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Oleanolic acid exerts bone anabolic effects via activation of osteoblastic 25-hydroxyvitamin D 1-alpha hydroxylase

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ABSTRACT

Oleanolic acid (OA) is previously shown to exert bone protective effects in aged animals. However, its role in regulating osteoblastic vitamin D bioactivation, which is one of major causes of age-related bone loss, remains unclear. Our results revealed that treatment of OA significantly increased skeletal CYP27B1 expression and circulating $1,25(OH)_2D_3$ in ovariectomized mice (p <0.01). Moreover, OA upregulated CYP27B1 protein expression and activity, as well as the vitamin D-responsive bone markers alkaline phosphatase (ALP) activity and osteopontin (OPN) protein expression, in human osteoblast-like MG-63 cells (p<0.05). CYP27B1 expression increased along with the osteoblastic differentiation of human bone marrow derived mesenchymal stem cells (hMSCs). CYP27B1 expression and cellular $1,25(OH)_2D_3$ production were further potentiated by OA in cells at mature osteogenic stages. Notably, our study suggested that the osteogenic actions of OA were CYP27B1 dependent. In summary, the bone protective effects of OA were associated with the induction of CYP27B1 activity and expression in bone tissues and osteoblastic lineages. Hence, OA might be a potential approach for management of age-related bone loss.

1. Introduction

According to World Health Organization (WHO), the elderly will make up 22% of world population by 2025 [1–3] . Osteoporosis is one of the major public health concerns that threaten the life of older adults [4]. Globally, approximately 70% of people over 80 suffer from osteoporosis, resulting in more than 8.9 million fractures in elderly annually [5]. With the increase in aging population worldwide, the consequent cost of treatment and long-term disability pose heavy economic and medical burden to the family and the society [6]. Therefore, strategies to manage aging and slow the age-related bone loss is of great demand.

Functional impairment of osteoblasts and decreased osteogenic differentiation of bone marrow mesenchymal stem cell (MSCs) in bone marrow are the major causes of age-related decline in bone formation [7]. Moreover, the disturbance in calcium homeostasis with age is also associated with the adverse skeletal outcomes in older adults [8]. Vitamin D is critical for maintaining calcium homeostasis by improving intestinal and renal calcium absorption, and thus facilitating bone mineralization. Nevertheless, recent studies indicated that vitamin D and calcium supplementation was not effective in reducing the incidence of fractures in older adults [9,10]. Possible reasons for such discrepancy might be the reduction in absorption and bioactivation of vitamin D in the elderly.

25-hydroxyvitamin D 1-alpha-hydroxylase (CYP27B1) is the predominant enzyme mediating the bioactivation of vitamin D into 1α, 25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$). Renal proximal tubules are the primary site expressing CYP27B1, thus serve as the major source of circulating $1,25(OH)_2D_3$ [11]. On the other hand, CYP27B1 presented in

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extra-renal sites are responsible for the local biosynthesis of 1,25$(OH)_2D_3$ and responsible for enhancing biological activities in a paracrine or autocrine manner in skeletal microenvironment [12]. 1,25$(OH)_2D_3$ generated in kidney binds to vitamin D receptor (VDR) presented in the target tissues to initiate the transcription of genes encoding calcium transporters. For osteoblasts, 1,25$(OH)_2D_3$/VDR system exerts anabolic activities by modulating expressions of osteogenic factors such as osteopontin (OPN), alkaline phosphatase (ALP) and bone sialoprotein (BSP) [13]. Accumulating studies suggested an reduction in expression and activity of CYP27B1 in renal and osteoblast lineages were responsible for the decreased circulating level of 1,25$(OH)_2D_3$ and impaired osteogenesis in aging [14,15]. Thus, regulating renal and extra-renal CYP27B1, especially those in osteoblasts, is important for retarding the development of age-related bone loss. Renal CYP27B1 expression and 1,25$(OH)_2D_3$ synthesis is strictly regulated by hormonal factors like parathyroid hormone (PTH) and fibroblast growth factor 23 (FGF23) [16–18]. However, the regulation of CYP27B1 in bone remains unclear and controversial [19].

Oleanolic acid (OA) is a naturally occurring triterpenoid commonly found in olive oil, fruit, and medicinal herbs [20]. Bone-protective effects of OA have been reported by numerous studies showing its actions on improving bone mass and bone microarchitecture in osteoporotic animals induced by estrogen deficiency and glucocorticoid [21–23]. Moreover, our previous work clearly showed that OA markedly attenuated bone loss and negative calcium balance in aged rats [24]. Such effects of OA might be associated with its actions on modulating renal CYP27B1 and circulating 1,25$(OH)_2D_3$ [24]. However, the actions of OA on osteoblastic CYP27B1 and its involvement in the osteogenic effects of OA is far from clear.

In the present study, we hypothesized that OA exerted bone anabolic effects via its actions on vitamin D metabolism in bone. To investigate whether the bone protective effects of OA are related to its effects on CYP27B1 in bone, *in vivo* and *in vitro* actions of OA on expressions of CYP27B1 and osteogenic markers were determined in ovariectomized (OVX) mice and human osteoblast-like cells, respectively. Actions of OA on age-related decline in CYP27B1 expression and osteogenesis were studied by evaluating expression of CYP27B1 and cellular production of 1,25$(OH)_2D_3$ at different stages of osteoblastic differentiation of human MSCs (hMSCs) from aged subjects. We also evaluated the dependence of osteogenic effects of OA on CYP27B1 by silencing the expression of CYP27B1 by transfection of siRNA. It is anticipated that this study would increase our understanding of the regulation of extra-renal CYP27B1 and age-related bone loss by naturally occurring small molecule.

2. Materials and methods

2.1. Animal study and sample collection

Animal study was conducted according to the protocol approved by the Animal Ethics Committee of the Hong Kong Polytechnic University (ASESC No.: 19–20/4-ABCT-R-STUDENT). Forty 6-month-old female C57BL/6 mice weighing were purchased and housed in centralized animal facilities (CAF) of the Hong Kong Polytechnic University on a 12 h light and dark cycle. After the sham or ovariectomy (OVX) operation, mice were randomly assigned to 5 groups (n=8/group) and were pair fed with 3 g phytoestrogen-free AIN-93 M rodent diet (Research diets, New Brunswick, NJ, USA) (Table S1), the minimum daily intake of mice. For each treatment group, the sham-operated and OVX mice were treated with vehicle (Sham and OVX), other OVX mice were orally administrated with 17β-estradiol (E_2, 200 μg/kg/day), high calcium diet (HCD, 1% calcium in AIN-93 M based rodent diet) and OA (200 ppm in AIN-93 M based rodent diet) for 8 weeks. 17β-estradiol was purchased from Sigma-Aldrich (MO, USA). OA with purity over 98% was from Shanghai Winherb Medical Technology Co. (Shanghai, China) and embedded in the control diet. The concentrations of OA in the diet were designed based on the dosage used in our previous study and daily dietary intake of mice [24]. E_2 and HCD were employed as positive controls to evaluate the actions of OA on bone metabolism and vitamin D metabolism in OVX mice. Body weight of the animals were monitored on a weekly basis throughout the study. Before sacrifice, 24-hour urine were collected individually by using metabolic cages. The mice were then sacrificed by cardiac stick exsanguinations following anesthesia. Serum was aliquoted and stored at −80 °C for biochemical measurements. The intact lumbar vertebra was collected and wrapped in saline-soaked gauze and stored at - 20 °C for micro-computed tomography (μCT) analysis. Iliac crests were collected stored at −80 °C for determination of CYP27B1 expression in bone tissues.

2.2. Micro-computed tomography

The bone mineral density and microarchitectural properties of trabecular bone at the fourth lumbar vertebrae (L4) and proximal tibia were determined by μCT system (viva-μCT40; Scanco Medical, Switzerland) as previously described [24]. Briefly, a total of 100 continuous slides were scanned in the middle part of the lumbar vertebrae (L4) (middle point ± 50 slices) with energy of 70 kVp and intensity of 114 μA. For the proximal tibia, 100 slices were acquired from the growth plate to metaphysis, 50 serials slices were selected for analysis. The volume of interest (VOI) was evaluated using a three-dimensional direct model with a constant threshold of 375. Trabecular bone parameters including volumetric bone mineral density (vBMD, mg HA/cm^3), bone volume fraction (BV/TV, %), trabecular number (Tb.N, 1/mm), trabecular thickness (Tb.Th, mm), trabecular separation (Tb.Sp, mm), and connectivity density (Conn.D, 1/mm^3) were evaluated.

