J Appl Biomed
DOI: 10.32725/jab.2021.016
journal homepage: http://jab.zsf.jcu.cz

Journal of Applied Biomedicine

Original research article

A-769662 stimulates the differentiation of bone marrow-derived mesenchymal stem cells into osteoblasts via AMP-activated protein kinase-dependent mechanism

Basem M. Abdallah *, Abdullah M. Alzahrani

King Faisal University, College of Science, Biological Sciences Department, Al-Ahsa, Saudi Arabia

Abstract
AMP-activated protein kinase (AMPK) signaling shows an important role in energy metabolism and has recently been involved in osteogenic and adipogenic differentiation. In this study we aimed to investigate the role of AMPK activator, A-769662, in regulating the differentiation of mesenchymal stem cells derived from bone marrow (BMSCs) into osteoblastic and adipocytic cell lineage. The effect of A-769662 on osteogenesis was assessed by quantitative alkaline phosphatase (ALP) activity, matrix mineralization stained with Alizarin red, and gene expression analysis by quantitative polymerase chain reaction (qPCR). Adipogenesis was determined by Oil Red O staining for fat droplets and qPCR analysis of adipogenic markers. A-769662 activated the phosphorylation of *AMPKα1* during the osteogenesis of mBMSCs as revealed by western blot analysis. A-769662 promoted the early stage of the commitment of mouse (m) BMSCs differentiation into osteoblasts, while inhibiting their differentiation into adipocytes in a dose-dependent manner. The effects of A-769662 on stimulating osteogenesis and inhibiting adipogenesis of mBMSCs were significantly eliminated in the presence of either *AMPKα1* siRNA or Compound C, an inhibitor of AMPK pathway. In conclusion, we identified A-769662 as a new compound that promotes the commitment of BMSCs into osteoblasts versus adipocytes via AMPK-dependent mechanism. Thus our data show A-769662 as a potential osteo-anabolic drug for treatment of osteoporosis.

Keywords: A-769662; Adipocyte; AMPK; BMSC; Osteoblast; Stem cells

Highlights:
- The study shows the stimulatory effect of AMPK activator, A-769662 on the differentiation of mesenchymal stem cells into osteoblast.
- Data demonstrated the inhibitory effect of A-769662 on adipogenesis.
- A-769662 induces osteogenesis via AMPK dependent mechanism.

Introduction

Mesenchymal stem cells derived from bone marrow (BMSCs; known as skeletal stem cells) are a group of adult stem cells that share the same microenvironment with hematopoietic stem cells in the bone marrow niche. BMSCs are known to have both self-renewal capacity and differentiate potential which falls into three-mesoderm lineages including adipocytes, osteoblasts and chondrocytes (Abdallah and Kassem, 2008; Abdallah et al., 2015; Bianco and Robey, 2015). Preclinical and clinical studies showed the potential role of BMSCs in promoting bone and dental regeneration after trauma in bone diseases, including osteoporosis, dental, facial and long bone defects (Luby et al., 2019; Zheng et al., 2019).

Several intracellular kinase pathways regulate the stage specific differentiation of BMSCs into osteoblasts. These include: transforming growth factor β (TGFβ) activated kinase 1 (TAK1) (Chen et al., 2012); phosphoinositide 3-kinase (PI3-kinase)/Akt (Amantea et al., 2008); AMP-activated protein kinase (AMPK) (Kanazawa et al., 2009; Kim et al., 2012; Shah et al., 2010); and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) (Abdallah et al., 2015; Ge et al., 2007; Kanno et al., 2007). Thus, identifying compounds that could target any of these osteogenic-specific protein kinases will promote the differentiation of BMSCs into osteoblasts.

AMPK is a serine/threonine protein kinase complex, it consists of α, β and the regulatory γ subunits. AMPK has been reported to regulate cellular energy metabolism (Hardie, 2014; Mihaylova and Shaw, 2011; Wang et al., 2012), cellular autophagy (a caspase-independent cell degradation process), and cell cycle arrest (Tamargo-Gomez and Marino, 2018). In this context, several reports have investigated AMPK as a novel

* **Corresponding author:** Basem M. Abdallah, Associate Professor, King Faisal University, College of Science, Biological Sciences Department, P.O Box 380, Al-Ahsa, 31982, Saudi Arabia; e-mail: babdallallah@kfu.edu.sa
http://doi.org/10.32725/jab.2021.016
Submitted: 2020-12-30 • Accepted: 2021-06-21 • Prepublished online: 2021-07-01
J Appl Biomed 19/3: 159–169 • EISSN 1214-0287 • ISSN 1214-021X

therapeutic target for type 2 diabetes, atherosclerosis, obesity and cancer (Babkov et al., 2019; Day et al., 2017; Thirupathi and Chang, 2019).

Thienopyridone (A-769662) is an established AMPK activator that allosterically activates AMPK without affecting kinases upstream of the AMPKβ subunit (Kim et al., 2016). A-769662 was found to suppress adipogenesis in a pre-adipocyte cell line (Zhou et al., 2009), reduce body weight gain and decrease plasma glucose in ob/ob mice (Cool et al., 2006), and inhibit mesenchymal stem cell (MSC) proliferation in a p27-dependent manner (de Meester et al., 2014). Additionally, A-769662 reportedly protects osteoblasts against hydrogen peroxide (H_2O_2)-induced apoptosis (Zhu et al., 2014). However, the effect of A-769662 on BMSC differentiation into osteoblasts has not yet been reported.

In this study, we investigated the effect of A-769662 on BMSCs differentiation into osteoblasts versus adipocytes. Interestingly, A-769662 significantly stimulated osteogenesis while suppressing adipogenesis of BMSCs via an AMPK-dependent mechanism.

Materials and methods

Cell cultures and reagents

Primary mBMSCs were isolated from male C57BL/6J mice as previously described (Abdallah et al., 2018). Animals were treated according to the national and institutional guidelines for the ethical and legal treatment of animals. The Standing Research Ethics Committee, King Faisal University, Saudi Arabia, approved the extraction of BMSCs from mice. For BMSCs isolation, mice were sacrificed at the age of 2 months, and this was followed by immediate dissection. In brief, cells flushed out from the bone marrow of mouse tibia and femur were filtrated using 70 μm nylon mesh filter and cultured in RPMI-1640 medium supplemented with 12 μM L-glutamine (Invitrogen), 1% penicillin/streptomycin (P/S) (Gibco Invitrogen, USA), and 12% FBS (Gibco Invitrogen). Non-adherent cells were removed after 24 h, washed and cultured in the above medium at 37 °C, CO_2 incubator.

Human BMSC cells were purchased from Cell Applications Inc. (San Diego, CA). hBMSCs were cultured in DMEM medium (Dulbecco's modified Eagle medium) (Sigma-Aldrich) containing 1% penicillin/streptomycin and 10% FBS (Gibco Invitrogen), according to the manufacturer's instructions. Medium was changed every 2–3 days.

Compound C and A-769662 were purchased from Calbiochem (Darmstadt, Germany) and Cayman Chemical (Ann Arbor, MI, USA) respectively.

