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Secreted Clusterin protein inhibits osteoblast differentiation of bone marrow mesenchymal stem cells by suppressing ERK1/2 signaling pathway

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ABSTRACT

Secreted Clusterin (sCLU, also known as Apolipoprotein J) is an anti-apoptotic glycoprotein involved in the regulation of cell proliferation, lipid transport, extracellular tissue remodeling and apoptosis. sCLU is expressed and secreted by mouse bone marrow-derived skeletal (stromal or mesenchymal) stem cells (mBMSCs), but its functional role in MSC biology is not known. In this study, we demonstrated that Clusterin mRNA expression and protein secretion in conditioned medium increased during adipocyte differentiation and decreased during osteoblast differentiation of mBMSCs. Treatment of mBMSC cultures with recombinant sCLU protein increased cell proliferation and exerted an inhibitory effect on the osteoblast differentiation while stimulated adipocyte differentiation in a dose-dependent manner. siRNA-mediated silencing of *Clu* expression in mBMSCs reduced adipocyte differentiation and stimulated osteoblast differentiation of mBMSCs. Furthermore, the inhibitory effect of sCLU on the osteoblast differentiation of mBMSCs was mediated by the suppression of extracellular signal-regulated kinase (ERK1/2) phosphorylation. In conclusion, we identified sCLU as a regulator of mBMSCs lineage commitment to osteoblasts versus adipocytes through a mechanism mediated by ERK1/2 signaling. Inhibiting sCLU is a possible therapeutic approach for enhancing osteoblast differentiation and consequently bone formation.

1. Introduction

Bone marrow skeletal (also known as stromal or mesenchymal) stem cells (BMSCs) are a subpopulation of adult stem cells that reside in the bone marrow within a specific perivascular niche and are characterized by their ability for self-renewal and multipotent differentiation into mesodermal cells, including osteoblast, adipocytes, and chondrocytes [1–4]. Several pre-clinical and clinical studies have suggested the possible use of BMSC-based therapy for enhancing bone regeneration in a number of conditions, such as non-union fracture, bone reconstruction and augmentation in cranial, oral, maxillo-facial and long bone defects [5]. Thus, understanding the regulatory mechanisms underlying the differentiation of BMSCs into bone-forming osteoblastic cell lineage is important to provide novel therapeutic targets that can be used to direct the differentiation of BMSCs into the osteoblastic lineage to enhance bone formation.

In this context, we and others have demonstrated that the regulation of BMSCs differentiation into osteoblasts is mediated by the secreted factors produced by BMSCs [3,6]. These osteogenic secreted factors include the secreted Frizzled-related protein 1 (sFRP-1) [7], Delta like-1/Fetal antigen 1 (Dlk1/FA1) [8,9], Leukemia inhibitor factor (LIF) [10], Vascular endothelial growth factor A (VEGF) [11], WNT1-induced Secreted Protein-1 (WISP1) [12], Semaphorin 3A (Sema3A) [13] and Nel-Related Protein 1, NELL-1 [14]. We have also previously employed global, hypothesis-generating methods of transcriptomics or proteomics to identify novel factors important for BMSCs commitment to osteoblastic cells and to bone formation [15,16]. By comparing the transcriptome and secretome of BMSC-derived osteoprogenitor cells versus adipoprogenitor cells [17], we identified Clusterin and found that its expression was significantly upregulated in BMSCs-derived adipocytes (Abdallah BM and Kassem M, unpublished data).

Clusterin (CLU, also known as Apolipoprotein J), is a heterodimeric protein, that found in two forms: nuclear form (nCLU) and soluble form (sCLU). sCLU is ubiquitously expressed in many tissues including

Abbreviations: ALP, alkaline phosphatase; Runx2, runt-related transcription factor 2; Col1a1, collagen type 1; OCN, osteocalcin; OPN, osteopontin; PPARγ2, peroxisome proliferator-activated receptor gamma 2; C/EBP-α, CCAAT/enhancer-binding protein alpha; aP2, adipocyte lipid-binding protein 2; LPL, lipoprotein lipase; APM1, adiponectin; MSX2, msh homeo box 2; DLX5, distal-less Homeobox 5.

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brain, liver, testis, ovary, and heart and is present in the circulation and in all biological fluids as a component of high density lipoprotein (HDL) complex with Apolipoprotein A-1 (ApoA1) [18–20]. sCLU is a pro-cell survival factor, that is involved in the regulation of cell proliferation, apoptosis, tissue remodeling, complement inhibition, lipid transport, and carcinogenesis [18,21,22]. sCLU has been reported to be protective against oxidative stress-induced apoptotic cell death in a variety of cells including BMSCs [23], [24] [25]. Furthermore, increased expression of CLU was shown to be associated with oxidative stress and inflammation in many diseases including neurodegenerative diseases, cancers and inflammatory diseases [26]. The function of sCLU as an anti-apoptotic factor is mediated by the modulation of NF-κB, PI3K/AKT and ERK1/2 signaling pathways [27–29]. Regarding bone metabolism, sCLU was reported to inhibit osteoclast bone resorption by suppressing macrophage colony-stimulating factor, M-CSF-mediated ERK activation [30]. However, the role of sCLU in osteoblast differentiation from BMSCs and in bone formation has not been reported. In this study, we demonstrated that *sClu* is expressed by BMSCs and that its steady-state gene expression is increased during adipocyte differentiation and decreased during osteoblast differentiation. Functional analysis revealed that sCLU stimulates cell proliferation and the early commitment of BMSCs toward the adipocytic lineage at the expense of the osteoblastic lineage, an effect mediated via ERK1/2 phosphorylation.

2. Materials and methods

2.1. Animals

C57BL/6 mice were originally purchased from Charles River. Mice were bred and housed under standard conditions (21 °C, 55% relative humidity) on a 12-h light/12-h dark cycle at the animal housing unit and the Physiology Laboratory, College of Science, King Faisal University, Saudi Arabia, in accordance with the protocol approved by the Standing Research Ethics Committee. Ad libitum food (Altromin® Spezialfutter GmbH & Co. KG, Lage, Germany) and water were provided. Sera were collected from young female mice (2 months old) and old female mice (18 months old).

