

Effects of the 1, 4-dihydropyridine L-type calcium channel blocker benidipine on bone marrow stromal cells

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Abstract Osteoporosis (OP) often increases the risk of bone fracture and other complications and is a major clinical problem. Previous studies have found that high blood pressure is associated with bone formation abnormalities, resulting in increased calcium loss. We have investigated the effect of the antihypertensive drug benidipine on bone marrow stromal cell (BMSC) differentiation into osteoblasts and bone formation under osteoporotic conditions. We used a combination of in vitro and in vivo approaches to test the hypothesis that benidipine promotes murine BMSC differentiation into osteoblasts. Alkaline phosphatase (ALP), osteocalcin (OCN), runt-related transcription factor 2 (RUNX2), β -catenin, and low-density lipoprotein receptor-related protein 5 (LRP5) protein expression was evaluated in primary femoral BMSCs from C57/BL6 mice cultured under osteogenic conditions

for 2 weeks to examine the effects of benidipine. An ovariectomized (OVX) mouse model was used to investigate the effect of benidipine treatment for 3 months in vivo. We found that ALP, OCN, and RUNX2 expression was up-regulated and WNT/ β -catenin signaling was enhanced in vitro and in vivo. In OVX mice that were intragastrically administered benidipine, bone parameters (trabecular thickness, bone mineral density, and trabecular number) in the distal femoral metaphysis were significantly increased compared with control OVX mice. Consistently, benidipine promoted BMSC differentiation into osteoblasts and protected against bone loss in OVX mice. Therefore, benidipine might be a suitable candidate for the treatment of patients with postmenopausal osteoporosis and hypertension.

Z.-p.M. and J.-c.L. contributed equally to this work.

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Introduction

Several studies have reported that high blood pressure is associated with bone formation abnormalities, resulting in increased calcium loss, plus secondary activation of the parathyroid gland and increased calcium removal from bone (Brickman et al. 1990; Gadallah et al. 1991; Grobbee et al. 1988; Hvarfner et al. 1987; McCarron et al. 1980; Strazzullo et al. 1983; Young et al. 1992). Similarly, research conducted in hypertensive animal models has shown that hypercalciuria and subsequent hyperparathyroidism reduces growth and decreases total bone mineral content later in life (Cirillo et al. 1989; Izawa et al. 1985). Clinically, approximately 40 % of women over the age of 50 will suffer a fracture related to postmenopausal osteoporosis during their lifetime (Melton et al. 1992). Recently, higher blood pressure in elderly Caucasian women has also been reported to be associated with increased bone loss at the femoral neck possibly contributing to bone fracture (Cappuccio et al. 1999).

Benidipine (BD; Fig. 1a) is a dihydropyridine-type calcium channel blocker that has been widely used in hypertension therapy. Calcium channel blockers primarily inhibit calcium influx through L-type voltage-dependent calcium channels on smooth muscle cell vessels, thereby disrupting the excitation contraction process (Katz et al. 1984). Previous studies have demonstrated that BD positively affects bone metabolism (Nishiya and Sugimoto 2001; Nishiya et al. 2002; Wang et al. 2014). However, the mechanism remains unclear, and few studies have examined the effects of antihypertensive drugs on bone function in animal models of postmenopausal osteoporosis.

Bone marrow stromal cells (BMSCs) are composed of progenitor and multipotent skeletal stem cells and are able to differentiate into osteocytes, adipocytes, and chondrocytes in vitro. During the aging process, BMSC differentiation into osteoblasts decreases, whereas BMSC differentiation into adipocytes increases, thereby resulting in decreased osteogenesis and bone loss. Nevertheless, osteoblasts play a pivotal role in the regulation of bone formation. During differentiation, osteoblasts express osteocalcin (OCN), alkaline phosphatase (ALP), runt-related transcription factor 2 (RUNX2), and other bone matrix proteins and ultimately undergo mineral deposition. Therefore, BMSCs have an important role in bone metabolism. Moreover, because BMSCs can differentiate into skeletal cell phenotypes (Bianco et al. 2006), they are a good tool for studying the metabolism of osteoblast differentiation.

Osteoblast differentiation is predominantly regulated by WNT/ β -catenin signaling (the canonical WNT pathway), which acts as the master regulator of osteogenesis (Baron

and Rawadi 2007; Canalis et al. 2007). Canonical WNT signaling also functions in the fate determination of mesenchymal stem cells (Baron and Rawadi 2007). WNT/ β -catenin signaling plays a critical role in bone tissue by controlling the differentiation of stem cells into mature osteoblasts, rather than chondrocytes and adipocytes (Rossini et al. 2013). In the absence of β -catenin, these cells do not differentiate into mature OCN-expressing osteoblasts (Hu et al. 2005; Rodda and McMahon 2006). In addition, low-density lipoprotein receptor-related protein 5 (LRP5), a downstream effector of WNT signaling, can promote bone formation in humans and mice (Boyden Lynn et al. 2002; Gong et al. 2001; Little et al. 2002). Thus, the WNT/ β -catenin signaling pathway is central to osteogenesis and bone formation (Canalis et al. 2007).

In order to understand the mechanism of action of BD during bone formation, the effect of BD on BMSC function needs to be further characterized. To examine BMSCs, we have used ovariectomized (OVX) C57/BL6 mice as an animal model that mimics postmenopausal osteoporosis in humans in order to investigate whether BD affects bone density and OCN and RUNX2 expression in vivo.

