Article

Wnt/β-Catenin Pathway Is Involved in Cadmium-Induced Inhibition of Osteoblast Differentiation of Bone Marrow Mesenchymal Stem Cells

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Abstract: Cadmium is a common environmental pollutant that causes bone damage. However, the effects of cadmium on the osteogenic differentiation of bone marrow mesenchymal stem cells (BMMSCs) and its mechanism of action in this process are unclear. Here, we determined the effects of cadmium chloride (CdCl2) on the osteogenic differentiation of BMMSCs and the potential mechanism involved in this process. As determined in the present investigation, CdCl2, in a concentration-dependent manner, affected the viability of BMMSCs and their cytoskeletons. Exposure to 0.1 or 0.2 µM CdCl2 inhibited osteogenic differentiation of BMMSCs, which was reflected in the down-regulation of osteoblast-related genes (ALP, OCN, Runx2, OSX, and OPN); in suppression of the protein expression of alkaline phosphatase (ALP) and runt-related transcription factor 2 (Runx2); and in decreased ALP activity and capacity for mineralization. Moreover, mRNA microarray was performed to determine the roles of these factors in BMMSCs treated with CdCl2 in comparison to control BMMSCs. As determined with the microarrays, the Wingless-type (Wnt), mothers against decapentaplegic and the C. elegans gene Sam (SMAD), and Janus kinase-Signal Transducers and Activators of Transcription (JAK-STAT) signaling pathways were involved in the effects caused by CdCl2. Moreover, during differentiation, the protein levels of Wnt3a, β-catenin, lymphoid enhancer factor 1 (LEF1), and T-cell factor 1 (TCF1) were reduced by CdCl2. The current research shows that CdCl2 suppresses the osteogenesis of BMMSCs via inhibiting the Wnt/β-catenin pathway. The results establish a previously unknown mechanism for bone injury induced by CdCl2.

Keywords: osteogenesis; bone marrow mesenchymal stem cells; cadmium; Wnt/β-catenin pathway
1. Introduction

Cadmium, a heavy metal and persistent environmental toxicant, has a biological half-life of 10–30 years [1,2]. It is released into the environment through natural processes and human activities, such as mining and smelting, which may lead to contamination of soils and to increased cadmium uptake by crops and vegetables that are grown for human consumption [3]. A cluster of adverse health effects, including bone damage and nephrotoxicity, occur with long-term exposure to water and food contaminated with cadmium. Exposure to high levels to cadmium reduces bone mineral density (BMD) manifested as osteoporosis and osteomalacia [4]. In addition, population-based studies from several countries reveal an association between decreased BMD and low-level environmental exposure to Cd [3]. The molecular and genetic basis by which CdCl$_2$ causes diseases, however, remains largely unknown.

As a rigid organ, bone is in a dynamic balance and is self-renewed by the activities of osteoblasts and osteoclasts [5]. Osteoclasts resorb bone and then recruit bone marrow mesenchymal stem cells (BMMSCs) for subsequent differentiation and bone formation, which is responsible for continuous bone remodeling [6]. BMMSCs, as potential progenitor cells regulated by transcription factors, differentiate into a variety of cell lineages including osteoblasts, chondrocytes, and adipocytes [7,8]. Based on the properties mentioned above, MSCs play a vital role in tissue repair and regeneration and have great potential applications in tissue engineering and regenerative medicine [9–13]. Compared to immortalized cell lines, which have abnormal characteristics after transformation, using BMMSCs to assess the toxicity of cadmium has many advantages [5]. Therefore, we choose BMMSCs to investigate the toxicity of CdCl$_2$ on bone formation.

Various cytokines, growth factors, and signaling pathways, including the Wnt/β-catenin pathway, are essential in the regulation of osteoblast differentiation [14]. There has many research reporting that Wnt/β-catenin pathway is associated with differentiation of BMSCs. Recently, a research revealed that the inhibition of Wnt pathway suppresses osteogenic differentiation of BMSCs during osteoporosis [15]. In BMSCs, insulin-like growth factor binding protein 7 (IGFBP7) regulates the osteogenic differentiation via Wnt/β-catenin pathway [16]. The activation of canonical Wnt signaling leads to cytoplasmic stabilization and increased nuclear translocation of β-catenin, which activates T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors and switches from repression to activation of TCF/LEF target genes [17]. To date, there has been no report on the relationship between the Wnt pathway and the inhibition of osteogenic differentiation of BMMSCs induced by CdCl$_2$. Knowledge of the regulatory Wnt pathway and its role in the osteogenic differentiation of BMMSCs is limited.

The present study investigated the inhibition of osteogenesis in BMMSCs by CdCl$_2$. The expression patterns of mRNAs in BMMSCs and CdCl$_2$-treated BMMSCs (CdCl$_2$-BMMSCs) were analyzed. For CdCl$_2$-BMMSCs, changes of signaling pathways that may be involved in the suppression of osteogenic differentiation of BMMSCs induced by CdCl$_2$ were addressed. Further, during osteogenic differentiation, CdCl$_2$ inhibited the Wnt pathway. The results provide an understanding of bone dysfunction induced by CdCl$_2$.