2.2. Isolation and cultivation of BMSCs

Mouse BMSCs were isolated from the bone marrow of wild-type 8-weeks-old male C57BL/6 J mice as described previously [31]. In brief, The femur and tibia were dissected from mice and bone marrow was flushed out with a 21-gauge syringe containing, complete isolation media (CIM), which consists of RPMI-1640 (Roswell Park Memorial Institute) supplemented with 10% fetal bovine serum (FBS; GIBCO), 100 U/mL penicillin (GIBCO, Thermo Fisher Scientific, Darmstadt, Germany) and 100 μg/mL streptomycin (GIBCO). Cells were filtered, washed with PBS and cultured in 40 mL CIM in a 175-cm2 flask in 5% CO2 incubator at 37 °C. Non-adherent cells were removed after 24 h by washing with PBS, and adding 30 mL of fresh CIM. Cells were passaged every 1 week with using 0.25% trypsin/1 mM EDTA [32]. BMSCs cultures were used between passages 2 to 4 only.

Recombinant mouse Clusterin Protein was purchased from R&D Systems GmbH (Wiesbaden, Germany).

2.3. Cell proliferation study

Short-term in vitro cell growth was determined by culturing the cells at 2000 cells/well in 4 well plates. Cells were trypsinized and counted by the hemocytometer. We measured 4–6 biological replicates for each time point.

2.4. Osteoblast differentiation

Cells were cultured at 15,000 cells/cm^2 in CIM medium. At 70% cell confluence, cultured media were changed to osteogenic-induction medium (OIM) consists of: α-minimum essential medium (α-MEM; Gibco) containing 10% FBS, 100 U/mL of penicillin, 100 mg/mL of streptomycin, 50 μg/mL of vitamin C (Sigma-Aldrich), 10 nM dexamethasone and 10 mM β-glycerol-phosphate (Sigma-Aldrich). Cells were cultured in OIM for 12 days (or as indicated). The media were changed every 2–3 days during the time course of osteoblast differentiation.

2.5. Adipocyte differentiation

Cells were cultured at 15,000 cells/cm^2 in CIM medium. At 100% cell confluence, cultured media were replaced by adipogenic-induction medium (AIM) consists of: DMEM supplemented with 9% horse serum, 450 μM 1-methyl-3-isobutylxanthine (IBMX), 250 nM dexamethasone, 5 μg/mL insulin (Sigma-Aldrich) and 1 μM rosiglitazone (BRL 49653, Cayman Chemical). Cells were cultured in AIM for 12 days (or as indicated). The media were changed every 2–3 days during the time course of adipocyte differentiation.

2.6. Alkaline phosphatase (ALP) activity assay and number of viable cells measurement

Number of viable cells was determined using the Cell Titer-Blue cell viability assay according to the manufacturer's instructions (Promega, USA) at OD 579. ALP activity was determined following the manual instructions of ALP assay kit (Abcam plc, Cambridge, UK). The color of the reaction was measured at 405 nm. ALP activity was normalized to cell number (measured by number of viable cells) and then represented as fold change over control non-induced cells [33].

2.7. Alizarin red staining for mineralized matrix

Cells were fixed with 70% ice-cold ethanol for 1 h at −20 °C, and stained with 40 mM Alizarin red S (AR-S; Sigma-Aldrich), pH 4.2 for 10 min at room temperature. For the quantification of mineralized matrix in culture, Alizarin red stain was eluted using 10% (*w*/*v*) cetylpyridinium chloride solution (Sigma-Aldrich) with shaking for 20 min and the absorbance of the eluted dye was measured at 570 nm.

2.8. Oil Red O staining and quantification

Cells were fixed in 4% paraformaldehyde for 10 min at room temperature, then stained with Oil Red O (0.5 g in 60% isopropanol) (Sigma-Aldrich) for 1 h at room temperature to stain the fat droplets. Lipids were quantified by elution of Oil Red O in isopropanol for 10 min at room temperature. The absorbance of the extracted dye was detected at 490 nm. Oil Red O measurements were normalized to cell umber (measured by number of viable cells) and then represented as fold change over control non-induced cells.

2.9. RNA extraction and real-time PCR analysis

Total RNA was extracted from tissues and cells using a single-step method of TRIzol (Thermo Fisher Scientific). cDNA was synthesized from 1 μg of total RNA using revertAid H minus first strand cDNA synthesis kit (Fermentas). Quantitative real time PCR was performed with Applied Biosystems 7500 Real-Time system using Fast SYBR® Green Master Mix (Applied Biosystems, California, USA) with specific primers (Supplementary Table 1). The expression of each target gene was normalization to β-Actin and *Hprt* mRNA expression as reference genes, using a comparative CT method [(1/(2delta-CT)) formula, where delta-CT is the difference between CT-target and CT-reference] with Microsoft Excel 2007® as described [34].

2.10. siRNA transfection

For silencing *Clu* gene expression in mBMSCs, we used *Clu* siRNA (si*Clu*, s64077, GAAGAAGUCUCUAAGGAUAtt) (Silencer Select® siRNA, Ambion, USA) and non-targeting control siRNA (Thermo Fisher Scientific) as a negative control. Cells were transfected with siRNA by a reverse transfection protocol using Lipofectamine 2000 transfection reagent according to the manufacturer's instructions (Thermo Fisher Scientific) and as descried previously [35].

2.11. ELISA measurement of sCLU

Serum free conditioned media were collected from cultured mBMSCs during their in vitro differentiation at different time points. sCLU was measured in collected conditioned media using by ELISA using mouse Clusterin Quantikine ELISA Kit (R&D Systems GmbH,Wiesbaden, Germany), according to the manufacture instruction's.