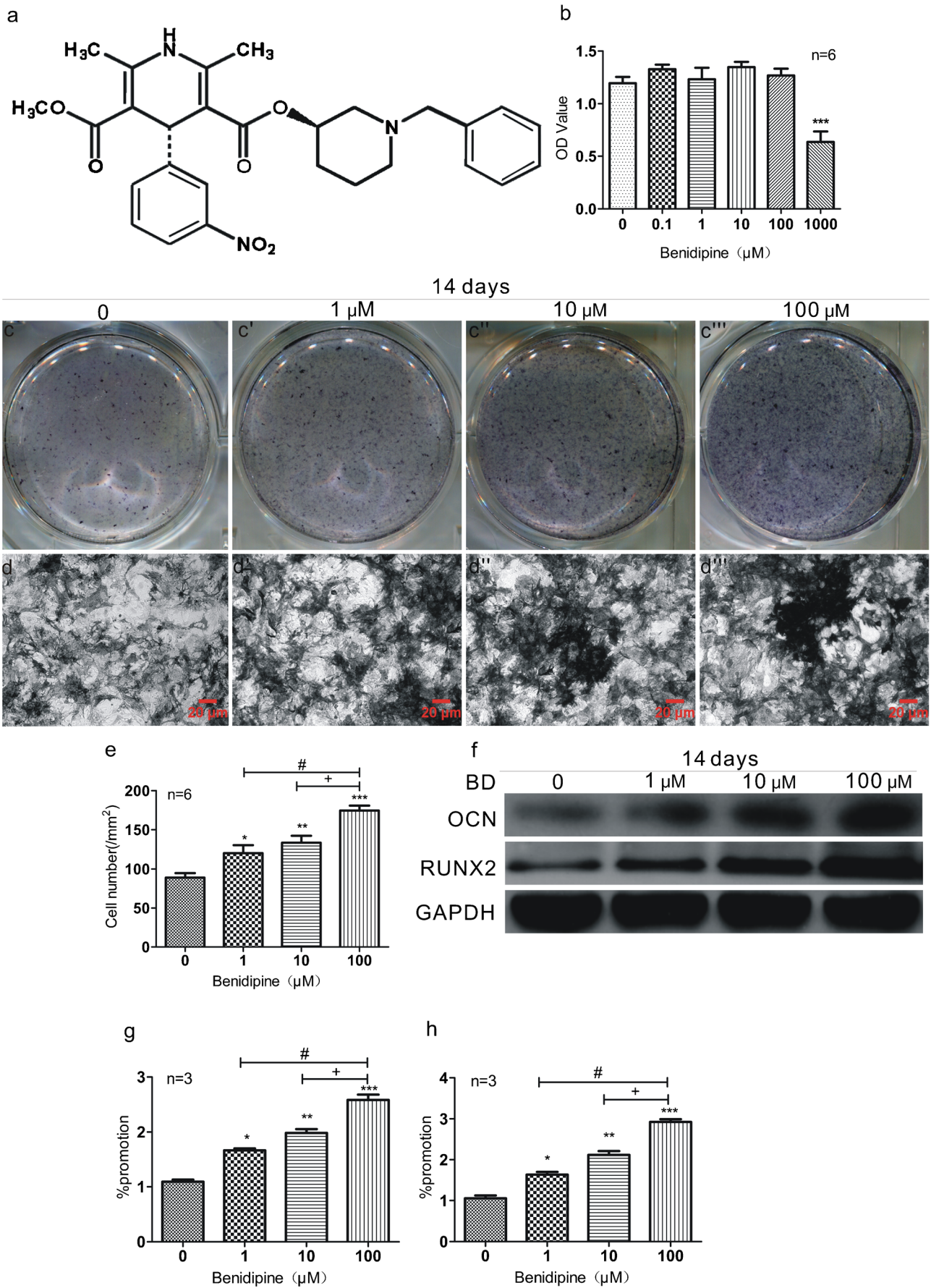
In the present study, we have found that BD increases ALP activity in long-term cultures of BMSCs and augments OCN and RUNX2 accumulation. In BD-treated OVX mice, trabecular thickness (Tb.Th), bone mineral density (BMD), and trabecular number (Tb.N) are significantly increased, and WNT/ β -catenin signaling is up-regulated.

Materials and methods

BD preparation

A solution of BD (molecular weight: 505,5622; Sigma) was prepared by dissolving solid BD in dimethylsulfoxide (DMSO, Sigma) solvent. The stock solution was stored at -20°C .

