Original Research

Cardiomyocyte differentiation induced in cardiac progenitor cells by cardiac fibroblast-conditioned medium

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Abstract

Our previous study showed that after being treated with 5-azacytidine, Nkx2.5$^+$ human cardiac progenitor cells (CPCs) derived from embryonic heart tubes could differentiate into cardiomyocytes. Although 5-azacytidine is a classical agent that induces myogenic differentiation in various types of cells, the drug is toxic and unspecific for myogenic differentiation. To investigate the possibility of inducing CPCs to differentiate into cardiomyocytes by a specific and non-toxic method, CPCs of passage 15 and mesenchymal stem cells (MSCs) were treated with cardiac ventricular fibroblast-conditioned medium (CVF-conditioned medium). Following this treatment, the Nkx2.5$^+$ CPCs underwent cardiomyogenic differentiation. Phase-contrast microscopy showed that the morphology of the treated CPCs gradually changed. Ultrastructural observation confirmed that the cells contained typical sarcomeres. The expression of cardiomyocyte-associated genes, such as alpha-cardiac actin, cardiac troponin T, and beta-myosin heavy chain (MHC), was increased in the CPCs that had undergone cardiomyogenic differentiation compared with untreated cells. In contrast, the MSCs did not exhibit changes in morphology or molecular expression after being treated with CVF-conditioned medium. The results indicated that Nkx2.5$^+$ CPCs treated with CVF-conditioned medium were capable of differentiating into a cardiac phenotype, whereas treated MSCs did not appear to undergo cardiomyogenic differentiation. Subsequently, following the addition of Dkk1 and the blocking of Wnt signaling pathway, CVF-conditioned medium-induced morphological changes and expression of cardiomyocyte-associated genes of Nkx2.5$^+$ CPCs were inhibited, which indicates that CVF-conditioned medium-induced cardiomyogenic differentiation of Nkx2.5$^+$ CPCs is associated with Wnt signaling pathway. In addition, we also found that the activation of Wnt signaling pathway was accompanied by higher expression of GATA-4 and the blocking of the pathway inhibited the expression of GATA-4 in CVF-conditioned medium-incubated Nkx2.5$^+$ CPCs. This finding suggests that Wnt signaling pathway may alter GATA-4 expression and activate the cardiogenic program in the regulation of differentiation. In conclusion, Nkx2.5$^+$ CPCs have enormous potential for cardiomyogenic differentiation and the CVF-conditioned medium specifically induces CPCs to differentiate into a cardiac phenotype. Wnt signaling pathway is involved in CVF-conditioned medium-induced cardiomyogenic differentiation of Nkx2.5$^+$ CPCs.

Keywords: Cardiac progenitor cells, cardiomyocytes, conditioned medium, differentiation, cardiac fibroblasts

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Introduction

Stem cell transplantation is an alternative therapy for treating patients with end-stage heart failure. Ideally, the cells used in the therapy should be pluripotent and possess the ability to differentiate into cardiomyocytes upon appropriate stimulation. It has been confirmed that a variety of cell types, such as embryonic stem cells (ESCs),[1] induced pluripotent stem cells,[2] mesenchymal stem cells (MSCs),[3] and

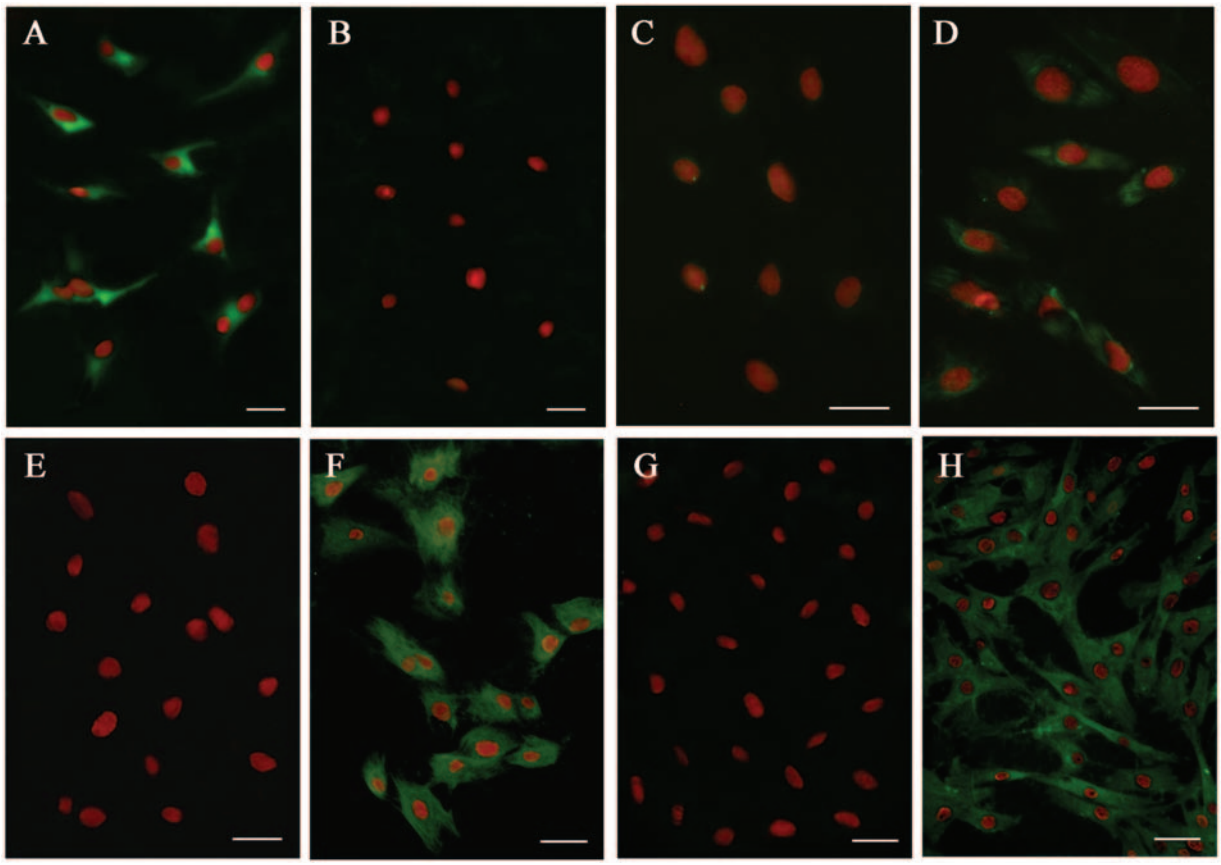

Figure 1 Expression of vimentin, alpha-cardiac actin, factor VIII, and desmin in cardiac fibroblasts. (a) Representative positive staining of vimentin in cardiac fibroblasts; (b) blank control group; (c) representative negative staining of alpha-cardiac actin in cardiac fibroblasts; (d) alpha-cardiac actin staining of the positive control group, rat neonatal cardiomyocytes; (e) representative negative staining of factor VIII in cardiac fibroblasts; (f) factor VIII staining of the positive control group, rat endothelial cells; (g) representative negative staining of desmin in cardiac fibroblasts; (h) desmin staining of the positive control group, rat smooth muscle cells. Nuclei were counterstained with propidium iodide (PI). Scale bar: 30 μm. (A color version of this figure is available in the online journal.)