2.12. Western blot assays

Cells were collected at different time points post treatment, and lysed in cell lysis buffer supplemented with protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Protein (30 μg) was separated on 8% precast polyacrylamide gel, NuPAGE® Novex® Bis-Tris gel systems (Thermo Fisher Scientific, Darmstadt, Germany). After blocking (by 5% milk powder in Tris-buffered saline (TBS, pH 8.0) for 1 h, the membrane was probed with antibodies and incubated with peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Heidelberg, Germany). Proteins were visualized with the ECL system (Amersham bioscience, UK). Specific antibodies for phosphor p38 MAPK (Thr180/Tyr 182) and JNK (Thr183/Tyr185) were purchased from Cell Signaling Technology (Leiden, Netherlands). Antibodies (for total or phosphor) ERK1/2 (sc-7383) was purchased from Santa Cruz Biotechnology, Inc. Rabbit monoclonal anti-Clusterin antibody was purchased from Abcam (Cambridge,

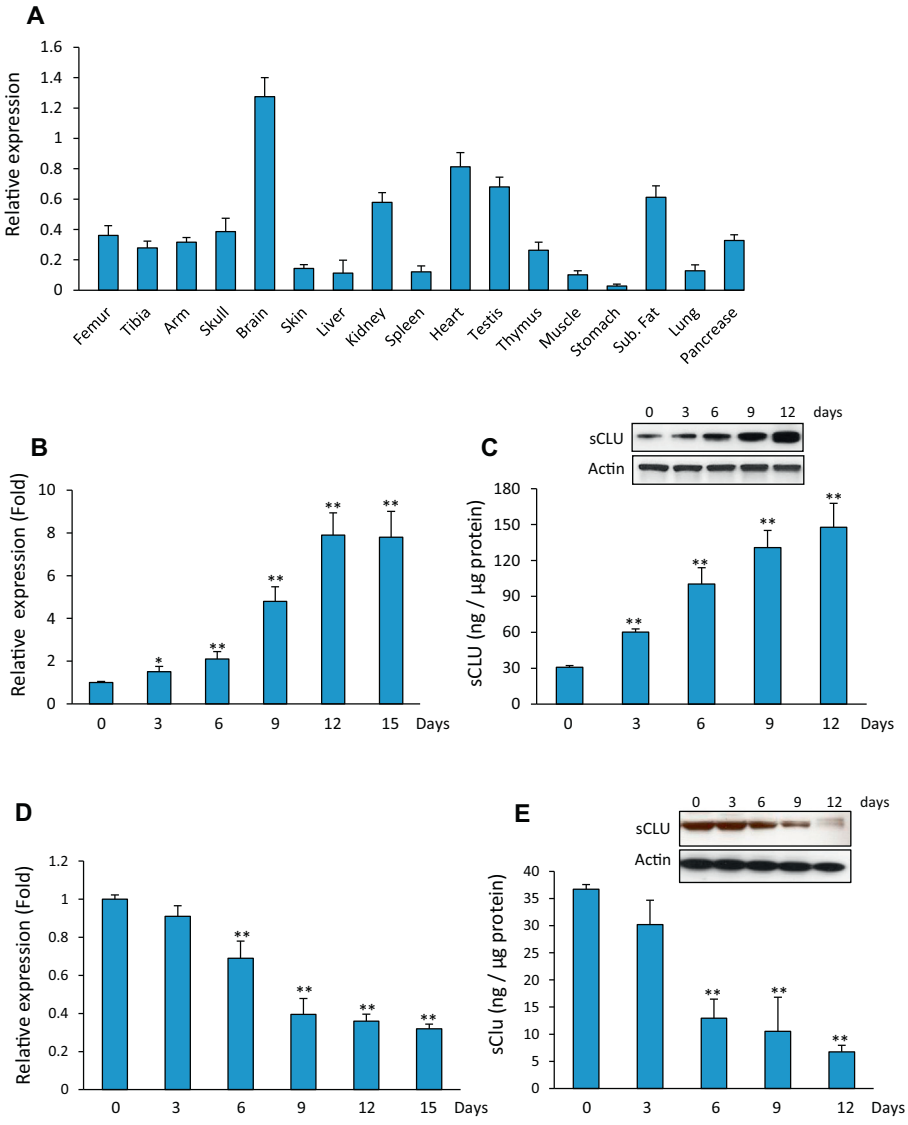

Fig. 1. Expression and secretion of sCLU during mBMSCs differentiation. (A) Quantitative real time RT-PCR (qPCR) analysis of *Clu* mRNA expression by adult mouse tissues derived from 2 months old mice. *Clu* mRNA expression was represented as relative gene expression after normalization to reference genes as described in M&M. (B) qPCR analysis of *Clu* mRNA expression at different time points during the adipocyte differentiation of primary mBMSCs. (C) ELISA measurements and western blot analysis of the sCLU protein secreted in the conditioned medium (CM) collected from mBMSC cultures during adipogenesis. (D) qPCR analysis of *Clu* mRNA expression at different time points during the osteoblast differentiation of primary mBMSCs. (E) ELISA measurements and western blot analysis of the sCLU protein secreted in the conditioned medium (CM) collected from mBMSCs cultures during osteogenesis. Values are mean ± SD of three independent experiments, (*$p < 0.05$, **$p < 0.005$, compared to control non-induced day 0 for panel B-E).

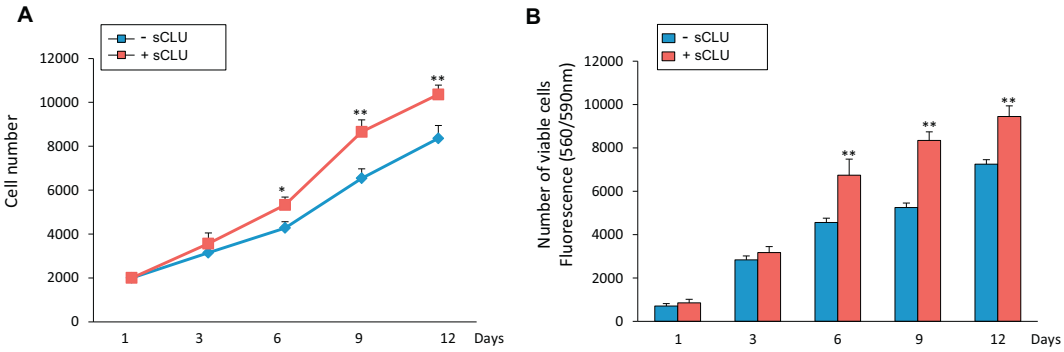

Fig. 2. sCLU promotes the cell proliferation of mBMSCs. (A) Effect of sCLU on short term cell proliferation of primary mBMSCs. Cells were cultured under basal culture condition for 12 days in absence (control) or presence of sCLU recombinant protein (5 μg/mL). Cell number was counted using hemocytometer. (B) Effect of sCLU on cell number as measured by number of viable cells of primary mBMSCs during their culture under basal culture condition. Number of viable cells was measured as described in M&M. Values are mean ± SD of three independent experiments, (*$p < 0.05$, **$p < 0.005$, as compared to non-treated control).

