Bone morphogenetic protein 4 as a biomarker for cadmium-associated bone damage

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Research Article

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Abstract

Background

Cadmium is a well-characterized bone toxic agent and can induce bone damage via inhibiting osteogenic differentiation. However, only a few studies have addressed the mechanism on cadmium-associated bone damage and none of them has used a panel of sensitive and specific biomarkers for the detection of cadmium-associated bone damage. BMP/SAMD signaling pathway can mediate osteogenic differentiation, but the association between cadmium and BMP/SAMD signaling pathway is yet to be illuminated.

Methods

We treated human bone marrow mesenchymal stem cells (BMSCs) with CdCl2 in vitro to detect the expression of BMPs and SAMDs. And we also enrolled 67 cases of bone damage and 67 cases without bone damage. Urinary cadmium concentration and the concentrations of BMP-2 and BMP-4 of subjects were detected. Mediation analyses was used to estimate the influence of urinary cadmium and BMP-4 on bone damage, adjusting for a set of confounders.

Results

Cd exposure significantly promoted adipogenic differentiation of hBMSCs, and inhibited its' osteogenic differentiation by inhibiting the expression of BMP-2/4, SAMD4, and p-SAMD1/5/9 complex. BMP-4 mediated 22.92% (95%CI 6.37, 46.00) of the total association between cadmium and the risk of osteoporosis.

Conclusions

We found BMP-4 can be a diagnostic biomarker and therapeutic target of cadmium-associated bone damage.

Background

Osteoporosis is a bone damage disease which is characterized by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture [1]. It is estimated that nearly 22 million women and 5.5 million men are suffering from osteoporosis, leading to increased risk of fractures in women aged 55 years or older and men aged 65 years or older coupled with increased mortality and health-care costs [1, 2]. Numerous studies have investigated the risk factors of osteoporosis, which found apart from the individuals' risk factors of age, body mass index (BMI), smoking and heavy drinking, heavy metal pollution especially cadmium (Cd) also has a serious influence...
on osteoporosis [3, 4, 5]. Since the 1950s, findings have suggested that Cd could be associated with adverse effects on bone structures [6, 7, 8, 9]. A series of cross-sectional and prospective studies of different populations have demonstrated an association between cadmium exposure and low bone mineral density, as well as an increased risk of bone damage [10, 11, 12]. In conjunction with population-based research, in vivo and in vitro experiments have indicated that Cd could directly affect bone formation and bone resorption by inhibiting osteoblast differentiation [13, 14, 15]. Bone marrow-derived mesenchymal stromal cells (BMSC), as the progenitors of osteoblasts, which growing evidence has indicated exhibit reduced capacity to differentiate into osteoblasts and an increased capacity to differentiate into adipocytes during osteoporosis development results in a reduction in bone formation [16, 17, 18, 19, 20, 21, 22]. But how cadmium inhibits BMSC osteoblast differentiation is not clear.

In most species, bone morphogenetic proteins (BMPs) can direct the commitment of marrow-derived mesenchymal stromal cells (MSCs) into osteoprogenitors and further stimulate osteogenic differentiation and bone mineralization [23, 24, 25]. Both human and mouse genetic studies have demonstrated that BMPs perform positive roles in postnatal bone homeostasis including osteoblast expansion, differentiation, and bone formation [23, 26, 27]. The response to the activation of BMPs mainly through BMP-Smad signaling pathway to regulate stem cell renewal, differentiation, migration, and apoptosis, and controls embryo development and postnatal tissue homeostasis [28, 29]. However, the complex regulated network relationship among cadmium, BMPs, and SMADs is still unclear. In this study, we combined human bone marrow mesenchymal stem cells with a case-control study to explore the effect of BMPs and SMADs in CdCl2 regulated osteogenic differentiation of human bone marrow mesenchymal stem cells and the possibility of BMPs as a biomarker of cadmium-associated bone damage.

Method

Isolation and culture of hBMSCs

Human bone marrow mesenchymal stem cells (hBMSCs) gifted from the Zhongshan Medical University were isolated from fresh human bone marrow aspirates from patients undergoing fracture management. The patients which including two women and a man were aged 23–34 years old. Under sterile conditions, hBMSCs were maintained in alpha minimal essential medium (α-MEM; Sigma-Aldrich CO, St. Louis, MO, USA) and supplemented with 10% fetal bovine serum (FBS, Thermo Fisher scientific, Waltham, MA, USA) and 1% penicillin-streptomycin (Corning, Sigma-Aldrich, St. Louis, MO, USA). The cells were cultured at 37°C in a humidified incubator containing 5% CO2. The medium was changed every 2 days until confluence. Cultures were serially passaged by replating confluent cells at half their confluent density, growing the cells to confluence, and replating again at half their confluent density. Cells were used at passage 3-6.

Cell viability assay

Cell viability was assessed with Cell Counting Kit-8 (CCK-8, Beyotime, Nangjing, China) following the manufacturer's instructions. HBMSCs were seeded in 96-well plates at a density of 5000 cells/well. After
24 hour, they were exposed to 0, 0.625, 1.25, 2.5, 5.0, 10.0, 20.0, 40.0, 80.0 or 160.0 µM CdCl₂ (Sigma Aldrich, USA) for 24 hour. Subsequently, a 100 µl volume of CCK-8 solution was added to each, followed by incubation for 2 hour at 37°C, 5% CO₂. Consequently, absorbance at 450 nm was measured on a microplate reader (Biotek, Winooski, VT, USA).

**CdCl₂ treatment**

For osteogenic differentiation of hBMSCs, CdCl₂ (Sigma Aldrich, USA) at concentrations of 0, 2.5, or 5.0 µM was added into the osteogenic induction medium to replace the growth medium.

