

# $\alpha$ 2-antiplasmin modulates bone formation by negatively regulating osteoblast differentiation and function

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**Abstract.**  $\alpha$ 2-antiplasmin ( $\alpha$ 2AP) is known to be a physiological inhibitor of plasmin. Previously, we showed that  $\alpha$ 2AP displays various functions, such as promotion of extracellular matrix production, cell growth, and cell differentiation that are not promoted by its function as a plasmin inhibitor. We herein investigated the role of  $\alpha$ 2AP in bone formation by examining calcein incorporation after its injection in  $\alpha$ 2AP-deficient mice. We found that  $\alpha$ 2AP deficiency enhanced the bone formation rate in mice. We also found that the osteocalcin expression and alkaline phosphatase activity were elevated in the femur and serum of the  $\alpha$ 2AP-deficient mice. Intriguingly,  $\alpha$ 2AP deficiency promoted osteoblast (OB) differentiation of primary calvarial OBs. In contrast,  $\alpha$ 2AP attenuated OB differentiation of mouse osteoblastic the MC3T3-E1 cells. Furthermore,  $\alpha$ 2AP attenuated Wnt-3a-induced  $\beta$ -catenin expression and low-density lipoprotein receptor-related protein 6 activation in the MC3T3-E1 cells. These results suggest that  $\alpha$ 2AP negatively affects OB differentiation and function by inhibiting the Wnt/ $\beta$ -catenin pathway. These findings provide a basis for clinical strategies to improve various bone disorders.

## Introduction

Bone homeostasis is regulated by an appropriate balance between resorption of old bone and formation of new bone, which is known as bone remodeling. Osteoblasts (OBs), which are responsible for bone formation, arise from mesenchymal stem cells (MSCs) as OB progenitors, and OB differentiation and function are positively regulated by several signaling pathways including Wnt/ $\beta$ -catenin-mediated signaling which

targets the expression of osteogenic transcription factor Runx2 (1,2). Additionally, OBs are associated with differentiation and activation of osteoclasts (OCs) which are responsible for bone resorption (3).

$\alpha$ 2-antiplasmin ( $\alpha$ 2AP) is known to be synthesized in various tissues, and functions as the principal inhibitor of plasmin, a main component of the fibrinolytic system (4,5). As a new function of  $\alpha$ 2AP, we previously found that  $\alpha$ 2AP is associated with tissue remodeling, angiogenesis, extracellular matrix (ECM) production, cell growth and cell differentiation (6-11).  $\alpha$ 2AP is most phylogenetically closely related to the non-inhibitory serine protease inhibitor, pigment epithelium-derived factor (PEDF) (12), and they have very similar structure (3  $\beta$ -sheets and 9  $\beta$ -helices) (13,14). Furthermore, Shiomi *et al* recently reported that  $\alpha$ 2AP deficiency attenuated ovariectomy (OVX)-induced bone loss, and  $\alpha$ 2AP is associated with osteoclast formation (15). These observations suggest that  $\alpha$ 2AP exhibits various functions not only as a plasmin inhibitor, but also as a modulator of bone metabolism. However, the mechanisms underlying  $\alpha$ 2AP-regulated bone metabolism remain to be clarified at the cellular and molecular levels.

We herein investigated the roles of  $\alpha$ 2AP in bone metabolism, particularly in regards to how  $\alpha$ 2AP affects OB differentiation and bone formation.

## Materials and methods

**Animals.** The  $\alpha$ 2AP-deficient ( $\alpha$ 2AP<sup>-/-</sup>) mice were generated by homologous recombination using embryonic stem cells, as previously described (16). Wild-type ( $\alpha$ 2AP<sup>+/+</sup>) and  $\alpha$ 2AP<sup>-/-</sup> mice littermates were housed in groups of 2-5 in filter-top cages with a fixed 12-h light and 12-h dark cycle.

The animal experiments were approved by the Animal Research Committee of Doshisha Women's College of Liberal Arts (approval ID, Y15-024). All experiments were performed in accordance with relevant guidelines and regulations.

