

## Efficient cardiomyogenic differentiation of bone marrow mesenchymal stromal cells by combination of Wnt11 and bone morphogenetic protein 2

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### Abstract

Wnt11 and bone morphogenetic protein 2 (BMP-2) are key signaling factors for stem cell differentiation into functional cardiomyocytes (CMs). In this study, we elucidate the biological effect of BMP-2 and Wnt11 on bone marrow mesenchymal stromal cells (BM-MSCs) that differentiate into myocardial-like cells in a simulated myocardial microenvironment *in vitro*. A cell co-culture system was established with recombinant Wnt11 treatment of NIH/3T3 cells and CMs. BMP-2 was added in a diverse schedule to induce cardiomyogenic differentiation of BM-MSCs co-cultured under various conditions. The levels of cardiac-specific markers Nkx2.5,  $\alpha$ -myosin heavy chain ( $\alpha$ -MHC),  $\beta$ -myosin heavy chain ( $\beta$ -MHC) and cardiac troponin I (cTnI) were determined by reverse transcriptase polymerase chain reaction and immunocytochemistry to evaluate cardiomyogenic differentiation. Wnt11 or BMP-2 used on their own to differentiate BM-MSCs resulted in no expression of  $\alpha$ -MHC and cTnI. Wnt11 alone in a myocardial microenvironment enhanced cardiomyogenic differentiation. BMP-2 demonstrated a dose-dependent effect on BM-MSC differentiation into myocardial-like cells. Addition of BMP to BM-MSCs at various time points resulted in varying effects on cardiomyogenic differentiation. The combination of Wnt11 and BMP-2 treatment in a temporal manner significantly enhanced cardiomyogenic differentiation of BM-MSCs, with high expressions of  $\alpha$ -MHC,  $\beta$ -MHC, Nkx2.5 and cTnI upon co-culture with CMs. Our study demonstrates that the combination of Wnt11 and BMP-2 effectively promotes cardiomyogenic differentiation of BM-MSCs *in vitro*. The synergistic effect of Wnt11 and BMP-2 on the cardiomyogenic differentiation of BM-MSCs is further enhanced in a myocardial microenvironment.

**Keywords:** BMP-2, Wnt11, differentiation, cardiomyocyte, BM-MSCs

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### Introduction

Bone morphogenetic protein-2 (BMP-2) and Wnt11 are key signaling factors for cardiomyogenic differentiation of stem cells. Wnt11 is a member of the Wnt family and promotes the differentiation of stem cells into cardiomyocytes (CMs) via non-classical Wnt/ $\text{Ca}^{2+}$  and Wnt/JNK signaling pathways.<sup>1,2</sup> Previous studies confirmed that Wnt11, via the non-classical Wnt/ $\text{Ca}^{2+}$  pathway, induces the expression of the cardiac-specific markers Nkx2.5 and GATA-4, as well as promoting differentiation of bone marrow mesenchymal stromal cells (BM-MSCs) into CMs. However, the differentiated cells induced by Wnt11 lack  $\alpha$ -myosin heavy chain ( $\alpha$ -MHC) expression, and functional CMs cannot be formed.<sup>3,4</sup>

BMP signaling is crucial during mesoderm induction and cardiac formation.<sup>5</sup> BMP-2, a member of the transforming growth factor  $2\beta$  protein family and a ligand protein, has been used to differentiate embryonic stem cells into CMs, with a dose-dependent effect on receptor signaling *in vitro*.<sup>6,7</sup> Temporal BMP expression during differentiation of embryonic stem cells results in various effects. However, thus far, there have been no reports on whether BMP-2 exerts a similar effect during differentiation of BM-MSCs into CMs. Xu *et al.*<sup>8</sup> showed that BM-MSCs co-cultured with CMs transdifferentiated into cells with a cardiac phenotype, suggesting that the myocardial microenvironment perhaps plays an important role in the differentiation of BM-MSCs into myocardial-like cells.

We hypothesized that a combination of Wnt11 and BMP-2 would have a synergistic effect on the differentiation of BM-MSCs into CMs in a myocardial microenvironment. In the present study, we established a new system for cardiomyogenic differentiation of BM-MSCs using a combination of Wnt11 and BMP-2 in conjunction with a myocardial microenvironment, and attempted to elucidate the biological effects and significance of Wnt11 and BMP-2 on the differentiation of BM-MSCs into myocardial-like cells in an *in vitro* myocardial microenvironment.

## Materials and methods

### Animals

Sprague-Dawley neonate rats aged between one and three days were obtained from the Experimental Animal Center of Xuzhou Medical College (Jiangsu, China). Animals received humane care in compliance with the Guidelines for the Care and Use of Laboratory Animals published by Jiangsu Province, China.

### BM-MSC culture

Rat BM-MSCs were purchased from Cyagen Biosciences Inc., Guangzhou, China. Cells were cultured in L-Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), penicillin (100 U/mL; Beyotime, Shanghai, China) and streptomycin (100 U/mL; Beyotime) at 37°C/5% CO<sub>2</sub>. Culture medium was changed every two days. Identification of BM-MSCs was performed by flow cytometry. Cells at passage 3 were used for experimentation.