Cell toxicity assay

MTT cell proliferation assay kit (Sigma-Aldrich) and CellTiter-Blue® Cell Viability Assay (Promega, USA) were used to evaluate the cell viability according to the manufacturer's instructions kit. Cells were incubated with MTT solution to metabolize to formazan and with CellTiter-Blue® solution to reduce resazurin to resorufin. Absorbance was measured at a wavelength of 550 nm for MTT assay and at 579 nm for CellTiter-Blue® assay.

Differentiation of BMSCs into osteoblasts

mBMSCs were stimulated to differentiate into osteoblasts using osteogenic induction medium (OIM) containing α-minimum essential medium (α-MEM; Gibco) supplemented with 100 U/ml of penicillin/streptomycin (P/S), 10% FBS, 10 mM β-glycerol-phosphate (Sigma-Aldrich ApS), and 50 mg/ml of vitamin C (Sigma-Aldrich ApS).

Differentiation of BMSCs into adipocytes

mBMSCs were stimulated to differentiate into adipocytes with DMEM supplemented with 100 U/ml of P/S, 9% horse serum, 250 nM dexamethasone, 450 μM 1-methyl-3-isobutylxanthine (IBMX), 1 μM rosiglitazone (BRL 49653, Cayman Chemical, USA), and 5 μg/ml insulin (Sigma-Aldrich).

Measurement of alkaline phosphatase (ALP) activity

mBMSCs were cultured and induced with OIM in 96 well plate. The quantification of ALP activity was measured by incubating the cells with P-nitrophenyl phosphate (1 mg/ml) at 37 °C for 20 min. The reaction was terminated by 3M NaOH. The absorbance was measured at 405 nm. CellTiter-Blue® cell viability assay was used to determine cell viability. Samples were measured in 6 replicates. Values were normalized to cell viability and represented as fold change over control including non-induced cells.

Cytochemical staining

ALP staining

mBMSCs were stimulated to differentiate into osteoblasts as mentioned above. Cells were fixed in acetone/citrate buffer pH 4.2 (1.5 : 1) at RT for 5 min. Staining was performed in Napthol-AS-TR-phosphate solution (Sigma-Aldrich ApS) at RT for 1 h. Napthol-AS-TR-phosphate is diluted 1 : 5 in H_2O and mixed 1 : 1 with Fast Red TR (Sigma-Aldrich ApS) diluted 1 : 1.2 in 0.1 M Tris buffer, pH 9.0.

Quantification of Alizarin Red S staining

mBMSCs were stimulated with OIM for 10–12 days. Cells were fixed with 70% ice-cold ethanol at –20 °C for one hour. The mineralized matrix produced by the cells upon osteoblast differentiation was stained with Alizarin red solution (Sigma-Aldrich ApS, 40 mM, pH = 4) at RT for 10 min. For quantification of mineralization, cells stained with AR-S were incubated with 10% cetylpyridinium chloride (Sigma-Aldrich ApS) at RT for 1 hour. The eluted dye was measured spectrophotometrically at 570 nm.

Quantification of lipid accumulation by Oil Red O staining

mBMSCs were induced to differentiate into adipocytes as mentioned above. After one week of induction, cells were fixed in 4% paraformaldehyde at RT for 10 min, and lipid droplets were stained with Oil Red O solution (prepared by dissolving 0.5 g in 60% isopropanol) (Sigma-Aldrich). For lipids quantification, Oil Red O was eluted with isopropanol for 10 min at RT. The extracted dye was measured spectrophotometrically at OD 490 nm. Values were normalized to the number of viable cells and represented as fold change over control.

RNA extraction and real-time PCR analysis

Total RNA was isolated from cells using TRIzol (Thermo Fisher Scientific, Roskilde, Germany). 1 μg of total RNA was used to synthesize cDNA using the RevertAid H minus first strand cDNA synthesis kit (Fermentas, St Leon-Rot, Germany). The qPCR was performed using Fast SYBR® Green Master Mix in Applied Biosystems 7500 Real-Time system (Applied Biosystems, California, USA). Suppl. Table 1 shows the primer sequences used. The expression value of each target gene was normalized to the mRNA expression values of reference genes *β-Actin* and *Hprt*, using a comparative CT method [(1/(2delta-CT) formula] with Microsoft Excel 2007® (Abdallah et al., 2018).

Western blot analysis

Cells were lysed in cell lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM sodium chloride, 1% NP-40, 0.1% SDS, 1 mM EDTA, 1 mM phenyl-methylsulfonyl fluoride, 1 mM NaF, 1 mM Na_3VO_4), supplemented with protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). 20 µg of protein was separated on 8–12% NuPAGE® Novex® Bis-Tris gel systems (Thermo Fisher Scientific, Germany) followed by transfer to PVDF membrane (Millipore, USA). The membrane was blocked and probed with antibodies (dilution 1 : 1000) and incubated with secondary anti-rabbit horseradish peroxidase-conjugated antibody (Santa Cruz Biotechnology, Heidelberg, Germany). Antibodies against total and phospho *AMPKα1* (Thr172), p-ACC (Ser79), ERK, p38, AKT and Smad 1/5/8 were purchased from Cell Signalling Tech. (Denver, MA, USA). Total Smad1/5/8 was purchased from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany) and α-tubulin from Sigma (USA).

siRNA transfection of mBMSCs

The sequence of siRNA used for silencing *AMPKα1* was, GCAUAUGCUGCAGGUAGA UdTdT (Thermo Fisher Scientific). As a negative control, the non-targeting control siRNA was used. The reverse transfection protocol was used to transfect the mBMSCs with 100 nM siRNA using Lipofectamine 2000 (ThermoFisher Scientific) as described previously (Jafari et al., 2015).

Statistical analysis

All values are presented as the mean value ± SD (standard deviation) of 3 values of 3 independent experiments. Unpaired Student's *T*-test (2-tailed) was used, assuming equal variation in the two groups. Differences were considered statistically significant at $^{*}P < 0.05$, and $^{**}P < 0.005$.

Results

AMPK activation in primary mBMSCs by A-769662

We studied the cytotoxic effect of A-769662 on mice (m) BMSCs. We measured the cell viability of the primary culture of mBMSCs in the presence of different A-769662 doses using the MTT assay and CellTiter-Blue. A-769662 exerted a toxic effect on cell viability at concentrations above 10 µM (Fig. 1A, 1B). Therefore, A-769662 was used at concentrations between 0.1–10 µM throughout this study. Further, we examined AMPK signaling activation by A-769662 in mBMSCs over a 6-day culture period. Treatment of mBMSCs with A-769662 significantly activated AMPK signaling, as revealed by the activation of the phosphorylation of AMPKα and its substrate p-acetyl CoA carboxylase (ACC) via western blot assay (Fig. 1C). To examine, whether A-769662 is targeting any other known osteogenic signaling pathways, we studied the effect of A-769662 on the activation of ERK, p-38, AKT and Smad 1/5/8 proteins. Interestingly, treatment of mBMSCs with A-769662 did not show any significant effect on the phosphorylation of the above proteins during osteogenesis as compared to non-treated cells (Suppl. Fig. 1A). In addition, A-769662 did not promote the stimulatory effect of any known signaling molecules (including TGFβ1, BMP2, Wnt3a, bFGF, and PDGF) on osteogenesis in mBMSCs (Suppl. Fig. 1B). This finding confirmed the specificity of A-769662 in targeting AMPK signaling during osteogenesis.

