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Original Paper

Osteocalcin Induces Proliferation via Positive Activation of the PI3K/Akt, P38 MAPK Pathways and Promotes Differentiation Through Activation of the GPRC6A-ERK1/2 Pathway in C2C12 Myoblast Cells

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Key Words

Osteocalcin • Myoblast • Proliferation • Differentiation • GPRC6A • PI3K/Akt • P38 MAPK • ERK1/2

Abstract

Background/Aims: Sarcopenia is characterized by an age-related decline in skeletal muscle plus low muscle strength and/or physical performance. Despite the clinical significance of sarcopenia, the molecular pathways underlying sarcopenia remain elusive. The recent demonstration that undercarboxylated osteocalcin (ucOC) favours muscle function related to insulin sensitivity and glucose metabolism raises the question of whether this hormone may also regulate muscle mass. The present study explored the promotive effects of ucOC in proliferation and differentiation processes of C2C12 myoblasts as well as the possible signalling pathways involved. ***Methods:*** The effects of exogenous ucOC on C2C12 myoblasts proliferation were assessed using CCK8 and immunohistological staining assays. C2C12 cells were pretreated with PI3K/Akt or P38 MAPK inhibitors to investigate the possible involvement of the PI3K/Akt and P38 MAPK pathways in proliferation. The levels of Akt, phosphorylated-Akt (p-Akt), P38, and phosphorylated-P38 (p-P38) were measured by Western Blotting. The effects of ucOC on myoblast differentiation were quantified by morphological analysis. A silencing experiment was conducted in which the expression of GPRC6A in C2C12 myoblasts was modified. The expression of GPRC6A, myosin heavy chain (MyHC) and the related ERK1/2 signalling pathway in C2C12 myoblasts were monitored by qRT-PCR and Western Blotting. ***Results:*** We showed that treatment with exogenous ucOC stimulated the priming

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of C2C12 myoblasts proliferation. Inhibition of Akt phosphorylation by wortmannin or inhibition of P38 MAPK phosphorylation by SB203580 decreased C2C12 cell proliferation. Wortmannin also reduced P38 MAPK phosphorylation, whereas SB203580 did not affect Akt activation. Furthermore, ucOC promoted C2C12 myoblast differentiation**.** Inhibition of ERK1/2 phosphorylation with U0126 decreased C2C12 cell differentiation. Finally, GPRC6A expression was substantially increased after ucOC treatment of C2C12 cells. GPRC6A silencing inhibited Akt, P38 MAPK phosphorylation in C2C12 cells, and ERK1/2 phosphorylation in C2C12 myotubes; GPRC6A silencing also decreased cell proliferation, decreased cell differentiation, and downregulated MyHC expression. ***Conclusions:*** The present data suggest that ucOC induces myoblast proliferation via sequential activation of the PI3K/Akt and p38 MAPK pathways in C2C12 myoblast cells. Moreover, ucOC enhances myogenic differentiation via a mechanism involving GPRC6A-ERK1/2 signalling.

Introduction

During ageing, a progressive loss of skeletal muscle mass and a parallel decrease in muscle strength and endurance occur [1]. This condition, termed sarcopenia, has important healthcare and socioeconomic implications for humans as it contributes to frailty, functional loss, disability, high healthcare costs, and premature death [2-4]. Despite the clinical significance of sarcopenia, the mechanisms through which sarcopenia develops are complex and still remain to be completely elucidated, and standardized intervention programmes are still lacking. Bone and muscle are neighbours with close ties. We and others have shown that decreases in muscle and bone mass accompany ageing [5-7]. Given the scope of the problem, it is critical to obtain a better understanding of how these two tissues integrate and crosstalk as a basis for identifying new therapeutics that can be used to prevent the loss of muscle and bone with ageing.

We already know that the interactions between muscle and bone are mediated by a variety of hormones, cytokines and nutrients [8]. However, the precise mechanisms responsible for synchronizing bone and skeletal muscle mass remain unclear. Although basic studies have helped dissect the multiple influences of skeletal muscle on bone through cytokines such as myostatin, insulin-like growth factor 1 (IGF-1), fibroblast growth factor 2 (FGF-2), interleukin-6 (IL-6), IL-15, myostatin, osteoglycin (OGN), FAM5C, and osteoactivin [9-14], few osteokines involved in the feedback control of muscle by bone have been identified, with the exception of the osteocyte-specific Wnt family member 3a (Wnt3a), prostaglandin E2 **(**PGE2), osteoblast-derived IGF-1 and osteocalcin [15-18].

Osteocalcin is a bone matrix protein that was initially believed to be a measure of bone mineralization [19]. Recent increasing data have emerged that cast osteocalcin in a new light; these data suggest a broader role for osteocalcin that extends to the regulation of energy metabolism, reproduction, and cognition [20-23]. An undercarboxylated form of osteocalcin (ucOC), which is the hormonally active isoform, acts in a feed-forward loop to increase β-cell proliferation as well as insulin production and secretion, and skeletal muscle and adipose tissue respond to ucOC by increasing their sensitivity to insulin [24]. More recently, an additional role for ucOC in maintaining muscle mass and function in older mice has been suggested because mice lacking osteocalcin (Ocn-/-) display decreased muscle mass and strength compared to their WT littermates [25]. Moreover, mice lacking a neuromuscular gap junction protein (connexin43) had increased muscle area and grip strength after injection with human ucOC compared to the same knockout mice injected with saline [26]. It is known that muscle mass is associated with the number and/or differentiation efficiency of myoblasts, which are the main contributors to myogenesis. However, the precise role of osteocalcin in the biological processes that occur in muscle remains to be deciphered.

