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TGF-β1 combined with Sal-B promotes cardiomyocyte differentiation of rat mesenchymal stem cells

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Abstract. Transforming growth factor β1 (TGF-β1) and salvianolic acid B (Sal-B) are key signaling factors for stem cell differentiation into cardiomyocytes (CMs). The present study compared the biological effect of TGF-β1 and Sal-B, alone or in combination, on bone marrow mesenchymal stromal cells (BMSCs) that differentiate into myocardial-like cells in a simulated myocardial microenvironment *in vitro*. BMSCs were isolated from bones of limbs of 10 male Sprague Dawley rats and cultured. The 2nd-generation BMSCs were co-incubated with TGF-β1 and Sal-B, alone or in combination, for 72 h. The control group was BMSCs cultured without any inductive substance. The levels GATA binding protein 4 (GATA4) and homeobox protein NKx2.5 were determined by reverse-transcription quantitative polymerase chain reaction and immunofluorescence staining was used to evaluate α-sarcomeric actin and cardiac troponin I (cTNI) as cardiomyogenic differentiation markers. The ultrastructure of BMSCs in each group was also observed. BMSCs were initially spindle-shaped with irregular processes. The cells gradually increased in number 24 h post-inoculation and proliferated 7 days later. Compared with the control group, BMSCs in the treatment groups had fusiform shapes, orientating with one accord and were connected with adjoining cells forming myotube-like structures on day 28. The morphology and architecture/myotubes of BMSCs was similar among the treatment groups, but the amount of cells in the combined group was comparatively higher. The results of immunofluorescence staining revealed the expression of the CM-specific proteins α-sarcomeric actin and cTNI in these cells. The expression of these cardiac-specific markers in the combined group was significantly higher than that in the other groups ($P<0.01$ or $P<0.05$). In addition, the transcriptional expression of GATA4 and NKx2.5 in the treatment groups was stable and significantly higher than that in the control group on day 7. Transmission electron microscopy showed that BMSCs in the treatment groups all had myofilaments, rough endoplasmic reticulum and mitochondria in the cytoplasm when compared with the control group. Taken together, these results indicated that the combination of TGF-β1 and Sal-B effectively promotes cardiomyogenic differentiation of BMSCs *in vitro* and their application may represent a therapeutic strategy for the treatment of ischemic heart disease.

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Introduction

Acute myocardial infarction (AMI) is the most important manifestation of ischemic heart disease and is one of the leading causes of major morbidity and mortality in the modern world. Recently, with the emergence of myocardial tissue engineering, the delivery of *ex vivo* bone mesenchymal stem cells (MSCs) to the infarcted heart has been successfully performed (1-3).

MSCs are multipotent progenitor cells, which are easy to purify and amplify (4-6). They are able to differentiate into cardiomyocytes (CMs) or CM-like cells (CLCs) *in vivo* and *in vitro* (7,8). 5-azacytidine is a classic inducer that enhances differentiation of MSCs into CMs by random demethylation. However, it has been demonstrated that 5-azacytidine is toxic and produces relatively low differentiation ratios (9).

Transforming growth factor β1 (TGF-β1) is a pleiotropic cytokine with numerous complex effects in cell and tissue physiology. It is a multifunctional cytokine involved in the differentiation, growth and survival of a variety of cells (10). Salvianolic acid B (Sal-B) is a water-soluble phenolic acid extracted from *Salvia miltiorrhiza*, which is a Chinese herbal medicine used in the treatment of various heart diseases for hundreds of years. Therefore, its safety as an inducer of differentiation may be comparatively higher than that of 5-azacytidine. In the present study, a new system for cardiomyogenic differentiation of bone marrow mesenchymal stromal cells (BMSCs) was established by using a combination of TGF-β1 and Sal-B and electron microscopy, immunofluorescence as well as reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis were used to elucidate the biological effects and significance of TGF-β1 and Sal-B regarding the differentiation of BMSCs into CMs or CLCs.

