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Role and mechanism of PTEN in adiponectin-induced osteogenesis in human bone marrow mesenchymal stem cells

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Human bone marrow-derived stromal cells (hBMSC) are multi-potent stem cells that can differentiate into osteogenic and adipogenic lineages. Adiponectin (APN) is an adipocyte-derived hormone that modulates a series of metabolic processes. Recent studies revealed a relationship between APN and bone regeneration, though the underlying mechanism was not fully examined. Phosphatase and tensin homolog deleted on chromosome ten (PTEN) is a tumor suppressor and a therapeutic target for the metabolic syndrome. Its deletion mutants increase osteoblast activity and bone mineral density. Both APN and PTEN are involved in osteogenic differentiation. However, whether PTEN is involved in APN-induced bone metabolism remains unclear. This project was designed to study whether PTEN was involved in APN-mediated osteogenesis of hBMSCs. We found that APN downregulated PTEN expression and that both it and an inhibitor of PTEN (SF1670) increased the expression of osteogenic markers such as osteocalcin, alkaline phosphatase, and runt-related transcription factor-2 in APN-treated hBMSCs. Our results suggested that APN enhanced osteogenic differentiation of hBMSCs in vitro partially by inhibiting PTEN expression. APN could be a therapeutic agent in tissue regeneration engineering and bone regeneration by inhibiting PTEN expression and then promoting the osteogenic differentiation of hBMSCs.

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1. Introduction

Human bone marrow-derived stromal cells (hBMSCs) are multi-potent stem cells that exhibit multiple functions including the capability of self-renewal, differentiation (osteogenic, chondrogenic, neurogenic, and adipogenic), hematopoiesis support, and immune regulation [1–4]. They are considered as one of the most important components in tissue regeneration engineering, and play a key role in bone regeneration [5–7]. Studies on acquiring and retaining safe and reliable lineages for osteogenic differentiation of hBMSCs are particularly important for clinical translation of tissue engineering strategies.

Adiponectin (APN, also referred to as GBP-28, apM1, AdipoQ, and Acrp30) is a 244 amino acid adipocyte-derived hormone that modulates a number of metabolic processes including energy homeostasis, insulin sensitivity, and glucose and lipid metabolism [8,9]. Recent studies report a link between APN and bone homeostasis in bone-forming cells [10–14]. We reported previously that APN regulates bone metabolism via central and peripheral mechanisms through APPL1/phosphoinositide 3-kinase (PI3K)/Akt-mediated pathways by inhibiting osteoclastic differentiation and promoting osteoblastic commitment in vitro and vivo [15–19].

Phosphatase and tensin homolog (PTEN) is one of the most common tumor suppressors lost in human cancers [20,21]. Studies have reported its important role in suppressing osteosarcoma; PTEN in stem cells is associated with its osteosarcoma suppressive function [22–25]. Thus, PTEN may also be related to osteogenesis. Osteo-chondro progenitor cells and osteoblasts show an enhanced differentiation ability and exhibit greatly reduced apoptosis leading to remarkable matrix overproduction by activating the PI3K/Akt signal transduction pathway [26,27]. Recently, Burgers et al. found

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that mice lacking PTEN in osteoblasts had improved intramembranous and late endochondral fracture healing [28]. This was further confirmed by Collins et al. who showed that healed fractures in mice lacking PTEN had better mechanical properties of the femur [29]. Except for its function in bone metabolism, PTEN haploinsufficiency appears to enhance insulin sensitivity, leading to an increased risk of obesity and cancer, but a decreased risk of type 2 diabetes through the PI3K-Akt pathway [30].

Because APN and PTEN are both involved in insulin sensitivity and bone metabolism through the PI3K/Akt signaling pathway, the present study was designed to explore whether PTEN was involved in APN-induced osteogenic differentiation of hBMSCs, and the related mechanisms.

