The Efficacy of Enhanced Growth by Ectopic Expression of Ghrelin and Its Variants Using Injectable Myogenic Vectors*

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ABSTRACT : Ghrelin is an acylated peptide recently identified as the endogenous ligand for the growth hormone (GH) secretagogues receptor 1a (GHS-R1a) and is involved in a novel system for regulating GH release. To understand the long-term effects of ghrelin, here we constructed six myogenic expression vectors containing the cDNA of swine mature ghrelin (pGEM-wt-sGhln, pGEM-wt-hGhln), ghrelin mutant of Ser^3 with Trp^3 (pGEM-mt-sGhln, pGEM-mt-hGhln) and truncated ghrelin derivative (pGEM-tmt-sGhln, pGEM-tmt-hGhln) encompassing the first 7 residues of ghrelin (including Ser^3 substituted with Trp^3) and adding a basic amino acid, Lys (K) in the C-terminus. The constructs, pGEM-wt-sGhln, pGEM-mt-sGhln and pGEM-tmt-sGhln were linked with the ghrelin leader sequence, while the pGEM-wt-hGhln, pGEM-mt-hGhln and pGEM-tmt-hGhln were linked with a leader sequence from the human growth hormone releasing hormone (hGHRH). Intramuscular injection of 200 μg pGEM-wt-sGhln or pGEM-tmt-sGhln augmented growth over 3 weeks in normal rats and peaked at day 21 or 14 post-injection respectively, whose body weight gains were on average approximately 6% or 19% heavier over controls. However, other injectable vectors had no such enhanced growth effects. Our results suggested that the efficacy of the ghrelin leader sequence was more effective than that of hGHRH in our system. Moreover, the results indicated that skeletal muscle might have the ability to posttranslationally modify the *in vivo* expressed ghrelin. And the most strikingly, the short ghrelin analog seems to mimic the biological effects more efficiently when compared with the full-length ghrelin. ***(Asian-Aust. J. Anim. Sci. 2004. Vol 17, No. 1 : 146-152)***

Key Words : Ghrelin, GH, Myogenic Vector, Gene Medicine, Skeletal, Rat

INTRODUCTION

Ghrelin, a peptide originally discovered predominantly in endocrine cells within rat stomach, is the ligand for growth hormone (GH) secretagogues (GHS) receptor 1a (GHS-R1a). This 28 amino acid peptide possesses a unique serine residue at the third position (Ser^3) that is modified by n-octanoic acid. Acylation is essential for ligand binding to the receptor and subsequent ghrelin activities (Kojima et al., 1999). Recently, it has also been reported that short peptide encompassing the first 4-5 residues (including acylated serine) of ghrelin is the "core" required for its efficient binding and activation (Bednarek et al., 2000; Matsumoto et al., 2001). The isolation of ghrelin can be considered a landmark in the GH field, which opens up the possibility of obtaining a greater insight into understanding of mechanism involved in the regulation of GH secretion and somatic growth. Ghrelin has been shown to exert a very potent and specific GH-releasing activity both *in vitro* and *in vivo* (Date et al., 2000; Takara et al., 2000; Toll et al., 2001). However, it is already clear that ghrlein is much more than simply a natural GH secretagogue. The accumulating evidence in the rat and human suggests that, in addition to regulating GH release, ghrelin also affects feeding (Nakazato et al., 2001; Lawrence et al., 2002), gastrointestinal function (Masuda et al., 2000; Date et al., 2001), energy metabolism (TschÖp et al., 2000; Horvath et al., 2001), cardiovascular function (Nagaya et al., 2001a; 2001b) and even antiproliferative effects (Cassoni et al., 2001).

GH enhances protein synthesis, lypolysis and epiphyseal growth, and is implicated in the regulation of the immune system. Regulated expression of the GH pathway is essential for optimal linear growth, as well as for homeostasis of carbohydrate, protein and fat metabolism. The GH secretion by the somatotroph cells depends upon the interaction between hypothalamic regulatory peptides, target gland hormones and a variety of growth factors acting in a paracrine and autocrine fashion (Nou et al., 2003; Tanwattana et al., 2003). The occurrence of ghrelin in both rat and human indicates that GH release from the pituitary gland may be controlled not only by hypothalamic growth hormone releasing hormone (GHRH) and somatostatin, but also by ghrelin. Interestingly, this peptide appears to be much more potent, on a molar basis, than GHRH in stimulating GH secretion (Pombo et al., 2001). It is possible that, like GHRH (Draghia-Akli et al., 1997, 1999), the ectopically secreted ghrelin in both mature and

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Table 1. Sequences of oligonucleotide and primer