Table 1 Analysis of rat MSCs surface antigen by flow cytometry

	Passage4 (%)	Passage 7 (%)	Passage 10 (%)
CD29	98.1 ± 1.3	99.0 ± 0.8	98.6 ± 1.1
CD54	97.7 ± 2.0	96.6 ± 2.3	97.5 ± 1.9
CD90	98.3 ± 1.6	99.4 ± 0.3	99.2 ± 0.7
CD31	1.5 ± 0.2	0.9 ± 0.1	0.4 ± 0.1
CD34	0.8 ± 0.1	1.1 ± 0.2	0.5 ± 0.1
CD45	1.7 ± 0.3	1.9 ± 0.4	0.7 ± 0.2

Values are mean ± SE from triplicate determinations repeated in four separate experiments.

adipose-derived stem cells,[4,5] can differentiate into cardiomyocytes *in vitro*. However, many of the methods that induce cardiomyogenic differentiation in various types of cells are unspecific, such as treatment with 5-azacytidine, dimethylsulfoxide, retinoic acid, and other compounds. These methods direct stem cells toward the expected cardiomyogenic fate, which is due to random reprogramming of gene expression by drugs such as 5-azacytidine and dimethylsulfoxide rather than to the cell's potential for cardiomyogenic differentiation.[6–9]

We previously reported that Nkx2.5$^+$ human cardiac progenitor cells (CPCs) derived from embryonic heart tubes could differentiate into cardiomyocytes after being treated with 5-azacytidine.[10] Although 5-azacytidine is a classical agent to induce myogenic differentiation in various types of cells, the drug is toxic and unspecific for myogenic differentiation. Accordingly, the potential of CPCs for cardiomyogenic differentiation remains to be determined. In this study, both CPCs and MSCs were treated with cardiac ventricular fibroblast-conditioned medium (CVF-conditioned medium) to explore the possibility of the two cell types differentiating into cardiomyocytes under specific and non-toxic conditions. The purpose was to investigate the potential of CPCs for cardiomyogenic differentiation by comparing it to that of MSCs.

Methods

Isolation and cultivation of Nkx2.5$^+$ CPCs and MSCs

All of the experimental procedures were conducted in accordance with the institutional guidelines for the care and use of laboratory animals of the Second Military Medical University (Shanghai, China) and conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The methods used for culturing Nkx2.5$^+$ CPCs were previously described.[11] Briefly, 11-day-old postfertilized embryos from timed