2. Results

2.1. Purity and Potential to Differentiate of BMMSCs

Primary BMMSCs were isolated from rat bone marrow and passaged. Cells adhered to the plastic culture dishes and demonstrated a typical small spindle-shaped morphology (Figure 1A). BMMSCs have positive and negative markers, including Sca-1, CD44, CD73, CD90, CD105, and CD45 [18–20]. Confirming the identity of BMMSCs, flow cytometry analysis showed that the cells had high levels of CD44 (99.80 ± 0.19%) and CD90 (96.54 ± 0.46%) but limited expression of the hematopoietic marker CD45 (1.93 ± 0.56%) (Figure 1B). The data confirm that the isolated cells were positive for mesenchymal-associated markers. Multipotency is another criterion for defining BMMSCs [21]. After 21 days of adipogenic induction, numerous lipid droplets were detected by staining with Oil Red O (Figure 1C). After 14 days of osteogenic induction, numerous mineralized
nodules were evident, as revealed by alizarin red S staining (Figure 1D). These results indicated that the primary BMMSCs cultured had adequate purity and a high potential to differentiate.

2.2. Low Concentrations of Cadmium Have No Effect on Cell Viability, but High Concentrations of Cadmium Reduce Cell Viability of BMMSCs in a Concentration-Dependent Manner

To evaluate CdCl₂ cytotoxicity, BMMSCs were incubated with 0, 0.25, 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, 10, or 20 µM CdCl₂ for 24 h. For low concentrations (≤1.0 µM), there were no significant differences in cell viability between the CdCl₂-treated and control groups (Figure 2A). However, high concentrations (≥2.0 µM) of CdCl₂ reduced viability of BMMSCs in a concentration-dependent manner. Since we evaluated the effects CdCl₂ on osteogenic differentiation for 7 days or more, we also assessed cell viability after incubation with various concentrations of CdCl₂ for 7 days. BMMSCs were incubated with 0, 0.1, 0.2, 0.5, 1.0, 2.0, 3.0, 4.0, or 5.0 µM CdCl₂ for 7 days. Low concentrations (≤0.2 µM) had no effect on viability of BMMSCs, but high concentrations (≥0.5 µM) reduced the viability of BMMSCs in a concentration-dependent manner (Figure 2B). In addition, 5-ethynyl-2′-deoxyuridine (EdU) assay was performed to detect the proliferation rate of BMMSCs treated by CdCl₂ for 24 h or 7 days. We found that the results of EdU assay were consistent with that of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Figure 2C). Therefore, we chose concentrations of 0.1 and
0.2 µM CdCl₂ to treat BMMSCs in further studies. Furthermore, we treated the cells with 0, 0.1, or 0.2 µM CdCl₂ for 24 h, after which immunofluorescence and confocal microscope analysis showed that CdCl₂ changed the morphology and atrophied the skeleton of BMMSCs (Figure 2D). These data indicated that CdCl₂ had no effect on cell viability but damaged cell morphology of BMMSCs, and that high concentrations inhibited cell viability in a concentration-dependent manner.

![Figure 2](image)

**Figure 2.** Low concentrations of cadmium have no effect on cell viability, and high concentrations of cadmium reduce cell viability of BMMSCs. BMMSCs were exposed to 0, 0.25, 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, 10, or 20 µM CdCl₂ for 24 h. (A) The viability of BMMSCs was detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) analysis (mean ± SD, n = 5). * p < 0.05, ** p < 0.01, different from control BMMSCs. BMMSCs were treated with 0, 0.1, 0.2, 0.5, 1.0, 2.0, 3.0, 4.0, or 5.0 µM CdCl₂ for 7 days. (B) The viability of BMMSCs was detected by MTT analysis. (mean ± SD, n = 5). ** p < 0.01, different from control BMMSCs. BMMSCs were exposed to 0, 0.5, 1, 2, 4, and 8 µM CdCl₂ for 24 h, or 0, 0.1, 0.2, 0.5, 1, and 2 µM CdCl₂ for 7 days. (C) The proliferation rate of BMMSCs was detected by 5-ethynyl-2′-deoxyuridine (EdU). Scale bar, 100 µm. BMMSCs were exposed to 0, 0.1, or 0.2 µM CdCl₂ for 24 h. (D) Cytoskeleton of BMMSCs were shown by immunofluorescence and confocal microscopic analyses. Nuclei were stained blue with 4′,6-diamidino-2-phenylindole (DAPI), and cytoplasm was stained green and red with α-tubulin and F-actin. Scale bar, 20 µm.