### Isolation, culture and identification of neonatal rat CMs

Neonate rat hearts were removed under inhalation anesthesia. CMs were separated by digestion with 0.125% trypsin and 0.08% type I collagenase, then centrifuged at 300 × *g* for five minutes. To selectively enrich CMs, dissociated cells were plated for two hours to allow non-CMs to attach. The CM suspension was then transferred to a Transwell chamber (Corning, Corning, NY, USA). Bromodeoxyuridine (100 μmol/L) was added during the first 24–36 h to prevent proliferation of non-CMs. Then, CMs were cultured in H-DMEM supplemented with 20% FBS, penicillin (100 U/mL) and streptomycin (100 U/mL) at 37°C/5% CO<sub>2</sub>. Culture medium was changed every two days. CM purity was evaluated by cardiac troponin I (cTnI) immunocytochemistry and the cells were used for experimentation after 96 h in culture.

### Generation of stably transfected Wnt11 3T3 cell sublines

The Wnt11 expression vector (MSCVneo-wnt-11) was a gift from Prof. Thomas Brau (Max Planck Institute for Heart and Lung Research, Bad Nauheim, Germany). Mouse embryonic fibroblasts (NIH/3T3) were purchased from the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China. To generate Wnt11-conditioned

medium, NIH/3T3 cells were transfected with MSCVneo-Wnt-11 using Lipofectamine 2000 (Invitrogen, Grand Island, NY, USA). Stable transfectants (designated Wnt11/3T3) were selected using G418 (Gibco) resistance. Expression of the Wnt11 protein in the conditioned medium was verified by an enzyme-linked immunosorbent assay (ELISA; CUSABIO, Carlsbad, CA, USA).<sup>9</sup>

### Establishment of a co-culture system and cardiomyogenic differentiation

The co-culture system was established as described previously.<sup>10</sup> The third-generation BM-MSCs ( $5 \times 10^6$ /mL) were seeded on six-well tissue plates after digestion with 0.25% trypsin in 0.02% ethylenediaminetetraacetic acid at room temperature. CMs were inoculated in Transwell chambers, and then these were moved to the corresponding well of the co-culture and maintained at 37°C/5% CO<sub>2</sub>.

For cardiomyogenic differentiation, BM-MSCs were cultured in the lower chamber of a Transwell chamber. The CMs or Wnt11/3T3 cells were placed in the top chamber. BMP-2 (Corning), WIF-1 (a Wnt11 inhibitor; Corning) or Noggin (a BMP-2 inhibitor; Corning) was added to the culture medium. To determine the optimal concentration of BMP-2 for cardiomyogenic differentiation, four BMP-2 concentrations (0.25, 0.5, 1 and 10 ng/mL) were used. After 14 days of induction, Nkx2.5, α-MHC, β-myosin heavy chain (β-MHC) and cTnI expressions were analyzed by reverse transcriptase polymerase chain reaction (RT-PCR).

Cardiomyogenic differentiation was performed in three steps. The first step involved Wnt11 treatment of eight groups: the positive controls (CMs); negative controls (BM-MSCs); Wnt11/3T3+BM-MSCs; Wnt11/CM+BM-MSCs; 3T3+BM-MSCs; Wnt11/3T3+WIF-1; Wnt11/CM+WIF-1; and CM+BM-MSCs. The second step involved BMP treatment of six groups: positive controls; negative controls; BM-MSCs+BMP-2; BM-MSCs+CM+BMP-2; BM-MSCs+CM+Noggin; and BM-MSCs+CM+Noggin+BMP-2. The final cardiomyogenic differentiation step involved both Wnt11 and BMP-2 treatment of seven groups: the positive controls; negative controls; Wnt11+Noggin+BMP-2+BM-MSCs; CM+Wnt11+Noggin+BMP-2+BM-MSCs; CM+Wnt11+BM-MSCs; CM+Noggin+BMP-2+BM-MSCs; and CM+BM-MSCs.

Cell cultures were maintained at 37°C/5% CO<sub>2</sub> in saturated humidity. After 14 days of induction, RT-PCR was used to determine Nkx2.5, cTnI, α-MHC and β-MHC mRNA expressions. To identify the impact of the myocardial microenvironment on the BMP-2 signaling pathway, expression of BMPRI-α, a membrane receptor of the BMP signaling pathway, and Smad1, an important downstream cytokine of the BMP signaling pathway, was also determined by RT-PCR in the BM-MSCs controls, Wnt11/3T3+BM-MSCs, CM+BM-MSCs and Wnt11/CM+BM-MSCs groups. In addition, immunocytochemistry staining was performed to identify expression of the cardiac-specific markers cTnI, α-MHC and Nkx2.5.

## RT-PCR analysis

To measure the mRNA expression levels of Nkx2.5, cTnI,  $\alpha$ -MHC,  $\beta$ -MHC, BMPRI- $\alpha$  and Smad1, total RNA was extracted from differentiated BM-MSCs on day 14 by using a DNA-free Kit (Qiagen, Hilden, Germany). Total RNA (1  $\mu$ g) was reverse-transcribed by using a First-Strand Synthesis Kit (Invitrogen) and then a 1/100 volume of the cDNA was subjected to PCR amplification for 30–42 cycles. Cycling parameters consisted of a denaturation step at 94°C for 30 s, annealing at the appropriate temperature for 90 s and extension at 72°C for 60 s using the primer sets (Supplementary Figure 1; please see <http://ebm.rsmjournals.com/lookup/suppl/doi:10.1258/ebm.2012.011291/-/DC1> for all supplementary figures). Amplified cDNA was electrophoresed on 2% agarose gels containing ethidium bromide and the quantities were analyzed by densitometry with NIH Image software (National Institutes of Health, Bethesda, MD, USA).

