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Research article

Bone morphogenetic protein 4 is involved in cadmium-associated bone damage

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Abstract

Cadmium (Cd) is a well-characterized bone toxic agent and can induce bone damage via inhibiting osteogenic differentiation. Bone morphogenetic protein (BMP)/SMAD signaling pathway can mediate osteogenic differentiation, but the association between Cd and BMP/SMAD signaling pathway is yet to be illuminated. To understand what elements of BMPs and SMADs are affected by Cd to influence osteogenic differentiation and if BMPs can be the biomarkers of which Cd-induced osteoporosis, human bone marrow mesenchymal stem cells (hBMSCs) were treated with cadmium chloride ($CdCl_2$) *in vitro* to detect the expression of BMPs and SMADs, and 134 subjects were enrolled to explore if the BMPs can be potential biomarkers of Cd-associated bone damage. Our results showed that Cd exposure significantly promoted the adipogenic differentiation of hBMSCs and inhibited its osteogenic differentiation by inhibiting the expression of BMP-2/4, SMAD4, and p-SMAD1/5/9 complex. And mediation analyses yielded that BMP-4 mediated 39.32% (95% confidence interval 7.47, 85.00) of the total association between the Cd and the risk of Cd-associated bone damage. Moreover, during differentiation, BMP-4 had the potential to enhance mineralization compared with $CdCl_2$ only group. These results reveal that BMP-4 can be a diagnostic biomarker and therapeutic target for Cd-associated bone damage.

Keywords: cadmium; bone damage; osteogenic differentiation; BMP; SMAD.

Osteoporosis is a bone damage disease that is characterized by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture (Lane *et al.*, 2000). It is estimated that nearly 22 million women and 5.5 million men are suffering from osteoporosis, leading to an increased risk of fractures in women aged 55 years or older and men aged 65 years or older coupled with increased mortality and healthcare costs (Lane *et al.*, 2000; Fuggle *et al.*, 2019). Numerous studies have investigated the risk factors of osteoporosis, which found apart from the 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 (Amuno *et al.*, 2018; Lavado-Garcia *et al.*, 2017; Satarug *et al.*, 2010). Since the 1950s, findings have suggested that Cd could be associated with adverse effects on bone structures (Kido *et al.*, 1989; Nambunmee *et al.*, 2010; Nawrot *et al.*, 2010; Schutte *et al.*, 2008). A series of studies including cross-sectional and prospective studies of different populations have demonstrated an association between cadmium exposure and low bone mineral density and an increased risk of bone damage (Alfvén *et al.*, 2002; Buha *et al.*, 2019; Chen *et al.*, 2009). 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 (Akesson *et al.*, 2006; Gallagher *et al.*, 2008; Ma *et al.*, 2021). Bone marrow-derived mesenchymal stromal cells (BMSCs) are the progenitors of osteoblasts which growing evidence has indicated during osteoporosis development exhibit a reduced capacity to differentiate into osteoblasts and an increased capacity to differentiate into adipocytes resulting in a reduction in bone formation (Bianco and Robey, 2015; Haddouti *et al.*, 2020; Kondrikov *et al.*, 2020; Li *et al.*, 2011, 2015; Moerman *et al.*, 2004; Yeung *et al.*, 2005). But how Cd inhibits BMSC osteoblast differentiation is not clear.

In most species, bone morphogenetic proteins (BMPs) can direct the commitment of marrow-derived mesenchymal stromal cells into osteoprogenitors and further stimulate osteogenic differentiation and bone mineralization (Hu *et al.*, 2018; Huang *et al.*, 2020; Salazar *et al.*, 2016). Both human and mouse genetic studies have demonstrated that BMPs perform positive roles in postnatal bone homeostasis including osteoblast expansion, differentiation, and bone formation (Dalmo *et al.*, 2020; Parrow and Fleming, 2014; Salazar *et al.*, 2016). The response to the activation of BMPs mainly through the BMP-SMAD signaling pathway regulates stem cell renewal, differentiation, migration, and apoptosis, controls embryo development and postnatal tissue homeostasis (de Kroon *et al.*, 2017; Lv *et al.*, 2017). However, the complex regulated network relationship among Cd, BMPs, and SMADs is still unclear. In this study, we combined human bone marrow mesenchymal stem cells (hBMSCs) with a case-control study to explore the effect of BMPs and SMADs in $CdCl_2$-regulated osteogenic differentiation of hBMSCs and the possibility of BMPs as biomarkers of Cd-associated bone damage.

Materials and methods

Isolation and culture of hBMSCs

hBMSCs gifted from the Zhongshan Medical University were isolated from fresh human bone marrow aspirates from patients undergoing fracture management. Flow cytometry analysis showed that hBMSCs were positive for the surface markers CD29, CD44, and CD90, but negative for the hematopoietic (CD34 and CD45) markers (Supplementary Figure 1). The osteogenic and adipogenic potentials of hBMSCs were demonstrated by Alizarin Red S and Oil Red O (Supplementary Figure 2). The patients which included 2 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, Missouri) and supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, Massachusetts) and 1% penicillin-streptomycin (Corning, Sigma-Aldrich, St. Louis, Missouri). The cells were cultured at 37°C in a humidified incubator containing 5% CO_2. 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 in passages 3–6.

Cell viability assay

Cell viability was assessed with Cell Counting Kit-8 (CCK-8, Beyotime, Nanjing, China) following the manufacturer's instructions. hBMSCs were seeded in 96-well plates at a density of 5000 cells/well. After 24 hours, 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 $CdCl_2$ (Sigma Aldrich) for 24 hours and 14 days. Subsequently, a 100 μl volume of CCK-8 solution was added to each cell, followed by incubation for 2 h at 37°C, 5%CO_2. Consequently, absorbance at 450 nm was measured on a microplate reader (Biotek, Winooski, Vermont).

