RNAi-mediated Ghrelin affects gastric H⁺-K⁺-ATPase activity and expression of GOAT-Ghrelin system *in vitro*

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Running title: RNAi of ghrelin on gastric function and GOAT expression

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Abstract Ghrelin has been implicated in the regulation of gastric functional development, and its physiological functions are mediated by Ghrelin-O-acyltransferase (GOAT) which is capable of generating the active form of this polypeptide hormone. However, whether and how ghrelin gene silencing may modify gastric acid secretion and GOAT-Ghrelin system is yet to be explored. The study was performed in gastric mucosal cells from weanling piglets *in vitro*. We evaluated the effect of ghrelin on gastric acid secretion, gene expression of GOAT and ghrelin as well as ghrelin levels by RNA interference assay. shGhrelin triggered the down-regulation of ghrelin mRNA expression ($P < 0.05$) via an RNAi mechanism, as observed by real-time RT-PCR. In addition, shGhrelin showed reduced total ghrelin production and secretion ($P < 0.05$) using ELISA *in vitro*. We also detected that GOAT mRNA expression was reduced in shGhrelin group ($P < 0.05$), compared with control groups. In accordance with the GOAT expression, acylated ghrelin production and secretion were reduced in gastric mucosal cells and culture medium ($P < 0.05$). Silencing of ghrelin gene achieved by RNAi-mediation inhibited the activity of H^+-K^+ -ATPase and pepsin ($P < 0.05$) in gastric mucosal cells. These results indicated that RNAi of Ghrelin gene inhibited the gastric acid secretion with decreased GOAT mRNA and acylated Ghrelin in gastric mucosal cells.

Key words: shGhrelin; H^+-K^+ -ATPase activity; acylated ghrelin; GOAT.

1. Introduction

Growth performance and health status of weanling piglets are issues of great concern to swine industry, because growth retardation and diarrhea reduce animal productivity and welfare. These problems are related, at least partly, to immature gastric function, especially gastric acid secretion, in nursing piglets (Hayashida et al., 2001). The proton pump, $H^+-K^+-ATPase$ is typically located in the parietal cells, mediating the electroneutral exchange of intracellular H^+ and extracellular K^+ to achieve acid secretion when parietal cells are under the stimulation of secretagogues (Yao and Forte, 2003). Therefore, $H^+-K^+-ATPase$ can serve as an accurate indicator for evaluating the ability of gastric acid secretion from parietal cells. The acidification of the stomach contents by $H^+-K^+-ATPase$ is essential for the activation of the digestive enzyme pepsinogen, which is the main enzyme degrading food proteins into peptides.

Gastric acid secretion is subjected to an important hormonal regulation. The bulk of ghrelin in the body occurs in the A-like cells of the acid-producing part of the stomach, and plasma ghrelin-like immunoreactivity levels in totally gastrectomized patients were reduced to 35% of those in normal controls (Li et al., 2011), which suggested that ghrelin played the important role in regulating gastric acid secretion. Ghrelin is a 28-amino acid polypeptide produced and secreted from endocrine X/A-like cells in oxyntic glands of the stomach (Hayashida et al., 2001). Subsequently, researches demonstrated that ghrelin has a wide spectrum of biological activities, such as stimulation of gastric motility, gastric acid secretion (Date et al., 2001; Masuda et

al., 2000;Yakabi et al., 2008) and stimulation of appetite (Kiewiet et al., 2009). Biological activities of ghrelin are mainly dependent on the attachment of octanoate to the third serine residue, a reaction catalyzed by Ghrelin *O*-acyltransferase (GOAT) (Gutierrez et al., 2008;Yang et al., 2008). Acylated ghrelin may then exert a stimulating effect and play a significant role in modulating gastric function development via a variety of mechanisms. Conflicting results are available about the effects of both ghrelin isoforms on gastric acid secretion (Sakurada et al., 2010). What is exactly the function of ghrelin in gastric acid secretion is still not clear. Furthermore, the exact mechanism of action and influence on gastric acid secretion remains unexplored. In the GOAT-Ghrelin system, GOAT plays a critical role through modulating the acyl ghrelin levels. This is important and the involvement of GOAT in ghrelin levels or its biological effects could represent an attractive therapeutic target (Kang et al., 2012).