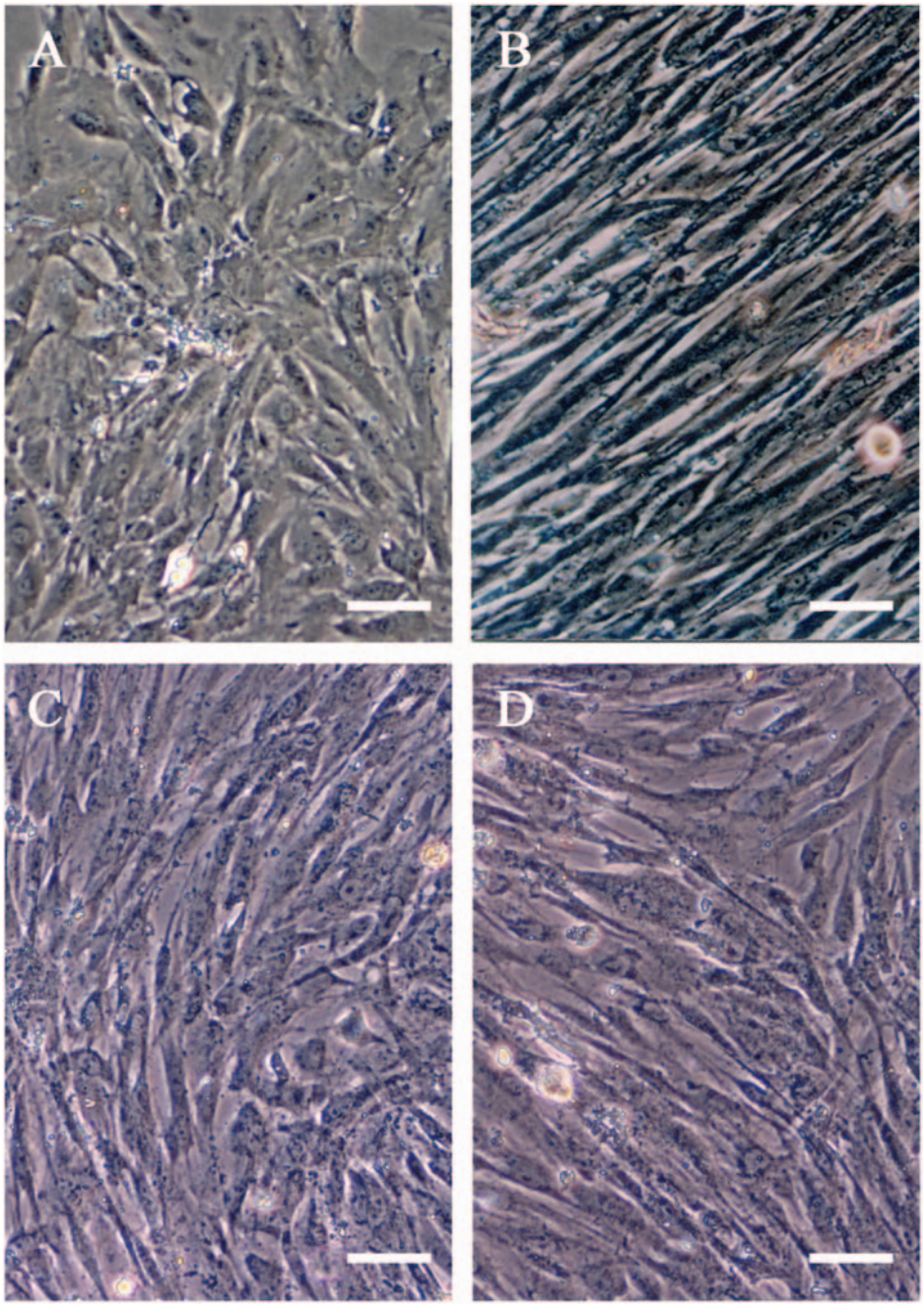

Figure 2 Shape changes of the Nkx2.5$^+$ CPCs and MSCs treated with CVF-conditioned medium after 12 days, as observed by phase-contrast microscopy. (a) Untreated Nkx2.5$^+$ CPCs and (b) Nkx2.5$^+$ CPCs treated with CVF-conditioned medium; (c) untreated MSCs and (d) MSCs treated with CVF-conditioned medium. Scale bar: 30 μm. (A color version of this figure is available in the online journal.)

mating of SD rats were dissected to obtain their heart tubes, which were then incubated and disaggregated in 0.25% trypsin (Gibco). The cells were pelleted by centrifugation, resuspended in medium at 10^5/mL, and seeded in 12-well cluster dishes (Corning). The medium used was dulbecco's modified eagle medium (DMEM) supplemented with 15% fetal calf serum (Gibco), 10 μg/L dulbecco's modified eagle medium (bFGF) (R&D Systems), 20 μg/L epidermal growth factor (EGF) (R&D Systems), 10^6 U/L leukemia inhibitory factor (LIF) (Chemicon International, Inc., Temecula, CA), 0.375% $NaHCO_3$, 100 U/mL penicillin G, 100 μg/mL streptomycin, and 2 mM L-glutamine (Amresco). After 5 or 7 days of primary culture, the cells derived from the rat heart tubes were dispersed using 0.25% trypsin. As before,[11] the Nkx2.5$^+$ CPCs were identified among these cells and used to establish clonal cultures.

The bone marrow of Sprague Dawley (SD) rats was isolated from femoral bones under aseptic conditions and dispersed into a single cell suspension. The cells were cultured at a density of 1×10^6/mL at 37°C, 5% CO_2 for 48 h, after which the supernatant containing the suspended cells was then discarded and the adherent cells were cultured. The medium used was L-DMEM supplemented with 20% fetal calf serum (Gibco), 100 U/mL penicillin G, 100 μg/mL

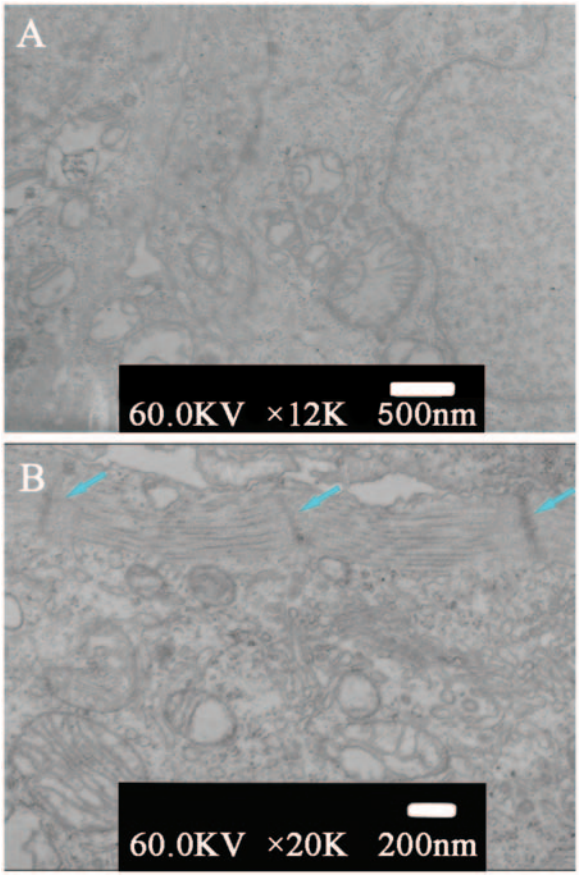

Figure 3 Nkx2.5$^+$ CPCs contained clearly evident typical sarcomeres after 12-day treatment with CVF-conditioned medium. (a) Untreated Nkx2.5$^+$ CPCs and (b) Nkx2.5$^+$ CPCs treated with CVF-conditioned medium. Arrows indicate sarcomeres in the cells. (A color version of this figure is available in the online journal.)

streptomycin, and 85 μg/ml amphotericin B. The cells were passaged for 4–10 generations when they reached 70–90% confluence. The MSCs were identified by flow cytometric analysis of surface antigens, including CD29, CD54, CD90, CD31, CD34, and CD45.

Preparation of CVF-conditioned medium and induction of Nkx2.5$^+$ CPCs and MSCs

Rat ventricular tissue was trimmed of excess fat, membranes, and vessels, washed several times with phosphate buffer solution (PBS) and minced into small pieces, which were then subjected to cell dissociation using collagenase (Gibco). The cells in the suspensions obtained in this way were pelleted by centrifugation at 800 × g for 5 min. The cells were then resuspended in DMEM supplemented with 15% fetal calf serum (Gibco). Subsequently, the CVFs were separated using selective adhesion procedures and maintained in a humidified atmosphere of 5% CO_2 at 37°C. Cells between passage 3 and 4 were used to prepare the CVF-conditioned medium.

After the CVFs were resuspended and incubated for 5 days, the medium, which was DMEM supplemented with 15% fetal calf serum, was renewed every other day for 1 week. The medium was collected and stored at -20°C before use. CVF-conditioned medium contained 80% medium derived from the CVF cultures, 15% DMEM, and 5% fetal calf serum. The Nkx2.5$^+$ CPCs and MSCs were incubated in the CVF-conditioned medium for 12 days to allow them to differentiate into cardiomyocytes. The conditioned medium was renewed every 3 days. Dkk1, the antagonist of Wnt signaling pathway, was added to the treated cells of the CVF-conditioned medium at a concentration of 20 ng/mL.