Fig. 1 In the presence of benidipine (BD), murine bone marrow stromal cells (BMSCs) increase osteogenesis. **a** Molecular structure of BD. **b** CCK8 assays show that BD does not significantly affect cell growth of primary mouse BMSCs at the concentrations used (1–100 μM) after treatment for 2 days (OD optical density). **c–c''**, **d–d''** Alkaline phosphatase (ALP) staining indicates that treatment with 1–100 μM BD increases ALP activity in BMSCs cultured in osteogenic differentiation media for 14 days. **e** Positive cell numbers were counted in ALP-stained plates. **f–h** BD increased runt-related transcription factor 2 (RUNX2) and osteocalcin (OCN) expression during osteoblast differentiation in cultured BMSCs. **f** Western blotting analyses show BD increases OCN (**g**) and RUNX2 (**h**) expression in BMSCs in a dose-dependent manner (GAPDH D-glyceraldehyde-3-phosphate dehydrogenase). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with the group without BD; # $P < 0.05$ compared with the group with 1 μM BD; + $P < 0.05$ compared with the group with 10 μM BD. Columns represent the means \pm SD from six wells per group (**e**, **g**, **h**)



Animals and drug treatment

Female C57/BL6 mice ($n=30$), aged 8 weeks and weighing 18–20 g, were purchased from Inner Mongolia Agricultural University (Huhhot, China). Mice were randomly divided into control (CON), CON+BD, sham, OVX, and OVX+BD groups. Mice in the OVX+BD group were intragastrically administered BD (15 mg/kg) each day for 5 days before ovariectomy and were maintained for 3 months after surgery. The CON and CON+BD groups differed from the sham and CON+BD groups. Mice in the control group were treated with vehicle, whereas those in the sham groups had some fat tissue around the ovaries removed.

Cell culture

BMSCs were isolated from C57BL/6 mice (aged 4 weeks). Briefly, mice femurs were dissected free of surrounding soft tissue. The bone marrow was flushed with α -MEM (Invitrogen, Carlsbad, Calif., USA). The marrow content from 4 to 6 bones was plated in culture flasks containing BMSC growth media [α -MEM containing 10 % fetal bovine serum (FBS), 100 U/ml penicillin, 100 mg/ml streptomycin sulfate (Gibco, New Zealand)]. Non-adherent cells were removed, and adherent BMSCs were cultured and expanded for further experiments. Primary cells were used in the experiments prior to the fourth passage. Cell culture media were replaced every 3 days.

Cell proliferation assays

Primary BMSCs were seeded in 96-well plates at a density of 1×10^4 cells/well. After 2 days in culture, cells were treated with BD at concentrations of 0.1 μ M, 1 μ M, 10 μ M, 100 μ M, and 1000 μ M for 48 h. Cell proliferation assays were performed by using Caspase-8 Colorimetric Assay Kits (CCK8; KeyGEN Biotech, China) according to the manufacturer's instructions. Absorbance was measured at 450 nm.

In vitro quantitative reverse transcription plus polymerase chain reaction analyses

Total RNA samples were isolated from BMSCs by using Trizol reagent according to the manufacturer's instructions

after 2 weeks osteogenic induction. Then, the total RNA products were immediately transcribed by reverse transcription (RT) into cDNA by using a PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, China). Polymerase chain reaction (PCR) amplification was performed in a Chromo4 Four-Color Real-Time PCR Detection System (Bio-Rad) by using the SYBR Premix Ex Taq II (Tli RNaseH Plus) kit (TaKaRa). Primer sequences (Life Technologies) for each gene used in this study are shown in Table 1.

In vitro osteoblastic differentiation

We induced osteoblastic differentiation by using a differentiation medium (α -MEM supplemented with 10 % FBS, 50 μ M ascorbic acid, 0.1 μ M dexamethasone, and 10 mM β -glycerol phosphate) after cells had been seeded in 6-well plates at a density of 1×10^4 cells/well. Then, we added BD (0.1 μ M, 1 μ M, 10 μ M, 100 μ M) to the differentiation medium. Media were changed every 3 days, and cellular differentiation was assayed 14 days after induction by using 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium (BCIP/NBT) alkaline phosphatase substrate solution (Sigma, USA).

Immunofluorescence analyses

Cell slides and paraffin-embedded sections were incubated overnight with rabbit polyclonal OCN antibody (1:50; Santa Cruz). Slides and sections were then incubated with goat anti-rabbit fluorescein isothiocyanate-conjugated IgG secondary antibody (1:100; Santa Cruz). Controls included substitution of primary antibody with rabbit IgG. Cells and femur histology were imaged by using laser-scanning confocal microscopy (FV1000; Olympus). OCN-positive cells were evaluated by using Image-Pro Plus software (Media Cybernetics, USA) to quantify cellular fluorescence intensity. Cells with a fluorescence intensity of ≥ 150 % of background were considered positive.

Immunostaining analyses

Cell slides and paraffin-embedded sections were prepared for immunostaining as follows. Slides were individually incubated with RUNX2 (1:100; CST), CD105 (1:50; CST), or CD106 (1:50; CST) for 1 h at room temperature. Secondary

Table 1 Primer sequences used in reverse transcription plus polymerase chain reaction (PCR) and in quantitative PCR for mice (*GAPDH* D-glyceraldehyde-3-phosphate dehydrogenase, *GSK* glycogen synthase kinase)

Target gene	GenBank accession number	Sequences (5'–3')	Product size
GAPDH	NM_001289726.1	Forward, CCCTTAAGAGGGATGCTGCC; reverse, TACGGCCAAATCCGTTTACA	124 bp
β -Catenin	XM_006511927.1	Forward, CTGCAACGACCTGACTGGTA; reverse, GGCCATGTCCAACCTCCATCA	103 bp
GSK-3 β	XM_006522425.1	Forward, AGAAGAGCCATCATGTCTGGG; reverse, CCAAAGCTGAAGGCTGCTG	83 bp

antibody staining was performed for 1 h at room temperature by using a biotin-labeled goat anti-rabbit antibody (1:100; Santa Cruz). Immunoreactivity was detected by using a diaminobenzidine (DAB) horseradish peroxidase color development kit (Sigma, USA) for 2–5 min. Slides were counterstained with Mayer's hematoxylin before dehydration and mounting. RUNX2 expression was analyzed by comparing the staining intensities between samples in the presence and absence of primary antibody under the same conditions.