2.3. Biochemistry assay of serum and urine samples

Serum and urinary levels of calcium (Ca) and phosphorus (P) were measured by standard colorimetric methods using commercial kits (Nanjing Jiancheng Bioengineering Institute, China). Urinary Ca and P levels were normalized with urinary creatinine (Cr) levels measured by picric acid methods following the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, China). Concentrations of 1,25$(OH)_2D_3$ in serum were determined by a mouse 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) ELISA kit (BlueGene Biotech, Shanghai, China). Serum levels of bone formation marker procollagen I N-terminal propeptide (PINP) and bone resorption marker C-terminal telopeptide of type I collagen (CTX-1) were tested by using mouse PINP ELISA kit (ABclonal, USA) and mouse cross linked CTX-I ELISA kit (CUSABIO technology, USA), respectively.

2.4. Cell culture and treatment

Human osteosarcoma MG-63 cells (CRL-1427™) were obtained from American Type Culture Collection (ATCC, USA) and cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) containing 10% heat-inactivated fetal bovine serum (FBS, Hyclone, USA) and 100 U/mL penicillin-streptomycin (Gibco, USA). For the experiment, cells were seeded in 6-well plates at a density of 2×10^5 cells/well to allow 80% confluence 24 hours later. Culture medium were then replaced by phenol red-free DMEM (Gibco, USA) containing 1% charcoal-striped FBS (Gibco, USA). 24 hours after changing medium, cells were treated with different concentrations of OA (1, 10, 100, 1000 nM) to determine the effects of OA on CYP27B1 and osteogenesis in osteoblast-like cells. Parathyroid hormone (PTH, 100 nM; Sigma-Aldrich, USA) was used as a positive control for the effects on CYP27B1 expressions and activity.

Human mesenchymal stem cells (hMSCs) from a 61-year-old female subject and a 60-year-old male subject [25], and the protocol was approved by the Joint Chinese University of Hong Kong-New Territories East Cluster Clinical Research Ethics Committee (CREC Ref. No. 2010.248). Cells were cultured in standard growth medium consisting of Minimum Essential Medium alpha (α-MEM, Gibco, USA) with 10% FBS

(Hyclone, USA) and 100 U/mL penicillin-streptomycin (Gibco, USA). For osteogenic differentiation, 2×10^5 cells/well were seeded in 6-well plates. Upon attaining confluence (day 0), culture medium was replaced with osteogenic induction medium which is composed of standard growth medium supplemented with 10 mM β-glycerophosphate (β-GP, sigma, USA), 1 nM dexamethasone (sigma, USA) and 50 μg/mL L-ascorbic acid (sigma, USA). The medium was changed every 3 days until they were differentiated for 7, 14 and 21 days. Upon reaching different osteogenic time points, culture medium was then replaced by phenol red-free α-MEM (Gibco, USA) containing 1% charcoal-striped FBS (Gibco, USA). Cells were treated with PTH (100 nM) or OA (1, 10, 100, 1000 nM) for 24 hours to determine the effects of OA on CYP27B1 and osteogenesis of hMSCs at different osteogenic differentiation stages.

2.5. CYP27B1 activity assay

Cells were seeded in 12-well plates until reaching 80% confluence. The culture medium was then changed to a phenol red-free and serum-free medium to minimize the interference from sterols for 24 hours before treatment. Then cells were treated with PTH or OA and supplemented with $25OHD_3$ (1 μM; Sigma, USA) as a substrate and N, N'-diphenylethylene-diamine (1, 2-dianilinoethane, 10 mM; Sigma, USA) as an antioxidant for 4 hours [26]. The CYP27B1 activity was evaluated by determining the cellular secreted $1,25(OH)_2D_3$ in culture medium [14]. Concentrations of $1,25(OH)_2D_3$ in supernatant was qualified with $1,25(OH)_2D_3$ ELISA kit by following manufacturer's instruction (Immundiagnostik AG, Germany). Total protein content was determined by Bradford assay (Bio-Red, CA, USA). CYP27B1 activity is defined by the level of $1,25(OH)_2D_3$ transformed from $25OHD_3$ by the per unit of protein per hour of $25OHD_3$ treatment.

2.6. RNA interference with CYP27B1 siRNA

To evaluate if the osteogenic effect of OA depended on the intracellular production of $1,25(OH)_2D_3$ in osteoblasts, small interfering RNAs (siRNAs) were used to silence the expression of CYP27B1. MG-63 cells were seeded in 6-well plates until reaching 80% confluence. Transfection was conducted by adding 25 nM SmartPool for human CYP27B1 specific siRNAs (Dharmacon, USA) or non-silencing control siRNA (Dharmacon, USA) with 4 μL/well Dharma-Fect-3 reagent (Dharmacon, USA) according to the manufacturer's instructions. Successful knockdown of CYP27B1 was confirmed by qRT-PCR and western blotting 48 hours after transfection.

2.7. Alkaline phosphatase (ALP) activity assay

Cells were seeded in 24-well plates until reaching 80% confluence, followed by treatment with different concentrations of OA for 7 days in phenol red-free medium with 1% charcoal-striped FBS (Gibco, USA). Medium and treatment were renewed every 3 days. Treated cells were lysed with passive lysis buffer (Promega, USA). ALP activity of the cell lysate was determined with a LabAssay ALP kit (Wako, Japan). Total protein content was measured by Bradford method to normalize ALP activity.

2.8. Real time quantitative RT- PCR analysis

Total RNA from animal tissues and cell cultures was isolated by TRIzol reagent (Invitrogen, USA). After reverse transcription by using PrimeScript™ RT Master Mix (TaKaRa, Japan), 500 ng cDNA product was added to PCR reaction mixture containing TB Green Premix Ex Taq II (TaKaRa, Japan). Real time PCR was performed in 7900HT Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) with the amplification conditions and procedures: initial denaturation at 95 °C for 30 s, 40 cycles of denaturation at 95 °C for 1 s and 60 °C for 20 s. The sequences of primers for target genes and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are listed in Table S2. Relative gene expression was calculated by $2^{-\Delta\Delta CT}$ method.

2.9. Western blot

Cells were lysed with lysis buffer (Beyotime, Shanghai, China) containing 1 mM phenylmethyl sulfonyl fluoride (PMSF) as a protease inhibitor. Following protein extraction, 30 μg proteins of each sample were loaded and separated in a 10% SDS-PAGE to polyvinylidene fluoride (PVDF) membranes (Millipore, Danvers, USA). The proteins were blocked with 5% Blotting-Grade Blocker (Bio-Rad, USA) in TBST and incubated with following primary antibodies: rabbit anti-CYP27B1 (1:1000, Invitrogen, USA), rabbit anti-osteopontin (1:1000, Invitrogen, USA), or mouse anti-β-Actin (1:5000, Abcam, USA), followed by anti-rabbit (1:3000, Abcam, USA) or anti-mouse (1:3000, Invitrogen, USA) IgG HRP conjugated secondary antibodies. The blots were then probed and visualized with ClarityTM Western ECL substrate (Bio-Rad, USA) using AzureTM C600 (Azure Biosystems, USA). The relative intensities of the bands were quantified by Image J software (National Institutes of Health, USA) and normalized with β-actin.

2.10. Statistical analyses

Data from both *in vivo* and *in vitro* experiments were presented as mean ± standard error of mean (SEM). Intergroup differences between different treatments in animals were analyzed by one-way analysis of variance (ANOVA) followed by post hoc Tukey's test for multiple comparisons (GraphPad Prism 8.0, USA). Influences of CYP27B1 siRNA on the osteogenesis of MG-63 and interactions of $25OHD_3$ and OA on different parameters were analyzed by two-way ANOVA analysis (GraphPad Prism 8.0, USA). A p value of less than 0.05 was considered statistically significant.