UK), and monoclonal anti-PPARγ antibody was from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany). Quantification of band intensity was measured using image J software and presented as relative to control.

2.13. Statistical analysis

All values are expressed as mean ± SD (standard deviation) of at least three independent experiments. Student's *t*-test was used for comparison between two groups. Differences were considered statistically significant at *$p < 0.05$, and **$p < 0.005$.

3. Results

3.1. Gene expression and protein secretion of sCLU during mBMSCs differentiation

We have recently employed a combination of microarray and secretome approaches to identify secreted factors regulating BMSC differentiation into osteoblasts and adipocytes. Among the factors identified and that have not been previously studied in the context of BMSC biology, we chose CLU to perform a detailed study. We examined the expression of *Clu* mRNA in flat and long bones, and we compared its

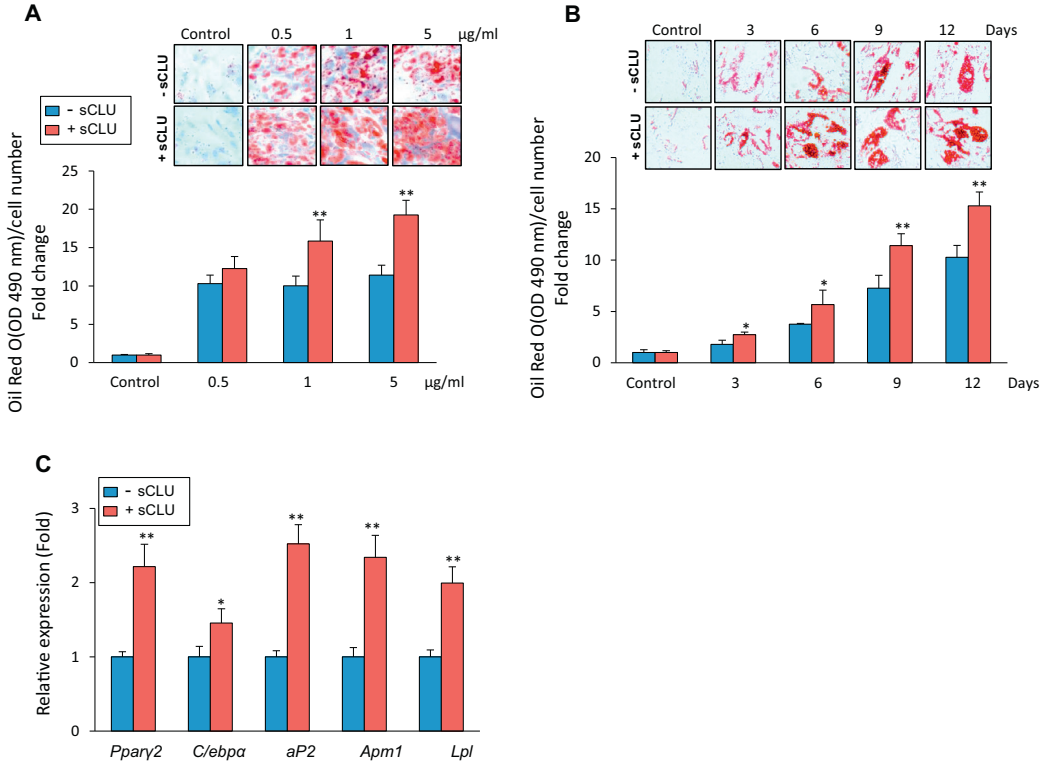

Fig. 3. sCLU exerts stimulatory effect on the adipocyte differentiation of mBMSCs. (A) Dose dependent induction effect of soluble CLU (0.5–5 μg/mL) on the adipocyte differentiation of mBMSCs as measured by quantitative Oil Red O staining for lipid accumulation after 10 days of adipogeneic induction. (B) Stimulatory effect of sCLU on the adipogenesis of mBMSCs during the differentiation course of 12 days. Cells were induced toward adipogenesis in the absence (− sCLU) or the presence of sCLU recombinant protein (5 μg/mL). Representative images of Oil Red O staining are shown at each time point during adipogenesis of mBMSCs. (C) qPCR analysis of the adipogenic markers expression in mBMSCs after 12 days of adipogenic induction in the absence (-sCLU) and the presence of sCLU (+sCLU). Each target gene was normalized to reference genes and presented as fold change over control non-treated cells. Values are mean ± SD of three independent experiments, (*$p < 0.05$, **$p < 0.005$, compared to non-treated cells).

expression levels to the one of other tissues in adult mice. As shown in Fig. 1A, *Clu* mRNA expression was expressed in adult mouse bones with a moderate expression level. Clusterin protein (sCLU) is expressed and secreted by mBMSCs. As shown in Fig. 1B, *Clu* mRNA was expressed in undifferentiated mBMSCs, and its expression significantly increased during the differentiation of mBMSCs into adipocytes, as assessed by qRT-PCR. In addition, the secretion of sCLU in the conditioned medium (CM) of mBMSC cultures was increased significantly during adipocyte differentiation, as measured by ELISA and Western blot analysis (Fig. 1C). In contrast, the expression of *Clu* mRNA and the secretion of sCLU protein were both downregulated during the differentiation of mBMSCs into osteoblasts (Fig. 1D & E).

3.2. sCLU promotes the cell proliferation of mBMSCs

Since CLU was shown to promote the cell proliferation of different cell types and since its expression increased during tissue regeneration [36,37], we examined the paracrine effect of sCLU on the cell proliferation of mBMSCs in short-term cultures. As shown in Fig. 2A, treatment of mBMSCs with recombinant sCLU protein stimulated their cell proliferation, as assessed by cell count analysis and number of viable cells.