$CdCl_2$ and BMP-4 treatment

For $CdCl_2$ exposure of hBMSCs during osteogenic and adipogenic differentiation, $CdCl_2$ at concentrations of 0, 2.5, or 5.0 μM was added into the osteogenic/adipogenic induction medium to replace the growth medium. For adipogenic differentiation, hBMSCs were incubated for 10 days. For osteogenic differentiation, hBMSCs were incubated for 14 days. During the osteogenic and adipogenic differentiation, the osteogenic/adipogenic induction medium added with or without $CdCl_2$ was changed every 2 days. For BMP-4 groups, the medium included 50 ng/ml of recombinant BMP-4 (MedChemexpress).

For siRNA-mediated knockdown, predesigned siRNAs specific for BMP-4 and nontargeting control siRNA were obtained from RIBOBIO (R10043.8, control siRNA: siN0000001-1-5, siBMP-4: stB0003737B-1-5, China). Lipofectamine 3000 transfection reagent (Invitrogen) was used to transfect hBMSCs with siRNAs according to the supplier's recommendations. Afterward, the cells were cultured for an additional 48 hours to allow robust knockdown of protein before experiments were carried out. After transfection, hBMSCs were incubated with an osteogenic induction medium for 14 days, the medium was replaced every 2 days. The target sequence for BMP-4 is as follows: CTTCCACCGTATAAACATT.

Alizarin Red S and Oil Red O staining

The hBMSCs were seeded into 12-well plates at a density of 2×10^5 cells/well and exposed to $CdCl_2$ 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) solution at room temperature for 20 min. After discarding the solutions and washing the plates with PBS for 4 times, images were taken under an optical microscope at 10× magnification (Zeiss, Germany).

Oil Red O powder (Sigma) 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 12-well plates, return the 12-well plates 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, and 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, the mouth of the dish was sealed with a parafilm, and the image was taken on a light microscope (Zeiss, Germany) at 10× 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, China), and quantified by bicinchoninic acid protein assay kit (KeyGEN BitoTECH). Mixtures of cellular proteins and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (5×, Panera, Guangzhou, China) 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, Massachusetts) using the wet-transfer system at 100 V for 50 min. 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) and an antibody for Runx2 (Cell Signaling Technology USA), Osterix (Abcam Cambridge, UK), BMP-2/-4/6/7 (ABclonal, Wuhan, China), SMAD1/4/5, p-SMAD1, and p-SMAD1/4/9 complex (Cell Signaling Technology). Membranes were thereafter rinsed 5 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, California) for 1.5 hours at room temperature. After washing, strips in membranes were visualized using chemiluminescent peroxidase substrate (Millipore) and Tanon-5200 chemical luminescence developing system (Tanon, Shanghai, China). GAPDH served as the internal reference. The relative expressions of Runx2, Osx, BMP-2/-4/6/7, SMAD1/4/5, p-SMAD1, and p-SMAD1/4/9 complex proteins in treatment groups were determined by grey value analysis using Image J software (https://imagej.net/). ELISA kits were used to detect the concentration of BMP-2 (Abcam, Cambridge, UK) and BMP-4 (Thermo, Frederick), respectively.

Participants

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 to Cd. The population was divided into 2 groups: namely bone damage and non-bone damage group depending on DEXA results (T-score) as follows: bone damage patients with T-score ≤ -2.5 and non-bone damage patients with T-score > -2.5 (Kanis, 1994). Both bone damage and non-bone damage group included 67 subjects (male 28, female 39).

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

Collection of samples and analytical method

Peripheral blood and first-morning urine samples were collected before breakfast. Blood samples were centrifuged at 1500 g 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. Urinary cadmium (U-Cd) concentrations were determined using inductively coupled plasma mass spectrometry (7700 × ICP-MS, Agilent Technologies). 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 the relative standard deviation was 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, Vermont) at a wavelength of 546 nm, using a Creatinine assay kit (sarcosine oxidase, Nanjing Jiancheng, China). ELISA kits were used to detect human BMP-4 in plasma (Thermo, Frederick) and BMP-2 in serum (Abcam), respectively.

Determination of bone mineral density

Bone mineral density at the forearm was measured using peripheral dual-energy X-ray absorptiometry (DXA; 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 a reference for the highest bone density.

Statistical analysis

GraphPad 7.0 was applied for statistical analyses. Descriptive statistical analyses were performed for the 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 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 $p < .05$ were considered statistically significant.

Results

Effects of cadmium chloride on the viability and osteogenesis and adipogenic differentiation of hBMSCs

To support the toxicity of Cd in hBMSCs, cells were incubated with different doses of $CdCl_2$ for 24 hours and 14 days, and the medium added with or without $CdCl_2$ was changed every 2 days. We established the effect of $CdC1_2$ on cell viability using the CCK-8 assay (Figure 1A). $CdCl_2$ caused a significant doses dependent reduction in the viability with 10–100 μM $CdC1_2$, but not with 5 μM $CdC1_2$ 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 $CdC1_2$ as representative of cytotoxic dose for further studies.