### 2.3. Cadmium Suppresses the Osteogenesis of BMMSCs

When BMMSCs seeded into plates reached 60% confluence, CdCl₂ was supplemented into osteogenic induction medium to determine if CdCl₂ affected the osteogenesis of BMMSCs. BMMSCs were exposed to
0, 0.1, and 0.2 µM CdCl₂ for various times. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) and RT-PCR revealed that the osteogenic-related mRNA levels of alkaline phosphatase (ALP), osteocalcin (OCN), runt-related transcription factor 2 (Runx2), osterix (OSX), and osteopontin (OPN) in CdCl₂-BMMSCs decreased after 3 days and 7 days (Figure 3A–G). ALP activity in cells treated with CdCl₂ was inhibited in a concentration-dependent manner (Figure 3H). Western blot assays demonstrated that CdCl₂ suppressed the protein expressions of ALP and Runx2 in BMMSCs (Figure 3I,J). In CdCl₂-BMMSCs, ALP staining was faint compared to the control after 14 days of osteo-induction (Figure 3K). Alizarin red S staining was used to reveal the effects of CdCl₂ on the mineralization potential of BMMSCs. Since mineralized nodules are typically observed at terminal differentiation [22], cells were osteo-induced for 14 days. In control BMMSCs, there were numerous dark brown nodules; CdCl₂ caused lower numbers of relatively smaller nodules (Figure 3K). These data demonstrated that CdCl₂ suppress the osteogenesis of BMMSCs.

**Figure 3.** Cadmium inhibits the osteogenic differentiation of BMMSCs. BMMSCs were exposed to 0, 0.1, or 0.2 µM CdCl₂ for 1, 3, or 7 days. (A–E) The mRNA levels of the osteogenic differentiation markers, such as alkaline phosphatase (ALP) (A), osteocalcin (OCN) (B), runt-related transcription factor 2 (Runx2) (C), osterix (OSX) (D), and osteopontin (OPN) (E) were measured by quantitative Reverse-Transcriptase Polymerase Chain Reaction (qRT-PCR) (mean ± SD, n = 3). * p < 0.05, ** p < 0.01, different from control BMMSCs. BMMSCs were exposed to 0, 0.1, or 0.2 µM CdCl₂ for 7 days. (F) and (G) The mRNA levels of the osteogenic differentiation markers, ALP, OCN, Runx2, OSX, and OPN were determined by RT-PCR analysis (mean ± SD, n = 3). * p < 0.05, ** p < 0.01, different from control BMMSCs. BMMSCs were exposed to 0, 0.1, or 0.2 µM CdCl₂ for 1, 3, or 7 days. (H) ALP activity was detected by ALP assay (mean ± SD, n = 3). * p < 0.05, ** p < 0.01, different from control BMMSCs. BMMSCs were exposed to 0, 0.1, or 0.2 µM CdCl₂ for 7 days. (I) Western blots were performed, and (J) relative protein levels of ALP and Runx2 were determined (mean ± SD, n = 3). * p < 0.05, ** p < 0.01, different from control BMMSCs. BMMSCs were exposed to 0, 0.1, or 0.2 µM CdCl₂ and subjected to osteogenic differentiation for 14 days. (K) The ALP content and the numbers of mineralization nodules were evaluated by ALP staining (upper) and alizarin red S staining (lower). Scale bar, 100 µm.
2.4. The Effects of Cadmium on mRNA Expression Profiles in BMMSCs

The expression of global genes was evaluated in a gene microarray performed with BMMSCs incubated in osteogenic differentiation medium containing 0.2 μM CdCl₂. A heat map showed the top 40 down-regulated and the 40 up-regulated mRNAs in CdCl₂-BMMSCs relative to control BMMSCs and the color indicator means gene expression, the darker the color, the higher the gene expression (Figure 4A). The detailed information of these top regulated mRNAs is presented in Supplementary Table S1. mRNAs differentially expressed between control and CdCl₂-treated cells were identified through scatter plots (Figure 4B). The red dots meant upregulated genes (>2.0-fold change) and the blue dots meant downregulated genes (>2.0-fold change). In CdCl₂-BMMSCs, 242 mRNAs were down-regulated, and 230 were up-regulated. Gene ontology (GO) analysis showed that pathways related to osteogenic differentiation, including the Wnt signaling pathway, the mothers against decapentaplegic and the C. elegans gene Sam (SMAD) signaling pathway, and the Janus kinase-Signal Transducers and Activators of Transcription (JAK-STAT) signaling pathway, and pathways associated with cell motility were affected by CdCl₂ (Figure S1). The detailed information of Wnt signaling related differentially mRNAs in BMMSCs treated with CdCl₂ is presented in Supplementary Table S2. Through these pathways, CdCl₂ may regulate the osteogenic differentiation of BMMSCs.