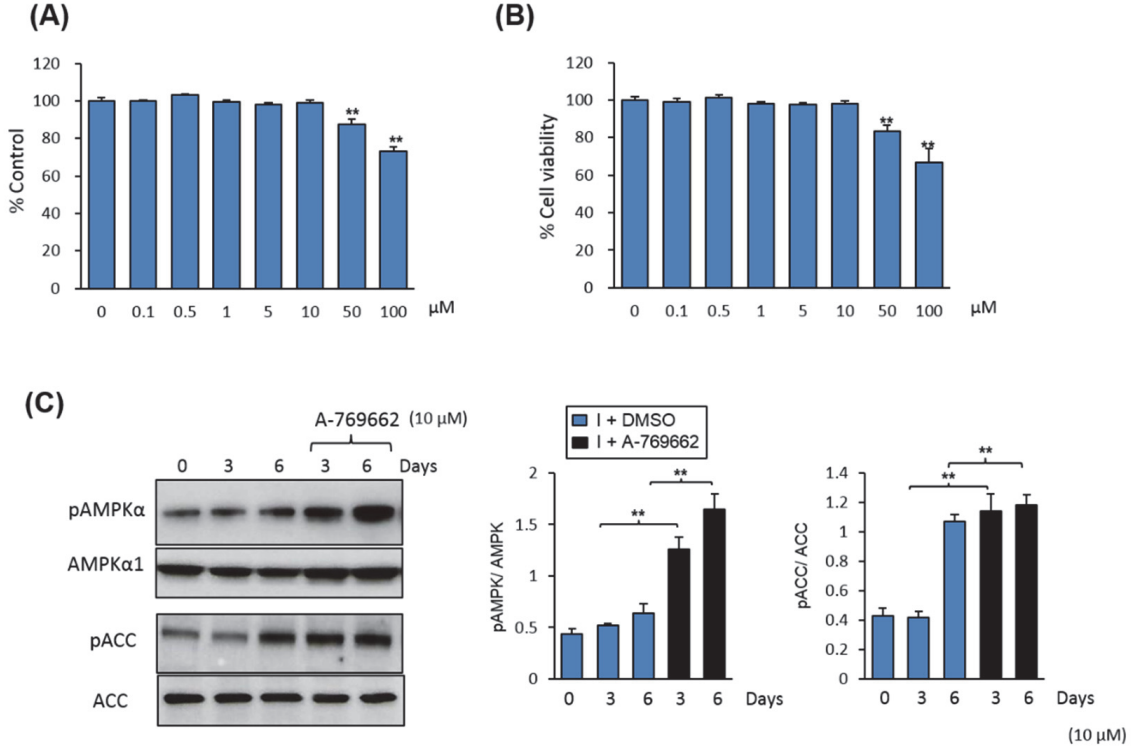

Fig. 1. Activation of AMPK in mBMSCs by A-769662. (**A**) Cytotoxicity of A-769662 on mBMSCs as measured by MTT assay and CellTiter-Blue® assay (**B**). Cells were treated either with DMSO (0) as control or different concentrations of A-769662 for 3 days in culture. (**C**) Western blot analysis for activation of AMPK by A-769662 (10 µM) in mBMSCs. Values for each phospho protein normalized to corresponding total protein and are presented in the graphs. Values are mean ± SD of triplicates; ** $p < 0.005$ compared to control cells with DMSO.

A-769662 promotes the differentiation of mBMSCs into osteoblasts

The effect of A-769662 was examined on osteoblast differentiation of mBMSCs. ALP activity quantification revealed the stimulatory effect of A-769662 on the osteoblast differentiation of mBMSCs in a dose-dependent manner (Fig. 2A). In addition, quantification of matrix mineralization by Alizarin Red staining after 12 days of induction was significantly and dose dependently increased by A-769662 treatment (Fig. 2B). At the molecular level, treatment of mBMSCs with A-769662 for 12 days during osteogenesis, significantly upregulated the mRNA expression of the osteoblast master transcription regulator, *Runx2*, and osteoblast-related genes, including *Alp, Col1a1*, *Ocn* and *Opn* by (≥1.5-fold; Fig. 2C).

A-769662 promotes early commitment of mBMSCs into osteoblasts

To understand the mechanism of A-769662-induced osteogenesis, we treated mBMSCs with A-769662 at different osteoblast differentiation stages: early commitment (days 0–3), extracellular matrix development (days 3–9) and matrix mineralization (days 9–12). ALP activity and Alizarin red quantification were performed on day 12. As shown in Fig. 2D, treatment of mBMSCs with A-769662 at both early (days 0–12) and middle stages (days 3–12) increased ALP activity by 98% and 63% respectively, while treatment during the late middle stage and late stage (days 6–12 and days 9–12) did not alter ALP activity. This effect was further supported by quantifying matrix mineralization during the same treatment strategies after 12 days of induction (Fig. 2D, E). We also evaluated the effect of AMPK inhibition on osteogenesis using Compound C (Comp. C), a potent AMPK inhibitor. Treatment of mBMSCs with 10 μM Comp. C significantly blocked AMPKα phosphorylation and its substrate (ACC), as shown in Fig. 3A. Comp. C supressed the mBMSCs differentiation into osteoblasts, as denoted by a significant decrease in ALP activity and Alizarin red quantification by 62% and 57%, respectively, compared to control cells treated with DMSO (Fig. 3B, C). Furthermore, siRNA-mediated silencing of *AMPKα1 (siAMPKα1)* suppressed the *AMPKα1* mRNA expression in mBMSCs by 82% (Fig. 3D). Silencing of *AMPKα1* showed to inhibit the mBMSCs differentiation into osteoblasts as measured by reduced ALP activity by 38% compared to siRNA control cells (Fig. 3E).