Histological analyses

Femurs were fixed in buffered aqueous formalin, embedded in paraffin, sectioned at 2 μm , and stained with hematoxylin and eosin (HE).

Micro-computed tomography analyses

We obtained long bones from mice, dissected them free of soft tissue, fixed the bones overnight in 4 % paraformaldehyde, and analyzed them by using high-resolution micro-computed tomography (micro-CT; μCT 80, Scanco Medical, Brüttisellen, Zurich, Switzerland). We set the scanner at a voltage of 89 kV, a current of 112 μA , and a scan thickness of 20 μm . We established cross-sectional images of the proximal tibiae and femora in order to perform three-dimensional histomorphometric analyses of the trabecular bone. Our analyses included various bone parameters: Tb.Th, BMD, and Tb.N.

Western blot analyses

Proteins isolated from 6-well plates were subjected to SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene (PVDF) membranes. Membranes were probed with rabbit polyclonal antibodies to RUNX2 (1:2000; CST), OCN (1:1000; Santa Cruz), CD105 (1:2000; CST), CD106 (1:2000; CST), GAPDH (1:1000; Santa Cruz), β -catenin (1:1000; CST), LRP5 (1:1000; Santa Cruz), and goat anti-rabbit second antibody (1:1000; Santa Cruz). PVDF membranes were incubated with primary antibodies for 8 h at 4 $^{\circ}\text{C}$ and washed three times with TRIS-buffered saline with Triton X-100 (TBST; 5 min per wash). The secondary antibody was incubated with the membranes for 1 h at room temperature, followed by three washes with TBST (5 min per wash). Bound antibodies were detected by enhanced chemiluminescence (ECL) with Amersham ECL Plus Western Blotting Detection Reagent according to the manufacturer's instructions (ECL Plus Kit, GE Healthcare, UK).

Statistical analyses

Data were analyzed by using one-way analyses of variance (ANOVA). Homogeneity of variance tests was used to evaluate data homogeneity (IBM SPSS Statistics 21.0 software). If the variances were equal, the least-significant difference test was employed. If the variances were unequal, Dunnett's test was used. Results are presented as the means \pm SD. $P < 0.05$ was considered statistically significant.

Results

Effect of BD on cell proliferation

As shown in Fig. 1b, we measured the effect of BD on primary murine BMSC proliferation by using CCK8 assays. We found that BD at concentrations of 1–100 μM did not significantly affect cell growth after treatment for 2 days.

Effect of BD on BMSC in vitro osteogenesis

First, we supplemented the negative effect of BD alone on BMSC differentiation as shown in Supplementary Fig. 1a. Moreover, BD was unable to promote significant changes in osteogenic markers such as OCN (Supplementary Fig. 1b) and RUNX2 (Supplementary Fig. 1c) in the absence of differentiation medium for 2 weeks. However, to investigate the consequences of adding BD in vitro further, BMSCs from C57/BL6 mice were cultured for 14 days in osteogenic media. The addition of BD increased the expression of ALP (Fig. 1c–c'', d–d'') by approximately 10–25 % (Fig. 1e) in cultured BMSCs. RUNX2 was also up-regulated as shown by western blotting (Fig. 1f, h) and in the immunocytochemistry experiments (Fig. 2b–b'', d). In addition, BD increased the expression of OCN as demonstrated by western blotting (Fig. 1f, g) and immunofluorescence (Fig. 2a–a'', c) analyses. Therefore, our data suggest that BD promotes in vitro osteogenesis.

Effect of BD on femur bone microstructure morphometrics and histologic appearance

To evaluate the effect of BD on mice bone formation, we performed μCT analyses on sham (Fig. 3b, c, d–f), OVX (Fig. 3b', c', d–f), and OVX+BD (Fig. 3b'', c'', d–f) mice. Micro-CT results from long bones of OVX+BD (Fig. 3b'', c'', d–f) mice demonstrated a clear trend in which Tb.Th (Fig. 3d) was increased relative to that of the OVX group (Fig. 3d). Moreover, BMD (Fig. 3e) and Tb.N (Fig. 3f) in OVX+BD bones were significantly higher than in OVX mice (Fig. 3e, f). However, these parameters were not significantly different between the OVX and sham groups (Fig. 3e, f). HE staining of femur bone sections (Fig. 3a–a'') indicated that the number

of trabeculae in OVX mice (Fig. 3a') was significantly decreased compared with that in the OVX+BD group (Fig. 3a'').