**ALP, Alizarin red S and Oil red O staining**

Leukocyte Alkaline Phosphatase Kits (ALP, Sigma Aldrich, USA) was used for ALP staining according to the manufacturer's instructions. HBMSCs were seeded in 12-well plates at a density of 2×10⁵ cells/well in a growth culture medium. When their confluence reached 80%, hBMSCs were exposed to CdCl₂ with osteogenic differentiation for 14 days. After that, cells were fixed with 4% formaldehyde and 5% citrate in acetone at room temperature for 30 seconds. The fixed cells were washed with PBS and incubated with 0.2% naphthol AS-BI and 0.2% diazonium salt at room temperature for another 15 min. After washing the plates with phosphate buffer saline (PBS), images were taken at 10× magnification under an optical microscope (Zeiss, German).

The hBMSCs were seeded into 12-well plates at a density of 2 × 10⁵ cells/well and exposed to CdCl₂ with osteogenic differentiation for 14 days. The cells were washed with PBS, fixed with 10% formaldehyde at room temperature for 10 min, and incubated with 40 mM alizarin red S (Sigma Aldrich, USA) solution at room temperature for 20 minutes. After discarding the solutions and washing the plates with PBS for 4 times, images were taken under an optical microscope at 10× magnification (Zeiss, German).

Oil red O powder (Sigma, USA) was used to make oil red O mother liquor, and oil red O dyeing was performed. Take out hBMSCs that have induced adipogenic differentiation from the incubator, discard the old medium, and wash 3 times with PBS, 1 ml each time. Add 3 ml 4% paraformaldehyde to the cell culture dish (6cm), return the dish to the incubator, and fix the cells for 30 min. Discard the paraformaldehyde, wash the cells 3 times with PBS, and add 3 ml of oil red O application solution (prepared in advance and kept at room temperature for later use). Return the dish to the incubator again and stain for 60 min. Discard the oil red O application solution, wash the cells 3 times with 60% isopropanol, 1 ml each time, and then wash the cells 3 times with sterilized water, 1 ml each time. Finally, 2 ml of 4% paraformaldehyde was added, and the mouth of the dish was sealed with a parafilm, and the image was taken on a light microscope (Nikon, Japan) at 10x magnification.

**Protein isolation and western blot**

Total cellular proteins were extracted using ice cooled strong RIPA lysis buffer containing 1 mmol/L phenylmethanesulfonyl fluoride and 1 mmol/L phosphatase inhibitor cocktails (all from KeyGEN BitoTECH, Nanjing, CHN), and quantified by bicinchoninic acid protein assay kit (KeyGEN BitoTECH,
Nanjing, CHN). Mixtures of cellular proteins and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (5×, PanEra, Guangzhou, CHN) were heated at 100°C for 10 min. Approximately 40 µg of denatured proteins were loaded and separated by SDS-PAGE (12% acrylamide), and then transferred to the polyvinylidene difluoride membranes (0.45 µm, Millipore, Bedford, MA, USA) using a wet-transfer system at 100 V for 50 minutes. After blocking with 5% nonfat milk which was dissolved in Tris-buffered saline-Tween (TBST, 0.1% Tween), membranes were incubated overnight at 4°C with a 1:1000 dilution of anti-glyceraldehyde 3-phosphate dehydrogenase (Cell Signaling Technology, USA) and an antibody for Runx2 (Cell Signaling Technology, USA), Osterix (Abcam, USA), BMP-2/-4/-6/-7(ABclonal, Wuhan, CHN), SMAD1/4/5, p-Smad1 and p-Samd1/4/9 complex (Cell Signaling Technology, USA). Membranes were thereafter rinsed five times with TBST washing solution, followed by incubating with corresponding horseradish peroxidase co-conjugated secondary antibodies (1:2000 dilution for anti-mouse IgG and 1:5000 dilution for anti-rabbit IgG, all from Santa Cruz, CA, USA) for 1.5 h at room temperature. After washing, strips in membranes were visualized using chemiluminescent peroxidase substrate (Millipore, Bedford, MA, USA) and Tanon-5200 chemical luminescence developing system (Tanon, Shanghai, CHN). GAPDH served as the internal reference. The relative expressions of Runx2, Osx, MP-2/-4/-6/-7, SMAD1/4/5, p-Smad1 and p-Samd1/4/9 complex proteins in treatment groups were determined by grey value analysis using Image J software (https://imagej.net/) and normalized to the control. ELISA kits were used to detect the concentration of BMP-2(Abcam, Cambridge, UK) and BMP-4(Thermo, Frederick, USA) respectively.

Participant

The target population comprised people, between 40 and 80 years of age, who lived in southern China Guangdong province for 15 years or longer without occupational exposure of cadmium. The population was divided into two groups: namely bone damage and non-bone damage groups depending on DEXA results (T-score) as following: bone damage patients with T-score ≤ − 2.5 and non-bone damage patients with T-score > − 2.5[29]. Both bone damage and non-bone damage groups included 67 subjects (male 28, female 39).

Residents in the area used the cadmium-polluted river water to irrigate their elds and rice is the main food. Participants were excluded who had received any drug known to alter bone metabolism, such as corticosteroids. Written informed consent was obtained from each participant. Variables, such as gender, age, BMI, and smoking status (never/ever) which may act as potential confounders were collected. BMI was calculated as weight in kg divided by squared height in m.

