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Adiponectin Promotes Human Jaw Bone Marrow Stem Cell Osteogenesis

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Abstract

Human jaw bone marrow mesenchymal stem cells (h-JBMMSCs) are multipotent progenitor cells with osteogenic differentiation potential. The relationship between adiponectin (APN) and the metabolism of h-JBMMSCs has not been fully elucidated, and the underlying mechanism remains unclear. The aim of the study was to investigate the effect and mechanism of APN on h-JBMMSC metabolism. h-JBMMSCs were obtained from the primary culture of human jaw bones and treated with or without APN (1 μg/mL). Osteogenesis-related gene expression was evaluated by real-time polymerase chain reaction (PCR), alkaline phosphatase (ALP) activity assay, and enzyme-linked immunosorbent assay (ELISA). To further investigate the signaling pathway, mechanistic studies were performed using Western blotting, immunofluorescence, lentiviral transduction, and SB202190 (a specific p38 inhibitor). Alizarin Red staining showed that APN promoted h-JBMMSC osteogenesis. Real-time PCR, ALP assay, and ELISA showed that ALP, osteocalcin (OCN), osteopontin, and integrin-binding sialoprotein were up-regulated in APN-treated cells compared to untreated controls. Immunofluorescence revealed that adaptor protein containing a pleckstrin homology domain, phosphotyrosine domain, and leucine zipper motif (APPL1) translocated from the nucleus to the cytoplasm with APN treatment. Additionally, the phosphorylation of p38 mitogen-activated protein kinase (MAPK) increased over time with APN treatment. Moreover, knockdown of APPL1 or p38 MAPK inhibition blocked the expression of APN-induced calcification-related genes including ALP, Runt-related transcription factor 2 (RUNX2), and OCN. Furthermore, Alizarin Red staining of calcium nodes was not increased by the knockdown of APPL1 or p38 inhibition. Our data suggest that this regulation is mediated through the APPL1–p38 MAPK signaling pathway. These findings collectively provide evidence that APN induces the osteogenesis of h-JBMMSCs through APPL1-mediated p38 MAPK activation.

Keywords: APPL1, p38 mitogen-activated protein kinases, osteocalcin, osteopontin, RUNX2, IBSP

Introduction

Adiponectin (APN) is a 30-kDa secretory protein that was first identified in 1995 (Scherer et al. 1995). APN has been studied extensively in obesity and insulin resistance, as serum levels are often inversely proportional to body fat in both sexes and the onset of insulin resistance (Ryo et al. 2004). Decreased APN levels are involved in the progression of insulin resistance (Yamauchi et al. 2001).

Recently, the relationship between APN and osteogenesis has received significant attention. APN knockout mice exhibit a significant retardation of bone growth, reduced trabecular bone volume, decreased cortical bone, and increased osteoclast numbers in bone explants as determined by histological analysis, micro–computed tomography, and tartrate-resistant acid phosphatase staining (Tu et al. 2011). In vivo, mice treated with an APN-expressing adenovirus for 2 wk showed an increase and decrease in trabecular bone mass and the number of osteoclasts and the level of plasma NTx (a bone resorption marker), respectively, when compared with control mice (Oshima et al. 2005). Moreover, sustained APN release induced osteogenesis in rabbits and suppressed osteoclastic activity both in vitro and in vivo (Luo et al. 2012). Other studies have reported that APN is sufficient to stimulate the osteoblastic differentiation of mesenchymal progenitor cells (Lee et al. 2009), suppress sympathetic tone, and promote trabecular bone mass in mice (Wu et al. 2014) and that it promotes bone formation in rabbits with mandibular osteodistraction (Jiang et al. 2011).

In this study, human jaw bone marrow mesenchymal stem cells (h-JBMMSCs) were used to investigate the osteogenic pathway. Compared to iliac crest mandibular bone marrow mesenchymal stem cells, h-JBMMSCs exhibit a greater ability

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to proliferate and show higher levels of alkaline phosphatase (ALP) and calcium deposition when cultured in vitro (Akintoye et al. 2006). In addition, h-JBMMSCs were previously shown to produce higher bone volume than appendicular bone marrow mesenchymal stem cells in vivo (Akintoye et al. 2006; Yamaza et al. 2011). Furthermore, h-JBMMSCs are readily accessible because they can be easily aspirated from alveolar bone with minimal pain.