**In vivo calcein labeling.** Calcein (Nacalai Tesque, Inc., Kyoto, Japan) in saline was intraperitoneally injected into eight-week-old  $\alpha$ 2AP<sup>+/+</sup> and  $\alpha$ 2AP<sup>-/-</sup> mice (20 mg/kg). Two injections were given 3 days apart. The undecalcified sections

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of femurs from eight-week-old  $\alpha 2\text{AP}^{+/+}$  and  $\alpha 2\text{AP}^{-/-}$  mice were prepared by the Tohkai Cytopathology Institute (Gifu, Japan). Bone formation was visualized using a calcein incorporation assay as described by Naylor *et al* (17). Briefly, the mineral apposition rate ( $\mu\text{m/day}$ ) *in vivo* was calculated by identifying newly formed bone via calcein labeling. The data of double-labeled regions were obtained by using fluorescence microscopy, and the mineral apposition rate was calculated as the distance of the double-labeled regions.

**Immunohistochemical staining of osteocalcin.** Paraffin-embedded tissue of femurs in eight-week-old  $\alpha 2\text{AP}^{+/+}$  and  $\alpha 2\text{AP}^{-/-}$  mice was serially sectioned at 4–7  $\mu\text{m}$  distance. Then, the sections were labeled with anti-rabbit osteocalcin antibody (cat. no. SC-30045; Santa Cruz Biotechnology, Inc., Santa Cruz CA, USA), and then secondarily labeled with Cy3-conjugated anti-rabbit IgG (cat. no. A10520; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The signals were then detected using a laser scanning microscope. The stained images obtained from separate fields on the specimens were analyzed using ImageJ software.

**Enzyme-linked immunosorbent assay (ELISA).** The osteocalcin in the serum from eight-week-old  $\alpha 2\text{AP}^{+/+}$  and  $\alpha 2\text{AP}^{-/-}$  mice was then measured using a mouse osteocalcin EIA kit (Biomedical Technologies, Stoughton, MA, USA). The absorbance of the ELISA samples was measured at 450 nm using Multiskan JX (Thermo LabSystems, Beverly, MA, USA).

**Measurement of alkaline phosphatase (ALP) activity.** We measured ALP activity in the serum and osteoblasts from  $\alpha 2\text{AP}^{+/+}$  and  $\alpha 2\text{AP}^{-/-}$  mice as previously described (18). ALP activity was determined using *p*-nitrophenyl phosphate (Sigma-Aldrich, Steinheim, Germany) as a substrate. The absorbance of the samples was measured at 405 nm using Multiskan JX (Thermo LabSystems).

**Cell culture.** Primary OBs derived from  $\alpha 2\text{AP}^{+/+}$  and  $\alpha 2\text{AP}^{-/-}$  mouse calvaria were obtained as previously described (19). Primary OBs or MC3T3-E1 cells were maintained in minimum essential medium (MEM) (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Biowest, Nuaille, France) and 1% penicillin-streptomycin (Invitrogen Life Technologies) at 37° in a humidified atmosphere of 5%  $\text{CO}_2$ /95% air.

**OB differentiation.** OB differentiation in the primary OBs derived from  $\alpha 2\text{AP}^{+/+}$  and  $\alpha 2\text{AP}^{-/-}$  mouse calvaria and MC3T3-E1 cells were induced as previously described (19). Briefly, primarily cultured OBs or MC3T3-E1 cells were cultured for 14 days in the differentiation media supplemented with 10 mM  $\beta$ -glycerophosphate and 10 nM dexamethasone (both from Sigma-Aldrich), and 50  $\mu\text{g/ml}$  ascorbic acid (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in 6-well plates. After 14 days, the cells were then washed with phosphate-buffered saline (PBS), and cell proteins were extracted with a lysis buffer (10 mM Tris-HCl, pH 7.5, 0.1% Triton X-100).

**Reverse transcription-polymerase chain reaction (RT-PCR).** We performed RT-PCR as previously described (19).

First-strand cDNA was synthesized from total RNA using the High Fidelity RT-PCR kit (Toyobo, Osaka, Japan). Quantitative RT-PCR (RT-qPCR) was performed on the IQ5 real-time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with SYBR-Green technology on cDNA generated from the reverse transcription of purified RNA. The 2-step PCR reactions were performed as 92°C for 1 sec and 60°C for 10 sec. *Runx2* mRNA expression was normalized against *GAPDH* mRNA expression using the comparative cycle threshold method. We used the following primer sequences: *Runx2* forward, 5'-GAATGGCAGCAGCTATTAAATCC-3' and reverse, 5'-GCCGCTAGAATTCAAAACAGTTGG-3'; *GAPDH* forward, 5'-TTCATTGACCTCAACTACATG-3' and reverse, 5'-GTGGCAGTGATGGCATGGAC-3'.