Immunofluorescence staining

Vimentin, factor VIII, desmin, and alpha-cardiac actin were detected in the CVFs using immunofluorescence staining methods. The positive control cells included rat endothelial cells, rat smooth muscle cells, and rat neonatal cardiomyocytes. Alpha-cardiac actin labeling of Nkx2.5$^+$ CPCs and MSCs treated with CVF-conditioned medium or CVF-conditioned medium/Dkk1 was compared with untreated cells stained using the same method. These cells were plated at 50% confluence in 24-well plates, rinsed twice with PBS, and fixed in 4% paraformaldehyde for 20 min at room temperature. After washing three times with PBS, the cells were incubated at 4°C overnight with the primary antibodies, including with rabbit antivimentin (1/150, Santa Cruz), antidesmin (1/200, Chemicon), and antibodies directed against factor VIII and alpha-cardiac actin (1/100, Wuhan Boster Biological Technology, Wuhan, China). After three washes with PBS for 5 min each, the cells were incubated with of fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG secondary antibodies (Sigma-Aldrich) diluted 1:150 in PBS/1% bovine serum albumin (BSA) for 1 h at room temperature. Blank control staining was performed by substituting PBS/1.0% BSA for the primary antibody. The percentage of positive cells was determined by counting the positively stained cells and the total cells in one visual field under a microscope. At least three fields (approximately 300 cells) were analyzed for each sample. The average data, presented as the mean values ± SD, were compared using Dunnett's test.

Surface antigens of rat MSCs

Rat MSCs of passages 3, 7, and 10 were treated with 0.25% trypsin–ethylene diamine tetraacetic acid (EDTA), harvested, and washed twice with PBS. The cells were incubated on ice with FITC-conjugated rabbit anti-rat antibodies directed against CD29, CD31, CD34, CD45, CD54, and CD90. The control groups were incubated with FITC-conjugated antibodies directed against rabbit IgG. The labeled cells were analyzed by flow cytometry.

Transmission electron microscopy (TEM)

After being treated with CVF-conditioned medium or CVF-conditioned medium/Dkk1, the Nkx2.5$^+$ CPCs and MSCs were fixed in 4.0% glutaraldehyde/0.1 M sodium cacodylate, postfixed in 1% osmium tetroxide/0.1 M sodium cacodylate, and then stained *en bloc* using 0.5% aqueous uranyl acetate. This process was followed by dehydration in a graded alcohol series, with infiltration and embedding in epoxy resin. Thin sections were obtained using an LKB-I

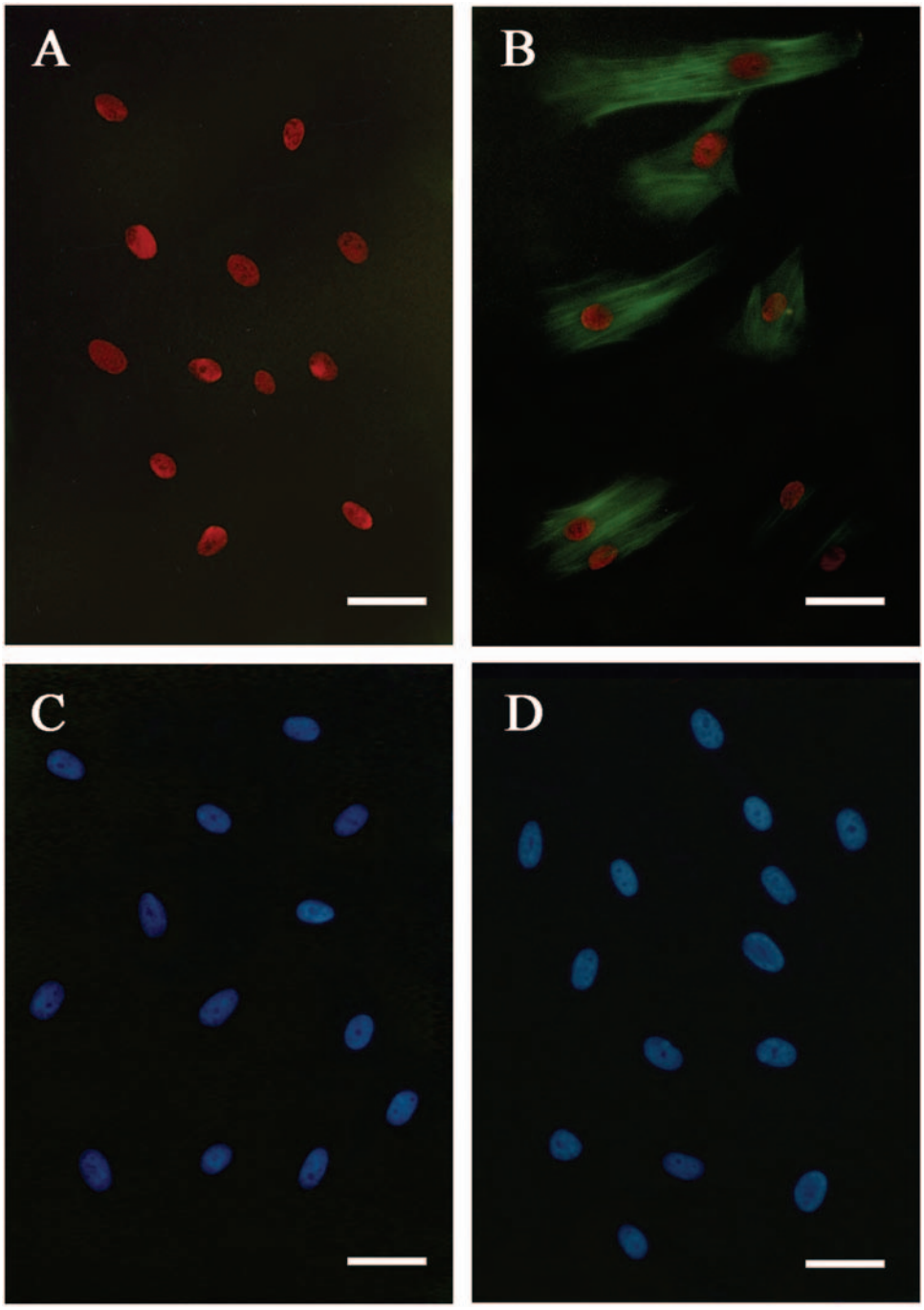

Figure 4 Expression of alpha-cardiac actin in Nkx2.5$^+$ CPCs and MSCs treated with CVF-conditioned medium after 12 days. (a) Representative negative staining of alpha-cardiac actin in untreated Nkx2.5$^+$ CPCs (nuclei were stained red with PI); (b) representative positive staining of alpha-cardiac actin in Nkx2.5$^+$ CPCs treated with CVF-conditioned medium (nuclei were stained red with PI); (c) representative negative staining of alpha-cardiac actin in untreated MSCs (nuclei were stained blue with 4',6-diamidino-2-phenylindole (DAPI)); (d) representative negative staining of alpha-cardiac actin in MSCs treated with CVF-conditioned medium (nuclei were stained blue with DAPI). Scale bar: 30 μm. (A color version of this figure is available in the online journal.)

ultramicrotome equipped with a diamond knife and were collected on uncoated 200-mesh copper grids, poststained with lead citrate and examined by JEM-1200EX TEM.