![Figure 4](image-url)

Figure 4. The effects of cadmium on mRNA expression profiles. BMMSCs were exposed to 0 or 0.2 μM CdCl₂ for 24 h, and a mRNA microarray was accomplished with control BMMSCs and CdCl₂-BMMSCs according to the standard protocols of Arrayster. (A) A heat map of the 40 down-regulated and 40 up-regulated mRNAs in control BMMSCs and CdCl₂-BMMSCs. (B) The scatter plots were for variation of expression of mRNAs between control BMMSCs and Cd-BMMSCs.

2.5. Cadmium Inhibits the Wnt/β-Catenin Pathway in BMMSCs

The Wnt signaling appears to be involved in regulating the differentiation BMMSCs [23,24]. The activation of Wnt signaling facilitates osteogenic differentiation [25]. Moreover, an mRNA microarray indicated that the Wnt signaling pathway are involved in the inhibition of osteogenic differentiation of BMMSCs induced by CdCl₂. Therefore, we hypothesized that CdCl₂ causes a Wnt/β-catenin dysfunction and thus affects osteogenesis. Wnt3a, β-catenin, LEF1, and TCF1 are molecules in the Wnt/β-catenin pathway. Exposure of BMMSCs to CdCl₂ for 24 h decreased protein...
levels of these proteins (Figure 5A,B). After BMMSCs were exposed to the osteogenic medium containing Wnt3a, the decreased protein levels of Wnt3a, β-catenin, LEF1, and TCF1 were rescued (Figure 5C,D). In order to exclude the potential effects of the multi-factors in osteogenic medium, we also checked the expression of Wnt3a, β-catenin, LEF1, and TCF1 in BMMSCs treated with CdCl2 (without osteogenic medium), which showed that the protein expression levels increased (Figure 5E,F). Thus, in BMMSCs, Wnt/β-catenin pathway is inhibited by CdCl2.

![Figure 5. The effects of cadmium on Wnt/β-catenin pathway. BMMSCs were exposed to 0, 0.1 or 0.2 µM CdCl2 for 24 h. (A) Western blots were performed, and (B) relative protein levels of Wnt3a, β-catenin, LEF1 and TCF1 were determined (mean ± SD, n = 3). * p < 0.05, ** p < 0.01, different from control BMMSCs. BMMSCs were exposed to 0, 0.2 µM CdCl2, or 50 ng/mL Wnt3a with the treatment of 0.2 µM CdCl2 in osteogenic medium for 24 h. (C) Western blots were performed, and (D) relative protein levels of Wnt3a, β-catenin, LEF1 and TCF1 were determined (mean ± SD, n = 3). * p < 0.05, different from BMMSCs treated with CdCl2. BMMSCs were exposed to 0, 0.1 or 0.2 µM CdCl2, or 50 ng/mL Wnt3a with the treatment of 0.2 µM CdCl2 in growth medium for 24 h. (E) Western blots were performed, and (F) relative protein levels of Wnt3a, β-catenin, LEF1 and TCF1 were determined (mean ± SD, n = 3). * p < 0.05, different from BMMSCs treated with CdCl2.](image)

2.6. Wnt/β-Catenin Pathway is Involved in the Suppression of Osteogenesis Induced by Cadmium in BMMSCs

To further investigate the role of Wnt/β-catenin pathway in the suppression of osteogenesis induce by CdCl2, BMMSCs were exposed to CdCl2 at concentrations of 0 or 0.2 µM with or without the treatment of Wnt3a and induced to differentiate into osteoblasts by osteogenic induction medium. After the treatment of Wnt3a for 7 days, Western blots showed that the protein levels of ALP and Runx2 were increased (Figure 6A,B), and the ALP activity was also increased (Figure 6C). After the treatment of Wnt3a for 14 days, the ALP staining showed that the faint staining induced by 0.2 µM CdCl2 was rescued, and the alizarin red s staining determined that the smaller and fewer dark-brown colored nodules were increased (Figure 6D). These data demonstrate that, in BMMSCs, Wnt/β-catenin pathway is involved in the inhibition of osteoblast differentiation caused by CdCl2.
3. Discussion

In this work, we found that CdCl₂ suppressed the osteogenic differentiation of BMMSCs. After treatment of BMMSCs with CdCl₂, the expression of osteogenic-related genes was reduced, and ALP staining and alizarin red staining confirmed that CdCl₂ had an adverse effect on the osteogenic differentiation capacity of BMMSCs. In addition, we also performed mRNA microarray to identify biological processes that may be involved in the suppression of the osteogenesis and found that several classical signaling pathways may be involved. We measured the expressions of related molecules involved in the Wnt pathway and found that key molecules in this pathway were low in the presence of CdCl₂, indicating that activation of the Wnt pathway was inhibited.