**Western blot analysis.** Western blot analysis was performed as previously described (20). Briefly, cells were washed twice with cold PBS, harvested, and then sonicated in lysis buffer containing 10 mM Tris-HCl buffer (pH 7.5), 1% SDS, 1% Triton X-100, and a protease inhibitor cocktail (Roche, Mannheim, Germany). The protein concentration in each lysate was measured using a BCA protein assay kit (Pierce, Rockford, IL, USA). Proteins in the supernatant were separated by electrophoresis on 10% SDS-polyacrylamide gels and transferred to a PVDF membrane. We detected  $\beta$ -catenin, phospho-lipoprotein receptor-related protein 6 (p-LRP6), LRP6 and GAPDH by incubation with anti-rabbit  $\beta$ -catenin antibody (cat. no. Rb-1491; NeoMarkers, Fremont, CA, USA), anti-rabbit phospho-LRP6 antibody (cat. no. bs-2905R) and anti-rabbit LRP6 antibody (cat. no. bs-3253R) (both from Bioss Inc., Woburn, MA, USA), and anti-rabbit GAPDH antibody (cat. no. SAB2100894; Sigma-Aldrich) followed by incubation with horseradish peroxidase-conjugated antibodies to rabbit IgG (cat. no. NA934-1ML; Amersham Pharmacia Biotech, Uppsala, Sweden).

**Spontaneous secretion of VEGF in primary OBs.** Spontaneous secretion of VEGF in primary OBs was measured as previously described (6). The OBs were maintained in MEM $\alpha$  containing 10% FBS. After 6 days, the medium was exchanged for serum-free MEM $\alpha$ . After 24 h, the conditioned medium was collected, and VEGF in the medium was then measured by VEGF ELISA kit (R&D Systems, Minneapolis, MN, USA). The absorbance of the ELISA samples was measured at 450 nm using Multiskan JX (Thermo LabSystems).

**Statistical analysis.** All data are expressed as the means  $\pm$  SEM. The significance of the effect of each treatment ( $P < 0.05$ ) was determined by analysis of variance (ANOVA) followed by the least significant difference test.

## Results

**Effect of  $\alpha 2\text{AP}$  deficiency on bone formation in mice.** We investigated the effect of  $\alpha 2\text{AP}$  deficiency on bone formation by examining calcein incorporation after its injection in the  $\alpha 2\text{AP}^{+/+}$  and  $\alpha 2\text{AP}^{-/-}$  mice. The double calcein-labeled regions in the femurs from the eight-week-old  $\alpha 2\text{AP}^{+/+}$  and  $\alpha 2\text{AP}^{-/-}$  mice are shown in Fig. 1A. The mineral apposition rate was calculated as the distance of the double calcein-labeled