2. Materials and methods

2.1. Antibodies and reagents

Human BMSCs were obtained from ScienCell (San Diego, CA, USA). Human global APN was from Aviscera Bioscience (Santa Clara, CA, USA). The PTEN inhibitor, SF1670, and the COX-2 inhibitor, celecoxib, were from Selleck (Houston, TX, USA). Antibodies against PTEN, runt-related transcription factor-2 (RUNX2), and osteocalcin (OCN) were from Cell Signaling Technology (Beverly, MA, USA) and Santa Cruz Biotechnology (Dallas, TX, USA) respectively. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), the penicillin/streptomycin mixture, and 0.125% trypsin-EDTA were from Gibco (Gaithersburg, MD, USA). All other reagents were from Sigma-Aldrich (St. Louis, MO, USA) and were of analytical grade.

2.2. hBMSC culture and osteogenic induction

hBMSCs were cultured in DMEM containing 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin at 37 °C in an incubator with an atmosphere of 95% air, 5% CO_2, and 100% relative humidity. The fourth and fifth passages of the cells were used. For osteoblast differentiation, cells were seeded at 2×10^4 per well in 12-well plates and induced using conditioned medium consisting of α-MEM, 10% FBS, 10 nM dexamethasone, 10 mM β-glycerophosphate, and 50 μg/mL L-ascorbic acid (Sigma-Aldrich). The cells were further cultured for the indicated times with or without 1 μg/mL APN. SF1670 was used at 5 μM and celecoxib at 15 and 20 μM.

2.3. Alkaline phosphatase (ALP) assays

ALP activity was measured using an assay kit (Nanjing Jianchen Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. For microscopic views, we used the nitroblue tetrazolium/5-bromo-4-chloro-3'-indolyphosphate staining kit (Beijing ComWin Biotech, Beijing, China) to detect ALP activity. Cells were washed once with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) containing 0.05% Tween 20, then fixed with 10% formalin for 1 min and stained in the dark for 10–15 min. Cells were then washed once with PBS buffer and pictures were taken. Positive staining is a dark blue color.

2.4. RNA extraction, reverse transcription, and the quantitative real-time polymerase chain reaction (qRT-PCR)

After hBMSCs were seeded in 6-well plates at the indicated densities with or without osteoinduction medium, APN was added daily at 1 μg/mL. SF1670 (5 μM) was added 3 h after APN treatment. Total mRNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). mRNA was reverse-transcribed into complementary DNA (cDNA) using a PrimeScript 1stStrand cDNA Synthesis kit (TaKaRa Bio, Shiga, Japan) according to the manufacturer's instructions. Quantification of all gene transcripts was performed by qRT-PCR using the Power SYBR Green PCR Master Mix and an ABI PRISM 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA). Relative mRNA expression levels were normalized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase. Cycle threshold values were used to calculate the fold differences. The primers used are listed in Table 1. All primer sequences were determined through established GenBank sequences.

2.5. Western blot analyses

hBMSCs were harvested and total protein was extracted using radioimmunoprecipitation assay buffer (Applygen, Beijing, China). Cells were washed twice with ice-cold PBS and scraped from the dish into microfuge tubes containing mammalian lysis buffer. The cells were also sonicated (Sonics & Materials, Newtown, CT, USA) to maximize protein recovery. Proteins were denatured at 100 °C for 5 min and the concentration determined using the bicinchoninic acid reagent (Thermo Fisher Scientific, Rockford, USA). Protein samples (20 μg/lane) in loading buffer were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). After blocking by incubation in non-fat dried milk, the membrane was incubated with the primary antibody (1:1000) overnight. The membrane was then incubated with the secondary antibody, marked by IRDye800, and scanned with an Odyssey® CLx Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA) to visualize the immunoreactive protein bands.

2.6. Statistical analyses

Data are expressed as means ± standard deviation. Statistically significant differences ($p < 0.05$) among the various groups were evaluated using one-way ANOVA. All statistical analyses were performed using SPSS 19.0 software (IBM, Chicago, IL, USA).

3. Results

3.1. Exposure to APN downregulates PTEN in hBMSCs

To investigate whether PTEN was involved in APN-induced osteogenesis, hBMSCs were treated with 1 μg/mL APN. APN-treated hBMSC cells experienced a rapid decrease in PTEN expression compared with control cells (Fig. 1a and b). Within 30 min post-stimulation, PTEN protein levels were decreased (~33% by densitometric scanning) in response to APN. This decrease continued to 1 h. PTEN protein levels showed little difference between stimulated and non-stimulated cells at 2 h (Fig. 1a and b). These data demonstrated that APN regulated PTEN expression in a time-dependent manner.