Western blot analysis for GSK-3β, beta-catenin, GATA-4, cardiac troponin T, and beta-MHC

The cells treated with CVF-conditioned medium or CVF-conditioned medium/Dkk1 and the untreated cells were lysed in RIPA lysis buffer or nuclear and cytoplasmic protein extraction reagent (Beyotime). Then, the extracted proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto polyvinylidene fluoride (PVDF) membranes (Beyotime). The proteins of interest were detected using anti-GSK-3β, beta-catenin, GATA-4, cardiac troponin T, and beta-MHC by the ECL (Beyotime) chemiluminescence

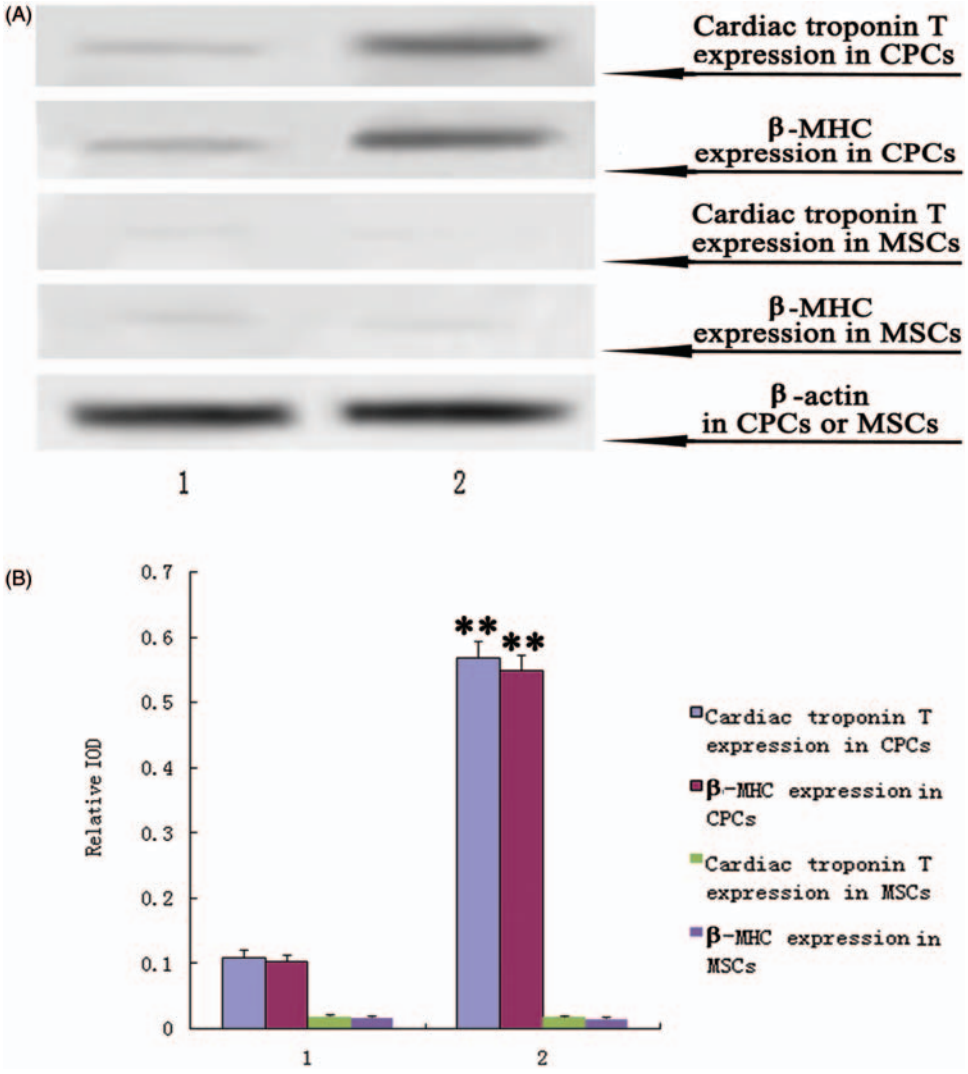

Figure 5 Western blotting analysis of cardiac troponin T and beta-MHC expression in Nkx2.5$^+$ CPCs and MSCs treated with CVF-conditioned medium after 12 days. (a) Western blotting analysis of the expression of cardiac troponin T and beta-MHC proteins in cells treated with CVF-conditioned medium and in untreated cells; (b) quantitative assessment of cardiac troponin T and beta-MHC protein levels using integrated optical density analyses. 1 = untreated cells; 2 = cells treated with CVF-conditioned medium. *$P < 0.05$; **$P < 0.01$ compared to the corresponding values for the untreated cells. (A color version of this figure is available in the online journal.)

detection method. The intensities of the labeled bands were analyzed using Multi-Analyst software. The level of beta-actin was used to normalize the signal intensities. The intensity data were then subjected to statistical analysis. The differences were assessed using Dunnett's test. A value of $P < 0.05$ was considered statistically significant.

Results

Identification of CVFs and MSCs

Phase-contrast light microscopy showed that the CVFs exhibited the morphological characteristics of fibroblasts, as previously described.[12] Briefly, these cells were spindle-shaped during the initial phases of culture. As they approached confluence, the cardiac fibroblasts developed a stellar shape. As assessed by immunofluorescence staining, the cardiac fibroblast cultures from passage 3 were positive for vimentin and negative for factor VIII, desmin, and alpha-cardiac actin (Figure 1), indicating that ventricular myocytes, smooth muscle cells, and endothelial cells were absent.

The rat MSCs were identified by flow cytometric analysis of their surface antigens. They were positive for CD29, CD54, and CD90 and negative for other surface markers, including CD31 (endothelial), CD34, and CD45 (hematopoietic markers). These cells maintained their phenotype from passage 3 to passage 10 (Table 1).

Morphological changes of Nkx2.5$^+$ CPCs and MSCs after treatment with CVF-conditioned medium

The Nkx2.5$^+$ CPCs displayed a fibroblast-like morphology before they were incubated with the CVF-conditioned medium, and this phenotype was maintained during further passages under non-stimulating conditions. During treatment with the conditioned medium, the morphology of the Nkx2.5$^+$ CPCs gradually changed.