Effect of BD on RUNX2 and OCN expression

Above all, we found that BD did not have a significant effect on endogenous stem cells as shown by immunohistochemistry (Supplementary Fig. 2) with characteristic markers of marrow stromal cells, such as CD105 (Supplementary Fig. 2a-a'') and CD106 (Supplementary Fig. 2b-b''). Thus, endogenous stem cells did not change significantly after BD treatment. In

addition, CD105- (Supplementary Fig. 2c) and CD106- (Supplementary Fig. 2d) positive cells were evenly distributed in all groups, as revealed microscopically by immunohistochemical staining, and exhibited no significant differences. In order to examine the molecular and cellular changes associated with BD-mediated protection against bone loss in vivo, we examined the epiphyseal growth plate in which bone formation occurs on a cartilaginous template (Karsenty 2003; Kronenberg 2003). RUNX2 (Fig. 4a-a'') and OCN (Fig. 4b-b'') expression was markedly reduced in OVX (Fig. 4a', b', c, d) mice compared with OVX+BD (Fig. 4a'', b'', c, d) mice. However, no statistically significant differences

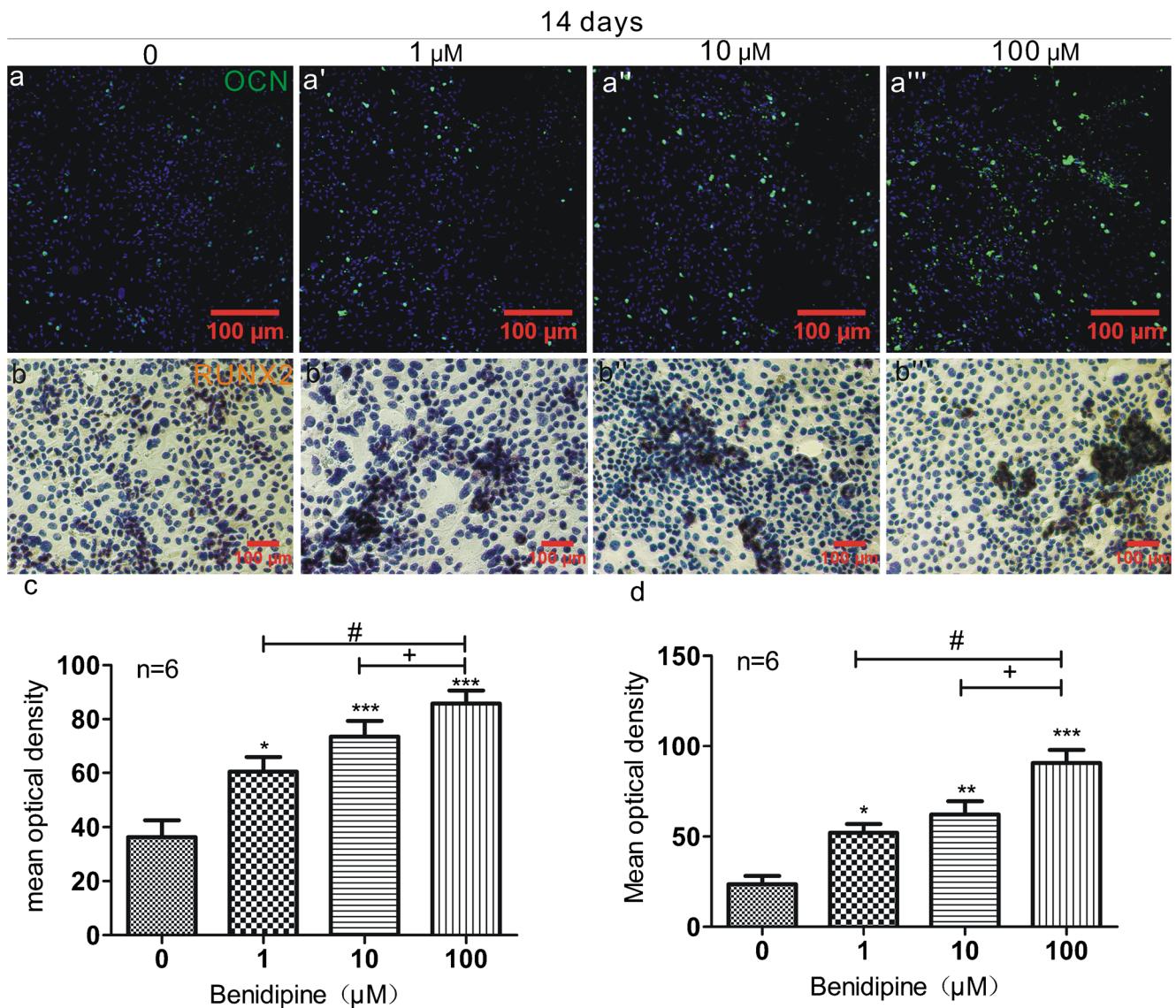
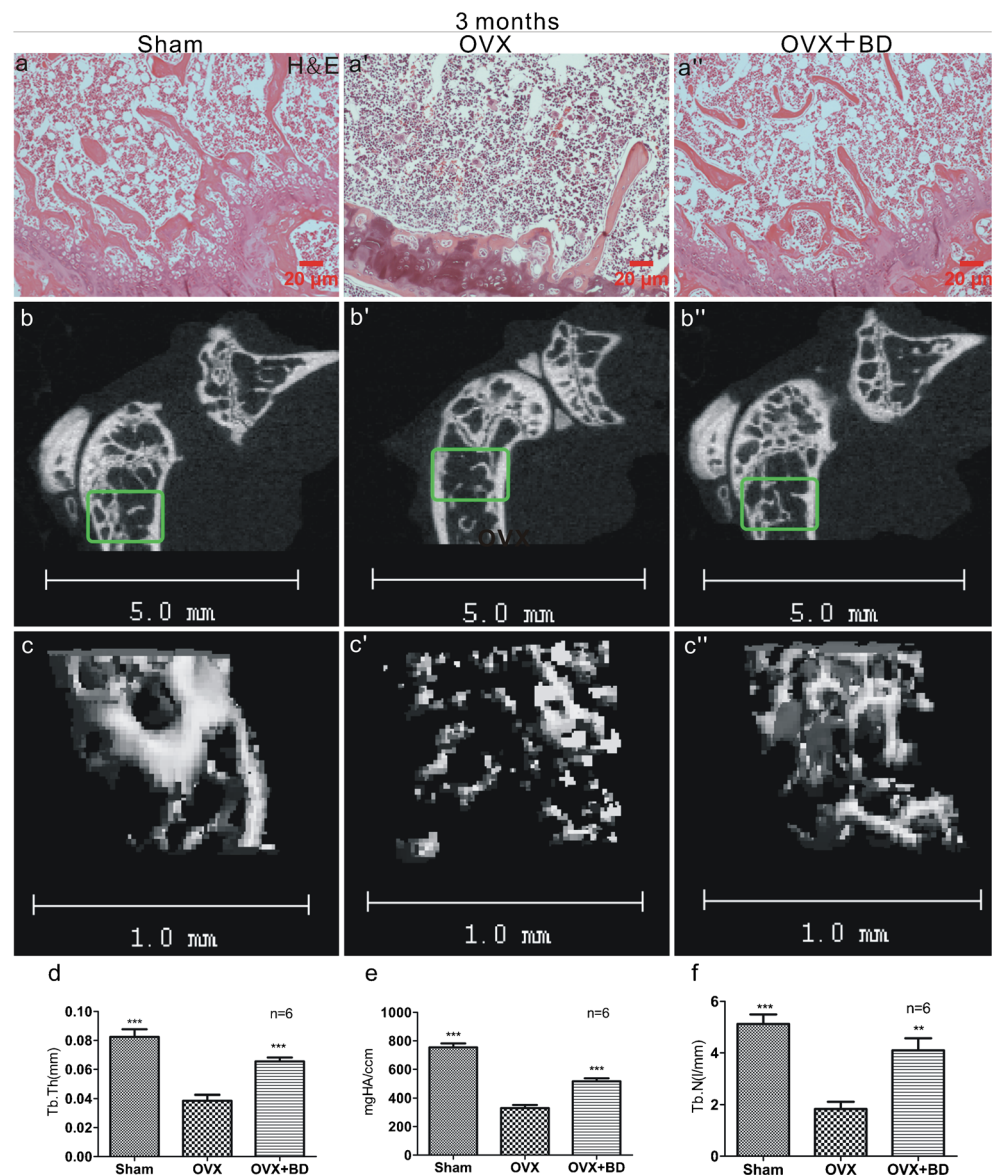


