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Original Article

Regulation of osteogenic differentiation by DNA methylation of the dishevelled gene in bone marrow mesenchymal stem cells

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Abstract: Bone marrow mesenchymal stem cells (BMSCs) are stem cells with multidirectional differentiation potential, which can be used as seed cells to repair and reconstruct many types of tissues and organs following injury or disease. Osteogenic differentiation involves a variety of pathway and factors, including cytokines, growth factors, and hormones. In the present study, we investigated the potential role of Dishevelled in osteogenic differentiation of BMSCs in induction medium containing the methyltransferase inhibitor 5-aza-2'-deoxycytidine. The expression of Dishevelled was analyzed using the reverse transcriptase-polymerase chain reaction (RT-PCR) and a Western blot. The methylation degree of the CpG island in the promoter region of the Dishevelled gene was analyzed, and protein expression levels of Wnt, Glycogen synthase kinase-3 (GSK3), axin, Dishevelled, and β-catenin were increased after the addition of the methyltransferase inhibitor. The expression of Dishevelled increased in accordance with the differentiation of osteoblasts, and the degree of methylation of the promoter affected its expression level. In conclusion, regulating the methylation degree of the Dishevelled gene promoter region appears to influence the expression of Dishevelled and therefore the osteogenic differentiation of BMSCs.

Keywords: BMSC, dishevelled, methylation, osteogenic differentiation

Introduction

Mesenchymal stem cells originate in mesoderm and exist primarily in bone marrow [1-4]. Bone marrow mesenchymal stem cells (BMSCs) exhibit self-proliferation and multidirectional differentiation potential [1-4]. They also possess pluripotent activity and are as "universal cells," with roles in osteogenesis and repair of the nervous system [4]. Various studies of Alzheimer's disease, Parkinson's disease, and stroke, in addition to animal models of spinal cord injury, have shown that stem cells effectively improved symptoms of these diseases [5]. Research also reported good osteogenic activity of BMSCs based on osteoblast-specific staining and the reverse transcriptase-polymerase chain reaction method [6]. Previous studies demonstrated that alkaline phosphatase (AKP) activity increased at different stages of osteogenesis induction in accordance with the time of induction, which indicated that the induction of differentiation was important for the maintenance of the bone metabolic balance [7-9]. Osteogenic differentiation activity of BMSCs holds promise for the development of novel drugs for the treatment of bone nonunion, bone defects, osteolytic bone disease, and other bone-related diseases.

The osteogenic differentiation of BMSCs involves a complex regulatory network and many signaling pathways [10-12]. Marie reported that the fibroblast growth factor signaling pathway played an important regulatory role in the development of the skeletal system, and they were mutually activated/inhibited and closely linked, although the underlying mechanism remains to be elucidated [13]. Further studies of interaction mechanisms and signaling pathways, including upstream and downstream products and targets, involved in osteogenic differentiation of BMSCs could provide a basis for the clinical treatment of bone disease.

In the present study, we investigated the potential role of Dishevelled in osteogenic differentiation of BMSCs in induction medium containing the methyltransferase inhibitor 5-aza-2'-deoxycytidine. Both the expression of Dishevelled and regulatory factors were detected by the RT-PCR method and a Western blot. The methylation degree of the Dishevelled gene affected the expression of Wnt, GSK3, axin, Dishevelled, β-catenin, and PP2A and regulated the process of osteogenic differentiation.

Material and methods

Cell culture

BMSCs were purchased from Saiye Biotechnology Co., Ltd (Beijing, China) and cultured in growth medium and osteogenic induction medium (Saiye Biotechnology Co.). The growth medium contained Dulbecco Modified Eagle Medium (DMEM) and was supplemented with 10% Fetal Bovine Serum (FBS) (Beijing Baird Biotechnology Co., Ltd, Beijing, China), 2 mM of L-glutamine, 10 μg/mL of penicillin, and 10 μg/mL of streptomycin (Sigma, CA, USA). The osteogenic induction medium contained DMEM and was supplemented with 10% FBS, 2 mM of L-glutamine, 10 μg/mL of penicillin, 10 μg/mL of streptomycin, 100 nM of dexamethasone, 10 mM of β-glycerophosphate, and 50 μM of L-ascorbic acid-2-phosphate (Sigma).

