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

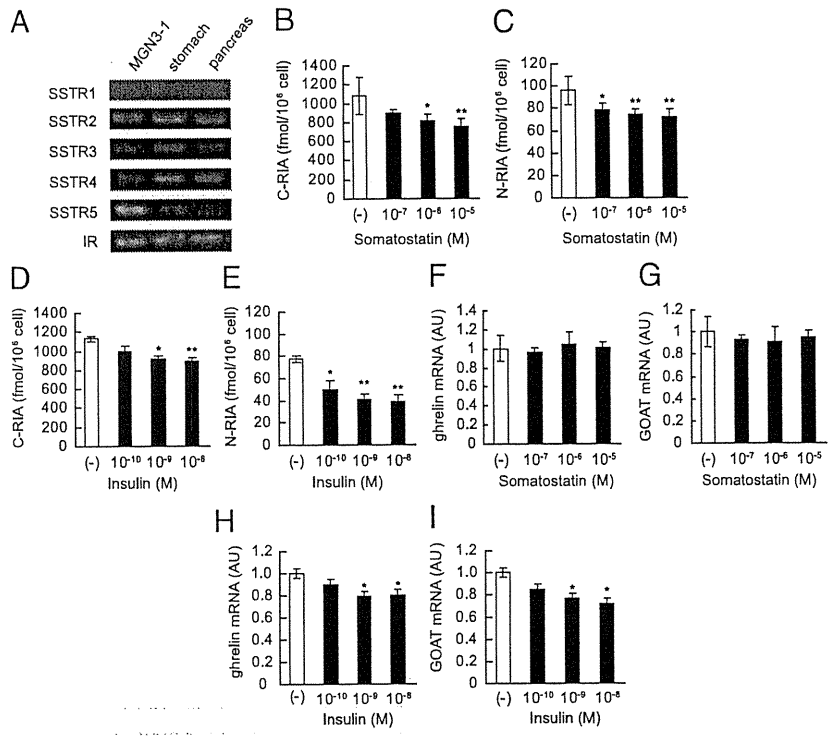

FIG. 4. The effect of somatostatin and insulin on ghrelin secretion and expression in MGN3-1 cells. A, RT-PCR analysis of somatostatin receptor (SSTR) types 1–5 and insulin receptor (IR) mRNA expressions in MGN3-1 cells. B–F, Ghrelin secretion was suppressed when MGN3-1 cells were incubated in DMEM supplemented 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 (−).

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.

Acknowledgments

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

Address all correspondence and requests for reprints to: Hiroshi Iwakura, M.D., Ph.D., 54 Shogoin Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan. E-mail: hiwaku@kuhp.kyoto-u.ac.jp.

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

Disclosure Summary: All authors have nothing to declare.

