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# Transcription factors GATA4 and TBX5 promote cardiomyogenic differentiation of rat bone marrow mesenchymal stromal cells

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**Summary.** Bone marrow mesenchymal stromal cells (BMSCs) have potential applications in cell and gene therapies for cardiac disease. The cardiac-specific transcription factors GATA-binding protein 4 (GATA4) and T-Box protein 5 (TBX5) are considered to be pivotal in cardiogenesis. The aim of this study was to investigate the effects of GATA4 and TBX5 on cardiomyogenic differentiation of BMSCs. The BMSCs were initially isolated and identified. Vectors harboring cardiac transcription factor genes GATA4 and TBX5 or empty vectors were transferred into BMSCs. Cardiomyogenic cells differentiated from BMSCs were identified by expression of cardiac-specific markers including cardiac troponin T, connexin 43, β-myosin heavy chain, and myosin light chain-2 using immunocytochemical staining, western blotting, and quantitative real-time PCR. The ultrastructures of the differentiated cells were examined by transmission electron microscopy, which were similar to those of fetal cardiomyocytes. The differentiated cells exhibited L-type calcium current activities reflective of the electrophysiological characteristics of cardiomyocytes. These findings indicate that exogenous expression of cardiac-specific transcription factors GATA4 and TBX5 enhance cardiomyogenic differentiation of BMSCs.

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## Introduction

Cell- and gene-based therapies are attractive and viable options for tissue regeneration and repair after injuries such as myocardial infarction (Collins and Russell, 2009; Seidel et al., 2009). Candidate cell sources include embryonic stem cells (ESCs) and adult stem cells (ASCs). Bone marrow mesenchymal stromal cells (BMSCs) are ASCs that have been widely investigated in cell/gene-based therapies (Hamada et al., 2005).

BMSCs have potential applications in several therapies because of their unique biological characteristics. BMSCs are capable of differentiating along multiple lineages and, at least *in vitro*, have a significant expansion capability. It is noteworthy that allogeneic BMSCs do not induce host immunoreactivity upon local transplantation or systemic administration. It is also feasible to enhance some of their features through gene modification. Furthermore, BMSCs are an ideal carrier to deliver genes into tissues of interest for gene therapy applications (Baksh et al., 2004).

Cardiogenesis is a complex process involving the commitment of anterior lateral plate mesoderm cells to the cardiogenic lineage. These committed precursors differentiate into cardiomyocytes and subsequently proliferate (Gittenberger-de Groot et al., 2005). There are a number of transcriptional and signaling programs involved in cardiogenesis (Olson, 2006).

During heart development, cardiac transcription factors GATA-binding protein 4 (GATA4) and T-Box protein 5 (TBX5) are master regulators. GATA transcription factors are zinc-finger DNA-binding proteins that regulate diverse pathways associated with embryonic morphogenesis and cellular differentiation (Patient and McGhee, 2002). In cardiogenesis, GATA4 is pivotal for the formation of ventral folding in the embryo and a single ventral heart tube (Molkentin et al., 1997). There is further evidence that overexpression of GATA4 enhances the differentiation of P19 embryonal carcinoma cells into cardiomyocytes, whereas its downregulation suppresses this effect (Hu et al., 2010). Forced expression of Csx/Nkx2.5 and GATA4 increases the frequency of cardiomyogenic differentiation of mesenchymal stromal cells (Yamada et al., 2007). Transplantation of mesenchymal stromal cells overexpressing Csx/Nkx2.5 and GATA4 represents a new treatment strategy with the potential to improve cardiac function after myocardial infarction (MI) (Gao et al., 2011). TBX5 is a member of the T-box family of transcription factors, which are required for normal vertebrate patterning, differentiation, and heart development (Goetz et al., 2006; Hoogaars et al., 2007). It has been suggested that TBX5 plays a role in the function of the cardiac conduction system (Arnolds et al., 2012). TBX5 was recently reported to induce cardiac transdifferentiation of noncardiomyocytes in combination with other transcription factors (Takeuchi and Bruneau, 2009; Ieda et al., 2010; Inagawa et al., 2012; Qian et al., 2012; Song et al., 2012; Zhou et al., 2012). Clinical studies have provided direct evidence for the roles of human GATA4 and TBX5 in heart development. Deletion of any of these two genes results in the failure of normal heart development and cardiomyocyte maintenance, and is associated with some familial and isolated congenital heart disease cases such as GATA4 in atrial septal defects and TBX5 in Holt-Oram syndrome (Mandel et al., 2005; Stennard and Harvey, 2005; Rajagopal et al., 2007).

There have been few reports of the individual effects of GATA4 and TBX5 on cardiac differentiation of BMSCs. In the present study, we investigated the effects of short-term increases in the expression of cardiac-specific transcription factors GATA4 or TBX5 on cardiomyogenic differentiation of BMSCs, providing an effective strategy for differentiation of BMSCs into myocardial cells.

## Materials and methods

### *Isolation and cultivation of BMSCs*

Three-week-old Sprague-Dawley (SD) rats (20-25 g) were purchased from the Experimental Animal Center at Hebei Medical University, China. The experiments were carried out according to the guidelines of the Beijing Laboratory Animal Center and were approved by the Ethics Committee of Hebei Medical University. All efforts were made to minimize animal suffering. BMSCs were harvested from the bone marrow of the femurs and tibias of the SD rats by inserting a 21 G needle into the shaft of the bone and flushing with 30 ml complete medium [Dulbecco's modified Eagle's medium/Ham's F12 medium (DMEM/F12) (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) (PAA, Morningside, Australia), 100 U/ml penicillin, and 100 μg/ml streptomycin]. Cells were passed through a 100-μm nylon filter, and the cells from one rat [defined as passage 0 (P0)] were plated into one 75-$cm^2$ culture flask. The medium was replaced at 48 h and then every 2 or 3 days. The adherent spindle-shaped BMSCs at 80% confluence were serially passaged and expanded. The cells were monitored daily by phase-contrast microscopy. P3 cells were used in experiments.

### *Characterization of BMSC phenotypes*

The cell surface markers of BMSCs were analyzed by flow cytometry. After trypsinization, the detached cells were washed and re-suspended in phosphate buffered saline (PBS). Approximately $1\times10^6$ cells were incubated with the following monoclonal antibodies: phycoerythrin (PE)-conjugated CD29 (BD PharMingen, SanDiego, CA, USA), peridinin-chlorophyll-protein/cyanine5.5 (PerCP/Cy5.5)-conjugated anti-CD90, and fluorescein isothiocyanate (FITC)-conjugated anti-CD45 (both purchased from Biolegend, SanDiego, CA, USA). All incubations were performed at room temperature for 20 min. Control samples were incubated with a PE-conjugated Armenian hamster IgM isotype antibody or PerCP/Cy5.5- and FITC-conjugated mouse IgG1 isotype antibodies. After incubation and washing, the cells were analyzed by an EPICS XL flow cytometer (Beckman Coulter, Brea, CA, USA).