To detect the effects of $CdCl_2$ on osteogenesis and adipogenic differentiation of hBMSCs, cells were treated as in Figure 1B, and the osteogenic/adipogenic induction medium added with or without $CdCl_2$ was changed every 2 days. Then Oil Red O staining (Figure 1C) showed that 2.5 and 5 μM $CdCl_2$ can increase intracellular lipid droplet formation. Alizarin Red staining (Figure 1D) showed that 2.5 and 5 μM $CdCl_2$ can extremely reduce intracellular calcification nodules quantity and volume. And 5 μM $CdCl_2$ can downregulate 75% of the protein expression levels of Runx2 and 86% of OSX ($p < .05$, Figs. 1E and 1F), suggesting that $CdCl_2$ can inhibit hBMSCs osteogenic differentiation.

Effects of cadmium chloride on BMPs and SMADs

In our study, the expression of BMP-2/4/6/7, SMAD1/4/5, p-SMAD1, and p-SMAD1/4/9 complex were detected. Results showed that the expression of BMP-2 was reduced by 69.76% compared with 0 μM, and BMP-4 was also reduced by 76.77% ($p < .05$, Figs. 2A and 2B), but the expression of BMP-6 and BMP-7 had no statistical difference with 0 μM. The expression of SMAD4 was reduced by 52% compared with 0 μM and p-SMAD1/5/9

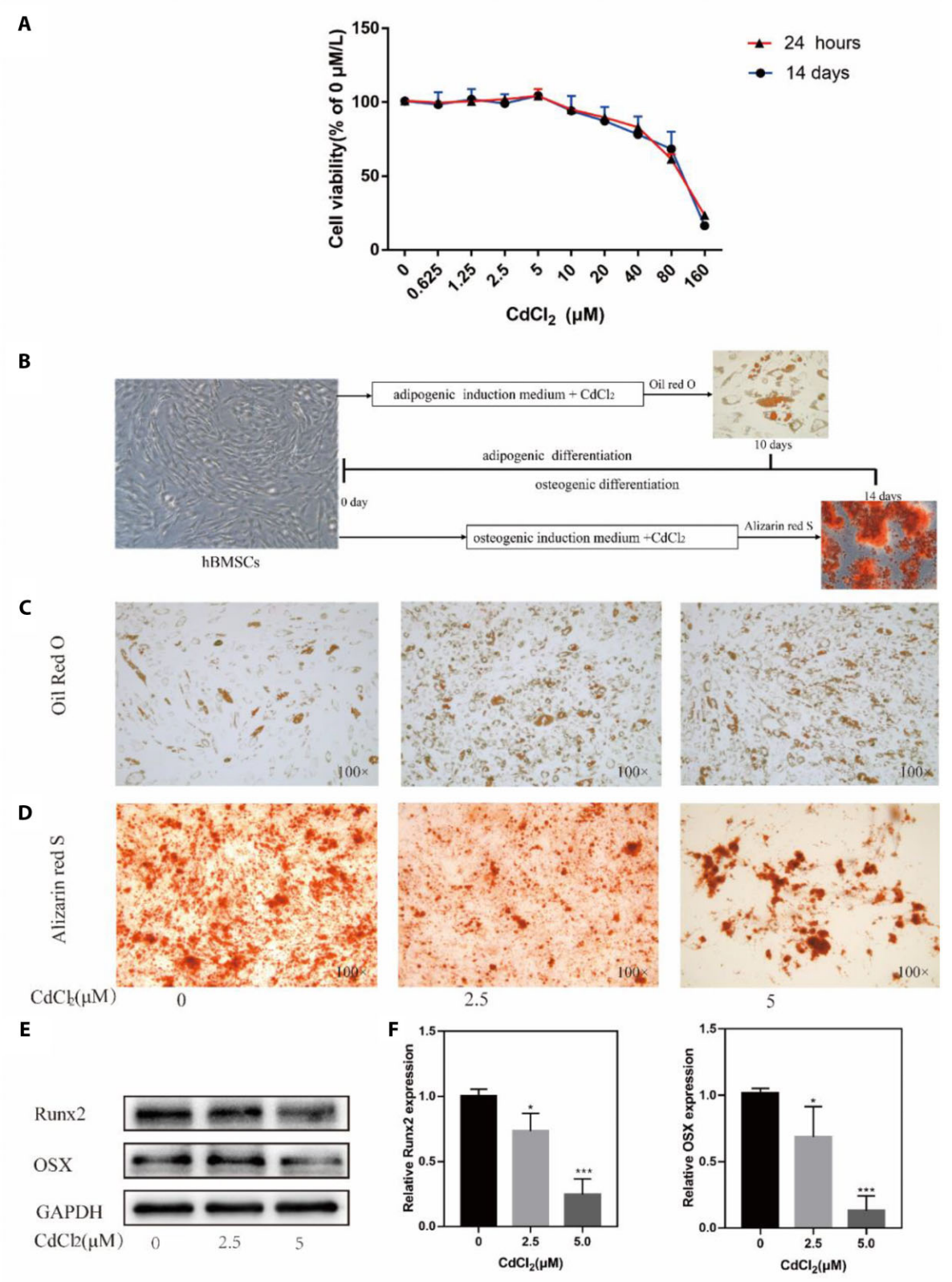

Figure 1. Effects of $CdCl_2$ on the viability, osteogenic differentiation, and adipogenic differentiation of hBMSCs. A CCK-8 assay was used to determine the cytotoxicity of $CdCl_2$ on hBMSCs cells. Calculate cell viability according to the equation. A, hBMSCs cells were exposed to different concentrations of $CdCl_2$ (0, 0.625, 1.25, 2.5, 5.0, 10.0, 20.0, 40.0, 80.0, or 160.0 μM). To detect the effects of $CdCl_2$ exposure on hBMSCs osteogenic and adipogenic differentiation, hBMSCs were treated as (B), with the $CdCl_2$ concentrations of 0, 2.5, or 5.0 μM based on the results of CCK-8. For adipogenic differentiation, hBMSCs were differentiated for 10 days. C, The numbers of adipose droplets were evaluated by Oil Red O staining. For osteogenic differentiation, hBMSCs were differentiated for 14 days. D, Mineralization nodules were determined by Alizarin Red S staining. E and F, Relative protein levels of Runx2 and OSX were determined. These are the results from 3 independent biological replicates experiments (mean ± SD, $n = 3$). $^{*}p < .05$, $^{***}p < .001$ different from hBMSCs in the absence of $CdCl_2$. Abbreviations: CCK-8, Cell Counting Kit-8; hBMSC, human bone marrow mesenchymal stem cell.