To date, the relationship between APN and h-JBMMSCs has not been fully elucidated. Previous reports showed that APN exerts its effects through 2 membrane receptors: AdipoR1 and AdipoR2. AdipoR1 is expressed mostly in skeletal muscle, whereas AdipoR2 is most abundant in the liver (Yamauchi et al. 2003). The adaptor protein containing a pleckstrin homology domain, phosphotyrosine domain, and leucine zipper motif (APPL1) could directly interact with the intracellular region of the APN receptors and then mediate APN signaling and its effects on metabolism (Mao et al. 2006). The p38 mitogen-activated protein kinase (MAPK) pathway plays an important role in the regulation of fatty acid metabolism (Fang et al. 2010; Park et al. 2013). However, it is unknown whether these key metabolic signaling pathways are involved in the regulation of h-JBMMSC osteogenesis by APN.

Many studies have emphasized a key role of APN in tissue regeneration and have shown that APN deficiency greatly inhibits the mechanisms underlying tissue renewal (Fiaschi et al. 2014). Previous reports showed that bone growth was retarded in APN knockout mice (Tu et al. 2011). In addition, APN was shown to promote pancreatic islet regeneration (Ye et al. 2015). In a mouse bone defect model, APN was found to induce the migration of bone marrow–derived mesenchymal stem cells (BMSCs) to the defect area and promote tissue regeneration (Yu et al. 2015). We previously reported that APN could be used as a novel anabolic agent for tissue-engineered bone in extraction sockets of beagle dogs, but the mechanism of action remained unclear (Hu et al. 2015). In this study, we confirmed that APN induced ossification and demonstrated its ability to induce osteogenesis via activation of the APPL1–p38 MAPK pathway.

Materials and Methods

Cells and Cell Culture

The study protocol was approved by the Medical Ethical Commission of the Peking University School and Hospital of Stomatology (PKUSSIRB-201520026). All patients provided informed consent. The resected bone mass from patients undergoing orthognathic surgery was cut into small fragments (<1 mm^3) and cultured in T25 flasks (Corning, Corning, NY, USA) with α-MEM (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) (Gibco) in a 37 °C humidified incubator with a 5% CO_2 atmosphere. Passage 3 (P3) or P4 h-JBMMSCs were used for all analyses following phenotypic confirmation by flow cytometry analysis as previously described. h-JBMMSCs were positive for CD73, CD90, and CD105 but negative for CD34, CD11b, CD19, CD45, and HLA-DR (Hu et al. 2015) (data not shown). Osteoblast differentiation was induced using conditional medium consisting of α-MEM, 10% FBS, 10 nM dexamethasone, 10 mM β-glycerophosphate, and 50 μg/mL L-ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA). The cells were cultured with or without 1 μg/mL APN (Z03072; Genscript, Piscataway, NJ, USA) for 7 d to examine ossification-related gene expression or 21 d for Alizarin Red staining. For the mechanistic analyses, cells were starved for 12 h in α-MEM without FBS and then treated with or without 1 μg/mL APN. Cells were treated with SB202190 (DMSO vehicle; Selleck, Houston, TX, USA) for 2 h before APN treatment as noted.