## Immunofluorescence

Cardiomyogenic differentiation was confirmed by expressions of cardiac-specific markers cTnI,  $\alpha$ -MHC and Nkx2.5. The following antibodies were used: sheep polyclonal anti-mouse Nkx2.5 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit monoclonal anti-mouse  $\alpha$ -MHC (1:150; Abcam, Cambridge, MA, USA) and mouse monoclonal anti-rat cTnI (1:100; Santa Cruz Biotechnology). Immunocytochemistry was performed according to previously published methods.<sup>11</sup> Photomicrographs of the immunocytochemical results were acquired with a DP71 fluorescence microscope (Olympus, Tokyo, Japan). For quantitative assessment of cardiomyogenic differentiation, the total number of cells per field of view was counted by nuclear staining with Hoechst 33258. Cells expressing cardiac-specific intracellular contractile proteins were counted and the percentage of cells that differentiated into CMs were calculated from the number of cells positive for cardiac-specific markers divided by the total number of nuclei in the field.

## Statistical analysis

All data are expressed as means  $\pm$  standard deviation (SD), and were analyzed by analysis of variance and Student–Newman–Keuls test. *P* values less than 0.05 were considered statistically significant.

## Results

### Myocardial cTnI immunocytochemistry and BM-MSC identification

Cell morphology was round prior to adhesion, and cells adhered after 24 h with stretching pseudopods as well as strong refraction. After 3–4 days, cell clusters formed and beating CMs were observed. Nuclei were stained blue and CMs appeared red. Myocardial cell purity was 86% (Supplementary Figure 2a, b). The flow cytometry identification results showed that the ratios of CD34<sup>+</sup>, CD45<sup>+</sup>,

CD90<sup>+</sup>, CD44<sup>+</sup> and CD11b<sup>+</sup> cells were 0.57, 0.33, 99.4, 99.4 and 95.29%, respectively (Supplementary Figure 2c).

### Wnt11 plasmid transfection

After 48 h, Wnt11 expression as measured by ELISA, showed that the Wnt11 concentrations were  $28.29 \pm 1.31$  ng/mL in the Wnt11/3T3 group,  $6.69 \pm 0.26$  ng/mL in the CM group and  $8.34 \pm 0.29$  ng/mL in the Wnt11/CM group. The Wnt11 concentration in the Wnt11/3T3 group was significantly higher than in the Wnt11/CM and the CM groups (*P* < 0.01). The concentration of Wnt11 in the Wnt11/CM group was higher compared with that of the CM group (*P* < 0.05).

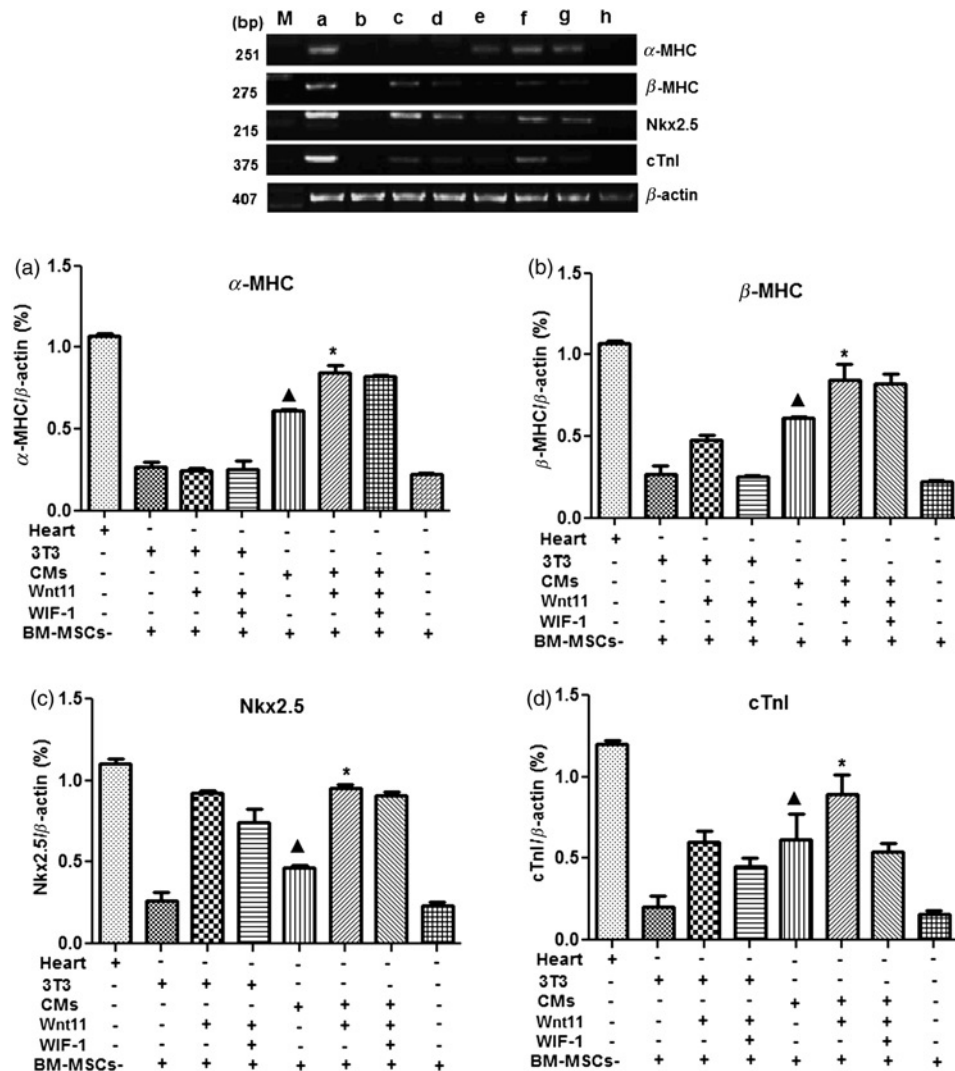
### Wnt11 enhances cardiomyogenic differentiation of BM-MSCs