RNA interference (RNAi) is a mechanism responsible for transcriptional (Janowski et al., 2006;Calero-Nieto et al., 2010) and post-transcriptional gene silencing (Elbashir et al., 2001). The silencing mechanisms can lead to the degradation of a target mRNA, as induced by small interfering RNAs (siRNAs) or short hairpin RNAs (shRNAs). shRNAs are introduced into the nuclei of target cells using either bacterial or viral vectors that, in some cases, can stably integrate into the genome. But siRNAs typically require perfect homology to induce degradation. Advantages of shRNA over siRNA include the ability to use viral vectors for delivery to overcome the difficulty of transfecting certain cell types, the option to control

shRNA expression using inducible promoters, and the ability to co-express them with a reporter gene (Rao et al., 2009).

In this work, we evaluated the effects of ghrelin on gastric acid secretion *in vitro* using gastric mucosal cells models, by investigating the activity of H⁺-K⁺-ATPase and pepsin, as well as to investigate the biological role of GOAT involvement in regulation mechanisms by RNA interference assay.

2. Materials and methods

2.1 Design of shRNA and Plasmid construction

The siRNA sequence targeting ghrelin gene (GenBank accession number: AF308930) was designed using Dharmacon siRNA Design tool (<http://www.dharmacon.com>). The siRNA sequences used in this study are as follows: siGhrelin: 5'-CAAGAAGCCAGCAGCCAAAU-3'. A siRNA with a scrambled sequence, 5'-CAGAACAGAGCCACACAGAU-3', unable to induce degradation in any cellular mRNA, was used as negative control of specificity (shNC). All these DNA oligonucleotides were chemically synthesized by Invitrogen (Shanghai, China). A widely used siRNA expression vector, pGPU6/GFP/Neo (GenePharma, Shanghai, China) was selected to be the parental plasmid.

2.2 Cell culture and RNA transfection

Gastric mucosa was obtained from 21-week-old weanling Suzhong piglets with average body weight of 10 kg and cells were dispersed as described previously (Terano et al., 1982) with minor modifications. Briefly, the tissue was washed thoroughly in D-Hank's solution containing antibiotics (100 U penicillin/ml and 100

U streptomycin/ml) for about 1h and digested with 0.15% trypsin at 37°C for 30 min, filtered and centrifuged (1000 r/min, 5 min). Thereafter cells were cultured at a density of 2×10^6 per well (37°C, 5% CO₂) in DMEM/F-12 supplemented with 10% fetal bovine serum, 5 mM HEPES buffer, and 100 U/ml antibiotics.

For transfection experiments, 1×10^5 gastric mucosal cells were cultured in six-well plates to 80–90% confluency. Cell transfection was performed using Lipofectamin 2000 (Invitrogen, Shanghai, China) according to the manufacturer's instructions. Briefly, for each well, 3 µg of the respective shRNA plasmid was incubated with 250 µL serum-free medium for 5 min. Subsequently, a mixture of 9 µL Lipofectamine 2000 and 241 µL serum-free medium incubated for 5 min was added slowly to the plasmid mixture. After incubation for 20 min at room temperature, 1 mL serum-free medium was added to the dilution, mixed gently and then added to the well. After incubation at 37°C for 5 h, the medium containing the transfection mix was replaced with growth medium. After 48 h of incubation, the plates were freeze/thawed three times and supernatants and cells were collected for real-time RT–PCR analysis and ELISA. At the same time, non- transfection normal cells were used as black group (BC). All experiments in this study were performed in triplicate.