### *Evaluation of BMSC multipotency*

For adipogenic differentiation, BMSCs were induced for 2 weeks in DMEM/F12 supplemented with 10%FBS, 1 μmol/L dexamethasone, 10 μg/mL insulin, 200 μmol/L indomethacin, and 500 μmol/L isobutyl-methylxanthine (all purchased Sigma, St. Louis, MO, USA). The medium was replaced twice a week. At the end of the culture, the cells were fixed in 4% paraformaldehyde for 15 min, stained with a 0.5% Oil Red O solution for 30 min at room temperature to show lipid droplets in the induced cells, and then counterstained with hematoxylin for 1 min.

For osteogenic differentiation, BMSCs were incubated in DMEM/F12 supplemented with 10%FBS, 0.1 μmol/L dexamethasone, 50 μg/mL ascorbate-2-phosphate, and 10mmol/L β-glycerophosphate (all purchased from Sigma). After incubation in differentiation medium for 2 weeks, the cells were stained with 0.1% Alizarin Red-Tris-HCl (pH 8.3) to reveal aggregates of matrix mineralization.

For chondrogenic differentiation, BMSCs were

maintained in DMEM/F12 supplemented with 0.1 μmol/L dexamethasone and 50 ng/ml thyroxine (Sigma) for 2 weeks prior to analysis. Chondrogenesis was identified by Alcian Blue-HCl (pH 1) staining of cell nodules.

### Experimental groups

BMSCs were divided into five experimental groups: (1) GATA4-transfected group (G group), P3 BMSCs transfected with pVP22-GATA4/myc-His; (2) GATA4 empty plasmid group (GE group), P3 BMSCs transfected with pVP22/myc-His; (3) TBX5 transfected group (T group), P3 BMSCs transfected with pcDNA3.1-TBX5; (4) TBX5 empty plasmid group (TE group), P3 BMSCs transfected with pcDNA3.1; (5) blank group, P3 BMSCs.

### Plasmid amplification and purification

Plasmids used for transfection were pVP22-GATA4/myc-His (a gift from Dr. Marc Penn, Cleveland Clinic, USA) (Bian et al., 2007), pcDNA3.1-TBX5 (a gift from Dr. David Brook, Queen's Medical Centre, UK) (Ghosh et al., 2001), pVP22/myc-His (Invitrogen, Carlsbad, CA, USA), and pcDNA3.1 (Invitrogen).

Plasmid DNAs (pDNAs) were obtained by growing *E.coli* cultures overnight in 25mL LB medium containing 50μg/mL kanamycin in 250mL shaker flasks. Plasmid purification was performed using a JETSTAR2.0 Plasmid Midiprep Kit (Invitrogen) according to the manufacturer's protocol. The concentrations of the purified pDNAs were analyzed at 260 nm by a Nanodrop (Thermo Scientific, Wilmington, DE, USA).

### Cell transfection and culture for cardiomyogenic induction

P3 BMSCs were seeded at a density of $4\times10^4/cm^2$ in 6-well plates and cultured for 24 h. Lipofectamine™ 2000-mediated (LF2000) (Invitrogen) transient transfection was performed according to the manufacturer's protocol. Briefly, 4 μg pDNA (pVP22-GATA4/myc-His for the G group, pVP22/myc-His for the GE group, pcDNA3.1-TBX5 for the T group, and pcDNA3.1 for the TE group) and 10 μl LF2000 were diluted in 250 μl DMEM/F12 and incubated at room temperature for 20 min. Then, adherent cells were covered with the mixture and cultured for 4 h. The medium was then replaced with fresh complete medium. After incubation for 48 h, western blot analysis was performed to verify expression of the target proteins. Each group of transfected cells was incubated in complete medium for 4 weeks. Then, western blot analysis, immunocytochemistry, quantitative real-time PCR (qRT-PCR), and transmission electron microscopy (TEM) were performed to detect the cardiomyogenic differentiation of BMSCs induced by transfection of cardiac-specific transcription factors.

### Western blot analysis

Equal amounts of proteins (60 μg) were electrophoresed on sodium dodecyl sulfate-polyacrylamide gels and then transferred to nitrocellulose membranes. The membranes were blocked and incubated with primary antibodies for 12 h. Rabbit anti-GATA4 (ZSGB-BIO, Beijing, China) and rabbit anti-TBX5 (Aviva Systems Biology, San Diego, CA, USA) primary antibodies were used to detect protein expression at 48 h after lipofection, and a mouse anti-cardiac troponin T (cTnT) primary antibody (Abcam, Cambridge, MA, USA) was used to detect cardiomyogenic induction of BMSCs after 4 weeks of culture. The membranes were then incubated with goat anti-rabbit IgG (KPL, Gaithersburg, Maryland, USA) or goat anti-mouse IgG (KPL, Gaithersburg, Maryland, USA) secondary antibodies in blocking buffer for 1 h, followed by exposure to Enhanced Chemiluminescence Luminal reagent (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 min. The blots were imaged with a Gel-Pro analyzer (Media Cybernetics, Bethesda, MD, USA). β-actin was used as a housekeeping gene. All western blot experiments were repeated at least three times.

### Immunocytochemistry

At 4 weeks after lipofection, immunocytochemical staining was performed on the cultured cells. Briefly, cells were fixed with 4% paraformaldehyde for 30 min and then incubated in 3% $H_2O_2$-methanol for 10 min. Then, the cells were permeabilized with 0.1% Triton X-100 in PBS for 30 min, followed by blocking of non-specific binding with 10% goat serum for 30 min. After blocking, the cells were incubated overnight with mouse anti-cTnT (Abcam) or rabbit anti-connexin 43(Cx43) (Invitrogen) primary antibodies. The cells were incubated with biotin-labeled goat anti-mouse or anti-rabbit IgG secondary antibodies for 1 h at room temperature and then peroxidase-conjugated streptavidin for 1 h. Finally, immune reactivities were detected by 3,3'-diaminobenzidine.

Eight fields were chosen randomly in each sample and the average optical density (OD) was analyzed with Image-Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD, USA).