3. Results

3.1. OA improved calcium homeostasis, bone turnover and properties in OVX mice

Actions of OA on bone properties and osteogenic markers in OVX mice were characterized firstly. As expected, ovariectomy induced significant uterine atrophy (Table 1, $p<0.001$ *vs.* sham) and reduced trabecular vBMD and micro-structural bone properties (Table 2, $p<0.001$ *vs.* Sham), indicating successful construction of osteoporotic animal model. E_2, but not high calcium diet (HCD) nor OA, increased uterine index in OVX mice (Table 1, $p<0.001$ *vs.* OVX). Serum Ca and P levels in mice were not altered by all the interventions in the study. In contrast, OVX resulted in the increase in urinary excretion of Ca, but not P, ($p<0.001$ *vs.* sham) in mice. Treatment with E_2 and OA significantly reduced urinary Ca excretion in OVX mice (Table 1, $p<0.01$ *vs.* OVX). Surprisingly, the increase in dietary calcium did not affect urinary Ca excretion, but significantly suppressed urinary excretion of P in OVX mice (Table 1, $p<0.01$ *vs.* OVX). Effects of treatment on bone turnover were evaluated by measuring procollagen I N-terminal propeptide (PINP, bone formation marker) and C-telopeptide of type I collagen (CTX-1, bone resorption marker) in serum. OVX significantly decreased serum PINP ($p<0.001$ *vs.* sham) and increased serum CTX-1 ($p<0.001$ *vs.* sham) levels in mice (Table 1). E_2 and OA significantly restored serum PINP (Table 1, $p<0.05$ vs. OVX), and suppressed serum CTX-1 to the level equivalent to that of sham-operated mice (Table 1, $p<0.001$ vs. OVX) in OVX mice. HCD significantly increased serum PINP levels ($p<0.01$ vs. OVX) but did not alter CTX-1 levels in OVX mice. Circulating $1,25(OH)_2D_3$ was not affected by OVX (*vs.* Sham) nor E_2 (*vs.* OVX) but was markedly suppressed by HCD ($p<0.01$ *vs.* OVX) in OVX mice. In contrast, serum $1,25(OH)_2D_3$ levels were significantly increased in OVX mice in response to treatment with OA (Table 1, $p<0.01$ *vs.* OVX),

Table 1
Effects of 17β-estradiol (E_2), high calcium diet (HCD) and oleanolic acid (OA) on the body weight gain, uterine index, and serum and urinary biochemical parameters in ovariectomized (OVX) mice.

	Sham	OVX	E_2	HCD	OA
Body weight and uterus index					
Weight gain, %	2.70 ± 1.22*	7.91 ± 0.95	-1.98 ± 0.44 ***	3.52 ± 1.21	4.28 ± 1.35
Uterine index, mg/g	2.74 ± 0.22***	0.65 ± 0.07	3.06 ± 0.26 ***	0.80 ± 0.08	0.73 ± 0.10
Serum chemistry					
Ca, mg/dL	7.51 ± 0.05	7.83 ± 0.11	7.66 ± 0.09	7.69 ± 0.08	7.91 ± 0.05
P, mg/dL	7.26 ± 0.52	7.35 ± 0.71	8.90 ± 0.41	7.36 ± 0.76	7.44± 0.43
PINP, ng/mL	37.05 ± 1.02 ***	31.52 ± 1.08	35.07 ± 0.64 *	35.93 ± 0.71 **	36.26 ± 0.57 **
CTX-I, ng/mL	0.37 ± 0.03 ***	0.55 ± 0.02	0.35 ± 0.03 ***	0.48 ± 0.02	0.35 ± 0.02 ***
$1,25(OH)_2D_3$, pg/mL	64.76 ± 4.69	59.06 ± 5.66	59.73 ± 2.13	35.96 ± 4.36 **	79.37 ± 3.97 **
Urine chemistry					
Ca/Cr, mg/mg	0.22 ± 0.02*	0.37 ± 0.03	0.21 ± 0.03 **	0.44 ± 0.04	0.18 ± 0.03 ***
P/Cr, mg/mg	2.01 ± 0.19	1.95 ± 0.16	1.87 ± 0.09	1.23 ± 0.05 **	1.97 ± 0.12

Six-month-old sham operated mice were fed with phytoestrogen-free AIN-93 M diet, the ovariectomized (OVX) mice were orally administrated with vehicle, E2 (200 μg/kg/day), HCD (1% calcium in AIN-93 M based rodent diet) and OA (200 ppm in AIN-93 M based rodent diet) for 8 weeks. Uterine index was calculated from the wet weight of uterus over the body weight. The urine calcium (Ca) and phosphorous (P) levels were normalized with urine creatinine (Cr) concentration. Procollagen I N-terminal propeptide (PINP), C-telopeptide of type I collagen (CTX-1). Data are demonstrated as mean ± SEM and analyzed by one-way ANOVA followed by Tukey's multiple comparison tests. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. OVX (n=8/group)

confirming the actions of OA on vitamin D and Ca homeostasis.

The effects of different treatments on trabecular bone properties at proximal tibia and lumbar vertebrae in OVX mice were evaluated (Table 2). As expected, E_2 significantly improved BMD, bone volume/total volume (BV/TV), trabecular thickness (Tb.Th) at both bone sites ($p<0.05$ *vs.* OVX), improved trabecular number (Tb.N) and connectivity density (Conn.D) and decreased trabecular separation (Tb.Sp) at lumbar vertebrate ($p<0.05$ *vs.* OVX) in OVX mice. HCD and OA could significantly increase BMD, BV/TV and Tb.Th at both sites, and also increased Conn.D and reduced Tb.Sp at lumbar vertebrate in OVX mice ($p<0.05$ *vs.* OVX).

3.2. OA upregulated CYP27B1 expression and osteogenesis in iliac crests of OVX mice

The effects of OA on vitamin D bioactivation and osteogenesis in renal and extra-renal tissues were further evaluated in kidney and iliac crests, respectively. As shown in Fig. 1A, CYP27B1 protein expression in kidney was markedly suppressed by OVX ($p<0.001$ *vs.* Sham) in mice. Such a decrease in renal CYP27B1 expression was significantly reversed by treatment with E_2 ($p<0.05$ *vs.* OVX) and OA ($p<0.001$ *vs.* OVX) in OVX mice. HCD further decreased renal CYP27B1 expression in OVX mice (Fig. 1A, $p<0.05$ *vs.* OVX). The regulation of CYP27B1 in iliac crest appeared to be different. Neither ovariectomy nor E_2 treatment affected CYP27B1 protein expressions in iliac crests (Fig. 1B). In contrast, CYP27B1 protein expressions in iliac crest were significantly induced in response to HCD by 2-fold (Fig. 1B, $p<0.05$ *vs.* OVX) and OA by more than 4-fold (Fig. 1B, $p<0.001$ *vs.* OVX) in OVX mice. To determine the *in vivo* effects of OA on osteogenesis, the mRNA expressions of OPN and ALP in iliac crest were studied. OA significantly upregulated the mRNA

Table 2
Effects of 17β-estradiol (E_2), high calcium diet (HCD) and oleanolic acid (OA) on trabecular bone properties at proximal tibia and fourth lumbar vertebrae (L4) in ovariectomized (OVX) mice.