2.3 Quantitative Real-time RT-PCR (qRT-PCR)

Total RNA from cells was extracted using a Trizol kit (TaKaRa, China). Total RNA concentration was then quantified by measuring the absorbance at 260 nm in a photometer (Eppendorf Biophotometer). Ratios of absorption (260:280 nm) of all preparations were between 1.85 and 2.01. Trace DNA contamination was removed by

DNase digestion (TaKaRa, China). Aliquots of RNA samples were subjected to electrophoresis through a 1.4% agarose-formaldehyde gel to verify their integrity. cDNA was synthesized from aliquots (1 µg) of DNAase-treated total RNA with the PrimeScript 1stStrand cDNA Synthesis kit (TaKaRa, China). It is a routine practice to confirm the absence of genomic DNA contamination in samples used for Real Time PCR analysis. Primers were designed to span an intron so any genomic DNA contamination can be easily reported by an extra PCR product in melting curve analysis.

The real-time RT-PCRs of ghrelin and GOAT cDNA and GAPDH as internal control were carried out using the SYBR-Green I chemistry (TaKaRa Co. Nanjing, China). Amplification primers of the three genes were synthesized by TaKaRa Co. (Nanjing, China) as follows: ghrelin F: 5'- GCTCCTCATGGCAGACTTGG -3' and R: 5'- TGGACTCCTTTCTCTGCTGC -3', GOAT F: 5'- TTCAGATGAGCTGGCAGACG -3' and R: 5'- CGCTTCCTACAGCTTCCGAT -3', and GAPDH F: 5'- CGTCCCTGAGACACGATGGT -3' and R: 5'- CCCGATGCGGCCAAAT -3'. Two microliters of diluted cDNA (1:10) were used in each real-time PCR assay with the 7500 Fast Real-Time PCR System (Applied Biosystems). The cycle profile was programmed as follows: 30 sec at 95°C (initial PCR activation); and 40 cycles of 5 sec at 95°C (denaturation), 30 sec at 63°C (annealing); the final dissociation step was 15 sec at 95°C, 30 sec at 63°C, 15 sec at 95°C. Results were analyzed with the Ct ($2^{-\Delta\Delta C_t}$) method to compare the transcriptional levels of the target genes relative to BC group.

2.4 Biochemical Analysis

Whole cells samples were prepared by homogenization and mild sonication in cold RIPA buffer with antiproteases (Sigma, Shanghai, China). Tissue lysates were centrifuged for 15 min at 12,000 *g* in a microfuge at 4°C. Culture medium and cells lysates supernatant were collected in a tube containing AEBSF (BEST, Chengdu, China) to a final concentration of 1 mg/mL, let at room temperature for 30 min, and immediately centrifuged at 4°C. Cultural medium and supernatant were acidified with HCl to a final concentration of 0.05M. Total and active ghrelin levels in the cells culture medium and cells were determined using a Sandwich ELISA kit (EMD Millipore, Hong Kong, China) according to the manufacturer's instructions. The limit of assay sensitivity was 40 pg /mL for total and 8 pg /mL for active ghrelin.

2.5 H^+-K^+ -ATPase and pepsin activity

H^+-K^+ -ATPase activity (Catalogue number A069) and Pepsin (Catalogue number A080-1) in gastric mucosal cells were respectively measured with commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The activity of H^+-K^+ -ATPase was analyzed by measured the inorganic phosphate release after hydrolysis of ATP. The lysates of gastric mucosal cells was made and centrifuged at 3500 rpm for 15min. The amount of inorganic phosphate released per milligram of protein per hour ($\mu\text{mol Pi/mg prot/h}$) was determined from the resulting supernatant spectrophotometrically at 660nm. Haemoglobin was diluted with HCl (pH1.5-5.0), as at such acidic conditions, all of the pepsinogen was activated, so the gastric pepsin activity measured accounted for the total pepsin content in the culture

medium of gastric mucosal cells by spectrophotometer at 660nm. The protein concentration was detected by BCA protein detection kit (Catalogue number W041) from the resulting supernatant spectrophotometrically at 562nm.

$$\text{Pepsin (U per } \mu\text{g)} = \text{pepsin (U) per cell culture medium (mL)} / \text{protein (}\mu\text{g) per cell culture medium (mL)}.$$

2.6 Statistical analysis

All data were expressed as mean $\pm$ SEM. All statistical analyses were performed with SPSS 16.0 for Windows. Comparisons among groups were performed using one-way analysis of variance (ANOVA) followed by a Least-Significant Difference (LSD) test for individual comparisons. All experiments were carried out in triplicate. The differences were considered statistically significant when $P < 0.05$ in all analyses.