3.2. Inhibition of PTEN promotes osteogenic differentiation of hBMSCs

We determined whether the PTEN inhibitor, SF1670, could enhance the osteogenic effects of APN. After 7 days of osteoinduction, significant matrix mineralization was found in the osteoinduction conditioned medium group (Fig. 2). SF1670-treated hBMSCs showed more ALP staining than the APN alone group. ALP activity was measured to determine if the PTEN inhibitor regulated APN-induced osteogenesis. We observed a clear augmentation of ALP activity in the SF1670-treated group compared to the other groups. Taken together, these data show that inhibition of PTEN

Table 1
Primer sequence.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
GAPDH	CAATGACCCCTTCATTGACC	TGGACTCCACGACGTACTCA
RUNX-2	ACTACCAGCCACCGAGACCA	ACTGCTTGCAGCCTTAAATGACTCT
OCN	AGCCACCGAGACACCATGAGA	AGCCACCGAGACACCATGAGA
OPN	CATACAAGGCCATCCCCGTT	ACGGCTGTCCCAATCAGAAG
OSX	CCTCTGCGGGACTCAACAAC	TAAAGGGGGCTGGATAAGCAT

promoted APN-induced osteogenesis in hBMSCs.

3.3. Osteogenesis-related mRNA and protein expression in hBMSCs treated with APN and SF1670

The transcription profiles of osteoblast markers in APN- and PTEN inhibitor-treated groups were identified by qRT-PCR (Fig. 3a, b, c, d). After 7 days of osteogenic induction, the levels of RUNX2 and OCN mRNA in hBMSCs showed a considerable upregulation in the SF1670-treated group compared with the other groups (Fig. 3a and b). After 14 days, the expression of OSX and OPN mRNA also showed an increase in the PTEN inhibitor-treated group (Fig. 3c and d). We also sought to determine if the protein level of RUNX2 was upregulated in the PTEN inhibitor-treated group. Compared to the other groups, the RUNX2 protein level showed a significant upregulation in the APN and APN plus SF1670 groups after 24 h of osteoinduction (Fig. 3e).

3.4. PTEN mediates the osteogenic differentiation of hBMSCs

To understand the role of the PTEN pathway in the process of hBMSC osteogenic differentiation, we investigated changes in the expression of osteogenic markers after the activation of PTEN. Celecoxib, a selective COX-2 inhibitor, suppresses cancer stemness and progression by upregulating PTEN [31,32]. The expression of RUNX2 and OCN proteins was decreased significantly after treatment of hBMSCs with celecoxib (Fig. 4a, b, c). SF1670 enhanced the expression of the RUNX2 and OCN proteins (Fig. 4d and e). These results suggested that PTEN was involved in osteogenic differentiation of hBMSCs by downregulating the expression of RUNX2 and OCN.

4. Discussion

Because both APN and PTEN are involved in insulin sensitivity and bone metabolism through the PI3K/Akt signaling pathway, the present study explored whether PTEN was involved in APN-induced osteogenic differentiation of hBMSCs and the related mechanisms. The results demonstrated that the addition of APN to hBMSCs decreased PTEN expression and induced the osteogenic commitment of hBMSCs. Furthermore, the PTEN inhibitor, SF1670, increased the expression of osteogenic markers in APN-treated hBMSCs including OCN, ALP, and RUNX2. These results suggested that PTEN was involved in the hBMSC osteoblast differentiation process and may be a molecular mechanism underlying APN-mediated osteoinduction.