Osteoblast Differentiation

h-JBMMSCs were cultured in osteoblast-inducing conditional medium in 24- or 6-well plates with or without APN, and the supernatants were collected for enzyme-linked immunosorbent assays (ELISAs). Total protein was extracted using RIPA buffer (Applygen, Beijing, China), and the concentration was determined using bicinchoninic acid reagent (Thermo Fisher Scientific, Waltham, MA, USA). Cells were cultured for 1 wk and treated with APN 3 times (once every 3 d) during the culturing process. Real-time polymerase chain reaction (PCR) was used to examine target gene expression as previously described (Hu et al. 2015). ALP activity was measured using an ALP assay kit (A059-2; Nangjing Jianchen Bioengineering Institute, Nangjing, China) according to the manufacturer's instructions. ELISAs were used to assess osteocalcin (OCN) (Cat. DSTCN0; R&D Systems, Minneapolis, MN, USA), osteopontin (OPN) (Cat. DOST00; R&D Systems), and integrin-binding sialoprotein (IBSP) (Cat. SEB092Hu; Cloud-Clone Corp., Houston, TX, USA) secretion levels according to the respective manufacturer's instructions. After 21 d, cells in the 24-well plates were fixed with 95% alcohol, washed in phosphate-buffered saline (PBS) 3 times for 10 min, treated with 1% Alizarin Red S (Sigma-Aldrich) at room temperature for 20 min, and then washed with PBS 3 times for 10 min to stain calcium nodes. The results were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Immunofluorescence and Western Blotting

For immunofluorescence studies, h-JBMMSCs were cultured on coverslips to 30% to 50% confluence before APN treatment. For Western blotting, h-JBMMSCs were cultured in 60-mm dishes, starved for 12 h after reaching 90% confluence, and then treated with APN at the indicated time points. The primary antibodies used were as follows: APPL1 (3858S; Cell Signaling Technology [CST], Danvers, MA, USA) at 1:2,000 for Western blotting or 1:100 for immunofluorescence; p-p38 (4511P; CST) at 1:1,000; p38 (8690P; CST) at 1:1,000; actin (g0314; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:1,000; and Runt-related transcription factor 2 (RUNX2) (8486S; CST) at 1:1,000. Anti-rabbit (7074S; CST) secondary antibody at 1:5,000 for Western blotting and anti-rabbit (4412S; CST) secondary antibody at 1:200 for immunofluorescence

were used as described previously (Chen et al. 2015). The nuclei were stained with DAPI. The coverslips were scanned using an Axiovert 650 confocal microscope (Zeiss). The laser excitation was 488 nm (green, APPL1) and 405 nm (blue, DAPI). The Western blots were analyzed using ImageJ software (National Institutes of Health). For immunofluorescence, 10 fields of approximately 10 cells were scored.

Lentiviral Transduction

Lentiviral transduction was used to knock down APPL1 in h-JBMMSCs. The target sequence (5′-3′) of lentivirally expressed siRNA was CAGTCAGAAGAGAGTGATT, whereas the control sequence was TTCTCCGAACGTGTC ACGT (Genechem, Shanghai, China). h-JBMMSCs were cultured in 1×10^6 TU/mL virus for 12 h, and then the medium was replaced with fresh medium. After culturing for 48 h, cultures with transduction efficiencies >90% were used in analyses.

Statistical Analysis

Data are shown as the mean ± standard deviation. Statistically significant differences ($P < 0.05$) among the groups were evaluated using a 1-way analysis of variance or the Student's *t* test. All statistical analyses were performed using SPSS 19.0 software (IBM Corp., Armonk, NY, USA).

Results

APN Promoted Osteogenesis of h-JBMMSCs In Vitro and Up-regulated Ossification-related Genes

Alizarin Red staining of h-JBMMSCs was used to confirm the biological effect of APN on osteogenesis. Results showed a significant increase in Alizarin Red staining in APN-treated h-JBMMSCs when compared to the control group (Fig. 1A).

After the culture of h-JBMMSCs in osteoblast-inducing differentiation medium for 1 wk, ossification-related gene expression was evaluated via real-time PCR, ALP activity assay, and ELISA. Real-time PCR results showed that the APN-treated cells expressed ALP (2.73-fold), IBSP (3.49-fold), OCN (1.58-fold), and OPN (2.73-fold) at significantly higher levels compared to control cells ($P < 0.05$) (Fig. 1B). Similarly, ALP activity was increased by 2.21-fold compared to untreated cells ($P < 0.01$) (Fig. 1C). ELISA also showed that the APN-treated cells expressed OCN (49 ng/mL/mg v. 18.5 ng/mL/mg, respectively), OPN (54.2 ng/mL/mg v. 21 ng/mL/mg, respectively), and IBSP (81.5 ng/mL/mg v. 29 ng/mL/mg, respectively) at higher levels compared to the control cells ($P < 0.01$) (Fig. 1D).