Epidemiological studies have evaluated effects of cadmium among various populations exposed by environmental routes. These studies demonstrate that increased cadmium exposure correlates with decreased bone mineral density (BMD) [26]. For rats, those exposed to cadmium show low BMD and pathological changes in the microstructure of bone, including increased trabecular separation and decreased trabecular connectivity. [27]. Cadmium has both an indirect and direct mode of influencing bone turnover [28]. Cadmium reduces bone formation through inhibition of osteoblast activity and promotes bone resorption, which is related to the impairment of bone cell metabolism [29,30]. However, the specific mechanism for bone damage caused by CdCl₂ is unclear, especially from the
The Wnt/β-catenin signaling pathway is related to the osteogenic differentiation of BMMSCs, and the expression of osteoblast-related genes were lower in the group exposed to CdCl2 relative to the control group. Further, the inhibitory effect of CdCl2 on osteogenic differentiation of BMMSCs was reflected in lower ALP activity, lower protein levels of ALP and Runx2, and less calcification.

Bone remodeling is an indispensable condition for maintaining bone balance, and bone formation is destroyed in the pathological state of bone. The imbalance between osteoblast-mediated bone formation and osteoclast-mediated bone resorption is an important cause of reduced bone mass and altered bone structure [39]. The formation and calcification of osteoblast plays a crucial role during bone modeling and remodeling, which can be regulated by the Wnt/β-catenin pathway. The results of many investigations about the Wnt pathway in the context of bone generally show increases in canonical signaling through this pathway, leading to increased osteoblastogenesis and bone formation [40]. Most components of the Wnt signaling pathway are functional in BMMSCs, which are often used as a model for bone [41].Canonical Wnt signaling is involved in self-renewal and in lineage-selective determination of BMMSCs. In humans and in mice, there is a relationship between Wnt signaling and bone mass. Canonical Wnt signaling promotes bone formation by mechanisms including stem cell renewal [42], stimulation of osteoblast proliferation, and induction of osteoblastogenesis [43]. Therefore, we hypothesized that CdCl2 damages Wnt signaling and thereby affects osteogenesis.

The canonical Wnt/β-catenin pathway is triggered when the Wnts are combined with Wnt ligands. The binding of Wnt with Frizzled and LRP5/6 initiates to form a complex of proteins, which subsequently recruit the Axin complex. The protein complex, which consists of axin, glycogen synthase kinase 3 (GSK3), and the adenomatos polyposis coli (APC) protein, stabilizes β-catenin. β-catenin entering into the nucleus activates many target genes, such as the lymphoid enhancer-binding factor/T cell factor (LEF/TCF) transcription factor family, which promotes the expression of many osteoblast-related genes [44,45]. In the present study, we established two groups of cells, normal BMMSCs and CdCl2-BMMSCs, and performed mRNA microarray to find altered genes. The bioinformatics analysis indicated that Wnt/β-catenin signaling is involved in the suppression of osteogenesis induced by CdCl2. Therefore, we examined expression of proteins involved in the Wnt/β-catenin pathway, including Wnt3a, β-catenin, LEF1, and TCF1. The expressions of these were lower in CdCl2-BMMSCs than in controls, and the proteins in the group treated with 0.2 μM Cd were lower than those of the group treated with 0.1 μM CdCl2. Activation of the Wnt/β-catenin signaling pathway is related to the osteogenic differentiation of BMMSCs, but, in this study of BMMSCs, the expression of proteins in the Wnt/β-catenin pathway decreased after their treatment with CdCl2. CdCl2 affected activation of the Wnt/β-catenin pathway. After the treatment of Wnt3a, the protein levels of Wnt3a, β-catenin, LEF1, and TCF1 and the degrees of osteoblast differentiation of BMMSCs were rescued. Thus, inhibition of osteogenic differentiation of BMMSCs is associated with inhibition of activation of the Wnt/β-catenin pathway by CdCl2.
4. Materials and Methods

4.1. Isolation and Culture of Primary BMMSCs

Primary BMMSCs were harvested from 3-week-old Sprague-Dawley (SD) rats as described previously [5]. Briefly, the femurs and tibias of rats were dissected, and bone marrow was flushed out with syringes and centrifuged at 800 rpm for 5 min to collect the cells. Cells were cultured in Modified Eagle medium-Alpha (\(\alpha\)-MEM) (Corning Cellgro, New York, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA), 1% L-glutamine (Gibco, Gaithersburg, MD, USA), 1% penicillin-streptomycin (Gibco, USA), and 1% HEPES (Gibco, USA) and were maintained in a humidified 37 °C/5% CO\(_2\) incubator with changes of medium every other day. When the confluence reached 80–90%, BMMSCs were harvested by treating them with 0.25% trypsin-EDTA (Gibco, USA) and were passaged for further culture. BMMSCs, in passages 3–5, were used for experiments. All of the experiments and procedures involving animals were approved by the Institutional Animal Care and Use Committee (IACUC) of Nanjing Medical University (Identification code:11015; Approval Number: IACUC-1705014; Date: 24th April 2017).