	Sham	OVX	E_2	HCD	OA
Proximal tibia					
BMD, mg HA / cm^3	185.33 ± 17.22 ***	76.73 ± 7.56	144.49 ± 5.56 ***	128.00 ± 4.55 **	115.54 ± 7.35 *
BV/TV, %	14.60 ± 2.00 ***	5.34 ± 0.80	11.44 ± 0.57 **	9.91 ± 0.65 *	9.65 ± 0.77 *
Tb. N, mm^{-1}	4.43 ± 0.14	4.22 ± 0.07	3.99 ± 0.05	4.13 ± 0.07	4.50 ± 0.22
Tb. Th, μm	59.70 ± 3.03 ***	47.49 ± 1.04	61.06 ± 1.35 ***	57.80 ± 1.43 ***	56.13 ± 0.96 **
Tb. Sp, μm	233.26 ± 6.83	250.66 ± 4.14	244.30 ± 3.76	243.87 ± 3.55	236.19 ± 6.40
Conn. D, mm^3	77.90 ± 6.98	32.50 ± 2.92	74.08 ± 6.05	51.32 ± 1.35	57.19 ± 4.79
Lumbar vertebrae					
BMD, mg HA / cm^3	300.82 ± 26.59 ***	159.47 ± 9.99	233.97 ± 11.02 **	241.45 ± 6.71 **	234.13 ± 18.63 **
BV/TV, %	30.00 ± 3.82 ***	12.81 ± 1.40	21.09 ± 1.39 *	22.05 ± 0.77 *	21.94 ± 2.60 *
Tb. N, mm^{-1}	3.86 ± 0.22 ***	2.73 ± 0.08	3.20 ± 0.10 *	3.15 ± 0.07	3.13 ± 0.06
Tb. Th, μm	76.23 ± 3.01 ***	57.09 ± 1.10	73.20 ± 2.07 ***	72.44 ± 1.85 ***	65.29 ± 1.73 *
Tb. Sp, μm	247.54 ± 15.81 ***	348.99 ± 14.63	295.70 ± 11.62 *	305.34 ± 8.66 *	303.18 ± 5.37 *
Conn. D, mm^3	79.01 ± 4.62 ***	46.66 ± 3.58	67.50 ± 4.71 **	65.56 ± 2.29 **	65.73 ± 4.06 **

Six-month-old sham operated mice were fed with phytoestrogen-free AIN-93 M diet, the ovariectomized (OVX) mice were orally administrated with vehicle, E2 (200 μg/kg/day), HCD (1% calcium in AIN-93 M based rodent diet) and OA (200 ppm in AIN-93 M based rodent diet) for 8 weeks. Trabecular bone properties at proximal tibia and fourth lumbar vertebra (L4) including bone mineral density (BMD), bone volume/total volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), and connectivity density (Conn.D) were evaluated by microcomputed tomography (μCT). Data are demonstrated as mean ± SEM and analyzed by one-way ANOVA followed by Tukey's multiple comparison tests. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. OVX (n=8/group).

expression of OPN (Fig. 1C, $p<0.001$ *vs.* OVX) and ALP (Fig. 1D, $p<0.05$ *vs.* OVX), the vitamin D-responsive osteogenic markers, in OVX mice. To characterize the relationship between renal and extra-renal CYP27B1 and bone formation induced by OA, correlation between mRNA expression of CYP27B1 in kidney and iliac crest and serum level of PINP was studied. As shown in Fig. 1E and Fig. 1F. the expression of iliac crest CYP27B1 mRNA, but not those in kidney, was significantly correlated with serum level of bone formation marker PINP ($p<0.01$) in mice. The results suggested the role of locally expressed CYP27B1 in mediating the effects of OA on bone metabolism.

3.3. OA upregulated expression and activity of CYP27B1 in osteoblast-like cells

We further investigated the actions of OA on CYP27B1 expression and activity in human osteoblastic cells MG-63 *in vitro*. As shown in Fig. 2, PTH (100 nM) significantly upregulated CYP27B1 protein (Figs. 2A, 2B, $p<0.05$ *vs.* control) and mRNA (Fig. 2C, $p<0.05$ *vs.* control) expressions in MG-63 cells upon treatment for 24 hours. Protein expression of CYP27B1 could also be stimulated by 1 nM to 1μM OA (Fig. 2A and B, $p<0.05$ *vs.* control) while mRNA expression level of CYP27B1 could be induced by 1 nM OA (Fig. 2C, $p<0.05$ *vs.* control) in MG-63 cells. The effect of OA on CYP27B1 activity in MG-63 cells was determined. As expected, no detectable $1,25(OH)_2D_3$ level in MG-63 cells was observed unless adequate $25OHD_3$ (1 μM) substrate was

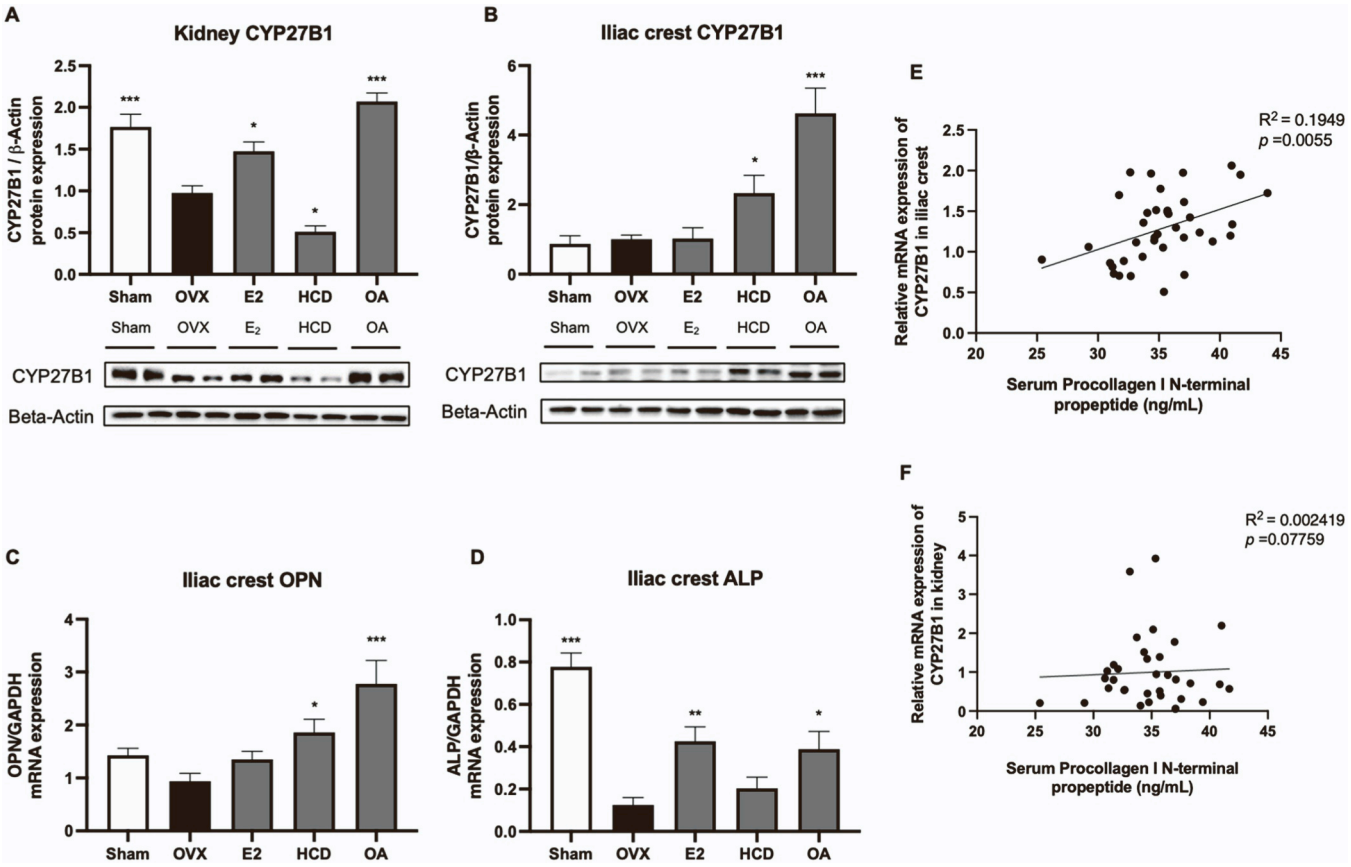

Fig. 1. Effects of 17β-estradiol (E_2), high calcium diet (HCD) and oleanolic acid (OA) on expression of CYP27B1 in kidney and iliac crests of OVX mice. Six-month-old sham operated mice were fed with phytoestrogen-free AIN-93 M diet, the ovariectomized (OVX) mice were orally administrated with vehicle, E_2 (200 μg/kg/day), HCD (1% calcium in AIN-93 M based rodent diet) and OA (200 ppm in AIN-93 M based rodent diet) for 8 weeks. mRNA and protein were isolated from kidney and iliac crests. Protein expression of CYP27B1 (A) and iliac crests (B) were determined by western blotting. mRNA expression of osteopontin (OPN, C) and alkaline phosphatase (ALP, D) in iliac crests were determined by RT-PCR. Correlation between mRNA expression of CYP27B1 in iliac crest (E) and kidney (F) and serum level of procollagen I N-terminal propeptide (PINP) was calculated by linear regression. Data are expressed as mean ± SEM, n=8. Differences between groups were analyzed by one-way ANOVA followed by Tukey's multiple comparison tests. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. OVX (n=8/group).

added (Fig. 2D). Our results showed that both PTH (0.1μM) and OA (1 nM) significantly upregulated $1,25(OH)_2D_3$ synthesis in MG-63 cells (Fig. 2D, $p<0.05$ *vs.* $25OHD_3$).