3. Results

3.1 Effect of shGhrelin on ghrelin and GOAT mRNA expression in vitro.

As shown in Figure 1a, compared with BC and NC group, ghrelin mRNA expression was decreased significantly by 11.1 fold and 16.7 fold, respectively by the RNA interference pathway in treatment group ($P < 0.05$); And it is interesting to note that RNA interference for ghrelin gene induced lower gastric GOAT mRNA expression by 4.8 fold and 6.7 fold in gastric mucosal cells compared to BC and NC control groups, respectively (Fig. 1b).

Fig. 1

3.2 Effect of shGhrelin on total and acylated ghrelin secretion in culture medium and production in gastric mucosal cells.

As shown in Figure 2, shGhrelin treatment caused significant decreased total ghrelin secretion in culture medium when compared to the BC (Fig. 2a), and it was fold change of -0.65. Compared with BC and NC group, total ghrelin synthesis in gastric mucosal cells also decreased significantly, and fold change was -0.43 and -0.50 respectively (Fig. 2b). Acylated ghrelin secretion in culture medium (Fig. 2c) and synthesis in gastric mucosal cells (Fig. 2d) significantly decreased in shGhrelin group when compared to BC and NC group. Fold change in acylated ghrelin secretion was -0.49 and -0.60, in acylated ghrelin synthesis levels was -0.74 and -0.63 between shGhrelin and BC, NC group, respectively.

Fig. 2

3.3 Effect of shGhrelin on H^+-K^+ -ATPase activity in vitro.

As shown in Figure 3, gastric H^+-K^+ -ATPase activity in mucosal cells was significantly lower in shGhrelin group when compared to BC and NC group (Fig. 3). It was a fold change of -0.46 and -0.53, respectively.

Fig. 3

3.4 Effect of shGhrelin on pepsin activity in vitro.

As shown in Figure 4, compared to BC and NC group, significant changes were detected at the pepsin activity in shGhrelin group (Fig. 4). It gave a fold change of -0.69 and -0.71, respectively.

Fig. 4

4. Discussion

In the present study, our results indicated that ghrelin mRNA and protein expression were significantly reduced through RNAi pathway. Intriguingly, the

silencing expression of ghrelin gene resulted in the knockdown in GOAT mRNA levels in gastric mucosal cells. shGhrelin reduced gastric H⁺-K⁺-ATPase and pepsin activity, which may be associated with decreased acylated ghrelin levels in culture medium and gastric mucosal cells.

Ghrelin is unique in that it requires a post-translational modification in which the third serine residue is covalently linked to a medium-chain fatty acid, typically octanoate (Kojima et al., 1999). The *O*-*n*-octanoylation of ghrelin is essential for binding to and activation of the GHS-R1a receptor (Banks et al., 2002). Majority of ghrelin circulates in the unacylated form which was considered to be devoid of any endocrine action (Chen et al., 2005). Studies have reported that the physiological effects of ghrelin are carried out by the acylated form (AG) and not by unacylated form (UAG). Acyl ghrelin enhanced gastrin-stimulated acid secretion while desacyl ghrelin did not (Sakurada et al., 2010). Ghrelin *O*-acyltransferase (GOAT) was identified as a specific acyl-transferase for ghrelin (Yang et al., 2008). GOAT is highly co-expressed with ghrelin in cells of the mouse gastric mucosa (Sakata et al., 2009), which manifested its significance in the regulation of ghrelin secretion (Gutierrez et al., 2008), because the amount and/or activity of this specific enzyme likely affect the level of acyl ghrelin. Analysis of GOAT mRNA expression in *vitro* studies suggests the influence of silenced ghrelin gene, which was consistent with reported (Goebel-Stengel et al., 2013), which might be attributed to an observed decrease in acylated ghrelin levels. Also inhibition of GOAT gene had been shown to reduce acylated ghrelin secretion and cells acylated ghrelin, an effect notably seen in

present study. In present study, shGhrelin significantly down-regulated GOAT mRNA expression in gastric mucosal cells. The result contradict the reported that 100nM ghrelin significantly decreased GOAT mRNA expression two-fold in the PC3 prostate cancer cell line, while no effect in the DU145 and LNCaP prostate cancer cell lines (Seim et al., 2013). Many factors contribute to such contradiction including doses, duration and method of administration, and specificity of cell-type. It is likely that the effect of ghrelin on GOAT is negative as pharmacological pathway, while positive by physiological pathway.

