KLF4 positively regulates human ghrelin expression

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Ghrelin, an endogenous ligand of the GH (growth hormone) secretagogue receptor, influences many metabolic processes including GH secretion, food intake, energy balance, insulin secretion and adipogenesis. Although ghrelin exhibits a variety of biological functions, the mechanism by which ghrelin expression is regulated is unknown. Ghrelin is expressed in the gastrointestinal tract, predominantly in the stomach, as is KLF4 (Krüppel-like factor 4). Therefore we investigated whether ghrelin expression is associated with KLF4, and found that the tissue distribution of ghrelin corresponded with that of KLF4. Furthermore, treatment with butyrate, an inducer of KLF4 expression, stimulated ghrelin expression, and fasting, which induces ghrelin expression, also increased KLF4 expression, suggesting that ghrelin expression is associated with KLF4. Then, we investigated the effects of KLF4 on the human ghrelin-promoter activity and identified a KLF4-responsive region in the promoter. KLF4 expression specifically stimulated human ghrelin-promoter activity in a dose-dependent manner in human gastric-cancer AGS cells. However, this effect was not seen in response to a mutant KLF4 construct. Transfection studies using mutant constructs containing 5′-deletions in the human ghrelin promoter revealed that the KLF4-responsive element is located between −1228 and −1105. Electrophoretic mobility shift assays using oligonucleotides containing −1165/−1146 revealed the binding of KLF4 to the human ghrelin promoter. Finally, deletion of the −1165/−1146 region abrogated KLF4-induced transactivation of the ghrelin promoter. Collectively, these results indicate that KLF4 positively regulates human ghrelin expression via binding to a KLF-responsive region in the promoter.

Key words: gastrointestinal tissue, gene expression, ghrelin, Krüppel-like factor 4 (KLF4), promoter.

INTRODUCTION

The gastrointestinal tract plays a crucial role in homeostasis through its effects on the digestion, absorption and assimilation of ingested nutrients. Ghrelin, an orexigenic hormone composed of 28 amino acids that is secreted from the stomach during fasting, stimulates the release of GH (growth hormone) from the pituitary gland and regulates both food intake and energy balance [1–3]. The effect of ghrelin on appetite is mediated by the hypothalamus through the stimulation of NPY (neuropeptide Y), AGRP (Agouti-related protein) and orexin release [4,5]; the inhibition of Somatostatin and its analogues, as well as urocortin-1, a potent anorexigenic peptide, also suppress ghrelin expression [11,12]. GH deficiency decreases the mRNA expression of ghrelin in the stomach and plasma ghrelin levels [13], suggesting that GH from the pituitary regulates ghrelin production in the stomach. Clinical data show that plasma ghrelin is associated with obesity [14]. In Prader–Willi syndrome, which is the most common form of human syndromic obesity, plasma ghrelin is increased 3- to 4-fold [15]. In contrast, ghrelin levels are reduced or normal in patients with other genetic obesities, such as leptin-receptor mutations or melanocortin-4-receptor mutations [16,17].

The human ghrelin promoter has been isolated and characterized [18], and two transcriptional initiation sites are known to exist within human ghrelin: one at −80 and the other at −555. Both transcripts are expressed at equal levels in the human stomach, whereas the longer transcript is expressed primarily in the thyroid medullary carcinoma TT cell line. The basic helix–loop–helix transcription factors USF1 (upstream transcription factor 1) and USF2 specifically bind to E-boxes in the human ghrelin promoter as a heterodimer, and may play a role in the regulation of human ghrelin expression; however, the regulatory mechanism of ghrelin transcription is unknown. KLF4 (Krüppel-like factor 4), also known as GKLKF (gut-enriched Krüppel-like factor), is a zinc-finger-containing transcription factor that binds to GC-rich DNA with a consensus binding sequence of CACCC [19,20]. KLF4 is a member of...
the KLF family, which contains at least 16 members that are highly expressed in the gastrointestinal tract (especially the colon and small intestine) and other epithelial tissues, including skin, lung, testis and thymus [21]. KLF4 contains both transcriptional activation and repression domains, and is known to activate and repress gene expression [22]. Analyses of KLF4 target genes have shown that KLF4 regulates cell growth and differentiation in these tissues by promoting the down-regulation of the ODC (ornithine decarboxylase gene) promoter [23] and the up-regulation of several keratin-encoding genes [24,25]. In addition, KLF4 is required for p53-mediated G1/M cell-cycle arrest following DNA damage [26] and the terminal differentiation of goblet cells in the colon [27]. KLF4 is also required for normal gastric epithelial proliferation and differentiation.