APN Altered APPL1 Localization and Enhanced p38 MAPK Phosphorylation in h-JBMMSCs

Western blot analysis revealed that p38 MAPK phosphorylation increased in response to APN treatment over time from

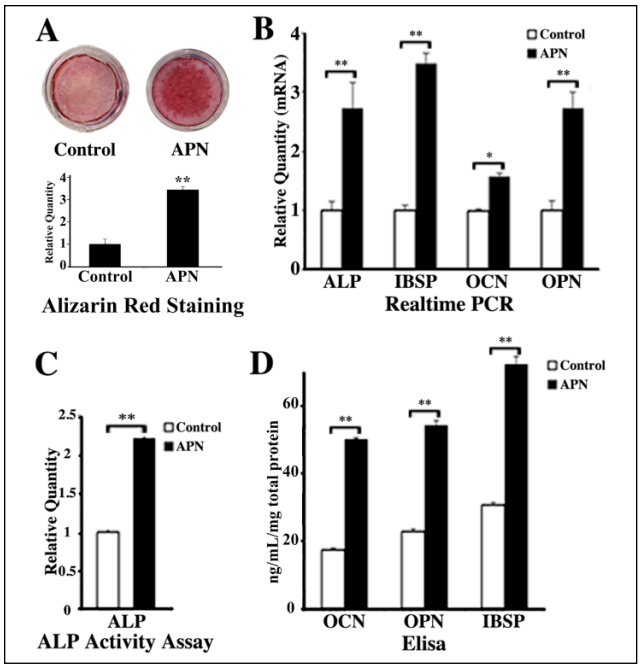

Figure 1. Adiponectin (APN) promoted the osteogenesis of human jaw bone marrow mesenchymal stem cells (h-JBMMSCs) in vitro and up-regulated ossification-related genes. h-JBMMSCs were cultured in osteoblast-inducing conditional medium and treated with or without APN at 1 μg/mL once every 3 d. (**A**) After 21 d of culture in 12-well plates, cells were examined by Alizarin Red staining (upper panel), and the data were quantified (lower panel). (**B**) After 7 d of culture in 6-well plates, RNA was extracted using Trizol reagent and used to examine the expression of the following osteogenesis-related genes: alkaline phosphatase (ALP), OCN, OPN, and IBSP. (**C**) After 7 d of culture in 24-well plates, total protein was extracted in RIPA buffer to assess the ALP activity. (**D**) After 7 d of culture in 24-well plates, cell supernatants were collected to examine the secretion of osteogenesis-related proteins. Protein concentrations were normalized to the relative quantity in each sample (*$P < 0.05$, **$P < 0.01$).

5 min to 60 min of APN treatment but had no significant effect on APPL1 (Fig. 2A). However, confocal microscopy showed that APPL1 translocated from the nucleus to the cytoplasm after 1 min of APN stimulation (Fig. 2B).

APPL1 Is Required for APN-induced p38 MAPK Phosphorylation and h-JBMMSC Osteogenesis

APPL1 was knocked down using lentiviral transduction to determine its effect on p38 phosphorylation and ossification-related gene expression in h-JBMMSCs. Western blotting revealed sparse APPL1 expression in the APPL1 knockdown group, whereas expression was normal in mock-transduced controls. Notably, APN-induced p38 phosphorylation was markedly increased in the mock group but remained the same in the APPL1 knockdown group. The expression of RUNX2, a key transcription factor associated with osteoblast differentiation, was increased in the APN-treated group but unchanged in the APPL1 knockdown group (Fig. 3A). Furthermore, in the ALP activity assay and ELISA, the activity of ALP and expression of OCN and OPN were 1.89-fold, 1.91-fold (37.1 ng/mL/