References

1. Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K 1999 Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature 402:656–660
2. Cummings DE, Purnell JQ, Frayo RS, Schmidova K, Wisse BE, Weigle DS 2001 A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans. Diabetes 50:1714–1719
3. Tschöp M, Wawarta R, Riepl RL, Friedrich S, Bidlingmaier M, Landgraf R, Folwaczny C 2001 Post-prandial decrease of circulating human ghrelin levels. J Endocrinol Invest 24:RC19–RC21
4. Tschöp M, Weyer C, Tataranni PA, Devanarayan V, Ravussin E, Heiman ML 2001 Circulating ghrelin levels are decreased in human obesity. Diabetes 50:707–709
5. Ariyasu H, Takaya K, Tagami T, Ogawa Y, Hosoda K, Akamizu T, Suda M, Koh T, Natsui K, Toyooka S, Shirakami G, Usui T, Shimatsu A, Doi K, Hosoda H, Kojima M, Kangawa K, Nakao K 2001 Stomach is a major source of circulating ghrelin, and feeding state determines plasma ghrelin-like immunoreactivity levels in humans. J Clin Endocrinol Metab 86:4753–4758
6. Kanamoto N, Akamizu T, Hosoda H, Hataya Y, Ariyasu H, Takaya K, Hosoda K, Saijo M, Moriyama K, Shimatsu A, Kojima M, Kangawa K, Nakao K 2001 Substantial production of ghrelin by a human medullary thyroid carcinoma cell line. J Clin Endocrinol Metab 86:4984–4990
7. De Vriese C, Delporte C 2007 Autocrine proliferative effect of ghrelin on leukemic HL-60 and THP-1 cells. J Endocrinol 192:199–205
8. De Vriese C, Grégoire F, De Neef P, Robberecht P, Delporte C 2005 Ghrelin is produced by the human erythroleukemic HEL cell line and involved in an autocrine pathway leading to cell proliferation. Endocrinology 146:1514–1522
9. Gutierrez JA, Solenberg PJ, Perkins DR, Willency JA, Knierman MD, Jin Z, Witcher DR, Luo S, Onyia JE, Hale JE 2008 Ghrelin octanoylation mediated by an orphan lipid transferase. Proc Natl Acad Sci USA 105:6320–6325
10. Iwakura H, Ariyasu H, Li Y, Kanamoto N, Bando M, Yamada G, Hosoda H, Hosoda K, Shimatsu A, Nakao K, Kangawa K, Akamizu T 2009 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
11. Iwakura H, Hosoda K, Doi R, Komoto I, Nishimura H, Son C, Fujikura J, Tomita T, Takaya K, Ogawa Y, Hayashi T, Inoue G, Akamizu T, Hosoda H, Kojima M, Kangawa K, Imamura M, Nakao K 2002 Ghrelin expression in islet cell tumors: augmented expression of ghrelin in a case of glucagonoma with multiple endocrine neoplasm type I. J Clin Endocrinol Metab 87:4885–4888
12. Hosoda H, Kojima M, Matsuo H, Kangawa K 2000 Ghrelin and des-acyl ghrelin: two major forms of rat ghrelin peptide in gastrointestinal tissue. Biochem Biophys Res Commun 279:909–913
13. Iwakura H, Ariyasu H, Kanamoto N, Hosoda K, Nakao K, Kangawa K, Akamizu T 2008 Establishment of a novel neuroblastoma mouse model. Int J Oncol 33:1195–1199
14. Iwakura H, Hosoda K, Son C, Fujikura J, Tomita T, Noguchi M, Ariyasu H, Takaya K, Masuzaki H, Ogawa Y, Hayashi T, Inoue G, Akamizu T, Hosoda H, Kojima M, Itoh H, Toyokuni S, Kangawa K, Nakao K 2005 Analysis of rat insulin II promoter-ghrelin transgenic mice and rat glucagon promoter-ghrelin transgenic mice. J Biol Chem 280:15247–15256
15. Akamizu T, Shinomiya T, Irako T, Fukunaga M, Nakai Y, Kangawa K 2005 Separate measurement of plasma levels of acylated and desacyl ghrelin in healthy subjects using a new direct ELISA. J Clin Endocrinol Metab 90:6–9
16. Yang J, Brown MS, Liang G, Grishin NV, Goldstein JL 2008 Identification of the acyltransferase that octanoylates ghrelin, an appetite-stimulating peptide hormone. Cell 132:387–396
17. Zhu X, Cao Y, Voogd K, Voogd K, Steiner DF 2006 On the processing of proghrelin to ghrelin. J Biol Chem 281:38867–38870
18. Nishi Y, Hiejima H, Hosoda H, Kaiya H, Mori K, Fukue Y, Yanase T, Nawata H, Kangawa K, Kojima M 2005 Ingested medium-chain fatty acids are directly utilized for the acyl modification of ghrelin. Endocrinology 146:2255–2264
19. Shimada M, Date Y, Mondal MS, Toshinai K, Shimbara T, Fukunaga K, Murakami N, Miyazato M, Kangawa K, Yoshimatsu H, Matsuo H, Nakazato M 2003 Somatostatin suppresses ghrelin secretion from the rat stomach. Biochem Biophys Res Commun 302:520–525
20. Silva AP, Bethmann K, Raulf F, Schmid HA 2005 Regulation of ghrelin secretion by somatostatin analogs in rats. Eur J Endocrinol 152:887–894
21. Norrelund H, Hansen TK, Orskov H, Hosoda H, Kojima M, Kangawa K, Weeke J, Moller N, Christiansen JS, Jorgensen JO 2002 Ghrelin immunoreactivity in human plasma is suppressed by somatostatin. Clin Endocrinol (Oxf) 57:539–546
22. Broglio F, Koetsveld Pv P, Benso A, Gottero C, Prodam F, Papotti M, Muccioli G, Gauna C, Hofland L, Deghenghi R, Arvat E, Van Der Lely AJ, Ghigo E 2002 Ghrelin secretion is inhibited by either somatostatin or cortistatin in humans. J Clin Endocrinol Metab 87:4829–4832
23. Sugino T, Yamaura J, Yamagishi M, Kurose Y, Kojima M, Kangawa K, Hasegawa Y, Terashima Y 2003 Involvement of cholinergic neurons in the regulation of the ghrelin secretory response to feeding in sheep. Biochem Biophys Res Commun 304:308–312
24. Broglio F, Gottero C, Van Koetsveld P, Prodam F, Destefanis S, Benso A, Gauna C, Hofland L, Arvat E, van der Lely AJ, Ghigo E 2004 Acetylcholine regulates ghrelin secretion in humans. J Clin Endocrinol Metab 89:2429–2433
25. Grinspoon S, Miller KK, Herzog DB, Grieco KA, Klibanski A 2004 Effects of estrogen and recombinant human insulin-like growth factor-I on ghrelin secretion in severe undernutrition. J Clin Endocrinol Metab 89:3988–3993
26. Koutkia P, Canavan B, Breu J, Johnson ML, Grinspoon SK 2004 Nocturnal ghrelin pulsatility and response to growth hormone secretagogues in healthy men. Am J Physiol Endocrinol Metab 287: E506–E512
27. Basa NR, Wang L, Arteaga JR, Heber D, Livingston EH, Taché Y 2003 Bacterial lipopolysaccharide shifts fasted plasma ghrelin to postprandial levels in rats. Neurosci Lett 343:25–28
28. Murdolo G, Lucidi P, Di Loreto C, Parlanti N, De Cicco A, Fatone C, Fanelli CG, Bolli GB, Santeusanio F, De Feo P 2003 Insulin is required for prandial ghrelin suppression in humans. Diabetes 52:2923–2927
29. Saad MF, Bernaba B, Hwu CM, Jinagouda S, Fahmi S, Kogosov E, Boyadjian R 2002 Insulin regulates plasma ghrelin concentration. J Clin Endocrinol Metab 87:3997–4000