Oligonucleotide/pimer	Sequence (5' to 3')	Sense/antisense
Sequqnce 1	ATGCCCTCCACGGGGACCATTTGC AGCCTGCTGCTCCTCAGCGTGCTC CTCATGGCAGACTTGGCCATGGCG GGCTCCAGCTTCTTAAGCCCCGAA CACC	Sense
Sequence 2	ATGCCACTCTGGGTGTTCTTCTTTG TGATCCTCACCCTCAGCAACAGCT CCCACTGCTCCCCACCTCCCCCTTT GACCCTCAGGATGCGGCGGGGCTC CAGCTTCTTAAGCCC	Sense
Sequence 3	GCAACTAGTCTACCGGGGCTTCAGT TTGGCTGCTGGCTTCTTGGACTCCT TTCTCTGCTGCACTTTCTGGTGTTCG GGGCTTAAGAAGCTGGAGCC	Antisense
Primer 1	ATGCCCTCCACGGGGACCATTT	Sense
Primer 2	ATGCCACTCTGGGTGTTCTTCTTT	Sense
Primer 3	GCAACTAGTCTACCGGGGCTTC	Antisense
Primer 4	TTCGAGCTCGGTACCCGG	Sense
Primer 5	GGGGCTTAAGAACCAGGAGC	Antisense
Primer 6	GCAACTAGTCTACTTGGGGCTTAA	Antisense

Sequence 1 includes swine ghrelin signal peptide and 1-28 bp of mature swine ghrelin cDNA; Sequence 2 includes hGHRH signal peptide and 1-20 bp of mature swine ghrelin cDNA; Sequence 3 includes 1-84 bp of mature ghrelin sequence, a stop codon and a Spe I site. In the swine ghrelin mature sequence, only the codon of the fifth amino acid, Leu (L), TTG was replaced with TTA to create the Afl II site. Recognition sequences of Afl II, Spe I and Sac I restriction enzyme were underlined.

its variants may be also biologically active and can even produce acromegaly. Previous studies on GH-releasing activity of ghrelin *in vivo* include frequent subcutaneous, intravenous or intracerebroventricular administration of synthetic ghrelin and its analogs (TschÖp et al., 2000; Nakazato et al., 2001), for ghrelin has a short half-life in the circulation. Therefore, one would expect to study long-last biological effects of ghrelin by alternatives such as gene medicine.

For gene medicine, the ghrelin cDNA could be targeted to peripheral organs where the peptide is processed, secreted and transported to systemic circulation, where it could be biologically active. Skeletal muscle is an attractive target for transfection *in vivo* by direct plasmid DNA injection, where it can be expressed at significant levels for long-term (Draghia-Akli et al., 1997, 1999). In this study, six expression vectors containing natural mature ghrelin and its analogs cDNA were constructed. The effects of signal peptides of either ghrelin itself or human GHRH were also tested. After intramuscular injection of a single dose plasmid DNA, the ectopically expressing efficacy of these gene medicines was observed. Intramuscular injection of pGEM-wt-sGhln or pGEM-tmt-sGhln resulted in greatly increased body weight gain in normal puberty rats over 3 weeks compared with controls.

MATERIALS AND METHODS

Cloning of swine mature ghrelin and its variants cDNA

Three oligonucleotides and six primers were synthesized (Sangon). The oligonucleotide and primer sequences were shown as Table 1. Firstly, sequence 1 was annealed with sequence 3 and then PCR amplified with primer 1 and 3. The PCR products were cloned to sequencing vector pMD18-T (Takara) to yield pMD18-T-wt-sGhln. Secondly, the PCR reactions were performed using pMD18-T-wt-sGhln as templates by primer 4 and 5. The products of PCR were inserted into SacI/AflII sites of pMD18-T-wt-sGhln to create pMD18-T-mt-sGhln. Thirdly, the pMD18-T-mt-sGhln was double cut by Sac I/Afl II and small fragments were restored, and then amplified from this small fragments by PCR primer 4 and 6. The products of PCR were inserted into Sac I/Spe I sites of pMD18-T-mt-sGhln to yield pMD18-T-tmt-sGhln. The same operations were also performed between sequence 2 and sequence 3 as described above to create the pMD18-T-wt-hGhln, pMD18-T-mt-hGhln, pMD18-T-tmt-hGhln with the corresponding primer 2 and 3, primer 4 and 5 and primer 4 and 6. The anneal action were performed in a total volume 20 μl using sense and antisense oligonucleotides 25 μM of each, 0.25 mMdNTP, Taq DNA polymerase 0.25 U/μl by 94°C, 4 min, 58 °C, 40 s and 72°C, 1 min, in one cycle. The conditions of PCR all above were the same in 30 cycles with initial denaturating step at 94°C for 5 min, 30 s annealing at 60°C, 30 s, elongation at 72°C and 30 s denaturing at 94°C.

