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Undercarboxylated osteocalcin downregulates pancreatic lipase expression in an ATF4-dependent manner in pancreatic acinar cells

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ABSTRACT

Osteocalcin is an osteoblast-specific secreted protein that has been associated with endocrine roles in multiple aspects of energy metabolism. We examined whether undercarboxylated osteocalcin (ucOC) downregulates pancreatic lipase (PNLIP) expression in pancreatic acinar cells and then identified the downstream signaling pathway involved. We previously demonstrated that β adrenergic blockade attenuates body weight/fat mass gain in high-fat diet-fed mice and that this effect is associated with decreased PNLIP expression in pancreatic acinar cells. In the present study, we first confirmed that the serum ucOC level is inversely correlated with PNLIP expression, i.e., mice exhibiting high serum levels of ucOC showed low PNLIP levels in the pancreas. In in vitro experiments using primary pancreatic acinar and 266-6 cells, ucOC downregulated PNLIP expression. cAMP/PKA signaling inhibitors significantly reversed ucOC-induced downregulation of PNLIP expression. ucOC promoted the phosphorylation of cAMP response element-binding protein 2 (ATF4). Overexpression of ATF4 significantly suppressed PNLIP expression. Knockdown of ATF4 by siRNA reversed the ucOC-induced downregulation of PNLIP expression. A luciferase reporter assay showed that ucOC suppressed PNLIP promoter transactivation. Chromatin immunoprecipitation and a luciferase reporter assay demonstrated that ATF4 directly bound to the CRE on the mouse PNLIP promoter and suppressed PNLIP transactivation. Knockdown of G-protein coupled receptor 6A (Gprc6a), a candidate receptor for mediating the response to ucOC in the bone-pancreas endocrine loop, by siRNA reversed the downregulating effect of ucOC on PNLIP expression.

Taken together, ucOC downregulates pancreatic lipase expression in a cAMP/protein kinase A/ATF4-dependent manner. Gprc6a is a potential osteocalcin-sensing receptor that regulates PNLIP expression in pancreatic acinar cells.

1. Introduction

Osteocalcin is one of the most abundant proteins produced in bone. This protein is released into the blood stream when new bone is formed and is considered as a bone turnover marker [1].

Mature osteocalcin contains three glutamic acid residues. Once γ-carboxylated, they are in charge of binding to calcium and hydroxyapatite. Upon binding of the γ-carboxylated glutamatic acid residue to calcium, a disulfide bond between cysteine residues stabilizes the 3 dimensional structure of the osteocalcin molecule [2]. Glutamate carboxylation events occur by binding of the immature peptide to γ-glutamyl carboxylase. To complete the carboxylation of the partially carboxylated vitamin K-dependent propeptide, vitamin K is converted to an epoxide and then reduced by vitamin K epoxide reductase [3].

Undercarboxylated osteocalcin (ucOC) does not appear to bind to calcium, but may interact with its recently identified osteocalcin sensing receptor, G-protein coupled receptor family C group 6 member A (Gprc6a) [4]. Previous studies showed that Gprc6a mediates the effect of ucOC in various tissues [5–7]. Analyzing mutant mouse strains lacking *Osteocalcin* and/or its receptor *Gprc6a*, Mera P et al. demonstrated cAMP-CREB pathway as one mediator of osteocalcin signaling through Gprc6a in myofibers [8]. Pi M et al. has shown that Gprc6a is a candidate for mediating the response to ucOC in β-cells in vitro and pancreas in vivo regulating insulin signaling [9].

Carboxylated osteocalcin interacts with hydroxyapatite crystals and modulates bone turnover, whereas ucOC functions as a hormone that affects multiple aspects of glucose and energy metabolism, male fertility, and cognition [6,7,10]. In the pancreas, circulating ucOC increases β-cell proliferation and insulin production/secretion, while ucOC increases insulin sensitivity in the skeletal muscle and adipose tissue

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[11–13]. Lee et al. demonstrated that ESP (Ptprv), a tyrosine phosphatase expressed only in the bone, negatively regulates the carboxylation of osteocalcin, thus affecting insulin secretion in the pancreas [14]. Lee et al. demonstrated that lacking ESP are hypoglycemic and are protected from obesity due to an increase in β-cell proliferation, insulin secretion [14].

Before intestinal absorption, ingested dietary fat is converted into a fatty acid and 2-monoacylglycerol by pancreatic lipase (PNLIP) [15]. Inhibition of PNLIP and subsequent suppression of dietary fat absorption is considered to be an effective approach for obesity prevention [5,6]. PNLIP expression increases with high-fat diet feeding [16]. High-fat diet feeding, which is the major cause of obesity, has been shown to stimulate the sympathetic nervous system [17]. We previously demonstrated that propranolol, a nonselective inhibitor of the β1/β2-adrenergic receptor, mitigates dietary fat absorption and obesity development and that this effect is associated with suppression of PNLIP expression in pancreatic acinar cells [18].

Clinical studies have shown that serum carboxylated osteocalcin and ucOC levels are inversely correlated with obesity, diabetes, or aging [19–21]. Many animal studies have demonstrated that ucOC administration improves glucose metabolism and prevents obesity and glucose intolerance [22,23].

Even though there has been reports that osteocalcin improves glucose/energy metabolism, it has not yet been elucidated the direct mechanism how osteocalcin suppresses weight gain in relation to obesity. In general, main mechanisms associated with weight control can be thought of as follows: (i) inhibiting lipid digestion, absorption and intake and (ii) promoting lipid metabolism to reduce lipogenesis and enhance lipolysis, and decreasing lipid accumulation by suppressing the proliferation and differentiation of adipocytes [24,25]. In the present study, we noted the possibility of ucOC inhibition of dietary fat absorption. In the pancreas, several lipid metabolism-related enzymes are secreted. PNLIP is generally considered as a principal regulator of dietary fat absorption. Given these previous findings and the endocrine role of ucOC in targeting the pancreas, we hypothesized that ucOC has another endocrine role beyond regulating β-cell function, such as regulating PNLIP expression in pancreatic acinar cells.