The GPCR class C group 6 subtype A (GPRC6A) protein is a member of a family of G protein-coupled receptors that are sensitive to pertussis toxin [27]. GPRC6A, as the putative receptor for osteocalcin, is expressed by a wide variety of cell types, including pancreas,

testes and other tissues that respond to circulating osteocalcin [28, 29]. It appears likely that GPRC6A also mediates osteocalcin's functions in muscle [30]. For the first time, we investigated the role of ucOC in myoblast proliferation and differentiation using C2C12 cells, a recognized *in vitro* model of skeletal muscle. Here, we show that exogenous ucOC is sufficient to induce proliferation in C2C12 cells. Furthermore, during the differentiation stage, osteocalcin promotes myogenic differentiation. These results expand the importance of the regulation of muscle physiology by bone-derived hormones and suggest a basis for novel and adapted therapies to treat age-related muscle wasting.

Materials and Methods

Cell culture

The murine myoblast cell line C2C12 was obtained from the American Type Culture Collection (Manassas, VA, USA). C2C12 cells were cultured in 100-mm culture dishes in growth medium (GM) consisting of Dulbecco's modified Eagle's medium (DMEM, HyClone, Logan, UT, USA) with 10% foetal bovine serum (GIBCO, Grand Island, NY, USA), 100 U/mL penicillin and 100 μg/mL streptomycin (Solarbio, Beijing, China) under a humidified atmosphere of 5% CO2 and 95% air at 37°C. The medium was replaced every 72 hours. To induce differentiation, the cells were grown to 75% confluence, and the medium was then replaced with differentiation medium (DM) consisting of DMEM containing 2% horse serum (Invitrogen, Carlsbad, CA, USA), 100 U/mL penicillin and 100 μg/mL streptomycin, which was replenished everyday. The ucOC concentration in WT adult mice is approximately 7 ng/mL. Thus, in these experiments, we used amounts of ucOC ranging from 50-fold lower than its physiological concentration to 7-fold higher. Uncarboxylated osteocalcin (AnaSpec Inc, Fremont, CA, USA) was resuspended and diluted in phosphate-buffered saline (PBS) to obtain final concentrations of 0.1-50 ng/mL. To examine the effect of ucOC on cell proliferation and differentiation, the medium was replaced with serum-free medium or with DM containing ucOC (0-50ng/mL) at the indicated times. In some experiments, C2C12 cells were pretreated with the phosphatidylinositol-3-kinase (PI3K) inhibitor wortmannin (SB203580 or U0126, Sigma, St. Louis, MO, USA) 30 min prior to ucOC stimulation. GPRC6A siRNAs were obtained from Santa Cruz (Santa Cruz Biotechnology Inc., California, USA), and C2C12 cells were transfected with the siRNAs using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

Growth curves

C2C12 myoblasts were plated in 96-well plates at 10, 000-12, 000 cells per well. Twenty-four hours later, the culture medium was changed to serum-free medium containing varying doses of ucOC (0 ng/mL, 0.1 ng/mL, 1ng/mL, 10ng/mL or 50ng/mL). The experiment was continued until the control cells reached subconfluence (72 hours). The cells were counted using the CCK8 assay, and the average cell counts for each day were used to plot the growth curves of the ucOC-treated myoblasts.

Cell proliferation assay

At the indicated times, C2C12 cells were treated with CCK8 (10 μL/well, Sigma) for an additional 1 h. Finally, we recorded the absorbance at 450 nm using a microplate absorbance reader (Tecan, Safire II, Switzerland). The OD value (absorbance at450 nm) was detected using an enzyme-linked immunosorbent assay (ELISA) (BIO-RAD, USA).

Immunohistological staining

Growing adherent cells on coverslips were obtained each day and fixed in 4% formaldehyde. The fixed cells were incubated overnight at 4°C with a primary antibody against PCNA (cell proliferation marker, 1:100; Santa Cruz Biotechnology). The next day, biotin-labelled secondary antibodies were applied and were detected with diaminobenzidine (Sigma). Then, nuclei were stained with haematoxylin (Sigma). PCNA-positive nuclei were stained brown, and PCNA-negative nuclei were stained blue. The proportion of PCNA-positive nuclei was determined through image analysis of the cells. Photomicrographs were captured and analysed using Image-Pro-Plus 5.0 software (Media Cybernetics, Silver Spring, MD, USA). The area of PCNA-positive nuclei per high-power field was calculated.

Differentiation experiments

C2C12 cells were treated with ucOC at 0 ng/mL, 0.1 ng/mL, 1 ng/mL, 10ng/mL, and 50 ng/mL in DM. The medium was exchanged daily for fresh DM containing ucOC. For morphological analysis, the cells were fixed with methanol/acetone (Sigma), and the number of multinucleated myotubes present in a field (1 μm2 ×100) was counted using a phase-contrast microscope (OLYMPUS, CX41, Tokyo) 72 h after switching to DM.

Western Blotting

Cells were lysed for 30 min on ice in radioimmunoprecipitationassay (RIPA) lysis buffer (Solarbio, Beijing, China) containing 0.1mM PMSF and a protease inhibitor (Roche).The samples were subjected to 12% SDS-PAGE and transferred to nitrocellulose membranes. Blots were probed using primary antibodies, including anti-phospho-Akt (Cell Signaling Technology, CST, Boston, MA, USA), anti-Akt (CST), anti-phospho-P38 (CST), anti-P38 (CST), anti-phospho-ERK1/2 (CST), anti-ERK1/2 (CST), anti-GPRC6A (SantaCruz), anti-MyHC (Santa Cruz), and anti-GAPDH (CST). The lysates were subjected to standard Western Blotting analysis. The images were obtained usingTanon-5200 (Tanon, Shanghai, China). The relative densities of the specific bands were quantified using the image detection software Image Lab 5.0 (Bio-Rad Laboratories Inc., California, USA).