Collection of samples and analytical method

Peripheral blood and first-morning urine samples were collected before breakfast. Blood samples were centrifuged at 1500g for 15 min at 4°C, and serum was separated. Obtained blood and urine samples were subdivided and kept frozen at -80 °C until analysis. Those urine samples intended for Cd analysis were acidified and kept at room temperature until analysis. First-morning urine was defined as the first sample collected from an individual at or after 5:00 in the morning. U-Cd concentrations were determined
using inductively coupled plasma mass spectrometry (7700 × ICP-MS, Agilent Technologies, USA). Multi-
element calibration standards were prepared from an environmental calibration standard (Agilent part
number: 5183 – 4688). Then, 10 µg/mL internal standard mix (Agilent part number: 5183 – 4680) was
diluted to 1.0 µg /mL with 5% (v/v) HNO3. Further, 10 ng/mL tuning solution (Agilent part number: 5184 –
3566) was diluted to 1 ng/mL with 5% (v/v) HNO3. The inductively coupled plasma mass spectrometry
(ICP-MS) was operated in helium collision mode (for interference removal). Percent recovery was between
95% and 105%, and relative standard deviation less than 10%. The commercial QC sample (Seronorm
Trace Elements Urine L-2, SERO AS, Norway) was analyzed for every 20 samples to ensure instrument
performance. U-Cd concentrations were either adjusted or divided by urine creatinine (µg/g). Urinary levels
of creatinine were determined using a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA) at a
wavelength of 546nm, which using Creatinine assay kit (sarcosine oxidase, Nan jing Jian Cheng, China).
ELISA kits were used to detect human BMP-4 in plasma (Thermo, Frederick, USA) and BMP-2 in serum
(Abcam, Cambridge, UK), respectively.

**Determination of bone mineral density**

Bone mineral density at the forearm was measured using peripheral dual-energy x-ray absorptiometry
(pDXA; model EXA-3000, OsteoSys, Korea). The site used was forearm bone density. The apparatus was
calibrated before each use, and measurements were performed by an experienced operator. The T-score
was derived by comparing the DXA scan result of the subject with that of a healthy adult of the same
gender and race. The standard data on Asian adults were used as reference for the highest bone density.

**Statistical analysis**

Graphpad 7.0 was applied for statistical analyses. Descriptive statistical analyses were performed for
characteristics of the study population. Continuous variables were shown as the mean ± standard
deviation, and differences between cases and controls were examined using t-tests for normally
distributed data or the Mann-Whitney U tests for non-normally distributed data. Categorical variables were
presented as numbers and percentages, and intergroup comparisons were analyzed using chi-square
tests. Spearman was used to examine the possible relationship between U-Cd, BMP-4 and T value.
Mediation analyses were performed to further investigate the influence of U-Cd and BMP-4 on T value.

Data analyses were performed using SPSS25.0 and R(ggplot2, mediation). Two-sided values of P < 0.05
were considered statistically significant.

**Results**

To support the toxicity of Cd in HBMSCs, cells were incubated with different doses of CdCl2 for 24 h. We
established the effect of Cd in the cell viability using the CCK-8 assay (Fig. 1). CdCl2 caused a significant
doses dependent reduction in the viability with 10–100 µM Cd, but not with 5 µM Cd treatment; therefore,
to minimize the potential impact of the decline in cell viability on the experimental results we selected 2.5
µM and 5 µM Cd as representative of cytotoxic dose for further studies.
Oil red O staining (Fig. 2a) showed that 2.5 and 5 µM cadmium chloride can increase intracellular lipid droplet formation. Alkaline phosphatase (ALP) and alizarin red staining (Fig. 2b-c) showed that 2.5 and 5 µM cadmium chloride can extremely reduce intracellular calcification nodules quantity and volume. And 5 µM cadmium chloride can down-regulate 25% of the protein expression levels of Runx2 and 33% of OSX (P < 0.05, Fig. 2d-e), suggesting that cadmium chloride can inhibit hBMSCs osteogenic differentiation.

In our study, the expression of BMP-2/4/6/7, SMAD1/4/5, p-Smad1 and p-Samd1/4/9 complex were been detected. Results showed that the expression of BMP-2 reduced by 58% compared with 0 uM, and BMP-4 was also reduced 38% (P < 0.05, Fig. 3a, b), but the expression of BMP-6 and BMP-7 had no statistical difference with 0 uM. The expression of SMAD4 reduced by 52% compared with 0 uM and p-SMAD1/5/9 complex was reduced by 31% (P < 0.05, Fig. 3c, d), and the expression of SMAD1, SMAD5 and p-SAMD1 had no statistical difference with 0 uM. The concentration of BMP-2 and BMP-4 of cells culture supernatant, results showed that the BMP-4 was decreased 52% after treated by cadmium chloride (p < 0.05, Fig. 3e,f).

The clinical characteristics of the study participants are summarized according to the cases and control groups in Table 1. There was no significant difference in sex, BMI, and smoking status among the two groups. However, individuals who suffered from bone damage had a higher urinary cadmium(U-Cd) concentration than those in the non-bone damage group (p < 0.05). Moreover, the average concentration of BMP-4 in the non-bone damage group was higher than the bone damage group (p < 0.05).
Table 1
Demographic Characteristics of Subjects from Cd-Polluted and non-Cd-Polluted Areas

| Characteristics | osteoporosis | P-value |
|-----------------|--------------|---------|
|                 | No           | Yes     |     |
| Number (%)      | 67(50%)      | 67(50%) | -    |
| Age (years)     | 58.69(57.41,59.96) | 58.10(56.83,59.34) | 0.5250 |
| Gender n (%)    | 67(50%)      | 67(50%) | 1.00 |
| Male            | 28(41.79%)   | 28(41.79%) | -    |
| Female          | 39(58.21%)   | 39(58.21%) | -    |
| BMI (kg/m²)     | 22.74(22.05,23.41) | 22.24(21.58,22.91) | 0.30 |
| Smoking status n (%) | 67(50.00%) | 67(50.00%) | 0.86 |
| Yes             | 26(38.81%)   | 27(40.30%) | -    |
| No              | 41(61.19%)   | 40(59.70%) | -    |
| Urinary cadmium (mg/g cr) | 7.14(3.87,10.41) | 12.30(9.02,15.57) | 0.0293 |
| BMP4(pg/ml)     | 624.80(597.84,651.77) | 544.14(517.17,571.11) | < 0.0001 |

Results were reported as means and standard deviation. Variables with a non-normal distribution were described by median and the 5th to 95th percentile interval.