APN is related to bone homeostasis in bone-forming cells [14] and may regulate osteogenesis and osteoclastogenesis via an effect on stromal cells [11,33,34]. Our previous research showed that APN activates MC3T3-E1 cell PI3K signaling via APPL1 and induces osteogenic differentiation of human jaw bone marrow mesenchymal stem cells through APPL1-mediated activation of p38 mitogen-activated protein kinase [17,18]. PTEN encodes a dual-specificity phosphatase that recognizes protein and phosphatidylinositol substrates, and modulates cellular functions such as

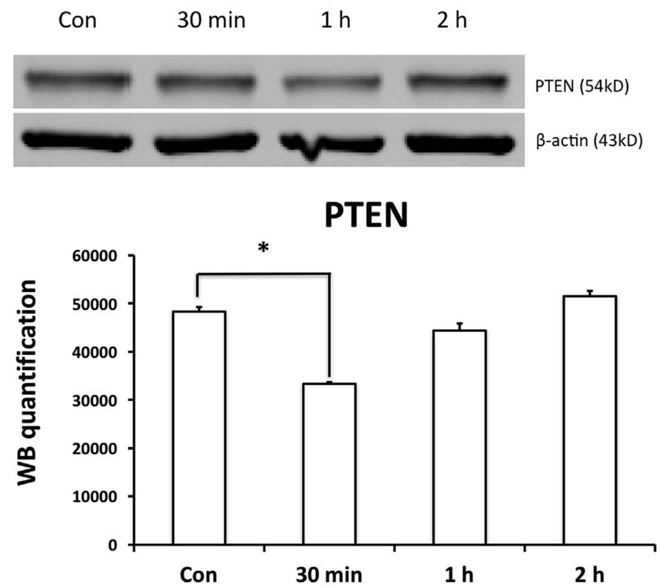


Fig. 1. Adiponectin (APN) downregulates PTEN expression in human bone marrow stromal cells (hBMSCs). hBMSCs were cultured for 12 h in DMEM, then treated with 1.0 μg/mL APN for 30 min, 1, and 2 h. A representative western blot of the PTEN protein levels is shown in the upper panel. The data were analyzed quantitatively in the lower panel and are expressed as means ± SD. *P < 0.01 compared with the control (Con) group.

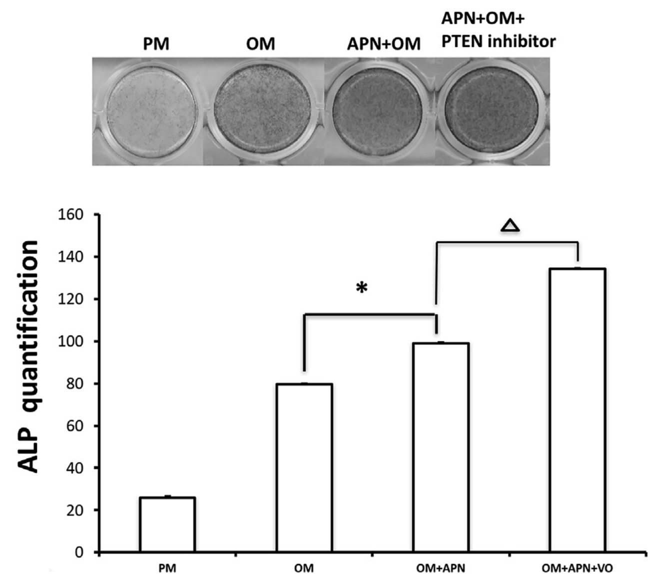


Fig. 2. Inhibition of PTEN promotes adiponectin (APN)-related osteogenesis in human bone marrow stromal cells (hBMSCs). After 7 days of culture in 12-well plates, osteogenesis was examined by assessing alkaline phosphatase (ALP) staining (a) and ALP activity (b). *P < 0.05 compared with the osteoinduction conditioned medium (OM) group. Quantitative data are expressed as means ± SD. ΔP < 0.01 compared with the APN group.

osteoblast and osteoclast differentiation [28,29,35]. However, it is unclear whether PTEN is involved in APN-mediated osteogenesis.