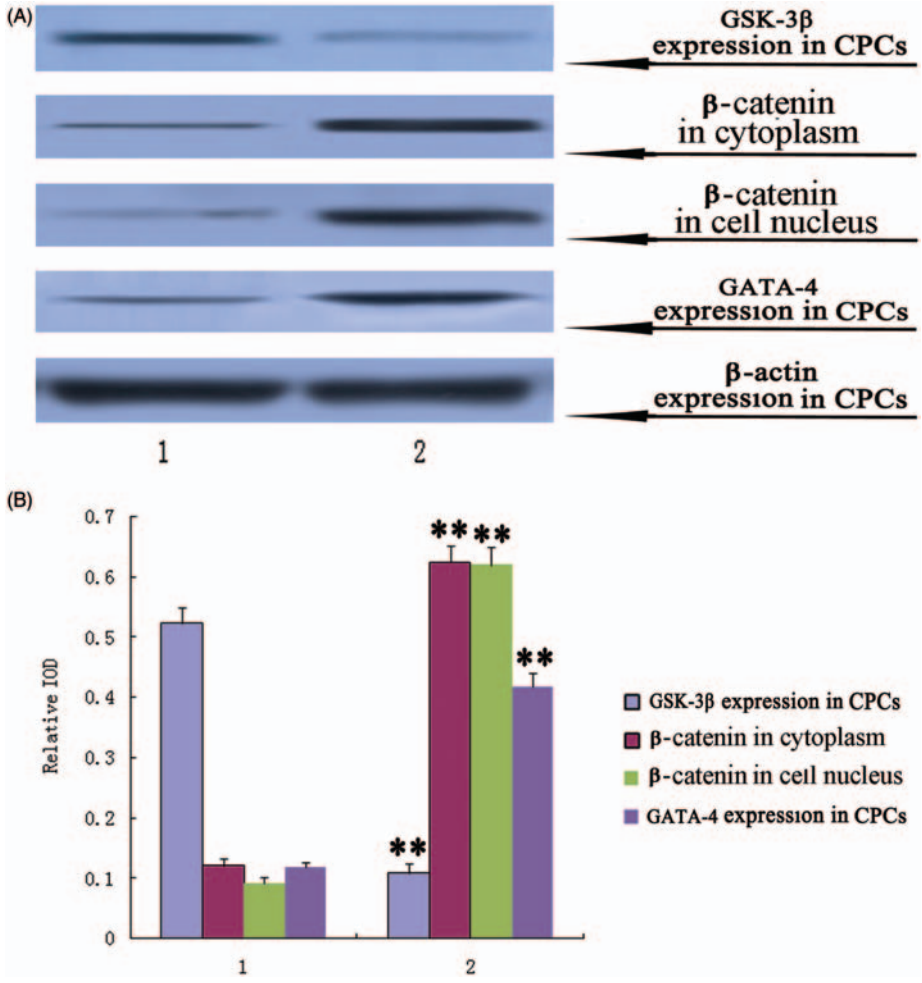

Figure 6 Western blotting analysis of GSK-3β, beta-catenin, and GATA-4 expression in Nkx2.5$^+$ CPCs treated with CVF-conditioned medium after 5 days. (a) Western blotting analysis of the expression of GSK-3β, beta-catenin, and GATA-4 proteins in cells treated with CVF-conditioned medium and in untreated cells; (b) quantitative assessment of GSK-3β, beta-catenin, and GATA-4 protein levels using integrated optical density analyses. 1 = untreated cells; 2 = cells treated with CVF-conditioned medium; GSK-3β expression in CPCs = GSK-3β expression in the treated cells with CVF-conditioned medium; beta-catenin in cytoplasm = beta-catenin expression in cytoplasm of the treated cells with CVF-conditioned medium; beta-catenin in cell nucleus = beta-catenin expression in nucleus of the treated cells with CVF-conditioned medium; GATA-4 expression in CPCs = GATA-4 expression in the treated cells with CVF-conditioned medium. *$P < 0.05$; **$P < 0.01$ compared to the corresponding values for the untreated cells. (A color version of this figure is available in the online journal.)

Phase-contrast microscopy showed that the treated cells lengthened in the longitudinal axis and became more orderly compared to the untreated cells. In contrast, there was no significant difference between the morphology of the MSCs treated with CVF-conditioned medium and the untreated MSCs (Figure 2). In addition, after the Nkx2.5$^+$ CPCs were incubated with CVF-conditioned medium, they clearly contained typical sarcomeres (Figure 3), whereas the treated MSCs did not reveal any cardiomyocyte-like ultrastructural features when observed by TEM.

Nkx2.5$^+$ CPCs showed significantly increased expression of cardiomyocyte-associated genes after treatment with CVF-conditioned medium

Immunofluorescence staining showed that over 80% of the Nkx2.5$^+$ CPCs treated with CVF-conditioned medium stained positively for alpha-cardiac actin, and their untreated counterparts did not demonstrate alpha-cardiac actin staining (Figure 4). Western blotting demonstrated that cardiac troponin T, beta-MHC, and GATA-4 expression were significantly increased in the treated cells compared with untreated cells ($P < 0.01$, $n = 5$; Figures 5 and 6). In contrast, the MSCs treated with CVF-conditioned medium did not exhibit positive staining for alpha-cardiac actin, as was the case for the untreated MSCs (Figure 4). There was no significant difference in the expression of cardiac troponin T and beta-MHC between the MSCs treated with CVF-conditioned medium and the untreated cells ($P > 0.05$, $n = 5$; Figure 5).