3.3. sCLU enhanced the differentiation of mBMSCs into adipocytes

To study the effect of sCLU on mBMSC differentiation, we examined the effect of recombinant sCLU protein on adipocyte formation. As shown in Fig. 3A, sCLU stimulated the differentiation of mBMSCs into the adipocytic lineage in a dose-dependent manner, as assessed by quantitative Oil Red O staining, which revealed the formation of mature lipid-filled adipocytes. In addition, we showed the stimulatory effect of sCLU (used at 1 μg/mL) on mature adipocyte formation, at different time points during the course of adipocyte differentiation of mBMSCs (Fig. 3B). Consistently, mBMSCs treated with sCLU significantly upregulated the early (*Pparγ2*) and late (*aP2*, *Apm1*, *Lpl*) adipocytic markers compared to control non-treated cells (Fig. 3C).

3.4. sCLU inhibited the differentiation of mBMSCs into osteoblasts

We examined the effect of sCLU on the osteoblastic differentiation potential of mBMSCs. Treatment of mBMSCs with recombinant sCLU protein inhibited both ALP activity and the formation of mineralized matrix in a dose-dependent manner, as assessed by quantitative ALP activity and Alizarin red staining, respectively (Fig. 4A & B). In addition, significant downregulation of early (*Runx2*, *Msx2*, *Col1a1*, *Dlx5*, and *Alp*) and late osteoblastic markers (*Ocn* and *Opn*) was observed (Fig. 4C). These data suggest that sCLU functions as a negative regulator of the osteoblastic differentiation of mBMSCs.

3.5. Identification of sCLU as a regulator of mBMSCs differentiation

To verify the role of CLU as a novel regulator of mBMSC lineage commitment and differentiation, we examined the effect of *Clu* loss-of-function by siRNA on the osteoblast and adipocyte differentiation of mBMSCs. siRNA-mediated silencing of *Clu* (si*Clu*) inhibited *Clu* expression at the mRNA and protein levels by approximately 70% (Fig. 5A). As shown in Fig. 5B, *Clu* gene silencing inhibited the adipocytic differentiation of mBMSCs by approximately 50%, as revealed by quantitative Oil Red O staining of lipid-filled mature adipocytes and by the

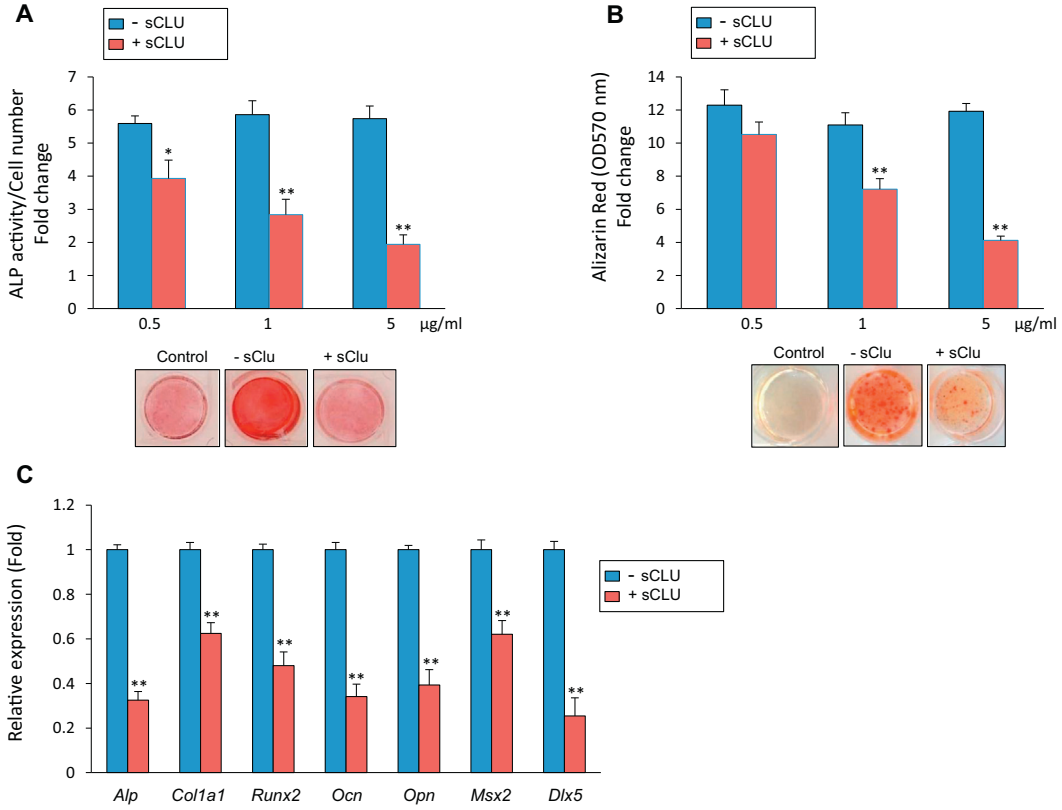

Fig. 4. sCLU inhibits osteoblast differentiation of mBMSCs (A) Dose dependent inhibitory effect of the sCLU on the osteoblast differentiation of mBMSCs as measured by quantitative alkaline phosphatase activity (ALP) and (B) Alizarin red staining after 6 days and 12 days of osteogenic induction respectively. ALP and Alizarin red staining images are shown. ALP and Alizarin red measurements were normalized to the cell number. (C) qPCR analysis of the osteogenic markers expression after 12 days of induction for mBMSCs in the absence (-sCLU) and the presence of sCLU (+sCLU) (5 μg/mL). Each target gene was normalized to reference genes and presented as fold change over control non-treated cells. Values are mean ± SD of three independent experiments, ($^{*}p < 0.05$, $^{**}p < 0.005$, compared to non-treated cells). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

downregulation of adipocytic gene expression (Supplemental Fig. 1A). In contrast, si*Clu* transfection enhanced the osteoblastic differentiation of mBMSCs by approximately 20%, as measured by quantitative ALP activity and mineralized matrix formation (Fig. 5C & D). In addition, si*Clu* transfection in mBMSCs increased the expression of osteoblastic markers, as assessed by qRT-PCR (Supplemental Fig. 1B).