Short interfering RNA (siRNA) and transfection

siRNA transfection was used to downregulate GPRC6A expression. All the siRNAs were purchased from Santa Cruz Biotech (Santa Cruz, CA, USA). The GPRC6A-targeted siRNA (si GPRC6A) and control siRNA (si Ctrl) were dissolved separately in Optimem I (Invitrogen). After 10 min of equilibration at room temperature, each RNA solution was combined with an equal volume of Lipofectamine™ 2000 solution, mixed gently, and allowed to form siRNA liposomes for 20 min.

Isolation of RNA and RT-PCR analysis

Total RNA was extracted from C2C12 cells using a Nucleo SpinRNA kit (TakaraBio, Shiga, Japan) according to the manufacturer's instructions. Isolated total RNA (100ng) was subjected to reverse transcription with or without Prime Script Reverse Transcriptase (Takara Bio) and PCR analysis using primers for GPRC6A, 5'-CCAGACGACCACAAATCCAG- 3' and 5'-GATTCATAACTCACCTGTGGC-3', and for GAPDH, 5'ACCACAGTCCATGCCATCAC-3'and 5'-TCCACCACCCTGTTGCTGTA-3'.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA). Unless indicated otherwise, the data are presented as the mean ± S.E.M. Comparison between two groups was analysed using Student's t-test. One-way ANOVA was utilized for multiple comparisons followed by Tukey's post hoc test. A p value <0.05 was defined as significant.

Results

ucOC induces proliferation of myoblasts

In the proliferative phase, we analysed the effect of ucOC on C2C12 cell proliferation. Serum-deprived C2C12 cells were incubated with GM or with various concentrations of ucOC (0-50ng/mL). Morphological changes in the C2C12 cells and in the areas of PCNA-positive cells were evaluated using immunohistological analysis. Cells treated with ucOC showed increased proliferation and a phenotype very similar to that of cells treated with GM (Fig. 1A and 1B), although treatment with standard GM was much more effective. We found that enlargement of the area of PCNA-positive cells occurred at ucOC concentrations as low as 0.1ng/mL. At 10 ng/mL, ucOC triggered up to a 1.36-fold increase in the area of PCNA-positive cells. At a concentration of 50 ng/mL, the effect of ucOC was reduced (Fig. 1B).

We further studied the stimulatory effect of ucOC on C2C12 cell growth using a CCK8 assay. The average cell counts for each day were used to plot a growth curve (Fig. 1C). The results indicated that in the presence of ucOC the number of cells increased at 24, 48, and 72 h. The results demonstrate that ucOC induces C2C12 cell proliferation with a dose-response