Among the major organs, the gastrointestinal tract shows the highest level of KLF4 expression; thus we investigated whether KLF4 contributes to ghrelin expression in the stomach. We found that KLF4 increases ghrelin transcription in the stomach, which may play an important role in the tissue-specific or fasting-mediated expression of ghrelin.

**EXPERIMENTAL**

**Cell culture and plasmid constructs**

HEK-293 cells (human embryonic kidney cells) and AGS cells (human gastric cancer cells; both obtained from ATCC) were cultured in Dulbecco’s modified Eagle’s medium containing glucose (Invitrogen) supplemented with 10% (v/v) fetal bovine serum (Gibco BRL). Sodium butyrate was obtained from Sigma–Aldrich.

The regions of the human ghrelin promoter corresponding to nucleotides −997 to −33 and −997 to −495 were amplified by PCR from human genomic DNA and inserted into the KpnI/Xhol sites in pGL3-Basic vector (Promega). Deletions in the 5′-flanking regions of the promoter were constructed by PCR using pairwise combinations of the sense primers 5′-GGTACCTCTGCTGGGCTTCACTTGGAG-3′ [for P(−107)–Luc (luciferase)], 5′-GGTACCGTTCCAGGACAGCTGGGAG-3′ [for P(−778)–Luc], 5′-GGTACCTCTCCGGAGAGGAT-3′ [for P(−1105)–Luc], 5′-GGTACCGGACAAAGGCTGGTGGGAG-3′ [for P(−1228)–Luc], and 5′-GGTACCTTTTCCTCCGCGGAATAAAAGGA-3′ [for P(−1997)–Luc] with the antisense primers 5′-CTCGAGAGCTGTCAGCAACAGGTC-3′ [for P(−495)], and 5′-CTCGAGGAGGAGAGGCTGTCCT-3′ [for P(−33)].

**Transient transfection and luciferase assay**

HEK-293 cells grown in six-well plates were transiently transfected with human ghrelin promoters and KLF4 expression plasmid using Lipofectamine™ reagent (Invitrogen) according to the manufacturer’s instructions. At 48 h post-transfection, the cells were lysed in reporter lysis buffer (Promega) and luciferase activity was measured using a Luciferase Assay System (Promega). To normalize the transfection efficiency, a PMCV-β-gal vector (Promega) was included in each transfection as an internal-control plasmid and luciferase activity was normalized to β-galactosidase activity in each transfection.

**EMSA (electrophoretic mobility shift assay)**

Probes were annealed with the following oligomers: the sense oligomers 5′-CTGGTGAGGGAGGCAAGGCA-3′ (for site 1), 5′-TGAAAAAGCCAGGCAGGAGC-3′ (for site 2) and 5′-GGTACATTCTGAGCTTGGGAC-3′ (for site 3); and the antisense oligomers 5′-GGTACATTTCTCCCTGGAGC-3′ (for site 1), 5′-GGTACCTGAGCTGCTTGA-3′ (for site 2), and 5′-ATTTCGAGGTGTCATGAC-3′ (for site 3). Each set of oligomers was mixed with 5× sequencing buffer (200 mM Tris/HCl, pH 7.5, 100 mM MgCl2, and 250 mM NaCl) and then heated at 99°C for 5 min. The oligomers were cooled naturally and loaded on to a special agarose gel [3:1 (w/v) NuSieve agar/agar (Promega)] for elution using a MREmaid Kit (Qiagen). EMSAs were performed as previously described [28]. For competition experiments or antibody-supershift assays, nuclear extracts (5 μg) were incubated with unlabelled competitors or antibodies for 30 min at room temperature (25°C), followed by incubation with radiolabelled probe for 30 min at room temperature, and then loaded on to 5% native polyacrylamide gels for separation by SDS/PAGE. The dried gels were exposed at −70°C for more than 24 h. Antibodies for KLF4 and ATF3 (activating transcription factor 3) were purchased from Santa Cruz Biotechnology.