Construction of myogenic specific expression vectors

The plasmid DNA backbone pGEM, derived from pGEM-5zf (Promega), was reformed previously in our lab.

Table 2. Peptide structure of swine ghrelin and its variants encoded by corresponding expression vectors

Expression vector	Structure
pGEM-wt-sGhln	MPSTGTICSLLLLSVLLMADLAMA*GSSFLSPEHQKVQQRKESKKPAAKLKPR
pGEM-mt-sGhln	MPSTGTICSLLLLSVLLMADLAMA*GSWFLSPEHQKVQQRKESKKPAAKLKPR
pGEM-tmt-sGhln	MPSTGTICSLLLLSVLLMADLAMA*GSWFLSPK
pGEM-wt-hGhln	MPLWVFFFVILTLSNSSHCSPPPPLTLRMRR*GSSFLSPEHQKVQQRKESKKPAAKLKPR
pGEM-mt-hGhln	MPLWVFFFVILTLSNSSHCSPPPPLTLRMRR*GSWFLSPEHQKVQQRKESKKPAAKLKPR
pGEM-tmt-hGhln	MPLWVFFFVILTLSNSSHCSPPPPLTLRMRR*GSWFLSPK

Amino acids before * represent signal peptide.

The pGEM-A5f3f contains a 2.857 bp Nco I/Mlu I fragment, which includes 1.910 bp porcine skeletal muscle α-actin (SKA) 5'-flanking region, the first exon and the first intron (Reecy et al., 1996) and the 3' untranslated region of hGH cDNA in a 613 bp Sac I/Nsi I fragment. To construct expression vectors, pGEM-A5f3f was first cut by Mlu I, then blunted using Klenow Fragment (Takara) and then cut by Spe I; The wild-type ghrelin and its variants cDNA were obtained by PCR amplification from pMD18-T-Ghln constructs respectively and were inserted into blunt-ended Mlu I/Spe I to create pGEM-wt-sGhln, pGEM-mt-sGhln, pGEM-tmt-sGhln, pGEM-wt-hGhln, pGEM-mt-hGhln and pGEM-tmt-hGhln. The amino acid sequence of ghrelin and its variants encoded by the vectors above were shown as Table 2. The PCR reactions were performed as mentioned above.

Intramuscular injection of expression vectors in puberty rats

Wistar male SPF rats (National Institute for Control of Pharmaceuticals and Biological Products, Beijing, China) were housed and cared for in the animal facility of Institute for Space Medical Engineering (Beijing, China). All animal experimentations were performed in accordance with guidelines for the care and use of laboratory animals, under environmental condition of 10 h light/14 h darkness. On day 28th after born, rats were weighted and the left regenerating quadriceps muscle was exposed and injected with 200 μg plasmid expression vectors in 200 μl phosphate buffer saline (PBS, pH 7.4) or 200 μl PBS as negative control. The animals were weighted two times every week post-injection. Blood samples were collected via tail vein respectively at day 21, 28, 35 or 42 after injection, centrifuged after 40 mins at room temperature and stored at -80°C prior to analysis. Finally, animals were sacrificed and injected and control organs were removed and frozen in liquid nitrogen.

Rat GH RIA

Serum rGH concentrations were measured by radioimmunoassay (RIA) using kit provided by the NHPP, NIDDK, NICHHD, USDA, USA. Values are expressed in terms of rat GH-RP-2 standard (potency 2 IU/mg) as ng/ml of serum. The sensitivity of the assay was 0.1 ng/ml. To avoid inter assay variations, all samples from each experiment were run in one single assay.

Gene expression analysis by RT-PCR

Total RNAs of muscle, stomach and other tissues of Wistar rats were extracted using the TRIzol reagent (Invitrogen). First cDNAs were synthesized from 10 units of DNase I (Promega) treated total RNA using M-MLVRT (Life Technology) for polymerase chain reaction (RT-PCR) according to manufacture's instruction. In (-) tube, the reverse transcriptase was omitted. Specific oligonucleotides were used to amplify pGEM-tmt-sGhln cDNA either in 89bp fragment: 5'-TCCACGGGGACCATTTG-3' (sense) and 5'-GGGCTTAAGAACCAGGA-3' (antisense) or in 254 bp fragment: 5'-CGGCCTTGCTGATCTTG-3' (sense) and 5'-GAGCAGATTCGTCGTCC-3' (antisense). The RT-PCR conditions were 35 cycles, 94°C for 30 s, gradient annealing 52-60°C for 30 s, 72°C for 30 s in 25 μl volume. A 327 bp fragment of rat ghrelin and 630 bp fragment of rat β-actin cDNA as control were performed as described before (Raff et al., 1997; Kojima et al., 1999).