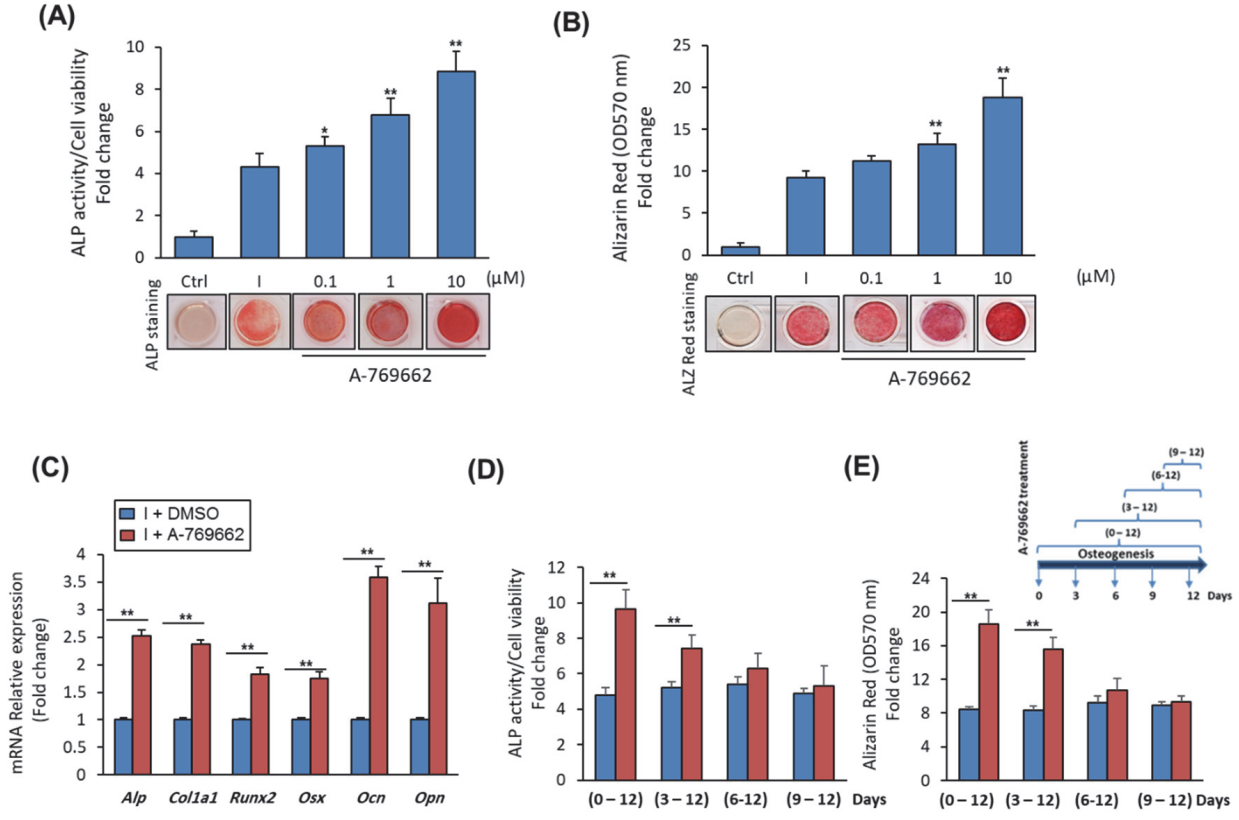

Fig. 2. A-769662 stimulates mBMSCs differentiation into osteoblasts. (**A**) Stimulatory effect of A-769662 on mBMSCs differentiation into osteoblast as assessed by quantification of ALP activity and (**B**) matrix mineralization stained with Alizarin red after osteogenic induction for 7 and 12 days respectively. mBMSCs were induced with osteogenic medium without (I+DMSO) or with different concentrations of A-769662. Images for staining were shown. (**C**) Osteogenic mRNA expression analysis by qPCR in mBMSCs treated without or with A-769662 (10 μM) during osteoblast induction for 12 days. Expression values were normalization to reference genes and presented as fold change. (**D**) Effect of A-769662 on mBMSCs at different stages during osteogenesis, measured by ALP activity and (**E**) Alizarin Red staining. Cells were induced to differentiate into osteoblasts and then treated without or with A-769662 (10 μM) at different time points (day 0, 3, 6, and 9) as shown by the schematic timeline. Values are mean ± SD of triplicates; * $p < 0.05$, ** $p < 0.005$ compared to induced cells without A-769662.

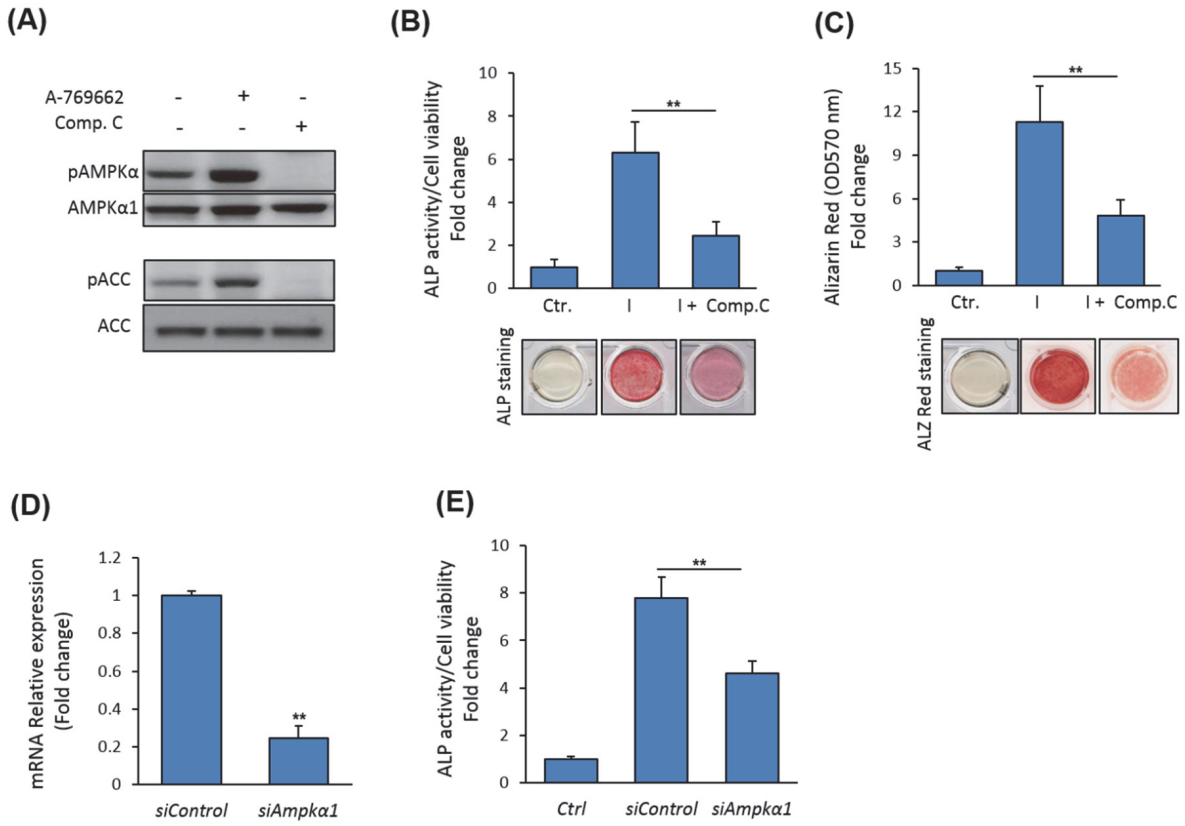

Fig. 3. A-769662 promotes early commitment of mBMSCs in osteoblast lineage. (**A**) Analysis of the inactivation of AMPKα and ACC phosphorylation in mBMSCs with Compound C (Comp. C) (10 μM) by Western blot. (**B**) Comp. C suppressed the osteoblast differentiation of mBMSCs as shown by reduced ALP activity and (**C**) Alizarin red staining. Cells were analyzed after 7 days and 12 days of induction respectively. (**D**) mRNA expression analysis of *Ampkα1* in mBMSCs transfected without (siControl) or with *Ampkα1* siRNA *(siAmpkα1)*. The qPCR was performed 6 days post transfection. (**E**) Effect of *Ampkα1* siRNA on mBMSCs differentiation measured by quantitative ALP activity after 7 days of induction. Cells were reverse transfected with siRNA, and after 24 h, the cells were induced to differentiate; ** $p < 0.005$ compared to I+DMSO. Values are mean ± SD of triplicates.