**Preparation of nuclear protein**

Nuclear extracts were prepared from AGS cells as described previously [29]. The plates were scraped and centrifuged, then mixed with 1 ml of cold Buffer A [25 mM HEPES, pH 7.0, 25 mM KCl, 0.05 mM EDTA, 5 mM MgCl2, 10% (v/v) glycerol, 0.1% (v/v) Nonidet P40 and 1 mM DTT (dithiothreitol) containing protease and phosphatase inhibitors]. The samples were resuspended gently and ultracentrifuged at 40000 rev./min for 15 min in a Beckman TLA rotor. They were then mixed with a 1:10 vol. of cold Buffer B (0.3 M HEPES, pH 7.6, 1.4 M KCl, 30 mM MgCl2, and 1 mM DTT containing protease and phosphatase inhibitors) and additional cold Buffer C [50 mM HEPES, pH 7.6, 50 mM KCl, 0.1 mM EDTA, 1 mM DTT and 10% (v/v) glycerol containing protease and phosphatase inhibitors] to bring the final volume to 315 μl. After adding 35 μl of 3 M ammonium sulfate, the samples were rocked for 30 min in a cold room. The pelleted samples were resuspended in 30–50 μl of Buffer C and frozen at −70°C.

**RT–PCR (reverse transcription–PCR)**

Total RNA was extracted using QIAzol Lysis Reagent (Qiagen). The mRNA in the samples was reverse-transcribed using a Superscript™ II First Strand cDNA Synthesis Kit (Invitrogen). The resulting cDNA was amplified by PCR using the primer pairs ghrelin-F (5′-ATCTGAGTTGTGCTGCT-3′) and ghrelin-R (5′-GATACTGACCTGACGAC-3′) and KLF4-F (5′-CACAAAGATTCCACATCTCAA-3′) and KLF4-R (5′-CTCCTAT- GTGTAAGGCGAGG-3′). The housekeeping gene β-actin was amplified using the sense primer 5′-GTCGGTACACCTGGCATTTGT-3′ and the antisense primer 5′-CTCCTAGCTTGAGGGTTGAA-3′. RT–PCR blots were quantified using Tina 2.0 software (Raytest) and normalized to β-actin.

**Experimental animals**

All protocols were approved by the Animal Care and Use Committee of the National Institute of Health and KFDA (Korea: Food & Drug Administration). Control mice (8-week-old male

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Positive regulation of ghrelin expression by KLF4

Figure 1 Ghrelin expression is correlated with KLF4 expression

(A) mRNA expression in various tissues of 8-week-old male ICR mice was determined by RT–PCR using primers specific for ghrelin and KLF4 respectively. Tissues: 1, stomach; 2, liver; 3, large intestine; 4, kidney; 5, muscle. Mean values indicated by different labels (a, b, c, d) are significantly different from each other (P = 0.05), on the basis of Duncan’s multiple-range test. (B) Butyrate stimulates ghrelin expression. AGS cells were treated with various concentrations (0, 1, 2 and 4 mM) of butyrate for 12 h. mRNA expression was determined by RT–PCR. (C) siRNA-mediated knockdown of KLF4 results in the suppression of induction of ghrelin expression by butyrate. AGS cells were transfected with KLF4 siRNA or with scrambled RNA (siRNA whose sequence was scrambled from that of KLF4 siRNA, as a negative control) and then treated with 4 mM butyrate for 12 h. mRNA expression of ghrelin was determined by RT–PCR. The results were compared with those of no siRNA without butyrate. (D) KLF4 overexpression increases ghrelin expression. AGS cells were transfected with various amounts (0, 0.25, 0.5 and 1 μg) of pcDNA-3.1–KLF4 DNA. mRNA expression of ghrelin was determined by RT–PCR (left), and the protein level of KLF4 was estimated by Western-blot analysis (right). All values represent the mean ± S.E.M. from three independent experiments. *, P < 0.05; **, P < 0.001.

ICR) were obtained from the Experimental Animal Resource Laboratory of the KFDA. After 48 h of fasting and 24 h of refeeding, the mice were killed, and their stomachs, livers, kidneys, muscles and large intestines were collected and stored at −80°C for mRNA isolation.

Statistical analysis

All experiments were performed at least three times. The results were expressed as the mean ± S.E.M. Statistical analysis was performed using Student’s t test. (The results in Figure 1A were...
analysed using Duncan's multiple-range test. The results were considered statistically significant at $P < 0.05$.