Statistical analysis

Data were analyzed with the General Linear Models Procedure of SAS software. Values are expressed as mean±standard error of the mean (SEM). The statistical significance of differences between plasmid DNA injection groups and control group was evaluated independently with Ducan's multiple range test, preceded by the analysis of variance (ANOVA). A p value of less than 0.05 was considered to be significant.

RESULTS

In vivo activity of myogenic expression vectors

Previous studies showed that continuous infusion of ghrelin durably increases body weight in adult rats (TschÖp et al., 2000; Nakazato et al., 2001). We then asked if a single injection of pGEM-wt-sGhln, pGEM-wt-hGhln in adolescent rats would be sufficient to elicit augmented GH systemic levels and enhance growth. Since N-octanoyl posttranslational modification at serine residue 3 of ghrelin is required for biological activity, and at present, there do not provide any information whether skeletal muscle can

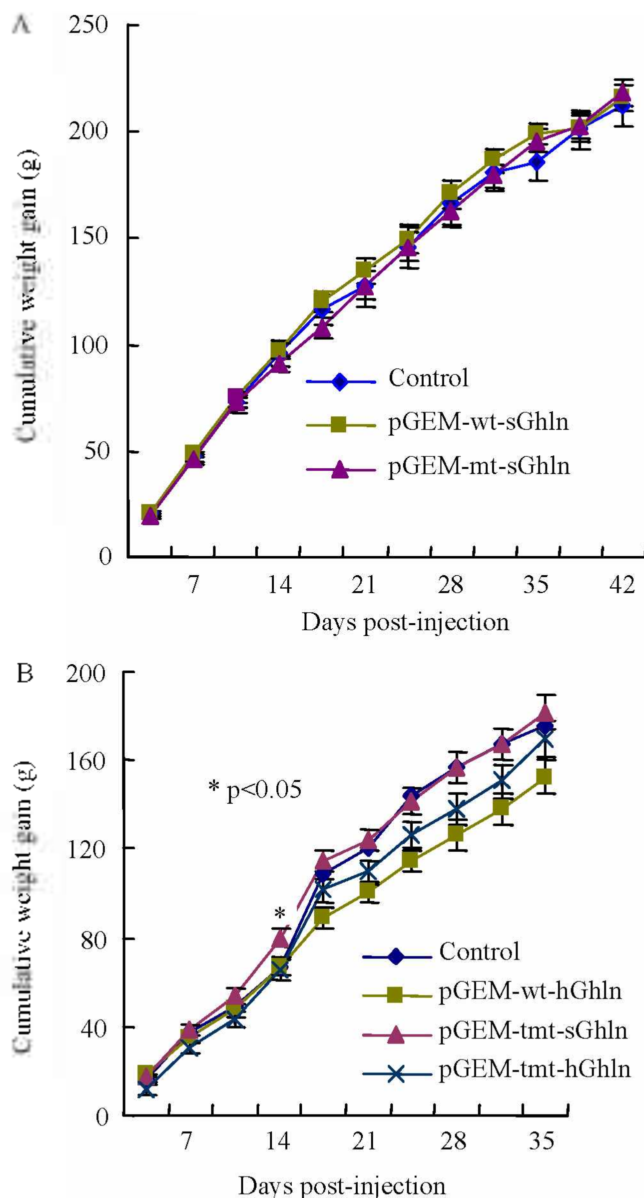

Figure 1. Increase in body weight after a single dose plasmid DNA injection in the regenerating left quadriceps muscle of puberty rats. The results are presented as means±SEM. (A) Injection of pGEM-wt-sGhln (n=18), pGEM-mt-sGhln (n=18), age-matched control animals (n=8). (B) Injection of pGEM-wt-hGhln (n=18), pGEM-tmt-sGhln (n=18), pGEM-tmt-hGhln (n=16), age-matched control animals (n=16). *$p<0.05$ was observed at day 14 of pGEM-tmt-sGhln compared with control group.

perform the cellular machinery for this acylation, so the pGEM-mt-sGhln and pGEM-mt-hGhln replacing the octanoylated serine with an aromatic amino acid. Trp(W) were constructed. Using a Blast program, we found that N-terminal of ghrelin, especially in the first 7 amino acids is highly homologous within all mammalian species studied. Previous studies had also reported that short peptides encompassing the first 4-5 residues of ghrelin were found to activate the GHS-R1a almost as efficiently as the full-length ghrelin (Bednarek et al., 2000; Matsumoto et al., 2001), so the pGEM-tmt-sGhln and pGEM-tmt-hGhln encoding the short peptides encompassing the first 7 amino acids (including serine residue 3 substituted with Trp) with amidation of the C-terminal by a basic amino acid. Lys (K) were also constructed. We then want to test whether a single injection of those vectors encoding ghrelin variants can mimic the effects of mature ghrelin if ghrelin ectopically produced in skeletal muscle cannot be acylated.