Differentiation and identification of osteogenic potential of BMSCs

The BMSCs were placed in osteogenic induction medium and cultured in a cell incubator at 37°C and 5% CO_2 for 7 days, 10 days, 14 days, and 21 days. Osteoblast differentiation was identified by alizarin red staining. AKP activity was examined by using Alkaline phosphatase detection kit according to the instructions of the manufacturer (Tiangen, Beijing, China).

Expression of Dishevelled in osteogenic differentiation of BMSCs

The BMSCs were divided into four groups: a positive control group and groups in which differentiation was induced for 7, 14, and 21 days. The expression of the Dishevelled gene was detected by the RT-PCR method. A Western blot was used to detect the protein level of Dishevelled.

Methylation of the Dishevelled gene promoter region during osteogenic differentiation of BMSCs

Methylation specific PCR (MSP) was used to detect the methylation status of the CpG island in the promoter region of the Dishevelled gene before and after osteogenic differentiation of human marrow mesenchymal stem cells (hMSCs). Cell DNA was extracted using a blood DNA kit (Tiangen) according to the instructions of the manufacturers. DNA was bisulfite modified and purified using a CpGenome DNA Modification Kit (Tiangen) in accordance with the manufacturer's instructions. PCR amplification was carried out using Dishevelled methylation primers and nonmethylated primers

Regulation of Dishevelled DNA methylation in BMSCs during osteogenic differentiation

The BMSCs were divided into two groups. One group was transfected with an empty vector (control), and differentiation was induced in the other group for 14 days. The mRNA and protein expression of the Dishevelled and PP2A genes were detected by the RT-PCR method and Western blotting, respectively.

Expression levels of Wnt, GSK3, axin, Dishevelled, β-catenin, and PP2A following the addition of the methyltransferase inhibitor 5-aza-2'-deoxycytidine

The BMSCs were divided into the following eight groups:

Group 1: Uninduced BMSCs (10 μM of DMSO added, continued to culture for 72 h); Group 2: Uninduced BMSCs (10 μM of 5-aza-2'-deoxycytidine added, continued to culture for 72 h); Group 3: Induced osteogenic differentiation of BMSCs after 7 days (10 μM of DMSO added, continued to culture for 72 h); Group 4: Induced osteogenic differentiation of BMSCs after 7 days (10 μM of 5-aza-2'-deoxycytidine added, continued to culture for 72 h); Group 5: Induced osteogenic differentiation of BMSCs after 14 days (10 μM of DMSO added, continued to culture for 72 h); Group 6: Induced osteogenic differentiation of BMSCs after 14 days (10 μM of 5-aza-2'-deoxycytidine added, continued to culture for 72 h); Group 7: Induced osteogenic differentiation of BMSCs after 21 days (10 μM of DMSO added, continued to culture 72 h); Group 8: Induced osteogenic differentiation of BMSCs

