Over-expression of Nkx2.5 and/or cardiac α-actin inhibit the contraction ability of ADSCs-derived cardiomyocytes

Lili Zhao · Dapeng Ju · Qian Gao · Xueli Zheng ·
Gongshe Yang

Abstract Adipose tissue-derived stromal cells (ADSCs) can differentiate into cardiomyocytes, which provide a source of new cardiomyocyte progenitors for tissue engineering. Here, we showed that ADSCs isolated from subcutaneous adipose tissues of mouse were largely negative for CD31, CD34, but positive for CD105. About 1.62% cells in these cells can spontaneously differentiate into cardiac-like cells (cells expressing cardiac marker proteins) when cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented only with penicillin, streptomycin, and 20% newborn bovine serum (NBS), expressed cardiac markers such as MF20, Connexin45, cMHC, cTnT, a-actin, Nkx2.5, and GATA4, and part of these cells (account for about 0.47% of inoculated cells) showed spontaneous contractions accompanied by transient Ca^{2+} activity in culture. In vitro, although over-expression of *Nkx2.5* and/or *cardiac α-actin* increased the number of cardiac-like cells expressing cardiac-specific proteins, but while inhibited the contraction function of ADSCs-derived cardiomyocytes.

Keywords Adipose · Stromal cells · Cardiomyogenic differentiation · Cardiomyocytes · Nkx2.5 · Cardiac α-actin

L. Zhao · D. Ju · Q. Gao · X. Zheng · G. Yang (✉)
Laboratory of Animal Fat Deposition and Muscle Development, College of Animal Science and Technology, Northwest A&F University, 22 Xinong Road, Yangling, Shaanxi Province 712100, People's Republic of China
e-mail: gsyang999@hotmail.com

Introduction

Myocardial infarction caused by myocardial necrosis seriously damages human health—for instance, by irreversibly leading to heart failure. In recent years, efforts have been focused on developing therapies that allow replacement of damaged cardiomyocytes. Candidate cells include embryonic stem cells [1], cardiogenic progenitor cells [2], bone marrow stem cells (BMSCs) [3], cardiac side population cells [4], and induced pluripotent stem (iPS) cells [5], which are known to differentiate into cardiac myocytes in vivo and/or in vitro. Because there are strong objections to using human embryonic stem cells in both research and therapy, an increasing amount of attention has been focused on adult stem cells. These can be isolated from important organs such as skeletal muscle, skin, brain, liver, and bone marrow, but the process is expensive for patients and accompanied by the risk of donor site morbidity [6]. Because adipose tissue is easier to sample and associated with fewer ethical questions [7], it appears to be a better source of stem cells.

Multi-potential adipose tissue-derived stromal cells (ADSCs) can differentiate into a variety of cell types, including adipocytes, skeletal muscle cells, cardiac cells, smooth muscle cells, nerve cells, cartilage cells, and osteoblasts [6–13]. ADSCs are known to have a close relationship with cardiovascular cells, and can differentiate into beating cardiomyocytes; however, this process requires specific culture conditions. For example, 5-azacytidine treatment [14] or culturing in semisolid methylcellulose medium (MethoCult GF M3534, main recipes are rh-Insulin, 2-Mercaptoethanol, rm-Stem Cell Factor, rm-IL-3, rh-IL-6, etc.) [10] have been shown to result in the differentiation of rabbit and mouse ADSCs, respectively, into beating cardiomyocytes. Human ADSCs have also

been shown to differentiate into beating cardiomyocytes when co-cultured with beating cardiomyocytes [15]. Worth attention, mouse dedifferentiated fat (DFAT) cells can spontaneously differentiate into beating cardiomyocytes when cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented only with 20% FBS, penicillin, and streptomycin without adding any other factors [16]. The pluripotency of DFAT cells has been confirmed [17, 18], but appears to be lower than that of ADSCs. Thus, it seems likely that ADSCs can spontaneously differentiate into beating cardiomyocytes under the same simple culture conditions, although this possibility has not yet been investigated.

Nkx2.5/Csx (cardiac-specific homeobox) is one of the earliest essential transcription factors regulating cardiomyocyte differentiation, cardiovascular formation/cyclization, and ion channels activation involved in cardiac conduction and contraction [19]. In a previous experiment, the forced expression of cardiomyocyte-specific transcription factors such as Csx/Nkx2.5 and GATA4 destined bone marrow-derived stromal cells to a cardiomyocytic lineage [20]. In addition, being predominantly expressed throughout heart development [21], cardiac α-actin is a target of Nkx2.5 [22] and the major contractile protein. Although these transcription factors and cytoskeletal proteins are critical for the cardiac differentiation program, their effects on elevating the ratio of cardiac differentiation of adult stem cells are still not sure.

In present study, we firstly found that primary mouse ADSCs could spontaneously differentiated into beating cardiomyocytes in basic culture medium, without adding any other factors and cardiac differentiation inducers. The changes of cell morphology, expression of cardiac marker proteins and Ca^{2+} ion channel were also analyzed before and after the formation of ADSCs derived cardiomyocytes. Furthermore, we examined the effects of Nkx2.5 and cardiac α-actin over-expression on ADSCs' cardiac differentiation. Taken together, our results showed the presence of cardiac progenitor cells in ADSCs, which could differentiate into beating cardiomyocytes in our mentioned culture condition. Although over expression of Nkx2.5 and cardiac α-actin could elevate the cardiac marker proteins expression, inhibited the contraction ability of ADSCs-derived cardiomyocytes.

Materials and methods

Isolation and culture of ADSCs

Cells were isolated from stromal vascular fraction (SVF) using previously described methods [15, 23] with slight modifications. Briefly, when C57BL/6 mice (Fourth

Military Medical University, China) were 6–8 weeks old, adipose tissue was removed from their inguinal subcutaneous fat depots under sterile conditions. The isolated tissue was minced into 1-mm^3 pieces and digested with 0.1% collagenase type I [1 mg/ml collagenase type I (Invitrogen, Carlsbad, CA, USA) and 20 mg/ml BSA] for 60 min at 37°C, then filtered through 30-μm nylon mesh. After centrifugation at 1,500 rpm for 7 min, precipitates were treated with red cell lysis buffer (154 mmol/l NH_4Cl, 10 mmol/l $KHCO_3$, and 0.1 mmol/l EDTA) for 10 min at room temperature, precipitated by centrifugation at 1,500 rpm for 7 min, resuspended in phosphate buffered saline buffer (PBS), and reprecipitated by centrifugation at 1,500 rpm for 7 min. Finally, the pellets were resuspended in DMEM (Invitrogen) supplemented with 20% newborn bovine serum (NBS; Sijiqing, Hangzhou, China), 50 U/ml penicillin (GIBCO/BRL, Carlsbad, CA, USA), and 50 U/ml streptomycin (GIBCO/BRL). Cells were plated (Day 0) at a density of 1×10^4/cm^2 in 35-mm dishes (Corning Inc., NY, USA) and maintained at 37°C in a 5% CO_2 humidified atmosphere. After 24 h, nonadherent cells were removed, and the plastic adherent cells were named ADSCs [24] (Day 1), the medium were changed every 2 days, and the cells used in this study were not passaged. All procedures were approved by the Institutional Animal Care and Use Committee of Northwest A&F University.