In the present study, we explored whether ucOC downregulates PNLIP expression in pancreatic acinar cells and then identified the intracellular downstream signaling pathway involved. We also explored whether the effects of ucOC downregulating PNLIP expression in pancreatic acinar cells were mediated by Gprc6a.

2. Materials and methods

2.1. Animals experiments

Animal samples to observe the correlation between serum ucOC level and PNLIP level were gathered from previous studies (Suppl Fig. 1) [18]. In brief, forty C57BL/6mice, aged 5 weeks at purchase (Orient Bio Inc., Seoul, Korea), were block-assigned by body weight into four groups of ten animals each: ad-lib fed controls treated with vehicle (CONVEH) or β-blocker (CONBB), and high fat diet mice treated with vehicle (HIGHVEH) or β-blocker (HIGHBB). The CON group was fed ad-lib for the twelve-week protocol. For the high fat diet (60% calories from fat) experiments, mice were fed a high fat diet for 12 weeks. β-Blocker groups were administered propranolol (0.5 g/liter drinking water) via drinking water. Animal procedures were approved by the Seoul National University Institutional Animal Care and Use Committee (SNU-110531-2).

ucOC daily injection experiment was conducted as follows: Six-week-old male C57BL/6 mice (Orient Bio Inc. Seoul, Korea) were assigned into four groups of five animals each: ad lib-fed controls treated with the vehicle (CONVEH) or undercarboxylated osteocalcin (CONucOC) and mice fed high fat diet (60% of calories from fat) treated with the vehicle (OBVEH) or undercarboxylated osteocalcin (OBucOC). Recombinant osteocalcin was freshly diluted in saline solution (0.9% NaCl) at a concentration of 0.3 ng/μl and mice were injected once a day intraperitoneally (i.p.) with 10 μl/g of this solution or with saline solution (vehicle). In control diet groups, daily injections were initiated at 6 weeks of age. In high-fat diet groups, mice were fed a high-fat diet (60% of calories from fat) starting at 6 weeks of age and daily injections of osteocalcin or vehicle were initiated 4 weeks later. Animal procedures were approved by the Gangneung-Wonju National University Institutional Animal Care and Use Committee (GWNU-2019-4).

2.2. Reagents and antibodies

The PCR primers were synthesized by Cosmogenetech (Seoul, Korea). LipofectAMINE™ reagent was purchased from Invitrogen (Carlsbad, CA). Dual-Glo luciferase assay kit was purchased from Promega (Madison, WI). Pancreatic lipase antibodies (sc-98992), Actin antibodies (sc-27778), and were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Gprc6a antibodies (orb 157283) and HRP-conjugated secondary antibodies (31640) were purchased from biorbyt (Cambridge, UK) and Thermo Fisher Scientific (IL, USA), respectively. For the Western blot analysis, the Supex reagent was ordered from Dyne-Bio (Sungnam, Korea). The Dual-Glo luciferase assay kit was purchased from Promega (Madison, WI). Undercarboxylated form of osteocalcin was purchased from BACHEM (Torrance, CA/H-6552). L-arginine was purchased from Sigma-Aldrich (St. Louis, MO). ELISA was performed with the ucOC (CUSABIO Biotech/CSB-E1768lm) and PNLIP (CUSABIO Biotech/CSB-E16930m) assay kit according to the manufacturer's instructions.

2.3. Cell culture

Primary islets were isolated as described [14,26]. Briefly, after rinsing twice in Hank's Balanced Salt Solution (HBSS) 1× (Sigma Aldrich), pancreas was sliced in small pieces of 1 to 3 mm^3. After centrifugation, the pancreas sections were injected with 10 ml of a collagenase 1A solution (HBSS 1× containing 10 mM HEPES (Sigma Aldrich), 200 U/ml of collagenase 1A (Sigma Aldrich), and 0.25 mg/ml of trypsin inhibitor (GIBCO)) and digested for 30 min. After then, cell mixture was filtrated by allowing it to pass through a 100 μm filter. Pancreatic acinar structures (acinus of 10–15 cells) were then seeded in a 6-well culture dish. After 24 h, the isolated primary acini cells were transferred into the type 1 collagen-coated 6-well culture dish. Media was replaced every three days.

266-6 cells, a murine pancreatic acinar cell line, were maintained in Dulbecco's Modified Eagles Medium (DMEM; Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT). Culture plates were coated prior to use 0.1% Gelatin Type A (Gelatin; Sigma-Aldrich, St. Louis, MO, USA) in distilled water for 30 to 60 min. HEK293T cells was maintained in DMEM supplemented with 10% FBS.

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

To evaluate mRNA expression, real time RT-PCR was performed as previously described [27]. Mouse genes and the sequences of the PCR primers used for real time-PCR are shown in Suppl Table 1. For quantification, internal mouse GAPDH was used as the reference for the normalization of each sample. For quantification, internal mouse GAPDH was used as the reference for the normalization of each sample. Real-time PCR data are presented as the mean ± SD of triplicates.

2.5. Western blot analysis

The cells were washed with PBS and scraped into lysis buffer (consisting of 10 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM EDTA (pH 8.0), 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM sodium fluoride, 0.2 mM sodium orthovanadate, 1 mM PMSF,