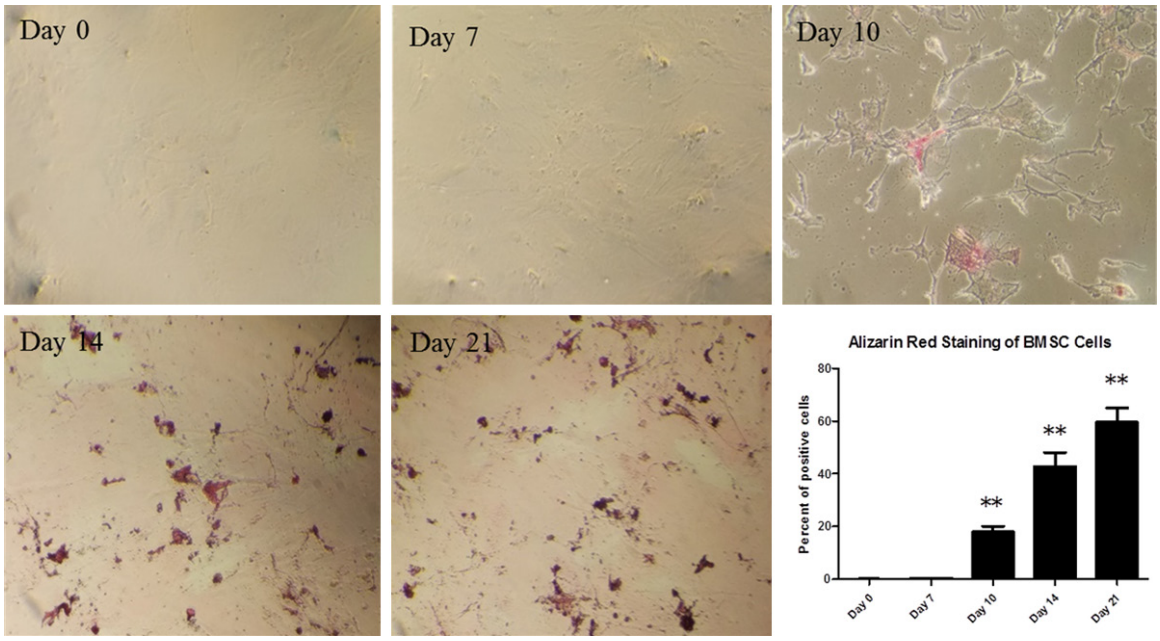

Figure 1. Differentiation and identification of BMSCs. Osteogenic differentiation of BMSCs after 0, 7, 10, 14, and 21 days was detected by alizarin red staining. $^{**}P < 0.01$.

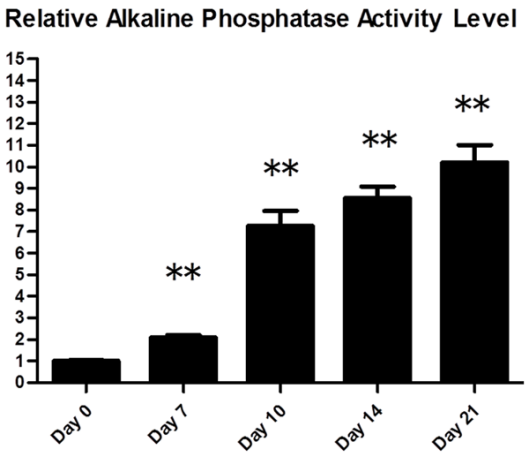

Figure 2. AKP levels markedly increased in accordance with the induction time. The expression of AKP increased in accordance with osteogenic differentiation of BMSCs after 0, 7, 10, 14, and 21 days, $^{**}P < 0.01$.

after 21 days (10 μM of 5-aza-2'-deoxycytidine added, continued to culture for 72 h).

Dishevelled gene expression was detected by the RT-PCR method. A Western blot was used to detect the protein expression level of Dishevelled.

Statistical analysis

The results are presented as the mean ± standard deviation (SD). Comparisons between more than two groups were performed by conducting an analysis of variance (a one-way ANOVA), and $p < 0.05$ was considered statistically significant.

Results

Differentiation and identification of BMSCs

After 7, 10, 14, and 21 days of osteogenic differentiation, the BMSCs were induced to differentiate in the induction medium, as shown by the detection of alizarin red staining in **Figure 1**, the degree of osteogenic differentiation increased in accordance with time. AKP levels also markedly increased in accordance with the induction time (**Figure 2**).

Expression of Dishevelled in BMSCs

The expression of Dishevelled was detected by the RT-PCR method, and a western blot was used to detect the protein level of the gene. The results demonstrated that the mRNA and protein expression of Dishevelled increased over time in the osteogenic differentiation medium (**Figure 3**).