Construction of recombinant adenovirus expression vectors and ADSCs infection

Nkx2.5 and cardiac α-actin CDS sequences were cloned from the heart tissue of 8-week-old C57BL/6 mice. Recombination and propagation of adenovirus expressing Nkx2.5 or cardiac α-actin were performed as previously described [25]. Briefly, ORFs were cloned into a pAd-Track-CMV shuttle vector between the HindIII (Takara Biotechnology Inc., Dalian, China) and XbaI (Takara) sites. pAdEasy-1 was used to perform homologous recombination in Escherichia coli BJ5183 component cells, after which recombined plasmids were identified using PCR and restriction endonuclease PacI (New England Biolabs, Inc., Beverly, MA, USA), and the results of sequencing show the sequences are right (Beijing Sunbiotech Co., Ltd. China). To produce the adenovirus, the identified plasmids were linearized with PacI and transfected into a HEK293 cells with liposome 2000 (Invitrogen). The propagation procedure was repeated three times, then the virus was collected for identification and titer determination. The titer was estimated with the Kärber formula, and the final titrations were 9×10^8 PFU/ml (Ad-Nkx2.5) and 6×10^8 PFU/ml (Ad-cardiac α-actin).

To explore the effects of Nkx2.5 and cardiac α-actin on spontaneous differentiation of ADSCs into cardiomyocytes,

we infected ADSCs (confluence: 80–90%) with Ad-Nkx2.5 (5 μl/35-mm dish), Ad-cardiac a-actin (5 μl/35-mm dish), or both viruses simultaneously [(5 μl Ad-Nkx2.5 and 5 μl Ad-cardiac a-actin)/35 mm dish]. Uninfected cells and Ad-GFP-infected cells were used as controls.

Gene expression analysis

Total RNA was extracted using RNAiso plus reagent (Takara). 500 ng of total RNA was processed into single strand cDNA using reverse transcription kits (Takara) with random primers. Primers used for each of the genes studied listed below. β-actin sense: GGCTGTATTCCCCTCCA TCG and CCAGTTGGTAACAATGCCATGT (154 bp, NM_007393.2), Nkx2.5 sense: GACAAAGCCGAGACG GATGG and CTGTCGCTTGCACTTGTAGC (222 bp, NM_008700.2), cardiac a-actin sense: CTGGATTCTGGC GATGGTGTA and CGGACAATTTCACGTTCAGCA (173 bp, NM_009608.2). Real-time PCR reactions were performed in triplicate using the SYBR green kit (Takara) with a Bio-rad iQ^{TM}5 system. The $2^{-\Delta\Delta CT}$ method was used to analyze the relative expression of each gene. Briefly, equivalent amount of cDNA template was used for transcripts amplification and processed in triplicate. Average CT values were calculated and the ΔCT values were calculated by subtracting the β-actin average CT value for each sample. Subsequently, $\Delta\Delta$CT values were calculated by subtracting the average ΔCT values of the control group. The final fold differences were calculated as $2^{-\Delta\Delta CT}$ for each gene among treatments. These measurements were repeated three times.

Western blot analysis

Whole proteins were extracted from cultured cells with RIPA buffer (Pierce, Rockford, IL, USA) supplemented with a protease inhibitor (Halt Protease Inhibitor Cocktail Kit, Pierce). Equivalent amounts (30 μg) of protein were subjected to electrophoresis, then electro-transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% skim milk in TBST (Tris Buffered Saline and 0.1% Tween 20) for 2 h at room temperature, then incubated at 4°C overnight with primary antibodies, including rabbit anti-Nkx2.5 (Santa Cruz, CA, USA), rabbit anti-cardiac α-actin (Bister, Wuhan, China), mouse anti-β-actin was used as an internal control. The membranes were then incubated with HRP-conjugated rabbit anti-goat and goat anti-mouse secondary antibodies (Santa Cruz) for 2 h at room temperature. All antibodies were used according to the manufacturers' recommendations. Signals were detected using a chemiluminescent ECL western blot detection system (Millipore Corp., Bedford, MA, USA). Quantity one 4.6.3 imaging software was used for densitometric analysis of the expressed protein bands.

Flow cytometry

At day 4, the first beating cells were detected, we detected the surface antigens and cardiac markers by flow cytometry respectively. The cells were digested with 0.25% trypsin, fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and incubated for 1 h at 4°C with anti-CD31 (Bister, Wuhan, China), anti-CD34 (Bister, Wuhan, China), anti-CD105 (Bister, Wuhan, China), anti-DHPR (1:50, Santa Cruz), anti-RYR$_2$ (ryanodine receptor; 1:50, Chemicon International, London, UK), anti-cMHC (1:100, Biosynthesis, Wuhan, China), anti-cTnI (1:100, Biosynthesis), and anti-Connexin45 (1:100, Bister) specific primary antibodies. The cells were then incubated with RBITC (Rhodamine B isothiocyanate; Biosynthesis, Whan) and phycoerythrin/PE (Proteintech, Chicago, IL, USA) conjugated secondary antibodies for 1 h at 4°C. Finally, cells were washed with PBS and resuspended in 4% paraformaldehyde for flow cytometry. The negative control tubes were not incubated with primary antibody but with fluorescently-labeled second antibody. Data were analyzed by the FlowJo and DiVa package.

Ca^{2+} imaging

Free intracellular Ca^{2+} imaging recordings were obtained from cells loaded with fluo-3 AM (5 μmol/l; Beyotime, Guangzhou, China), a fluorescent Ca^{2+} indicator. Cells were incubated with fluo-3 AM at 37°C for 30 min, then washed with standard Tyrode's buffer and incubated with DMEM at 37°C for 10 min. Video images were made by a digital camera (Canon G10, Canon, Tokyo, Japan) through a fluorescent microscope with a Nikon TE 2000 microscope (Nikon, Tokyo, Japan).