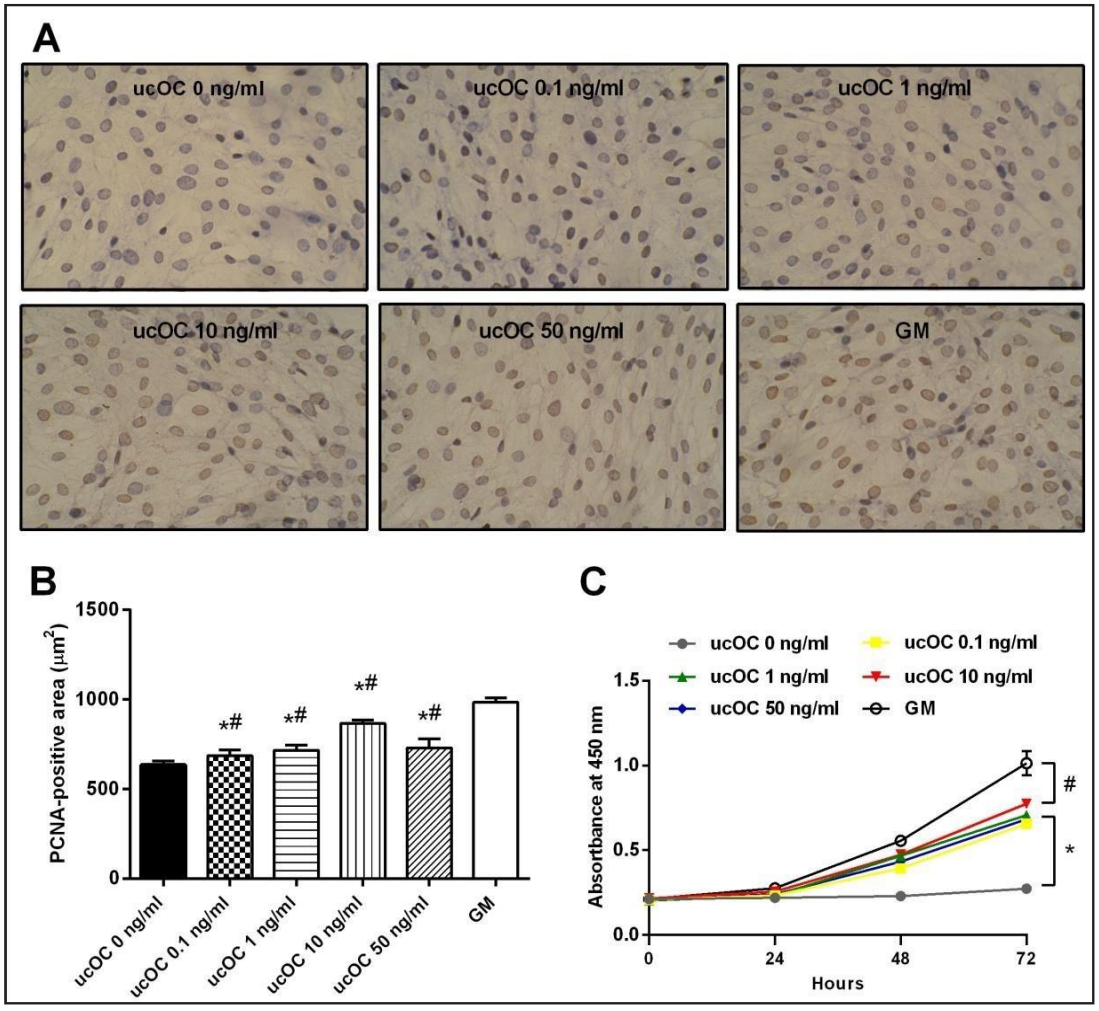

Fig. 1. ucOC induces proliferation of C2C12 cells in vitro. (A), (B) Immunohistochemical analysis of PCNA in C2C12 cells. (A) The proportion of PCNA-positive nuclei and (B) the calculated area of PCNA-positive nuclei were significantly higher in the ucOC (0.1-50ng/ml) groups than in the control group (ucOC 0 ng/ml group). (C) Growth curves result represent the effects of ucOC on cell growth in C2C12. The absorbance value was detected at 24, 48, and 72 hours. Data are representative of three independent experiments performed in triplicate. *P<0.05 versus control group, #P<0.05 versus GM group.

relationship similar to that observed for the area of PCNA-positive cells. Thus, these data show that low concentrations of ucOC act as a powerful regulator of C2C12 proliferation.

PI3K/Akt and p38 MAPK signalling are involved in ucOC-mediated priming of myoblast proliferation

To examine the molecular mechanisms responsible for the proliferative effects of ucOC in myoblasts, we evaluated ucOC-dependent activation of the ERK1/2, PI3K/Akt and P38 MAPK pathways. These pathways play major roles in regulating cell growth, survival, and differentiation. C2C12 cells were serum-deprived for 24 h and then treated with 10ng/mL ucOC for various times (10min, 6 h, and 24 h). We found that ucOC treatment significantly increased the levels of p-Akt and p-P38 MAPK in the cells at 24h (Fig. 2A).

To address the role of Akt in ucOC-induced cell proliferation, C2C12 cells were pretreated with wortmannin, a reversible inhibitor of Akt. After wortmannin treatment, the phosphorylation status of Akt was attenuated. Accordingly, C2C12 cell proliferation