Blocking Wnt signaling pathway inhibits CVF-conditioned medium-induced morphological changes and expression of cardiomyocyte-associated genes of CPCs

When Nkx2.5$^+$ CPCs were treated with CVF-conditioned medium, after 5 days beta-catenin expression was increased and GSK-3β expression was significantly decreased

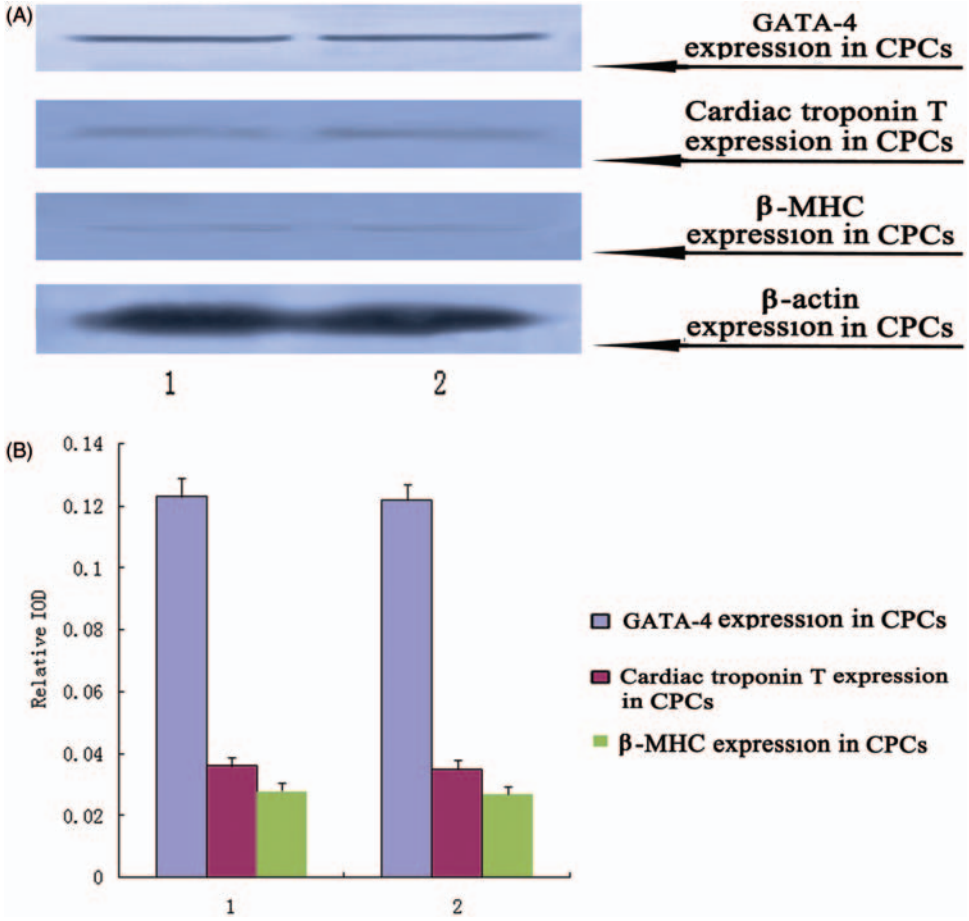

Figure 7 Western blotting analysis of GATA-4 expression in Nkx2.5$^+$ CPCs treated with CVF-conditioned medium after 5 days and cardiac troponin T/beta-MHC expression in the treated cells after 12 days when being added to DKK1. (a) Western blotting analysis of the expression of GATA-4, cardiac troponin T, and beta-MHC proteins in cells treated with CVF-conditioned medium and in untreated cells; (b) quantitative assessment of GATA-4, cardiac troponin T, and beta-MHC protein levels using integrated optical density analyses. 1 = untreated cells; 2 = cells treated with CVF-conditioned medium; GATA-4 expression in CPCs = GATA-4 expression in the treated cells when being added to DKK1; cardiac troponin T expression in CPCs = cardiac troponin T expression in the treated cells when being added to DKK1; beta-MHC expression in CPCs = beta-MHC expression in the treated cells when being added to DKK1. *$P < 0.05$; **$P < 0.01$ compared to the corresponding values for the untreated cells. (A color version of this figure is available in the online journal.)

significantly in the treated cells compared with untreated cells ($P < 0.01$, $n = 5$; Figure 6), indicating that Wnt signaling pathway was activated. Following the addition of Dkk1 to the cells, no significant difference in the expression of cardiac troponin T, beta-MHC, and GATA-4 was observed between Nkx2.5$^+$ CPCs treated with CVF-conditioned medium and the untreated cells ($P > 0.05$, $n = 5$; Figure 7). Immunofluorescence staining showed that the Nkx2.5$^+$ CPCs treated with CVF-conditioned medium/Dkk1 did not exhibit positive staining for alpha-cardiac actin, as was the case for the untreated cells (Figure 8). These findings are consistent with the results obtained using TEM, which indicates that after the Nkx2.5$^+$ CPCs were treated with CVF-conditioned medium/Dkk1, they did not reveal any sarcomeres, as was the case for the untreated cells. Phase-contrast microscopy showed that there was no significant difference between the morphology of the Nkx2.5$^+$ CPCs treated with CVF-conditioned medium/Dkk1 and the untreated MSCs (Figure 8). These results suggest that blocking Wnt signaling pathway inhibited CVF-conditioned medium-induced morphological changes and expression of cardiomyocyte-associated genes of Nkx2.5$^+$ CPCs.

Discussion

The cardiomyogenic differentiation of stem cells has been shown to require a paracrine pathway in the heart.[13] This finding suggests that the microenvironment plays an important role in inducing stem cells to differentiate into cardiomyocytes. In particular, the cellular microenvironment created by the secretions of cardiac non-myocytes, which mimics the *in vivo* physiological environment, is important in directing the differentiation of stem cells into cardiomyocytes. For example, culturing ESCs in medium conditioned by visceral endoderm-like cells greatly enhanced their cardiogenic differentiation.[14] Similarly, when medium conditioned by mouse embryonic fibroblasts was used, the homogeneity of the beating EBs was significantly improved.[15] The cardiac fibroblast is the main factor in the formation and regulation of the