After 14 days of induction,  $\alpha$ -MHC,  $\beta$ -MHC, Nkx2.5 and cTnI expressions in the CM+Wnt11+BM-MSCs group were significantly higher compared with that in the BM-MSCs, Wnt11/3T3+BM-MSCs and the CM+BM-MSCs groups (*P* < 0.05). Cardiac-specific marker expression in the CM+BM-MSCs group was lower than that in the Wnt11+BM-MSCs group (*P* < 0.05). Additionally, cardiac-specific marker expression was decreased in the Wnt11/3T3+BM-MSCs and the Wnt11/CM+BM-MSCs groups after WIF-1 addition (*P* < 0.05). The  $\alpha$ -MHC marker was not expressed in the Wnt11/3T3+BM-MSCs group, but was expressed in the CM+Wnt11+BM-MSCs and CM+BM-MSCs groups. Its expression was higher in the Wnt11/CM+BM-MSCs group (*P* < 0.05); thus, we believe that Wnt11 and a myocardial microenvironment promote cardiomyogenic differentiation (Figure 1).

### BMP-2 enhances cardiomyogenic differentiation of BM-MSCs

To determine the optimal concentration of BMP-2 for cardiomyogenic differentiation, four BMP-2 concentrations (0.25, 0.5, 1 and 10 ng/mL) were used. After 14 days of induction, Nkx2.5,  $\alpha$ -MHC,  $\beta$ -MHC and cTnI expressions were analyzed by RT-PCR. Significant increases in Nkx2.5,  $\alpha$ -MHC and  $\beta$ -MHC expressions were observed using a BMP-2 concentration of 0.5 ng/mL. However, cTnI expression remained constant at various BMP-2 concentrations. Thus, the optimal BMP-2 concentration was 0.5 ng/mL. The results also showed that BMP-2 alone could not differentiate BM-MSCs into myocardial-like cells (Figure 2).

To determine the optimal condition for efficient cardiomyogenic differentiation, we added Noggin, a special BMP-2 inhibitor, and BMP-2, in a diverse schedule using the optimal BMP-2 concentration of 0.5 ng/mL. Noggin (50 ng/mL) was added at days 0–3 followed by removal and then BMP-2 was added from day 4. After 14 days, Nkx2.5, cTnI,  $\alpha$ -MHC and  $\beta$ -MHC mRNA expressions were increased in the BM-MSCs+CM and BM-MSCs+CM+Noggin+BMP-2 groups. Cardiac-specific marker expression in the BM-MSCs+CM+Noggin+BMP-2 group was higher compared with that in the CM+BM-MSCs group (*P* < 0.05).



**Figure 1** Wnt11 enhances cardiomyogenic differentiation of BM-MSCs. (a) Expression of  $\alpha$ -MHC in each group,  $^*P < 0.05$  versus CMs and Wnt11/CMs+WIF-1,  $^{\Delta}P < 0.05$  versus BM-MSCs. (b) Expression of  $\beta$ -MHC in each group,  $^*P < 0.05$  versus CMs and Wnt11/CMs+WIF-1,  $^{\Delta}P < 0.05$  versus Wnt11/3T3. (c) Nkx2.5 expression in each group,  $^*P < 0.05$  versus CMs and Wnt11/CMs+WIF-1,  $^{\Delta}P < 0.05$  versus Wnt11/3T3. (d) cTnI expression in each group,  $^*P < 0.05$  versus CMs and Wnt11/CM+WIF-1,  $^{\Delta}P < 0.05$  versus Wnt11/3T3. BM-MSC, bone marrow mesenchymal stromal cell; CM, cardiomyocyte;  $\alpha$ -MHC,  $\alpha$ -myosin heavy chain;  $\beta$ -MHC,  $\beta$ -myosin heavy chain; cTnI, cardiac troponin I

Cardiac-specific marker expression was very low in the BM-MSCs+CM+Noggin group and no difference was observed compared with the BM-MSCs group (Figure 3).

These results indicated that the addition of Noggin and BMP-2 at a specific time-point enhances cardiomyogenic differentiation. Moreover, BMP-2 together with a myocardial microenvironment had a significant effect on cardiomyogenic differentiation.