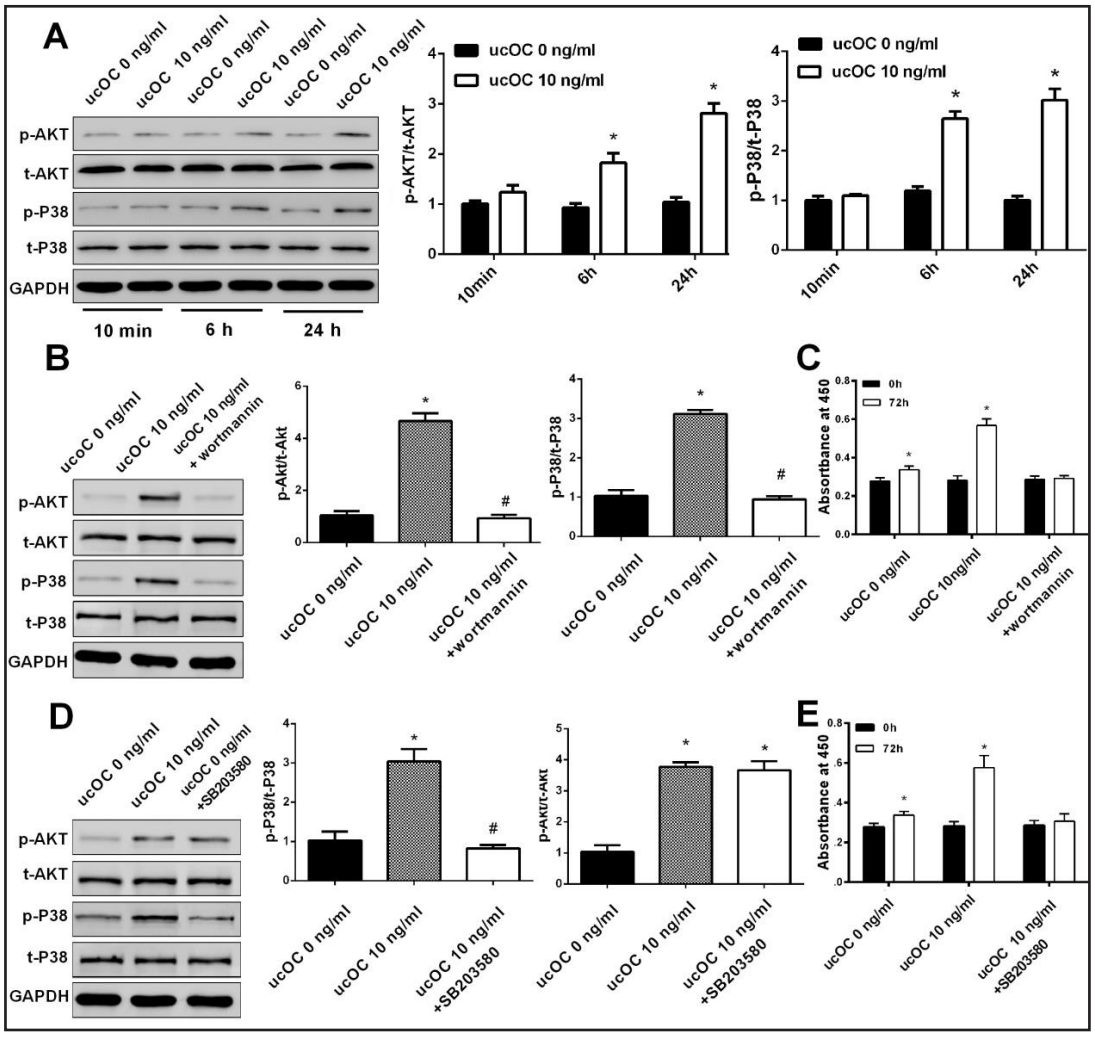

Fig. 2. PI3K/Akt and P38 MAPK signalling are involved in ucOC-mediated priming of C2C12 cells proliferation. (A) The expression of p-Akt, t-Akt, p-P38, t-P38 and GAPDH was detected in C2C12 cells by Western Blotting analyses. (B), (C) Wortmannin reduced Akt and P38 MAPK phosphorylation, inhibited the effect of ucOC on C2C12 cells proliferation. (D), (E) SB203580 reduced P38 MAPK phosphorylation, inhibited the effect of ucOC on C2C12 cells proliferation. (B), (D) Phosphorylation levels of Akt and P38 MAPK were determined by Western Blotting analysis at 24 hours. (C), (E) C2C12 cells were counted using CCK8 assay at 72 hours. GAPDH was used as an internal control. Data are representative of three independent experiments performed in triplicate. *P<0.05 versus control group (ucOC 0 ng/ml group), #P<0.05 versus ucOC 10 ng/ml group.

significantly decreased by 50% (Fig. 2B and 2C). We then tested whether P38 MAPK might also be involved in this pathway. As shown in Fig. 2D and 2E, inhibition of P38 MAPK by SB203580 also significantly inhibited the effect of ucOC on myoblast proliferation. In addition, wortmannin pretreatment reduced P38 MAPK phosphorylation. In contrast, SB203580 did not affect Akt activation, suggesting that P38 MAPK is a downstream signalling factor of Akt. However, no significant difference was detected in the level of p-ERK between groups following ucOC stimulation (data not shown). Taken together, these results suggest that ucOC stimulates the priming of myoblast proliferation through the sequential activation of Akt and p38 MAPK.

ucOC-induced myoblast proliferation is blocked by GPRC6A knockdown

We initially examined whether myoblasts express receptors for ucOC. mRNA analysis by qRT-PCR revealed that myoblasts express GPRC6A mRNA in a time-dependent manner during ucOC treatment, and this was confirmed by Western Blotting analysis (Fig. 3A). We speculated that GPRC6A would be stimulated by ucOC to activate Akt and P38 MAPK. Therefore, we used siRNA to knockdown GPRC6A to investigate the activation of Akt and P38 MAPK (Fig. 3B). The activation of Akt and P38 MAPK was inhibited by the knockdown of GPRC6A (Fig. 3C). Accordingly, C2C12 cell proliferation significantly decreased by 51% (Fig. 3D). In conclusion, we believe that GPRC6A functions as the receptor of ucOC and that it acts in the upper portion of the Akt and P38 MAPK signalling pathways to regulate myoblast proliferation.