To determine the effects of OA on vitamin D-responsive osteogenic markers, ALP activities (Fig. 2E) and OPN protein expression (Fig. 2A and F) in MG-63 cells were determined. $1,25(OH)_2D_3$ was found to dramatically increase ALP activity for more than 4-fold (Fig. 2E, $p<0.001$ *vs.* control) and modestly induce OPN protein expression (Fig. 2F, $p<0.01$ *vs.* control) in MG-63 cells. Similarly, OA could significantly induce ALP activity at 1–100 nM (Fig. 2E, $p<0.05$ *vs.* control) and increase OPN protein expressions at 1 nM to 1μM (Fig. 2F, $p<0.01$ *vs.* control) in MG-63 cells. These results suggested that the osteogenic effects of OA might be associated with its actions on modulating $1,25(OH)_2D_3$ production in osteoblasts.

3.4. OA improved cellular production of $1,25(OH)_2D_3$ and osteogenesis of hMSCs

To evaluate the effects of OA on vitamin D metabolism and osteogenesis in senescent cells, human mesenchymal stem cells (hMSCs) collected from aged subjects were employed and incubated in osteogenic medium for 0, 7, 14 and 21 days. Osteogenesis of hMSCs were assessed by visualization of the cellular morphology and time-dependent patterns of expressions of osteogenic markers during the differentiation (Fig. S1). Our results showed that the mRNA expression of osteogenic markers (Runx2, Col-1, BSP2, OPN) increased with osteogenic differentiation in hMSCs (Fig. S1A & B). Most importantly, CYP27B1 and VDR mRNA expression also increased with the induction of osteogenic differentiation in hMSCs (Fig. S1C). As shown in Fig. 3A & B, OA appeared to have little effect on the expression and activity of CYP27B1 when the cells were at the initial stages i.e. osteoprogenitors and preosteoblasts (day 0–7). However, CYP27B1 mRNA expression and cellular production of $1,25(OH)_2D_3$ were significantly upregulated in response to the treatment of PTH and OA when hMSCs were further differentiated into mature osteoblasts (day 14–21, Fig. 3A & B, $p<0.05$ vs. control of each stage). Expression of VDR in osteogenic cells derived from hMSCs increased markedly during the last two weeks of differentiation, which reached the highest level after differentiation for 21 days (Fig. 3A, $p<0.001$ vs. Day 0). As the major components of extracellular matrix, basal expression levels of type 1 collagen (Col-1), bone sialoprotein 2 (BSP2) as well as osteopontin (OPN) were much higher in the last stage of osteogenic differentiation in cells (Fig. 3A, $p<0.05$ vs. Day 0) and their expression were further improved by treatment with OA (Fig. 3A, day 14–21, $p<0.05$ vs. control). The effects of OA on bone mineralization of hMSCs were also determined (Fig. 3 C & D). Treatment of hMSCs with $1,25(OH)_2D_3$, PTH and OA (1 nM to 1μM) for 25 days in osteogenic medium significantly increased bone mineralization as revealed by Alizarin Red staining (Fig. 3 C & D, $p<0.05$ vs. control). Our results showed that OA significantly potentiated osteogenesis and bone mineralization of hMSCs at the mature osteogenic stages. The osteogenic actions of OA appeared to be associated with the increase in cellular synthesis of $1,25(OH)_2D_3$ induced by OA in mature osteoblasts.

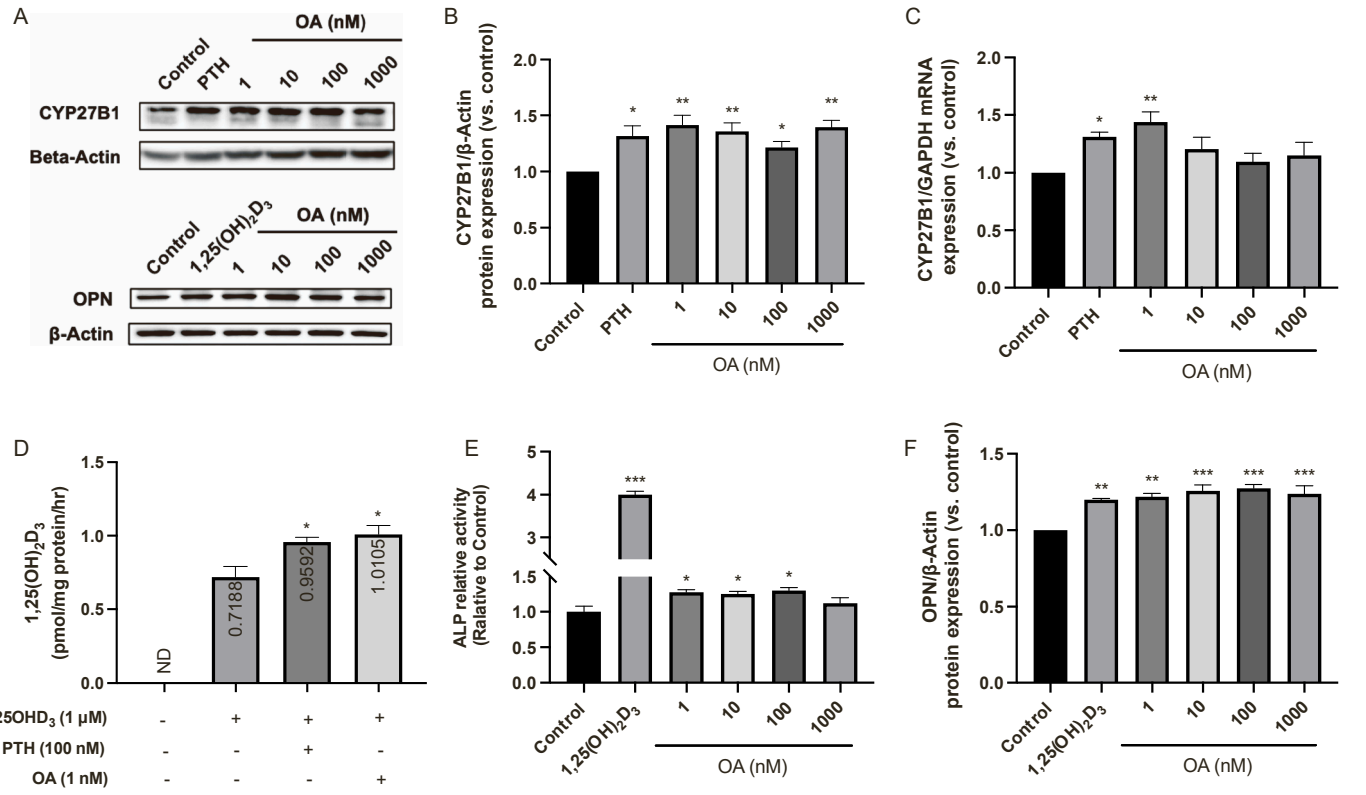

Fig. 2. Effects of OA on expressions and activity of CYP27B1 and osteogenic markers in MG-63 cells. Cells were exposed to PTH (100 nM), $1,25(OH)_2D_3$ (10 nM) or OA (1, 10, 100, 1000 nM) in phenol red-free medium for 24 hours. Protein expressions of CYP27B1 (A, B) and osteopontin (OPN, A, F) were determined by Western blotting. mRNA expression of CYP27B1 (C) was determined by RT-PCR. For CYP27B1 activity (D), cells were treated with PTH (100 nM) and OA (1 nM) in phenol red-free and serum-free medium in supplementation of $25(OH)D_3$ (1 μM) as substrate and N, N'-diphenylethylene-diamine (10 μM) as antioxidant for 4 hours. The CYP27B1 activity was determined by measuring cellular secreted $1,25(OH)_2D_3$ in supernatant per hour. None detected (ND). ALP activity (E) was determined after treatment of $1,25(OH)_2D_3$ or OA for 7 days. Data are expressed as mean ± SEM, n=3. Differences between treatment groups and control were analyzed by one-way ANOVA followed by Dunnett's multiple comparison tests. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control.