Immunofluorescence/immunohistochemistry

For immunocytochemical analyses, cells grown in culture dishes were washed with PBS, fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, blocked with 10% goat serum and 5% BSA in PBS, and incubated overnight at 4°C with anti-DHPR (1:50), anti-RYR$_2$ (1:50), anti-MHC (1:50), anti-cTnI (1:100), anti-MF20 (1:100), anti-Connexin45 (1:100), anti-Nkx2.5 (1:50), anti-cardiac α-actin (1:100), anti-MyoD (1:50; Santa Cruz), and anti-α-smooth muscle actin (1:100; Bister). At room temperature, cells were washed three times with PBS, incubated for 60 min with Cy3 or FITC-conjugated secondary antibodies, washed three times with PBS, and stained for 5 min with Hoechst 33342 (0.5 μg/ml, Sigma, St. Louis, MO,
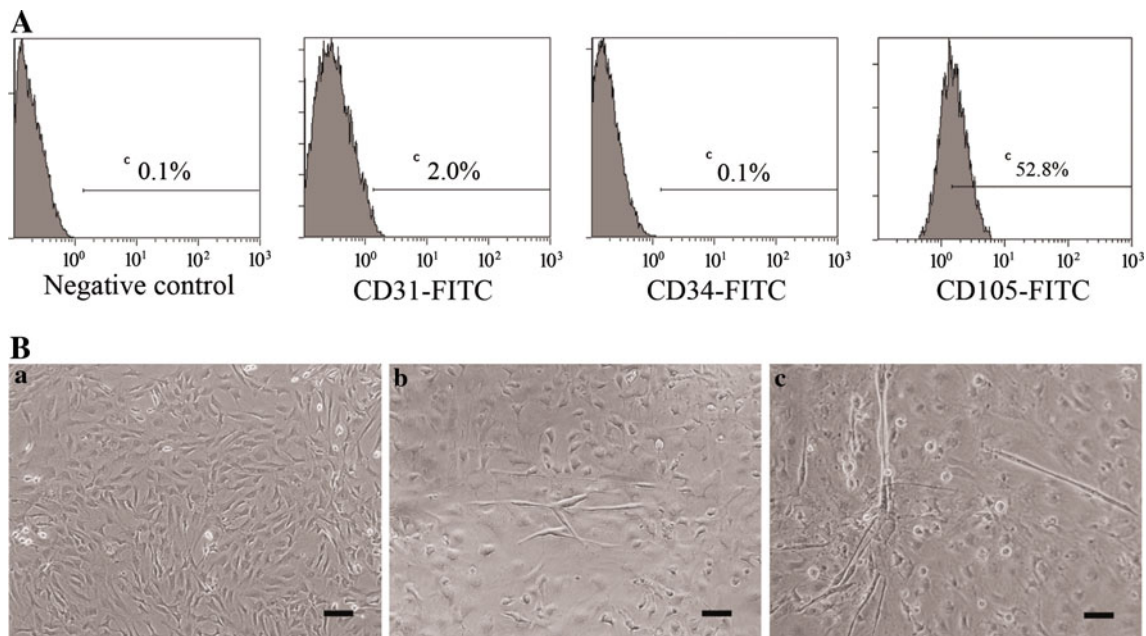
Fig. 1 Expression of surface antigens in ADSCs and morphological changes observed in ADSCs-derived cardiomyocytes. **A** Expression analysis of cell surface antigens in ADSCs. ADSCs were harvested primary dissociated, stained with FITC-conjugated antibodies to CD31, CD34, or CD105. Results showed that ADSCs did not express CD31 and CD34, but 51.52 ± 3.11% cells showed CD105 positive in ADSCs. **B** Morphological changes observed in ADSC-derived cardiomyocytes. Freshly isolated SVF cells were plated in DMEM supplemented with 20% NBS. The adherent cells are mainly fibroblast-like cells (Day 4, *a*). Shortly afterward, spindle cells developed (Day 5, *b*). These rapidly developed into spherical cells, rod-shaped cells, and long myotubes, some of which began beating (Day 7, *c*). *Scale bars* measure 100 µm

USA). Finally, the cells were again washed three times with PBS. Images were visualized and obtained by a Nikon TE 2000 microscope.

For immunohistochemistry, tissues were first incubated with primary antibodies, and then with biotin goat anti-mouse IgG or goat anti-rabbit IgG (1:200; Bister) secondary antibodies. Color development was performed using a liquid DAB substrate kit (Bister). Nuclei were stained with hematoxylin. Immunostained tissues that had not been incubated with primary antibodies were used to rule out non-specific binding. Paraffin-embedded sections of rat myocardium were used as staining positive controls of cMHC, cTnI and Connexin45, rat skeletal muscle was used as staining positive control of MyoD, rabbit bladder sections were used as staining positive control of α-SMA.

Counting

Cells grown in culture dishes were stained with Hoechst 33342. The total number of nuclei in growing areas was estimated by arbitrarily selecting six 2.75 mm^2 squares of culture dish in which to count the number of cells under fluorescent lighting; these values were then extrapolated to the total growth area. Additionally, beating cardiomyocytes were counted under a phase contrast microscope. Each round of counting was performed in triplicate by three

different individuals. Data are shown as means ± SE of four randomly selected dishes from three separate experiments.

Cultured cells were observed through an inverted-type phase-contrast video microscope (Nikon), and recorded by a Sony camera (Sony Corporation, Tokyo, Japan).

Statistical analysis

All experiments were performed at least three times. Main and interactive effects were analyzed by one-way ANO-VAs using SPSS version 16.0 software (SPSS, Inc., Chicago, IL, USA). Significance was defined as $P < 0.05$.

Results

Morphology analysis

Nonadherent cells were discarded 24 h after plating. The remaining adherent cells (ADSCs) mainly showed fibroblast-like morphology (from Day 1 onwards). We examined the expression of cell surface antigens of ADSCs by flow cytometry. The ADSCs were largely negative for CD31, CD34, but positive for CD105 (51.52 ± 3.11%)
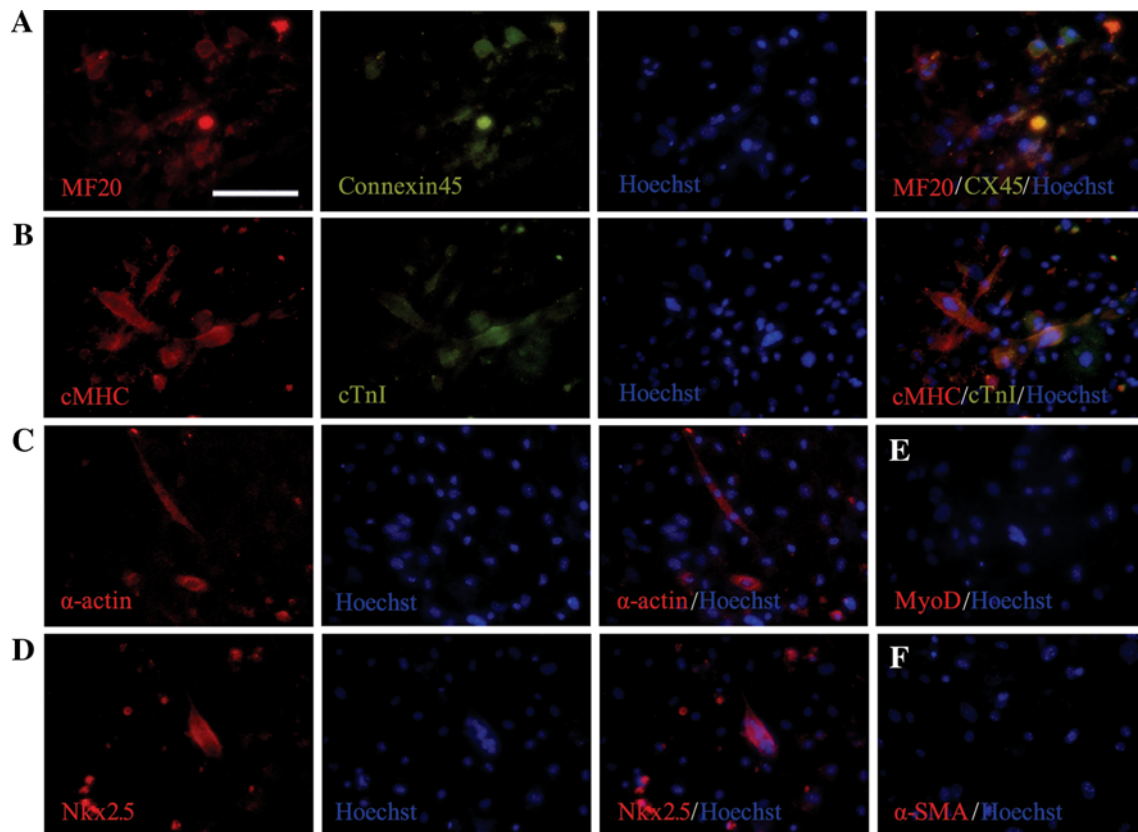
Fig. 2 Immunofluorescence analysis of muscle-specific proteins in ADSC-derived colonies. Four days after the cells began beating, they were double-stained for MF20 (*red*, **A**) and Connexin45 (*green*, **A**), cMHC (*red*, **B**) and cTnI (*green*, **B**), and α-actin (*red*, **C**) and Nkx2.5 (*red*, **D**). No immunofluorescence was detected in beating cells stained for MyoD (*red*, **E**) or α-SMA (*red*, **F**). Nuclei were stained with Hoechst 33342 (*blue*, **A–F**). *Scale bars* measure 100 μm. (Color figure online)