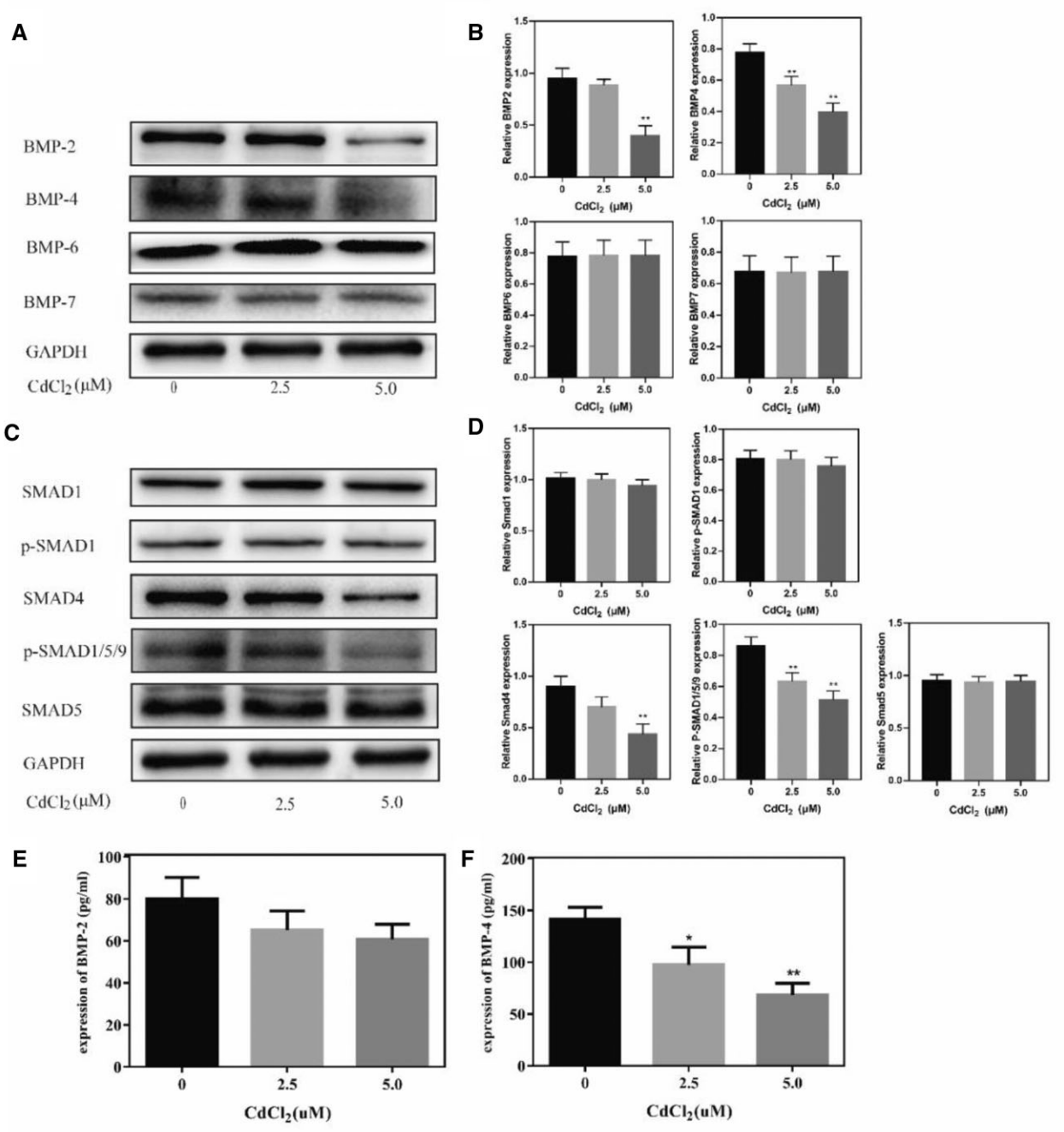

Figure 2. Effects of $CdCl_2$ on BMP/SMAD pathway. The hBMSCs were exposed to 0, 2.5, or 5.0 μM $CdCl_2$ and subjected to osteogenic differentiation for 14 days. A and C, Western blots were performed, and (B and D) relative protein levels of BMP-2, BMP-4, BMP-6, BMP-7, SMAD1, p-SMAD1, SMAD4, SMAD5, and p-SMAD1/5/9 were determined. E and F, Expression levels of BMP-2 and BMP-4 were detected by ELISA. These are the results from 3 independent biological replicates experiments (mean ± SD, $n = 3$). $^{*}p < .05$, $^{**}p < .01$, different from hMSCs in the absence of $CdCl_2$. Abbreviations: BMP, bone morphogenetic protein; hBMSC, human bone marrow mesenchymal stem cell.

complex was reduced by 31% ($p < .05$, Figs. 2C and 2D), and the expression of SMAD1, SMAD5, and p-SAMD1 had no statistical difference with 0 μM. The concentration of BMP-2 and BMP-4 in cells culture supernatant, results showed that the BMP-4 was decreased by 52% after being treated with $CdCl_2$ ($p < .05$, Figs. 2E and 2F).