Materials and methods

Animals. A total of 10 male Sprague Dawley (SD) rats (weight, 35-45 g; age, 3 weeks) were obtained from HuaFuKang Bioscience Co., Inc. Beijing (certificate no. 11401300032331). They were kept in plastic cages at a controlled temperature (18-21˚C) and humidity (55±5%) under a 12-h light/dark cycle. The animal experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of Hebei North University (Zhangjiakou, China).

Isolation and culture of BMSCs. Bone marrow was separated from the femur and tibial bones of SD rats following sacrifice by cervical vertebra dislocation. The marrows were collected and diluted with 5 ml Iscove's modified Dulbecco's medium-low glucose (IMDM-LG; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), supplemented with 15% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C in a humidified atmosphere with 5% CO_2. After 3 days, non-adherent hematopoietic cells were discarded and the adherent cells were washed twice with PBS. The culture medium was replenished every 3 days. When the density of the cell colonies reached ~90% confluence, the cells were detached with 0.25% trypsin (Amresco, Solon, OH, USA) and transferred to fresh flasks at a ratio of 1:2.

Flow cytometry analysis. Fourth generation BMSCs in the control group were identified using flow cytometry (FACSAria™IISORP; BD Biosciences, Franklin Lakes, NJ, USA). Stem cells at passage 4 were harvested and suspended in IMDM-LG medium (Gibco; Thermo Fisher Scientific, Inc.) containing 15% FBS (Gibco; Thermo Fisher Scientific, Inc.) at a concentration of $1x10^6$ cells/ml. Following a brief centrifugation (7 min, 400 x g) at room temperature (RT), cells resuspended in 100 μl of FACS buffer (PBS/2% FCS) and blocked with 1 μl FC-block (cat. no. 553142, BD Biosciences) for 10 min at RT. The following mouse anti-rat monoclonal antibodies were used (BD Biosciences) to characterize the BMSCs in the dark at 4˚C for 30 min: Fluorescein isothiocyabate- or PE-conjugated CD29 (1:10; cat. no. 562154), CD45 (1:20; cat. no. 561867) and CD90 (1:10; cat. no. 561404). Samples were analyzed using a flow cytometer FACS Calibur (BD Biosciences) operated using CellQuest Pro software (version 5.1; BD Biosciences) and at least 20,000 events were collected per sample. Data were analyzed using FlowJo software version 7.6 (Tree Star Inc., Ashland, OR, USA). Forward and side scatter profiles were obtained from the same samples.

Induction and differentiation of BMSCs. Previous studies by our group indicated that 5 ng/ml TGF-β1 or 250 μg/l Sal-B may be a suitable concentration for inducing BMSCs to differentiate into CMs (11,12). Therefore, BMSCs at the second passage were co-incubated with TGF-β1 (5 ng/ml) and Sal-B (250 μg/l), alone or in combination, for 72 h. Subsequently, the cells were washed three times with PBS and the medium was replaced with complete medium without any induction agent. BMSCs cultured without any inductive substance were used as a control group. The medium was changed every 3 days for 4 weeks and the cells were then prepared for the subsequent experiments.

Immunofluorescence staining of specific proteins of CMs. To evaluate cardiomyogenic differentiation of BMSCs in each group, immunofluorescence staining of CM-specific proteins, α-sarcomeric actin and cardiac troponin I (cTnI), was performed. The cells were transferred to sterile glass cover slips coated with 0.01% poly-L-lysine (cat. no. P4707; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). After 4 days, they were fixed with 4% formaldehyde for 15 min. Following blocking with 2% bovine serum albumin (100 μg/ml; cat. no. A2058; Sigma-Aldrich) at room temperature for 1 h, the cells were incubated with monoclonal rabbit anti-α-sarcomeric actin primary antibody (1:50; cat. no. ab-28052; Abcam, Cambridge, UK) and polyclonal goat anti-cTnI primary antibody (1:50; cat. no. ab47003; Abcam) at 4˚C for 24 h. The cells were then stained with rhodamine-conjugated anti-rabbit secondary antibody (1:100; cat. no. BA1001; Wuhan Boster Biological Technology, Ltd., Wuhan, China) and fluorescein isothiocyanate-conjugated anti-goat secondary antibody (1:100; cat. no. sc-2348; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 37˚C for 60 min and washed three times with PBS. Negative controls were also employed to offset the disturbance of the primary or secondary antibody. Cells were observed and images were captured by fluorescence microscopy (TCS-ST2; Leica Microsystems, Wetzlar, Germany).