Abbreviations: cr: creatinine; BMI: body mass index; BMP-4: Bone Morphogenetic Protein 4.

The U-Cd concentration was significantly positively correlated with BMP-4 (r = -0.22, p < 0.05), and significant inverse association was observed between BMP-4 concentration and T value (r = 0.33, p < 0.05) (Additional 3). In the mediation analysis, the model for T value and BMP-4 were combined to calculate the indirect effect explained by BMP-4 and the direct effect via U-Cd. There was a 25.24% (95% CI 9.44, 66.00) of the total effect of the BMP-4 on T value mediated by U-Cd (Fig. 4).

Discussion

Bone damage is the most frequent degenerative disease in the developed countries [30]. A number of studies have demonstrated that Cd exposure and low bone mineral density were correlated [10, 11, 12, 31]. For better knowledge of the underlying pathogenetic mechanisms and screening for useful diagnostic biomarkers of cadmium-associated bone damage, we investigated specific protein signatures for bone damage. In this study, we found cadmium could inhibit hBMSCs differentiation via BMP-2, BMP4, SMAD4 and p-Samd1/5/9 proteins. The present age-adjusted and gender-matched case–control study also showed that bone damage was associated with the increased U-Cd level and this relationship was mediated by BMP-4.
It is well known that Cd exposure has a potentially negative impact on human health. Following exposure, this toxic metal is retained in the body and causes damage to bone, which results in bone loss and increases susceptibility to fractures [32]. Our data also showed that the bone mineral density of the population exposed to cadmium is usually relatively low compared to those living in areas with less Cd exposures. Thus, identifying the biomarkers of cadmium toxicity in bone damage is significant for the development of prophylactic and therapeutic strategies.

Bone formation involves the commitment of MSCs to the osteoblastic lineage and their subsequent differentiation [33]. Many studies have found that bone damage is primarily driven by the dysregulated differentiation of BMSC into osteoblasts [34, 35, 36]. And in vitro evidence has demonstrated that long-term, low-dose Cd exposure can dramatically inhibit the differentiation of BMSCs into osteoblasts [36, 37]. In our study, osteogenic differentiation was greatly inhibited mineral nodules and the expression levels of related osteogenic markers (RUNX2, OSX) were decreased, while the adipogenic differentiation was increased, which is consistent with the previous research [38, 39, 40]. In fact, many studies have also demonstrated that Cd exposure suppresses BMSC osteogenesis through a series of signaling pathways, the detailed mechanism and sensitive and specific biomarkers is not clear [15, 19, 33, 37]. To find sensitive and specific biomarkers of Cd exposure inhibits osteoblast differentiation, we focused on the effect of BMPs and Smads, as it is highly expressed in osteoblasts and generally believed to induce osteogenic differentiation of BMSCs [42, 43, 44]. BMPs were originally named for their ability to induce ectopic bone formation. They have significant clinical significance and could accelerate fracture healing in patients with bone damage [41]. When the osteogenic were further analyzed, BMP-2, -4, -6 and -7 were shown to effectively induce osteogenic differentiation of MSCs in vitro and in vivo [45, 46, 47, 48, 49, 50]. But there is no study about cadmium, BMPs and bone damage. Our current study found that cadmium inhibited the expression of BMP-2 and BMP-4, but there were no effects on the expression of bone morphogenetic protein-6 (BMP-6) and bone morphogenetic protein-7 (BMP-7). Our results also showed that BMP-2/4 accumulated inintracellular matrices are essential for the osteoblastic differentiation of cells in the osteoblast lineage by upregulation of the osterix and Runx2 [51, 52, 53, 54, 55, 56]. Therefore, the regulatory mechanism of BMP-2/4 actions in osteoblastic cells is a principal issue to be elucidated for a better understanding of the pathogenesis of bone damage. And Smads are cytoplasmic signal transducers of BMPs. There are many SAMDs including SMAD1, SMAD2, SMAD3, SMAD4, SMAD5, P-SMAD1, SMAD1/5/8 and SMAD1/5/9 complex that can affect osteoblast function, among which SMAD1, SMAD4, SMAD5, P-SMAD1 and SMAD1/5/9 complex are regulated by BMP-2 and BMP-4 [57, 58, 59, 60, 61, 62]. Integrating with the results of BMPs, we also detected the expression of SMAD1, SMAD4, SMAD5, P-SMAD1 and SMAD1/5/9 complex in control group and CdCl2 treated groups. Studies have showed that BMP-2/4-induced osteoblast commitment is mediated by the phosphorylation of specific transactivators (p-SAMD1/5/9 complex) and SMAD4. Series studies have indicated p-SMAD1/5/9 forms a complex with SMAD4 and co-shifts into the nucleus, where they recruit cofactors and Runx2 to regulate the expression of osteogenic genes, (Runx2 and Osx, etc.) [41, 63, 64]. Our results showed a negative correlation between Cd and osteogenic differentiation inhibition explaining the connection between Cd and bone damage, which is as same as others [13, 14, 15]. Overall, these findings revealed that BMP-2 and
BMP-4 play an important role in Cd-induced osteoporosis and also be involved in regulating bone metabolism under physiological conditions.