Cadmium, BMP-4, and bone damage: a case-control study

The clinical characteristics of the study participants were summarized according to the cases and control groups in Table 1. There was no significant difference in sex, BMI, urinary calcium, urinary lead, and smoking status among the 2 groups. However, individuals who suffered from bone damage had higher U-Cd concentrations than those in the non-bone damage group ($p < .05$). Moreover, the average concentration of BMP-4 in the non-bone damage group was higher than in the bone damage group ($p < .05$).

The U-Cd concentration was significantly negative correlated with BMP-4 ($r = -0.22$, $p < .05$), and a significant positive association was observed between BMP-4 concentration and T value

Table 1. Demographic characteristics of subjects from osteoporosis and non-osteoporosis

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)	.53
Gender, *n* (%)	67 (50%)	67 (50%)	1.00
Male	28 (41.79%)	28 (41.79%)	—
Female	39 (58.21%)	39 (58.21%)	—
BMI (kg/m^2)	22.74 (22.05, 23.41)	22.24 (21.58, 22.91)	.30
Smoking status, *n* (%)	67 (50.00%)	67 (50.00%)	.86
Yes	26 (38.81%)	27 (40.30%)	—
No	41 (61.19%)	40 (59.70%)	—
Urinary calcium (mg/l)	124.87 ± 86.47	126.95 ± 79.51	.89
Urinary lead (μg/g cr)	7.21 ± 7.11	8.81 ± 9.46	.27
Urinary cadmium (mg/g cr)	7.14 (3.87, 10.41)	12.30 (9.02, 15.57)	.03
BMP4 (pg/ml)	624.80 (597.84, 651.77)	544.14 (517.17, 571.11)	<.01

Results were reported as means and standard deviations. Variables with a non-normal distribution were described by median and the 5th to 95th percentile interval.
Abbreviations: BMI, body mass index; BMP-4, bone morphogenetic protein 4; cr, creatinine.

($r = 0.33$, $p < .05$) (Supplementary Figure 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 39.32% (95% confidence interval 7.47, 85.00) of the total effect of the BMP-4 on the T value mediated by U-Cd (Figure 3).

BMP-4 promoted osteogenic differentiation inhibited by cadmium chloride

$CdCl_2$ decreased the level of BMP-4 (Figure 4A) to inhibit osteogenic differentiation. The presence of intracellular calcification nodules in quantity and volume suggested that supplied BMP-4 significantly promoted osteogenic differentiation inhibited by $CdCl_2$ (Figure 4B). Moreover, in the BMP-4 + $CdCl_2$ group, the protein expression levels of Runx2, OSX, SMAD4, and p-SMAD1/5/9 complex were significantly up-regulated than $CdCl_2$ only group (Figs. 4C and 4D).

BMP-4 siRNA inhibited osteogenic differentiation

$CdCl_2$ could reduce the expression of BMP-4. To clarify if BMP-4 was a potential biomarker of bone damage, we further established knockdown experiments of BMP-4 in the cell models. The presence of intracellular calcification nodules in quantity and volume suggested that reducing the expression of BMP-4 significantly inhibited osteogenic differentiation (Figure 5A). Moreover, the protein expression levels of Runx2, OSX, SMAD4, and p-SMAD1/5/9 complex were significantly downregulated (Figs. 5B and 5C).

Discussion

Bone damage is the most frequent degenerative disease in developed countries (Rodríguez and Mandalunis, 2016). Several studies have demonstrated that Cd exposure and low bone mineral density were correlated (Buha *et al.*, 2019; Chen *et al.*, 2009, 2011; Rodríguez and Mandalunis, 2016). For better knowledge of the underlying pathogenetic mechanisms and screening for useful diagnostic biomarkers of Cd-associated bone damage, we investigated specific protein signatures for bone damage. In this study, we found Cd could inhibit hBMSCs differentiation via BMP-2, BMP-4, SMAD4, and p-SMAD1/5/9 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. And BMP-4 can promote osteogenic differentiation inhibited by $CdCl_2$.

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 (Chen *et al.*, 2011; Rodríguez and Mandalunis, 2016). Our data also showed that the bone mineral density of the population exposed to Cd is usually relatively low compared with those living in areas with fewer Cd exposures. Thus, identifying the biomarkers of Cd toxicity in bone damage is significant for the development of prophylactic and therapeutic strategies.