4.2. Adipogenic Differentiation

BMMSCs were induced to adipogenic differentiation in Dulbecco’s modified Eagle medium-High glucose (DMEM-HG) (Hyclone, Logan, Utah, USA) supplemented with 10% FBS, 1 \(\mu\)Mmol/L dexamethasone, 100 \(\mu\)Mmol/L indomethacin (Sigma Aldrich, St. Louis, MS, USA), 0.5 mmol/L 3-isobutyl-1-methylxanthine (Sigma Aldrich, USA), 5 \(\mu\)Mg/mL insulin (Sigma Aldrich, USA), 1% L-glucose, 1% penicillin/streptomycin, and 1% HEPES.

4.3. Osteogenic Differentiation

BMMSCs were induced to osteogenic differentiation in DMEM-HG medium supplemented with 10% FBS, 10 nmol/L dexamethasone (Sigma Aldrich, USA), 10 mmol/L \(\beta\)-glycerol phosphate (Sigma Aldrich, USA), 50 \(\mu\)Mg/mL ascorbic acid (Sigma Aldrich, USA), 1% L-glucose, 1% penicillin-streptomycin, and 1% HEPES.

4.4. CdCl\(_2\) Treatment

For (Sigma Aldrich, USA) exposure of BMMSCs during osteogenic differentiation, CdCl\(_2\), at concentrations of 0, 0.1, or 0.2 \(\mu\)M, was added into the osteogenic induction medium before replacing the growth medium, once BMMSCs in the culture plates reached 60% confluence.

4.5. Cell Surface Marker Expression of BMMSCs

Expression of surface markers was assessed by flow cytometry using monoclonal antibodies for CD44 and CD90 (positive BMMSC markers) and CD45 (negative BMMSC marker) (BD Biosciences, San Jose, CA, USA). BMMSCs at passage 3 were suspended in PBS and incubated with each antibody at a concentration of 0.5 \(\mu\)Mg/mL, along with unstained BMMSCs and isotype controls.

4.6. Cell Proliferation Assays

Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyletetrazolium bromide (MTT) (Sigma Aldrich, USA) following the manufacturer’s instructions. BMMSCs (1 \(\times\) 10\(^4\) cells per well) were seeded in 96-well plates. 24 h later, BMMSCs were exposed to 0, 0.25, 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, 10, 20 \(\mu\)M or to 0, 0.1, 0.2, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0 \(\mu\)M CdCl\(_2\) for 24 h or for 7 days. After treatment, MTT reagent, diluted to a concentration of 0.5 mg/mL with osteogenic-inducing medium, was added to the plates, and they were incubated at 37 °C for 4 h. Next, the MTT was replaced by dimethyl sulfoxide (Sigma Aldrich, USA), and preparations were incubated at 37 °C for another 15 min, and then the plates were agitated softly for 15 min. The absorbance of each well was recorded at 490 nm by an Infinite M200 Pro instrument (TECAN, Switzerland). Each assay was repeated at least three times independently.
For the 5-ethynyl-2′-deoxyuridine (EdU) proliferation assay, treated BM-MSCs cells were seeded into 96-well plates for EdU assay to assess the proliferation ability, and each well contained 2 × 10⁴ cells. Then completed medium containing of 0.1% EdU A solution (RiboBio, Guangzhou, China) were added into 96-well after cells were attached to the well, and then incubated for two hours in an incubator. Subsequent experiments were carried out in strict accordance with the manufacturer’s instructions. Finally, photos were taken by fluorescence microscope.

4.7. Confocal Microscopic Analysis

Cells (7 × 10⁴ /well) were cultured on coverslips (In Vitro Scientific) and treated with 0, 0.1, or 0.2 µM CdCl₂ for 24 h, after which cells were fixed with 4% paraformaldehyde for 30 min. After treating of cells with 0.1% Triton X-100, their cytoskeletons were stained with anti-α-tubulin antibody (1:200, Beyotime, China) or anti-F actin antibody (1:200, Abcam, Cambridge, MA, USA), together with respective fluorescence secondary antibodies (Alexa Flour 488 and Alexa Flour 546) (1:200, Beyotime, Shanghai, China). Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (1:1000, Beyotime, Shanghai, China) for 5 min. Images were visualized and captured with a Nikon E800 confocal microscope (Nikon, Tokyo, Japan).

4.8. Oil Red O Staining

Cells (7 × 10⁴ /well) were seeded in 24-well plates, and adipogenesis was induced for 14 days. Cells were then fixed with 4% formalin solution for 15 min and incubated with 0.5% Oil Red O (Sigma Aldrich, USA) for 40 min. After washing the plates with 60% isopropanol, images were made at 10× magnification using an optical microscope (Nikon, Japan).