To ensure Cd exposure interacts with BMP-2/4 to induce bone damage, and if BMP-2 or BMP-4 can be a biomarker of cadmium-associated bone damage, we enrolled 134 subjects to evaluate whether BMP-2 or BMP-4 mediated the risk between Cd and bone damage. Surprisingly, BMP-4 expression level was reduced in bone damage. However, BMP-2 was not detected in both bone damage and non-bone damage people. Thus, we deduce that BMP-4 may be involved in the regulation of cadmium-associated bone damage. To verify the finding above, mediation analysis was used to detect the effects of BMP-4. These results showed that BMP-4 acted as mediators in cadmium and bone damage. Therefore, decreased BMP-4 level could be a plausible biological explanation of our findings. To the best of our knowledge, this is the first study to report that BMP-4 is involved in the cadmium-associated bone damage.

Some limitations of the present study should be addressed. Firstly, we didn't add inhibitor or siRNA of BMP-4 to verify the critical role of BMP-4 in cadmium-induced osteogenic differentiation inhibition for the cells not enough for this experiment. Secondly, our data has indicated that BMP-2 also plays an important role in cadmium-induced osteogenic differentiation inhibition in vitro, but we failed to detect it in our subjects. Thirdly, the individuals of this study mostly come from southern China, so it remains unclear whether these findings could be generalized to other populations. Therefore, the study should be replicated in a cohort with a larger sample size to confirm the current results.

**Conclusion**

Our study showed that CdCl2 suppresses the osteogenic differentiation of hBMSCs and addresses the effects of CdCl2 on BMP-2/4, SMAD4 and p-SAMD1/5/9 complex. And BMP-4 is the critical factor in cadmium-associated bone damage. Our findings not only explained how CdCl2 induces bone damage but also provide public health implications for developing strategies to reduce CdCl2 exposure and thereby mitigating its harmful effects.

**Abbreviations**

Cd  
cadmium  
BMSCs  
bone marrow mesenchymal stem cells  
BMI  
body mass index  
BMPs  
bone morphogenetic proteins  
MSCs  
marrow-derived mesenchymal stromal cells
CCK-8  
Cell Counting Kit-8  
Runx2  
runt-related transcription factor-2  
OSX  
Osterix  
U-Cd  
Urinary Cd  
ICP-MS  
inductively coupled plasma mass spectrometry  
95% CI  
95% confidence intervals  
BMP-2  
bone morphogenetic protein-2  
BMP-4  
bone morphogenetic protein-4  
BMP-6  
bone morphogenetic protein-6  
BMP-7  
bone morphogenetic protein-7

**Declarations**

**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from corresponding author on reasonable request.

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**Contributions**

Yu Wan, Li-jun Mo and Wu Lu designed the experiment and supervised the work. Yu Wan, Wu Lu, Dong-li Li, Hai-bin Huang and Qin-zhi Wei performed the experiments and assisted with sample collection. Yu Wan, Li-jun Mo, Wu Lu, Dong-li Li, Hai-bin Huang, Qin-zhi Wei and Da-peng Wang processed the experimental data and performed analyses. Yu Wan performed drafted the manuscript, and designed the
figures. Yu Wan, Li-jun Mo and Wu Lu contributed to the interpretation of the results. All authors read, edited, and approved the final manuscript.

**Ethics declarations**

Ethics approval and consent to participates were approved by the Fifth Affiliated Hospital of Southern Medical University Medical Ethics Committee (No.2019-YYK-004).

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare they have no competing interests.

**References**

1. Lane 1 JM, Russell L, Khan SN. Osteoporosis Clin Orthop Relat Res. 2000;372:139–50.

2. Nicholas R, Fuggle EM, Curtis KA, Ward NC, Harvey EM, Dennison C Cooper. Fracture prediction, imaging and screening in osteoporosis. Nat Rev Endocrinol. 2019;15: 535–547.

3. Soisungwan Satarug, Scott H, Garrett, Mary. Ann, Sens Donald A. Cadmium, environmental exposure, and health outcomes. Environ Health Perspect. 2010;118:182–90.

4. Jesus M, Lavado-García LM, Puerto-Parejo R, Roncero-Martín, Jose M, Moran JD, Pedrera-Zamorano, Ignacio J, Aliaga, et al. Dietary Intake of Cadmium, Lead and Mercury and Its Association with Bone Health in Healthy Premenopausal Women. Int J Environ Res Public Health. 2017;14:1437.

5. Amuno S, Al Kaissi A, Jamwal A, Niyogi S, Quenneville CE. Chronic arsenicosis and cadmium exposure in wild snowshoe hares (Lepus americanus) breeding near Yellowknife, Northwest Territories (Canada), part 2: Manifestation of bone abnormalities and osteoporosis. Sci Total Environ. 2018;612:1559–67.

6. Kido T, Nogawa K, Yamada Y, Honda R, Tsuritani I, Ishizaki M, et al. Osteopenia in inhabitants with renal dysfunction induced by exposure to environmental cadmium. Int Arch Occup Environ Health. 1989;612:1559–67.

7. Kowit Nambunmee R, Honda M, Nishijo W, Swaddiwudhipong H, Nakagawa. Werawan Ruangyuttikarn. Bone resorption acceleration and calcium reabsorption impairment in a Thai population with high cadmium exposure. 2010;201: 7–13.

8. Nawrot T, Geusens P, Nulens TS, Nemery B. Occupational cadmium exposure and calcium excretion, bone density, and osteoporosis in men. J Bone Miner Res. 2010;256:1441–5.