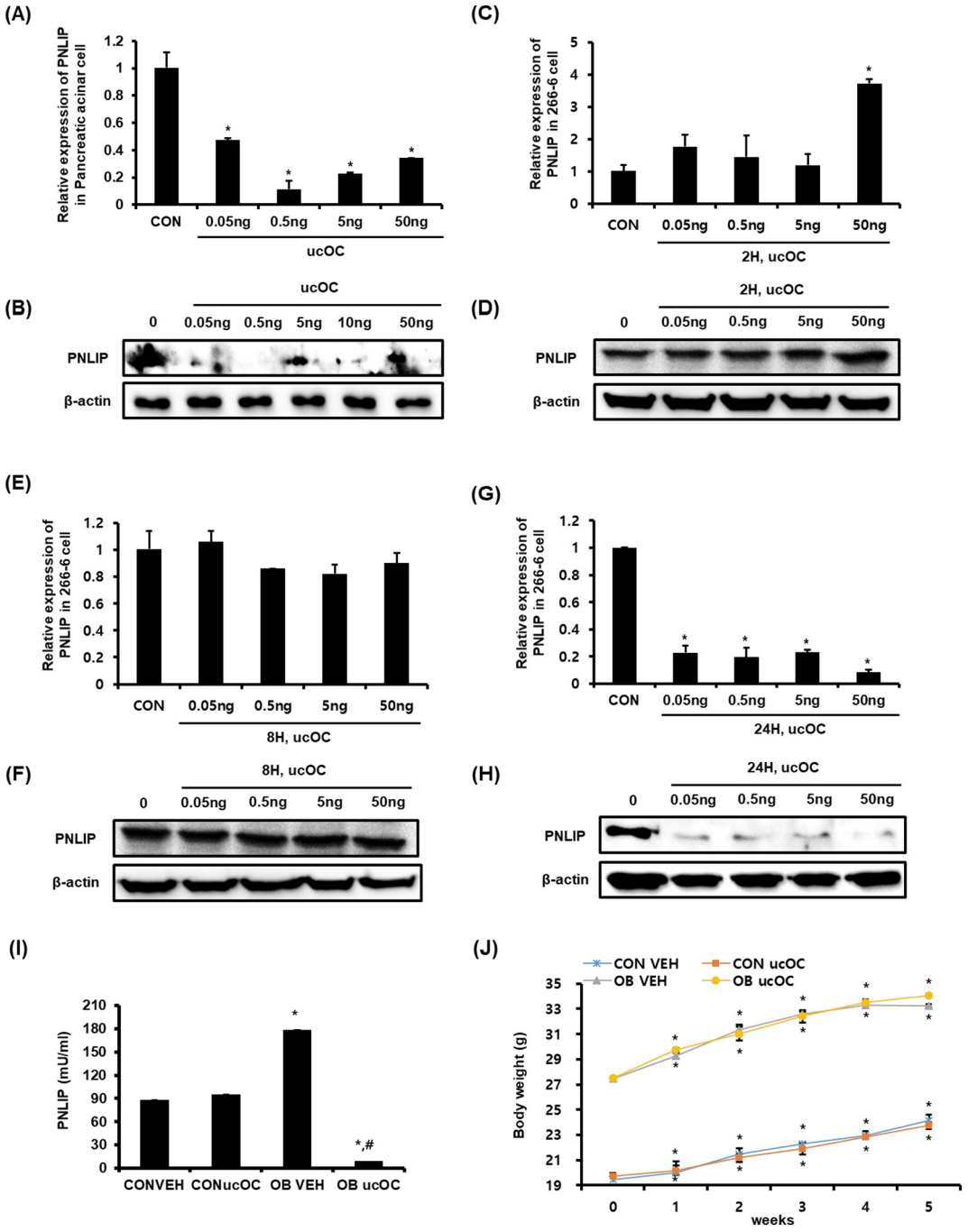

Fig. 1. ucOC downregulates PNLIP expression.
The primary cultured mouse pancreatic acinar cells (A, B) and the 266-6 pancreatic acinar cell line (C–H) were incubated with ucOC for 2, 8 and 24 hours at a range of concentration from 0.05 to 50 ng/ml. PNLIP expression levels were examined using quantitative RT-PCR (A, C, E, G) and western blot analysis (B, D, F, H). The data are presented as the means ± S.D. (*$p < 0.05$ vs. CON). The western blot data showed the results confirmed in at least 3 independent experiments. (I) The serum PNLIP level was examined using ELISA assay. Data are presented as the mean ± SD of duplicates. *$p < 0.05$ vs. CONVEH, $^{\#}p < 0.05$ vs. OB VEH. (J) Body weights over 5 weeks of the control diet (CON) or high fat diet (HIGH) in mice administered vehicle (VEH) or ucOC. *$p < 0.05$ vs. CONVEH.

1 μg/ml aprotinin, 1 μM leupeptin and 1 μM pepstatin) and sonicated briefly. Proteins were subjected to SDS-PAGE and subsequently electro-transferred onto a PVDF membrane. The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween20 and incubated with the indicated primary antibody followed by incubation with HRP-conjugated secondary antibody. Immune complexes were visualized with the Supex reagent, and luminescence was detected with a LAS1000 (Fuji PhotoFilm; Tokyo, Japan).

2.6. RNA interference of Gprc6a

siGENOME Smartpool mouse Gprc6a siRNA (sc-62414) and non-specific control scrambled siRNA (sc-37007) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). 266-6 cells were transfected using Dharmafect (Dharmacon) according to the manufacturer's instructions and the efficacy of knockdown was assessed by quantitative RT-PCR using a Gprc6a specific primers and western blot using Gprc6a antibodies.