(Fig. 1A). Various cell morphologies emerged between day 5 and 7, including clones of rounded cells, groups of small tube cells, fibroblast-like preadipocytes, and differentiated adipocytes with small lipid droplets. Myotube-like structures appeared during day 8–9, surrounded by some rounded cells. Some of the myotube-like structures and their associated rounded cells began to spontaneously contract (Fig. 1B and Supplemental Video 1). The number of myotube-like structures and beating cells increased slightly over the next 2–3 days. However, no synchronous contractions were observed, and cells gradually stopped beating between day 15 and 20.

When cells started to beat on the day 11 after ADSCs inoculation, we counted the total number of myotube-like structures and the number of beating cells. Cumulatively, at least 16.2 ± 0.6 myotube-like structures and rounded cells were obtained from every 1,000 plated cells. Among these myotube-like structures and rounded cells, approximately $29.1 \pm 1.2\%$ (account for about 0.47% of inoculated cells) exhibited spontaneous contractions. The contractions activity could be maintained for up to nearly a week.

Evidence that ADSCs differentiate into cardiomyocytes phenotypes

In order to confirm cardiomyocyte-like protein phenotypes, immunofluorescence was performed on the day 4 after the cells began beating. Positive staining was detected in response to antibodies for cardiac α-actin, MF20, Connexin45, cMHC, cardiac troponin I (cTnI), and Nkx2.5 (Fig. 2A–D), while no staining was observed in response to the skeletal muscle protein MyoD or α-smooth muscle actin (α-SMA) (Fig. 2E, F). Notably, Nkx2.5 was detected in both nuclei and plasma, indicating that it may not fully translocate from plasma into nuclei. Staining with nonimmune control IgG did not generate any microscopically-detectable fluorescent signals. The according negative staining, which were not incubated with primary antibodies were used to rule out non-specific binding, was shown in the right panel (data was not shown). The same type of specific tissue sections staining with nonimmune control IgG also did not generate any microscopically detectable positive signal (Fig. 3).

Fig. 3 Positive and negative controls for immunostaining. Paraffin-embedded sections of rat myocardium, rat skeletal muscle, and rabbit bladder. Tissues were first incubated with primary antibodies, and then with biotin goat anti-mouse IgG or goat anti-rabbit IgG secondary antibodies. *Color* development was performed using a liquid DAB substrate kit. Nuclei were stained with hematoxylin (*blue*). In the *left*, the *brown staining* reflects treatment with rat cardiomyocyte anti-cMHC (**A**), anti-cTnI (**B**), anti-Connexin45 (**C**), rat skeletal myoblast anti-MyoD (**D**), and rabbit bladder anti-α-SMA (**E**). The according negative stainings, which were not incubated with primary antibodies were used to rule out non-specific binding, was shown in the *right*. *Scale bars* measure 100 μm. (Color figure online)
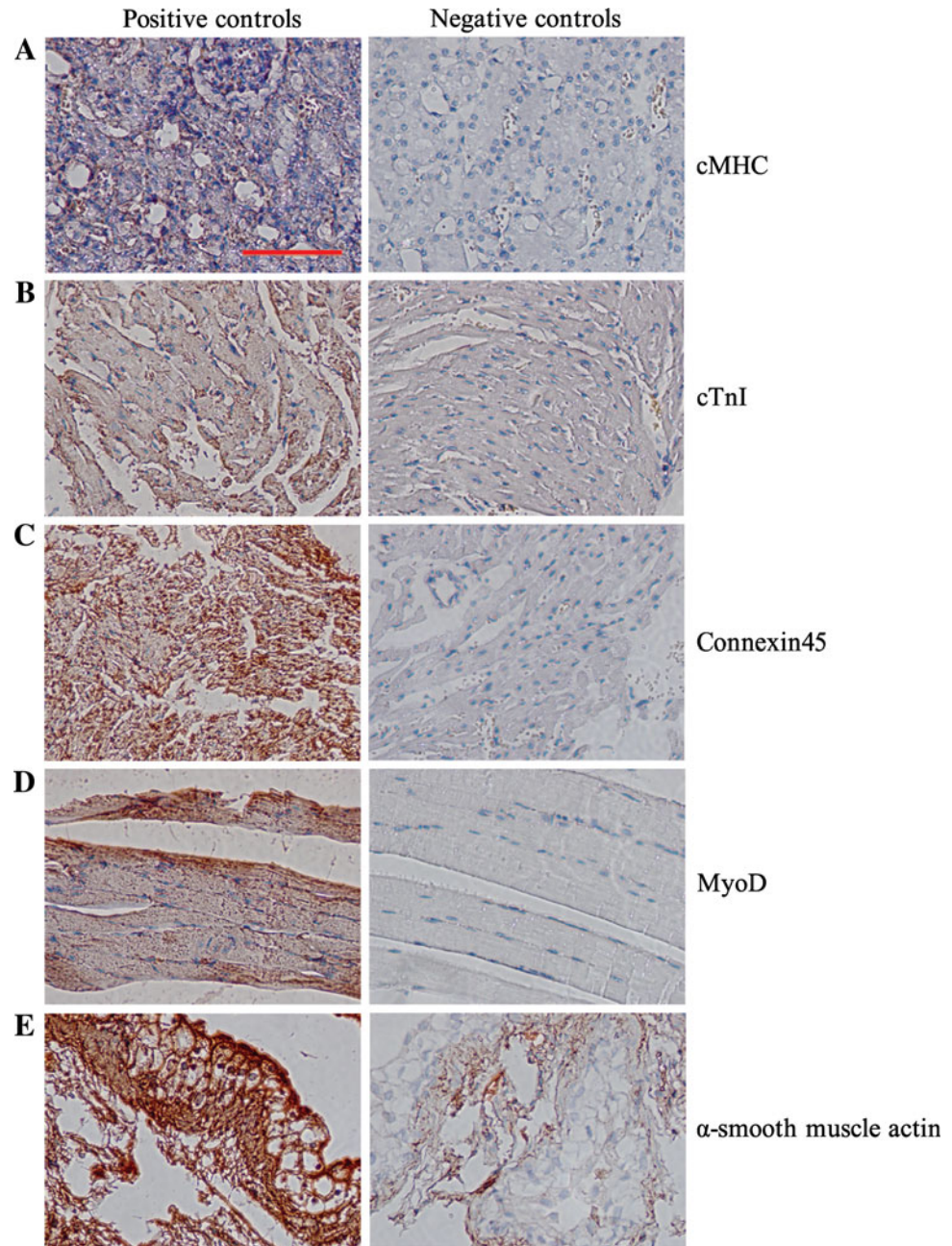