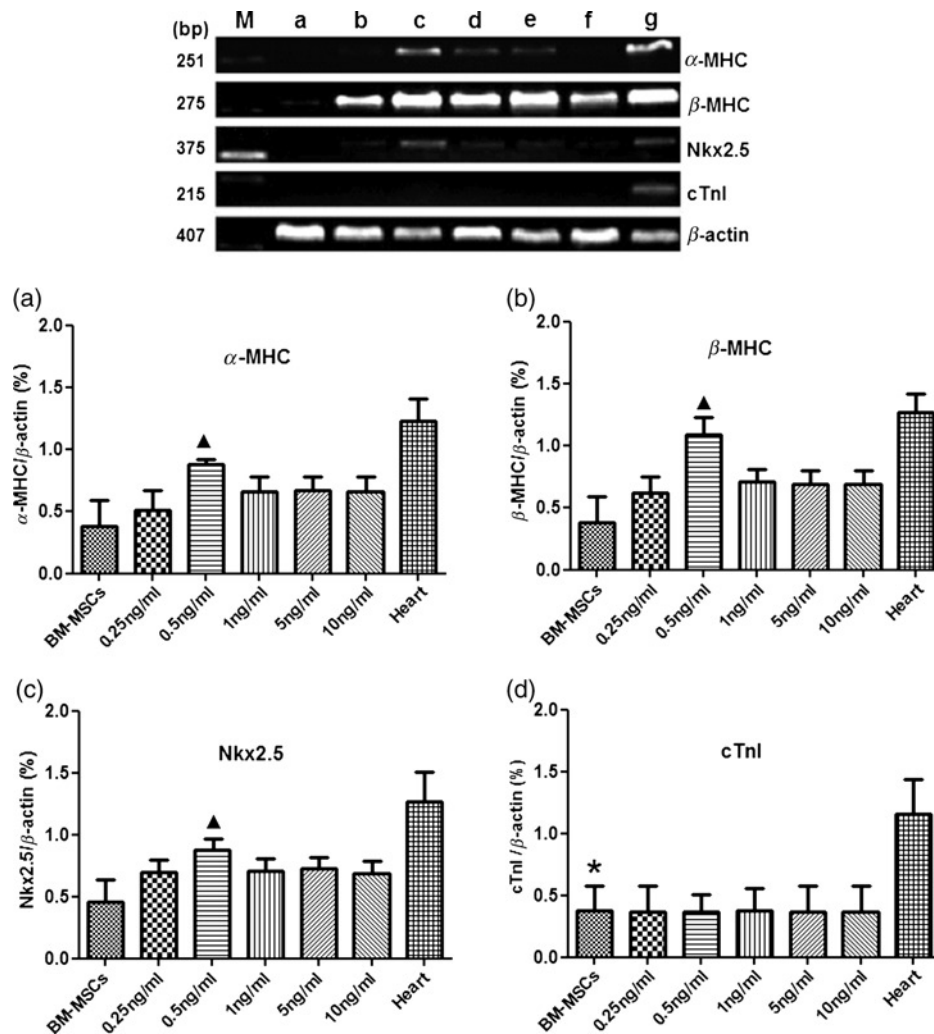
#### The myocardial microenvironment enhanced cardiomyogenic differentiation via BMP signaling pathway

Our results showed that the levels of BMPRI- $\alpha$  and Smad1 mRNA expressions were very low in the Wnt11 and the control groups. In contrast, expressions of BMPRI- $\alpha$  and Smad1 mRNA in the CM group were significantly higher compared with those in the Wnt11 and the control groups ( $P < 0.05$ ). Our results also showed that expressions

of BMPRI- $\alpha$  and Smad1 mRNA in the CM+Wnt11 group were significantly higher than that in the CM group ( $P < 0.05$ ) (Figure 3). The results indicated that the myocardial microenvironment enhanced cardiomyogenic differentiation of BM-MSCs via the BMP signaling pathway.

#### BMP-2 and Wnt11 play important roles in cardiomyogenic differentiation of BM-MSCs

BMP-2 and Wnt11 may coordinate their functions for cardiomyogenic differentiation during heart development. To investigate whether the combination of Wnt11 and BMP-2 further enhanced cardiomyogenic differentiation of BM-MSCs *in vitro*, we added both Wnt11 and BMP-2 using diverse schedules at the optimal conditions (0.5 ng/mL BMP-2 was added from the fourth day, 50 ng/mL Noggin was added 3 days before that) as determined by previous experiments.



**Figure 2** Determination of the optimal BMP-2 concentration. (a) Expression of  $\alpha$ -MHC in each group,  $^{\Delta}P < 0.05$  versus 0, 0.25, 1, 5 and 10 ng/mL. (b)  $\beta$ -MHC expression in each group,  $^{\Delta}P < 0.05$  versus 0, 0.25, 1, 5 and 10 ng/mL. (c) Expression of Nkx2.5 in each group,  $^{\Delta}P < 0.05$  versus 0, 0.25, 1, 5 and 10 ng/mL. (d) cTnI expression in each group,  $^{\Delta}P > 0.05$  versus 0, 0.25, 1, 5 and 10 ng/mL. BMP-2, bone morphogenetic protein 2;  $\alpha$ -MHC,  $\alpha$ -myosin heavy chain;  $\beta$ -MHC,  $\beta$ -myosin heavy chain; cTnI, cardiac troponin I

Compared with Wnt11 or BMP-2 treatment alone, increases in the expression levels of  $\alpha$ -MHC,  $\beta$ -MHC, Nkx2.5 and cTnI were observed in groups with a combination of both growth factors ( $P < 0.05$ ). In the myocardial microenvironment, both Wnt11 and BMP-2 significantly enhanced *in vitro* cardiomyogenic differentiation, with higher cardiac-specific marker expression compared with groups with a combination of both growth factors without CM co-culture ( $P < 0.05$ ) (Figure 4). Our results showed that the combination of Wnt11 and BMP-2 enhances the *in vitro* cardiomyogenic differentiation of BM-MSCs. Moreover, a myocardial microenvironment significantly enhances the effect of both growth factors.