### Total RNA isolation and qRT-PCR

To analyze the mRNA levels of Cx43, β-myosin heavy chain (β-MHC), myosin light chain-2 (MLC-2), total RNAs were extracted from cultured cells using TRIzol and reverse transcribed with a PrimeScript® RT reagent Kit (TaKaRa Biotechnology, Dalian, China) for qRT-PCR using SYBR® Premix Ex Taq™ (TaKaRa Biotechnology) following the manufacturer's instructions. The purity and amount of RNA were

determined by measuring the OD260/280 nm ratio. The primers used are listed in Table 1. The mRNA levels were normalized using β-actin as the housekeeping gene and analyzed by the $2^{-\Delta\Delta Ct}$ method. All reactions were independently repeated twice in duplicate to ensure the reproducibility of the results.

### *TEM analysis*

The cells from all groups and neonatal cardiomyocytes were fixed with 2.5% glutaraldehyde for 30 min. Then, the cells were collected by scraping and centrifuged at 7000 g for 15 min. After washing, the cell pellets were post-fixed with 1% osmium tetroxide for 1 h, dehydrated in acetone, and then embedded in Epon 812. Subsequently, 50 nm ultrathin sections were prepared using an ultramicrotome (Leica, Wetzlar, Hesse, Germany) and double stained with uranium acetate and lead citrate prior to viewing under an H-7500 transmission electron microscope (Hitachi, Tokyo, Japan).

### *Electrophysiological recordings*

Cells were chosen for electrophysiological recordings using whole-cell current and voltage clamps.

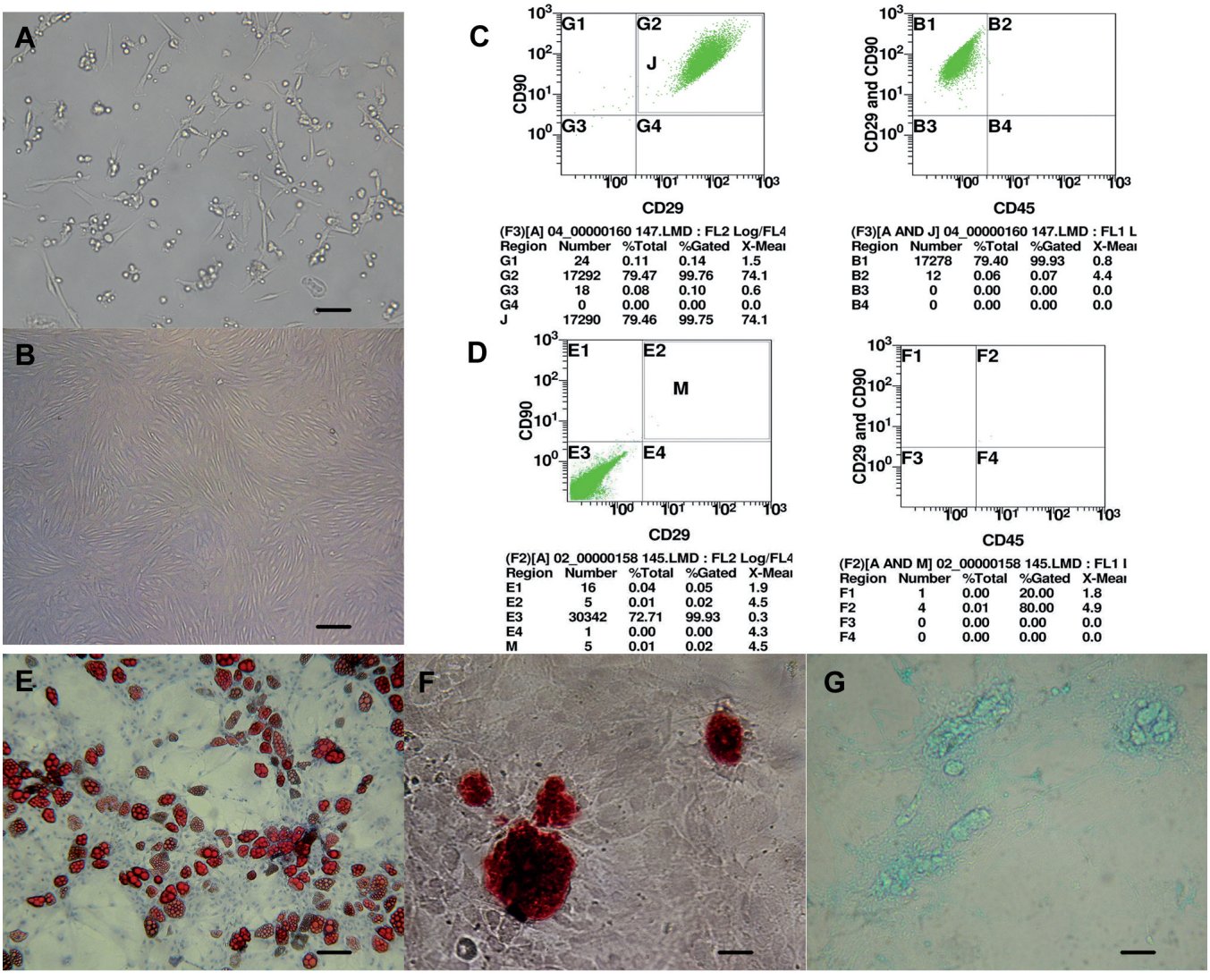



**Fig. 1.** Cultivation and characterization of BMSCs. Primary cultured BMSCs at 48 h after isolation **(A)** and P3 BMSCs **(B)** were observed by phase-contrast microscopy. To analyze phenotypes of the BMSCs, they were incubated with PE-, PerCP/Cy5.5- and FITC-conjugated antibodies and examined by flow cytometry. CD90+/CD29+/CD45- cells were up to 99% of P3 BMSCs **(C)**, but very few CD90+/CD29+/CD45- cells could be found in control samples **(D)**. To assess multipotency, adipogenic cells differentiated from BMSCs were stained with Oil Red O **(E)**, osteogenic cells differentiated from BMSCs were stained with Alizarin Red **(F)**, and chondrogenic cells differentiated from BMSCs were stained with Alcian Blue **(G)**. Scale bars: A, B, 200 μm; E, 100 μm; F, G, 50 μm.

All electrophysiological measurements were performed at 36°C. Data were acquired at a sampling rate of 10 kHz, filtered at 2 kHz, and analyzed off-line using a Pulsefit (HEKA) and Sigma 9.0 software (SPSS Inc. Chicago, IL, USA). To measure the L-type calcium current ($I_{Ca,L}$), the bath solution contained the normal Tyrode solution supplemented with 30 μM tetrodotoxin to eliminate $Na^+$ currents. The pipette solution contained 130 mM CsCl, 2 mM $MgCl_2$, 2 mM Mg-ATP, 2 mM $Na_2$-GTP, 10 mM EGTA, and 5 mM HEPES with the pH adjusted to 7.2 with CsOH.