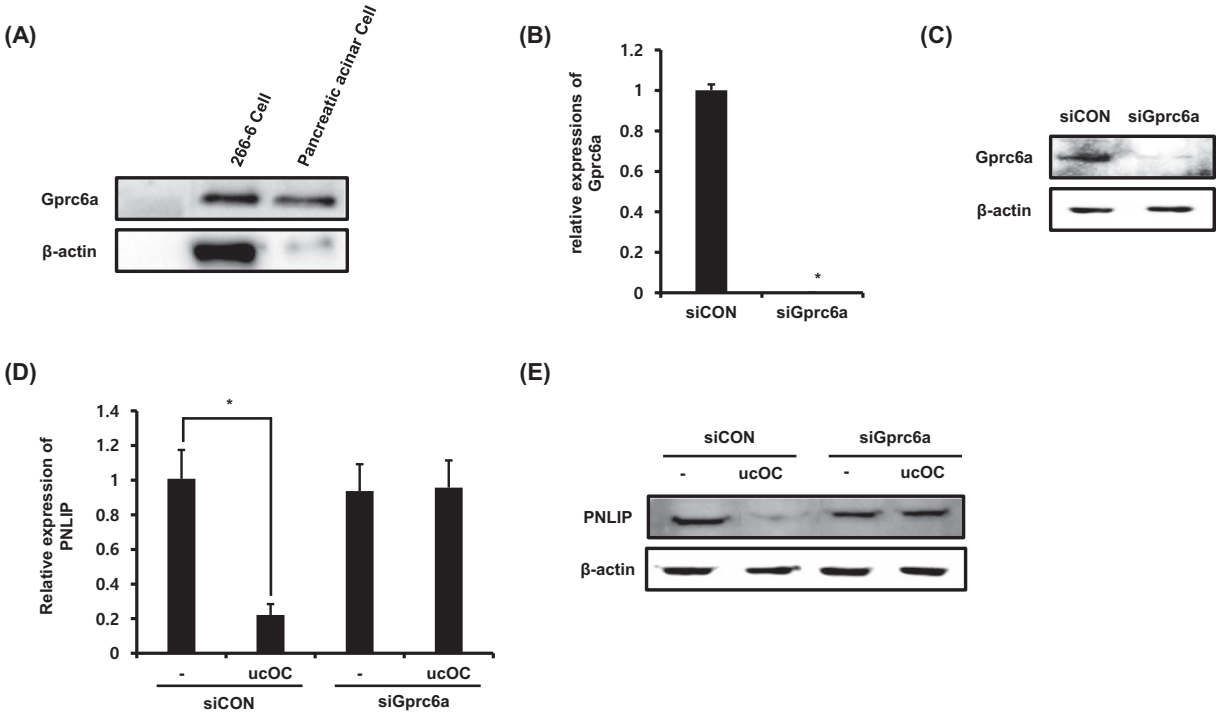

Fig. 2. ucOC downregulates PNLIP expressions through its interaction with an Gprc6a in pancreatic acinar cells.
(A) Western blot analysis was performed to identify the expression level of Gprc6a using whole lysate in 266-6 cell and primary cultured pancreatic acinar cells. (B, C) The 266-6 cells were transfected with non-targeting control siRNA (siCON) or siRNA targeting Gprc6a (siGprc6a). The efficiency of Gprc6a knockdown was confirmed by quantitative RT-PCR and Western blot analysis (*p < 0.05 vs. siCON). (D, E) The 266-6 cells transfected with siCON or siGprc6a were incubated for 24 h in the presence or absence of ucOC. The effect of Gprc6a knockdown on PNLIP mRNA or protein expression level was analyzed by quantitative RT-PCR and Western blot (*p < 0.05 vs. indicated pair). Data are presented as the mean ± SD. The western blot data showed the results confirmed in at least 3 independent experiments.

2.7. Plasmid construction

The mouse PNLIP promoter was constructed as previously described [18]. In brief, the mouse PNLIP promoter sequence (−1700 to +1 bp) was selected for cloning by searching mouse genomic sequences (http://genome.ucsc.edu/). The PNLIP promoter region was amplified by PCR using mouse genomic DNA as a template and subcloned into the (*Nhe*I) sites of pGL3b vector (PNLIP-WT-luc). Site-directed mutagenesis PCR (GTGAC ➔ GaGtg) was performed from −1587 to −1582 bp (MT), to generate function-defective reporter constructs (PNLIP-MT-luc) that contain mutations in the putative CRE binding site.

2.8. Transient transfection and reporter assay

HEK293 cells were transfected with the indicated plasmids using LipofectAMINE™ reagent (Invitrogen, Carlsbad, CA). In each transfection, 0.2 μg of reporter and the **Renilla** luciferase plasmid were used as indicated. After 24 h, the cells were incubated with drug (ISO) and ucOC for 8 h and luciferase activity was measured using the Dual-Glo luciferase assay kit (Promega, Madison, WI) according to the manufacturer's instructions. The relative luciferase activity was calculated after normalizing the transfection efficiency by Renilla luciferase activity.

2.9. Statistical analysis

To analyze the body weight values, PNLIP expression levels in pancreas and serum ucOC levels, two-way ANOVA was performed using the SAS (v.9.3) statistical package. In those cases where a significant omnibus F-test ($p < 0.05$) was obtained, the appropriate post hoc test was performed to explore the differences among individual means. The statistical significance of gene expression level and reporter activity was assessed by Student's *t*-test. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. Serum ucOC level is inversely correlated with PNLIP expression

In the present study, we first attempted to confirm whether the serum ucOC level is associated with PNLIP expression. High-fat diet, which is the major cause of obesity, is known to activate the sympathetic nervous system [17]. We previously demonstrated that propranolol, a nonselective inhibitor of the β1/β2-adrenergic receptor, attenuates body weight/fat mass gain in mice fed a high-fat diet and that propranolol decreased PNLIP expression in high-fat diet-fed mice, which increased fecal excretion of dietary fat [18] (Supple. Fig. 1A, B).

Our ELISA results confirmed that the serum ucOC level was lower (−45%, $p < 0.05$) in high-fat diet-fed mice than in control diet-fed mice. This reduction was totally rescued in propranolol-treated high-fat diet-fed mice (Supple. Fig. 1C) and inversely correlated with body weight and PNLIP expression levels, particularly in the high-fat diet-fed groups. These findings suggest that the serum ucOC level is inversely correlated with PNLIP expression.

Given with this data and the suggested role of ucOC in exerting favorable endocrine effects on energy metabolism [9], we set our novel hypothesis that ucOC may regulate the expression of PNLIP in pancreatic acinar cells.

3.2. ucOC downregulates PNLIP expression

Next, we treated cells with ucOC to confirm that osteoblast-derived osteocalcin controls PNLIP expression in pancreatic acinar cells. In vitro experiments using primary pancreatic acinar cells (Fig. 1A, B) and 266-6 cells (Fig. 1C–H) were conducted to evaluate PNLIP expression at the mRNA and protein levels. The serum level of ucOC is approximately

7 ng/ml in wild-type (WT) adult mice [14]. ucOC was employed to stimulate pancreatic acinar cells for 2 h, 8 h and 24 h over a wide concentration range of 0.05 to 50 ng/ml. ucOC did not induce a decrease in PNLIP expression level at 2 h and 8 h. At 24 h, compared to the untreated control, at all concentrations tested, ucOC significantly suppressed PNLIP expression, even at concentration as low as 0.05 ng/ml.