In the first animal experiment, animals were injected 200 μg pGEM-wt-sGhln and pGEM-mt-sGhln into the regenerating left quadriceps muscle respectively. Ghrlein and its mutant secreted into the systemic circulation after intramuscular injection. The pGEM-wt-sGhln enhanced growth up to 5 weeks after a single dose injection and cumulative weight gain peaked at day 21 about 6% heavier over controls (135.06±6.06 g vs. 127.24±6.22 g, $p<0.26$) as shown by Figure 1A, though, there had no statistical significance in their total body mass. In comparison, the effects of pGEM-mt-sGhln were a little inferior to that of control group during the same times.

In the second animal experiment, animals were injected 200 μg pGEM-wt-hGhln, pGEM-tmt-sGhln and pGEM-tmt-hGhln respectively. The ghrelin, its variants secreted into systemic circulation after plasmid DNA injection. The pGEM-tmt-sGhln was the most potent stimulus to enhance growth in normal rats over 3 weeks, as shown Figure 1B about 19% heavier than negative controls in cumulative weight gain peaked at day 14 post-injection (79.84±4.29 g versus 66.96±4.57 g, $p<0.05$) and there were no orgamegaly or associated pathology. In comparison, the growth in pGEM-wt-hGhln and pGEM-tmt-hGhln groups was inferior to that of control animals over the same duration. Taken together, our studies also showed the efficacy of leader/signal peptide of ghrelin itself was better than that of hGHRH with gene medicine approach.

GH-releasing activity in rats

All serum samples from each experiment were run in one single assay for rGH RIA. Serum rGH levels varied greatly between individuals either in plasmid DNA injected groups or in control animals (Figure 2A/B). This could be due to the possibility that GH secretion is pulsatile, i.e. a complex neuroendocrine regulation process. Further, time-course analysis of rGH as a response to myogenic vectors injection showed that collection of blood samples lagged behind the strongest effects of pGEM-wt-sGhln or pGEM-tmt-sGhln.

Tissue expression of rat ghrelin and pGEM-tmt-sGhln

At day 35 post-injection, the *in vivo* expression of pGEM-tmt-sGhln and rat ghrein were also assayed by RT-PCR on injected muscle, stomach, testis and heart. Total