A-769662 inhibits the mBMSCs differentiation into adipocytes

A-769662 dose dependently inhibited the differentiation of mBMSCs into adipocyte, as quantified by Oil red O staining for lipid accumulation (Fig. 4A). qPCR analysis consistently demonstrated the inhibitory effect of A-769662 on mRNA expression of adipocyte-specific markers for both early (*Pparγ2* and *C/ebpα*) and late (*aP2* and *Lpl*) genes in mBMSCs (Fig. 4B). On the other hand, AMPK inhibition by Comp. C significantly stimulated adipogenesis, as assessed by increased lipid accumulation and expression of adipogenic mRNA markers after 12 days of induction (Fig. 4C, D). Similarly, transfection of mBMSCs with *siAMPKα1* showed to stimulate their adipogenesis significantly as revealed by increased Oil red O staining for fat droplets by 42% compared to siRNA control cells (Fig. 4E).

AMPK activation was essential for A-769662-induced osteogenesis in mBMSCs

To investigate the involvement of AMPK in mediating A-769662-induced osteogenesis, we measured the effect of A-769662 on osteogenesis and adipogenesis of mBMSCs pre-treated with Comp. C. A-769662-induced osteogenesis was eliminated by Comp. C in mBMSCs, as shown by decreased levels of ALP activity after 7 days of osteogenic induction (Fig. 5A).

Similarly, A-769662-inhibited adipogenesis was rescued by Comp. C treatment, as denoted by Oil red O staining after 12 days of induction (Fig. 5B). Thus, AMPK activation is required to mediate the regulatory effect of A-769662 on the differentiation of mBMSCs.

The activation of AMPK was reported to regulate the differentiation of BMSCs into osteoblast versus adipocyte by upregulating *Runx2* and suppressing *Pparγ2* expression, the two main transcription factors for osteogenesis and adipogenesis respectively (Kim et al., 2012). Thus, we studied the regulation of *Runx2* and *Pparγ2* expression as downstream targets of A-769662-induced osteogenesis. As shown in Fig. 5C, the stimulatory effect of A-769662 on *Runx2* expression during osteogenesis was significantly reduced by Comp. C. On the other hand, the inhibitory effect of A-769662 on *Pparγ2* mRNA expression during adipogenesis of mBMSCs was rescued by Comp. C (Fig. 5D). Thus, A-769662 regulates the differentiation of mBMSCs into osteoblast or adipocyte by controlling the expression of downstream transcription factors, *Runx2* and *Pparγ2*.

A-769662 stimulates the hBMSCs differentiation into osteoblast versus adipocyte

Similar to its effect on mBMSCs, A-769662 induced an osteoblast lineage commitment for hBMSCs, as denoted by a sig-

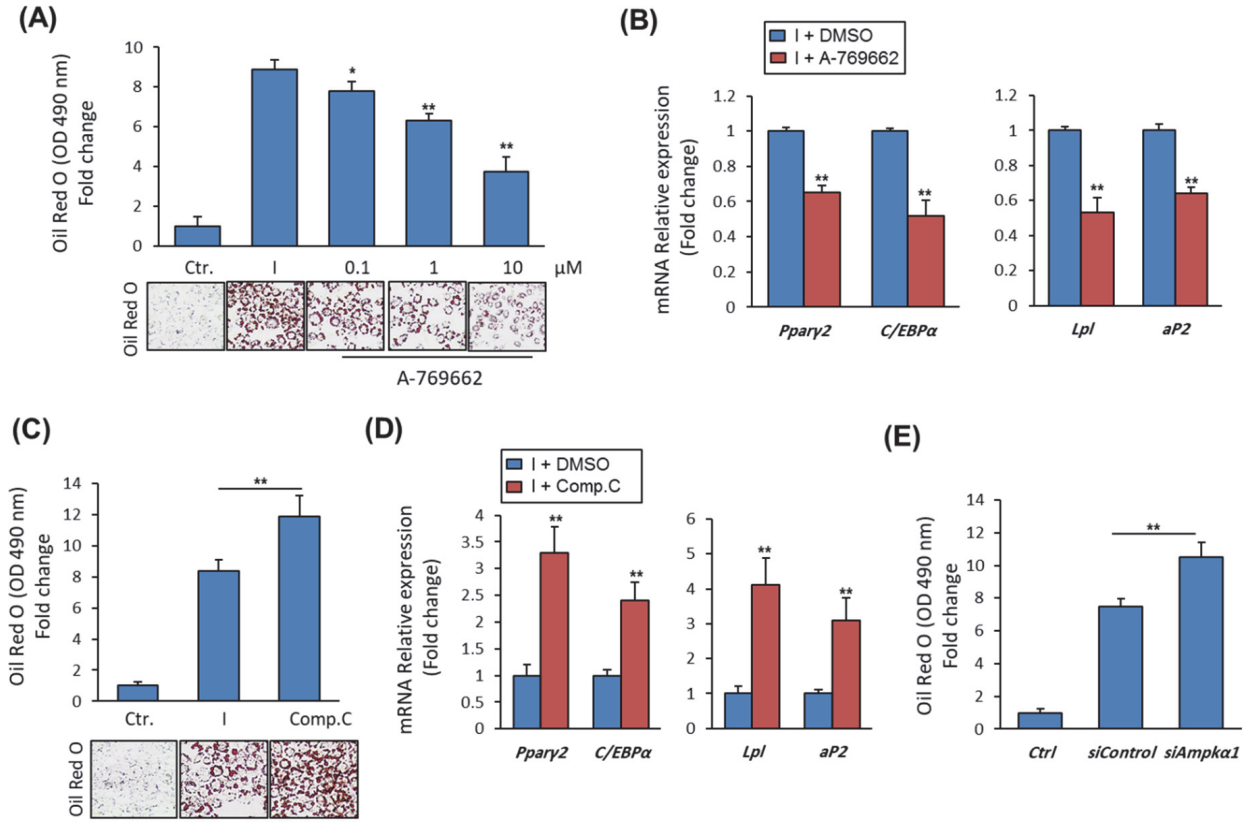

Fig. 4. A-769662 suppresses the differentiation of mBMSCs into adipocytes. (**A**) The inhibitory effect of A-769662 on adipocyte differentiation of mBMSCs as shown by quantitative Oil red O staining. Cells were induced to differentiate and then treated without (I+DMSO) as control or with different concentrations of A-769662 (10 μM) for 12 days. Staining images with Oil red O were shown. (**B**) mRNA expression of adipogenic markers by qPCR analysis at day 12 of mBMSCs differentiation with or without A-769662 (10 μM). (**C**) Stimulatory effect of Compound C on adipocyte differentiation as assessed by Oil red O staining and (**D**) upregulation of mRNA expression of adipogenic markers. (**E**) Stimulatory effect of *Ampkα1* siRNA on adipogenesis of mBMSCs as quantified by Oil red O staining. Values are mean ± SD of triplicates; * $p < 0.05$, ** $p < 0.005$ compared to control cells.

nificant dose-dependent increase in ALP activity after 7 days of osteogenic induction (Fig. 6A). In addition, A-769662 upregulated the expression of *Runx2* in mBMSCs in a dose dependent manner (Fig. 6B). On the other hand, A-769662 dose dependently suppressed hBMSC differentiation into adipocytes in association with the downregulation of *Pparγ2* gene expression, as assessed by quantification of Oil Red O staining (Fig. 6C) and qPCR analysis (Fig. 6D) respectively.