9. Schutte R, Nawrot TS, Richart T, Thijs L, Vanderschueren D, Kuznetsova T, et al. Bone resorption and environmental exposure to cadmium in women: a population study. Environ Health Persp. 2008;116:777–83.
10. Aleksandra Buha R, Jugdaohsingh V, Matovic Z, Bulat B, Antonijevic, Jemma G, Kerns, et al. Bone mineral health is sensitively related to environmental cadmium exposure-experimental and human data. Environ Res. 2019;176:108539.

11. Chen X, Zhu G, Jin T, Gu S. Effects of cadmium on forearm bone density after reduction of exposure for 10 years in a Chinese population. Environ Int. 2009;358:1164–8.

12. Alfvén T, Järup L, Elinder CG. Cadmium and lead in blood in relation to low bone mineral density and tubular proteinuria. Environ Health Perspect. 2002;110:699–702.

13. Akesson A, Bjellerup P, Lundh T, Lidfeldt J, Nerbrand C, Samsioe G, et al. Cadmium-induced effects on bone in a population-based study of women. Environ Health Perspect. 2006;114:830–4.

14. Gallagher CM, Kovach JS, Meliker JR. Urinary cadmium and osteoporosis in U.S. Women > or = 50 years of age: NHANES 1988–1994 and 1999–2004. Environ Health Perspect. 2008;116:1338–43.

15. Ma Y, Ran D, Cao Y, Zhao H, Song R, Zou H, et al. The effect of P2X7 on cadmium-induced osteoporosis in mice. J Hazard Mater. 2021;405:124251.

16. Moerman EJ, Teng K, Lipschitz DA, Lecka-Czernik B. Aging activates adipogenic and suppresses osteogenic programs in mesenchymal marrow stroma/stem cells: The role of PPAR-γ2 transcription factor and TGF-β/BMP signaling pathways. Aging Cell. 2004;3:379–89.

17. Li Y, He X, Li Y, He J, Anderstam B, Andersson G, et al. Nicotinamide phosphoribosyltransferase (Nampt) affects the lineage fate determination of mesenchymal stem cells: a possible cause for reduced osteogenesis and increased adipogenesis in older individuals. J Bone Miner Res. 2011;26:2656–64.

18. Yeung DK, Griffith JF, Antonio GE, Lee FK, Woo J, Leung PC. Osteoporosis is associated with increased marrow fat content and decreased marrow fat unsaturation: a proton MR spectroscopy study. J Magn Reson Imaging. 2005;22:279–85.

19. Li CJ, Cheng P, Liang MK, Chen YS, Lu Q, Wang JY, et al. MicroRNA-188 regulates age-related switch between osteoblast and adipocyte differentiation. J Clin Invest. 2015;125:1509–22.

20. Bianco P, Robey PG. Skeletal stem cells. Development. 2015;142:1023–7.

21. Haddouti EM, Randau TM, Hilgers C, Masson W, Pflugmacher R, Burger C, et al. Vertebral Bone Marrow-Derived Mesenchymal Stromal Cells from Osteoporotic and Healthy Patients Possess Similar Differentiation Properties In Vitro. Int J Mol Sci. 2020;21:8309.

22. Kondrikov D, Elmanski A, Bragg RT, Mobley T, Barrett T, Eisa N, et al. Kynurenine inhibits autophagy and promotes senescence in aged bone marrow mesenchymal stem cells through the aryl hydrocarbon receptor pathway. Exp Gerontol. 2020;130:110805.

23. Salazar VS, Gamar LW, Rosen V. BMP signalling in skeletal development, disease and repair. Nat Rev Endocrinol. 2016;12:203–21.

24. Huang D, Hou X, Zhang D, Zhang Q, Yan C. Two novel polysaccharides from rhizomes of Cibotium barometz promote bone formation via activating the BMP2/SMAD1 signaling pathway in MC3T3-E1 cells. Carbohydr Polym. 2020;231:115732.
25. Hu L, Yin C, Zhao F, Ali A, Ma J, Qian A. Mesenchymal Stem Cells: Cell Fate Decision to Osteoblast or Adipocyte and Application in Osteoporosis Treatment. Int J Mol Sci. 2018;19:360.

26. Parrow NL, Fleming RE. Bone morphogenetic proteins as regulators of iron metabolism. Annu Rev Nutr. 2014;34:77–94.

27. Dalmo E, Johansson P, Niklasson M, Gustavsson I, Neland S, Westermark B. Growth-Inhibitory Activity of Bone Morphogenetic Protein 4 in Human Glioblastoma Cell Lines Is Heterogeneous and Dependent on Reduced SOX2 Expression. Mol Cancer Res. 2020;18:981–91.

28. de Kroon LM, Narcisi R, van den Akker GG, Vitters EL, Blaney Davidson EN, van Osch GJ, et al. SMAD3 and SMAD4 have a more dominant role than SMAD2 in TGFβ-induced chondrogenic differentiation of bone marrow-derived mesenchymal stem cells. Sci Rep. 2017;7:43164.

29. Kanis JA. Assessment of fracture risk and its application to screening for postmenopausal osteoporosis: synopsis of a WHO report. WHO Study Group. Osteoporos Int. 1994;4:368–81.

30. Lv Y, Wang P, Huang R, Liang X, Wang P, Tan J, et al. Cadmium Exposure and Osteoporosis: A Population-Based Study and Benchmark Dose Estimation in Southern China. J Bone Miner Res. 2017;32:1990–2000.

31. Rodríguez J, Mandalunis PM. Effect of cadmium on bone tissue in growing animals. Exp Toxicol Pathol. 2016;68:391–7.

32. Chen X, Zhu G, Jin T, Qin B, Zhou W, Gu S. Cadmium is more toxic on volume bone mineral density than tissue bone mineral density. Biol Trace Elem Res. 2011;144:380–7.