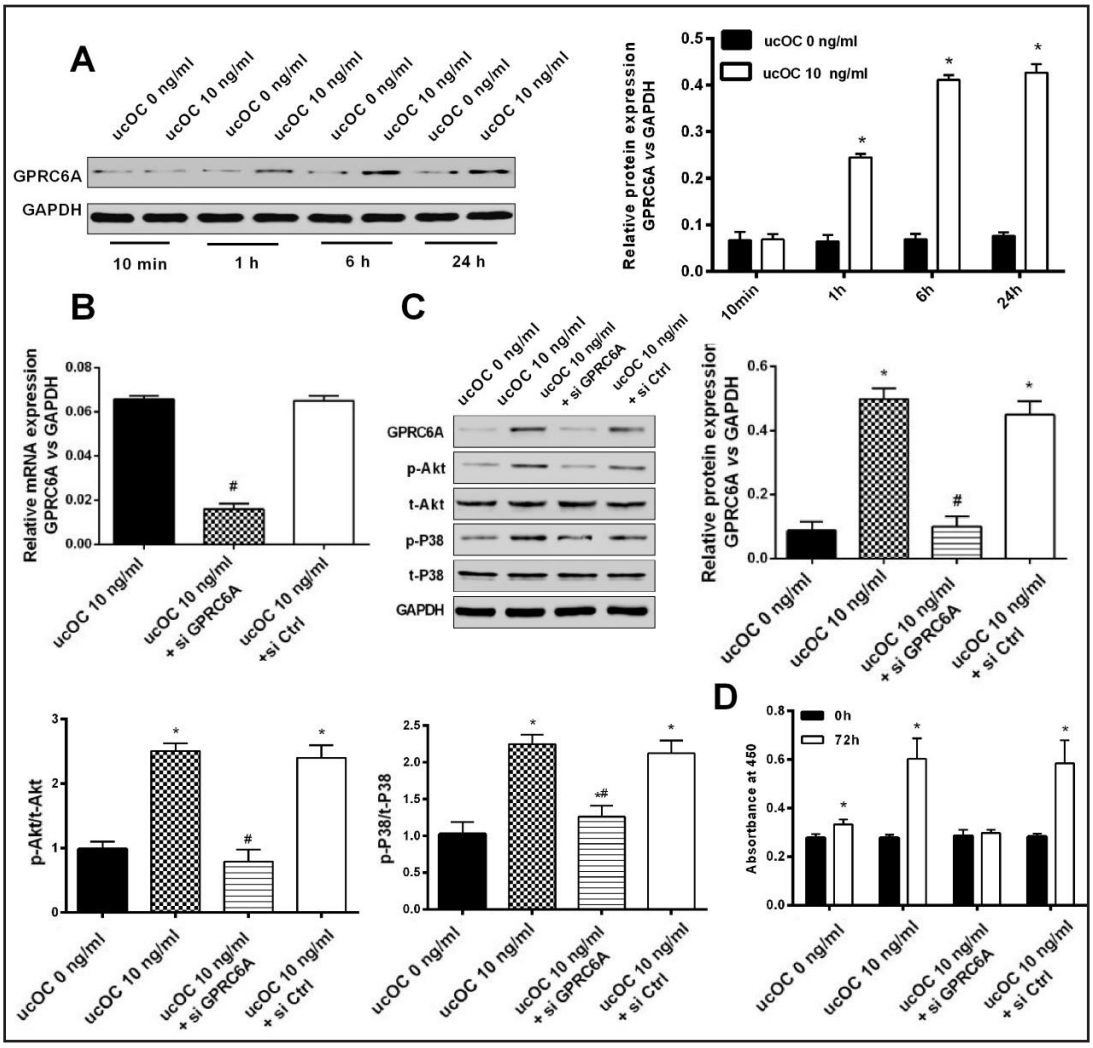

Fig. 3. GPRC6A knockdown blocks ucOC-induced myoblast proliferation in C2C12 cells. (A) Western Blotting analysis revealed that the expression of GPRC6A is time-dependent following ucOC treatment in C2C12 cells. (B) qRT-PCR suggested that the expression of GPRC6A gene was down-regulated by GPRC6A siRNA transfection in C2C12 cells. (C) The expression of GPRC6A protein, phosphorylation levels of Akt and P38 MAPK were determined by Western Blotting analysis at 24 hours. (D) C2C12 cells were counted using CCK8 assay at 72 hours. GAPDH was used as an internal control. Data are representative of three independent experiments performed in triplicate. *P<0.05 versus control group (ucOC 0 ng/ml group), #P<0.05 versus ucOC 10 ng/ml group.

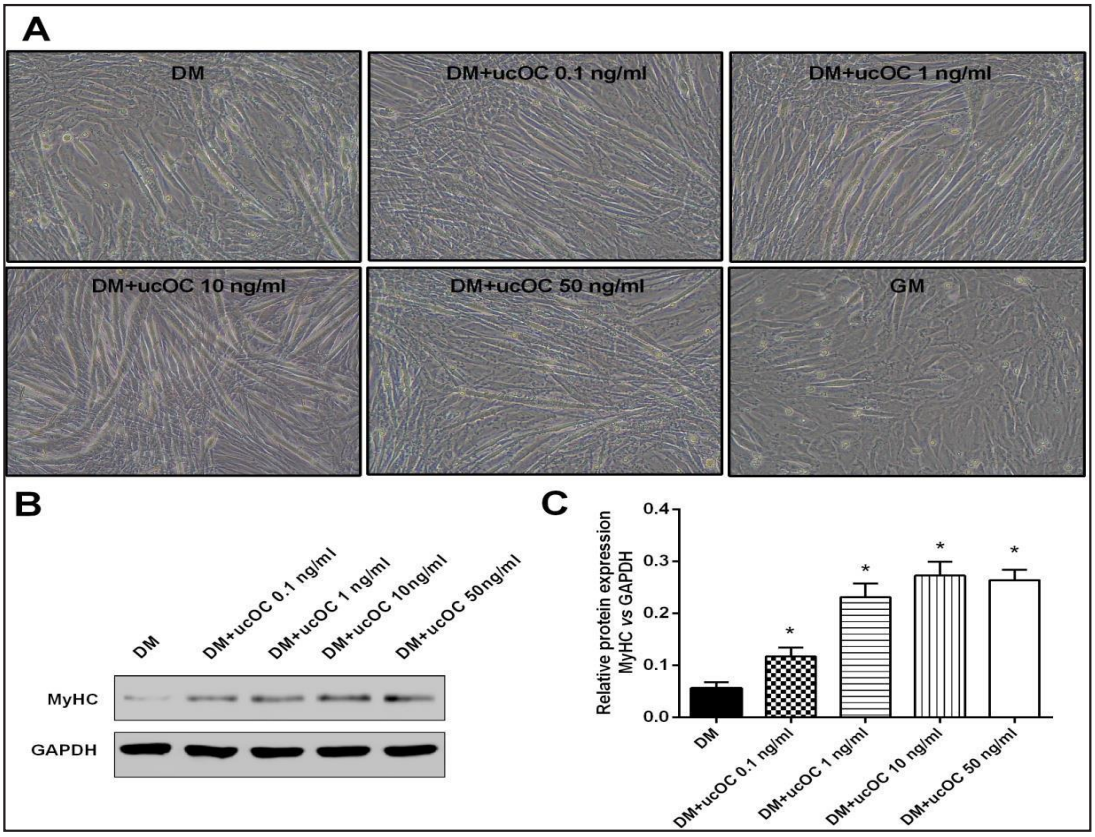

Fig. 4. ucOC enhances myogenic differentiation in C2C12 cells. (A) Representative views of the morphological changes of differentiated C2C12 cells at 72 hours. (B) Protein levels of muscle-specific protein MyHC was determined by Western Blot analysis at 24 hours. GAPDH was used as an internal control. Data are representative of three independent experiments performed in triplicate. *P<0.05 versus control group (DM group).

ucOC enhances the differentiation of myoblasts

The C2C12 cell line is commonly used as an *in vitro* model for myogenesis. To examine the effect of ucOC on myogenic differentiation, C2C12 myoblasts were induced to differentiate by switching the cells to DM in the presence of various concentrations of ucOC (0-50ng/mL) for 72 hours. We observed that the addition of ucOC to the DM resulted in the formation of larger myotubes with more nuclei per myotube in comparison to cells treated with standard DM (Fig. 4A). In addition, the expression of the muscle-specific protein MyHC was significantly upregulated by 2.13-, 4.0-, 5.0-, and 4.5-fold compared with that of the control by treatment with ucOC at 0.1, 1, 10, and 50 ng/mL, respectively (Fig. 4B). Taken together, these data indicate that ucOC treatment results in a statistically significant promotion of C2C12 cell differentiation at themorphological as well as at the biochemical level.