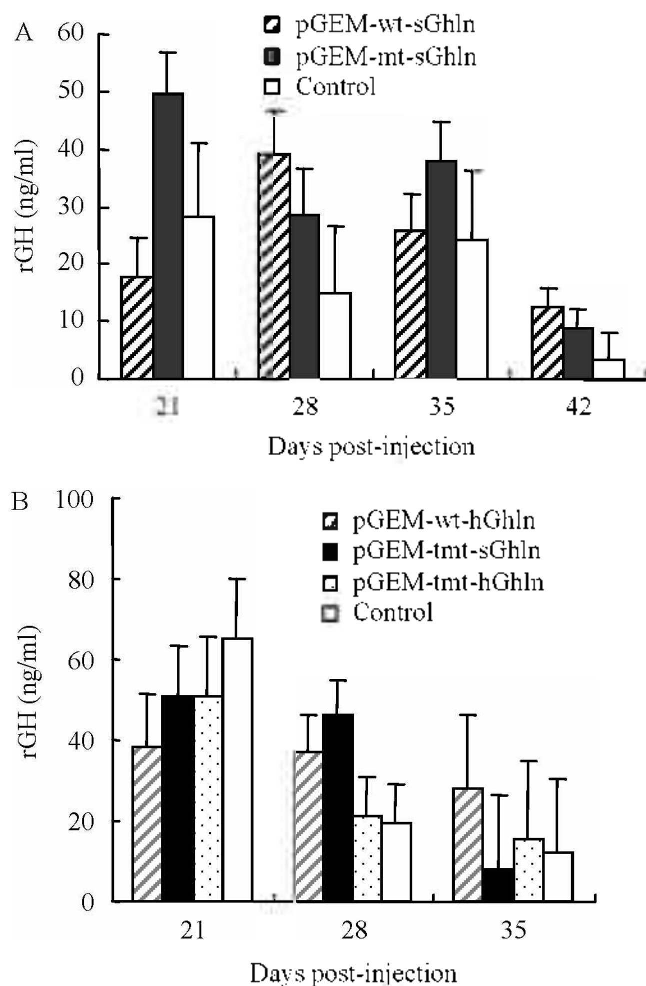

Figure 2. Serum rGH concentration measured by radioimmunoassay after direct intramuscular injection of pGEM-Ghln constructs in puberty rats. The results are presented as means±SEM. (A) Injection of pGEM-wt-sGhln, pGEM-mt-sGhln. (B) Injection of pGEM-wt-hGhln. pGEM-tmt-sGhln, pGEM-tmt-hGhln.

RNA was DNase I treated in order to eliminate the injected plasmid. A 327 bp fragment of rat ghrelin was only found in stomach (Figure 3). Further, our RT-PCR analysis indicates that muscle tissue does not express ghrelin in normal rats, though GHS-R exists in there. However, the expected fragment of pGEM-tmt-sGhln was not amplified in injected muscle (data not shown). Maybe, the injected pGEM-tmt-sGhln plasmid expressed transiently, for no more than 5 weeks. The efficiency of DNase treatment to eliminate plasmid DNA was determined by using RNA from tissue detected when the reverse transcriptase was omitted from the reaction and no amplification was observed in (-).

DISCUSSION

Initial studies focused on the action of ghrelin that stimulates growth hormone secretion. However, the

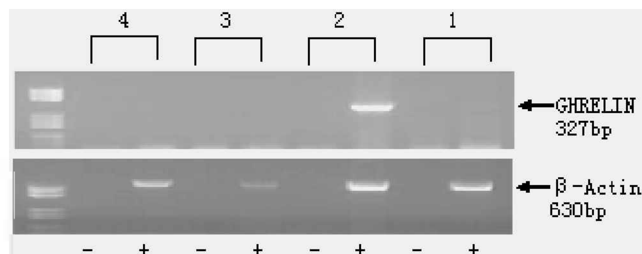

Figure 3. Tissue-specific expression of ghrelin assayed by RT-PCR in rats at day 35 after injection of pGEM-tmt-sGhln. RT-PCR analysis is as follows: 1. Heart 2. Stomach 3. Regenerating left quadriceps muscle 4. Testis. In (-) tubes the reverse transcriptase was omitted. Uper panel: PCR reaction using rat ghrelin cDNA specific oligonucleotides, Lower panel: PCR reaction using rat β-actin specific oligonucleotides.

discovery that infusion of ghrelin stimulates feeding and produces obesity in rodents, independently of changes in growth hormone secretion, rapidly changed the direction of research on ghrelin. Researcher began to examine the role of this gastric hormone in energy balance and its disorder, including obesity (Cummings et al., 2002). Potential targets for drug development in field of obesity treatment are now plentiful and ghrelin antagonists now have been included among them. Similarly, studies of ghrelin agonists will address the possible benefits for patients with cachexia or wasting disorder and maybe different disease states associated to alter GH secretion. Limitation of current studies includes the high cost of synthetic ghrelin peptide and its analogs, the short half-life of the peptide *in vivo*, and the requirement for frequent administration of either subcutaneous, intravenous or intracerebroventricular injection. Using pGEM-wt-sGhln or pGEM-tmt-sGhln injectable vectors, it might be possible to enhance growth in farm animal or to stimulate appetite for human with cachexia or wasting disorder for long-term in a more physiological and less expensive manner than classical therapies.

We have demonstrated the feasibility of delivering a peripheral hormone via a muscle-specific expression vectors, pGEM-wt-sGhln or pGEM-tmt-sGhln. Injected pGEM-wt-sGhln encoding natural mature ghrlein augmented growth up to 5 weeks. This may indicate that skeletal muscle may have the ability to perform posttranslational modification of ghrelin, which is necessary for its subsequent biological activities, but this hypothesis needs further confirmation. Injected pGEM-tmt-sGhln encoding truncated serine residue replacement ghrelin variant was the most potent stimulus to increase growth in normal rats. This may suggest that truncated ghrelin variant may have the potential to mimic the effects for receptor binding and biological activities as efficiently as full-length

ghrelin. This can be explained that short ghrelin analogue has low immunity and contains an amidation of the C-terminal with a basic amino acid. Lys (k), though there still exist the controversies whether the C-terminal part of the ghrelin molecule plays a role in establishing the bioactive conformation of the intact acylated ghrelin molecule (Torsello et al., 2002). Our current work is focused on optimizing plasmid purification and the deliver system to permit this tissue-specific gene medicine methodology to be applied to produce sustained levels of therapeutic proteins.