3.6. sCLU inhibited the osteoblast differentiation of mBMSCs by modulating ERK1/2 signaling pathway

To gain insight into the mechanism mediating the regulatory effect of sCLU on mBMSC lineage commitment, we examined the possibility that sCLU regulated mitogen-activated protein kinases, the MAPK/ERK1/2 signaling pathway, during mBMSCs differentiation [3,38]. Treatment of mBMSCs with sCLU displayed significant inhibition of ERK1/2 phosphorylation without affecting either p38 or JNK phosphorylation, as shown by Western blot analysis (Fig. 6A). Since ERK1/2 signaling pathway was shown to be activated during the osteoblastic differentiation of mBMSCs (Fig. 6B), we analyzed the effect of *Clu* gene silencing on the activity of ERK1/2 signaling pathway. Transfection of mBMSCs with si*Clu* increased ERK1/2 phosphorylation and significantly stimulated mBMSC differentiation into the osteoblastic lineage, as assessed by ALP activity measurements (Fig. 6C). In addition, inhibition of the ERK1/2 signaling pathway with the specific inhibitor U0126 significantly suppressed osteogenesis in mBMSCs, as assessed by ALP activity assay (Fig. 6D). On the other hand, we demonstrated that ERK1/2 phosphorylation was inhibited during the adipocyte differentiation of mBMSCs (Fig. 6E). Thus, we transfected mBMSCs with si*Clu* to activate the ERK1/2 signaling pathway and then examined the effect of ERK1/2 phosphorylation inhibition on si*Clu*-suppressed adipogenesis (Fig. 6F). Interestingly, treatment of si*Clu*-transfected mBMSCs with two different ERK1/2 phosphorylation inhibitors, U0126 and PD98059, significantly reverted the inhibitory effect of si*Clu* on the adipogenesis of mBMSCs, by increasing lipid accumulations by 66% and 36.6%, respectively, compared to non-treated siClu-transfected mBMSCs (Fig. 6F).

3.7. Serum levels of sCLU increased in aged mice

We further examined whether there was an association between the serum levels of sCLU and the increase in bone marrow fat occurring during aging, a physiological condition characterized by increased levels of bone marrow adipocytes at the expense of osteoblasts [39–41]. Thus, we measured the serum levels of sCLU in female young mice (2 months) compared to those of old mice (18 months). Interestingly, sCLU serum levels were elevated in old mice by 35.46% compared to young mice, as assessed by ELISA assay (Fig. 7).

4. Discussion

In this study, we have identified sCLU as a novel regulator of BMSC lineage commitment, which inhibits the differentiation of BMSCs into the osteoblastic cell lineage versus adipocytic cell lineage. Furthermore, we demonstrated that the inhibitory effect of sCLU on osteoblasts versus adipocyte differentiation, is mediated by the modulation of the ERK1/2 signaling pathway.

The ubiquitously expressed secreted glycoprotein sCLU plays an important role in cell proliferation and differentiation in many tissues. However, this study is the first to investigate the function of sCLU in stem cell biology and BMSC differentiation into osteoblasts and adipocytes. Our data demonstrated that the sCLU secretion is significantly