Transmission electron microscopy (TEM). After 4 weeks of differentiation, the cells were harvested and fixed with 3% glutaraldehyde and 1% osmium tetroxide, followed by embedding in epoxy resin. Ultra-thin sections were cut horizontally and double-stained with uranyl acetate and lead citrate. The cellular ultrastructure was observed using a JEM-2000EX transmission electron microscope (Jeol Ltd, Tokyo, Japan).

Analysis of cardiac differentiation-specific gene expression by RT-qPCR. The transcription factors including GATA binding protein 4 (GATA-4) and homeobox protein Nkx2.5 in each group were assessed by RT-qPCR on day 7. Total RNA was extracted using an RNA fast 200 kit (Fastagen, China) according to the manufacturer's protocol. RNA was then reverse-transcribed into complementary (c)DNA using an M-MLV RTase cDNA Synthesis kit (Invitrogen; Thermo Fisher Scientific, Inc.). Changes in mRNA expression levels were normalized to GAPDH levels. The cycle threshold (Cq) of each gene was analyzed. The fold-change between the treatment groups and the control group for target genes was calculated using the $2^{-\Delta\Delta Cq}$ method (13). Primers used for qPCR are listed in Table I.

Statistical analysis. Values are expressed as the mean ± standard deviation. One-way analysis of variance was used to analyze the differences in mRNA expression of the transcription factors associated with cardiomyogenic differentiation of BMSCs. Other comparisons were performed using the chi-square test. Statistical values were calculated using SPSS software 17.0 (SPSS, Inc., Chicago, IL, USA).

Table I. Primers used for polymerase chain reaction.

Gene	Primer sequence	Product length (bp)
GATA-4	Forward, 5'-GGCTATGTCCACCCCGCTCTG-3' Reverse, 5'-TGGCAGTTGGCACAGGAGAGG-3'	162
Nkx2.5	Forward, 5'-CCCCTGGATTTTGCATTCAC-3' Reverse, 5'-CGTGCGCAAGAACAAACG-3'	75
GAPDH	Forward, 5'-GAAGGTGAAGGTCGGAGTC-3' Reverse, 5'-GAAGATGGTGATGGGATTTC-3'	135

GATA-4, GATA binding protein 4.

Differences were considered statistically significant at $P<0.05$ with a 95% confidence interval.

Results

Morphological alterations of BMSCs. After 12 h of primary culture, BMSCs began to adhere to the culture bottle. After 3 days, BMSCs appeared as circular or short spindle-shaped cells with one nucleus. These cells began to proliferate on day 7 and gradually grew to form small colonies (Fig. 1A). After being subcultured, the cells were either polygonal or long and spindle-shaped.

After the BMSCs at second passage were induced by TGF-β1 and Sal-B, alone or in combination, for 72 h, the morphological differentiation from BMSCs to CLCs was initiated. The differentiated cells were spindle-shaped or branched, with one or two round nuclei located in the center. On day 28, BMSCs in the combined group had a fusiform shape, orientating with one accord and were connected with adjoining cells forming myotube-like structures (Fig. 1B, indicated by black arrows). The morphology and architecture/myotubes of BMSCs in the other treatment groups was similar to that in the combined group, but the amount of cells was relatively low.

Flow cytometric analysis revealed that CD29 and CD90 (fiber-connecting receptors) were present, while CD45 (hematopoietic stem cell marker) was not present on the surface of the fourth generation of BMSCs in the control group, which was indicative of mesenchymal stem cells (Fig. 1C).

CM-specific protein expression during BMSC differentiation. At week 4, most of the cells in the treatment groups expressed α-sarcomeric actin (red) and cTnI protein (green) (Fig. 2A). Quantitative analysis of the fluorescence intensity further revealed that in the combined group, the expression of α-sarcomeric actin and cTnI was significantly higher than that in the control group ($P<0.05$; Fig. 2B).