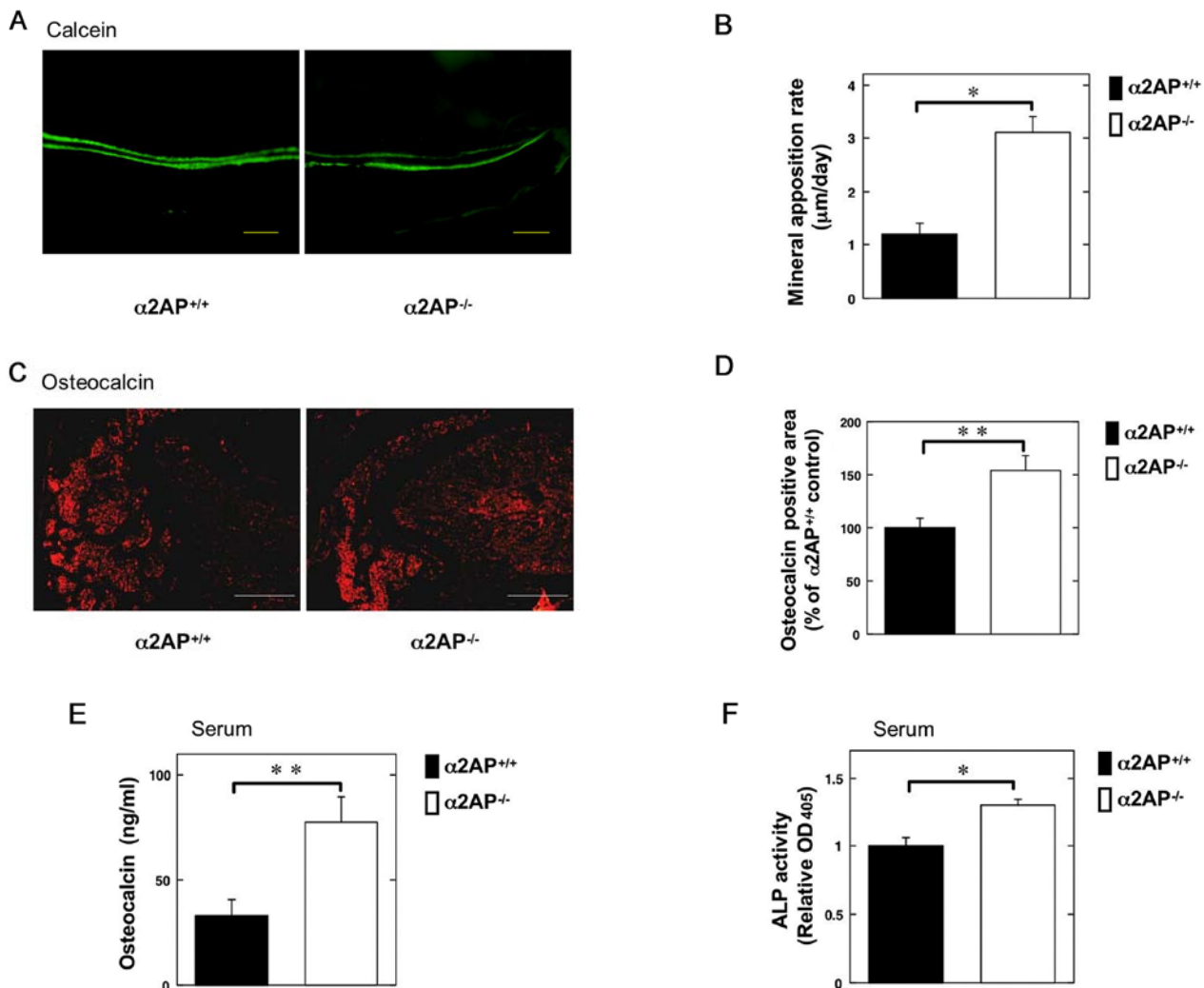


Figure 1. Effect of  $\alpha 2$ -antiplasmin ( $\alpha 2AP$ ) deficiency on bone formation in mice. (A) Eight-week-old  $\alpha 2AP^{+/+}$  and  $\alpha 2AP^{-/-}$  mice were given 2 injections of calcein 3 days apart to label mineralizing bone surfaces in the femur. Scale bar, 100  $\mu m$ . (B) The distance between these labels, measured in the trabecular bone at the epiphysis of femur sections, was determined as the mineral apposition rate (n=6). (C) The osteocalcin staining (red) of tissue section of femurs in eight-week-old  $\alpha 2AP^{+/+}$  and  $\alpha 2AP^{-/-}$  mice was performed as described in Materials and methods. Scale bar, 200  $\mu m$ . (D) The intensity of osteocalcin expression in the tissue sections was quantitatively evaluated as described in Materials and methods (n=4). (E) The quantitative level of osteocalcin in serum from the  $\alpha 2AP^{+/+}$  and  $\alpha 2AP^{-/-}$  mice was evaluated as described in Materials and methods (n=4). (F) The alkaline phosphatase (ALP) activity in serum from the  $\alpha 2AP^{+/+}$  and  $\alpha 2AP^{-/-}$  mice was evaluated as described in Materials and methods (n=4). The data represent the mean  $\pm$  SEM. \*P<0.01; \*\*P<0.05.

regions. The distance between the double calcein-labels in the femurs from the  $\alpha 2AP^{-/-}$  mice was larger than that from the  $\alpha 2AP^{+/+}$  mice (Fig. 1B). Additionally, we examined the expression of osteocalcin in the femurs from the  $\alpha 2AP^{+/+}$  and  $\alpha 2AP^{-/-}$  mice. The level of osteocalcin expression in the femurs from the  $\alpha 2AP^{-/-}$  mice was significantly higher than that of the  $\alpha 2AP^{+/+}$  mice at the protein level (Fig. 1C and D). Furthermore, we examined the levels of osteocalcin and ALP activity in the serum of the  $\alpha 2AP^{+/+}$  and  $\alpha 2AP^{-/-}$  mice. The levels of osteocalcin and ALP activity in the serum of the  $\alpha 2AP^{-/-}$  mice were significantly higher than those of the  $\alpha 2AP^{+/+}$  mice (Fig. 1E and F, respectively).