4.9. Alkaline Phosphatase (ALP) Assay

ALP activity was determined with Sensolyte®pNPP Alkaline Phosphatase Assay Kits (Anaspec, USA) according to manufacturer’s instructions. BM-MSCs were seeded in 96-well plates at a density of 1 × 10⁴ cells/well. At 24 h after plating, the cells were exposed to 0, 0.1, or 0.2 µM CdCl₂ with or without the treatment of Wnt3a, and osteogenic differentiation was induced as described above. Cells were washed twice with assay buffer, lysed with Triton-X-100, and collected in microcentrifuge tubes. After incubation at 4 °C for 10 min under agitation, cells were centrifuged at 2500 × g for 10 min to collect the supernatant. The supernatant was incubated with P-nitrophenyl phosphate (pNPP) substrate solution, and the absorbance was read at 405 nm with an Infinite M200Pro (TECAN, Switzerland). The ALP activity was normalized against protein concentration measured with BCA Protein Assay Kits (Beyotime Institute of Biotechnology, China).

4.10. Alkaline Phosphatase (ALP) Staining

Leukocyte Alkaline Phosphatase Kits (Sigma Aldrich, USA) were used for ALP staining according to the manufacturer’s instructions. BM-MSCs were seeded in 24-well plates at a density of 7 × 10⁴ cells/well in growth culture medium. Until the confluence reached 60%, BM-MSCs were exposed to CdCl₂ at concentrations of 0, 0.1, or 0.2 µM with or without the treatment of Wnt3a and induced to differentiate into osteoblasts by osteogenic induction medium. After 10 d of induction, the cells were fixed with 4% formaldehyde and 5% citrate in acetone at room temperature for 30 s. 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 PBS, images were taken at 10× magnification under an optical microscope (Nikon, Japan).

4.11. Alizarin Red S (ARS) Staining

BM-MSCs were seeded in 24-well plates at a density of 7 × 10⁴ cells/well and were exposed to CdCl₂ at concentrations of 0, 0.1, or 0.2 µM with or without the treatment of Wnt3a, and induced to
differentiate into osteoblasts by osteogenic induction medium for 14 days. The cells were then 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 min. After discarding the working solutions and washing the plates with PBS 4 times, images were made at 10× magnification under an optical microscope (Nikon, Japan).

4.12. RNA Preparation and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated from cells with TRIzol reagent (Invitrogen Life Technologies Co, Carlsbad, CA, USA) according to manufacturer’s protocol. The purity and concentration of total RNA was assessed with a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). Reverse transcription was accomplished using Prime Script™ RT Reagent Kits with gDNA Eraser (Perfect Real Time, Takara, Kusatsu, Japan) with 1 µMg of RNA according to the manufacturer’s instructions. qRT-PCR was performed with an ABI7900 Fast Real-Time System (Applied Biosystems, Waltham, MA, USA) using SYBR Premix Ex Taq™ Kits (Takara, Japan). GAPDH was used as an internal standard, and the relative expressions of genes were calculated by the 2^(-ΔΔCt) method [46]. Primers sequences are shown in Table 1.

**Table 1. Primer sequences used.**

| Gene  | Forward Primer (5′→3′) | Reverse Primer (5′→3′) |
|-------|------------------------|-----------------------|
| GAPDH | 5′-GGCACAGTCAAGGCTGAGAATG-3′ | 5′-ATGGTGGTGAGACAGCAGTA-3′ |
| ALP   | 5′-GGGACTGGTACTCGGACAAT-3′ | 5′-GGCCCTTCATCCAGTTCAT-3′ |
| OCN   | 5′-CATGAGGACCTCTCTCTGC-3′ | 5′-GGACCCAGCCCATAATG-3′ |
| Runx2 | 5′-TTGGAGCAGACATGCACAGTCA-3′ | 5′-GGCCTGTTTCCGTTGTGC-3′ |
| OSX   | 5′-AGCGACCACTTGACCAACAT-3′ | 5′-CTGCCCTTTCCGTTGTC-3′ |
| OPN   | 5′-CTTTTCACTCAATCGCCCTAC-3′ | 5′-CTGCCCTTTCCGTTGTC-3′ |

4.13. Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR)

Total RNA (1 µMg) was treated with 10 U of RNase R (Epicentre Technologies Corp., Madison, WI) in 1 × RNase R reaction buffer in a total volume of 10 µML. The mixture was incubated at 37 °C for 1 h. Total RNA (1 µMg) was transcribed into cDNA by HiScript II Q RT Supermix (Vazyme Biotech). The PCR reactions were evaluated by checking the PCR products on 2% w/v agarose gels. The intensities of bands were quantified with ImageJ software.