After differentiation, beating ADSC-derived cells expressed both DHPR and RyR$_2$, which indicated open cardiac-specific excitation–contraction coupling (E–C coupling) (Fig. 4). We also observed oscillations in fluo-3 AM fluorescence intensity in these cells (Supplemental Video 2). Results from the flow cytometry analyses indicated that the cells expressed cardiac marker proteins. Specifically, $9.13 \pm 0.23\%$, $7.13 \pm 0.15\%$, $6.27 \pm 0.61\%$, and $1.13 \pm 0.06\%$ of cells expressed GATA4, cTnI, Connexin45, and DHPR-RYR$_2$, respectively. Cumulatively, these data indicated that the beating ADSC-derived cells possessed the functional characteristics of cardiomyocytes in vitro (Fig. 5).

Effects of Nkx2.5 and/or cardiac α-actin over-expression on ADSCs differentiation

qPCR and western blot analysises showed that Nkx2.5 and cardiac α-actin were upregulated in cells infected with Ad-*cardiac a-actin* (mRNA by 6.23-fold and 13.60-fold, respectively, protein by 1.61-fold and 1.52-fold, respectively) and Ad-*Nkx2.5* (mRNA by 14.48-fold and 3.75-fold, respectively, protein by 1.87-fold and 1.16-fold, respectively) at 48 and 72 h post-infection, respectively. Co-infection with the two viruses increased Nkx2.5 and cardiac α-actin expression by mRNA by 14.92-fold and

Fig. 4 Cardiac-specific excitation–contraction coupling pathway analysis of beating colonies derived from ADSCs. Beating cells were double-stained for RyR_2 (*red*) and DHPR (*green*). Nuclei were stained with Hoechst 33342. *Scale bars* measure 100 μm. (Color figure online)
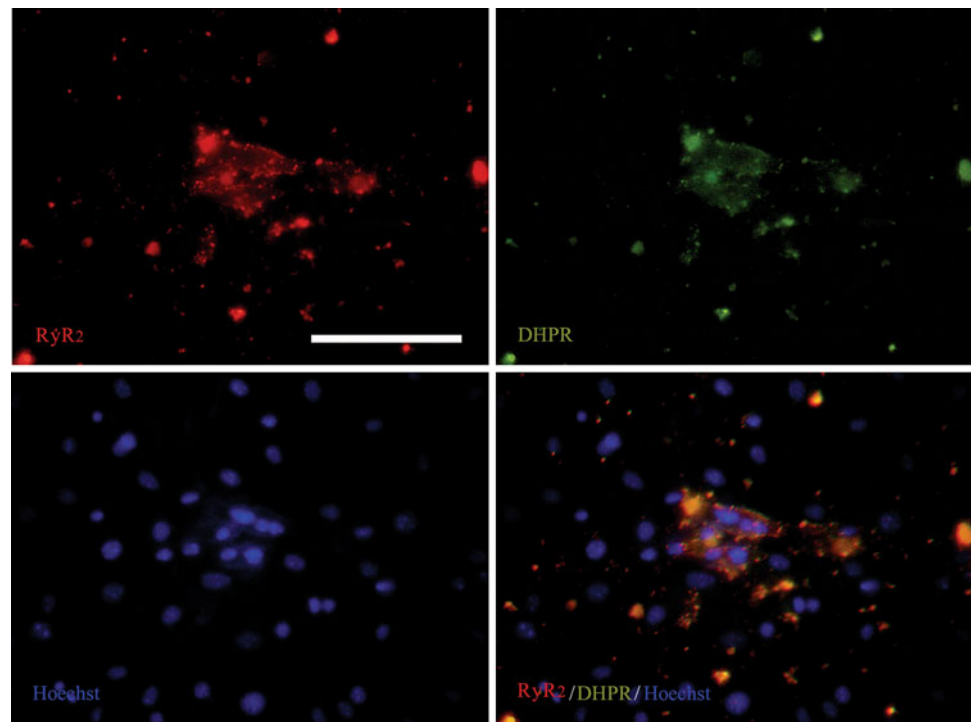
15.17-fold, respectively, and protein by 1.86-fold and 1.40-fold, respectively (Fig. 6A, B).

Results from the flow cytometry analyses indicated that the expression levels of cardiac maker proteins (including GATA4, cTnI, Connexin45, and DHPR/RYR_2) were significantly higher in cells treated with virus than in the controls, and the highest in cells infected with Ad-*Nkx2.5* (Fig. 5). Interestingly, the number of beating clones was significantly reduced when cultures were infected with the Ad-*cardiac a-actin* ($P < 0.05$) and/or Ad-*Nkx2.5* ($P < 0.05$) viruses; the fewest beating cells were found in the cultures infected with both viruses simultaneously ($P < 0.05$) (Fig. 6C).

Altogether, these experiments showed that overexpression of *Nkx2.5* and/or *cardiac a-actin* in mouse ADSCs increased the expression of cardiac-related proteins, but inhibited cell beating.

Discussion

We confirmed that mouse ADSCs can spontaneously differentiate into beating cardiomyocytes and expressed cardiac-specific markers when cultured in DMEM supplemented with NBS (without the addition of any cytokines, 5-azacytidine, or antioxidants). And over-expression of Nkx2.5 and cardiac α-actin were capable of increasing the number of cardioac-like cells expressing cardiac marker

proteins, however, we observed inhibition of beating function, which was contrary to our expect.