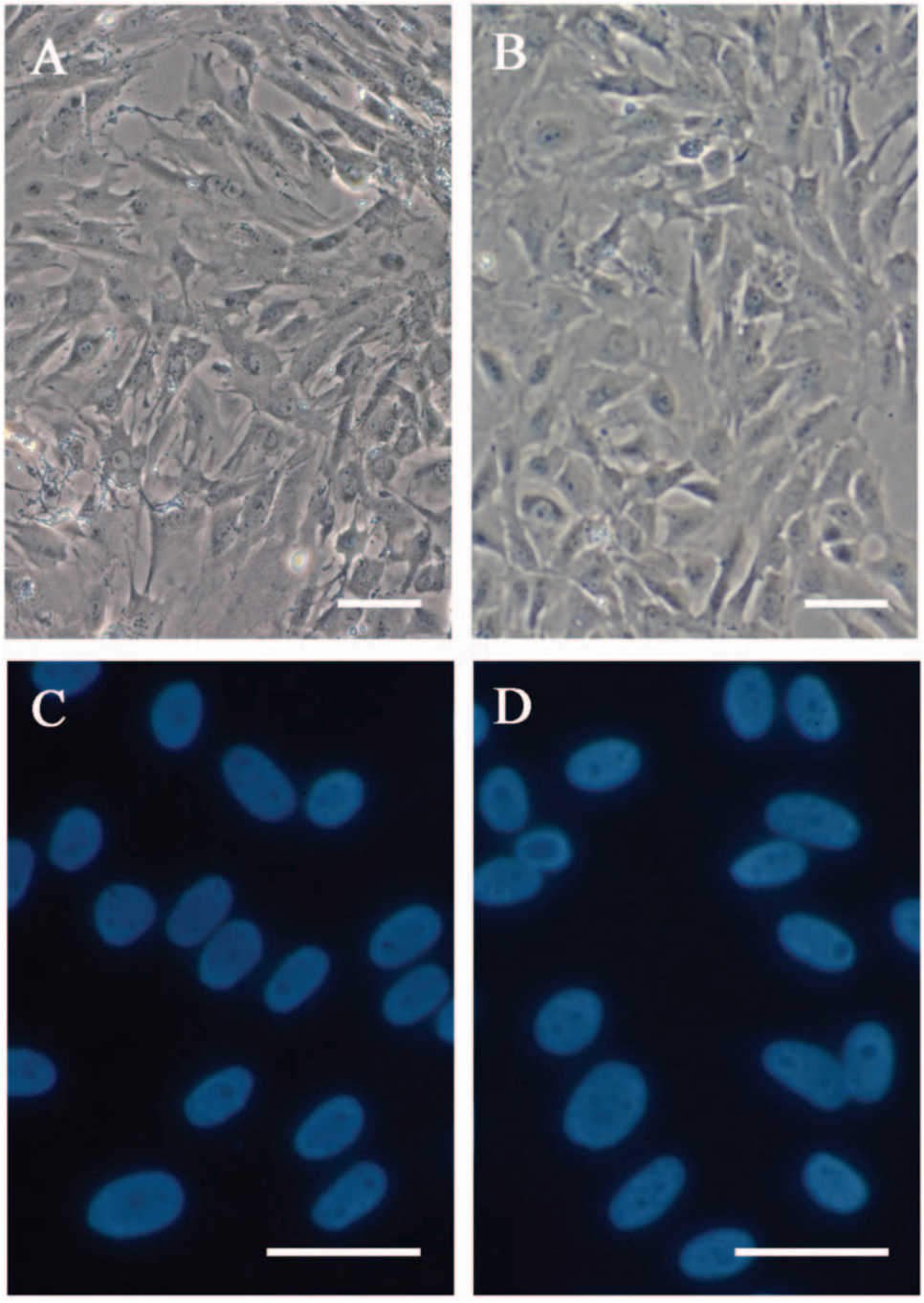

Figure 8 Shape changes and alpha-cardiac actin staining of the Nkx2.5$^+$ CPCs treated with CVF-conditioned medium and DKK1 after 12 days. (a) Untreated Nkx2.5$^+$ CPCs and (b) Nkx2.5$^+$ CPCs treated with CVF-conditioned medium and DKK1; (c) untreated Nkx2.5$^+$ CPCs; and (d) Nkx2.5$^+$ CPCs treated with CVF-conditioned medium and DKK1. Scale bar: 30 μm. (A color version of this figure is available in the online journal.)

cardiomyocyte microenvironment during cardiac development. Accordingly, a medium conditioned by cardiac fibroblasts may be a specific inducer of cardiomyogenic differentiation.

Our previous study demonstrated that after treatment with 5-azacytidine, Nkx2.5$^+$ human CPCs derived from embryonic heart tubes could differentiate into cardiomyocytes.[10] However, 5-azacytidine is toxic and unspecific for myogenic differentiation. Notably, almost every type of stem cell can be induced to differentiate into cells with a cardiac phenotype by 5-azacytidine treatment, indicating that this drug does not specifically determine the potential of CPCs for cardiomyogenic differentiation. To investigate the possibility that CPCs could be induced to differentiate into cardiomyocytes by a specific and non-toxic method, we established a CVF-conditioned medium that induced CPCs, but not MSCs, toward a cardiomyocyte fate. The results indicated that upon treatment with CVF-conditioned medium, Nkx2.5$^+$ CPCs were capable of differentiating into a cardiac phenotype.

In contrast, MSCs did not show any capacity for cardiomyogenic differentiation when provided with the same treatment. These results suggested that Nkx2.5$^+$ CPCs have an enormous potential for cardiomyogenic differentiation and that CVF-conditioned medium can specifically induce the differentiation of CPCs into cells with a myocardial phenotype.

Wnt signaling pathway is a conservative signaling pathway, playing very important roles in diverse physiological processes. Particularly, the signaling pathway involves a large number of proteins that are required for basic developmental processes, such as cell-fate specification. In this study, we found that after 5-day treatment of CVF-conditioned medium, Wnt signaling pathway was activated in Nkx2.5$^+$ CPCs. This finding suggests that Wnt signaling pathway may be involved in CVF-conditioned medium-induced cardiomyogenic differentiation of Nkx2.5$^+$ CPCs. Furthermore, following the addition of Dkk1 and the blocking of Wnt signaling pathway, CVF-conditioned medium-induced morphological changes and expression of cardiomyocyte-associated genes of Nkx2.5$^+$ CPCs were inhibited, which indicates that CVF-conditioned medium-induced cardiomyogenic differentiation of Nkx2.5$^+$ CPCs is associated with Wnt signaling pathway.

GATA-4 is a potent transcriptional activator of several cardiac muscle-specific genes and a key regulator of the cardiomyocyte gene program. The transcription factor regulated the expression of many cardiac genes and was tightly linked to cardiomyocyte-specific differentiation. In this study, we found that the activation of Wnt signaling pathway was accompanied by higher expression of GATA-4 and the blocking of the pathway inhibited the expression of GATA-4 in CVF-conditioned medium-incubated Nkx2.5$^+$ CPCs. This finding suggests that Wnt signaling pathway may alter GATA-4 expression and activate the cardiogenic program in the regulation of differentiation.

Author contributions: All authors participated in the design, interpretation of the studies, and analysis of the data and review of the manuscript. XZ, M-RS, and Z-DX conducted the experiments, participated in the design of the study, and drafted the manuscript. ZH participated in the part experiments. CC carried out flow cytometry analysis. Y-LC helped to draft the manuscript. ZK performed the statistical analysis. Z-FL participated in the reagent preparation. X-TL supplied animals. S-LG participated in the statistical analysis. S-HX and C-SZ conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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