**RESULTS**

**Ghrelin expression is correlated with KLF4 expression**

To analyse the correlation between ghrelin and KLF4 expression, we examined the expression patterns of ghrelin and KLF4 in various tissues (e.g., stomach, liver, kidney, muscle and large intestine) from 8-week-old male ICR mice by RT–PCR. As shown in Figure 1(A), ghrelin expression was highest in the stomach and large intestine, which is consistent with previous results (Figure 1A, left). In accordance with ghrelin, KLF4 displayed a similar expression pattern (Figure 1A, right). Butyrate, the most abundant short-chain fatty acid in the gastrointestinal tract, was previously reported to stimulate KLF4 expression [30]. Therefore, to determine whether butyrate also stimulates ghrelin expression through an increase in KLF4 expression, we treated AGS cells with butyrate for 12 h and analysed ghrelin expression. Butyrate treatment increased ghrelin expression (Figure 1B, left), concomitant with the induction of KLF4 expression in AGS cells (Figure 1B, right). To confirm the observation that KLF4 expression induced by butyrate leads to increased ghrelin expression, we inhibited KLF4 using siRNA and determined the effects of butyrate on ghrelin expression. KLF4 siRNA effectively inhibited expression of KLF4 (Figure 1C, left), and suppression of KLF4 by siRNA blocked the butyrate-induced
ghrelin expression (Figure 1C, right). Together, these results suggest that ghrelin expression is associated with KLF4 expression. Next, we examined how KLF4 overexpression affects the transcription of ghrelin in AGS cells using a pcDNA-3.1–KLF4 construct. As shown in Figure 1(D), overexpression of KLF4 led to an increase in the mRNA expression of ghrelin in proportion to the level of KLF4 protein.

**KLF4 increases the activity of the human ghrelin promoter**

Two transcripts may be produced from the human ghrelin promoter: a long transcript (transcript A) and a short transcript (transcript B). The transcription-initiation sites are located at −80 and −555 respectively. To investigate the effects of KLF4 on the expression of human ghrelin, we isolated the 5′-flanking regions of transcripts A and B respectively. Both the −1997/−33 upstream region of transcript A and the −1997/−495 upstream region of transcript B were isolated by PCR and inserted into pGL3-Basic to create 1997A and 1997B (Figure 2A). The constructs were then transiently transfected into HEK-293 cells with a KLF4 expression vector and luciferase activity was examined. As shown in Figure 2(B), HEK-293 cells transfected with KLF4 showed a dose-dependent increase in ghrelin-promoter activity. However, the increase was significantly inhibited by the co-transfection of a mutant KLF4 construct containing only the zinc-finger DNA-binding domain without the transcriptional regulatory domain or nuclear localization sequence (Figure 2C). Furthermore, we examined the effects of other KLF transcription factors on ghrelin expression in HEK-293 cells. As shown in Figure 2(D), although KLF2 and KLF5 increase the ghrelin promoter activity, KLF4 increased the promoter activity greatly compared with the increase observed using KLF2 or KLF5. These results indicate that KLF4 specifically stimulates the transcription of human ghrelin.

**Identification of the KLF4-responsive region**

To define the KLF4-responsive region in the ghrelin promoter, we introduced 5′ serial deletions into the ghrelin promoter and transfected the resulting constructs into HEK-293 cells. As shown in Figure 3, the deletion of nucleotides −1997 to −1228 did not affect the transactivation of the ghrelin promoter by KLF4. However, increasing the deletion to −778 resulted in a nearly complete loss of induction by KLF4 (Figure 3, upper). To pinpoint the KLF4-responsive region, a −1105 ghrelin promoter was constructed. The promoter showed almost complete resistance to induction by KLF4 (Figure 3, lower), suggesting that the KLF4-responsive region is located between nucleotides −1228 and −1105.

A computer-assisted search of the 120-bp region using TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html) revealed three putative KLF-binding sites, designated sites 1, 2 and 3 (Figure 4A). To determine whether KLF4 is capable of binding to these putative binding sites, we performed EMSAs using three labelled oligonucleotide probes covering each putative binding site and AGS-cell nuclear extracts. As shown in Figure 4(B), the probe including site 3 (−1165/−1146) showed binding to the extracts. Complex formation was competitively inhibited by an unlabelled homologous competitor but not by a non-homologous competitor (Figure 4C), suggesting that the binding was specific. To demonstrate the binding of site 3 by KLF4, a supershift assay was performed using an anti-KLF4 antibody. As shown in Figure 4(D), the antibody specifically eliminated the upper band of the complex, whereas a non-specific antibody had no effect, indicating that KLF4 specifically binds to site 3. Together, these results demonstrate that KLF4 interacts directly with the ghrelin promoter.