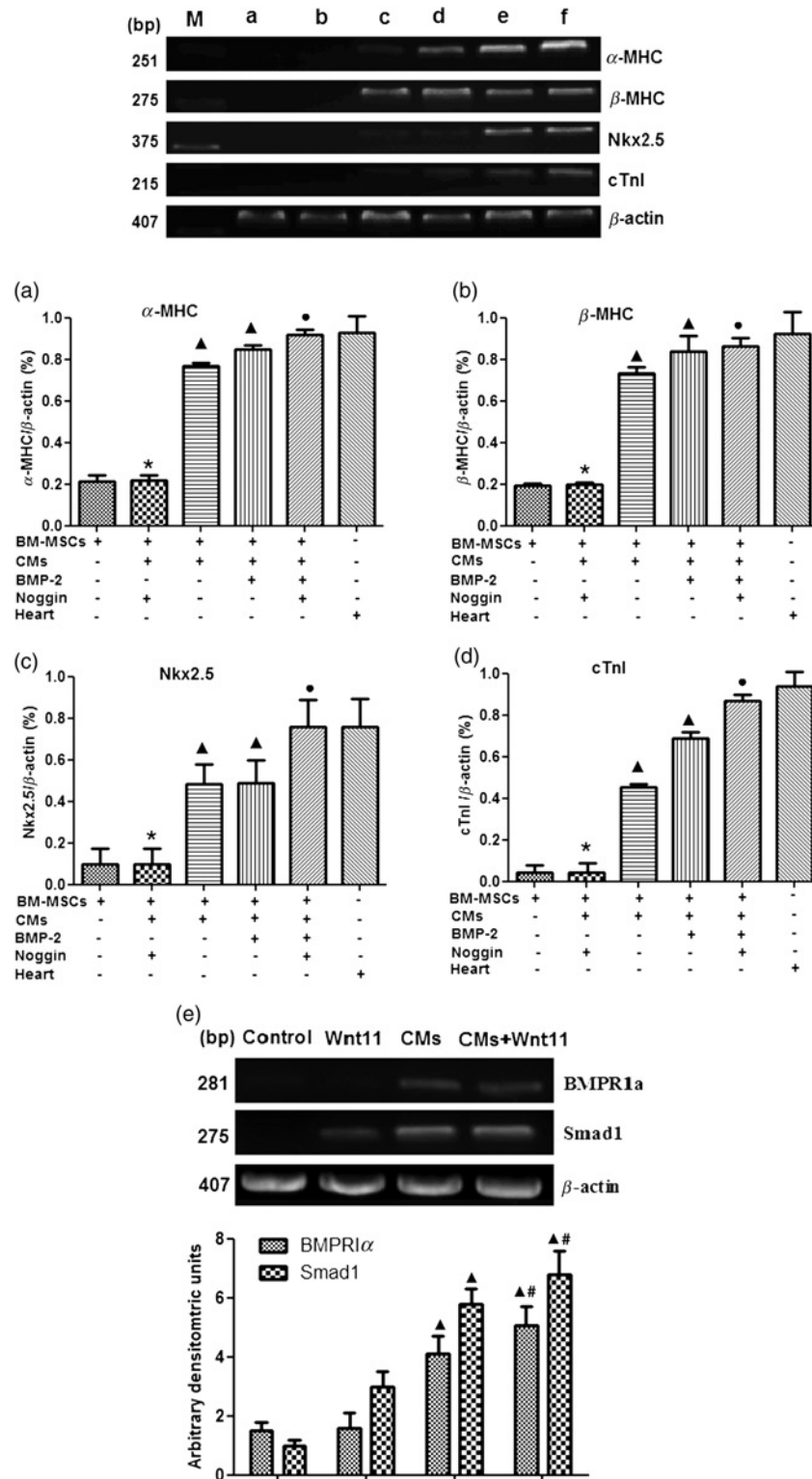
### Immunofluorescence

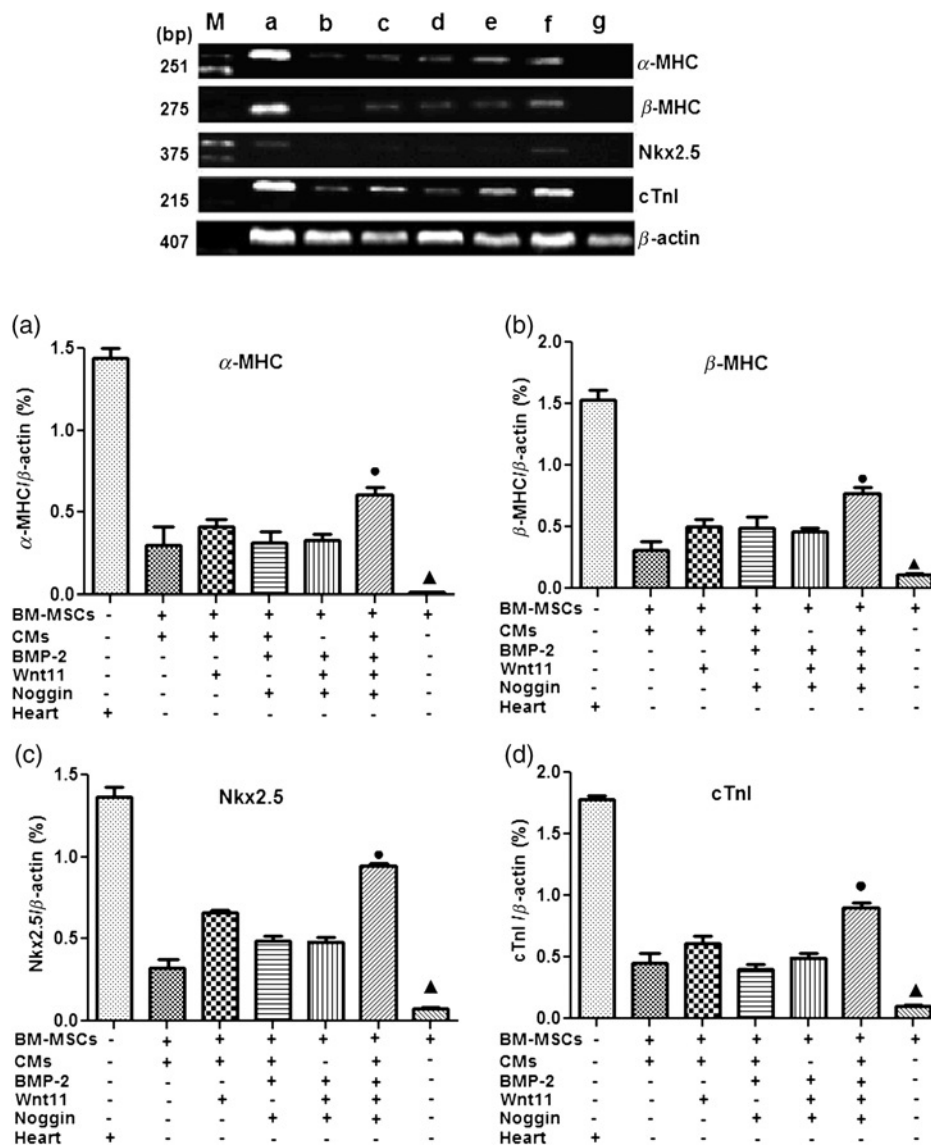
After 14 days of induction, the cells in the CM+Wnt11, CM+BMP-2, Wnt11+BMP-2 and CM+Wnt11+BMP-2 groups stained positive for some cardiac-specific markers, suggesting that the majority of cells possessed the features