33. Aslani S, Abhari A, Sakhinia E, Sanajou D, Rajabi H, Rahimzadeh S. Interplay between microRNAs and Wnt, transforming growth factor-β, and bone morphogenic protein signaling pathways promote osteoblastic differentiation of mesenchymal stem cells. J Cell Physiol. 2019;234:8082–93.

34. Clemens S, Aarts MG, Thomine S, Verbruggen N. Plant science: the key to preventing slow cadmium poisoning. Trends Plant Sci. 2013;18:92–9.

35. Ozkan ZS, Deveci D, Onalan Etem E, Yüce H. Lack of effect of bone morphogenetic protein 2 and 4 gene polymorphisms on bone density in postmenopausal Turkish women. Genet Mol Res. 2010;9:2311–6.

36. Knani L, Bartolini D, Kechiche S, Tortoioli C, Murdolo G, Moretti M, et al. Melatonin prevents cadmium-induced bone damage: First evidence on an improved osteogenic/adipogenic differentiation balance of mesenchymal stem cells as underlying mechanism. J Pineal Res. 2019. doi:10.1111/jpi.12597.

37. Ma Y, Ran D, Zhao H, Song R, Zou H, Gu J, et al. Cadmium exposure triggers osteoporosis in duck via P2X7/PI3K/AKT-mediated osteoblast and osteoclast differentiation. Sci Total Environ. 2021. doi:10.1016/j.scitotenv.

38. Lv YJ, Wei QZ, Zhang YC, Huang R, Li BS, Tan JB, et al. Low-dose cadmium exposure acts on rat mesenchymal stem cells via RANKL/OPG and downregulate osteogenic differentiation genes. Environ Pollut. 2019;249:620–8.

39. Wu L, Wei Q, Lv Y, Xue J, Zhang B, Sun Q, et al. Wnt/β-Catenin Pathway Is Involved in Cadmium-Induced Inhibition of Osteoblast Differentiation of Bone Marrow Mesenchymal Stem Cells. Int J Mol
40. Wang H, Chen H, Chernick M, Li D, Ying GG, Yang J, et al. Selenomethionine exposure affects chondrogenic differentiation and bone formation in Japanese medaka (Oryzias latipes). J Hazard Mater. 2020;387:121720.

41. Luppen CA, Chandler RL, Noh T, Mortlock DP, Frenkel B. BMP-2 vs. BMP-4 expression and activity in glucocorticoid-arrested MC3T3-E1 osteoblasts: Smad signaling, not alkaline phosphatase activity, predicts rescue of mineralization. Growth Factors. 2008;26:226–37.

42. Abe E, Yamamoto M, Taguchi Y, Lecka-Czernik B, O'Brien CA, Economides AN, et al. Essential requirement of BMPs-2/4 for both osteoblast and osteoclast formation in murine bone marrow cultures from adult mice: antagonism by noggin. J Bone Miner Res. 2000;15:663–73.

43. Nirmala FS, Lee H, Kim JS, Ha T, Jung CH, Ahn J. Green Tomato Extract Prevents Bone Loss in Ovariectomized Rats, a Model of Osteoporosis. Nutrients. 2020;12:3210.

44. Kang Q, Song WX, Luo Q, Tang N, Luo J, Luo X, et al. A comprehensive analysis of the dual roles of BMPs in regulating adipogenic and osteogenic differentiation of mesenchymal progenitor cells. Stem Cells Dev. 2009;18:545–59.

45. Luu HH, Song WX, Luo X, Manning D, Luo J, Deng ZL, et al. Distinct roles of bone morphogenetic proteins in osteogenic differentiation of mesenchymal stem cells. J Orthop Res. 2007;25:665–77.

46. Peng Y, Kang Q, Cheng H, Li X, Sun MH, Jiang W, et al. Transcriptional characterization of bone morphogenetic proteins (BMPs)-mediated osteogenic signaling. J Cell Biochem. 2003;90:1149–65.

47. Peng Y, Kang Q, Luo Q, Jiang W, Si W, Liu BA, et al. Inhibitor of DNA binding/differentiation helix-loop-helix proteins mediate bone morphogenetic protein-induced osteoblast differentiation of mesenchymal stem cells. J Biol Chem. 2004;279:32941–9.

48. Kanakaris NK, Petsatodis G, Tagil M, Giannoudis PV. Is there a role for bone morphogenetic proteins in osteoporotic fractures? Injury. 2009;40(Suppl 3):21-6.

49. Cheng HC, Liu SW, Li W, Zhao XF, Zhao X, Cheng M, et al. Arsenic trioxide regulates adipogenic and osteogenic differentiation in bone marrow MSCs of aplastic anemia patients through BMP4 gene. Acta Biochim Biophys Sin (Shanghai). 2015;47:673–9.

50. Lee SY, Kim GT, Yun HM, Kim YC, Kwon IK, Kim EC. Tectorigenin Promotes Osteoblast Differentiation and in vivo Bone Healing, but Suppresses Osteoclast Differentiation and in vivo Bone Resorption. Mol Cells. 2018;41:476–85.

51. Al Mamun MA, Hosen MJ, Islam K, Khatun A, Alam MM, Al-Bari MA. Tridax procumbens flavonoids promote osteoblast differentiation and bone formation. Biol Res. 2015;48:65.

52. Han Y, Kim YM, Kim HS, Lee KY. Melatonin promotes osteoblast differentiation by regulating Osterix protein stability and expression. Sci Rep. 2017;7:5716.