Bone formation involves the commitment of BMSCs to the osteoblastic lineage and their subsequent differentiation (Aslani *et al.*, 2019). Many studies have found that bone damage is primarily driven by the dysregulated differentiation of BMSCs into osteoblasts (Clemens *et al.*, 2013; Knani *et al.*, 2019; Ozkan *et al.*, 2010). And *in vitro* evidences have demonstrated that long-term, low-dose Cd exposure can dramatically inhibit the differentiation of BMSCs into osteoblasts (Knani *et al.*, 2019; Ma *et al.*, 2021). In our study, osteogenic differentiation greatly inhibited mineral nodules and the expression levels of related osteogenic markers (Runx2, OSX) were decreased the adipogenic differentiation was increased, which is consistent with the previous research (Lv *et al.*, 2019; Wang *et al.*, 2020; Wu *et al.*, 2019). Many studies have also demonstrated that Cd exposure suppresses BMSCs osteogenesis through a series of signaling pathways, but the detailed mechanism and sensitive and specific biomarkers are not clear (Clemens *et al.*, 2013; Li *et al.*, 2015; Lv *et al.*, 2019; Ma *et al.*, 2021). To find sensitive and specific biomarkers of Cd exposure that inhibit 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 (Abe *et al.*, 2000; Luppen *et al.*, 2008; Nirmala *et al.*, 2020). The BMP/SMAD signaling pathway has been widely reported in bone-related diseases. BMPs are a unique extracellular multifunctional signaling cytokine, belonging to the large transforming growth factor β (TGF-β) superfamily (Kitisin *et al.*, 2007). TGF-β superfamily signaling plays an important role in the regulation of cell growth, differentiation, and development in numerous biological systems. Signal transduction begins with ligand-induced oligomerization of serine/threonine receptor kinases and phosphorylation of cytoplasmic signaling molecules in the TGF-β/activin

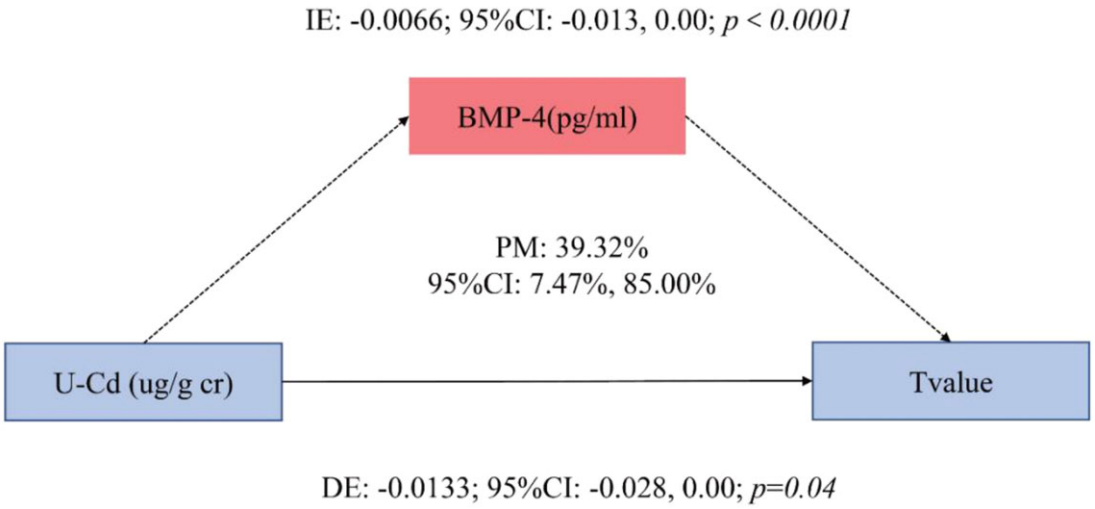

Figure 3. Association among Cd, BMP-4, and osteoporosis. The mediating role of plasma BMP-4 ligand in the relationship between U-Cd and occurrence of osteoporosis. Causal mediation analysis estimated the indirect effect of U-Cd on osteoporosis through plasma BMP-4 ligand. Abbreviations: BMP, bone morphogenetic protein; CI, confidence interval; DE, direct effect; IE, indirect effect; PM, proportion mediated; U-Cd, urinary cadmium.