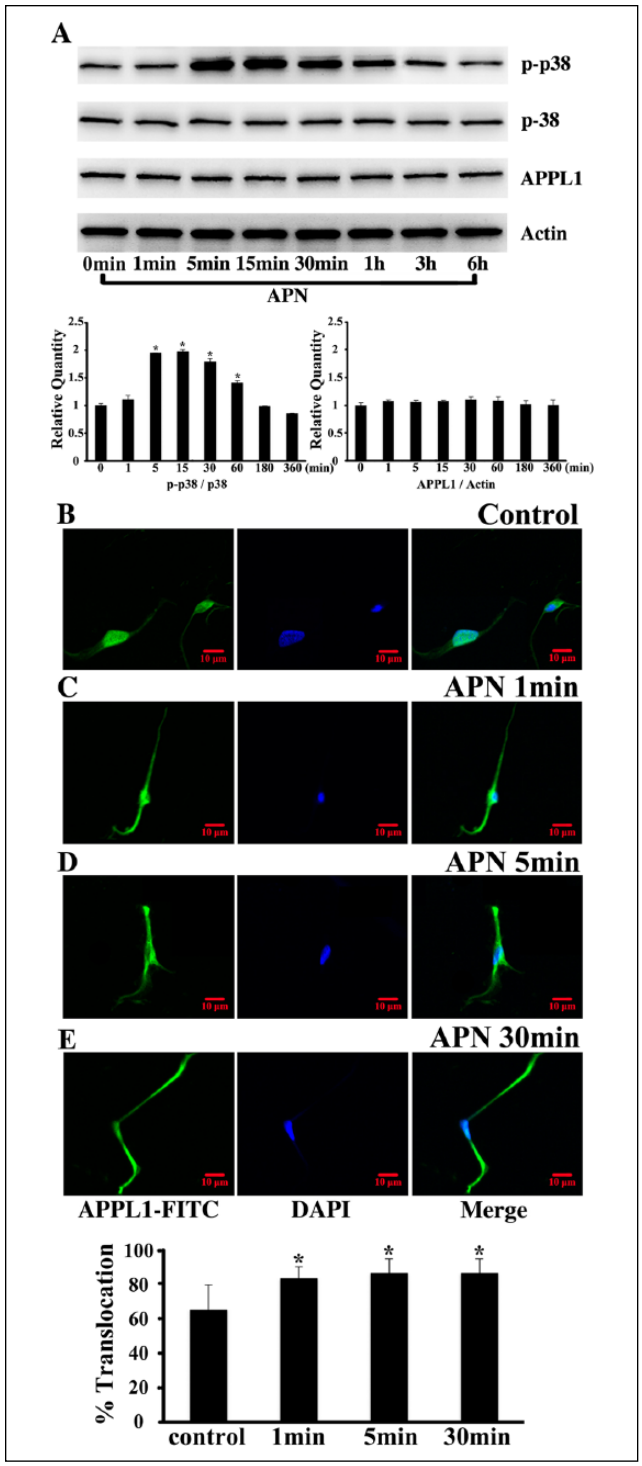

Figure 2. Adiponectin (APN) induced p38 MAPK phosphorylation and APPL1 cytoplasmic localization in human jaw bone marrow mesenchymal stem cells (h-JBMMSCs). (**A**) h-JBMMSCs were cultured in complete medium in 60-mm dishes, starved for 12 h in α-MEM, and then treated with or without APN at 1 μg/mL at the indicated time points. Then, phospho-p38, total p38, APPL1, and actin levels were examined by Western blotting (upper panel), and the data were quantitatively analyzed (lower panel). (**B**) Alternatively, FITC immunofluorescence was used to evaluate APPL1 localization using DAPI as a nuclear counterstain (upper panel), and the data were quantitatively analyzed (lower panel).

mg v. 19.4 ng/mL/mg, respectively), and 2.49-fold (70.2 ng/mL/mg v. 28.2 ng/mL/mg, respectively) higher in APN-treated cells than in the mock control cells, respectively, whereas there were no significant differences compared to the APPL1 knockdown group (Fig. 3B, C). Similarly, increased Alizarin Red staining was observed in APN-treated cells compared to control cells in the presence of APPL1. In the absence of APPL1, APN had no effect on the osteogenesis of h-JBMMSCs (Fig. 3D).