### *Statistical analysis*

Data are described as the mean ± standard deviation. SPSS 13.0 software (SPSS Inc.) was used for statistical analysis. One-way analysis of variance was applied for multiple comparisons. A P-value of less than 0.05 was considered to be statistically significant.

## Results

### *Cell morphology, phenotypic characterization, and multipotency of the BMSCs*

The isolated cells showed heterogeneity during the first 2 days of culture. When initially plated, the BMSCs appeared round in shape. At 48 h after plating, the cells were adherent, elongated, and spindle shaped (Fig. 1A). When the medium was changed, the suspended cells

**Table 1.** Primers used for qRT-PCR.

| Gene | Primer sequence (5'-3') | Accession No. | size (bp) |
|---|---|---|---|
| Cx43 | F: GGTGGGCACAGACACGAATAT<br>R: CTCAACAACCTGGCTGCGAAA | X06656.1 | 93 |
| β-MHC | F: ATCAAGGGAAAGCAGGAAGC<br>R: CCTTGTCTACAGGTGCATCA | NM_017240.1 | 91 |
| MLC-2 | F: TCACAATCATGGACCAGAACAGA<br>R: TGATCATCTCATCGATCTCTTCGT | X07314.1 | 98 |
| β-actin | F: TCATGAAGTGTGACGTTGACATCCGT<br>R: CCTAGAAGCATTTGCGGTGCACGATG | NM_031144.2 | 102 |

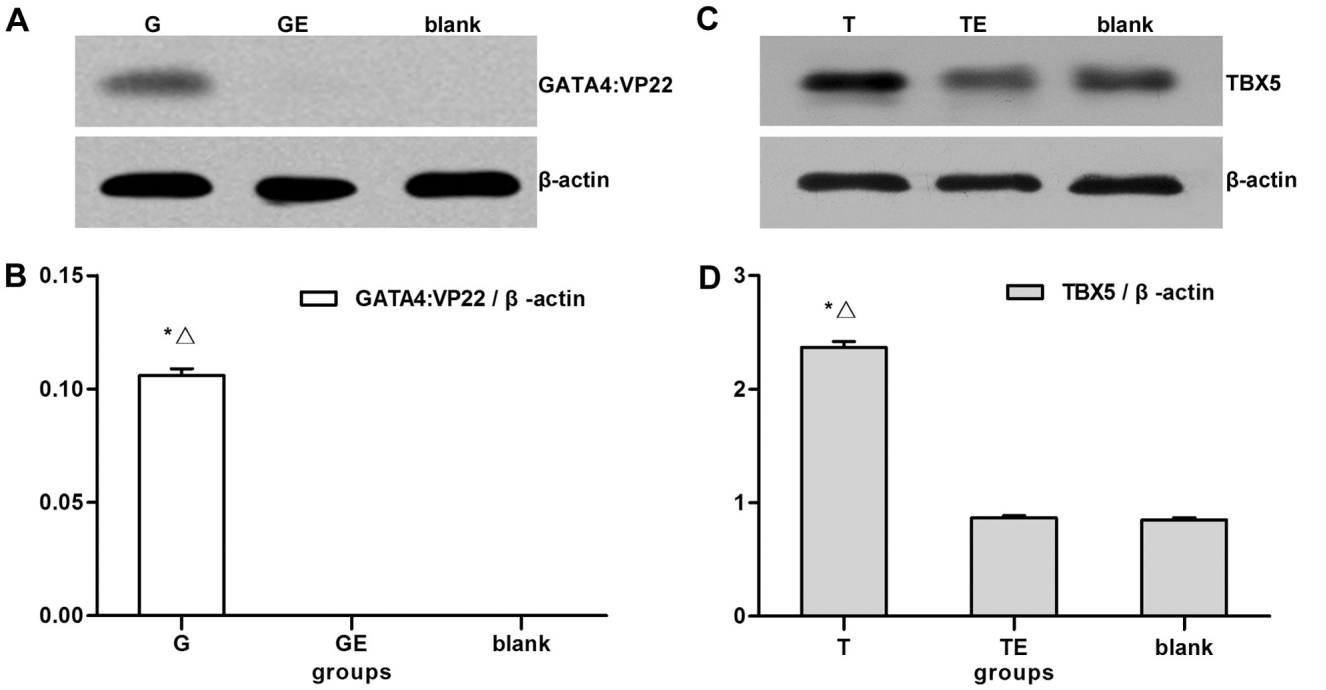
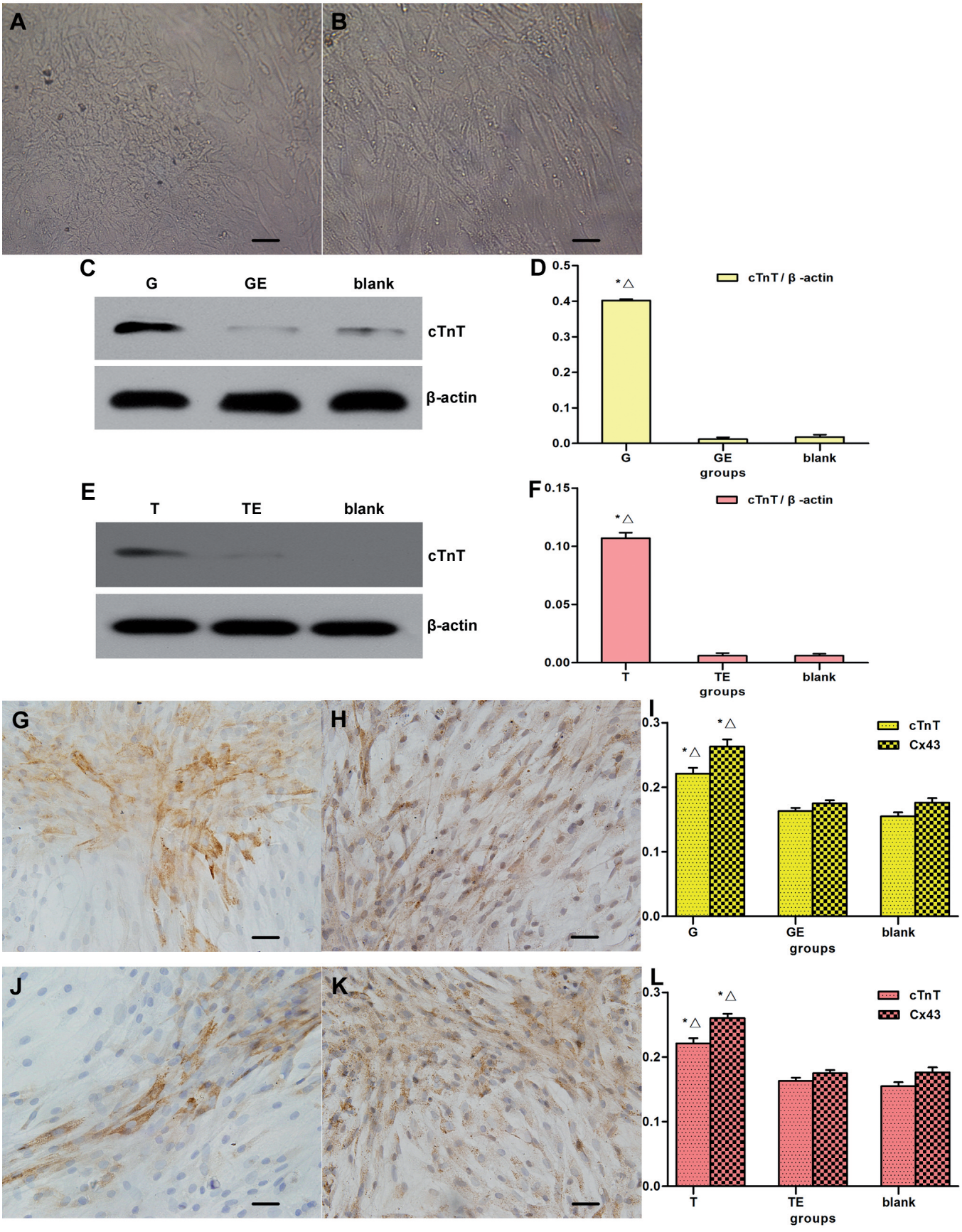