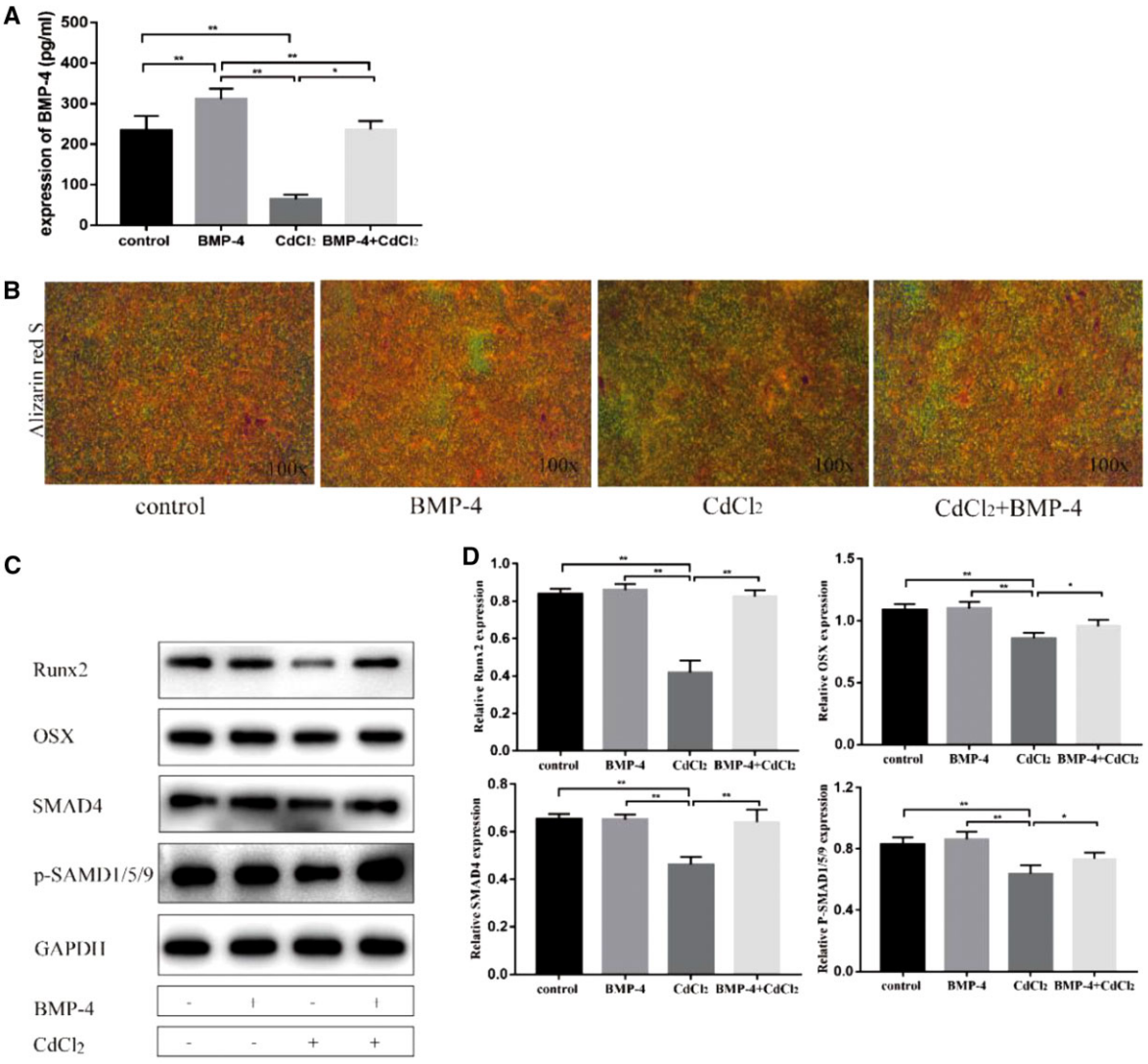

Figure 4. The osteogenic effect of BMP-4 on hBMSCs. The hBMSCs were treated with BMP-4 (50 ng/ml) and/or $CdCl_2$ (5.0 μM) for 14 days. A, The BMP-4 levels detected by ELISA. B, Mineralization was measured using Alizarin Red S staining. C, Western blots were performed. D, Relative protein levels of Runx2, OSX, SMAD4, and p-SMAD1/5/9 complex were determined. These are the results from 3 independent biological replicates experiments (mean ± SD, $n = 3$). $^{*}p < .05$, $^{**}p < .01$. Abbreviations: BMP, bone morphogenetic protein; hBMSC, human bone marrow mesenchymal stem cell.

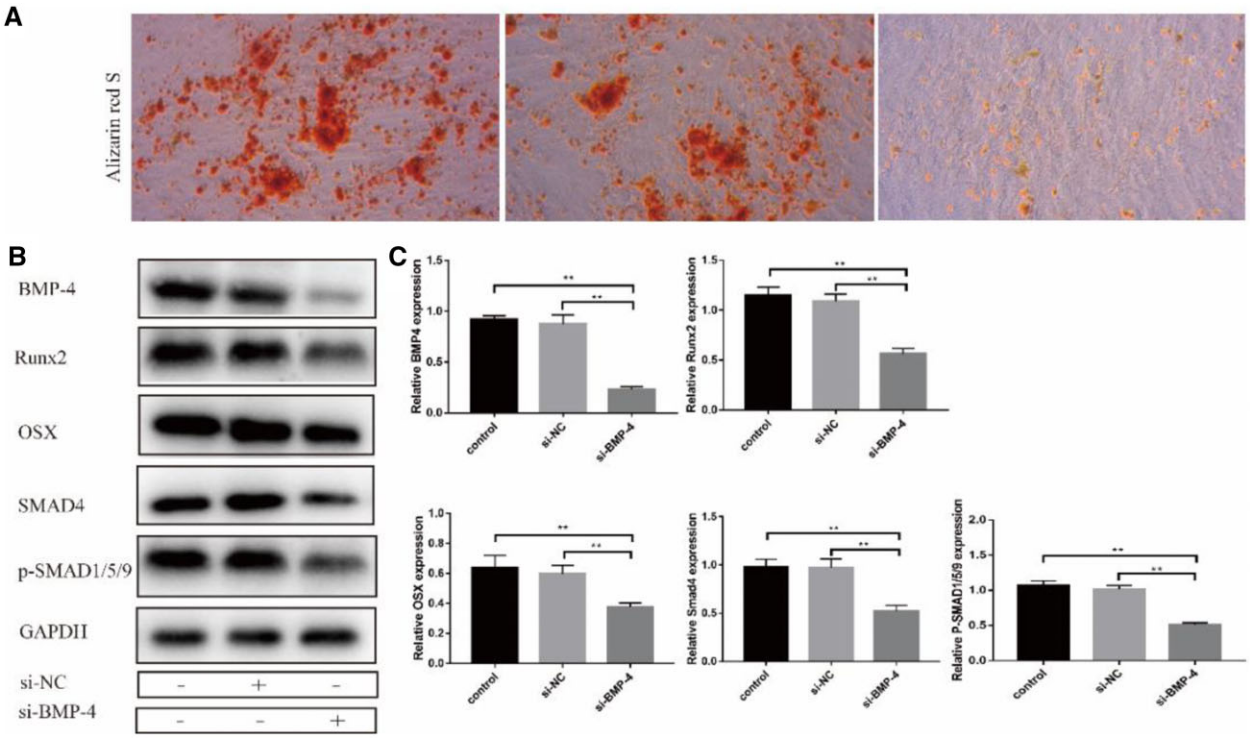

Figure 5. The effects of BMP-4 knockdown on osteogenic differentiation and SMADs. After si-BMP-4 transfect the hBMSCs were osteogenic induction for 14 days. A, Mineralization was measured using Alizarin Red S staining. B, Western blots were performed. C, Relative protein levels of BMP-4, Runx2, OSX, SMAD4, and p-SMAD1/5/9 complex were determined. These are the results from 3 independent biological replicates experiments (mean ± SD, $n = 3$). $^{**}p < .01$.