Blockade of p38 MAPK Phosphorylation Inhibited h-JBMMSC Osteogenesis

The p38 MAPK inhibitor SB202190 was used to determine the role of p38 MAPK in the APN-induced osteogenesis of h-JBMMSCs. As expected, Western blotting revealed elevated RUNX2 expression in APN-treated cells, and this increase was mitigated in cells pretreated with the p38 inhibitor (Fig. 4A). Similarly, ALP activity, OCN expression, and OPN expression were significantly lower in cells treated with the p38 inhibitor (Fig. 4B, C). On Alizarin Red staining, fewer positive red calcifying nodules were observed in the p38 MAPK inhibition group than in the other groups (Fig. 4D).

Discussion

It is well known that APN has insulin-sensitizing, anti-inflammatory properties. Along with its metabolic function, APN was previously shown to be involved in tissue regeneration, especially in osteogenesis (Yamauchi et al. 2001; Villarreal-Molina and Antuna-Puente 2012; Padmalayam and Suto 2013). APN was shown to promote human osteoblast proliferation and improve the osteogenic differentiation of BMSCs and adipose-derived stem cells (ASCs) (Scherer et al. 1995; Kanazawa et al. 2007; Lee et al. 2007; Chen et al. 2015). A recent study also reported that APN had a relationship with rheumatoid arthritis (Skalska and Kontny 2016). However, few studies have addressed the relationship between APN and h-JBMMSCs. We previously determined that APN promotes the ossification of extraction sockets in vivo (Hu et al. 2015). However, it is not clear whether APN could produce a similar effect in vitro. With APN treatment for 1 wk, h-JBMMSCs showed a significant up-regulation of osteogenic genes, including ALP, OCN, OPN, and IBSP. Luo et al. (2005) previously showed that with APN treatment for 24 or 48 h, osteoblasts expressed ALP and OCN at significant levels compared to controls. These findings collectively suggest that APN promotes osteogenesis by bone-forming cells.

Next, we investigated the mechanism of APN-driven osteogenesis in h-JBMMSCs. APPL1 is a key regulator of the APN signaling pathway and has been studied extensively in myoblasts, where it was previously shown to elicit p38 MAPK and AMPK phosphorylation in ASCs (Mao et al. 2006; Xin et al. 2011; Chen et al. 2015). Our study revealed similar APPL1 expression between APN-treated and control cells, as previously shown (Mao et al. 2006). Confocal microscopy showed that APPL1 translocated from the nucleus to the cytoplasm in

response to APN treatment. In a previous study, APPL1 translocated from the cytoplasm to the nucleus in HeLa cells in response to Rh-EGF (Miaczynska et al. 2004) and from the nucleus to the cytoplasm in ASCs in response to APN (Chen et al. 2015); thus, the distribution of APPL1 varies according to the cell type and stimulus applied (Miaczynska et al. 2004; Cheng et al. 2014).

Then, we knocked down APPL1 to explore the downstream signaling pathway. APPL1 knockdown in h-JBMMSCs showed no effect on basal p38 phosphorylation, as previously noted (Xin et al. 2011), and these cells showed no reaction in response to APN treatment, suggesting that APPL1 was not indispensable for the phosphorylation of p38 but was an essential mediator of APN-induced p38 MAPK pathway activation. Similar results were observed with respect to RUNX2 expression and osteogenesis-related protein expression, indicating that APN may promote h-JBMMSC osteogenesis via APPL1. Moreover, treatment with a p38 inhibitor decreased RUNX2 expression and blocked the APN-induced up-regulation of RUNX2, which has been observed in other studies (Kim and Kim 2010; Lee et al. 2011). Pretreatment with a p38 inhibitor decreased ALP activity, osteogenic protein expression, and the formation of calcifying nodules in h-JBMMSCs stimulated with or without APN. This finding suggests that APN promoted the osteogenesis of h-JBMMSCs via the p38 MAPK pathway.

Finally, we verified the potency of the signaling pathway using Alizarin Red staining. Unsurprisingly, the osteogenic differentiation ability of h-JBMMSCs was inhibited in APPL1 knockdown cells and SB202190-pretreated cells regardless of APN treatment.