Discussion

In this study, we demonstrated the dual role of an AMPK agonist, A-769662, in stimulating the early commitment of mBMSC differentiation into osteoblasts versus adipocytes. A-769662 exerted these effects via an AMPK-dependent mechanism and regulating the gene expression of *Runx2* and *Pparγ2*.

Several reports demonstrated the direct and specific effect of A-769662 on the activation of AMPK (Day et al., 2017; Kim et al., 2016). A-769662 was found to allosterically activate αβγ complex of AMPK and stabilizing the conformation of AMPK that resulted in the inhibition of dephosphorylation of Thr-172 in the AMPKα subunit (Sanders et al., 2007). In another mechanism, Ser108 phosphorylation in the AMPKβ1 subunit was shown to be required for the activation of AMPK by A-769662 (Scott et al., 2014). In addition, A-769662 was reported to activate AMPK without affecting the upstream kinases (Goransson et al., 2007).

Our data showed the stimulatory effect of A-769662 on the early commitment of mBMSCs differentiation into osteoblasts by upregulating the *Runx2* transcription factor and its downstream target genes that encode matrix proteins. Several *in vitro* and *in vivo* studies reported the stimulatory effect of AMPK activation on osteogenic differentiation (Kanazawa et al., 2009; Molinuevo et al., 2010; Shah et al., 2010). Consistent with our findings, activation of AMPK showed to promote osteogenesis by regulating *Runx2* mRNA expression (Kim et al., 2012) and phosphorylating RUNX2 (Chava et al., 2018). In addition, the AMPK activator, 5-aminoimidazole-4-carboxamide1-beta-d-ribonucleoside (AICAR) promoted osteoblast differentiation of osteoblastic MC3T3-E1 cells via inhibition of Rho-associated protein kinase (ROCK) (Kanazawa et al., 2009). AICAR and metformin, AMPK activators dose dependently increased the formation of trabecular bone nodule in rat calvaria-derived osteoblasts (Shah et al., 2010). Furthermore, *in vivo* metformin administration increased bone formation and suppressed the anti-osteogenic effect of rosiglitazone in rats (Molinuevo et al., 2010; Sedlinsky et al., 2011). In contrast, suppression of AMPK using either Comp. C or short

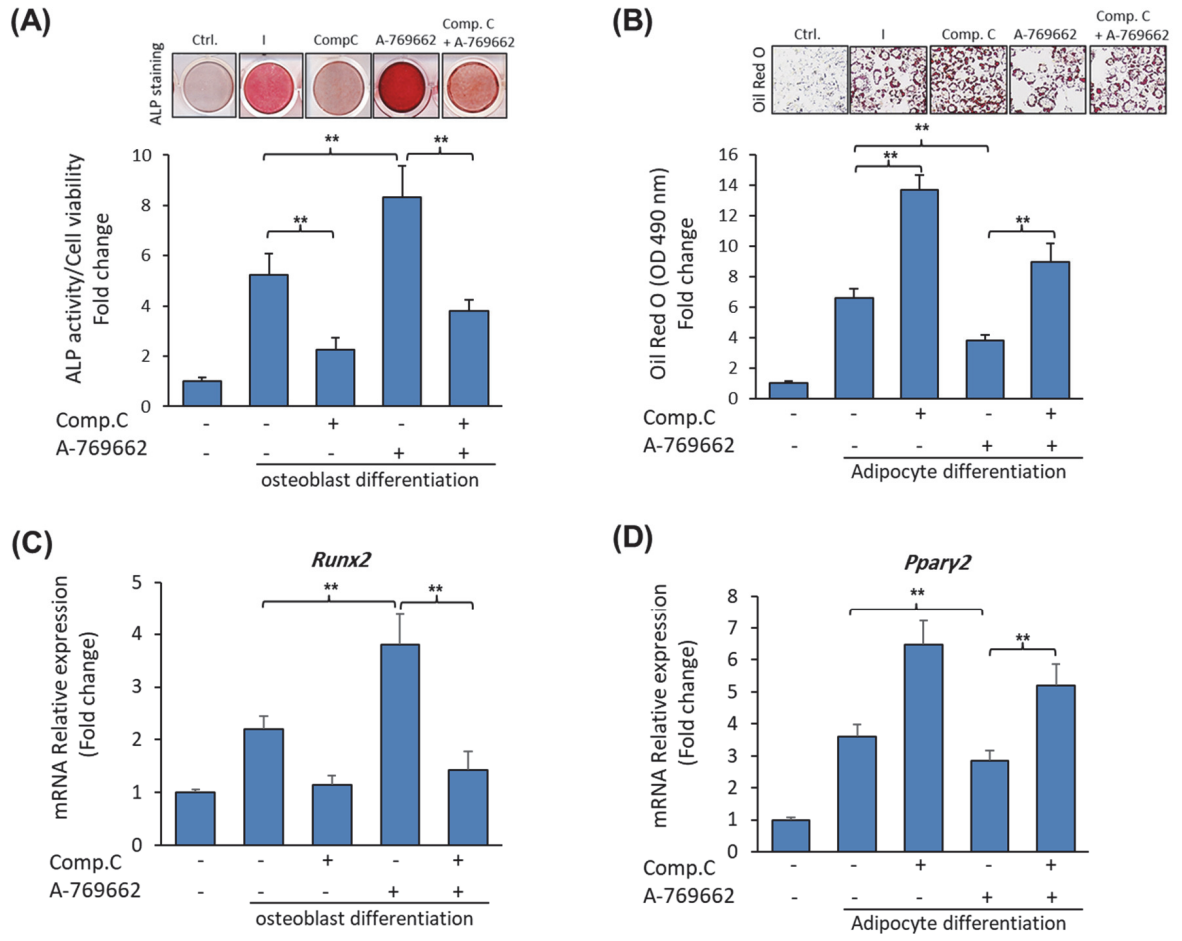

Fig. 5. The regulatory effect of A-769662 mBMSCs differentiation is mediated via AMPK-dependent mechanism. (**A**) The stimulatory effect of A-769662 on osteogenesis eliminated by Comp. C. ALP activity was determined in cells induced to osteoblastic lineage in the presence of Comp. C (10 μM) without or with A-769662 for 7 days. ALP staining images are presented. (**B**) The inhibitory effect of A-769662 on adipogenesis is rescued by Comp. C. Oil red O staining quantification performed in induced mBMSCs to adipocytes in the presence of Comp. C without or with A-769662 for 12 days. (**C**) qPCR analysis of *Runx2* gene expression in mBMSCs under the same experimental condition of panel A. (**D**) qPCR analysis of *Pparγ2* in mBMSCs under the same experimental condition of panel B; ** $p < 0.005$ compared to I+DMSO. Each experimental value is mean ± SD of independent triplicates.

hairpin RNA (shRNA)-mediated AMPK knockdown showed to inhibit osteogenesis by reducing their matrix mineralization and down-regulating the expression of *Runx2* expression (Kim et al., 2012).