SVF cells represent a heterogeneous cell population [7]; the adherent cell fraction remained after culture is known as ADSCs [26]. Several previous methods have been identified for inducing ADSCs or SVF cells to differentiate into cardiomyocytes; these include treatment with 5-aza-cytidine [14], culture in semisolid methylcellulose medium (MethoCult GF M3534, main recipes are rh-Insulin, 2-Mercaptoethanol, rm-Stem Cell Factor, rm-IL-3, rh-IL-6, etc.) [10], and co-culture with contracting cardiomyocytes [15]. We found that ADSCs could spontaneously differentiate into beating myotubes and rounded cells when cultured in DMEM supplemented only with 15% (data was not shown) or 20% NBS. In comparison to previous studies, we observed fewer beating cells with a shorter contracting period; however, our results showed that just very basic conditions (some unknown components existed in serum) were required for transformation of ADSCs into cardiomyocytes. Although these additional components (including 5-azacytidine, IL-3, IL-6, 2-Mercaptoethanol, etc.) are non-essential for the cardiac differentiation of ADSCs into cardiomyocytes, they could increase the differentiation rate via activating some key genes expression. Our results also supported previous speculation that some heretofore unknown serum components affected the ability and efficiency of ADSCs to spontaneously differentiate into cardiomyocytes, and that the source of serum may
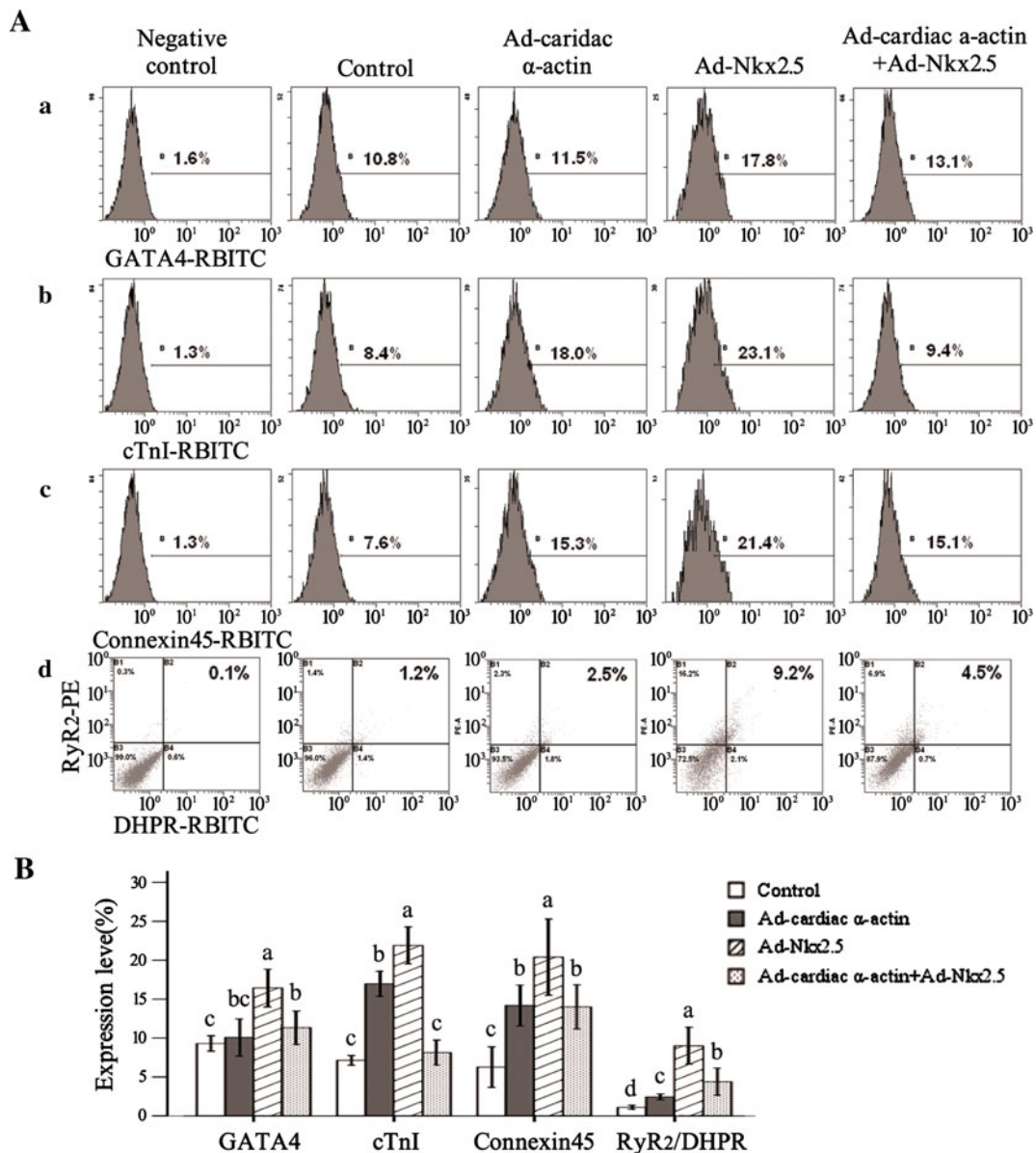
Fig. 5 Expression analysis of cardiac-specific proteins in ADSCs-derived cells. **A** Results from FACS analysis of cells from each of the different treatment groups, collected 3 days after the presence of beating cells was first detected. Cells were assayed for the presence of GATA4 (*a*), cTnI (*b*), Connexin45 (*c*), and RyR$_2$/DHPR (*d*) in order to generate a ratio of cardiac-specific protein-positive cells take up the total cells number that cultured in dish. **B** Quantitative analysis for **A**. *Different letters* show significant difference, $P < 0.05$. The results are the means of eight independent experiments for each group, respectively

directly affected the efficiency of the differentiation process [16].

Upregulation of cardiac-specific transcription factors is considered one of the first step of the commitment to cardiac differentiation [24]. In present study, we found approximately 6–10% primary ADSCs were positive for cardiac marker proteins (including GATA4, cTnI, Connexin45, etc.). However, only ∼1% of the ADSC-derived cells tested positive for cardiac-specific L-type Ca^{2+} channel flow (DHPR$^+$RYR$_2^+$). This discrepancy may be due to the fact that the presence of L-type Ca^{2+} channel