Ultrastructural characterization of the differentiated cells. TEM observation revealed that the cells in the treatment groups contained abundant organelles with oval nuclei located in the center of the cells (→). These organelles contained a large number of rough endoplasmic reticulum (←), mitochondria, glycogen and ribosomes (Fig. 3A). Myofilament was found to be parallel in the cytoplasm (←; Fig. 3B), suggesting characteristic myofilament installation during differentiation. Ultrastructural observation results in BMSCs of the combined group were more typical than in those of the other treatment groups. By contrast, no myofilaments were detected in the control group.

mRNA expression of transcription factors during cardiac differentiation of BMSCs. The mRNA expression of GATA-4 and Nkx2.5 was determined by RT-qPCR. A previous study showed that the expression of GATA4 and NKx2.5 started to increase at the beginning of cardiac differentiation, reached a plateau by day 7 of differentiation and remained at high levels for up to 8 weeks (14). Therefore, the gene expression levels were determined at day 7 of differentiation in the present study. The expression of GATA4 and NKx2.5 mRNA in the treatment groups was stable and significantly higher than that in the control group. For GATA4 mRNA, the expression in the TGF-β1 group, the Sal-B group and the combined group was 3.14-, 2.95- and 3.72-fold increased compared with that in the control group, respectively. For NKx2.5 mRNA, it was 2.55-, 2.36- and 2.93-fold increased compared with that in the control group, respectively (Fig. 4).

Discussion

The present study tested the hypotheses that i) TGF-β1 and Sal-B, alone or in combination, enhance the differentiation of BMSCs towards the cardiomyogenic phenotype and that ii) TGF-β1 combined with Sal-B may achieve better effects than each factor alone. The results indicated that the combination of TGF-β1 and Sal-B effectively promotes cardiomyogenic differentiation of BMSCs *in vitro* and they may represent a therapeutic strategy for the treatment of ischemic heart disease.

MSCs are non-specialized cells with the ability of self-renewal and pluripotent differentiation potential. They are easy to isolate from bone marrow, adapt to *ex vivo* expansion and differentiate into various cell lineages *in vitro* and *in vivo* without any ethical concerns or immunological rejection (15). *In vivo* and *in vitro* studies showed that the source of stem cells is crucial for successful implantation (16). MSCs harvested from young rodents demonstrated significantly increased cellular proliferation, greater resistance to hypoxic conditions and improved differentiation compared with MSCs obtained from older rodents. Thus, in the present study, 3-week-old SD