#### Effect of $\alpha 2AP$ deficiency on OB differentiation and function.

Next, to clarify the role of  $\alpha 2AP$  in OB differentiation, we examined the ALP activity in primary calvarial OBs from the  $\alpha 2AP^{+/+}$  and  $\alpha 2AP^{-/-}$  mice in the absence or presence of OB differentiation media. Intriguingly, the  $\alpha 2AP$  deficiency resulted in upregulation of ALP activity in OBs (Fig. 2A).

Additionally, we examined the expression level of *Runx2*, which is an essential transcription factor for OB differentiation, in OBs from the  $\alpha 2AP^{+/+}$  and  $\alpha 2AP^{-/-}$  mice in the presence of the OB differentiation media. The level of *Runx2* mRNA expression in the  $\alpha 2AP^{-/-}$  OBs was significantly higher than that in the WT OBs (Fig. 2B). It has been reported that PEDF, which is most phylogenetically closely related to  $\alpha 2AP$ , inhibits the Wnt/ $\beta$ -catenin pathway by blocking LRP6 (21). Therefore, to clarify whether or not  $\alpha 2AP$  is associated with the Wnt/ $\beta$ -catenin pathway, we examined the expression of  $\beta$ -catenin in OBs from the  $\alpha 2AP^{+/+}$  and  $\alpha 2AP^{-/-}$  mice. The expression of  $\beta$ -catenin in the  $\alpha 2AP^{-/-}$  OBs was significantly higher than that in the  $\alpha 2AP^{+/+}$  OBs at the protein level (Fig. 2C).

**Effect of  $\alpha 2AP$  on OB differentiation and function.** In order to clarify the roles of  $\alpha 2AP$  in the functions of OBs, we examined ALP activity in the  $\alpha 2AP$ -treated mouse osteoblastic MC3T3-E1 cells.  $\alpha 2AP$  treatment attenuated the OB differentiation media-induced ALP activation