Methylation of the Dishevelled gene promoter region in BMSCs

MSP was used to detect the methylation levels of the CpG island in the promoter region of the

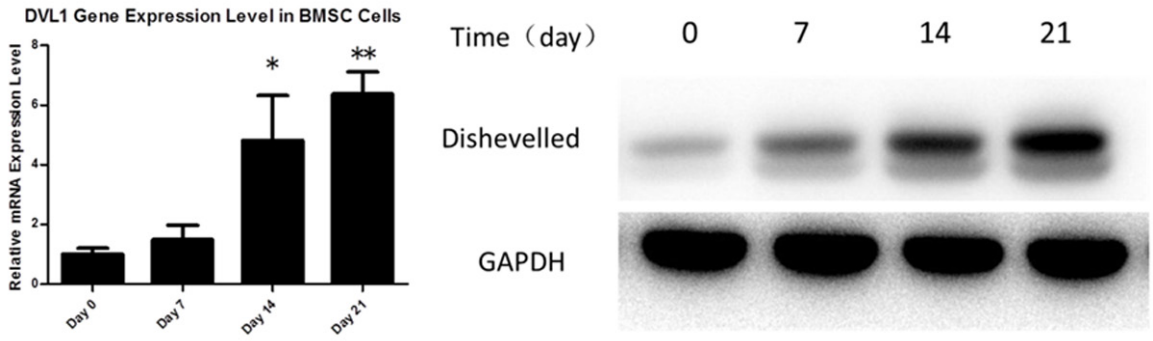

Figure 3. Expression of Dishevelled in BMSCs. The mRNA and protein expression levels of the Dishevelled gene during osteogenic differentiation of BMSCs was detected by the RT-PCR method and a Western blot after 0, 7, 10, 14, and 21 days. *$P < 0.05$, **$P < 0.01$.

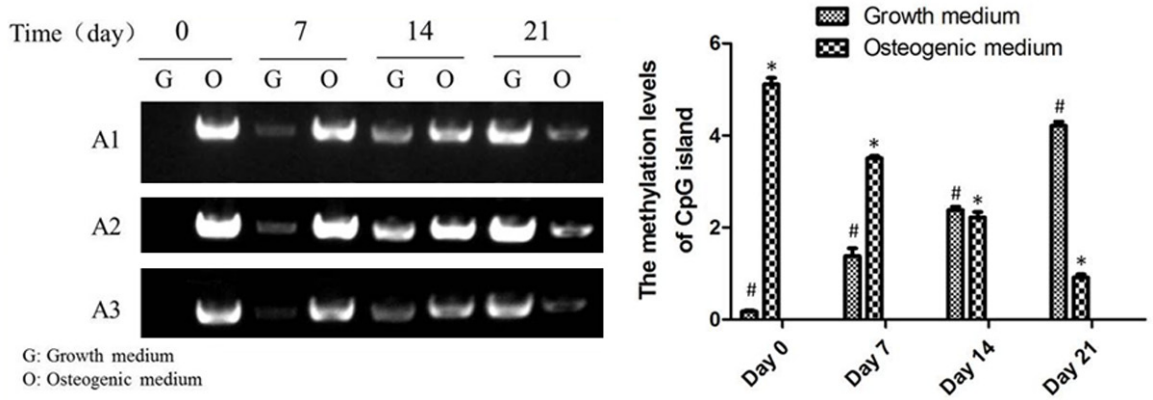

Figure 4. Methylation of the Dishevelled gene promoter region in BMSCs. The methylation levels of the CpG island during the process of osteogenic differentiation. *$P < 0.05$, #$P < 0.05$.

Dishevelled gene during osteogenic differentiation of BMSCs. As shown in **Figure 4**, the methylation levels of the CpG island at the Dishevelled gene promoter region decreased over time with the induction of differentiation. The degree of methylation was weakest on day 21, which resulted in elevated expression of Dishevelled.