Transgene expression in skeletal muscle has been demonstrated using plasmid backbone and reporter genes driven by porcine skeletal SKA promoters (Reecy et al., 1998). Among the nonviral techniques developed for gene transfer *in vivo*, the direct injection of plasmid DNA into muscle is simple, inexpensive and safe. One potential limitation for the SKA promoter to gene medicine application is that, in general, it yields lower levels expression *in vivo* than the stronger CMV viral promoter. Previously, these levels were insufficient to ensure systematic physiological concentration of secreted proteins such as hormones, neurotrophic factors or coagulation factors in large mammals. In order to obtain growth of large animals by gene medicines, we believed that it was necessary to increase the potency of myogenic vector system as reported (Li et al., 1999; Li et al., 1999). The control of cellular access and uptake, intracellular trafficking and nuclear retention of plasmids must also be achieved to improve efficiency of plasmid-based gene medicine.

The low cost, the possibility of industrial-scale production of plasmid DNA, combined with easy administration procedure should allow the development of this system in livestock and even for human clinical medicine. In addition, treated rats did not experience any adverse effects of therapy, had normal biological profiles and developed no associated pathology. In sum, the delivery of the muscle-specific expression constructs which encode the ghrelin, a peripheral hormone, ghrelin derivatives is a very promised approach to augment growth in livestock and for the gene therapeutic applications for human.

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REFERENCES

Bednarek, M. A., S. D. Feighner, S. S. Pong, K. K. McKee, D. L. Hreniuk, M. V. Silva, V. A. Warren, A. D. Howard, L. H. Van Der Ploeg and J. V. Heck. 2000. Structure-function studies on the new growth hormone-releasing peptide, ghrelin: minimal sequence of ghrelin necessary for activation of growth hormone secretagogue receptor 1a. J. Med. Chem. 43:4370-4376.

Cassoni, P., M. Papotti and C. Ghe. 2001. Identification, characterization and biological activity of specific receptors for natural (ghrelin) and synthetic growth hormone secretagogues and analogs in human breast carcinomas and cell lines. J. Clin. Endocrinol. Metab. 86:1169-1174.

Cummings, D. E., D. S. Weigle, R. S. Frayo, P. A. Breen, E. P. Dellinger and J. Q. Purnell. 2002. Plasma ghrelin levels after diet-induced weight loss or gastric bypass gastric surgery. N. Engl. J. Med. 346:1623-1630.

Date, Y., M. Nakazato, N. Murakami, M. Kojima, K. Kangawa and S. Matsukura. 2001. Ghrelin acts in the central nervous system to stimulate gastric acid secretion. Biochem. Biophys. Res. Commun. 280:904-907.

Date, Y., N. Murakami, M. Kojima, T. Kuroiwa, S. Matsukura, K. Kangawa and M. Nakazato. 2000. Central effects of a novel acylated peptide, ghrelin, on growth hormone release in rats. Biochem. Biophys. Res. Commun. 275:477-480.

Draghia-Akli, R., M. Fiorotto, L. A. Hill, P. B. Malone, D. R. Deaver and R. J. Schwartz. 1999. Myogenic expression of an injectable protease-resistant growth hormone-releasing hormone augments long-term growth in pigs. Nat. Biotechnol. 17:1179-1183.

Draghia-Akli, R., X. G. Li and R. J. Schwartz. 1997. Enhanced growth by ectopic expression of growth hormone releasing hormone using an injectable myogenic vector. Nat. Biotechnol. 15:1285-1289.

Horvath, T. L., S. Diano, P. Sotonyi, M. Heiman and M. TschÖp. 2001. Minireview: ghrelin and the regulation of energy balance-a hypothalamic perspective. Endocrinology. 142:4163-4169.

Kojima, M., H. Hosoda, Y. Date, M. Nakazato, H. Matsuo and K. Kangawa. 1999. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature. 402:656-660.

Lawrence, C. B., A. C. Snape, F. M. Baudoin and S. M. Luckman. 2002. Acute central ghrelin and GH secretagogues induce feeding and activate brain appetite centers. Endocrinology. 143:155-162.

Li, S., F. C. Maclaughlin, J. G. Fewell, Y. Li, V. Metha, J. L. Nordstrom, M. Coleman, N. S. Belagali, R. J. Schwartz and L. C. Smith. 1999. Increased level and duration of expression in muscle by co-expression of a transactivator using plasmid systems. Gene Therapy. 6:2005-2011.