pathway SMAD2 and SMAD3, and in the BMP pathway are SMAD1/5/9 (Schmierer and Hill, 2007). The carboxy-terminus of SMADs is phosphorylated by activated receptors, leading to binding to the common signal transduction factor SMAD4 and translocation into the nucleus. Activated SMADs can modulate various biological effects by binding to transcription factors, resulting in cell state-specific transcriptional regulation (Schmierer and Hill, 2007). 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 (Kang *et al.*, 2009). When the osteogenic were further analyzed, BMP-2, -4, -6, and -7 were shown to effectively induce osteogenic differentiation of BMSCs *in vitro* and *in vivo* (Cheng *et al.*, 2015; Kanakaris *et al.*, 2009; Lee *et al.*, 2018; Luu *et al.*, 2007; Peng *et al.*, 2003, 2004). But there is no study about cadmium, BMPs, and bone damage. Our current study found that Cd inhibited the expression of BMP-2 and BMP-4, but there were no effects on the expression of BMP-6 and BMP-7. Our results also showed that BMP-2/4 accumulated intracellular matrices are essential for the osteoblastic differentiation of cells in the osteoblast lineage by upregulation of the OSX and Runx2 (Al Mamun *et al.*, 2015; Han *et al.*, 2017; Kuo *et al.*, 2006; Matsumoto *et al.*, 2013; Suzawa *et al.*, 1999; van den Wijngaard A *et al.*, 2000). 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 SMADs 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 (Farhadieh *et al.*, 2004; Khanal *et al.*, 2008; Kim *et al.*, 2018; Yu *et al.*, 2002, 2007; Zhang *et al.*, 1997). Integrating with the results of BMPs, we also detected the expression of SMAD1, SMAD4, SMAD5, P-SMAD1, and SMAD1/5/9 complex in the control group and $CdCl_2$ treated groups. Studies have shown that BMP-2/4-induced osteoblast commitment is mediated by the phosphorylation of specific transactivators (p-SMAD1/5/9 complex) and SMAD4. Series studies have indicated that 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.) (Abe *et al.*, 2000; Salazar *et al.*, 2013). And 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 (Akesson *et al.*, 2006; Gallagher *et al.*, 2008; Ma *et al.*, 2021). 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 Cd-associated bone damage, we enrolled 134 subjects to evaluate whether BMP-2 or BMP-4 mediated the risk between Cd and bone damage. The participants that lived in the region where we investigated exposure to Cd through the consumption of staple food by local residents. In our previous study found a much higher average level of Cd was observed in rice and leafy vegetable samples, but the Cd levels in leafy vegetables were influenced by cooking practices (Xu *et al.*, 2021). Therefore, we can investigate if BMP-2 or BMP-4 mediated the risk between Cd and bone damage based on a case-control study. Surprisingly, the 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 and *in vitro* experiments were used to detect the effects of BMP-4. These results showed that BMP-4 acted as a mediator in cadmium and bone damage. And *in vitro* experiments, BMP-4 can significantly promote osteogenic differentiation inhibited by $CdCl_2$. And the knockdown experiment further clarified the value of BMP-4 in osteogenic differentiation. Therefore, decreased BMP-4 levels could be a plausible biological explanation for our findings. In summary, Cd can decrease the expression of BMP-4, which results in the phosphorylation of the p-SMAD1/5/9 complex being down-regulated and the binding of p-SMAD1/5/9 to the common signal transduction factor SMAD4 being inhibited. Moreover, the complex formed by p-SMAD1/5/9 with SMAD4 translocation into the nucleus of recruit cofactors regulates the expression of osteogenic genes also been restricted. To the best of our knowledge, this is the first study to report that BMP-4 is involved in cadmium-associated bone damage.

Some limitations of this study should be addressed. Firstly, our data have 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. The reason of what may be the ELISA kit of BMP-2 that we used has a tissue specificity. It has particularly abundant in the lung, spleen, and colon and at low levels in the heart, brain, placenta, liver, skeletal muscle, kidney, pancreas, prostate, ovary, and small intestine. And the range that can be detected is 31.2–2000 pg/ml. That may be why we could not detect the expression of BMP-2. Secondly, the individuals in 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.

Conclusions

Our study shows that Cd suppresses the osteogenic differentiation of hBMSCs and addresses the effects of Cd on BMP-2/4, SMAD4, and p-SAMD1/5/9 complex. And BMP-4 is the critical factor in Cd-associated bone damage. Our findings explain how Cd induces bone damage and provides public health implications for developing strategies to reduce Cd exposure and thereby mitigate its harmful effects.

Supplementary data

Supplementary data are available at *Toxicological Sciences* online.

Author contributions

Y.W., L.-j.M., and L.W.: conceptualization, writing—original draft, writing—review and editing. Y.W., L.-j.M., L.W., D.-l.L., J.S., Y.-k. H., H.-b.H., Q.-z.W., D.-p.W., J.-m.Q., and Z.-j.Z.: methodology, software, project administration, visualization, and investigation. X.-f.Y., D.-p.W., and Q.-z.W.: funding acquisition. X.-f.Y. and Q.-z.L.: supervision.

Ethics declarations

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

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Declaration of conflicting interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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