To investigate this possibility, we first determined whether the expression of PTEN was regulated by APN in hBMSCs. Our results showed a rapid and significant reduction of PTEN expression after APN treatment (Fig. 1). This suggested that the level of PTEN activity might be regulated at the post-transcriptional level and/or at the level of protein degradation. Our results were consistent with the

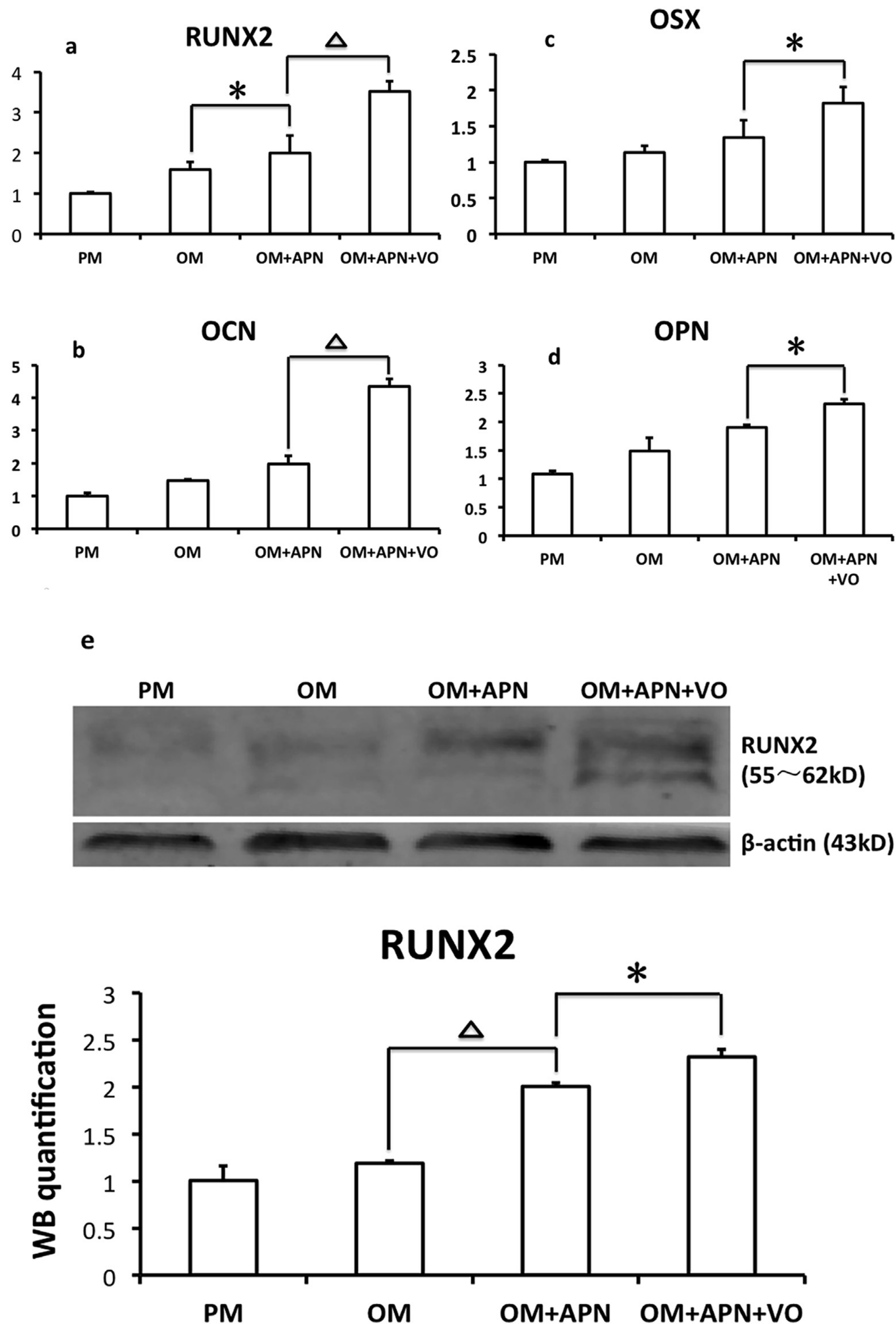


Fig. 3. Adiponectin (APN) combined with SF1670 promotes osteogenesis of human bone marrow stromal cells (hBMSCs). Cells were cultured in proliferation medium (PM) and osteoinduction conditioned medium (OM). The expression of osteogenic differentiation-related genes in hBMSCs cultured in OM with 1.0 μg/mL APN combined with 5 μM SF1670 at day 7 (a, b) and day 14 (c, d). (e) Upper panel: Representative western blot of the protein expression of RUNX2 and β-actin in hBMSCs cultured in PM, OM, OM with 1.0 