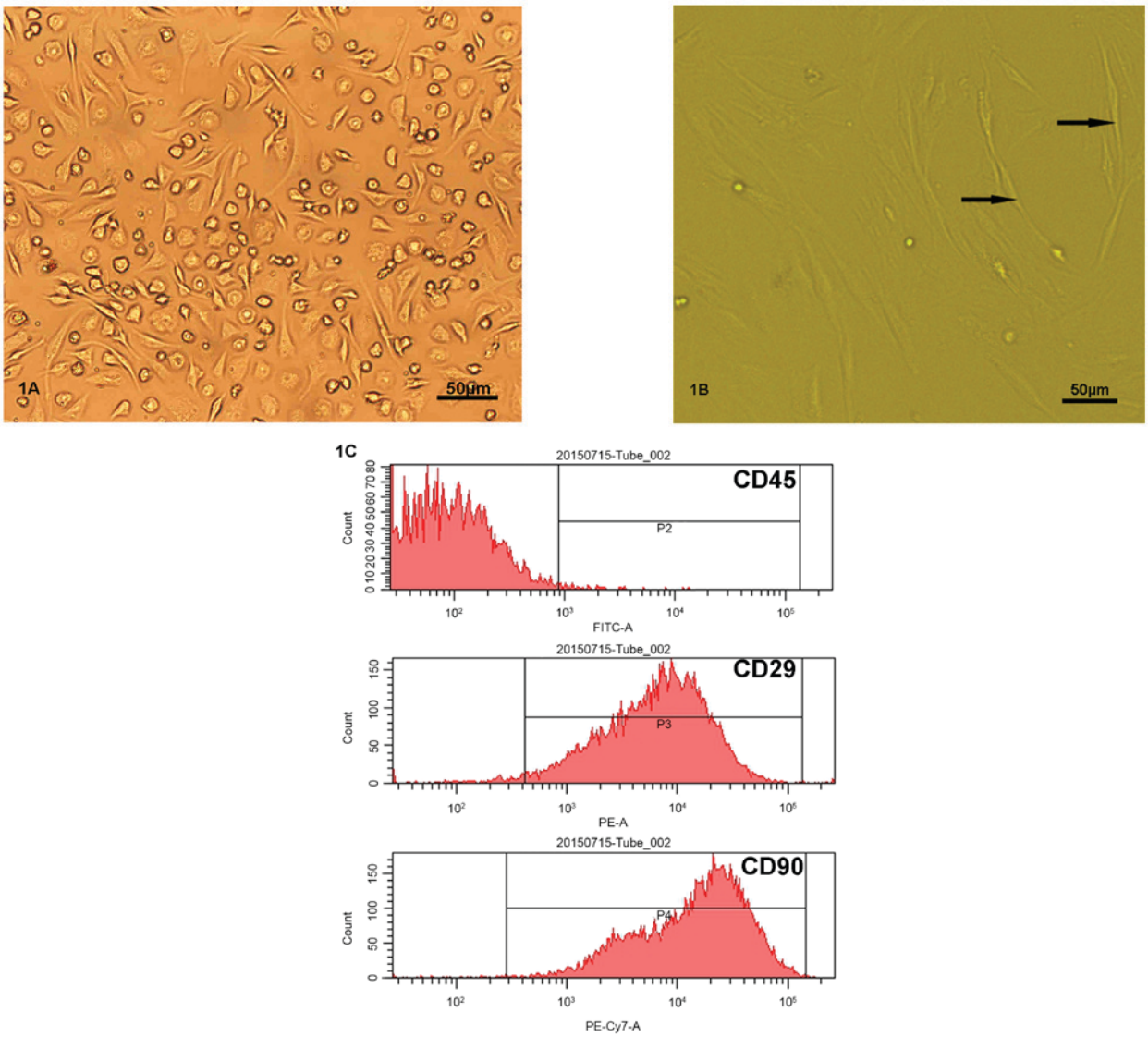

Figure 1. Morphological changes and identification of BMSCs. (A) Cultured BMSCs on day 7. (B) BMSCs treated with TGF-β1 + Sal-B at week 4. (C) Fourth generation BMSCs in the control group were identified using flow cytometry. Cells expressed CD29 and CD90, while being negative for CD45. Cells were observed using a microscope at a magnification of x200. BMSCs, bone marrow mesenchymal stromal cells; TGF, transforming growth factor; Sal, salvianolic acid; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

rats were selected in order to achieve optimal cellular proliferation and differentiation.

It has been demonstrated that MSCs are able to differentiate into CMs *in vivo* and *in vitro* (17,18). 5-azacytidine is a classic inducer for MSCs to differentiate toward CMs; however, it did not induce differentiation of BMSCs in the expected cardiomyogenic manner (19). The percentage of MSCs that differentiate toward CMs upon stimulation with 5-azacytidine is no more than 30% (20,21). Furthermore, the high concentration of 5-azacytidine required for cardiomyogenic differentiation (10 mM) produces toxicity and side effects (22). Therefore, it is required to identify novel inducers to safely increase the differentiation rate of MSCs.