We then conducted animal experiments to investigate the ucOC regulation in PNLIP expression in in vivo model. The increase in serum PNLIP level observed in high fat diet induced obese mice (OBVEH) mice was significantly mitigated in mice treated with ucOC (OBucOC). In the body weight results, no significant attenuation was observed yet in OBucOC mice compared to OBVEH mice during five weeks of experimental period (Fig. 1I, J).

3.3. ucOC downregulates PNLIP expression through its interaction with Gprc6a in pancreatic acinar cells

Next, we evaluated the signaling pathways that regulate PNLIP expression in pancreatic acinar cells. To determine whether this receptor plays a role in acinar cell biology, we first tested Gprc6a expression in acinar cells. As shown in Fig. 2, Gprc6a was expressed in pancreatic acinar cells and acinar cell lines (Fig. 2A).

To further verify the role of Gprc6a in mediating ucOC signaling, we knocked down the expression of Gprc6a in 266-6 cells with siRNA. The expression level of Gprc6a was efficiently reduced by Gprc6a–specific siRNA (Fig. 2B, C). Knockdown of Gprc6a completely abolished the downregulating effect of ucOC on PNLIP expression at both the mRNA and protein levels (Fig. 2D, E). This data suggests that Gprc6a mediates ucOC signaling downregulating PNLIP expression in pancreatic acinar cells.

3.4. ucOC downregulates PNLIP expression via cAMP/PKA signaling pathway

To characterize the intracellular events involved in ucOC regulation of PNLIP expression, we examined the effects of cAMP/PKA signaling inhibitors. Rp-cAMPs and KT5720 significantly reversed ucOC-induced PNLIP expression (Fig. 3A, B), indicating that the cAMP/PKA pathway is involved in ucOC-induced PNLIP expression.

In the downstream elements of the cAMP/PKA pathway, CREB is a representative transcription factor. We next investigated whether CREB mediates the ucOC regulation of PNLIP expression in pancreatic acinar cells. When 266-6 cells were treated with ucOC, ATF4 phosphorylation was induced within 1–15 min (Fig. 3C). To further verify the role of ATF4 in PNLIP expression, we knocked down the expression of ATF4 in 266-6 cells using siRNA. The efficiency of knockdown by ATF4 siRNA was confirmed by quantitative RT-PCR and western blot analysis (Fig. 3D, E). Knockdown of ATF4 rescued the ucOC-induced PNLIP suppression (Fig. 3D, E). These results suggest that the cAMP/PKA/ATF4 pathway is involved in ucOC regulation of PNLIP expression. We also investigated if the knockdown resulted in reduced effects of well-known Gprc6a ligands, L-arginine. L-arginine led to suppression in ATF4 phosphorylation. Knockdown of Gprc6a mitigated the capacity of L-arginine to suppress ATF4 phosphorylation in 266-6 cells (Fig. 3F)

3.5. pATF4 binds to regions −1587 to −1582 for suppressing PNLIP promoter activity

To investigate whether ATF4 directly regulates PNLIP expression, we performed *in silico* analysis to search for the CREB-binding element (GTGAC) in the mouse PNLIP promoter using the Transcription Element Search System and found one putative CRE at −1587 to −1582 bp within the 1.7-kb mouse PNLIP gene promoter region (Fig. 4A).

To examine whether ATF4 binds to these putative CRE binding motifs in vivo, we performed ChIP assay. The 266-6 cells were treated with ucOC for 24 h, and the DNA fragments were immunoprecipitated with an antibody to phospho-ATF4 or with control IgG. PCR amplification of the PNLIP promoter region encompassing −1587 to −1582 bp revealed that ucOC induced the binding of phospho-ATF4 to the PNLIP promoter (Fig. 4B).

We next performed a luciferase reporter assay to examine whether ucOC regulates PNLIP promoter transactivation. As a positive control, the cells were treated with 1 μM of isoproterenol [18]. Treatment of the cells with 1 μM of isopreterenol for 24 h significantly enhanced the reporter activity of PNLIP-WT-luc, which contains the 1.7-kb mouse PNLIP promoter sequence. ucOC significantly suppressed transcriptional activation of the PNLIP reporters (Fig. 4C).

Promoter reporter assays were conducted to examine whether ucOC treatment or ATF4 overexpression transactivated the PNLIP promoter. Overexpression of ATF4 significantly decreased the reporter activity of PNLIP-WT-luc, whereas mutations in CRE blocked ATF4-induced luciferase activity suppression (Fig. 4D). Taken together, these results indicate that ATF4 bound directly to the PNLIP promoter and suppressed transcription of the PNLIP gene.

4. Discussion

Our primary hypotheses were that ucOC downregulates PNLIP expression in pancreatic acinar cells. The mechanistic pathway of osteocalcin in pancreatic acinar cell biology was also investigated. Our results suggest that ucOC downregulates PNLIP expression in a cAMP/PKA/ATF4-dependent manner via its receptor candidate Gprc6a in pancreatic acinar cells. To our knowledge, this is the first study to demonstrate osteoblast-derived osteocalcin regulation of PNLIP expression.

In our study, ucOC-induced ATF4 activation suppressed the transcription activity of the PNLIP promoter. Based on the ChIP assay analysis data, a binding site for ATF4 is present in the promoter region of the PNLIP gene. In conditions of ATF4 overexpression or ucOC engaging, pATF4 bound to the promoter region −1587 to −1582 (Fig. 4A) and suppressed PNLIP transcription. Thus, ATF4 appears to be a critical transcription factor for downregulating PNLIP expression. ATF4 is a member of the CREB family and CREB can form a homodimer to bind to DNA sites [28]. Dimerization or tetramerization of ATF4 results in the formation of a loop at the PNLIP promoter leading to obstruct PNLIP transcription, which is a potential mechanism explaining how ATF4 suppresses PNLIP transcription [28].