**Fig. 2.** Western blot analysis of target protein expression after transfection. The expression of exogenous GATA4 (GATA4:VP22) in BMSCs transfected with pVP22-GATA4/myc-His was verified by western blotting (**A**: Lane 1, G group; Lane 2, GE group; Lane 3, blank group **B**: *P<0.05 vs blank group; ΔP<0.05 vs GE group). The expression of TBX5 in BMSCs transfected with pcDNA3.1-TBX5 was also verified by western blotting (**C**: Lane 1, T group; Lane 2, TE group; Lane 3, blank group **D**: *P<0.05 vs blank group; ΔP<0.05 vs TE group). The experiments were repeated three times with consistent results.


A
B
C
G
GE
blank
cTnT
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groups
E
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I
cTnT
Cx43
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L

were removed. The primary cultured cells reached about 80% confluence at 9-11 days after subculture at a ratio of 1:2. The subcultured cells were homogenous, spindle shaped, and arranged in swirls or radially (Fig. 1B).

To characterize the phenotypes of BMSCs, we performed flow cytometry. The results showed that CD90+/CD29+/CD45- cells were up to 99% of P3 BMSCs (Fig. 1C), whereas very few CD90+/CD29+/CD45- cells could be found in control samples (Fig. 1D).

The ability of BMSCs to differentiate into adipocytes, osteocytes, and chondrocytes was tested after incubation for 2 weeks. To identify adipogenic differentiation, Oil Red O staining revealed accumulated lipid droplets in some differentiated cells (Fig. 1E). During osteogenic induction for 2 weeks, the cells changed gradually to polygonal or irregularly shaped cells and grew intensively, showing mineralization in their deposited matrix. The induced cells were positive for Alizarin Red staining (Fig. 1F). Cells cultured in chondrogenic medium had differentiated into chondrocytes as suggested by positive Alcian Blue staining of nodules that formed in the induced cells (Fig. 1G), indicating an increase in proteoglycans.

### *Lipofection and verification of target protein expression by western blotting*

At 48 h after lipofection, transgene expression in BMSCs was examined by western blotting. The exogenous GATA4 protein (GATA4:VP22) was only expressed in the G group ($P<0.05$) (Fig. 2A,B). TBX5 protein expression was highest in the T group ($P<0.05$) (Fig. 2C,D). These results showed that the BMSCs could be transfected with pVP22-GATA4/myc-His and pcDNA3.1-TBX5 plasmids with a cationic liposome reagent.

### *Morphological characteristics of cardiomyogenic cells induced from BMSCs*

At 4 weeks after transfection of the cardiac-specific transcription factor genes, some transfected cells of the