μg/mL APN, and OM with both 1 μg/mL APN and 5 μM SF1670 for 24 h. Lower panel: The western blot data were analyzed quantitatively and expressed as means ± SD. $^{*}P < 0.05$ compared with the OM alone group; $\Delta P < 0.01$ compared with the OM + APN group.

previous finding that BMP2 altered PTEN protein levels in the MCF-7 breast cancer line after 1 h [36]. Those researchers showed that BMP2 regulated PTEN protein levels by inhibiting its association with the ubiquitin protein degradation pathway by downregulating the expression of the ubiquitin-conjugating enzymes UbCH7 and UbC9 [36] that mediate the transfer of activated ubiquitin to substrate proteins [37]. This finding suggested that APN affected PTEN expression. However, further investigation is needed to explore the underling mechanism.

Because activating RUNX2 gene expression in mesenchymal

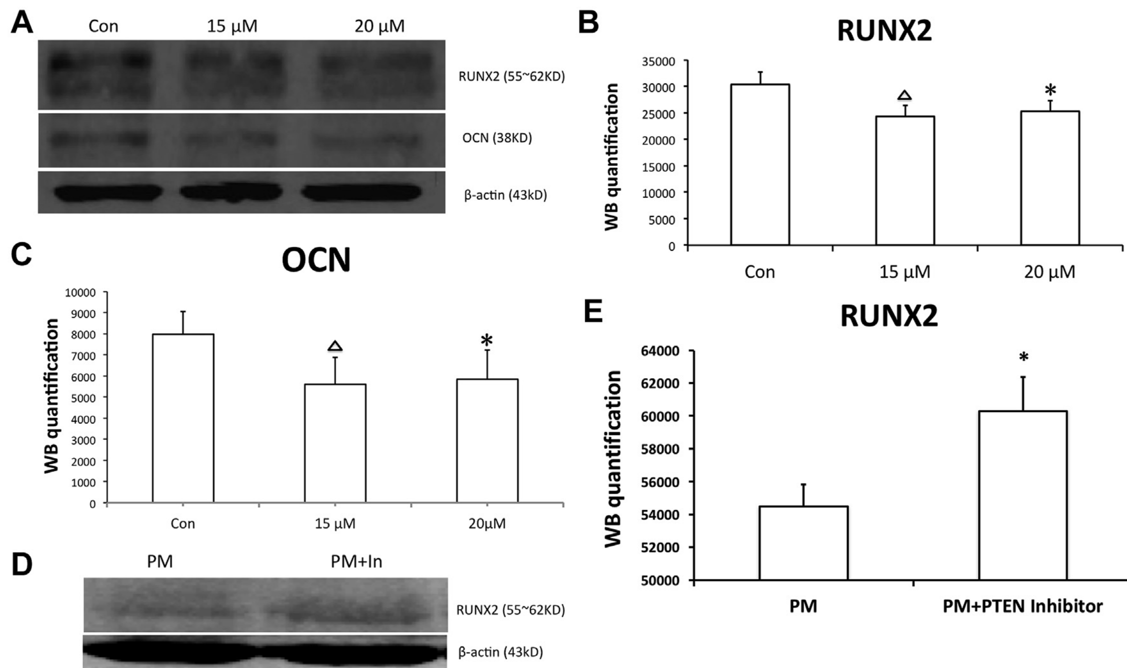
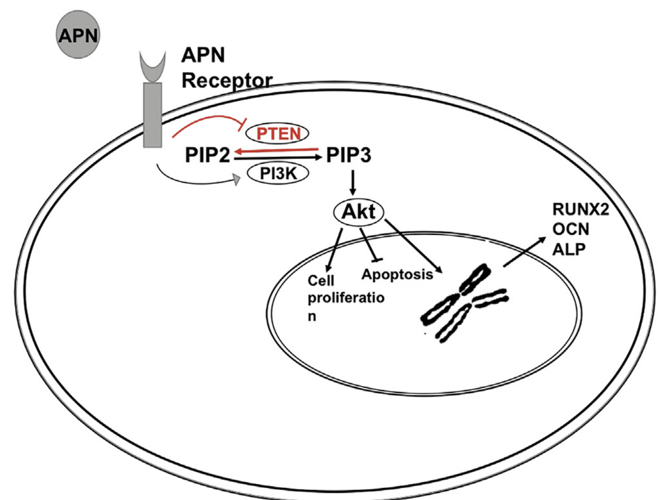