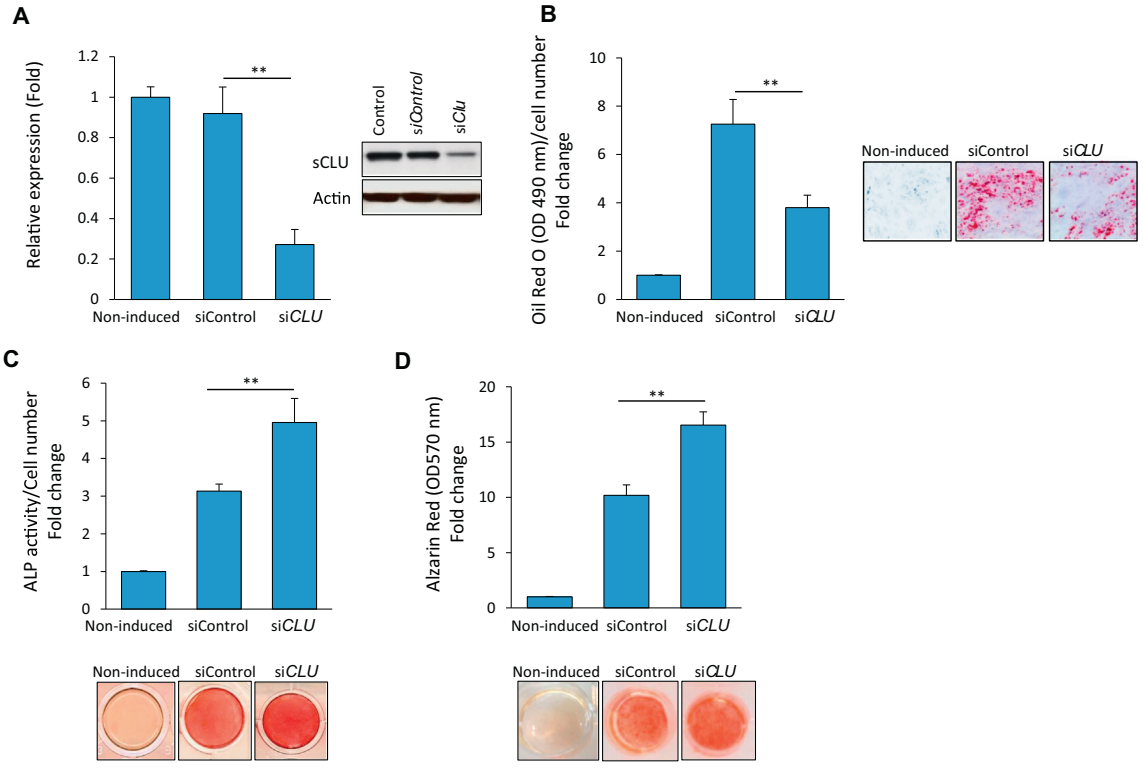

Fig. 5. siRNA-mediated silencing of *Clu* expression stimulates osteogenesis and inhibits adipogenesis of mBMSCs (A) qPCR analysis and Western blot analysis of *Clu* mRNA and protein expression respectively in mBMSCs. Cells were non-transfected (control) or reverse transfected with either control siRNA (siControl) or *Clu* siRNA (si*Clu*). Total RNA and cell lysates were analyzed at day6 post transfection. (B) Effect of *Clu* siRNA on the adipocyte differentiation of mBMSCs as measured by Oil red O quantification of lipid accumulation. Oil red O staining images are shown. (C) Effect of *Clu* siRNA on osteoblast differentiation of mBMSCs as measured by quantitative ALP activity and (D) matrix mineralization stained with Alizarin red staining. ALP and Alizarin red staining images are shown. Cells were reverse transfected with siRNA, and after 2 days culture media were replaced with either adipogenic or osteogenic induction media for 7 days. Cells without induction medium were used as non-induced control. Values are mean ± SD of three independent experiments, ($^{*}p < 0.05$, $^{**}p < 0.005$, compared to siControl cells). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

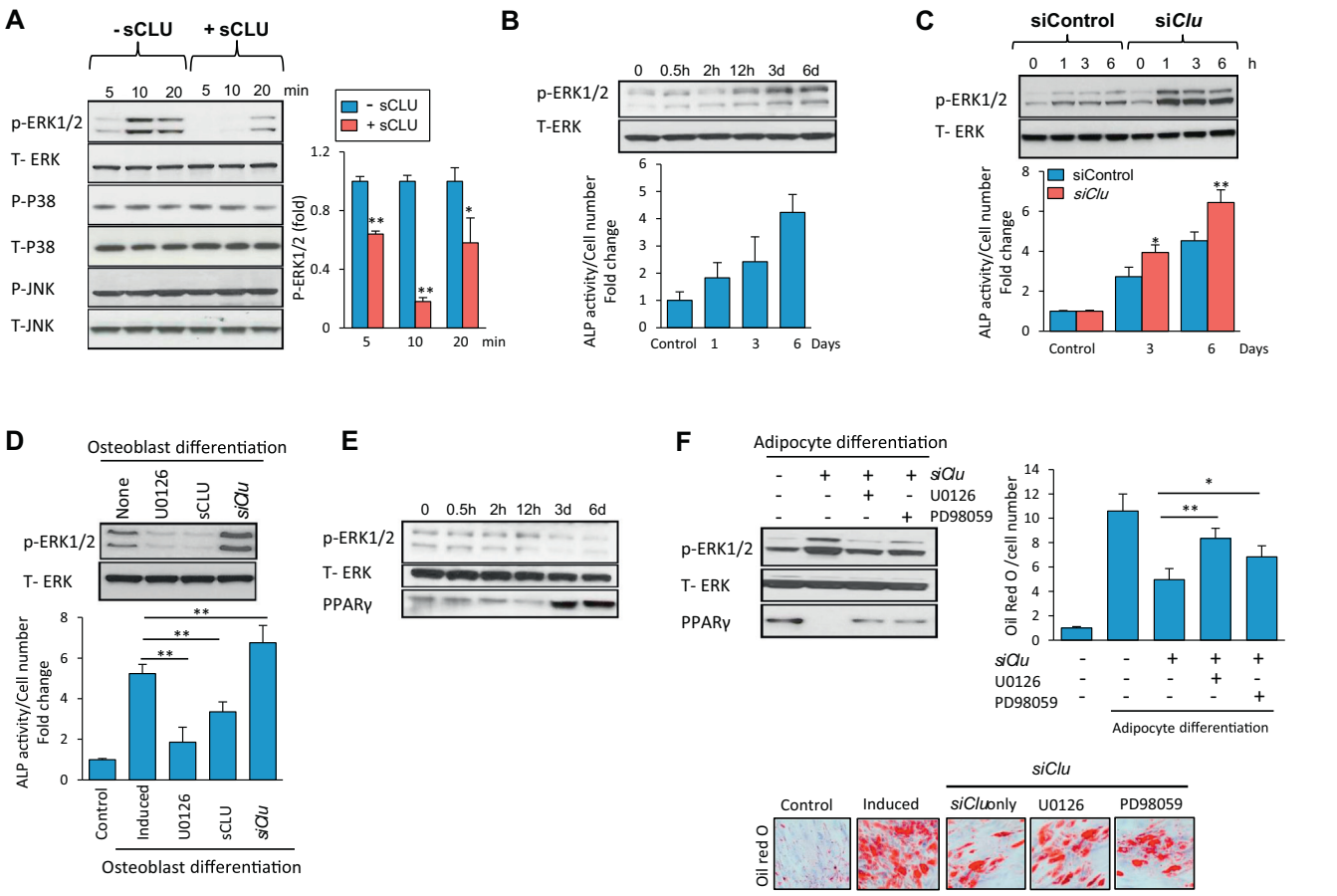

Fig. 6. sCLU regulates the differentiation fate of mBMSCs in ERK1/2-dependent mechanism. (A) Western blot analysis of ERK1/2, p38, AKT and JNK phosphorylation in cultured mBMSCs treated without (-sCLU) or with sCLU recombinant protein (+sCLU). Cells were cultured in 1% FBS and treated with sCLU in serum free medium. (B) Western blot analysis of ERK1/2 phosphorylation and quantification of ALP activity during the time course of the osteoblast differntiation of mBMSCs. (C) Western blot analysis of ERK1/2 phosphorylation and quantification of ALP activity during the osteoblast differntiation of primary mBMSCs, that transfected with either control siRNA (siControl) or *Clu* siRNA (*siClu*). Cells were reverse transfected with siRNA, and after 2 days, culture media were replaced with osteogenic induction media. (D) Western blot analysis of ERK1/2 phosphorylation and quantification of ALP activity in cultured mBMSCs that induced to osteogenic lineage in absence (None) or presence of U0126 (10 μM) (ERK1/2 inhibitor), sCLU (5 μg/mL) and *siClu*. Cell lysates were harvested at 10 min for western blot analysis, while ALP measurments were performed at day 6 of osteogenic induction. (E) Western blot analysis of ERK1/2 phosphorylation and PPARγ expression during the time course of adipocyte differentiation of mBMSCs. (F) Western blot analysis of ERK1/2 phosphorylation and PPARγ expression in mBMSCs, that revert transfected with *siClu* and after 2 days were induced to adipogenic lineage in absence or presence of U0126 (5 μM) and PD98059 (50 μM). Cell lysates were harvested at 10 min for western blot analysis, Oil red O measurments were performed at day 9 of adipogenic induction. Oil red O staining images are shown. Values are mean ± SD of three independent experiments ($^{*}p < 0.05$, $^{**}p < 0.005$, compared to: control non-induced, for panel A-C; induced (non-treated) for panel D; or induced (treated with *siClu* only) for panel F).