A number of *in vivo* studies with exogenous administration of ghrelin indicated that ghrelin plays important physiological roles in regulating gastric motility and gastric acid secretion in rats (Masuda et al., 2000; Date et al., 2001; Fukuda et al., 2004). We have previously reported that ghrelin could stimulate both mRNA expression and activity of $H^+-K^+-ATPase$ in gastric mucosal cell (Du et al., 2007). And ectopic over-expression of ghrelin by intramuscular transfection increased the expression and activity of $H^+-K^+-ATPase$ in mice (Du et al., 2013). Conflicting results implied that the role of ghrelin in regulating gastric acid secretion has not yet been established. Ghrelin has been reported to exert stimulatory effects on gastric acid secretion in a dose-dependently manner by ICV and Intravenous administrations (Masuda et al., 2000; Date et al., 2001). Gastric acid secretion was significantly inhibited 60% at the dose of 1pmol/rat ICV for ghrelin (Sibilia et al., 2002). But Dornonville reported that Ghrelin neither stimulated nor inhibited gastric acid secretion (Dornonville et al., 2004). Pepsinogens are synthesized, stored, and upon

appropriate stimulation released from gastric mucosal chief cells. Under the acidic conditions of the lumen of the stomach, the pepsinogens are converted to pepsin, which plays an important physiologic role as a digestive enzyme (Muller et al., 1990). High gastric acidity and pepsin can kill ingested microorganisms and may play a role in preventing bacterial overgrowth, enteric infection, and possibly spontaneous bacterial peritonitis (Schubert, 2014). We observed in the present study significant down-regulation of GOAT mRNA expression in gastric mucosal cells transfected shGhrelin, as well as the gastric H^+-K^+ -ATPase and pepsin activity were significantly inhibited *in vitro*, which suggested that ghrelin played an important role in stimulating secretion of chief cell. A systematic pathway through the mediation of GOAT to regulate gastric acid secretion can't be ruled out. Therefore, it suggested that ghrelin gene silencing may inhibit the H^+-K^+ -ATPase and pepsin activity through the mediation of GOAT. However, further experiments are required to clarify the exact signaling pathway for this action by GOAT silencing. GOAT-Ghrelin system regulates gastric acid secretion, and these findings suggest a novel function of GOAT involvement in the regulation of gastric acid secretion.

Conclusion

Our findings provide the first set of evidence that silencing expression of ghrelin gene resulted in the decreased GOAT mRNA levels compared with control group in gastric mucosal cells, which may contributed to reduced acylated ghrelin levels in gastric mucosal cells. The results were to further understand the relative physiological functions of the GOAT-Ghrelin system and provide insight into the mechanisms

behind the regulation of gastric acid secretion. If transferred to the human situation, may lead to novel insights and clues into the pathophysiology of and therapeutic approach to the treatment of Gastroenterology.

Declaration of interest

The authors declare that there is no conflict of interest about this research reported.

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Legend to Figures:

Fig. 1 Expression of ghrelin (a) and GOAT (b) mRNA in gastric mucosal cells using real-time RT-PCR. Values are mean $\pm$ S.E.M. Different letters indicate significant differences between control and treatment groups ($P < 0.05$), $n = 4$.

Fig. 2 Effect of shGhrelin on total (a) and acylated (c) ghrelin secretion in cultural medium and total (b) and acylated (d) ghrelin synthesis in gastric mucosal cells with ELISA methods. Values are mean $\pm$ S.E.M. * $P < 0.05$ and ** $P < 0.01$ indicate significant differences between control and treatment groups, respectively, $n = 4$. * The mean difference is significant at the 0.05 level; ** the mean difference is very significant at the 0.01 level.