Oury et al. reported that ucOC regulates testosterone synthesis in a CREB-dependent manner by promoting the expression of enzymes required for testosterone synthesis and germ cell survival in mice lacking CREB expression specifically in Leydig cells ($Creb_{Leydig}^{-/-}$ mice) [29]. Mera P et al. demonstrated that CREB mediates ucOC signaling in myofibers, which is necessary and sufficient for optimum adaptation to exercise [8]. They showed that CREB phosphorylation in myotubes was weaker in muscle-specific Gprc6a knockout mice ($Gprc6a_{Mck}^{-/-}$) than in control mice after exercise. Additionally, the exercise capacity of $Gprc6a_{Mck}^{+/-}$; $Creb_{Mck}^{+/-}$ mice was also declined as the capacity of $Gprc6a_{Mck}^{-/-}$ mice [8]. In pancreatic acinar cells, our data support the hypothesis that CREB, but ATF4, not CREB1, is the transcriptional effector of osteocalcin signaling.

Under normal physiological conditions, ucOC constitutes approximately 10% of the total circulating osteocalcin in adult mice [30,31]. Serum ucOC levels increase with osteoporosis and vitamin K deficiency [30,32,33]. Although one study showed that both ucOC and carboxylated osteocalcin modulate the glucose transport system and inflammation in adipocytes [34], most animal studies indicated that ucOC is the bioactive form of osteocalcin that exerts favorable endocrine effects on metabolism. Ferron M et al. reported that continuous administration of ucOC improved glucose metabolism and increased the beta cell mass and insulin secretion in high-fat diet-fed mice [23]. Moreover, the same research group demonstrated that intermittent intraperitoneal injection of ucOC induced full recovery from hepatic

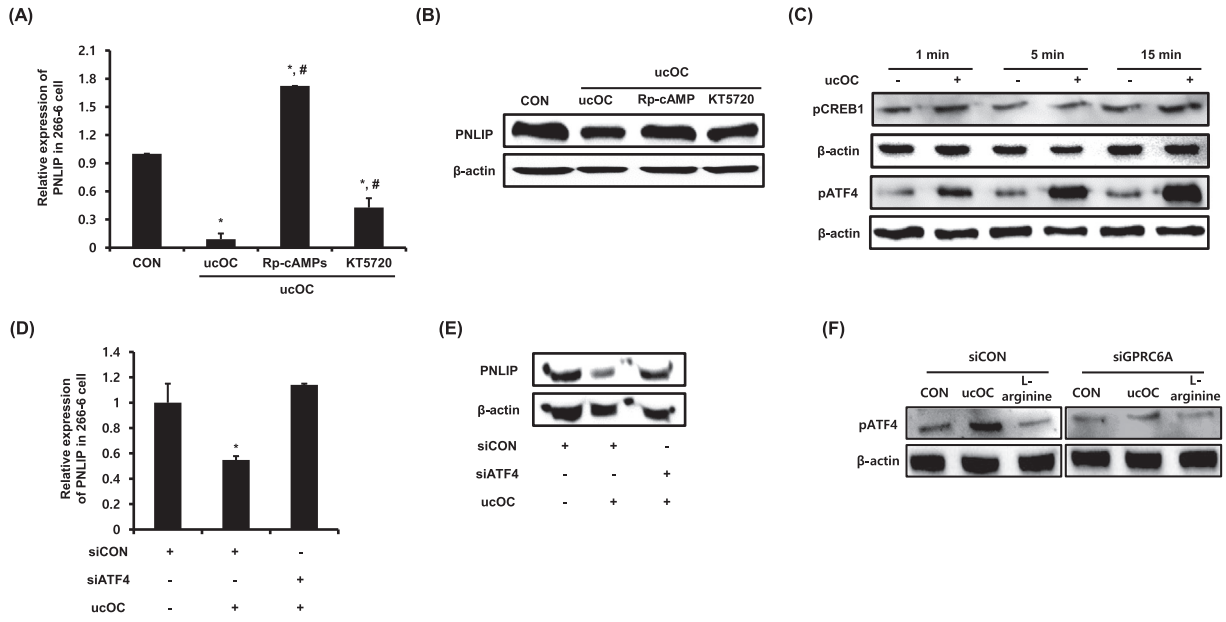

Fig. 3. ucOC downregulates PNLIP expressions via cAMP/PKA/ATF signaling pathway.
The 266-6 cells were incubated with ucOC (0.05 ng/ml) for 24h in the presence or absence of cAMP/PKA signaling inhibitors. The PNLIP mRNA or protein expression levels were examined using quantitative RT-PCR (A) and western blot (B) analyses. Data are presented as the mean ± SD of duplicates. *$p < 0.05$ vs. CON, $^{\#}p < 0.05$ vs. ucOC. Rp-cAMPs, 10 nM Rp-cAMPs; KT, 5 μM KT5720.
(C) The 266-6 cells were incubated in the presence or absence of ucOC for 1, 5, 15min. Western blot analysis was performed with an antibodies to phosphorylated CREB1 (pCREB1) or phosphorylated ATF4 (pATF4). (D, E) The 266-6 cell were transfected with non-targeting control siRNA (siCON) or siRNA targeting ATF4 (siATF4) and incubated for 24 h in the presence or absence of ucOC. *$p < 0.05$ vs. siCON. Data are presented as the mean ± SD. (F) The 266-6 cell were transfected with siCON or siGprc6a and incubated for 24 h in the presence or absence of ucOC (0.05 ng/ml) or L-arginine (30 mM). The western blot data showed the results confirmed in at least 3 independent experiments.

steatosis and improved glucose metabolism and insulin sensitivity in high-fat diet-fed mice [35]. Ferron also reported that osteoblast-specific inactivation of the genes for γ-carboxylase or vitamin K epoxide reductase, which are responsible for γ-carboxylation of osteocalcin, are associated with improved osteocalcin endocrine function [36].