flow may be a further sign of cardiac differentiation after the up-regulation of cardiac marker proteins, however, NBS alone may be insufficient to enable the further spontaneous differentiation of cells that has undergone the first step of cardiac differentiation. What's more important is that the results of immunofluorescence showed that the subcellular distribution of Nkx2.5 was detected in both nuclei and plasma, which was in accordance with that of Kodama H and Armiñán A's researches [27, 28], and prove the point that Nkx2.5 undergoes nuclear translocation [28]. Since Nkx2.5 and cardiac α-actin are closely involved in
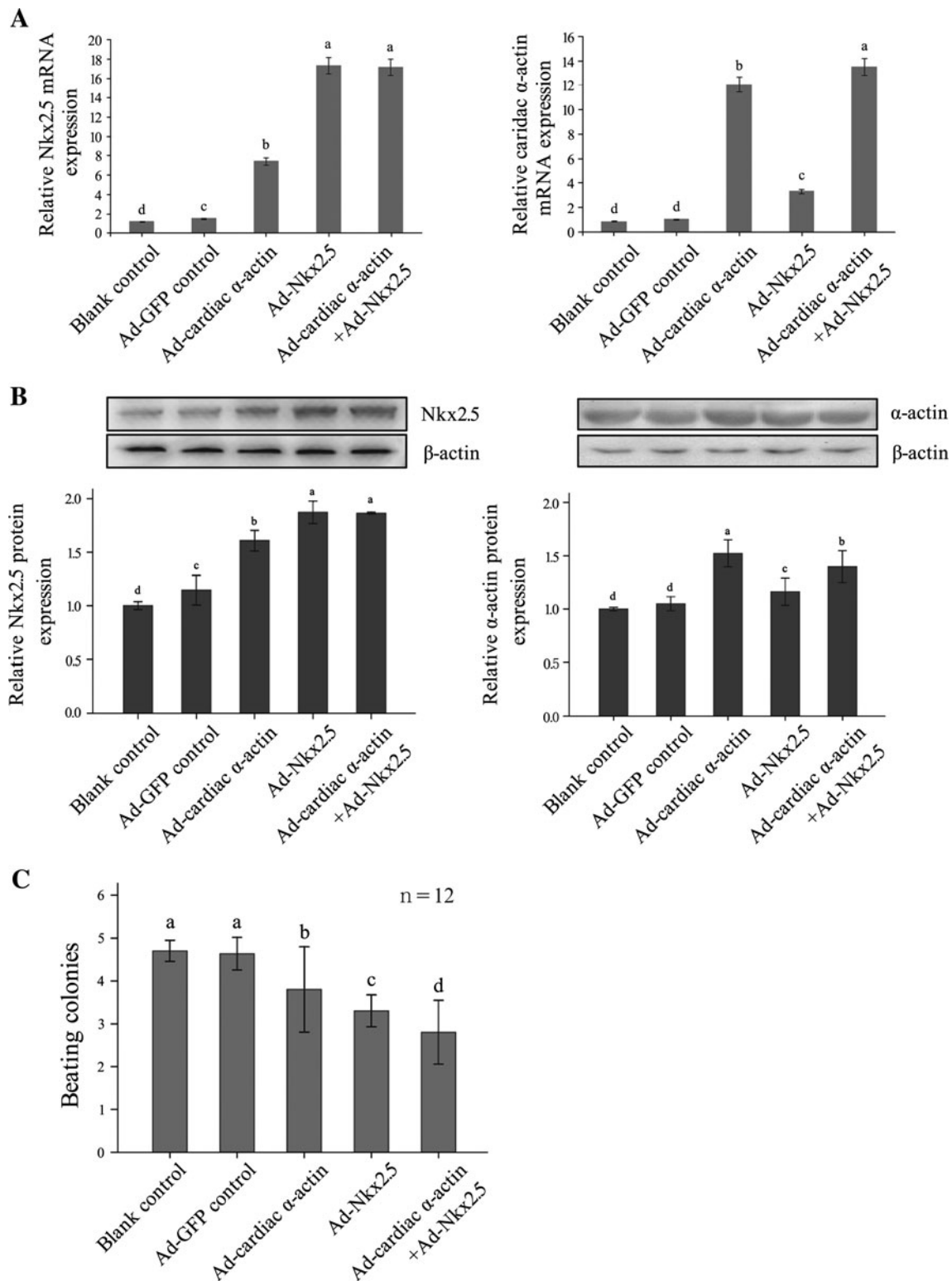
Fig. 6 Effect of Nkx2.5 and/or cardiac α-actin over-expression on the differentiation of ADSCs into cardiomyocytes. **A** Real time PCR analysis of Nkx2.5 (*left*) and cardiac α-actin (*right*) mRNA expression 48 h after infection with the *Ad-Nkx2.5* and/or Ad-*cardiac α-actin* virus. **B** Western blot analysis of Nkx2.5 (*left*) and α-actin (*right*) protein expression 72 h after infection with the *Ad-Nkx2.5*

and/or Ad-*cardiac α-actin* virus. **C** The average numbers of beating cells were obtained from every 1,000 plated cells in the five different treatment groups, as counted 5 days after beating cells were first detected in the control group. *Different letters* show significant difference, *P* < 0.05. The results are the means of eight independent experiments for each group, respectively

heart development and cardiomyocytes contraction, that over-expression of Nkx2.5 can promote the transformation of P19 cells into cardiomyocytes [29], and cardiac α-actin protein is an integral part of the contraction function, but inhibits it when activated too early [30], so we hope to improve the cardiac differentiation efficiency of ADSCs by over-expressing of the two proteins. In keeping with previous findings [20, 31–33], we found that over-expression of Nkx2.5 and cardiac α-actin can promote the expression of cardiac marker proteins in ADSCs, indicating that Nkx2.5 and cardiac α-actin could induce more ADSCs to differentiate into cardiocmyocyte-like cells. Consistent with the role of Nkx2.5 in regulating heart development related genes expression (such as ANF, MLC2V, MEF2c, α-sarcomeric actin, β-MHC, etc. [34–36]), we found that the cardiac differentiation efficiency in Ad-Nkx2.5 infected group was the highest among the treated groups. By counting the beating clones among adenovirials infected groups and control groups, respectively. Being unprecipitated, we found the two adenovirals respectively or together could not increase the formation of beating colonies and actually decreased cell contraction ability. Besides, we found that the contraction ability was lowest in cells infected with both viruses simultaneously. Wei [30] reported that a precocious activation of cardiac α-actin might promote formation of sarcomeric structures that could impair myocyte motility, however, we could not find a direct relationship between the number of beating cells and the expression level of Nkx2.5 or cardiac α-actin.

Cumulatively, we have shown that ADSCs could spontaneously differentiate into beating cardiomyocytes in vitro under the normal culture conditions. A very important finding is that the formation of beating clones is inhibited by overexpression of Nkx2.5 and cardiac α-actin. Further studies are needed to identify which signal pathways resulted in the contraction of cardiomyocytes, and what inhibited the first step of cardiac differentiation (the cardiomyocytes markers were much lower in Ad-Nkx2.5 and Ad-cardiac α-actin than that of Ad-Nkx2.5 treated group).