53. Matsumoto Y, Otsuka F, Takano-Narazaki M, Katsuyama T, Nakamura E, Tsukamoto N, et al. Estrogen facilitates osteoblast differentiation by upregulating bone morphogenetic protein-4 signaling. Steroids. 2013;78:513–20.
54. Kuo PL, Huang YT, Chang CH, Chang JK. Bone morphogenetic protein-2 and − 4 (BMP-2 and − 4) mediates fraxetin-induced maturation and differentiation in human osteoblast-like cell lines. Biol Pharm Bull. 2006;29:119–24.

55. van den Wijngaard A, Mulder WR, Dijkema R, Boersma CJ, Mosselman S, van Zoelen EJ, et al. Antiestrogens specifically up-regulate bone morphogenetic protein-4 promoter activity in human osteoblastic cells. Mol Endocrinol. 2000;14:623–33.

56. Suzawa M, Takeuchi Y, Fukumoto S, Kato S, Ueno N, Miyazono K, et al. Extracellular matrix-associated bone morphogenetic proteins are essential for differentiation of murine osteoblastic cells in vitro. Endocrinology. 1999;140:2125–33.

57. Yu Y, Yang JL, Chapman-Sheath PJ, Walsh WR. TGF-beta, BMPS, and their signal transducing mediators, Smads, in rat fracture healing. J Biomed Mater Res. 2002;60:392–7.

58. Khanal A, Yoshioka I, Tominaga K, Furuta N, Habu M, Fukuda J. The BMP signaling and its Smads in mandibular distraction osteogenesis. Oral Dis. 2008;14:347–55.

59. Farhadieh RD, Gianoutsos MP, Yu Y, Walsh WR. The role of bone morphogenetic proteins BMP-2 and BMP-4 and their related postreceptor signaling system (Smads) in distraction osteogenesis of the mandible. J Craniofac Surg. 2004;15:714–8.

60. Kim HY, Park SY, Choung SY. Enhancing effects of myricetin on the osteogenic differentiation of human periodontal ligament stem cells via BMP-2/Smad and ERK/JNK/p38 mitogen-activated protein kinase signaling pathway. Eur J Pharmacol. 2018;834:84–91.

61. Yu Y, Bliss JP, Bruce WJ, Walsh WR. Bone morphogenetic proteins and Smad expression in ovine tendon-bone healing. Arthroscopy. 2007;23:205–10.

62. Zhang Y, Musci T, Derynck R. The tumor suppressor Smad4/DPC 4 as a central mediator of Smad function. Curr Biol. 1997;7:270–6.

63. Salazar VS, Zarkadis N, Huang L, Norris J, Grimston SK, Mbalaviele G, et al. Embryonic ablation of osteoblast Smad4 interrupts matrix synthesis in response to canonical Wnt signaling and causes an osteogenesis-imperfecta-like phenotype. J Cell Sci. 2013;126:4974–84.

64. Salazar VS, Zarkadis N, Huang L, Watkins M, Kading J, Bonar S, Norris J, Mbalaviele G, Civitelli R. Postnatal ablation of osteoblast Smad4 enhances proliferative responses to canonical Wnt signaling through interactions with β-catenin. J Cell Sci. 2013;126:5598–609.

Figures
Figure 1

Effects of CdCl2 on viability of human bone marrow mesenchymal stem cells. A CCK-8 assay was used to determine the cytotoxicity of CdCl2 on hBMSCs cells. Calculate cell viability according to the equation. hBMSCs cells were exposed to different concentrations of CdCl2 (0, 0.625, 1.25, 2.5, 5.0, 10.0, 20.0, 40.0, 80.0 or 160.0 μM). * p < 0.05 (mean ± SD, n = 3).

![Graph showing cell viability](image)

Figure 2

Effects of CdCl2 on the osteogenic differentiation and the adipogenic differentiation of hBMSCs. The hBMSCs cells were exposed to 0, 2.5, or 5.0 μM CdCl2 and subjected to adipogenic differentiation for 10 days. (a) The numbers of adipose droplet were evaluated by oil red O staining. The human bone marrow mesenchymal stem cells were exposed to 0, 2.5, or 5.0 M CdCl2 and subjected to osteogenic
differentiation for 7 days. (b) The ALP content and the numbers of mineralization nodules were evaluated by ALP staining (upper) and alizarin red S staining (lower). The human bone marrow mesenchymal stem cells were treated for osteogenic induction for 14 days. (c) Mineralization nodules were determined by alizarin red S staining. (d-e) Relative protein levels of Runx2 and Osterix were determined (mean ± SD, n = 3). * p < 0.05, ** p < 0.01 different from hMSCs in the absence of CdCl2.

Figure 3

Effects of CdCl2 on BMP/Smad pathway. The hBMSCs were exposed to 0, 2.5 or 5.0 μM CdCl2 and subjected to osteogenic differentiation for 7 days. (a, c) Western blots were performed, and (b, d) relative
protein levels of BMP2, BMP4, BMP6, BMP7, Smad1, p-Smad1, Smad4, Smad5 and p-Smad1/5/9 were determined (mean ± SD, n = 3). * p < 0.05, ** p < 0.01, different from hMSCs in the absence of CdCl2.

\[
\text{IE: -0.0062 ; 95CI%: -0.0117, 0.00; } P < 0.0001
\]

**Figure 4**

Association among cadmium, BMP-4 and osteoporosis The mediating role of plasma BMP-4 ligand in the relationship between U-Cd and occurrence of osteoporosis. Causal mediation analysis was estimated the indirect effect of U-Cd on osteoporosis through plasma BMP-4 ligand. Note: CI, confidence interval; DE, direct effect; IE, indirect effect; PM, proportion mediated.

**Supplementary Files**

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