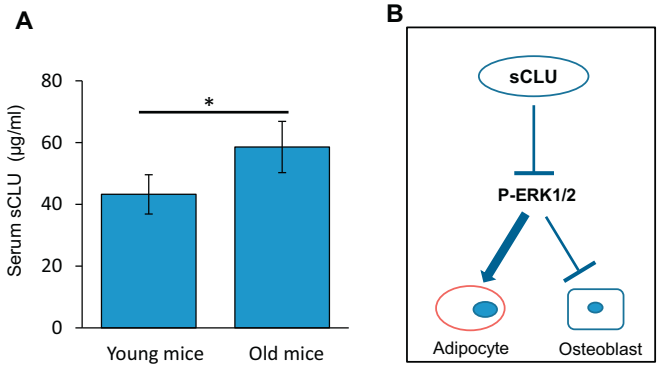

Fig. 7. Serum level of sCLU is elevated in aged mice. (A) Serum levels of sCLU were measured using ELISA in sera collected from young (2 months old) and old (18 months old) C57BL/6 female mice. Values are mean ± SD ($n = 10$ mice/group), ($^{*}p < 0.05$, $^{**}p < 0.005$). (B) The proposed mode of action of sCLU in the regulation of BMSCs differentiation. Secreted CLU stimulates the commitment of mBMSCs into adipogenic versus osteogenic cell lineage via suppressing ERK1/2 phosphorylation.

increased during the adipocyte differentiation of mBMSCs and exerts an inhibitory effect on the osteoblast differentiation of mBMSCs. These data support other in vitro and in vivo studies reporting the negative impact of adipocytes on osteoblasts in the bone marrow by secreting factors that negatively regulate the osteoblast differentiation of BMSCs. These factors include for example, pro-inflammatory cytokines [42], sFRP-1 [7], Pref-1 [9], and Chemerin [43]. Our finding that sCLU stimulated adipogenesis is in agreement with a previous study showing that the in vitro administration of sCLU stimulated mature adipocyte formation in mouse pre-adipocyte C2C12 and 3 T3-L1 cell lines [44]. Furthermore, sCLU was shown to be involved in lipid metabolism-related mechanisms including lipogenesis, lipid accumulation, and lipid transport [45]. At the clinical level, the increased plasma levels of sCLU were associated with obesity [46], while the reduced plasma levels of sCLU were associated with weight loss in obese adolescents [47]. In this context, we showed that the serum levels of sCLU were increased during aging in rodents, suggesting a positive association between sCLU serum levels and bone marrow fat levels; however, further studies are needed to confirm such correlation.

Our data demonstrated the stimulatory effect of sCLU on mBMSC proliferation. This is consistent with the reported function of sCLU in

stimulating the proliferation of several cell types including corneal epithelial cells [37], primary astrocytes [48] and renal tubular epithelial cells [36]. In addition, sCLU has been established as a pro-survival factor that protects cells from stress-induced apoptosis. In this context, the inhibition of sCLU was shown to sensitize cancer cells to chemotherapy [49], and sCLU inhibition was also recently investigated as a therapeutic target for treating autoimmune and cardiovascular diseases [50,22,51,26].

Our results demonstrated a role for the ERK1/2 signaling pathway in mediating the function of sCLU to regulate the fate of mBMSCs into adipocytic or osteoblastic cell lineages. Similarly, sCLU was shown to control other differentiation processes by regulating the ERK1/2 signaling pathway. For example, sCLU was found to stimulate the neuronal differentiation of neural precursor cells by modulating ERK phosphorylation [52], and to suppress the osteoclastogenesis of bone marrow-derived macrophages (osteoclast precursor cells) by inhibiting the macrophage colony-stimulating factor (M-CSF)-induced ERK activation [30]. The regulation of the commitment of BMSCs into osteoblasts or adipocytes was reported to be mediated by the MAPK/ERK signaling pathway [53]. Our data demonstrated the inhibitory effect of sCLU on ERK1/2 signaling pathway in order to promote the lineage commitment of mBMSCs into adipocytes at the expense of osteoblasts. Indeed, the differentiation of BMSCs into osteoblasts was associated with the activation of ERK, while the adipocyte differentiation of BMSCs was associated with reduced ERK activity [53–55]. This has also been verified in another context where cell shape-dependent activation of RhoA/ROCK signaling induced osteoblastogenesis and inhibited adipogenesis by activating the ERK/MAPK signaling pathway [56,57]. Furthermore, a recent study by Ge C, et al., 2016, demonstrated that the differentiation of BMSCs into osteoblasts or adipocytes is reciprocally regulated by the ERK/MAPK-dependent phosphorylation of Runx2 and PPARγ, two key transcription factors for osteoblast and adipocyte differentiation [38].

5. Conclusions

There is a need to identify the regulatory factors present within the BMSC niche and to determine their role in regulating BMSC lineage commitment and differentiation, as a pre-requisite to develop therapeutic strategies to enhance bone regeneration and bone formation [3]. Our study identified sCLU as a novel protein present within the BMSC niche, which controls the commitment of BMSCs into the adipogenic or osteogenic cell lineages. It is plausible that inhibiting sCLU within the BMSC niche could be used as a potential therapeutic strategy to enhance osteoblast differentiation and bone formation.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bone.2018.02.018.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All materials are available by the corresponding author.

Competing interests

The authors declare that he has no competing interests.

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Authors' contributions

BMA conceived the project, designed the study, performed experiments, analyzed data and wrote the manuscript. AZ performed experiments, analyzed data and edited the manuscript. MK conceived the project, analyzed the data and edited the manuscript.

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