3.5. The osteogenic effects of OA in mature osteoblasts were CYP27B1 dependent

To mimic the physiological conditions that $25OHD_3$ was supplied by the circulation, we further evaluated the effects of OA on CYP27B1 and osteogenesis in the presence of $25OHD_3$. MG-63 cells were pre-treated with $25OHD_3$ for 12 hours before exposure to OA. As shown in Fig. 4, pre-treatment of $25OHD_3$ significantly upregulated CYP27B1 expression (p <0.05 vs. control) as well as OPN expression and ALP activity (p <0.001 vs. control) in MG-63 cells. Such changes indicated the ability of MG-63 cells to convert $25OHD_3$ into $1,25(OH)_2D_3$ and thus respond to the elevated intracellular $1,25(OH)_2D_3$. More importantly, OA markedly potentiated the effects of $25OHD_3$ on CYP27B1 expression (Fig. 4B, p<0.01 vs. $25OHD_3$) in MG-63 cells. Addition of OA (10, 100 nM) significantly increased the protein expression of OPN as well as ALP activity in MG-63 cells when compared to treatment with $25OHD_3$ alone (Fig. 4B, p <0.05). These results further provide evidence for the correlation of upregulated CYP27B1 and osteogenic effects of OA in osteoblasts.

Next, we evaluated the role of CYP27B1 in the osteogenic effects of OA in mature osteoblast-like MG-63 cells. Here 1 nM and 10 nM were selected as the optimal concentrations of OA, because these concentrations are within the range of serum levels of OA (10 ng/mL) upon its administration to healthy adults [27]. As shown in Fig. 5 A & B, transfection of CYP27B1 siRNA significantly silenced the protein expression of CYP27B1 by 55% (p <0.01 vs. Non-silencing control siRNA) in MG-63 cells, indicating the effective knockdown of CYP27B1. The increased expression of CYP27B1 by OA were markedly attenuated with the knockdown of CYP27B1 (Fig. 5A & B, p <0.001 vs. Non-silencing control siRNA). Similarly, transfection of CYP27B1 siRNA suppressed osteogenesis of MG- 63 cells, as revealed by the decrease in protein expression of OPN and ALP activity as compared to the non-silencing control group (Fig. 5A, C & D, p <0.01). These results showed the important role of cellular produced $1,25(OH)_2D_3$ by CYP27B1 in the anabolic metabolism of MG-63 cells. Moreover, the upregulated OPN expression and ALP activity in response to OA were significantly reduced in response to knockdown of CYP27B1 in the cells (p <0.001). Our results clearly indicated the importance of CYP27B1 in mediating the osteogenic effects of OA in MG-63 cells.

4. Discussion

Reduction in osteogenesis and vitamin D bioactivation played important part in the development of age-related bone loss [14]. Our previous studies reported the effects of OA on restoring age-related bone loss and impaired calcium balance [24,28]. In the current study, we attempted to deepen our understanding of the effects of OA on vitamin D bioactivation in kidney and bone, and their contribution to osteogenic effects of OA.

Our results confirmed that administration of OA at 200 ppm (i.e. 25 mg/kg body weight/day) for 8 weeks effectively improved BMD and bone microarchitecture in mature OVX mice. Moreover, OA behaves like E_2 in retarding accelerated bone remodeling in OVX mice, as revealed by their actions on suppressing bone resorption marker CTX-1 and stimulating bone formation marker PINP. Such findings agree with previous reports showing the increase in osteoblast numbers and osteogenesis of BMSCs while the decrease in osteoclastic differentiation of bone marrow macrophages (BMMs) by OA in OVX mice [22,23,29]. Apart from its

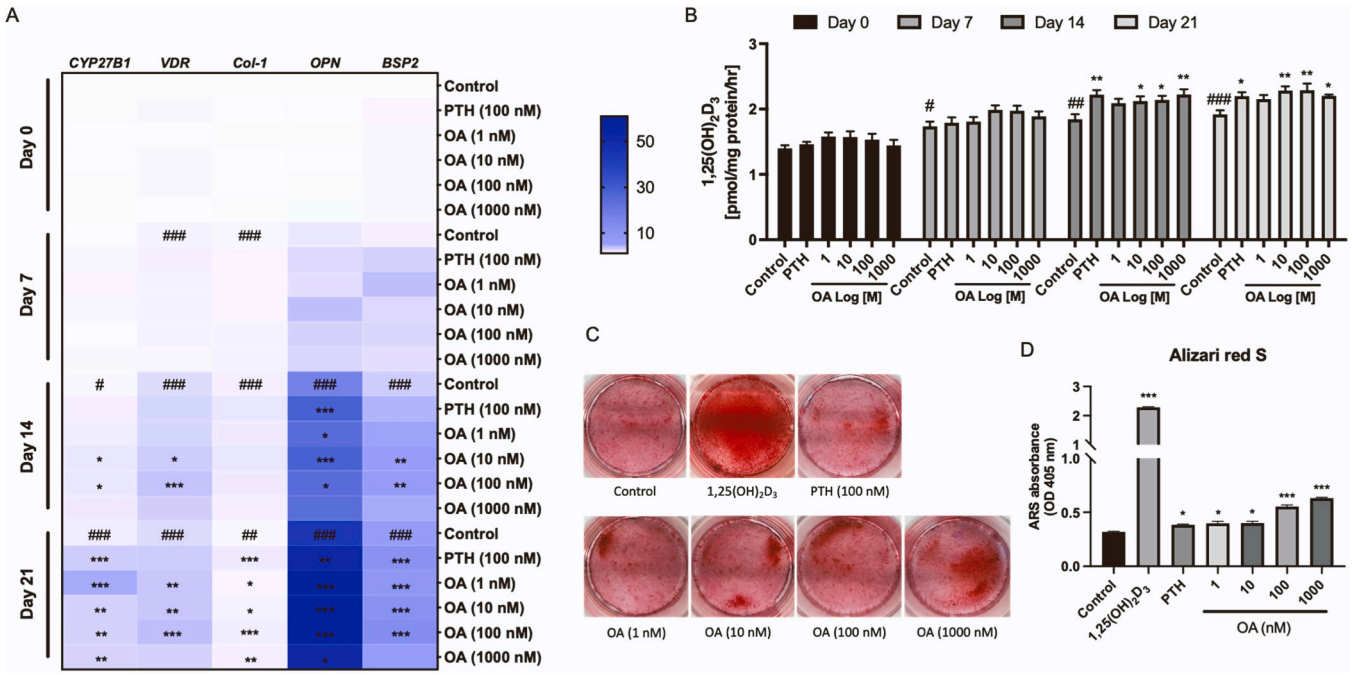

Fig. 3. Effects of OA on CYP27B1 and osteogenesis of human mesenchymal stem cells (hMSCs). hMSCs from a 61-year-old female and a 60-year-old male subjects were incubated in osteogenic induction medium for 7, 14, 21 days to obtain osteoprogenitors, pre-osteoblasts, and mature osteoblasts, respectively. At each of osteogenic stages, cells were treated with parathyroid hormone (PTH, 100 nM) or OA (1, 10, 100, 1000 nM) for 24 hours. mRNA expression levels of CYP27B1, vitamin D receptor (VDR), type I collagen (Col-1), osteopontin (OPN) and bone sialoprotein 2 (BSP2) (A) were determined by RT-PCR. Cells were treated with PTH (100 nM) or OA (1, 10, 100, 1000 nM) in phenol red-free and serum-free medium in supplementation of 25(OH)D_3 (1 μM) as substrate and N, N'-diphenylethylenediamine (10 μM) as antioxidant for 4 hours, cellular secreted 1,25$(OH)_2D_3$ (B) in supernatant was determined by ELISA kit. Cells were incubated in osteogenic medium in the presence of 1,25$(OH)_2D_3$ (10 nM), PTH (100 nM) or OA (1, 10, 100, 1000 nM) for 25 days. The mineral nodules (C) were stained with Alizarin Red S staining. The Alizarin Red S-stained particles (D) were quantified by acetic acid extraction. Data are expressed as mean ± SEM, n=3. Differences between treatment group and control were analyzed by one-way ANOVA followed by Dunnett's multiple comparison tests. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control of each osteogenic stage. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. control of Day 0.