Zhou et al. reported that intermittent injections of osteocalcin reversed autophagic dysfunction and endoplasmic reticulum stress resulting from diet-induced obesity in the vascular tissue via a nuclear factor-κB-p65-dependent mechanism, suggesting therapeutic strategies involving osteocalcin for cardiovascular dysfunction secondary to obesity [37]. In addition to increasing the beta cell mass and insulin secretion and improving insulin sensitivity, our results suggest another mechanistic pathway of osteocalcin involving modulation of PNLIP expressions in preventing obesity and glucose intolerance. Beyond linking two molecules, i.e., PNLIP and osteocalcin, these observations demonstrate that osteoblasts and pancreatic cells are metabolically connected in various manners to regulate energy metabolism. In the body weight results, no significant difference was observed yet between OB VEH mice and OB ucOC mice during five weeks of experimental period (Fig. 1J). It is presumed that the 5 weeks of experimental period was too short to observe that ucOC induced physiological change including PNLIP level leads to body weight change.

Our results show that ucOC increases PNLIP mRNA protein expression and that this effect was abolished by receptor blockade with Gprc6a siRNA. This data provides evidence Gprc6a mediates ucOC signaling downregulating PNLIP expression in pancreatic acinar cells. This endocrine paradigm, i.e. ucOC activates a widely expressed G-protein coupled receptor, Gprc6a, to exert its endocrine function, is supported by other recent reports; Wei et al. demonstrated that osteocalcin promotes β-cell proliferation during development and adulthood through Gprc6a [13]. An osteocalcin-Gprc6a endocrine network has been also proposed in hepatic, fat, and muscle tissues. In this respect, decreased glycogen storage and increased triglycerides, cholesterol levels, and hepatic steatosis were observed in Gprc6a$^{-/-}$ mice. Gprc6a$^{-/-}$ mice also exhibited increased visceral fat, decreased muscle mass and insulin resistance, and impaired insulin secretion. In contrast, ucOC improved insulin sensitivity possibly by activating Gprc6a in the liver, adipocytes, and skeletal muscle [11,12,38]. Mera et al. reported that osteocalcin is a systemic regulator of the adaptation to exercise in adult mice by signaling through Gprc6a in myofibers [8]. Regulation of ucOC in testosterone secretion by testicular Leydig cells [4] and vitamin D production in Leydig cell [39] through a Gprc6a-dependent pathway has been also reported. Khrimian et al. recently reported that ucOC mediates its influence on cognitive function and hippocampal-dependent memory by interacting with an orphan class C GPCR, Gpr158, which is expressed in neurons of the hippocampal CA3 region [40]. This study suggested that other types of receptors exist which recognize osteocalcin in different tissues, but our data showed that Gprc6a mediates ucOC signaling to modulate PNLIP expression at least in part through an ATF4-dependent pathway in pancreatic acinar cells.

Although class C GPCRs are activated by numerous ligands, however, previous investigations are not consistently supporting that osteocalcin or similar peptides activate class C GPCRs, including Gprc6a. For example, osteocalcin did not activate Gprc6a when expressed in Chinese Hamster Ovary cells [41] and the effects of osteocalcin in Leydig cells were not specifically mediated by Gprc6a [29]. Even previous animal models are not consistent with regards to potential effects of Gprc6a on glucose homeostasis with glucose intolerance and normal glucose tolerance in Gprc6a deficient mice [7,42]. Further studies are justified to identify another osteocalcin sensing receptors recognized by various cell types.

In addition, conflicting findings regarding Gprc6a signaling pathway have been reported. An earlier study published by Karsenty group has shown that osteocalcin leads to an increase in cAMP, indicating Gs coupling, but no osteocalcin mediated activation of the Gq or ERK pathways in TM3 leydig cells. Karsenty group also reported that cAMP-CREB pathway is as one mediator of osteocalcin signaling in myofibers [8]. Quarles group has shown that four Gprc6a agonists e.g.