TGF-β1 has an important role in AMI. A previous study indicated an association between TGF-β1 polymorphisms and risk of AMI in Iranian patients, suggesting that genetic polymorphisms in TGF-β1 may be helpful for determining susceptibility to AMI (23). Furthermore, TGF-β1 is one of the most commonly used biological agents for induction of cardiomyogenic differentiation of MSCs (24). The cardioprotective effects of MSCs include reduction of myocyte apoptosis and TGF-β1-conditioned human MSC-laden patches were reported to reduce myocyte apoptosis in the setting of AMI (25). A previous study by our group showed that TGF-β1 may induce MSCs to acquire the cardiogenic phenotype and that 5 ng/ml may be a suitable induction concentration (11), which is in good agreement with the experimental results by Li *et al* (26). Sal-B is a water-soluble phenolic acid extracted from *Salvia miltiorrhiza*. It suppresses the apoptotic effect of treatment with high glucose combined with hypoxia in embryonic stem cell-derived CMs. In particular, Sal-B inhibited the expression of hypoxia-inducible factor 1α and B-cell lymphoma 2/adenovirus E1B 19 kDa protein-interacting protein 3 as well as the levels of cleaved caspase 3, thereby suppressing apoptosis (27). Lin *et al* (28) detected autophagy and apoptosis of myocardial cells in hearts of AMI rats by immunofluorescence and terminal deoxynucleotidyl transferase dUTP nick end labeling. They

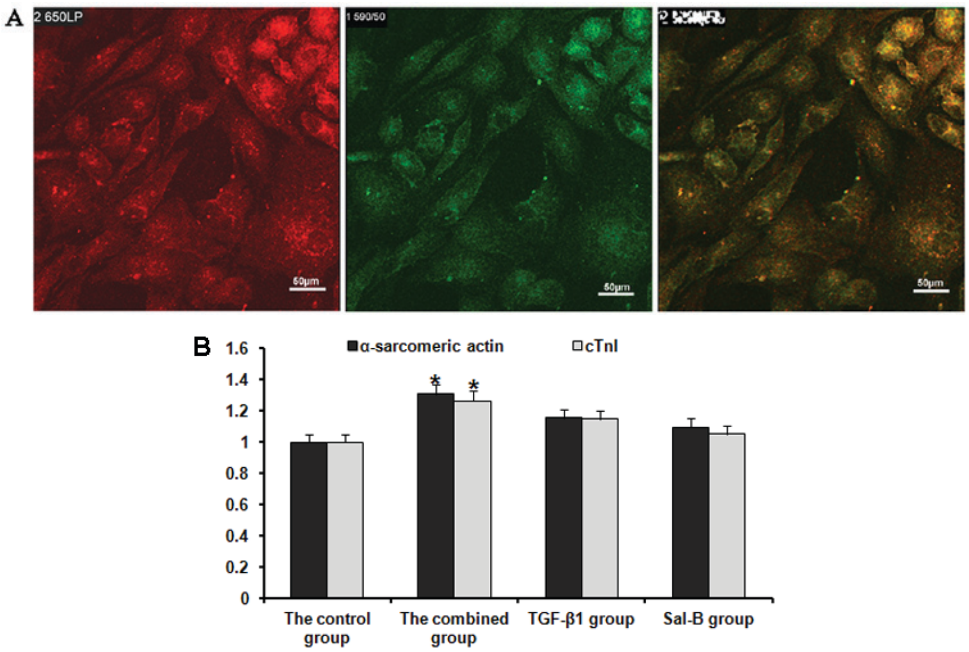

Figure 2. Laser scanning confocal microscopy was used to assess double immunofluorescence labeling of α-sarcomeric actin and cTnI of BMSCs treated with TGF-β1 + Sal-B. Cells were observed using a microscope at a magnification of x200. (A) α-sarcomeric actin was indicated by red fluorescence; Green fluorescence was indicative of cTnI; Merged image with double labeling displayed in yellow; (B) bar graph displaying the quantified double labeling of α-sarcomeric actin and cTnI in BMSCs of each group. *P<0.05 vs. Control. BMSCs, bone marrow mesenchymal stromal cells; TGF, transforming growth factor; Sal, salvianolic acid; cTnI, cardiac troponin I.