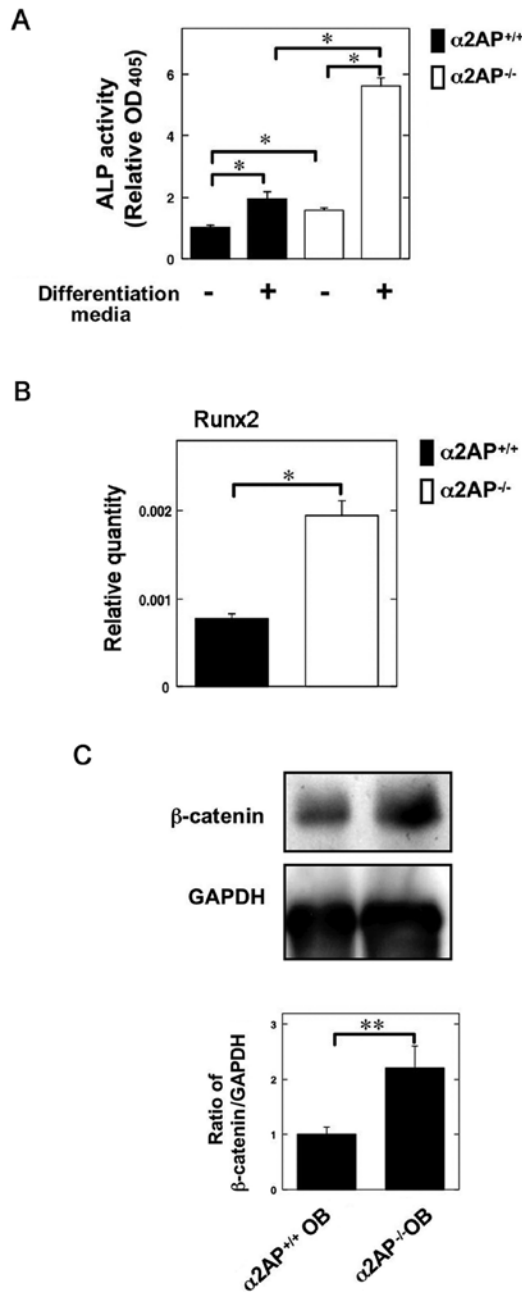


Figure 2. Effect of  $\alpha$ 2-antiplasmin ( $\alpha$ 2AP) deficiency on osteoblast (OB) differentiation and function. (A and B) OBs from the  $\alpha$ 2AP<sup>+/+</sup> and  $\alpha$ 2AP<sup>-/-</sup> mice were cultured for 14 days in the absence or presence of differentiation media. (A) Alkaline phosphatase (ALP) activity in OBs from the  $\alpha$ 2AP<sup>+/+</sup> and  $\alpha$ 2AP<sup>-/-</sup> mice was evaluated as described in Materials and methods (n=3). (B) Expression of *Runx2* mRNA in OBs from the  $\alpha$ 2AP<sup>+/+</sup> and  $\alpha$ 2AP<sup>-/-</sup> mice in the presence of differentiation media was evaluated as described in Materials and methods (n=3). (C) Expression of  $\beta$ -catenin in OBs from the  $\alpha$ 2AP<sup>+/+</sup> and  $\alpha$ 2AP<sup>-/-</sup> mice was evaluated by a western blot analysis. The histogram (bottom panel) shows quantitative representations of  $\beta$ -catenin obtained from densitometry analysis after normalization to the levels of GAPDH expression (n=3). The data represent the mean  $\pm$  SEM. \*P<0.01; \*\*P<0.05.

in the MC3T3-E1 cells (Fig. 3A). We also showed that  $\alpha$ 2AP treatment significantly attenuated the *Runx2* mRNA expression in the MC3T3-E1 cells in the OB differentiation media (Fig. 3B). Furthermore, we demonstrated that  $\alpha$ 2AP attenuated the Wnt-3a-induced  $\beta$ -catenin expression and LRP6 phosphorylation at the protein level (Fig. 3C).