Fig.3 Effect of shGhrelin on H⁺-K⁺-ATPase activity in gastric mucosal cells by biochemistry method. Values are mean $\pm$ S.E.M. Different letters indicate significant differences between control and treatment groups ($P < 0.05$), $n = 4$.

Fig.4 Effect of shGhrelin on pepsin activity in gastric mucosal cells by biochemistry method. Values are mean $\pm$ S.E.M. Different letters indicate significant differences between control and treatment groups ($P < 0.05$), $n = 4$.

REFERENCES

1. Banks, W.A., Tschop, M., Robinson, S.M., Heiman, M.L., 2002. Extent and direction of ghrelin transport across the blood-brain barrier is determined by its unique primary structure. *J.Pharmacol.Exp.Ther.* 302, 822-827.
2. Calero-Nieto, F.J., Bert, A.G., Cockerill, P.N., 2010. Transcription-dependent silencing of inducible convergent transgenes in transgenic mice. *Epigenetics.Chromatin.* 3, 3.
3. Chen, C.Y., Inui, A., Asakawa, A., Fujino, K., Kato, I., Chen, C.C., Ueno, N., Fujimiya, M., 2005. Des-acyl ghrelin acts by CRF type 2 receptors to disrupt fasted stomach motility in conscious rats. *Gastroenterology* 129, 8-25.
4. Date, Y., Nakazato, M., Murakami, N., Kojima, M., Kangawa, K., Matsukura, S., 2001. Ghrelin acts in the central nervous system to stimulate gastric acid secretion. *Biochem.Biophys.Res.Comm.* 280, 904-907.
5. Du, G.M., Liu, M.J., Parvizi, N., Zhao, R.Q., 2013. Ectopic expression of ghrelin affects gastric H(+)-K(+)-ATPase activity and expression of GHR/IGF-1 system in weaned mice. *Regul.Pept.* 186, 12-17.
6. Du, G.M., Shi, Z.M., Wei, X.H., Liu, M.J., Zhang, L., Zhao, R.Q., 2007. Expression of gastric ghrelin and H(+)-K(+)-ATPase mRNA in weanling piglets and effect of ghrelin on H(+)-K(+)-ATPase expression and activity in gastric mucosal cells in vitro. *Res.Vet.Sci.* 82, 99-104.
7. Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., Tuschl, T., 2001. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 411, 494-498.
8. Fukuda, H., Mizuta, Y., Isomoto, H., Takeshima, F., Ohnita, K., Ohba, K., Omagari, K., Taniyama, K., Kohno, S., 2004. Ghrelin enhances gastric motility through direct stimulation of intrinsic neural pathways and capsaicin-sensitive afferent neurones in rats. *Scand.J.Gastroenterol.* 39, 1209-1214.

9. Goebel-Stengel, M., Hofmann, T., Elbelt, U., Teuffel, P., Ahnis, A., Kobelt, P., Lambrecht, N.W., Klapp, B.F., Stengel, A., 2013. The ghrelin activating enzyme ghrelin-O-acyltransferase (GOAT) is present in human plasma and expressed dependent on body mass index. *Peptides* 43, 13-19.
10. Gutierrez, J.A., Solenberg, P.J., Perkins, D.R., Willency, J.A., Knierman, M.D., Jin, Z., Witcher, D.R., Luo, S., Onyia, J.E., Hale, J.E., 2008. Ghrelin octanoylation mediated by an orphan lipid transferase. *Proc.Natl.Acad.Sci.U.S.A* 105, 6320-6325.
11. Hayashida, T., Murakami, K., Mogi, K., Nishihara, M., Nakazato, M., Mondal, M.S., Horii, Y., Kojima, M., Kangawa, K., Murakami, N., 2001. Ghrelin in domestic animals: distribution in stomach and its possible role. *Domest.Anim Endocrinol.* 21, 17-24.
12. Janowski, B.A., Huffman, K.E., Schwartz, J.C., Ram, R., Nordsell, R., Shames, D.S., Minna, J.D., Corey, D.R., 2006. Involvement of AGO1 and AGO2 in mammalian transcriptional silencing. *Nat.Struct.Mol.Biol.* 13, 787-792.
13. Kang, K., Schmahl, J., Lee, J.M., Garcia, K., Patil, K., Chen, A., Keene, M., Murphy, A., Sleeman, M.W., 2012. Mouse ghrelin-O-acyltransferase (GOAT) plays a critical role in bile acid reabsorption. *FASEB J.* 26, 259-271.
14. Kiewiet, R.M., van Aken, M.O., van der, W.K., Uitterlinden, P., Themmen, A.P., Hofland, L.J., de Rijke, Y.B., Delhanty, P.J., Ghigo, E., Abribat, T., van der Lely, A.J., 2009. Effects of acute administration of acylated and unacylated ghrelin on glucose and insulin concentrations in morbidly obese subjects without overt diabetes. *Eur.J.Endocrinol.* 161, 567-573.
15. 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.
16. Li, J., Yi, S.Q., Wang, H.X., Yi, N., Ogawa, Y., Ozaki, N., Itoh, M., 2011. Immunolocalization of ghrelin in the stomach of Sprague-Dawley rat. *Anat.Histol.Embryol.* 40, 95-99.
17. Masuda, Y., Tanaka, T., Inomata, N., Ohnuma, N., Tanaka, S., Itoh, Z., Hosoda,