References

1. Vidarsson H, Hyllner J, Sartipy P (2010) Differentiation of human embryonic stem cells to cardiomyocytes for in vitro and in vivo applications. Stem Cell Rev Rep 6:108–120
2. Beltrami AP, Barlucchi L, Torella D, Baker M, Limana F, Chimenti S, Kasahara H, Rota M, Musso E, Urbanek K, Leri A, Kajstura J, Nadal-Ginard B, Anversa P (2003) Adult cardiac stem cells are multipotent and support myocardial regeneration. Cell 114:763–776
3. Pallante BA, Duignan I, Okin D, Chin A, Bressan MC, Mikawa T, Edelberg JM (2007) Bone marrow Oct3/4+ cells differentiate into cardiac myocytes via age-dependent paracrine mechanisms. Circ Res 100:1–11
4. Oyama T, Nagai T, Wada H, Naito AT, Matsuura K, Iwanaga K, Takahashi T, Goto M, Mikami Y, Yasuda N, Akazawa H, Uezumi A, Takeda S, Komuro I (2007) Cardiac side population cells have a potential to migrate and differentiate into cardiomyocytes in vitro and in vivo. J Cell Biol 176:329–341
5. Zhang J, Wilson GF, Soerens AG, Koonce CH, Yu J, Palecek SP, Thomson JA, Kamp TJ (2009) Functional cardiomyocytes derived from human induced pluripotent stem cells. Circ Res 104:30–41
6. Madonna R, Geng YJ, De Caterina R (2009) Adipose tissue-derived stem cells: characterization and potential for cardiovascular repair. Arterioscler Thromb Vasc Biol 29:1723–1729
7. Léobon B, Roncalli J, Joffre C, Mazo M, Boisson M, Barreau C, Calise D, Arnaud E, André M, Pucéat M, Pénicaud L, Prosper F, Planat-Bénard V, Casteilla L (2009) Adipose-derived cardiomyogenic cells: in vitro expansion and functional improvement in a mouse model of myocardial infarction. Cardiovasc Res 83:757–767
8. Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, Benhaim P, Lorenz HP, Hedrick MH (2001) Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng 7:211–228
9. Bacou F, el Andalousi RB, Daussin PA, Micallef JP, Levin JM, Chammas M, Casteilla L, Reyne Y, Nouguès J (2004) Transplantation of adipose tissue-derived stromal cells increases mass and functional capacity of damaged skeletal muscle. Cell Transplant 13:103–111
10. Planat-Bénard V, Menard C, André M, Puceat M, Perez A, Garcia-Verdugo JM, Pénicaud L, Casteilla L (2004) Spontaneous cardiomyocyte differentiation from adipose tissue stroma cells. Circ Res 94:223–229
11. Kim MR, Jeon ES, Kim YM, Lee JS, Kim JH (2009) Thromboxane A2 induces differentiation of human mesenchymal stem cells to smooth muscle-like cells. Stem Cells 27:191–199
12. Erickson GR, Gimble JM, Franklin DM, Rice HE, Awad H, Guilak F (2002) Chondrogenic potential of adipose tissue-derived stromal cells in vitro and in vivo. Biochem Biophys Res Commun 290:763–769
13. Gimble J, Guilak F (2003) Adipose-derived adult stem cells: isolation, characterization, and differentiation potential. Cytotherapy 5:362–369
14. Rangappa S, Fen C, Lee EH, Bongso A, Sim EK (2003) Transformation of adult mesenchymal stem cells isolated from the fatty tissue into cardiomyocytes. Ann Thorac Surg 75:775–779
15. Choi YS, Dusting GJ, Stubbs S, Arunothayaraj S, Han XL, Collas P, Morrison WA, Dilley RJ (2010) Differentiation of human adipose-derived stem cells into beating cardiomyocytes. J Cell Mol Med 14:878–889
16. Jumabay M, Zhang R, Yao Y, Goldhaber JI, Boström KI (2010) Spontaneously beating cardiomyocytes derived from white mature adipocytes. Cardiovasc Res 85:17–27
17. Matsumoto T, Kano K, Kondo D, Fukuda N, Iribe Y, Tanaka N, Matsubara Y, Sakuma T, Satomi A, Otaki M, Ryu J, Mugishima H (2007) Mature adipocyte-derived dedifferentiated fat cells exhibit multilineage potential. J Cell Physiol 215:210–222
18. Kazama T, Fujie M, Endo T, Kano K (2008) Mature adipocyte-derived dedifferentiated fat cells can transdifferentiate into skeletal myocytes in vitro. Biochem Biophys Res Commun 377: 780–785

19. Briggs LE, Takeda M, Cuadra AE, Wakimoto H, Marks MH, Walker AJ, Seki T, Oh SP, Lu JT, Sumners C, Raizada MK, Horikoshi N, Weinberg EO, Yasui K, Ikeda Y, Chien KR, Kasahara H (2008) Perinatal loss of Nkx2-5 results in rapid conduction and contraction defects. Circ Res 103:580–590

20. Yamada Y, Sakurada K, Takeda Y, Gojo S, Umezawa A (2007) Single-cell-derived mesenchymal stem cells overexpressing Csx/Nkx2.5 and GATA4 undergo the stochastic cardiomyogenic fate and behave like transient amplifying cells. Exp Cell Res 313: 698–706

21. Sassoon DA, Garner I, Buckingham M (1988) Transcripts of α-cardiac and α-skeletal actins are early markers for myogenesis in the mouse embryo. Development 104:155–164

22. Chen CY, Schwartz RJ (1996) Recruitment of the tinman homolog Nkx-2.5 by serum response factor activates cardiac a-actin gene transcription. Mol Cell Biol 16:6372–6384

23. Tholpady SS, Katz AJ, Ogle RC (2003) Mesenchymal stem cells from rat visceral fat exhibit multipotential differentiation in vitro. Anat Rec A Discov Mol Cell Evol Biol 272:398–402

24. Madonna R, De Caterina R (2008) In vitro neovasculogenic potential of resident adipose tissue precursors. Am J Physiol Cell Physiol 295:1271–1280

25. He TC, Zhou S, da Costa LT, Yu J, Kinzler KW, Vogelstein B (1998) A simplified system for generating recombinant adenoviruses. Proc Natl Acad Sci USA 95:2509–2514

26. Dubois SG, Floyd EZ, Zvonic S, Kilroy G, Wu X, Carling S, Halvorsen YD, Ravussin E, Gimble JM (2008) Isolation of human adipose-derived stem cells from biopsies and liposuction specimens. Methods Mol Biol 449:69–79

27. Kodama H, Hirotani T, Suzuki Y, Ogawa S, Yamazaki K (2002) Cardiomyogenic differentiation in cardiac myxoma expressing lineage-specific transcription factors. Am J Pathol 161:381–389

28. Armiñán A, Gandía C, Bartual M, García-Verdugo JM, Lledó E, Mirabet V, Llop M, Barea J, Montero JA, Sepúlveda P (2009) Cardiac differentiation is driven by NKX2.5 and GATA4 nuclear translocation in tissue-specific mesenchymal stem cells. Stem Cells Dev 18:907–918

29. Skerjanc IS, Petropoulos H, Ridgeway AG, Wilton S (1998) Myocyte enhancer factor 2C and Nkx2-5 up-regulate each other's expression and initiate cardiomyogenesis in P19 cells. J Biol Chem 273:34904–34910

30. Wei L, Roberts W, Wang L, Yamada M, Zhang S, Zhao Z, Rivkees SA, Schwartz RJ, Imanaka-Yoshida K (2001) Rho kinases play an obligatory role in vertebrate embryonic organogenesis. Development 128:2953–2962

31. Yamada Y, Yokoyama S, Wang XD, Fukuda N, Takakura N (2007) Cardiac stem cells in brown adipose tissue express CD133 and induce bone marrow nonhematopoietic cells to differentiate into cardiomyocytes. Stem Cells 25:1326–1333

32. Monzen K, Zhu W, Kasai H, Hiroi Y, Hosoda T, Akazawa H, Zou Y, Hayashi D, Yamazaki T, Nagai R, Komuro I (2002) Dual effects of the homeobox transcription factor Csx/Nkx2-5 on cardiomyocytes. Biochem Biophys Res Commun 298:493–500

33. Riazi AM, Lee H, Hsu C, Van Arsdell G (2005) CSX/Nkx2.5 modulates differentiation of skeletal myoblasts and promotes differentiation into neuronal cells in vitro. J Biol Chem 280: 10716–10720

34. Tanaka M, Chen Z, Bartunkova S, Yamasaki N, Izumo S (1999) The cardiac homeobox gene Csx/Nkx2.5 lies genetically upstream of multiple genes essential for heart development. Development 126:1269–1280

35. Chen CY, Schwartz RJ (1997) Competition between negative acting YY1 versus positive acting serum response factor and tinman homologue Nkx-2.5 regulates cardiac alpha-actin promoter activity. Mol Endocrinol 11:812–822

36. Morkin E (2000) Control of cardiac myosin heavy chain gene expression. Microsc Res Tech 50:522–531