In conclusion, our data suggest that APN could promote h-JBMMSC osteogenesis via APPL1-mediated p38 pathway activation and increase ossification-related gene expression. We are the first to verify this pathway in h-JBMMSCs; however, the full mechanism of APN-mediated h-JBMMSC osteogenesis is complicated and will require further investigation.

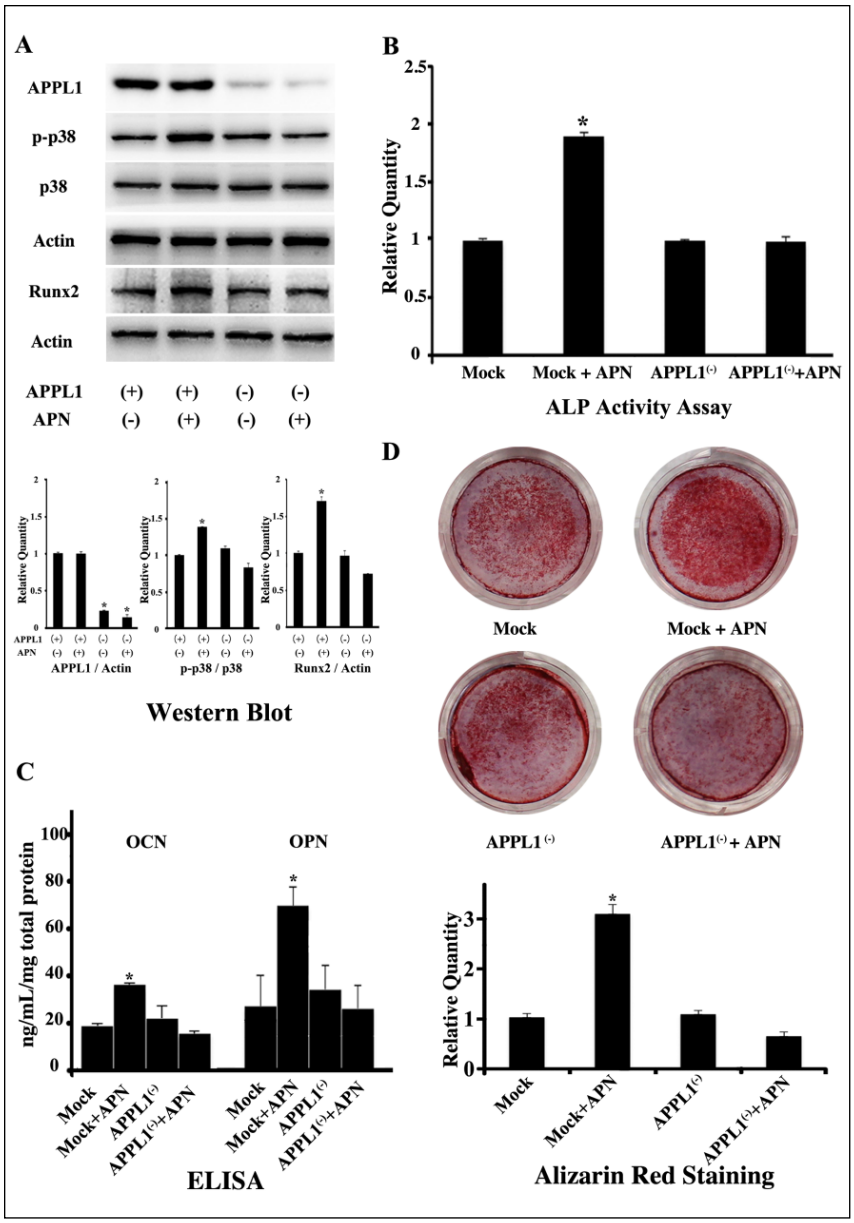

Figure 3. APPL1 was essential for adiponectin (APN)–induced p38 MAPK phosphorylation and human jaw bone marrow mesenchymal stem cell (h-JBMMSC) osteogenesis. (**A**) h-JBMMSCs were cultured in complete medium, starved for 12 h in α-MEM, and then treated with or without 1 μg/mL APN for 15 min. APPL1, phospho-p38, total p38, and actin levels were then examined. h-JBMMSCs were cultured in osteoblast-inducing conditional medium and treated with or without APN at 1 μg/mL once every 3 d. After 7 d, total protein was extracted to examine RUNX2 and actin expression (upper panel), and the data were quantitatively analyzed (lower panel). (**B**) After 7 d of culture in osteoblast-inducing conditional medium, total protein was extracted in RIPA reagent to monitor the ALP activity. The protein concentration was normalized to the relative quantity of each sample. (**C**) After 7 d of culture in 24-well plates, cell supernatants were collected to examine the secretion of osteogenesis-related proteins. Protein concentrations were normalized to the relative quantity in each sample. (**D**) After 21 d of culture, cells were used for Alizarin Red staining (upper panel), and the data were quantitatively analyzed (lower panel). APPL1$^{(+)}$, mock-transfected controls; APPL1$^{(-)}$, APPL1 knockdown. *$P < 0.05$.