Expression levels of Wnt, GSK3, axin, Dishevelled, β-catenin, and PP2A

The BMSCs were divided into eight parallel groups. The mRNA and protein expression levels increased slightly in accordance with the osteogenic differentiation time. In response to increased expression of Dishevelled, the expression of the differential gene (PP2A) was also elevated. PP2A expression seemed to be positively correlated with that of Dishevelled (**Figure 5**). In addition, as shown in **Figures 6** and **7**, the results of the RT-PCR and Western blot demonstrated that after the addition of the methylase inhibitor (5-aza-2'-deoxycytidine), the expression of Wnt, GSK3, axin, Dishevelled, and β-catenin significantly increased compared with that of the blank control (DMSO).

Discussion

BMSCs located in the medullary cavity are capable of self-renewal and multidirectional differentiation. Through the actions of different induction factors, they can differentiate into a range of cell types, including osteoblasts, adipocytes, and chondrocytes, which are important sources of osteoblasts. The balance of bone and adipose tissue in bone marrow is maintained by the differentiation of BMSCs into osteoblasts and adipocytes [14-15]. During the osteogenic differentiation of BMSCs, the expression of Dishevelled shows a tendency to increase, and the expression of associated regulatory genes and signal molecules shows the

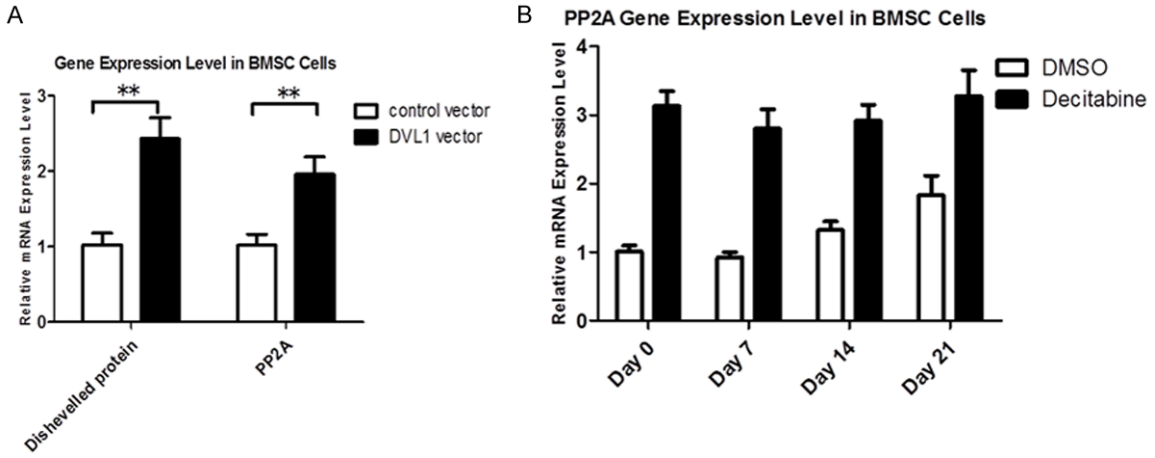

Figure 5. The expression of PP2A and Dishevelled during osteogenic differentiation. (A) Relationship between PP2A and Dishevelled expression. $^{**}P < 0.01$ vs. control vector. (B) Expression level of PP2A during osteogenic differentiation after the addition of the methylase inhibitor 5-aza-2'-deoxycytidine. $P < 0.05$.