Li, X., E. M. Eastman, R. J. Schwartz and R. Draghia-Akli. 1999. Synthetic muscle promoters: activities exceeding naturally occurring regulatory sequences. Nat. Biotchnol. 17:241-245.

Masuda, Y., T. Tanaka, N. Inomata, N. Ohnuma, S. Tanaka, Z. Itoh, H. Hosoda, M. Kojima and K. Kangawa. 2000. Ghrelin stimulates gastric acid secretion and motility in rats. Biochem. Biophys. Res. Commun. 276:905-908.

Matsumoto, M., H. Hosoda, Y. Kitajima, N. Morozumi, Y.

Minamitake, S. Tanaka, H. Matsuo, M. Kojima, Y. Hayashi and K. Kangawa. 2001. Structure-activity relationship of ghrelin: pharmacological study of ghrelin peptides. Biochem. Biophys. Res. Commun. 287:142-146.

Nagaya, N., K. Miyatake, M. Uematsu, H. Oya, W. Shimizu, H. Hosoda, M. Kojima, N. Nakanishi, H. Mori and K. Kangawa. 2001a. Hemodynamic, renal and hormonal effects of ghrelin infusion in patients with chronic heart failure. J. Clin. Endocrinol. Metab. 86:5854-5859.

Nagaya, N., M. Uematsu, M. Kojima, Y. Ikeda, F. Yoshihara, W. Shimizu, H. Hosoda, Y. Hirota, H. Ishida, H. Mori and K. Kangawa. 2001b. Chronic administration of ghrelin improves left ventricular dysfunction and attenuates development of cardiac cachexia in rats with heart failure. Circulation. 104:1430-1435.

Nakazato, M., N. Murakami, Y. Date, M. Kojima, H. Matsuo, K. Kangawa and S. Matsukura. 2001. A role for ghrelin in the central regulation of feeding. Nature. 409:194-198.

Nou, V., K. Tomoshi, H. inoue, N. Matsunaga, H. Kuwayama and H. Hidari. 2003. Effect of exogenous porcine GH administration on GH response to GH-releasing peptide-2 and GH-releasing hormone in swine. Asian-Aust. J. Anim. Sci. 16:1188-1192.

Pombo, M., C. M. Pombo, A. Garcia, E. Caminos, O. Gualillo, C. V. Alvarez, F. F. Casanueva and C. Dieguez. 2001. Hormonal control of growth hormone secretion. Horm. Res. 55 (Suppl. 1):11-16.

Raff, T., M. Van der Giet, D. Endemann, T. Wiederholt and M. Paul. 1997. Design and testing of β-actin primers for RT-PCR that do not co-amplify processed pseudogenes. Bio. Techniques. 23:456-460.

Reecy, J. M., C. A. Bidwell, G. P. Briley and A. L. Grant. 1996. Structure and regulation of the porcine skeletal α-actin-encoding gene. Gene. 180:23-28.

Reecy, J. M., C. A. Bidwell, O. M. Andrisani, D. E. Gerrard and A. L. Grant. 1998. Multiple regions of the porcine alpha-skeletal actin gene modulate muscle-specific expression in cell culture and directly injected skeletal muscle. Anim. Biotechnol. 9:101-120.

Takaya, K., H. Ariyasu, N. Kanamoto, H. Iwakura, A. Yoshimoto, M. Harada, K. Mori, Y. Komatsu, T. Usui, A. Shimatsu, Y. Ogawa, K. Hosoda, T. Akamizu, M. Kojima, K. Kangawa and K. Nakao. 2000. Ghrelin strongly stimulates growth hormone release in humans. J. Clin. Endocrinol. Metab. 85:4908-4911.

Tanwattana, P., S. Chanpongsang and N. Chaiyabutr. 2003. Effects of exogenous bovine somatotrophin on mammary function of late lactating crossbred Holstein cows. Asian-Aust. J. Anim. Sci. 16:88-95.

Tolle, V., P. Zizzari, C. Tomasetto, M. C. Rio, J. Epelbaum and M. T. Bluet-Pajot. 2001. *In vivo* and *in vitro* effects of ghrelin/motilin-related peptide on growth hormone secretion in the rat. Neuroendocrinology 73:54-61.

Torsello, A., C. Ghe, E. Bresciali, F. Catapano, E. Ghigo, R. Deghenghi, V. Locatelli and G. Muccioli. 2002. Short ghrelin peptides neither displace ghrelin binding *in vitro* nor stimulate GH release *in vivo*. Endocrinology.143:1968-1971.

TschÖp, M., D. L. Smiley and M. L. Heiman. 2000. Ghrelin induces adiposity in rodents. Nature 407:908-913.