suppressing effects on bone remodeling, OA significantly reduced urinary Ca loss in OVX mice. Moreover, OA appeared to mimic the effects of HCD in upregulating bone formation marker PINP, suggesting that the bone protective effects of OA might in part mediated by its actions on calcium homeostasis. In fact, our previous study reported that OA could improve Ca balance in aged female rats, possibly by induction of duodenal Ca transport protein expression [24]. It is possible that such effects of OA are associated with its stimulatory effects on the circulating level of 1,25$(OH)_2D_3$, rather than mimicking the effects of E_2.

The understanding of the regulation of CYP27B1 in bone is limited, although many studies showed that it was different from that in kidney [30–32]. Renal CYP27B1 is tightly regulated by hormonal factors like PTH, FGF23 and 1,25$(OH)_2D_3$ to maintain calcium and phosphate homeostasis, while the locally expressed CYP27B1 in bone could be stimulated in response to local requirement to exert multiple autocrine or paracrine activities in modulating bone metabolism [31,33,34]. In supporting of these findings, our results showed that OA stimulated the expression of CYP27B1 in both kidney and iliac crests with different potency, while HCD regulated CYP27B1 in these two sites with opposite effects. The more potent effects of OA on CYP27B1 expression in iliac crests suggested that the osteoprotective effects of OA might be associated with its regulation of CYP27B1 expression in bone tissues. Such actions of OA on local skeletal CYP27B1 expression might further contribute to its anabolic effects in bone tissue, as revealed by induction in osteogenic markers ALP and OPN. Our current results showed a strong positive correlation between serum level of PINP and CYP27B1 expression in iliac crest instead of kidney, indicating that CYP27B1 in bone tissues are important site for regulation and can be induced for exerting anabolic effects in bone.

In lining with previous findings, the present study clearly showed that osteoblastic cells were capable to generate 1,25$(OH)_2D_3$, when supplemented with adequate levels of 25OHD_3 [35–37]. Moreover, our results showed increased ALP activity and OPN expression in addition to upregulated CYP27B1 expression and 1,25$(OH)_2D_3$ production in MG-63 cells. In fact, such locally produced 1,25$(OH)_2D_3$ does not act via endocrine pathways, but functions through autocrine or paracrine mechanisms to induce osteoblastic differentiation and mineralization [36–38]. Notably, the effective dosage of OA was as low as 1 nM, its effects are comparable to those of 100 nM PTH, the known hormonal factor that induce expressions and enzymatic activity of CYP27B1 [14]. It is reported in a human pharmacokinetics study that oral administration of OA (40 mg) resulted in a peak plasma concentration (C_{max}) of 26 nM within 5.2 hours, and the half-life ($t_{1/2}$) of 8.7 hours [27], suggesting that similar actions of OA in osteoblasts are achievable in humans upon oral consumption.

Our study showed that CYP27B1 expression and cellular 1,25 $(OH)_2D_3$ production increased time-dependently along with the osteogenic process of hMSCs, suggesting that locally produced 1,25$(OH)_2D_3$ might be required for the differentiation and functions of osteoblasts [31]. These results were in line with those reported for vitamin D receptor (VDR) expression by us or others for its increase in hMSCs upon osteogenic induction [39], indicating the important role of vitamin D on osteogenesis. In fact, overexpression of VDR specifically in mature osteoblasts was known to increase BMD in transgenic mice model [40]. These observations suggested that the upregulation of CYP27B1 expression and activity in osteoblastic linages might be associated with the bone anabolic processes. In the present study, OA improved local production of 1,25$(OH)_2D_3$ at later stages of maturation and acted like 1, 25$(OH)_2D_3$ in modulating the expressions of vitamin D responsive genes in hMSCs derived from older adults. It appeared that the responses of

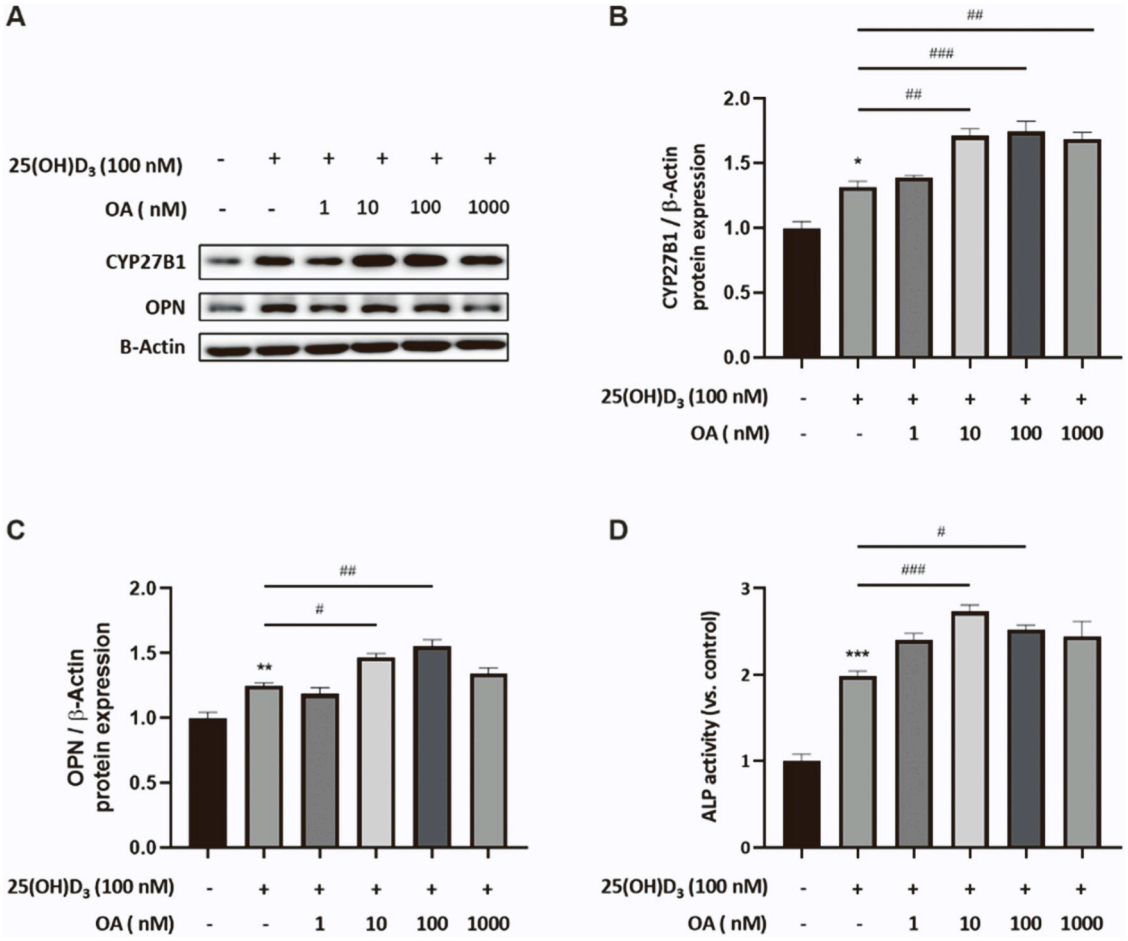

Fig. 4. Effects of OA on CYP27B1 and osteogenic markers in MG-63 cells in supplement with physiological levels of 25(OH)D_3. Cells were pre-treated with 25(OH)D_3 (100 nM) for 12 hours, followed by treatment of OA (1–1000 nM) for 24 hours. Protein expressions of CYP27B1 (A, B) and osteopontin (OPN; A, C) were determined by Western blotting. ALP activity (D) was measured in cells pre-treated with 25(OH)D_3 (100 nM) for 12 hours before further treatment with OA for 7 days. Data are expressed as mean ± SEM, n=3. Differences between treatment group and control were analyzed by two-way ANOVA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. 25(OH)D_3.