of CMs. These results were consistent with our RT-PCR results. Our results also showed that the cardiomyogenic differentiation in the CM+Wnt11 and CM+BMP-2 groups was enhanced with an increased percentage of BM-MSC-derived CMs. Additionally, a myocardial microenvironment had a greater effect on cardiomyogenic differentiation compared with using BMP-2 or Wnt11 alone, as indicated by cardiac-specific marker expression. The optimal conditions for cardiomyogenic differentiation were achieved using a combination of Wnt11 and BMP-2 in a myocardial microenvironment (Supplementary Figure 3).

### Discussion

Our results showed that the synergistic effects of Wnt11 and BMP-2 in a myocardial microenvironment optimally enhanced cardiomyogenic differentiation of BM-MSCs. Previous reports demonstrated that BM-MSCs change their environmental dependencies according to various survival





**Figure 4** BMP-2 and Wnt11 play important roles in cardiomyogenic differentiation of BM-MSCs. BMP-2 (0.5 ng/mL) was added from the fourth day and Noggin three days before that. (a) Expression of  $\alpha$ -MHC in each group; \* $P$  < 0.05 versus CMs, CMs+Wnt11, CMs+Noggin+BMP-2, Wnt11+Noggin+BMP-2 and BM-MSCs; and \* $P$  < 0.05 versus other groups. (b) Expression of  $\beta$ -MHC in each group; \* $P$  < 0.05 versus CMs, CMs+Wnt11, CMs+Noggin+BMP-2, Wnt11+Noggin+BMP-2 and BM-MSCs; and \* $P$  < 0.05 versus other groups. (c) Nkx2.5 expression in each group; \* $P$  < 0.05 versus CMs, CMs+Wnt11, CMs+Noggin+BMP-2, Wnt11+Noggin+BMP-2 and BM-MSCs; and \* $P$  < 0.05 versus other groups. (d) cTnI expression in each group; \* $P$  < 0.05 versus CMs, CMs+Wnt11, CMs+Noggin+BMP-2, Wnt11+Noggin+BMP-2 and BM-MSCs; and \* $P$  < 0.05 versus other groups. BM-MSC, bone marrow mesenchymal stromal cell; CM, cardiomyocyte; BMP-2, bone morphogenetic protein 2;  $\alpha$ -MHC,  $\alpha$ -myosin heavy chain;  $\beta$ -MHC,  $\beta$ -myosin heavy chain; cTnI, cardiac troponin I

and growth factors.<sup>12</sup> An organic environment is the assumed inducing agent for directed stem cell differentiation, including a myocardial microenvironment.<sup>13,14</sup> Xu *et al.*<sup>8</sup> showed that a myocardial microenvironment plays an important role in the differentiation of BM-MSCs into myocardial-like cells. In this study, we used a myocardial microenvironment to promote the efficiency of cardiomyogenic differentiation. With direct contact between cells, stimulation of electrical machinery and cytokines was possible. Therefore, this system eliminated any physical factors that could affect BM-MSCs by directly exposing cells to soluble secreted factors from cardiac cells<sup>15,16</sup> and may be favorable for observing biological effects during cardiomyogenic differentiation.

Under pathological conditions, Wnt11 signaling is involved in myocardial infarction repair. Orlic *et al.*<sup>17</sup> reported that transplanted stem cells differentiate in response to up-regulated Wnt11 expression in a myocardial infarction environment. Current data also demonstrate that Wnt11 is activated by a non-classical Wnt11 pathway and induces expressions of the early cardiac genes GATA4 and Nkx2.5 in *Xenopus* embryos and a P19 cell line.<sup>18,19</sup> Another report points out that supplementation of co-cultures with Wnt11-conditioned medium significantly enhances the differentiation of cardiac progenitor cells into CMs.<sup>20</sup> These studies suggest that Wnt11 may play an important role in the differentiation of BM-MSCs into myocardial-like cells.