- H., Kojima, M., Kangawa, K., 2000. Ghrelin stimulates gastric acid secretion and motility in rats. *Biochem.Biophys.Res.Comm.* 276, 905-908.
18. Muller, M.J., Defize, J., Hunt, R.H., 1990. Control of pepsinogen synthesis and secretion. *Gastroenterol.Clin.North Am.* 19, 27-40.
19. Rao, D.D., Vorhies, J.S., Senzer, N., Nemunaitis, J., 2009. siRNA vs. shRNA: similarities and differences. *Adv.Drug Deliv.Rev.* 61, 746-759.
20. Sakata, I., Yang, J., Lee, C.E., Osborne-Lawrence, S., Rovinsky, S.A., Elmquist, J.K., Zigman, J.M., 2009. Colocalization of ghrelin O-acyltransferase and ghrelin in gastric mucosal cells. *Am.J.Physiol Endocrinol.Metab* 297, E134-E141.
21. Sakurada, T., Ro, S., Onouchi, T., Ohno, S., Aoyama, T., Chinen, K., Takabayashi, H., Kato, S., Takayama, K., Yakabi, K., 2010. Comparison of the actions of acylated and desacylated ghrelin on acid secretion in the rat stomach. *J.Gastroenterol.* 45, 1111-1120.
22. Schubert, M.L., 2014. Gastric secretion. *Curr.Opin.Gastroenterol.* 30, 578-582.
23. Seim, I., Jeffery, P.L., de, A.L., Walpole, C.M., Fung, J., Whiteside, E.J., Lourie, R., Herington, A.C., Chopin, L.K., 2013. Ghrelin O-acyltransferase (GOAT) is expressed in prostate cancer tissues and cell lines and expression is differentially regulated in vitro by ghrelin. *Reprod.Biol.Endocrinol.* 11, 70.
24. Terano, A., Ivey, K.J., Stachura, J., Sekhon, S., Hosojima, H., McKenzie, W.N., Jr., Krause, W.J., Wyche, J.H., 1982. Cell culture of rat gastric fundic mucosa. *Gastroenterology* 83, 1280-1291.
25. Yakabi, K., Kawashima, J., Kato, S., 2008. Ghrelin and gastric acid secretion. *World J.Gastroenterol.* 14, 6334-6338.
26. Yang, J., Brown, M.S., Liang, G., Grishin, N.V., Goldstein, J.L., 2008. Identification of the acyltransferase that octanoylates ghrelin, an appetite-stimulating peptide hormone. *Cell* 132, 387-396.
27. Yao, X., Forte, J.G., 2003. Cell biology of acid secretion by the parietal cell. *Annu.Rev.Physiol* 65, 103-131.

Fig. 1a

Fig. 1b

Fig 2a

Fig 2b

Fig 2c

Fig 2d

Fig 3

Fig 4