Fig. 2 BD stimulates BMSC differentiation into osteoblasts. BD (1–100 μM) was added to primary mouse BMSCs in osteogenic differentiation media for 14 days. Immunofluorescence (a) and immunohistochemistry (b) assays were performed to examine the expression of osteoblast-specific proteins after BD treatment. a BD increased OCN expression (green) in BMSCs in a dose-dependent

manner, with a more pronounced effect at 100 μM BD (c). b BD dose-dependently enhanced RUNX2 (brown) activity in BMSCs, particularly at 100 μM BD (d). * P <0.05, ** P <0.01, *** P <0.001 compared with the group without BD; # P <0.05 compared with the group with 1 μM BD; + P <0.05 compared with the group with 10 μM BD. Columns represent the means \pm SD from six wells per group (c, d)

Fig. 3 BD decreased the rate of bone damage after treatment for 3 months. Hematoxylin and eosin staining (a–a'') shows that OVX decreases the number of bone trabeculae, whereas BD reduces this damage. Sagittal two-dimensional images (b–b'') and three-dimensional reconstruction (c–c'') of the green boxed areas in b–b'' of micro-computed tomography of distal femora reveal that the trabecular thickness (*Tb.Th*; d), BMD (e), and trabecular number (*Tb.N*; f) values in the OVX group are dramatically decreased compared with those of the sham and OVX+BD groups (*HA* hyaluronic acid). These three indicators are significantly improved upon BD treatment. $**P < 0.01$, $***P < 0.001$ vs. OVX. Columns represent the means \pm SD from six mice per group (d–f)



were seen between the sham (Fig. 4a–d) and OVX+BD (Fig. 4a'', b'', c, d) groups. Therefore, our *in vitro* and *in vivo* data indicate that BD-induced osteogenesis positively regulates bone formation.

Effect of BD on WNT/ β -catenin signaling

To gain further insight into the function of BD in osteogenic differentiation of cultured BMSCs, we examined the expression of β -catenin and LRP5 *in vitro*. Our western blotting results showed that β -catenin (Fig. 5a, b) and LRP5 (Fig. 5a, c) expression was enhanced. In addition, we investigated the β -catenin mRNA level (Fig. 5d) and glycogen synthase kinase-3 β (GSK-3 β) mRNA expression (Fig. 5e); they both increased in BMSCs after BD treatment. Collectively,

these data indicate that WNT/ β -catenin signaling is up-regulated in BMSCs in the presence of BD and suggest that BD cooperates with WNT/ β -catenin signaling to regulate ossification.