Previous data indicate that Wnt11 alone cannot efficiently induce BM-MSC differentiation into myocardial cells lacking  $\alpha$ -MHC expression.<sup>3–5</sup> It is well documented that  $\alpha$ -MHC possesses high ATPase activity and indicates the state of BM-MSC differentiation into CMs. Therefore,  $\alpha$ -MHC is an important marker for evaluating cardiomyogenic differentiation.<sup>21</sup> In the present study, we confirmed that Wnt11 induced the expression of cardiac-specific markers (Nkx2.5, cTnI,  $\beta$ -MHC) and promoted BM-MSC differentiation into myocardial cells via the non-classical Wnt11 pathway. However, the differentiated cells lack  $\alpha$ -MHC expression and functional myocytes cannot be formed. In our study, we found that differentiated cells from Wnt11-treated BM-MSCs subjected to *in vitro* simulation in a myocardial microenvironment not only expressed Nkx2.5, cTnI and  $\beta$ -MHC, but also  $\alpha$ -MHC. This would suggest that Wnt11 in a myocardial microenvironment further enhances the biological efficiency of BM-MSC differentiation into myocardial-like cells.

BMP signaling pathways are early key signaling pathways during cardiac development, which directly regulate many aspects of cell behavior including cell proliferation, differentiation and survival of cardiac progenitor cells.<sup>22,23</sup> Pathologically, BMP-2 expression is upregulated during myocardial infarction. BMP-2 not only acts on antiapoptosis pathways, but is also involved in the differentiation of cardiac progenitors into the myocardium.<sup>24</sup> Kawai *et al.*<sup>25</sup> found that BMP-2 induction of embryonic stem cell differentiation into myocardial cells was not efficient at low concentrations, while high concentrations inhibited differentiation. This observation indicates that BMP signaling pathways are dose-dependent for inducing stem cell differentiation. Some studies found that BMP signaling pathways are temporally affected in mouse embryonic stem cell differentiation, and that early temporary blocking of the BMP signaling pathway enhances differentiation into CMs.<sup>26,27</sup> In our study, we found that 50 ng/mL Noggin at days 0–3, followed by removal and subsequent addition of 0.5 ng/mL BMP-2 for 11 days, were the optimal conditions for BM-MSC differentiation into myocardial-like cells. With our treatment schedule for BMP-2 and Wnt11 induction, expressions of  $\alpha$ -MHC,  $\beta$ -MHC, Nkx2.5 and cTnI were at their highest levels, indicating that BMP-2 acts optimally under this condition. We also detected that the differentiated cells treated with 0.5 ng/mL BMP-2 alone expressed  $\alpha$ -MHC,  $\beta$ -MHC and Nkx2.5, but did not express cTnI. In comparison to the group treated with BMP-2 alone, BM-MSCs treated with BMP-2 in a myocardial microenvironment differentiated into cells with increased expression levels for  $\alpha$ -MHC,  $\beta$ -MHC, Nkx2.5 and cTnI. These results suggest that although BMP-2 alone induces BM-MSC differentiation into myocardial-like cells, differentiation is inefficient and a myocardial microenvironment further enhances BM-MSC differentiation. However, the detailed mechanism of BMP-2-induced BM-MSC differentiation into myocardial-like cells is yet to be fully elucidated. It has been suggested that via Smads, p38MAPK and PI3K pathways, the combination of BMP-2 and cognate receptors (BMPRI- $\alpha$  and BMPRII) activates downstream signaling molecules (i.e. Smad1, Tak1 and p85), then promotes

nuclear transcription factors that enhance the expression of Nkx2.5 and cTnI, and induce cardiomyogenic differentiation. Nonetheless, usage of the combined Wnt11/BMP-2 *in vivo*, especially in ischemic heart, should be further investigated.

In the present study, the use of Wnt11 or BMP-2 alone failed to differentiate BM-MSCs into myocardial-like cells. We noted that  $\alpha$ -MHC was not expressed following Wnt11 induction, but this was not the case in the myocardial microenvironment. We found that expressions of the BMP signaling factors, BMPRI- $\alpha$  and Smad1, were very weak in the absence of the myocardial microenvironment. In contrast, significantly increased expression levels of BMPRI- $\alpha$  and Smad1 were found, and  $\alpha$ -MHC was also expressed in the myocardial microenvironment, suggesting that the myocardial microenvironment is likely required to induce expression of key myogenic factors, such as  $\alpha$ -MHC. The upregulated expression of all cardiac-specific markers in the CMs+Wnt11 group was predominant compared with that in the CMs group. These results strongly indicate a correlation between BMP and Wnt11 signaling pathways in the myocardial microenvironment.

Our findings demonstrate that the synergistic effect of Wnt11 and BMP-2 induces cardiomyogenic differentiation, with high expressions of  $\alpha$ -MHC,  $\beta$ -MHC, Nkx2.5 and cTnI, which were enhanced further by co-culture with myocardial cells. Previous studies demonstrated a crosstalk between signaling pathways during stem cell differentiation. Yuasa *et al.*<sup>26</sup> pointed out that the crosstalk between FGF-2 and BMP-2 may efficiently enhance the cardiomyogenic differentiation of embryonic stem cells. Although our results showed a close relationship between Wnt11 and BMP-2 for cardiomyogenic differentiation of BM-MSCs, the detailed mechanisms for the crosstalk between Wnt11 and BMP-2, especially in the myocardial microenvironment, need to be further investigated.

In conclusion, Wnt11 and BMP-2 in a myocardial microenvironment play crucial roles in the early cardiomyogenic differentiation of BM-MSCs. It is possible that they may be useful in the development of BM-MSC-based therapies for treating heart disease, as well as for the basic study of cardiac development.

**Author contributions:** All authors participated in the design, interpretation of the studies, analysis of the data and review of the manuscript. ZfZ, HL, ZM, JF and PG conducted the experiments. HD and ZmZ directed the experiment.

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