To verify that site 3 is crucial for the KLF4-mediated transactivation of the ghrelin promoter, we deleted site 3 within the context of the −1997 promoter (Figure 5A) and transfected the resulting construct into HEK-293 cells. Transfection with the deletion mutant significantly abolished the induction of the ghrelin promoter by KLF4 (Figure 5B), indicating that site 3 is crucial for the transactivation of ghrelin by KLF4. However, the activity of the mutant promoter is still increased by KLF4 overexpression, although the increase is lower compared with the wild-type promoter, suggesting that alternative KLF4-binding sites unidentified in this study may be present within the promoter.

**KLF4 increases the expression of ghrelin during fasting in co-ordination with CREB (cAMP-response-element-binding protein)**

As ghrelin expression is elevated during fasting, we analysed the level of KLF4 transcription in the stomachs of fasted rats to determine whether KLF4 plays a role in the stimulation of ghrelin expression under fasting conditions. As shown in Figure 6(A), 48 h of fasting increased the transcription of KLF4 (Figure 6A, right) in parallel with an increase in ghrelin expression (Figure 6A, left), suggesting that fasting-induced ghrelin expression may be promoted by KLF4. CREB is a transcription factor responsible for the transactivation of various genes under fasting conditions. The increased expression of KLF4 during fasting raised the possibility that KLF4 and CREB may function in a co-ordinated manner to induce ghrelin expression. To assess this possibility, we performed a co-transfection study using KLF4 and CREB expression vectors. Transfection with 0.2 μg of KLF4 or 1 μg of CREB stimulated the activity of wild-type −1997 bp promoter 2- and 8-fold respectively, whereas co-transfection with KLF4 and CREB resulted in the synergistic activation of the promoter (approx. 16-fold; Figure 6B, left). However, even though CREB...
Figure 4  Identification of the KLF4-responsive region

(A) Three putative KLF4-binding sites (1, 2 and 3) were identified between nucleotides −1228 and −1105. (B) EMSAs were performed on the putative KLF4-binding sites using nuclear extracts from AGS cells. Site 3 formed a complex with these extracts. (C) Oligonucleotide competition experiments were performed. A 100-fold molar excess of each competitor was added. (D) A supershift assay was performed using an anti-KLF4 antibody or a non-specific antibody. The nuclear extracts were pre-incubated with each antibody and then with a labelled probe.

still increased the activity of the mutant promoter, synergistic activation was not observed on the mutant promoter (Figure 6B, right). Thus KLF4 increases the expression of ghrelin during fasting via a coordinated effort with CREB.

DISCUSSION

Ghrelin is produced mainly in the stomach, where it plays a regulatory role in a variety of metabolic processes; however, the mechanism underlying the regulation of ghrelin expression is poorly understood. The regulation of ghrelin and its effects may occur at several levels: transcription and translation, post-translational modification, and changes in the rate of secretion of ghrelin from cells in the stomach, hypothalamus and other sites. Fasting increases the expression of ghrelin mRNA in the stomach, but not in the pituitary or hypothalamus [4]. Hyperglycaemia suppresses ghrelin levels in humans [31], whereas a high-fat diet increases stomach-ghrelin expression in mice [32]. In humans, a carbohydrate-rich diet causes a larger drop in ghrelin levels than a high-fat diet [33]. Insulin and leptin have been shown to increase ghrelin expression [34], but both molecules have also been shown to decrease gastric-ghrelin expression [31,35]. GH-releasing hormone (GHRH) up-regulates ghrelin expression in rat pituitary [36]. Thyroid-hormone deficiency increases the level of circulating ghrelin as well as the transcription of gastric ghrelin [13]. PP (pancreatic polypeptide) significantly inhibits the mRNA expression of gastric ghrelin in rats deprived of food for 24 h [37], but the transcription factors responsible for the regulation of ghrelin expression are unknown.