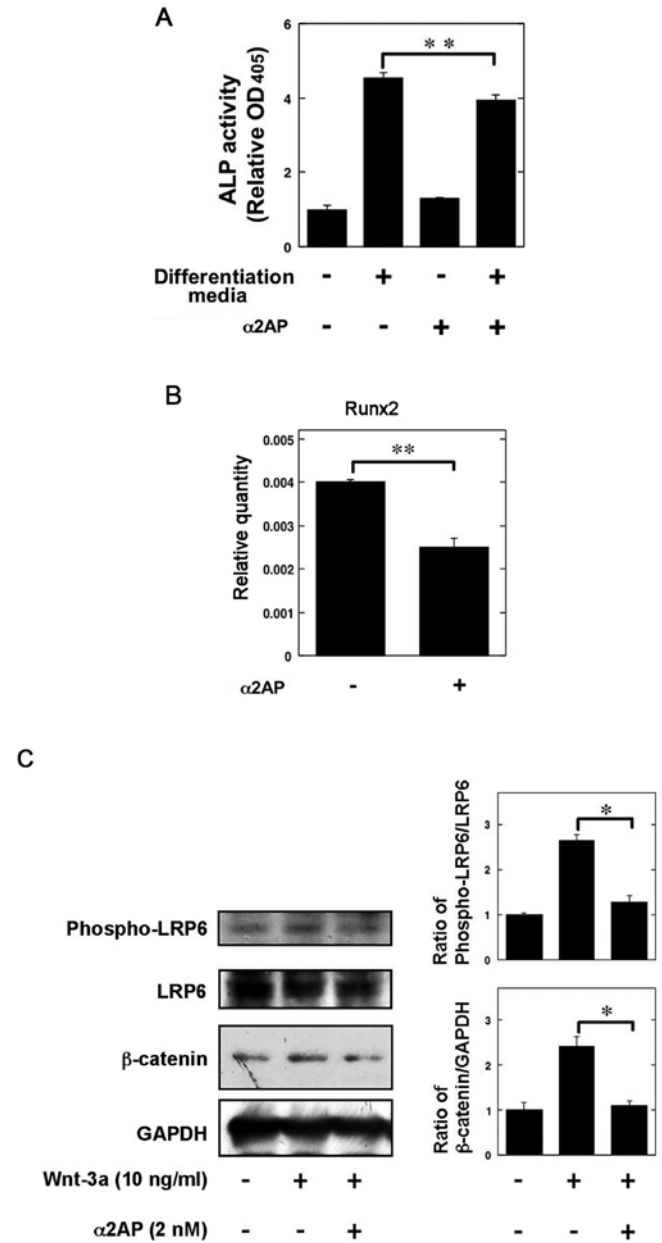


Figure 3. Effect of  $\alpha$ 2-antiplasmin ( $\alpha$ 2AP) on osteoblast (OB) differentiation and function. (A and B) MC3T3-E1 cells were cultured for 14 days in the absence or presence of differentiation media or  $\alpha$ 2AP (1 nM) as indicated. (A) Alkaline phosphatase (ALP) activity in MC3T3-E1 cells was evaluated as described in Materials and methods (n=3). (B) Expression of *Runx2* mRNA in MC3T3-E1 cells in the presence of differentiation media with or without  $\alpha$ 2AP (1 nM) was evaluated as described in Materials and methods (n=3). (C) MC3T3-E1 cells were cultured for 2 h in the absence or presence of Wnt-3a (10 ng/ml) or  $\alpha$ 2AP (2 nM) as indicated. Then, phosphorylation status of lipoprotein receptor-related protein 6 (LRP6) and  $\beta$ -catenin expression were examined by a western blot analysis. The histograms (right panels) show quantitative representations of phospho-LRP6 and  $\beta$ -catenin obtained from densitometry analysis after normalization to the levels of total LRP6 and GAPDH expression, respectively (n=3). The data represent the mean  $\pm$  SEM. \*P<0.01; \*\*P<0.05.

## Discussion

In the present study, we investigated the roles of  $\alpha$ 2AP in the statuses of differentiation and function of OBs that regulate bone formation. We found that the bone formation rate and osteocalcin expression in the femur and serum ALP activity were significantly

elevated in the  $\alpha$ 2AP-deficient mice compared with these parameters in the WT mice (Fig. 1). Additionally,  $\alpha$ 2AP deficiency promoted osteogenic transcription factor expression and ALP activity in OBs (Fig. 2A and B). In contrast, the  $\alpha$ 2AP treatment attenuated them (Fig. 3A and B). These data strongly suggest that  $\alpha$ 2AP negatively regulates OB differentiation and function.

Although  $\alpha$ 2AP is known to be a plasmin inhibitor, we previously found that  $\alpha$ 2AP regulates ECM production, cell growth, and cell differentiation in the absence of plasmin (7-10). We also showed that plasminogen deficiency did not affect OB differentiation (19). We herein showed that  $\alpha$ 2AP attenuated OB differentiation in the absence of plasmin (Fig. 3A and B). These observations suggest that  $\alpha$ 2AP-mediated OB differentiation is not carried out by its action as a plasmin inhibitor.

$\alpha$ 2AP is most phylogenetically closely related to PEDF (12), and they have very similar structure (3  $\beta$ -sheets and 9  $\alpha$ -helices) (13,14). It has been reported that PEDF inhibits the Wnt/ $\beta$ -catenin pathway by blocking LRP6, which is coreceptor for Wnts (21). The Wnt-3a-induced LRP6 activation results in inhibition of  $\beta$ -catenin degradation (22), and the Wnt/LRP6/ $\beta$ -catenin axis plays an important role in OB differentiation (23-25). We herein showed that the expression status of  $\beta$ -catenin was elevated in the OBs from the  $\alpha$ 2AP-deficient mice than in that from WT mice (Fig. 2C). In addition, the  $\alpha$ 2AP treatment attenuated Wnt-3a-induced  $\beta$ -catenin expression and LRP6 activation (Fig. 3C). These data strongly suggest that  $\alpha$ 2AP negatively modulates OB differentiation by inhibiting the Wnt/LRP6/ $\beta$ -catenin axis.

In a previous study, we showed that  $\alpha$ 2AP deficiency enhanced VEGF expression in fibroblasts (6).  $\alpha$ 2AP deficiency also enhanced VEGF expression in primary osteoblasts (data not shown). It has been reported that osteoblast-derived VEGF positively regulates OB differentiation and bone formation activity of OBs in autocrine or paracrine manners (26). Additionally, activation of the Wnt/ $\beta$ -catenin pathway induces VEGF production (27). The  $\alpha$ 2AP-mediated Wnt/ $\beta$ -catenin pathway may also regulate the production of VEGF production, and the  $\alpha$ 2AP-regulated VEGF production may be associated with bone homeostasis.

In conclusion,  $\alpha$ 2AP affects bone metabolism by negatively regulating OB differentiation and function. These findings provide a basis for therapeutic strategies for various bone disorders.

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