Generation of Transgenic Mice Overexpressing a Ghrelin Analog

Go Yamada, Hiroyuki Ariyasu, Hiroshi Iwakura, Hiroshi Hosoda, Takashi Akamizu, Kazuwa Nakao, and Kenji Kangawa

Department of Endocrinology and Metabolism (G.Y., K.N.), Kyoto University Graduate School of Medicine, and Ghrelin Research Project (H.A., H.I., T.A., K.K.), Translational Research Center, Kyoto University Hospital, Kyoto 606-8507, Japan; and Department of Biochemistry (H.H., K.K.), National Cardiovascular Center Research Institute, Osaka 565-8565, Japan

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 Ser^3 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 Ser^3 and could be synthesized *in vivo*. As the replacement of Ser^3 of ghrelin with Trp^3 (Trp^3-ghrelin) preserves a low level of ghrelin activity and Trp^3-ghrelin can be synthesized *in vivo*, we generated mice overexpressing Trp^3-ghrelin by using the hSAP (human serum-amyloid-P) promoter. Plasma Trp^3-ghrelin concentrations in the Tg mice were approximately 85-fold higher than plasma ghrelin concentrations in non-Tg littermates. Because Trp^3-ghrelin is approximately 1/10–1/20 less potent than ghrelin *in vivo*, plasma Trp^3-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-ontanoylic acid at the third Ser residue (Ser^3) (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 Ser^3 and is devoid of ghrelin activity. 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 Ser^3 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

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

doi: 10.1210/en.2010-0635 Received June 4, 2010. Accepted September 20, 2010.
First Published Online October 20, 2010

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; Trp^3-ghrelin, ghrelin analog with the third amino-acid residue (Ser^3) 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 Ser^3 with Trp^3 (Trp^3-ghrelin) preserved ghrelin activity with an EC_{50} of 31 nM in comparison with 1.3 nM for intact ghrelin. Replacement of Ser^3 with Val^3, Leu^3, or Ile^3 led to complete loss of ghrelin potency. Although ghrelin analog, in which the Ser^3 residue was replaced by Trp (Trp^3-ghrelin) is approximately 24-fold less active than native ghrelin *in vitro*, it can be synthesized *in vivo*. Thus, we selected Trp^3-ghrelin as a candidate ghrelin analog.

In this study, we examined whether Trp^3-ghrelin exerts ghrelin-like activity *in vivo*. After confirming this activity, we generated Tg mice overexpressing Trp^3-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 Trp^3-ghrelin

Eight-week-old male C57BL/6 mice were purchased from Japan CLEA. Ghrelin was obtained from Peptide Research Institute (Osaka, Japan). Trp^3-ghrelin, in which the Ser^3 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 Trp^3-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 Trp^3-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 Trp^3-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, Trp^3-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 *Eco*RI 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′-GCTCTGGGCTCAGGAACCAGGAGCCTGCCATGGCCATGTCC-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 *Sal*I and *Hin*dIII (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 Trp^3-ghrelin on food intake and GH secretion

To elucidate whether Trp^3-ghrelin has ghrelin-like potency *in vivo*, 8-wk-old male C57BL/6 mice were administrated vehicle, ghrelin, or Trp^3-ghrelin before determining food intake over a period of 2 h. Injection of ghrelin or Trp^3-ghrelin stimulated food intake in a dose-dependent manner (Fig. 1A). The 2-h food intake after injection of 3600 mcg/kg of Trp^3-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 Trp^3-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 Trp^3-ghrelin inhibited glucose-stimulated insuline release in a dose-dependent manner (Fig. 1C). The 10-min insulin response was significantly inhibited by 3600 mcg/kg Trp^3-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 Trp^3-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 (Trp^3-ghrelin needs about 20-fold amount for stimulation of food intake, about 10-fold amount for GH secretion, and about 10-fold amount for