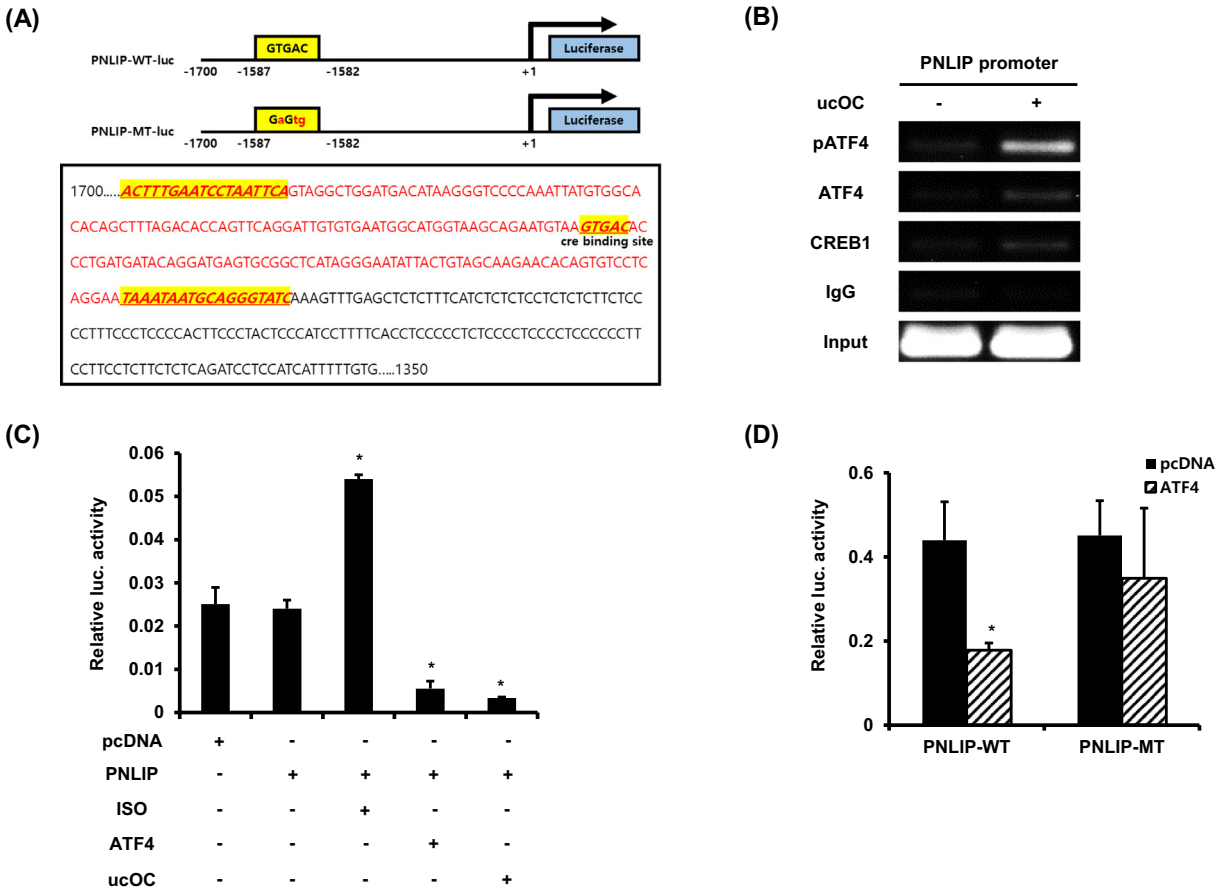

Fig. 4. pATF4 binds to regions −1587 to −1582 for suppressing PNLIP promoter activity.
(A) Schematic illustration of the luciferase (luc.) reporter containing the mouse PNLIP promoter. The highlighted bars show the nucleotide sequences of the putative CRE (-1587 to -1582). The mutant reporters include nucleotide substitutions (GTGAC → GaGtg). The amplified DNA region in the PNLIP promoter DNA sequence is highlighted in red. (B) The 266-6 cells were treated with ucOC for 24 h, and the chromatin immunoprecipitation was performed with antibodies to pATF4, ATF4, CREB1 or with normal IgG. (C, D) HEK 293 cells were transiently transfected with the indicated plasmids and incubated for 24 h, and luciferase activity was measured. (C) HEK 293 cells were treated with ISO (1 μM isoproterenol) for 24 h as a positive control. The data are shown as activity relative to the Renilla luciferase activity. Data are presented as the mean ± SD. *p < 0.05 vs. pcDNA. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

osteocalcin as well as divalent cations, L-arginine, testosterone lead to cAMP accumulation in the Gprc6a-HEK293 cell line and thus is likely to also be Gs coupled [5,43]. Pi M et al. have demonstrated that downstream serum-response element (SRE) and/or ERK are activated by the peptide osteocalcin (Gq pathway, Gi not investigated) using mGprc6a-transfected HEK293 cells [9]. Contrary to previous reports, Jacobsen S et al. reported that neither testosterone nor osteocalcin elicited any response in the ERK pathway or any of the other tested G protein signaling pathways using Chinese Hamster Ovary cell line that stably expresses mouse Gprc6a [41].

In the present study, ucOC led to ATF4 phosphorylation in 266-6 cells. Gprc6a deficiency attenuated the capacity of ucOC to stimulate ATF4 phosphorylation in 266-6 cells, indicating Gs coupled in pancreatic acinar cell response to osteocalcin. Thus, it is of significance this study provides information indicating the activation of Gs signaling pathway by osteocalcin in pancreatic acinar cells. L-arginine, a well-known Gprc6a ligand, led to suppression in ATF4 phosphorylation, suggesting thus is likely also to be Gi coupled. Knockdown of Gprc6a mitigated the capacity of L-arginine to suppress ATF4 phosphorylation in 266-6 cells. Further studies are thus needed to fully elucidate which ligand classes and G protein signaling pathways are utilized by the Gprc6a receptor in pancreatic acinar cells.

The serum ucOC level is known to be approximately 7 ng/ml in adult mice [14]. In humans, reference intervals for the serum OC concentration were studied as 15.4 ng/ml in men, 14.4 ng/ml in premenopausal women, and 18.6 ng/ml in postmenopausal women [44]. In the present study, we used osteocalcin concentrations ranging 140-fold lower to 7-fold higher than its physiological level. From a quite low concentrations of 0.05 ng/ml, ucOC suppressed PNLIP expression, indicating that it is a powerful regulator of PNLIP expression. Ferron et al. previously demonstrated that in vitro β-cell proliferation and insulin expression are significantly affected by relatively low concentrations of osteocalcin ranging from 0.03 to 0.3 ng/ml, i.e., 6–60 pM [31]. In vivo studies using mice implanted with pumps delivering 3, 10, and 30 ng/h of osteocalcin exhibited a significant and dose-dependent decrease in fat pad mass and serum triglycerides concentration [31]. However, in contrast to the observations in β cell proliferation and insulin secretion, insulin sensitivity was further improved in mice administered a higher concentration of 30 ng/h of osteocalcin. A significant decrease in the expression of perilipin and triglyceride lipase, two genes thought to be regulated by osteocalcin [14,35,45], was also observed in the white fat of mice administered 0.3 and 30 ng/h. Further studies are needed to determine the amounts of ucOC required to affect PNLIP expression.

Taken together, our data demonstrate that ucOC downregulates pancreatic lipase expression in a cAMP/PKA/ATF4-dependent manner. Gprc6a mediates ucOC signaling downregulating PNLIP expression in pancreatic acinar cells. These results support the new endocrine paradigm of the skeleton in regulating energy metabolism as well as other

physiological functions. Further studies of ucOC and Gprc6a-dependent endocrine networks are needed for the diagnosis and treatment of metabolic syndrome and related disorders.

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Authors' contributions

Study design: KB; data collection and data analysis: KB, DP, HG; Data interpretation: KB, DP, JB; Drafting manuscript: DP, KB; Revising manuscript content, approving final version: KB, JB.

Declaration of Competing Interest

None.

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