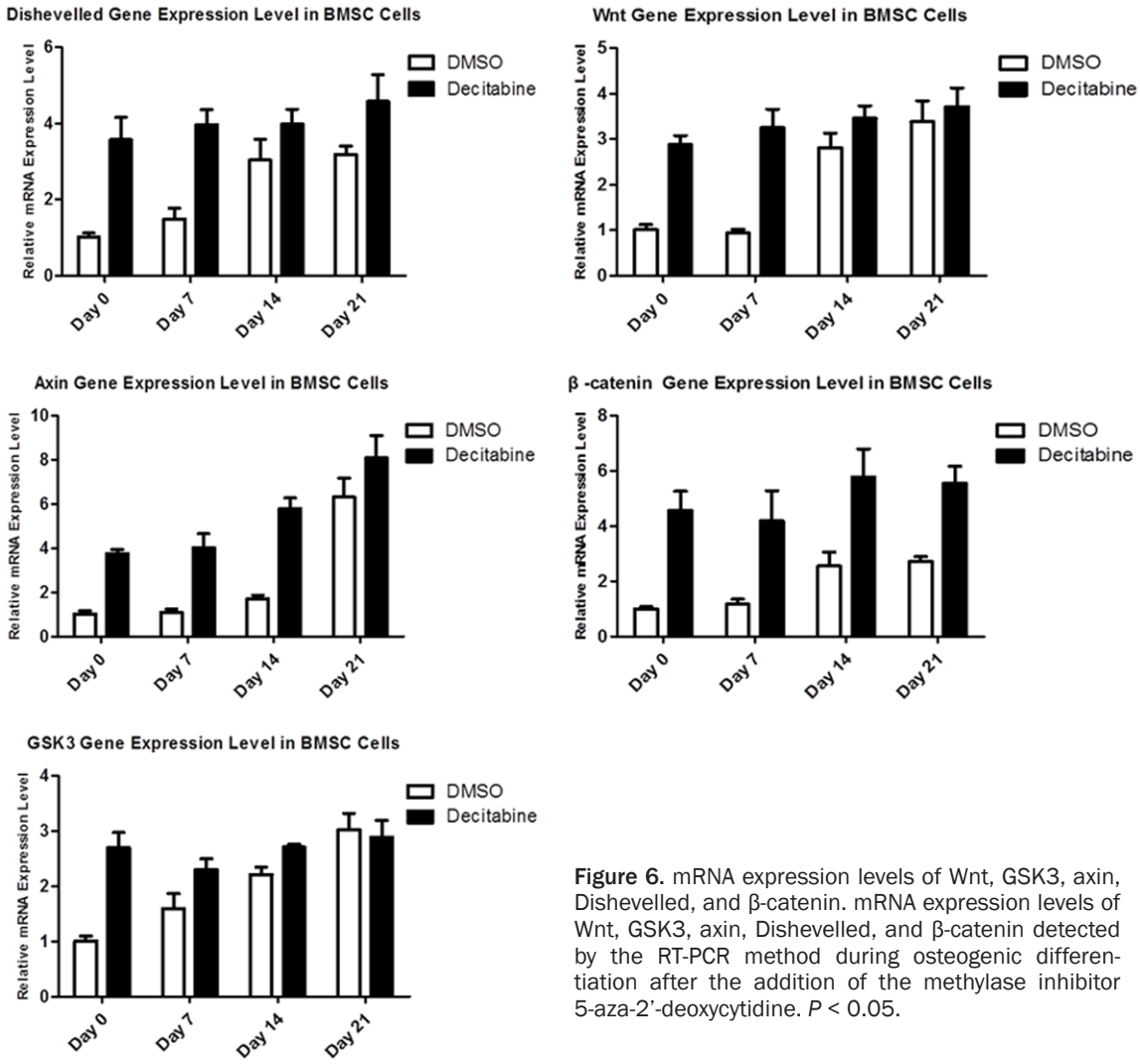

Figure 6. mRNA expression levels of Wnt, GSK3, axin, Dishevelled, and β-catenin. mRNA expression levels of Wnt, GSK3, axin, Dishevelled, and β-catenin detected by the RT-PCR method during osteogenic differentiation after the addition of the methylase inhibitor 5-aza-2'-deoxycytidine. $P < 0.05$.