Involvement of GPRC6A-ERK1/2 signalling in ucOC-mediated myogenic differentiation

To confirm the involvement of ucOC in the process of myogenesis, we treated C2C12 cells with 10 ng/mL ucOC and assessed the activation levels of the PI3K/Akt and P38 MAPK pathways, which our data show are involved in the ucOC-induced priming of myoblast proliferation. After ucOC treatment, the phosphorylation levels of these proteins remained almost unchanged (data not shown). We then analysed the ERK1/2 pathway, another well-known signalling pathway that has been analysed in many muscle studies by Western Blotting analysis using an antibody that recognizes an active, phosphorylated form of this kinase. The results confirmed that ucOC treatment significantly increased the levels of p-ERK1/2, whereas the total protein levels remained relatively constant (Fig. 5A). We

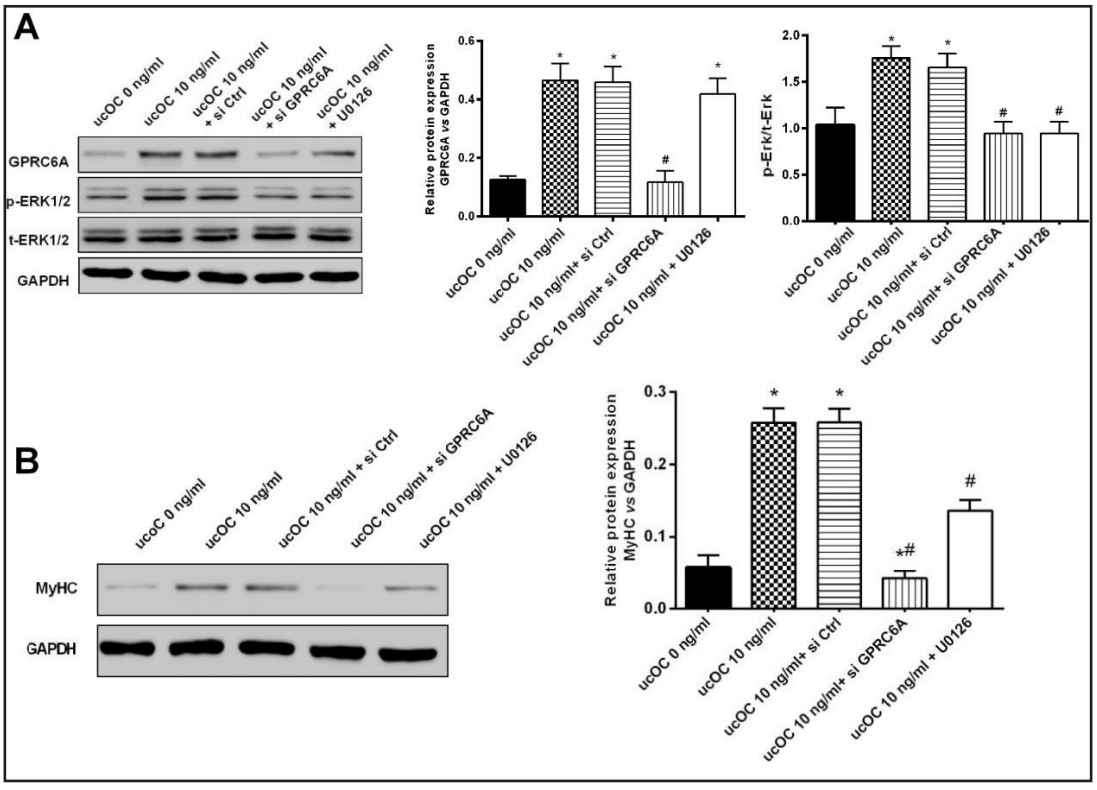

Fig. 5. GPRC6A-ERK1/2 signaling are involved in ucOC-mediated myogenic differentiation in C2C12 cells. (A), (B) C2C12 cells were transfected with GPRC6A siRNA or pretreated with ERK1/2 inhibitor U0126 respectively. Western Blotting analysis was performed to examine GPRC6A expression (A), phosphorylation level of ERK1/2 (A) and MyHC expression (B). GAPDH was used as an internal control. Data are representative of three independent experiments performed in triplicate. *P<0.05 versus control group (ucOC 0 ng/ml group), #P<0.05 versus ucOC 10 ng/ml group.

next assessed whether ucOC treatment overrides the block of myoblast differentiation by inhibition of ERK1/2. C2C12 cells were treated with the pharmacological inhibitor U0126 for 30 min prior to the addition of ucOC and then allowed to differentiate for 24 h, followed by Western Blotting analysis. As predicted, treatment with U0126 reduced the phosphorylation of ERK1/2 and decreased the expression of MyHC (Fig. 5B). In conclusion, our results demonstrate that ucOC accelerates myoblast differentiation at least partially through the activation of ERK1/2 signalling.

Next, we used siRNA to knockdown GPRC6A expression to investigate the activation of ERK1/2. The activation of ERK1/2 was inhibited due to the knockdown of GPRC6A (Fig. 5A). On the other hand, inhibition of ERK1/2 by U0126 did not affect GPRC6A. To confirm the involvement of GPRC6A in myogenic differentiation caused by ucOC, we examined the ucOC-induced expression of MyHC in the presence of siRNA for GPRC6A. The results demonstrated significant inhibition of the expression of MyHC in the presence of GPRC6A siRNA (Fig 5B). Thus, our data demonstrate that inhibition of GPRC6A blocks ucOC-induced myoblast differentiation and suggest that GPRC6A-ERK1/2 signalling pathways are involved in ucOC-stimulated myoblast differentiation.