We demonstrated the inhibitory effect of A-769662 on the adipocyte differentiation of mBMSCs. A-769662 was consistently shown to inhibit adipocyte differentiation of 3T3-L1 (Zhou et al., 2009) and C3H10T1/2 cell lines (Chen et al., 2017). Additionally, A-769662 displayed anti-adipogenic effects *in vivo* by protecting mice against high fat diet-induced obesity (Wu et al., 2018) and reducing levels of both plasma and liver triglyceride in ob/ob mice (Cool et al., 2006).

Our finding that the effect of A-769662 on regulating BMSCs differentiation is mediated through an AMPK-dependent mechanism is consistent with the reported mode of action of A-769662 in controlling some physiological processes. For example, the inhibitory effect of A-769662 on adipogenesis in 3T3-L1 cells (Zhou et al., 2009) was associated with protection against H_2O_2-induced apoptosis (Zhu et al., 2014).

Several clinical studies have demonstrated the association of increased bone marrow fat mass with reduced bone mass in osteoporotic patients (Hardouin et al., 2016; Rharass and Lucas, 2018; Shen et al., 2012). This phenomenon is elucidated by the existence of an inverse relationship between osteoblast and adipocyte differentiation of BMSCs (Gimble et al., 2006; Li et al., 2018; Taipaleenmaki et al., 2011). In this context, several signalling pathways were found to be involved in enhancing the commitment of BMSCs into osteoblasts on the expenses of adipocytes. These include, canonical and non-canonical Wnt signalling, Sonic hedgehog and TGF-β1 (Bennett et al., 2005; Zhao and Hantash, 2011).

A-769662 has a privilege as a direct and specific activator of AMPK, while other activators, like AICAR and metformin, that are used as drugs for cardiac ischemic injury (Corton et al., 1995) and diabetes (Towler and Hardie, 2007) were shown to function indirectly. For example, AICAR activator is a pro-drug that must be converted into the AMP analogue ZMP (Corton et al., 1995), and metformin showed to activate AMPK by increasing cellular AMP via inhibiting the mitochondrial respiratory chain complex I (Hardie, 2007). Thus, a drug that activates the AMPK directly and specifically like A-769662 would be more efficient in the treatment of metabolic diseases. However, more *in vivo* studies are needed to provide preclinical data on the therapeutic effect of A-769662 in disease animal models.

In this study, our data identified A-769662, an AMPK activator, as a novel compound that can plausibly be used as a the-

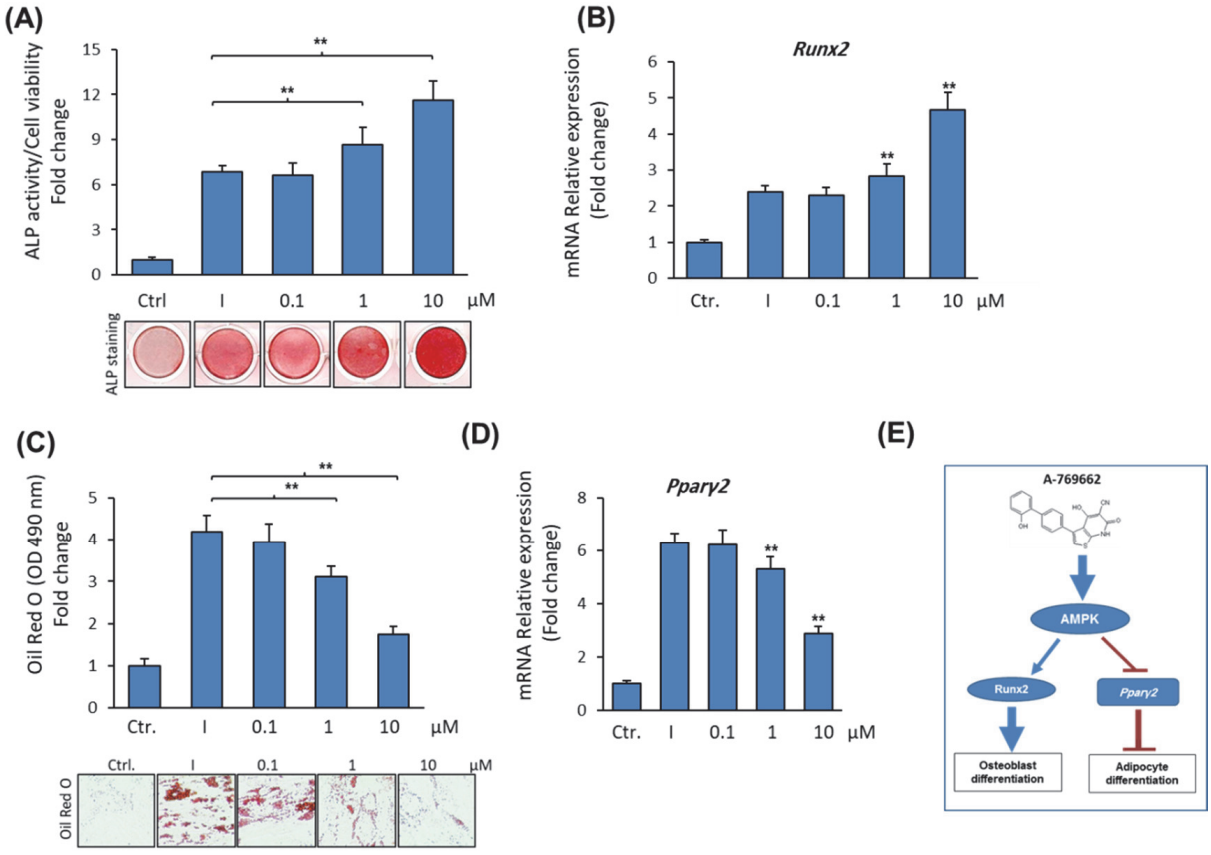

Fig. 6. A-769662 stimulates the differentiation of hBMSCs toward osteoblast versus adipocyte lineages. (**A**) A-769662 promotes hBMSCs differentiation into osteoblast in dose-dependent manner as measured by ALP activity quantification after 7 days of osteogenic induction. (**B**) qPCR analysis of *Runx2* in mBMSCs treated with different concentrations of A-769662 after 7 days of osteogenic induction. (**C**) A-769662 suppresses adipogenesis of hBMSCs in dose-dependent manner as measured by reduced Oil red O quantification after 12 days of adipogenic induction. Staining images were presented. (**D**) qPCR analysis of *Pparγ2* gene expression in mBMSCs induced to adipocyte differentiation in the presence of different concentrations of A-769662. (**E**) Schematic diagram for the mode of action on A-769662 in promoting osteogenesis in BMSCs via activating AMPK signaling and targeting the upregulation of *Runx2* and inhibition of *Pparγ2*; ** $p < 0.005$ compared to I+DMSO. Value of each experiment is mean ± SD of independent triplicates.

rapeutic drug for promoting bone formation via acting directly on stimulating the BMSCs differentiation into osteoblast. Further pre-clinical studies are required to provide *in vivo* date on the potential therapeutic effect of A-769662 on bone in a bone loss murine model.