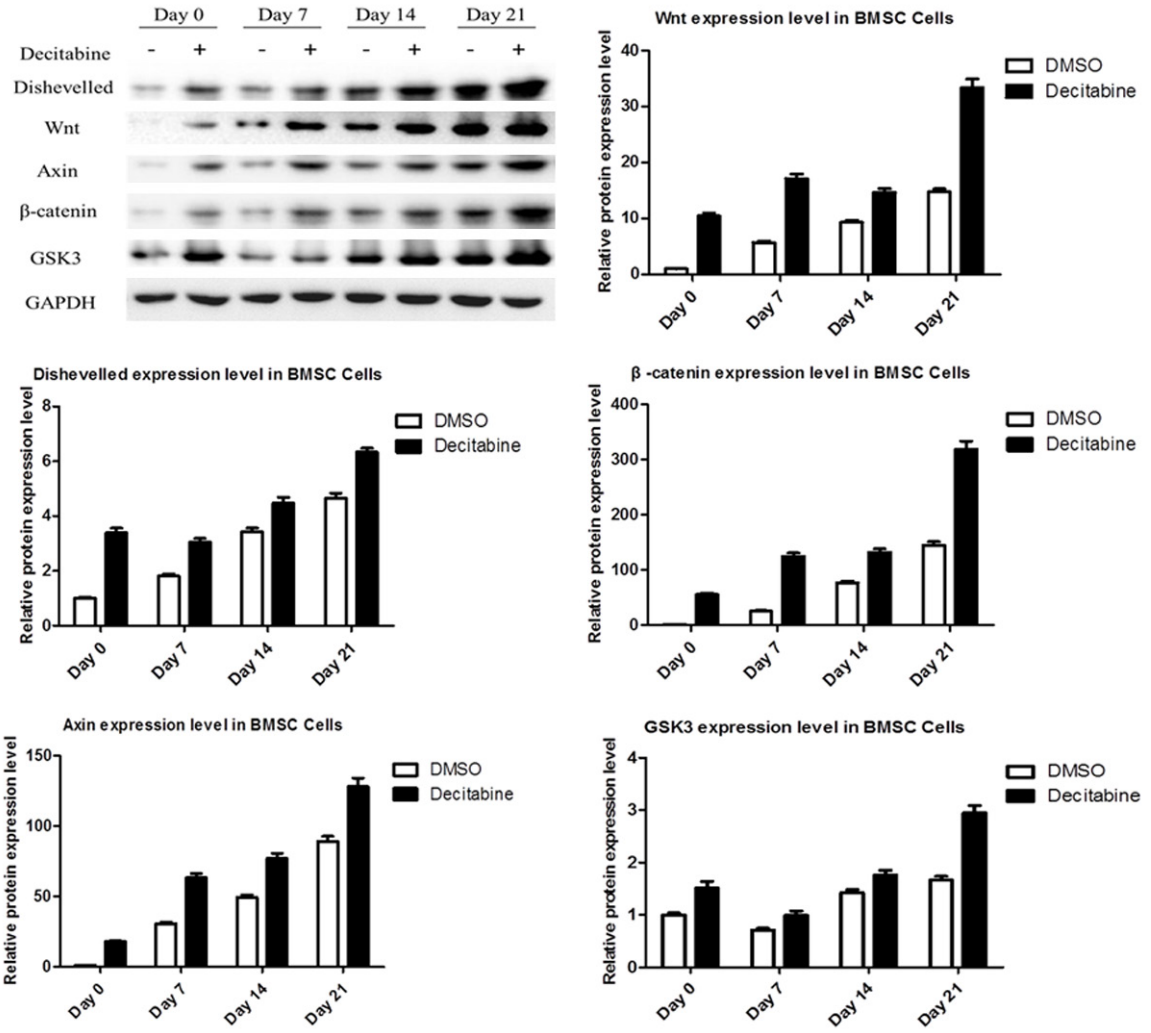

Figure 7. Protein levels of Wnt, GSK3, axin, Dishevelled, and β-catenin. The protein expression levels of Wnt, GSK3, axin, Dishevelled, and β-catenin were detected by a Western blot during osteogenic differentiation after the addition of the methylase inhibitor 5-aza-2'-deoxycytidine. $P < 0.05$.

same trend. In the present study, we investigated the potential role of Dishevelled in osteogenic differentiation of BMSCs in induction medium containing the methyltransferase inhibitor 5-aza-2'-deoxycytidine.