hMSCs to OA were dependent on the differentiation stages. Our result showed a robust in expressions of BSP2 and OPN in response to treatment of OA at later stages, but not at the early differentiation stages of hMSCs. It is worth noting that both BSP2 and OPN are matrix proteins that engage in the extracellular matrix mineralization and maturation of the osteoblasts [13, 41–44]. This is positively correlated with our results showing that OA act like 1,25$(OH)_2D_3$ and enhanced matrix mineralization of hMSCs. In viewing of the declined osteoblastic activity of CYP27B1 in older adults, OA could be a promising bone anabolic agent for management of age-related bone loss.

The beneficial effects of OA on osteogenesis appeared to be dependent on its actions on osteoblastic CYP27B1. In line with previous studies, our results showed exposure of mature osteoblastic cells to the physiological levels of 25(OH)D_3 resulted in not only the upregulation of CYP27B1 but also the induction of 1,25$(OH)_2D_3$ responsive markers in MG-63 cells [37,44]. More importantly, such induction in osteogenesis by 25(OH)D_3 were further enhanced by adding of OA as revealed by elevated OPN expression as well as ALP activity. On the other hand, silencing of CYP27B1 with CYP27B1 siRNA not only resulted in reduction of CYP27B1 expression and activity, but also lead to decreased bone anabolism in mature osteoblasts [35,37]. Moreover, such attenuation in osteoblast differentiation by CYP27B1 siRNA could not be restored even by OA. Again, our study showed that CYP27B1 in mature osteoblasts is biologically relevant, and it could be a major therapeutic target of OA in mediating bone metabolism.

Several limitations should not be ignored from present study. First of all, OVX-induced osteoporotic animals seem not the perfect model for studying age-related mechanisms of vitamin D metabolism, as OVX did not affect expression level of CYP27B1 in bone of such model. Natural aging mice or senescence accelerated models (SAMP8) could be the choices for future study. In addition, only one dose of OA was included in the animal study. Our previous study evaluated the effects of high dose (100 mg/kg/day) and low dose (50 mg/kg/day) of OA on systemic calcium balance and vitamin D metabolism [24]. In present study we selected the effective dose of OA based on previous findings. Moreover, considering the strong hydrophobicity and poor bioavailability, OA was incorporated into control diet for administration this time.

5. Conclusions

In summary, we demonstrated that OA effectively improved BMD and bone microarchitectures in OVX mice. The bone protective effects of OA partially come from the improved calcium balance via regulating circulating 1,25$(OH)_2D_3$ levels. On the other hand, OA induced expressions of CYP27B1 and local biosynthesis of 1,25$(OH)_2D_3$ in mature osteoblastic cells. To our knowledge, the present study was the first to report the modulatory effect of a naturally presented small molecule on vitamin D metabolism in bone cells. The findings extended our understandings of the paracrine or autocrine actions of 1,25$(OH)_2D_3$ in bone microenvironment and provides new insights into the approach for

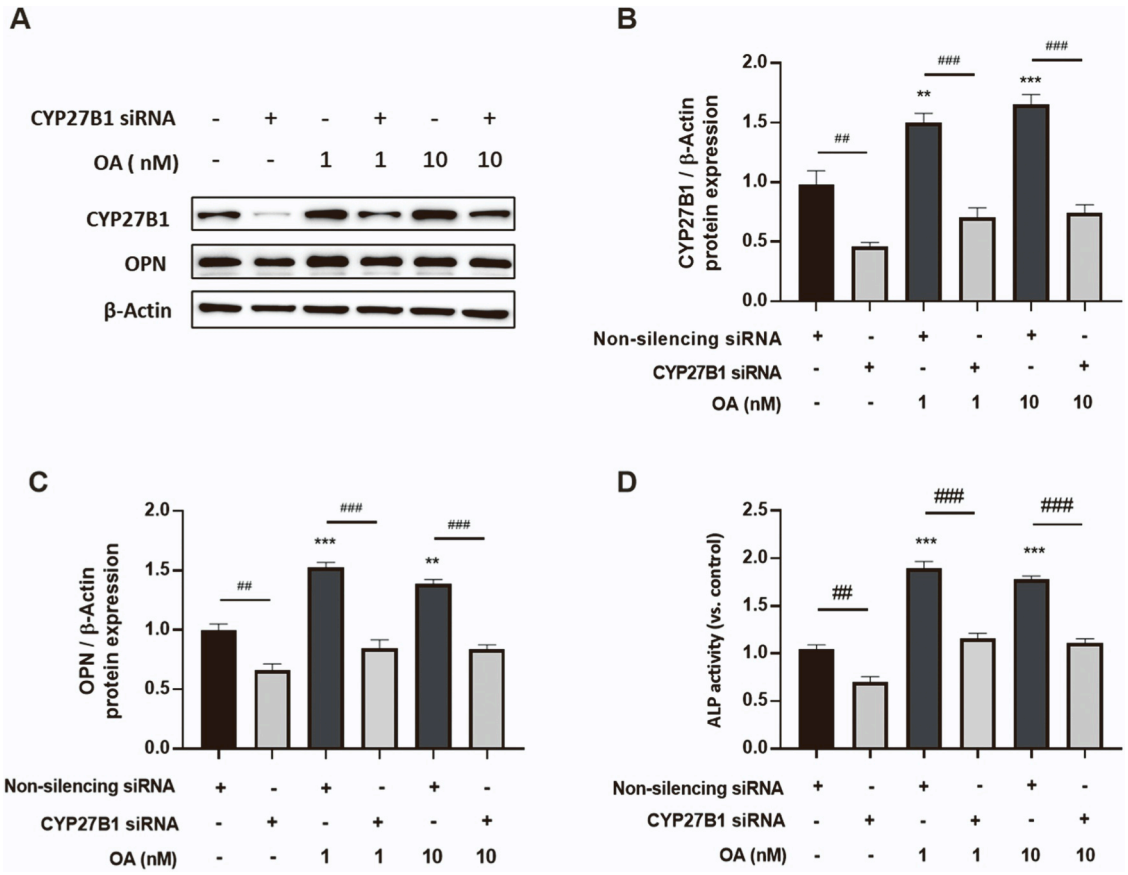

Fig. 5. Involvement of CYP27B1 in OA-induced osteogenesis of MG-63 cells. Cells were transfected with non-silencing control siRNA or CYP27B1 siRNA for 48 hours. Protein expressions of CYP27B1 (A, B) and osteopontin (OPN; A, C) in response to OA (1, 10 nM) in the presence or absence of CYP27B1 siRNA were determined in cells after treatment for 24 hours. (D) Alkaline phosphatase (ALP) activity was determined in cells treated with OA (1, 10 nM) for 7 days. Data are expressed as mean ± SEM, n=3. Differences between treatment group and control were analyzed by two-way ANOVA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. treatment group transfected with non-silencing control siRNA.

modulating CYP27B1 in bone cells, which might be a potential target for prevention and treatment of age-related bone loss.

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CRediT authorship contribution statement

Wen-Xuan Yu: Methodology, Project administration, Writing – original draft. **Christina Chui-Wa Poon:** Methodology. **Li-Ping Zhou:** Writing – review & editing. **Ka-Ying Wong:** Methodology. **Sisi Cao:** Supervision. **Chung-Yan Lam:** Project administration. **Wayne Yuk-wai Lee:** Methodology. **Man Sau Wong:** Resources, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2024.116402.

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