Discussion

In this study, we have performed experiments to examine the effect of BD on osteoporosis and to elucidate the molecular targets through which BD exerts its effects. Cells in previous studies were derived from neonatal mouse calvarias (Kosaka and Uchii 1998) or MC3T3-E1 cell lines (Nishiya and Sugimoto 2001; Nishiya et al. 2002). However, these cells are not a pure population of osteoblasts and thus cannot fully mimic osteoblast physiological function. Because BD treatment does not decrease bone absorption in cultured neonatal

Fig. 4 BD increased the expression of osteogenesis markers in OVX mice. Weak positive staining for RUNX2 (brown; **a**) and OCN (green; **b**) was observed, particularly on the surface of bone lacuna in the OVX group after 3 months. OCN (**d**) and RUNX2 proteins (**c**) were elevated after BD treatment. * $P < 0.05$, *** $P < 0.001$ vs. OVX. Columns represent the means \pm SD from six mice per group (**c**, **d**)

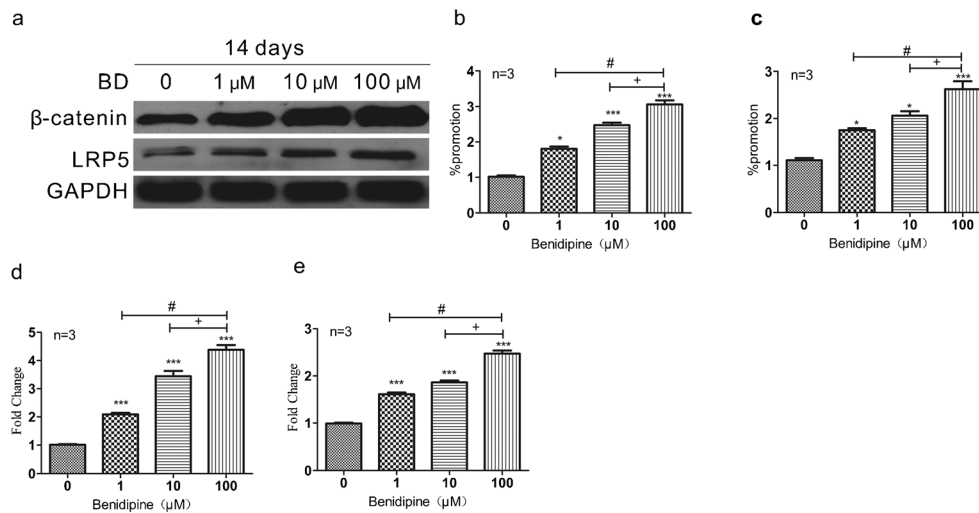
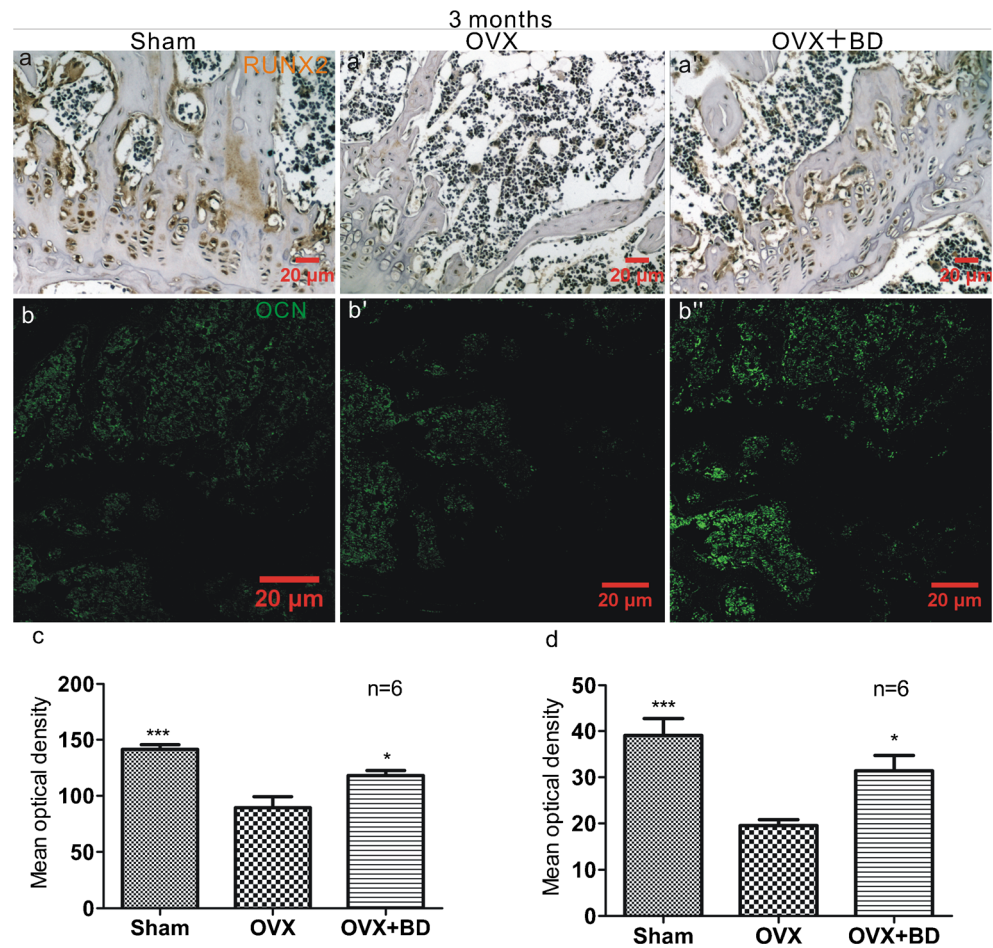