Author Contributions

Y. Pu, H. Wu, contributed to conception and design, drafted the manuscript; S. Lu, contributed to data acquisition, critically revised the manuscript; H. Hu, contributed to data analysis, critically revised the manuscript; D. Li, contributed to data interpretation, critically revised the manuscript; Y. Wu, Z. Tang, contributed to conception and design, critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

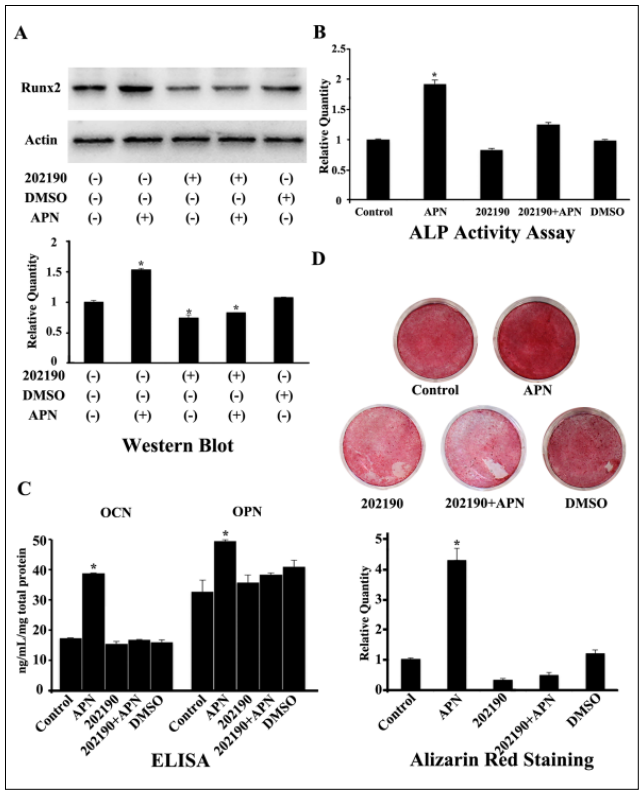

Figure 4. The blockade of p38 MAPK phosphorylation inhibited human jaw bone marrow mesenchymal stem cell (h-JBMMSC) osteogenesis induced by adiponectin (APN). h-JBMMSCs were cultured in osteoblast-inducing conditional medium and treated with or without APN at 1 μg/mL once every 3 d. SB202190 was applied 2 h before APN treatment. (**A**) After 7 d, the total protein was extracted to examine RUNX2 and actin expression (upper panel), and the data were quantitatively analyzed (lower panel). (**B**) After 7 d, the total protein was extracted in RIPA reagent to measure the ALP activity. The protein concentration was normalized relative to the quantity of protein in each sample. (**C**) After 7 d of culture in 24-well plates, cell supernatants were collected to examine the secretion of osteogenesis-related proteins. Protein concentrations were normalized to the relative quantity in each sample. (**D**) After 21 d of culture, cells were used for Alizarin Red staining (upper panel), and the data were quantitatively analyzed (lower panel) (*$P < 0.05$).

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