Fig. 4. PTEN downregulates the protein expression of osteogenic-related genes in human bone marrow stromal cells (hBMSCs). hBMSCs were cultured for 6 h before treatment with different concentrations of celecoxib for 24 h (a) A representative western blot of RUNX2, OCN, and β-actin protein levels is shown in the upper panel. The data were analyzed quantitatively in the lower panel and are expressed as means ± SD. (b) Cells were treated with different concentration of SF1670 for 3 h. A representative western blot of RUNX2 and β-actin protein levels is shown in the upper panel. The data were analyzed quantitatively in the lower panel and are expressed as means ± SD. *P < 0.05, ΔP <0.01 compared with the control (Con) group.

cells can induce osteoblast differentiation and skeletal development, and ALP is an important marker of bone metabolism, we assessed the expression of RUNX2 and OCN, and ALP activity, as osteogenesis markers [38–40]. Our results showed that ALP activity was increased indicating that the osteogenic differentiation ability of hBMSCs was enhanced in cells treated with both APN and a PTEN inhibitor, compared with cells treated with APN alone (Fig. 2). This suggested that APN promoted hBMSC osteogenesis by inhibiting PTEN.

In order to further confirm our hypothesis that PTEN is involved in APN-related osteogenesis, we assessed additional osteogenesis-related markers in hBMSCs. Unsurprisingly, in the APN-treated groups, the osteogenic ability of hBMSCs increased significantly when also treated with a PTEN inhibitor (Fig. 3). This result is consistent with previous studies showing that PTEN deletion mutants increase osteoblast activity and bone mineral density through PI3K/Akt signaling [28,29]. PTEN deletion mutants are associated with changes of related microRNAs, such as microRNA-1980, −214, and −23a [23–25]. Thus, PTEN deletion will influence cell behavior through signaling pathways, such as increasing focal adhesion kinase/matrix metalloproteinase 9 signaling, leading to an enhancement of the adhesion, migration, and invasion abilities of osteosarcoma cells [22], and activation of Akt and fibroblast growth factor signaling leading to increased osteoblast differentiation [23,35].

Overall, the results of our study demonstrated that PTEN played an important role in APN-mediated hBMSCs osteogenesis (Scheme 1). The higher protein levels of RUNX2 and OCN seen after treatment with celecoxib and the PTEN inhibitor indicated that PTEN blockade can improve osteogenic differentiation of hBMSCs. According to previous reports and our present data, there might be a connection between PTEN-regulated osteogenesis and the suppression of osteosarcoma. Importantly, RUNX2, a pro-osteogenic transcription marker in hBMSCs, is also a context-dependent



Scheme 1. Schematic diagram of adiponectin (APN)-promoted hBMSCs osteogenesis by inhibiting the PTEN pathway.

oncogene in osteosarcoma [41]. These data suggest that osteogenic regulation and osteosarcomagenesis may be two closely related processes. Thus, PTEN, the tumor suppressor, may also serve as a target for bone regeneration. However, this connection may be complicated and requires further investigation.

Collectively, the data we present here demonstrate that APN enhances osteogenic differentiation of hBMSCs in vitro by inhibiting the expression of PTEN. Our results also support the theory that APN could serve as a therapeutic agent for bone tissue engineering. However, the effect of downstream signaling pathway of PTEN on APN-mediated hBMSC osteogenesis requires further study.

Conflict of interest

No conflicts of interest, financial or otherwise, are declared by any of the authors.

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