Fig. 5 WNT/ β -catenin signaling is up-regulated by BD. Primary mouse BMSCs were treated with BD (1–100 μ M) under osteogenic conditions for 2 weeks. **a** BD dose-dependently enhanced β -catenin (see also **b**) and low-density lipoprotein receptor-related protein 5 (*LRP5*; see also **c**) activity in BMSCs, particularly at a concentration of 100 μ M. The β -catenin mRNA level (**d**) and glycogen synthase kinase-3 β (GSK-3 β)

mRNA expression (**e**) both increased in BMSCs after BD treatment. * $P < 0.05$, *** $P < 0.001$ compared with the group without BD; # $P < 0.05$ compared with the group with 1 μ M BD; + $P < 0.05$ compared with the group with 10 μ M BD. Columns represent the means \pm SD from three wells per group (**b–e**)

mouse calvarias in vitro (Kosaka and Uchii 1998), the action of BD might occur via the augmentation of bone formation by osteoblasts.

Marrow stem cells are contained within the bones. Some mesenchymal stem cells develop into osteoblasts and osteocytes (Danks and Takayanagi 2013; Owen 1988). Studies have revealed that the relationship between osteoporosis and osteoblast differentiation in BMSCs occurs concomitantly with decreased BMSC differentiation into osteoblasts in bone marrow in age-related osteoporosis (Chan and Duque 2002; Rodriguez et al. 1999).

In our present research, we utilized BMSCs to investigate the action of BD on bone metabolism. Cells were cultured under osteogenic conditions, which mimics human osteoporosis. First, we assessed cell viability and detected no obvious toxicity when BD was used at doses of up to 100 μM . However, at 1000 μM , BD showed toxic effects (Fig. 1b). Next, in our in vitro experiments, we found that an enhanced ALP activity dose dependently induced osteogenesis in BMSCs. To the best of our knowledge, this is the first report to confirm the action of BD on BMSC differentiation into osteoblasts (Fig. 6). This mechanism focuses on BMSCs and differs from currently available agents for osteoporosis that target mature osteoblasts, osteoclasts, or adipocytes (Davey et al. 2012; Sun et al. 2013).

To explore the signaling pathways involved in BD-induced BMSC osteogenesis, we examined the expression of β -catenin and LRP5, which are key factors in WNT signaling that regulate bone formation (Rossini et al. 2013). We found that β -catenin and LRP5 expression was enhanced by BD, indicating that BD promotes BMSC osteogenesis by promoting WNT signaling (Fig. 6).

Finally, to examine the effects of BD in vivo, we designed animal experiments. Micro-CT data showed that bone loss induced by OVX could be significantly rescued by BD treatment. We also examined, in femurs, the expression of OCN

and RUNX2, which are important cytokines during bone remodeling. RUNX2 and OCN expression were up-regulated (Fig. 6), suggesting that BD promotes bone formation and growth. Although our findings indicate a protective effect of BD on osteoporotic bone, we did not include a positive control group, such as estrogen treatment.

In summary, the present study suggests that BD promotes osteogenesis, which is dependent on increasing RUNX2 and OCN expression at the tissue and cellular levels, thereby increasing the lineage differentiation of BMSCs toward osteoblasts. Moreover, we suggest that WNT signaling, in part, might be the specific signaling pathway mechanism in this process. Furthermore, appropriate concentrations of BD might positively affect BMSC differentiation into osteoblasts and thus be a suitable candidate for the treatment of patients with postmenopausal osteoporosis and hypertension.

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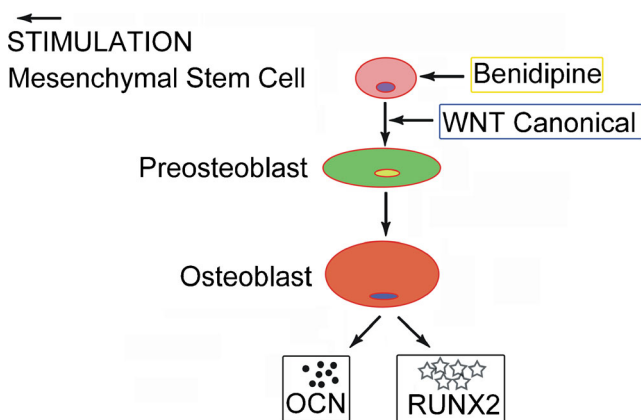


Fig. 6 Benidipine promotes BMSC differentiation into osteoblasts. WNT canonical signaling favors the maturation and survival of osteoblasts and promotes OCN and RUNX2 expression

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