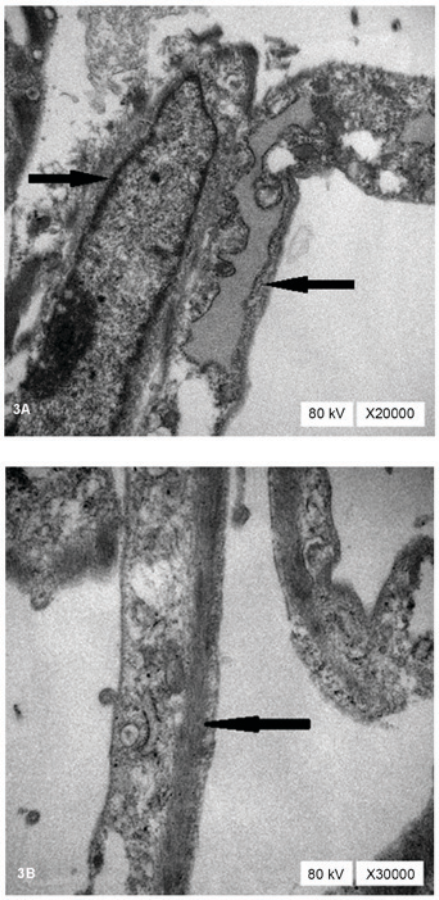

Figure 3. Induced cells were examined by transmission electron microscopy. (A) Abundant organelles such as rough endoplasmic reticulum (←), mitochondria in the cytoplasm with an oval nucleus (→) located in the center of the cell; (B) myofilament (←) was identified to be parallel in the cytoplasm with light and dark transverse striation (magnification, x20,000).

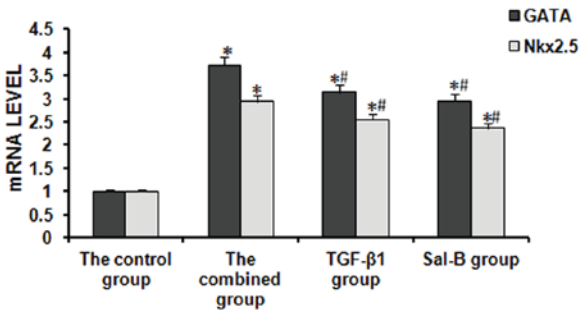

Figure 4. Transcriptional expression of the cardiomyocyte-specific genes GATA-4 and Nkx2.5 determined by reverse-transcription quantitative polymerase chain reaction analysis after 7 days of induction. *P<0.05 vs. Control, #P<0.05 vs. the combined group. GATA-4, GATA binding protein 4.

also examined the protein expression of proteins associated with apoptosis, autophagy and angiogenesis by western blot analysis, revealing that Sal B had a cardioprotective effect on AMI and that Sal B may be a promising candidate for AMI treatment. Since Sal B is a cardioprotective medicine, it is likely to be safer as an inducer than 5-azacytidine. Based on this, the present study established a novel system for cardiomyogenic differentiation of BMSCs using a combination of TGF-β1 and Sal-B.

To provide an unbiased comparison of the differentiation potency of BMSCs towards the cardiomyogenic phenotype using TGF-β1 and Sal-B, alone or in combination, *in vitro* observations were contrasted by electron microscopy, immunofluorescence and RT-qPCR. The myocardial markers α-sarcomeric actin, cTnI, Nkx2.5 and GATA-4 were detected in the experiments. cTnI has been demonstrated to be presented only in cardiac muscle and is a proven diagnostic and risk stratification

biomarker in patients with acute coronary syndromes. Nkx2.5 and GATA-4 are required for specification of the cardiac muscle phenotype (29). Nkx2.5 regulates the transcription of several cardiac genes, including α-sarcomeric actin and GATA-4 (30). The present study found that BMSCs treated with TGF-β1 and Sal-B, alone or in combination, showed an increased expression of cardiac-specific markers, including cTnI, GATA-4 and Nkx2.5, compared with that in the control group. Furthermore, the expression of these cardiac-specific markers in BMSCs of the combined group was significantly higher than that in the other groups. The synergetic effect between TGF-β1 and Sal-B may be achieved by reducing myocyte apoptosis.

In conclusion, the results of the present study indicated that the combination of TGF-β1 and Sal-B effectively promotes cardiomyogenic differentiation of BMSCs *in vitro* and that they may represent a therapeutic strategy for the treatment of ischemic heart disease.

Acknowledgements

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Competing interests

The authors declare that they have no competing interests.

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