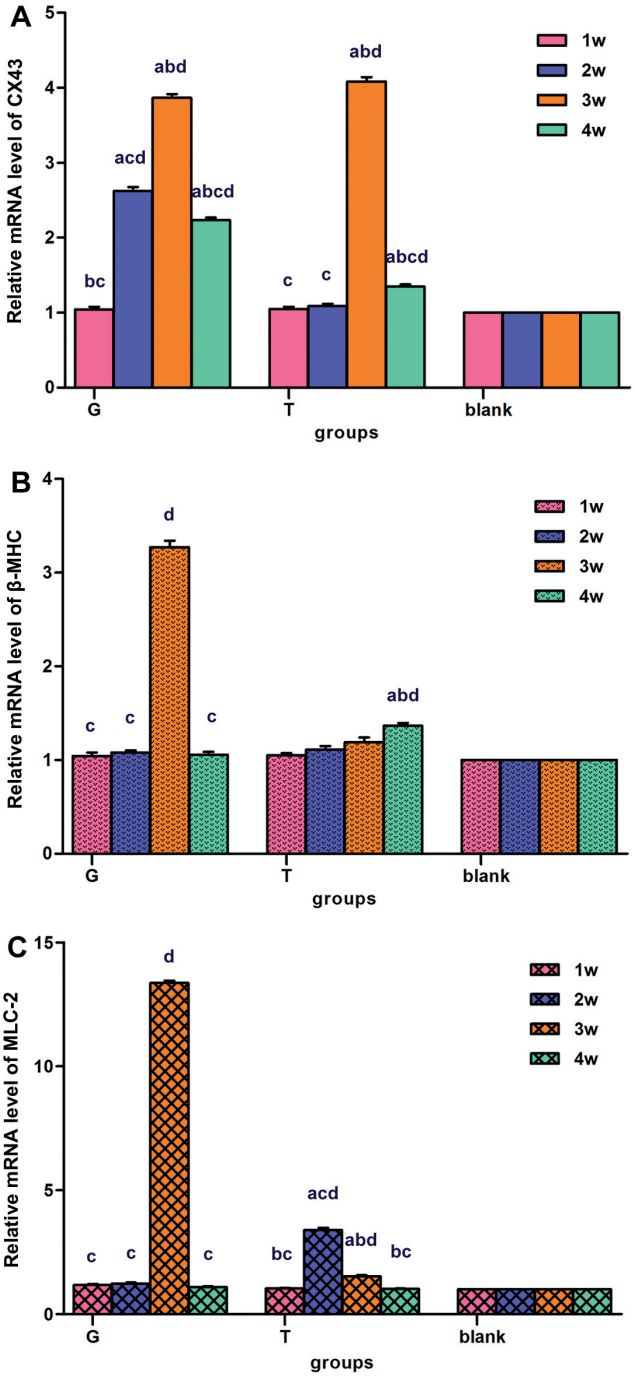


**Fig. 4.** qRT-PCR analyses of the mRNA levels of Cx43, β-MHC and MLC-2. Relative mRNA levels of Cx43 **(A)**, β-MHC **(B,)** and MLC-2 **(C)** ([a]$P<0.05$ vs 1 week; [b]$P<0.05$ vs 2 weeks; [c]$P<0.05$ vs 3 weeks; [d]$P<0.05$ vs blank group)

**Fig. 3** Differentiation of cardiomyocytes from BMSCs at 4 weeks after transfection. Morphological changes of cells in the G group **(A)** and T group **(B)** were observed by phase-contrast microscopy. Expression of the cardiac-specific protein cTnT was verified by western blotting of differentiated cardiomyocytes of the G group (**C**: Lane 1, G group; Lane 2, GE group; Lane 3, blank group **D**: *$P<0.05$ vs blank group; Δ$P<0.05$ vs GE group) and T group (**E**: Lane 1, T group; Lane 2, TE group; Lane 3, blank group **F**: *$P<0.05$ vs blank group; Δ$P<0.05$ vs TE group). The expression of cTnT was verified in differentiated cells of the G group **(G)** and T group **(J)** by immunocytochemical staining. There were brown yellow filament-like or granular structures in the cytoplasm of cTnT-positive cells. The expression of cardiac-specific protein Cx43 was verified in differentiated cells of the G group **(H)** and T group **(K)**. There were brown particles in the cytoplasm of Cx43-positive cells. Few positive cells were observed in the other groups. Statistical results of cTnT and Cx43 in the G group (**I**: *$P<0.05$ vs blank group; Δ$P<0.05$ vs GE group) and T group (**L**: *$P<0.05$ vs blank group; Δ$P<0.05$ vs TE group). Scale bars: A, 100 μm; B, G, H, J, K, 50 μm.

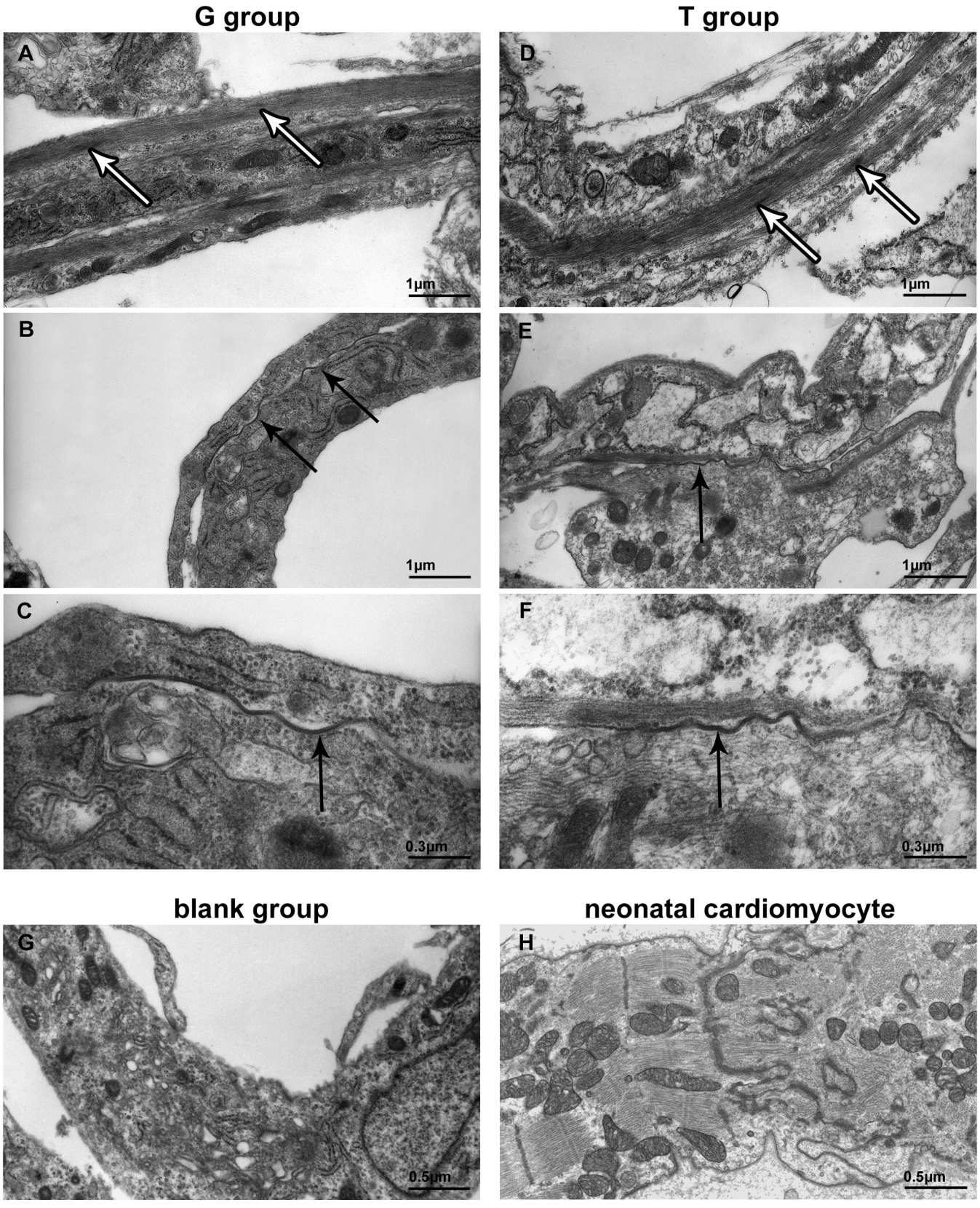


**Fig. 5.** TEM observations of the ultrastructures of differentiated cardiomyocytes from BMSCs in G and T groups. Hollow arrows indicate parallel myofilament-like structures with a dense zone in cytoplasm of cells in the G group **(A)** and T group **(D)**. Solid arrows indicate gap junctions between cells of the G group (**B, C)** and T group **(E, F)**. Blank group cells were spindle-shaped and gap junctions were barely visible between cells **(G)**. Neonatal cardiomyocytes had mature intercalated discs and branching columns of myofibrils. Numerous mitochondria occupied the intermyofibrillar spaces **(H)**. Scale bars: A, B, D, E, 1 μm; C, F, 0.3 μm; G, H, 0.5 μm.