4.14. Western Blots

Cell were lysed with RIPA (Beyotime Institute of Biotechnology, China) as described previously [5], and protein concentrations were quantified with BCA Protein Assay kits (Beyotime Institute of Biotechnology, China). Equal amounts (50 µMg) of protein were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). Membranes were incubated overnight at 4 °C with a 1:1000 dilution of anti-glyceraldehyde 3-phosphate dehydrogenase (anti-GAPDH, Beyotime) and an antibody for ALP (Abcam, USA), Runx2 (Cell Signaling Technology, Boston, MA, USA), Wnt3a (Abcam, USA), β-catenin (Abcom, USA), Lef1(Cell Signaling Technology, USA), or TCF1 (Cell Signaling Technology, USA). After additional incubation with a 1:1000 dilution of HRP-conjugated goat anti-mouse and goat anti-rabbit secondary antibodies (Jackson ImmunoResearch, USA) for 1 h, the immune complexes were detected by enhanced chemiluminescence (Cell Signaling Technology, USA). The intensities of bands were quantified with ImageJ software.
4.15. Microarray for Analysis of Gene Expression

BMMSCs were cultured with CdCl$_2$ at a concentration of 0.2 µM in osteogenic differentiation medium and each group included one sample that mixed by three parallel samples. Afterwards, cells were lysed, and total RNA was extracted for analysis of whole genome expression. Osteogenic differentiation medium without Cd was used as a control. The RNA samples were labeled and hybridized with 1.65 µMg of Cy3-labeled cRNA using Gene Expression Hybridization Kits (Santa Clara, USA) in a Hybridization Oven (Santa Clara, USA), according to the manufacturer’s instructions. After 17 h of hybridization, slides were washed in staining dishes (Thermo Shandon, US) with Gene Expression Wash Buffer Kits (Santa Clara, US). Samples were scanned with an Agilent Microarray Scanner (Santa Clara, US) with the default settings: Dye channel: Green, Scan resolution = 3 µMm, PMT 100%, 20 bit. Data were extracted with Feature Extraction software 10.7 (Santa Clara, CA, USA). Specific representations of heatmap and scatter plot were analyzed by bioinformatics using “pheatmap” package. The gene ontology was analyzed by bioinformatics using “ggplot2” package.

4.16. Statistical Analyses

All data values were expressed as means ± standard deviations (SD). Graphpad 7.0 was applied for statistical analyses. One-way analysis of variance (ANOVA) was used for comparisons of means among multiple groups, and a multiple-range least significant difference (LSD) was used for inter-group comparisons. All statistical analyses were performed with SPSS 19.2, and the data were marked with (*) for $p < 0.05$, or (**) for $p < 0.01$.

5. Conclusions

In summary, our investigations show that CdCl$_2$ suppresses the osteogenic differentiation of BMMSCs and address the effects of CdCl$_2$ on mRNA microarray. In this process, CdCl$_2$ caused decreases of Wnt3a, β-catenin, LEF1, and TCF1, which are involved in the Wnt pathway, which regulates differentiation of BMMSCs. Thus, in BMMSCs exposed to CdCl$_2$, the Wnt/β-catenin pathway may regulate inhibition of osteogenic differentiation (Figure 7). Our findings establish a mechanism for CdCl$_2$-induced bone damage and have public health implications for developing strategies to reduce CdCl$_2$ exposure and thereby to mitigate its harmful effects.

**Figure 7.** A schematic representation shows the proposed mechanism. The Wnt/β-catenin pathway is involved in the suppression of osteoblast differentiation of BMMSCs induced by CdCl$_2$. A schematic representation of the proposed pathway for Wnt/β-catenin, which is inhibited by CdCl$_2$, resulting in transcriptional reduction of Runx2 and the poor osteoblast differentiation of BMMSCs.
Supplementary Materials: The following are available online at http://www.mdpi.com/1422-0067/20/6/1519/s1, Figure S1: Gene ontology analysis of BMMSCs treated with CdCl$_2$, Table S1: The detailed information of top regulated mRNAs in BMMSCs with CdCl$_2$, Table S2: The detailed information of Wnt signaling related mRNAs in BMMSCs with CdCl$_2$.

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Conflicts of Interest: The authors have no conflicts of interest to disclose.

Abbreviations

CdCl$_2$  Cadmium chloride  
BMMSCs  Bone marrow mesenchymal stem cells  
p0  Primary bone marrow mesenchymal stem cells  
Adi-induction  Adipogenic induction  
Ost-induction  Osteogenic induction  
Wnt  Wingless-type  
JAK-STAT  Janus kinase–Signal Transducers and Activators of Transcription  
IGFBP7  Insulin-like growth factor binding protein 7 
MTT  3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide  
EdU  5-ethynyl-2′-deoxyuridine  
ALP  Alkaline phosphatase  
OCN  Osteocalcin  
Runx2  Runt-related transcription factor 2  
OSX  Osterix  
OPN  Osteopontin  
LEF1  Lymphoid enhancer-binding factor 1  
TCF1  T cell factor 1  
BMD  Bone mineral density  
ARS  Alizarin red S  
GO  Gene oncology  
GSK3  Glycogen synthase kinase 3  
APC  Adenomatosis polyposis coli

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