Conclusions

In this report, we have demonstrated the effect of the AMPK activator A-769662 in promoting the differentiation fate of murine and human BMSCs into osteoblastic versus adipocytic cell lineage. The regulatory effect of A-769662 on BMSCs differentiation is mediated via an AMPK-dependent mechanism and by targeting the upregulation of *Runx2* and downregulation of *Pparγ2* gene expression (Fig. 6E). Thus, our data identified A-769662 as a possible osteo-anabolic drug that can enhance bone formation in osteoporotic patients via directing the differentiation of BMSCs into osteoblasts versus adipocytes.

Availability of data and materials
All materials are available via the corresponding author.

Conflict of interests
The authors declare that they have no competing interests.

Funding
This work was funded by the Deanship of Scientific Research at King Faisal University, Saudi Arabia (Grant No. 180076).

Authors' contributions
BMA conceived and designed the project, and drafted the manuscript. BMA and AMZ performed experiments, data analysis, and revisions of the article, gave final approval of the version, and agreed to all aspects of the work.

Acknowledgements
The Authors acknowledge the Deanship of Scientific Research at King Faisal University for the financial support under the annual research project (Grant No. 180076).

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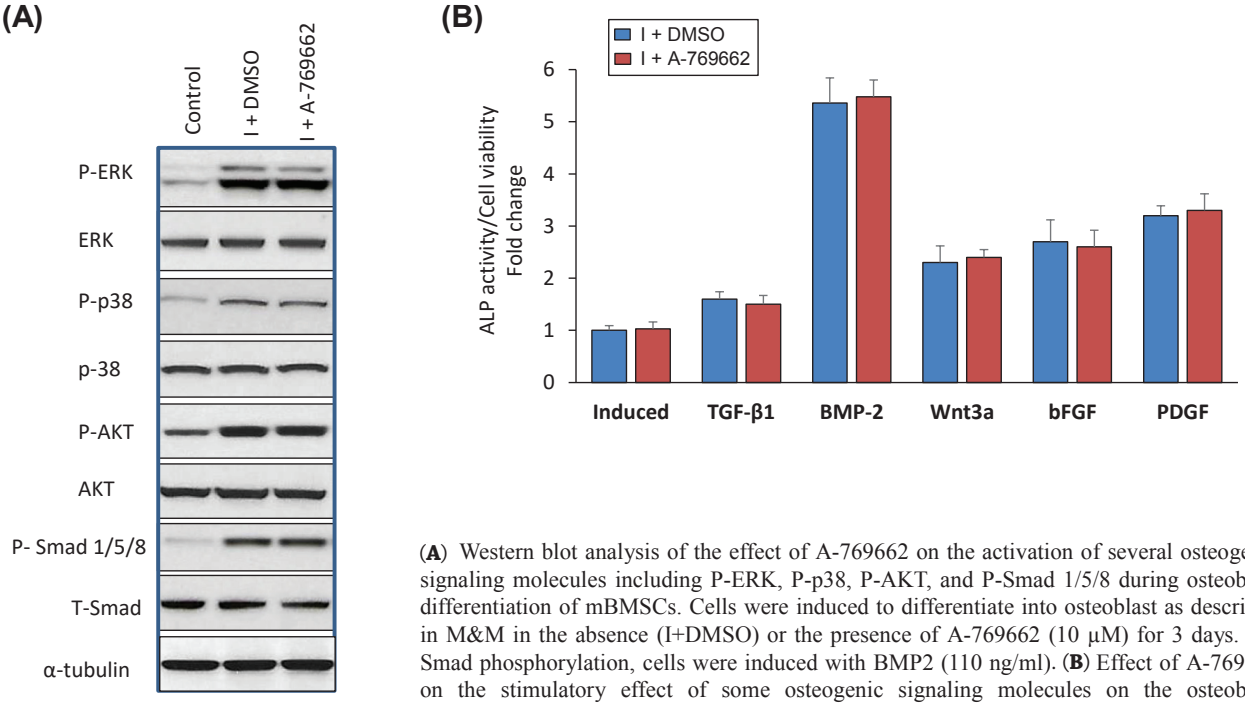
Supplementary materials

Suppl. Table 1. List of primers used for qPCR

Gene name	Forward primer 5'-3'	Reverse primer 5'-3'
β-Actin	GAT ATC GCT GCG CTG GTC GTC	ACG CAG CTC ATT GTA GAA GGT GTG G
Runx2	AGC AAC AGC AAC AAC AGC AG	GTA ATC TGA CTC TGT CCT TG
Ocn	CAG ACA AGT CCC ACA CAG CA	CTT TAT TTT GGA GCT GCT GT
Alp	GCC CTC TCC AAG ACA TAT A	CCA TGA TCA CGT CGA TAT CC
Osx	TATGCTCCGACCTCCTCAAC	AATAGGATTGGGCAGAAAG
Col1a1	GGT GAA CAG GGT GTT CCT GG	TTC GCA CCA GGT TGG CCA TC
Opn	GAA ACT CTT CCA AGC AAT TC	GGA CTA GCT TGT CCT TGT GG
Hprt	TCAGTCAACGGGGGACATAAA	GGGGCTGTACTGCTTAACCAG
Pparγ2	GGG TCA GCT CTT GTG AAT GG	CTG ATG CAC TGC CTA TGA GC
C/ebpα	AAG CCA AGA AGT CGG TGG A	CAG TCC ACG GCT CAG CTG TTC
aP2	CAA AAT GTG TGA TGC CTT TGT G	CTC TTC CTT TGG CTC ATG CC
Lpl	CTGCTGGCGTAGCAGGAAGT	GCTGGAAAGTGCCTCCATTG



(**A**) Western blot analysis of the effect of A-769662 on the activation of several osteogenic signaling molecules including P-ERK, P-p38, P-AKT, and P-Smad 1/5/8 during osteoblast differentiation of mBMSCs. Cells were induced to differentiate into osteoblast as described in M&M in the absence (I+DMSO) or the presence of A-769662 (10 μM) for 3 days. For Smad phosphorylation, cells were induced with BMP2 (110 ng/ml). (**B**) Effect of A-769662 on the stimulatory effect of some osteogenic signaling molecules on the osteoblast differentiation of mBMSCs. Cells were induced to osteoblast differentiation without or with A-769662 (10 μM) in the presence of TGFβ1 (10 ng/ml), BMP2 (100 ng/ml), Wnt3a (10 ng/ml), PDGF (100 ng/ml) bFGF (100 ng/ml) for 7 days. Values are mean ± SD of three independent experiments.

Suppl. Fig. 1. Effect of A-769662 on osteogenic signaling pathways