G group (Fig. 3A) and T group (Fig. 3B) became elongated and widened. Almost no cells in the other groups showed similar changes.

### *Western blot analysis of cardiomyogenic cells induced from BMSCs*

At 4 weeks after exogenous gene transfection, western blot results showed that cTnT expression in cardiac-specific transcription factor-transfected groups including the G group (Fig. 3C,D) and T group (Fig. 3E,F) was significantly higher ($P<0.05$) than that in empty plasmid groups and the blank group.

### *Immunocytochemistry of cardiomyogenic cells induced from BMSCs*

At 4 weeks after gene transfection, immunocytochemistry showed that cTnT and Cx43 were expressed in cells of the G group (Fig. 3G-I) and T group (Fig. 3J-L). There were brown yellow filament-like or granular structures in the cytoplasm of cTnT-positive cells, and brown particles in the cytoplasm of Cx43-positive cells. Few positive cells were observed in the other groups.

### *qRT-PCR of cardiomyogenic cells induced from BMSCs*

qRT-PCR results showed that the trends of Cx43 expression in G and T groups were similar. Cx43 expression levels increased gradually, reaching a peak at week 3, followed by a decrease in expression (Fig. 4A). β-MHC expression levels were highest at weeks 3 and 4 in G and T groups, respectively ($P<0.05$) (Fig. 4B). MLC-2 expression in the G group was highest at week 3 ($P<0.05$). The MLC-2 expression level in the T group was increased significantly at week 2 ($P<0.05$) and decreased gradually at weeks 3 and 4 (Fig. 4C).

### *TEM analysis of cardiomyogenic cells induced from BMSCs*

Ultrastructural analysis by TEM showed that the cells of G and T groups were spindle-shaped and enriched with many organelles such as mitochondria, ribosomes, rough endoplasmic reticulum, and lysosomes in the cytoplasm. Oval nuclei were located in the cell center. In addition, a major change was that many paralleled myofilament-like structures with dense zones were seen around the nucleus, and more myofilament-like structures appeared in the peripheral cytoplasm (Fig.

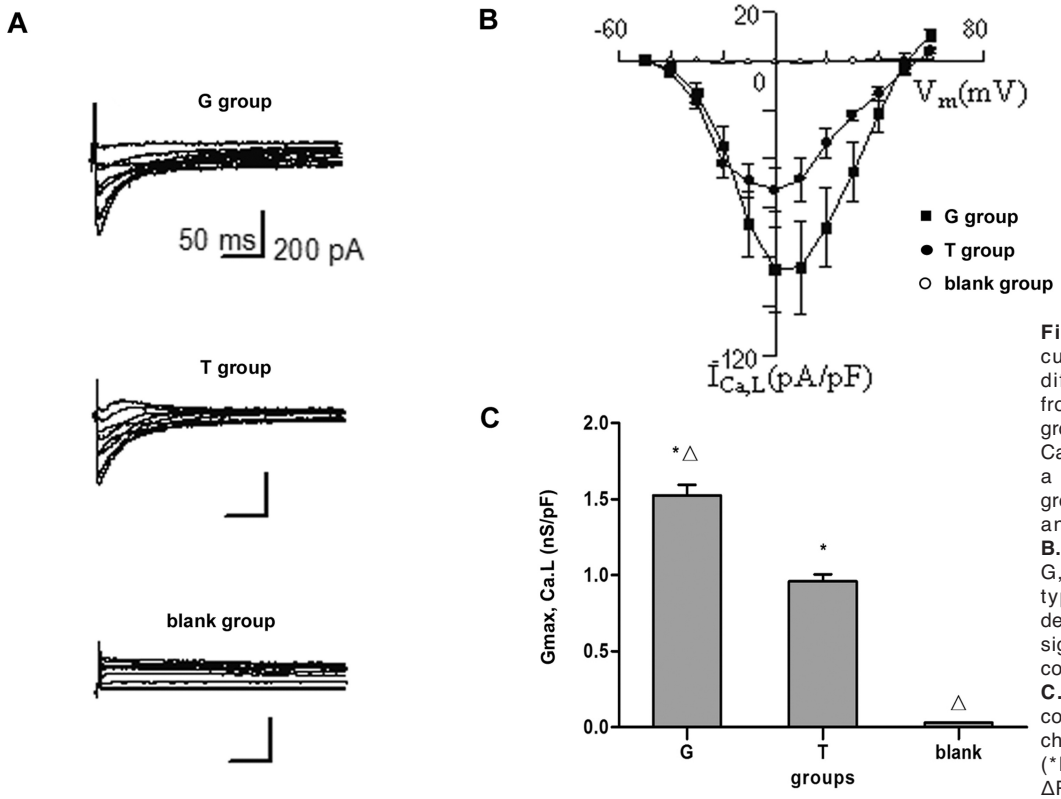


**Fig. 6.** L-type $Ca^{2+}$ channel current recordings in differentiated cardiomyocytes from BMSCs of G, T and blank groups. **A.** Representative L-type $Ca^{2+}$ channel current traces from a single isolated cell of the G group (upper), T group (middle), and blank group (lower). **B.** Current-voltage relationship in G, T and blank groups. The L-type $Ca^{2+}$ channel current densities at 0 mV were increased significantly in G and T groups compared with the blank group. **C.** Comparison of maximum conductance of the L-type $Ca^{2+}$ channel current in three groups (*$P<0.05$ vs blank group; Δ$P<0.05$ vs T group).

5A,D). More gap junctions were observed around the cells (Fig. 5B,C,E,F). Cells of the blank group were spindle shaped, and gap junctions were barely visible between cells (Fig. 5G). The neonatal cardiomyocytes had mature intercalated discs and branching columns of myofibrils. Numerous mitochondria occupied the intermyofibrillar spaces (Fig. 5H).