Discussion

Sarcopenia, a syndrome characterized by progressive and generalized loss of skeletal muscle mass and function, results in frailty, increased vulnerability to adverse health

outcomes, and decreased quality of life in the elderly population [31]. Several mechanisms may be involved in the onset and progression of sarcopenia. These mechanisms include protein synthesis, proteolysis, neuromuscular integrity and musculoskeletal coordination [32-35]. In the present study, we identified a novel role of the bone-derived hormone osteocalcin in musculoskeletal coordination. We showed that exogenous ucOC is sufficient to induce myoblast proliferation and enhance myogenic differentiation *in vitro* and that the ability of ucOC to induce myoblast proliferation depends on activation of the PI3K/Akt and p38MAPK pathways. In addition, GPRC6A-ERK1/2 signalling was shown to be involved in ucOC-mediated myogenic differentiation, and knockdown of GPRC6A by siRNA markedly attenuated the effects of ucOC.

Neither bone nor muscle tissue was previously suspected to perform endocrine functions, let alone to affect so many physiological processes in a significant manner [23]. Osteocalcin, the most abundant non-collagenous bone matrix protein, is a small g-carboxyglutamate protein that is preferentially expressed by osteoblasts and that regulates bone mineralization [19]. Accumulating results have implicated ucOC in glucose metabolism [20, 36]. Because skeletal muscle is a major site of glucose disposal [37], this suggests that the ucOC may be linked directly or indirectly to skeletal muscle mass and strength. At present, there is little evidence from animal experiments to show that ucOC is related to muscle mass. Paula et al. [25]. reported that osteocalcin signalling in myofibres is necessary to maintain muscle mass in older mice in part because it promotes protein synthesis in myotubes. Hua et al. [26]. indicated that connexin43 (Cx43), a gap junction protein that transduces mechanical signals in osteoblasts/osteocytes, indirectly modulates skeletal muscle growth and function, potentially via an endocrine effect of ucOC. Furthermore, another recent study showed that loss of muscle mass and strength under atrophic conditions induced by hindlimb immobilization is related to a reduction in ucOC levels in rats [38]. However, the mechanisms involved in the regulation of biological processes in muscle are poorly understood. In this study, we identified a novel function of ucOC and showed that it directly coordinates myoblast proliferation and the myogenic differentiation programme *in vitro*, suggesting that ucOC possesses myoblast proliferation and differentiation promoting effects under various conditions. Consistent with this, osteocalcin has been shown to induce proliferation of pancreatic beta cells and to stimulatethe differentiation of fat cells [24, 39].

MAPK signalling plays an important role in complex cellular processes such as proliferation, differentiation and survival [40]. At least 3 MAPK families have been characterized: ERK1/2, P38 MAPK and Jun kinase [41]. In addition to MAPK, PI3K/Akt is also an important signalling factor for cell survival and growth [42, 43]. In this study, we report a new mechanism by which ucOC stimulates myoblast proliferation via sequential activation of the Akt and P38 MAPK pathways. Using chemical inhibitors of Akt and P38 MAPK, we revealed that Akt and P38 MAPK are sequentially activated. During the differentiation phase, ucOC treatment enhanced the phosphorylation of ERK1/2, the key myogenic kinase that activates MyHC. The enhanced myoblast differentiation caused by ucOC treatment can be decreased by inhibition of ERK1/2 by U0126. Moreover, knockdown of GPRC6A by siRNA inhibited Akt, P38 MAPK and ERK1/2 activation and attenuated ucOC-induced myoblast proliferation and myogenic differentiation in C2C12 cells.

In general, ERK1/2 is essential for muscle cell proliferation, whereas the PI3K/Akt pathway mainly promotes differentiation of those cells [44, 45]. However, the findings of Yang et al. and of our team are inconsistent with these general ideas [46, 47]. The conflicting results may be due to differences in the experimental set ups used. In addition, because ucOC activates both ERK1/2 and Akt signalling pathways in C2C12 cells, we hypothesized that the ERK1/2 and Akt signaling pathways might interact with each other. It is also possible that ucOC stimulates a more complicated signaling network. Further research to test this hypothesis is warranted.

Myoblast proliferation and differentiation are early critical processes that regulate muscle growth and regeneration. Defects in satellite cell proliferation or differentiation are associated with a variety of muscular diseases. In the current study, we utilized a commonly

used *in vitro* model of muscle cells to study the mechanism of action of ucOC in muscle cells. We provide evidence supporting a role for ucOC as a factor that regulates the balance between muscle cell proliferation and differentiation by modulating signalling pathways. Our data identify ucOC as a potential therapeutic target for alleviating the reduction in muscle mass associated with muscle disorders and ageing. To the best of our knowledge, only a few human studies have investigated osteocalcin in relation to muscle mass and function, and these studies have led to inconsistent conclusions [48-50]; thus, the role of ucOC in muscle mass and physical function in humans is still unknown. Further research in humans is needed to confirm the data obtained inanimal studies.

In conclusion, our results provide the first evidence that osteocalcin induces proliferation via positive activation of the PI3K/Akt and P38 MAPK pathways and that it promotes differentiation through activation of the GPRC6A-ERK1/2 pathway. Age-related sarcopenia implies progressive deterioration of the molecular mechanisms responsible for cell viability, proliferation and differentiation. We believe that our data support the idea that osteocalcin plays a major role in sarcopenia by promoting myoblast cellular function. However, further investigation is required to translate these findings from *in vitro* experiments to humans. The salient findings from the present study provide us with a better understanding of osteocalcin's functions and offer exciting opportunities for the development of new therapeutic strategies for muscle diseases such as sarcopenia.

Acknowledgements

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Disclosure Statement

The authors declare no Disclosure Statement.

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