Studies conducted in recent years clarified the origin of osteoblasts, with research showing that they were derived from mesenchymal stem cells by multi- and controllable-directional differentiation [16]. Concrete evidence that postmenopausal-related osteoporosis was closely related to abnormal differentiation of BMSCs attracted widespread attention and has been the focus of much recent research. Studies demonstrated that excessive adipocyte differentiation was related to decreases in the number of osteoblasts and reduced bone formation, potentially leading to osteogenic metabolic diseases [16-17]. In clinical research and disease treatment, BMSCs have become important seed cells in the repair of osteogenic defects.

The fate of cell differentiation is regulated by differentiation factors, which induce a particular cell lineage, via a dynamic equilibrium process [18]. Osteoblasts derived from BMSCs and regulated by a variety of signaling pathways, rather than by the differentiation of BMSCs into adipocytes, fibroblasts, skeletal muscle, and tendon cells [18-21]. These regulatory factors include bone morphogenetic proteins (BMPs), growth hormone, transforming growth factor-β (TGF-β), mitogen-activated protein kinases (MAPK), insulin-like growth-fac-

tor-1, parathyroid hormone, fibroblast growth factors [22-26]. Osteogenic differentiation is modulated by a complex regulatory network. Classical signal transduction pathways associated with osteogenesis include MAPKs, TGF-β/BMPs, Wnt/β-catenin, notch, PI3K/Akt, and Hedgehog signaling pathways, with each of these pathways being interdependent and interrelated [27-30].

According to one study, β-catenin induced osteoblast differentiation by enhancing the effect of BMP-2 on mesenchymal stem cells. Research also showed that GSK3 played a critical role in the Wnt pathway [31]. After ERK-induced phosphorylation of Smad1, GSK recognized SmadI as a substrate and phosphorylated it, thereby promoting ubiquitination of SmadI and increasing Wnt pathway to regulate Smad1 [31]. Studies reported that mice injected with U0126 led to the early craniotomy in mice were saved to normal levels. One mechanism by which the ERK1/2 signaling pathway promoted osteoblast differentiation was suggested to be via the regulation of the synthesis of AP-1, and then accelerated the closure of skull sutures. In addition to activating the classical Smad pathway, research showed that a variety of osteogenic BMPs activated the MAPK pathway [32]. During the osteogenic differentiation of BMSCs, the activated ERK pathway interacted with the TGF-β/BMPs pathway and regulated the osteogenic transcription factor Runx2, which regulated osteogenic differentiation [32]. Thus, interactions of various key signal molecules and different signaling pathways play a role in the regulation of BMSC osteogenic differentiation. Multiple signaling pathways, including BMP/Smad, MAPK, and Wnt, influence the osteogenic differentiation of BMSCs and play important regulatory roles in bone remodeling. Through synergistic regulation of osteogenic differentiation, these pathways maintain the bone metabolic balance in the body. BMSCs are well suited to bone tissue engineering due to their ability to function as seed cells.

In conclusion, the present study investigated the potential role of Dishevelled in osteogenic differentiation of BMSCs in induction medium containing the methyltransferase inhibitor 5-aza-2'-deoxycytidine, and focusing on upstream regulatory factors and downstream target genes. We studied the interactions between a variety of regulatory factors and the mechanisms underlying these interactions. The results revealed that DNA methylation of the Dishevelled gene influenced the expression of regulatory genes and signaling molecules, thereby affecting the process of BMSC osteogenic differentiation. A better understanding of the mechanisms and signaling pathways involved in the regulation of bone growth could lead to new therapeutic targets for osteoporosis, bone tumors, and fractures, as well as novel methods for the treatment of related diseases.

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Disclosure of conflict of interest

None.

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