### *Electrophysiological recordings*

At 4 weeks after gene transfection, electrophysiological recordings showed that the cells of G and T groups displayed electrophysiological properties of an inward current that is representative of $I_{Ca,L}$. This current was not found in cells of the blank group (Fig. 6A). Compared with the blank group, the $I_{Ca,L}$ density at 0mV and maximum conductance were increased significantly in G and T groups (Fig. 6B,C).

## Discussion

Cell-based therapy is a promising therapeutic strategy based on the concept of cell-mediated restoration of damaged or diseased tissues. BMSCs are one of the main sources for cell-based therapies for cardiac repair and regeneration, because BMSCs are easily obtainable without ethical concerns and have little, if any, inherent immunogenicity (Battiwalla and Hematti, 2009; Wang et al., 2009). In the present study, we explored cardiac differentiation of BMSCs overexpressing GATA4 or TBX5, which may be helpful for therapeutic applications in cardiac repair and regeneration.

There are two classical methods to differentiate cardiomyocytes from BMSCs *in vitro*: induction by chemicals such as 5-azacytidine and co-culture with cardiomyocytes (Ye et al., 2006; Muscari et al., 2008; Psaltis et al., 2008; Armiñán et al., 2009; Tokcaer-Keskin et al., 2009). However, the efficacy of these techniques for cardiac differentiation of BMSCs is limited and highly variable (Psaltis et al., 2008). Therefore, it is critical to establish an effective method to enhance the differentiation of BMSCs into cardiomyocytes.

BMSCs are easy to modify genetically. This property allows specific differentiation pathways (Guillot et al., 2007). Previous studies have revealed several cardiac transcription factors called "cardiomyogenic master genes", such as GATA4 and TBX5, which regulate gene and protein expression during cardiac development. In the present study, we introduced GATA4 or TBX5 into BMSCs for cardiac differentiation. In particular, the effect of TBX5 on BMSCs had not been reported previously.

For transgene transfection, we used a lipofection protocol, because of its safety, negligible toxicity, non-immunogenicity, simple preparation, and ability to carry large genes (Park et al., 2003; Schaffert and Wagner, 2008; Madeira et al., 2010).

Differentiated cardiac cells express cardiac-specific markers. cTnT is the tropomyosin-binding subunit of the troponin complex that is located on the thin filament of cardiac muscles and regulates muscle contraction in response to alterations in the concentration of intracellular calcium ions. Mutations in cTnT have been associated with familial hypertrophic cardiomyopathy and dilated cardiomyopathy. In the present study, western blotting showed cTnT expression in BMSCs overexpressing GATA4 or TBX5, and immunocytochemistry revealed that the cTnT-positive cells contained brown yellow filament-like or granular structures in the cytoplasm, which is consistent with the characteristics of the myofilament-specific cTnT protein. Studies have shown that the gap junction protein Cx43, the principal connexin isoform, is enriched in cardiomyocytes and provides electrical coupling between cells (Vaidya et al., 2001). Cx43 is evenly distributed in cardiomyocytes at the early fetal stage and then gradually focuses to intercalated discs during heart development (Xie et al., 2005). In our study, immunocytochemistry showed brown particles in the cytoplasm of Cx43-positive cells in transcription factor-transfected groups, which is consistent with the features of early fetal cardiomyocytes. Cardiac β-MHC and MLC-2 are known as cardiac structural genes and are involved in the early stages of cardiogenesis during embryonic development (Sánchez et al., 1991; Planat-Bénard et al., 2004). Both genes are commonly used to identify cardiac differentiation of stem cells (Armiñán et al., 2009; Serena et al., 2012; Traister et al., 2012). In the present study, the mRNA levels of β-MHC and MLC-2 were increased by GATA4 or TBX5 overexpression, and then were down-regulated. It is reported that β-MHC is dominant essentially during fetal life in rodents. Then it decreases quickly around the time of birth and is replaced by α-MHC in adults (Lompre et al., 1981). In our study, the β-MHC expressional tendencies were consistent with that during fetal period or at birth. As far as MLC-2 is concerned, the expressions in different chambers are different. In the fetal stage, atrial myosin light chain-2 (MLC-2a) is primarily found in atria, while ventricular myosin light chain-2 (MLC-2v) is essentially restricted to ventricles (Franco et al., 1999). But in the early embryonic stage, MLC-2a is expressed in the presumptive ventricle, and its ventricular expression is subsequently down-regulated (Kubalak et al., 1994). In our study, the MLC-2 expressional tendencies were similar to that of MLC-2a in ventricle. However, because BMSCs do not originate from precardiac mesoderm, their cardiac-specific marker expression might be different from that of precardiac mesoderm cells. Further studies are needed to clarify the detailed mechanisms.

During fetal and postnatal stages, cardiomyocytes undergo a remarkable morphological change. In the early fetal stage, cardiomyocytes contain scattered mitochondria and sparse peripheral myofibrils with poorly developed intercalated discs and sarcoplasmic

reticulum, and absent transverse tubules. During late fetal and early postnatal stages, myofibrils extend into the myocyte interior and attain a mature appearance. Intercalated discs become increasingly complex and the sarcoplasmic reticulum and transverse tubules develop well (Smolich, 1995). In our study, cells of the G and T groups had parallel myofilament-like structures with a dense zone in the peripheral cytoplasm, and many extended into the myocyte interior around the nucleus. More gap junctions appeared in transcription factor-transfected cells. Gap junctions are a major constituent of intercalated discs. Compared with mature intercalated discs and the branching columns of myofibrils in neonatal cardiomyocytes, the ultrastructures of cells in the G and T groups were similar to those of early or slightly later fetal cardiomyocytes *in vivo*.

L-type calcium channels are the predominant route for calcium entry into cardiomyocytes and are key components in excitation-contraction coupling. Therefore, $I_{Ca,L}$ is highly expressed in cardiomyocytes (Scriven and Moore, 2013). $I_{Ca,L}$ is also expressed in human ESC-derived cardiomyocytes (Mummery et al., 2003; Liu et al., 2009). In our study, differentiated cells of the G and T groups exhibited $I_{Ca,L}$ activities reflective of the electrophysiological characteristics of cardiomyocytes.

In summary, the present study demonstrated that overexpression of cardiac-specific transcription factors GATA4 or TBX5 is capable of initiating the cardiogenic differentiation program and enhances the differentiation of BMSCs into early fetal cardiomyocytes. This study may provide a safe effective strategy to differentiate BMSCs into myocardial cells for applications in cell/gene-based therapies for non-inherited heart diseases.

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