KLFs are highly related zinc-finger proteins that take part in almost all facets of cellular function, including cellular proliferation, apoptosis, differentiation and neoplastic transformation, by regulating the expression of numerous genes with GC-rich promoters as activators or repressors. Most members of this family (which contains at least 16 proteins) have been identified
in mammals. KLFs are usually expressed in a tissue-selective manner [38]. The tissue distribution of KLF4 is enriched for the gastrointestinal tract, hence the name GKLF. Therefore, in the current study, we investigated the regulation of human ghrelin expression by KLF4 because ghrelin and KLF4 display similar tissue distributions. KLF4 efficiently stimulated human ghrelin-promoter activity, but that stimulation was blocked by a mutant KLF4 construct. Furthermore, the transactivation of ghrelin by KLF4 was specific; none of the other KLFs used in this study stimulated promoter activity, demonstrating that KLF4 specifically stimulates human ghrelin expression. The stimulation of ghrelin expression occurred as a result of the binding of KLF4 to the −1165/−1146 region of the human ghrelin promoter. The binding of KLF4 to the ghrelin promoter was revealed by an EMSA; a complex was formed with the −1165/−1146 nucleotide region in nuclear extracts from AGS cells. The binding was specific for KLF4 because an anti-KLF4 antibody specifically eliminated the upper band of the complex. When the biological importance of the KLF4-binding region was investigated using a mutant lacking the KLF4-binding site, the deleted mutant showed no stimulation of the ghrelin promoter by KLF4, indicating that the KLF4-binding site between −1165 and −1146 is crucial for the transactivation of ghrelin by KLF4.

KLF4 functions in the regulation of cellular growth and differentiation in various target tissues via the activation or repression of associated genes, including the down-regulation of ODC-promoter activity [23] and the up-regulation of several keratin-encoding genes [39]. In addition, KLF4 is involved in the p53-mediated G2/M cell-cycle arrest following DNA damage and in the terminal differentiation of goblet cells in the colon [40]. KLF4 is also associated with normal gastric epithelial proliferation and differentiation [41]. Our finding that KLF4 activates the expression of ghrelin, an appetite hormone, strongly...
suggests that KLF4 mediates metabolic functions through the regulation of ghrelin expression.

The plasma ghrelin level was shown to increase during fasting and decrease following refeeding in the present study and in previous studies [42]. However, the factors regulating ghrelin expression have not been clarified. We found that fasting increases KLF4 expression, which synergistically stimulates the CREB-mediated transactivation of ghrelin. CREB is a transcription factor known to activate diverse genes under fasting conditions. However, cAMP-response-element is not present within the −1997 upstream region of the human ghrelin gene, suggesting that CREB may stimulate ghrelin expression under fasting conditions indirectly through interaction with a cell-specific transcription factor. Therefore, KLF4 is a candidate transcription factor that stimulates ghrelin expression during fasting with the interaction of CREB, because the combination of KLF4 and CREB resulted in synergistic activation of the ghrelin promoter as well as KLF4-specific activation of ghrelin gene expression. The manner in which KLF4 regulates gene expression has been analysed in several studies. KLF4 inhibits the activity of the cytochrome P450 (CYP1A1) promoter via its ability to compete physically with Sp1, a strong activator of the CYP1A1 promoter [43]. In contrast, other studies have shown that KLF4 activates transcription on its own or in conjunction with other KLFs [44,45]. KLF4 is, therefore, a pleiotropic transcription factor that CREB may stimulate ghrelin expression under fasting conditions.

In summary, the present study demonstrates a clear relationship between ghrelin expression and KLF4. KLF4 positively regulates human ghrelin expression via binding to the promoter. These findings may help to clarify the regulation of human ghrelin expression.

AUTHOR CONTRIBUTIONS
Hyo Jung Lee performed the overall experiments in this study. Young Mi Kang performed the KLF4 siRNA knockdown experiment, Chang Suk Moon constructed several ghrelin-promoter-deleted reporters, Young Ho Seo constructed the wild-type ghrelin-promoter reporter, Myung Kuk Joe performed Western blotting for ghrelin expression under fasting conditions, Joo Hyun Lim performed RT–PCR in KLF4-overexpressed cells, Jihyun Song performed Duncan’s multiple-range test and overall statistical analysis (Student’s t-test), and Muyeong Ho Jung designed and supervised this study.

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