

# Directional differentiation of human embryonic stem cells into cardiomyocytes by direct adherent culture

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Myocardial infarction is a serious and common disease in clinics, with a poor prognosis and a high mortality in the long term. The latest research progress in stem cell research, which has been at the frontier of medical research in recent years, gives hope to the patients with heart disease. Researchers have been trying to find an efficient and practical differentiation procedure to induce embryonic stem cells (ESCs) to differentiate into cardiomyocytes. This study used a direct adherent-culture method to induce the differentiation of human ESCs (hESCs) into cardiomyocytes *in vitro* and its differentiation efficiency was detected. The hESCs were induced into cardiomyocytes by adherence culture using the inducers activin A and BMP4. The time of appearance of beating cardiomyocytes, the percentage of beating colonies, and the beating frequency of cardiomyocytes under the microscope were observed and counted; the specific cardiomyocyte marker cTnT was started by immunofluorescence, and the electrophysiological function of cardiomyocytes was detected by patch clamp experiment. An apoptosis-Hoechst staining kit was used to detect the apoptosis ratio of beating of cardiomyocytes which had been treated by hypoxia for 24 hours. Widespread spontaneous beating cardiomyocytes was typically observed by day 13 after differentiation. The statistical result was that the average time of appearance of beating cardiomyocytes was  $13.0 \pm 1.1$  days, the percentage of beating colonies was 66.7%, and the beating frequency of cardiomyocytes  $63.0 \pm 7.0$  times/min; beating cardiomyocytes were positive to cTnT staining. Spontaneous action potentials of beating cardiomyocytes were detected, and the apoptosis ratio of beating cardiomyocytes which had been treated by hypoxia for 24 hours was  $8.0 \pm 0.5\%$ . The direct adherent-culture method was successfully used to induce the differentiation of hESCs into cardiomyocytes. The adherent method of hESC induced with activin A + BMP4 was determined to be more simple and effective than other methods. The differentiation efficiency reached 66.7%, and the differentiation time was about 13 days.

**Keywords:** Adherence method, Cardiomyocytes, Differentiation, Human embryonic stem cells

## Introduction

Myocardial infarction is a serious and common disease in clinics, with a poor prognosis and a high mortality in the long term. The mature mammalian heart has limited regenerative capacity. If damage occurs to a significant number of cardiomyocytes, it can be irreparable, which can seriously impair ventricular function and eventually lead to heart failure. Although there has been noticeable progress in pharmacology, interventional therapy, and

surgical treatment, the prognosis of patients with heart failure remains poor. At present, organ transplantation is the only effective treatment for chronic heart failure. The morbidity of myocardial infarction increased obviously all over the world in recent years and better treatments are urgently needed in the clinic.<sup>1</sup> In view of the important medical issues, reconstructing the damaged heart with new cardiomyogenic cells is an attractive method. The latest progress in stem cell research, which has been at the frontier of medical research in recent years, gives hope to the patients with heart disease.<sup>2,3</sup> Embryonic stem cells (ESCs) have the greatest developmental totipotency and the highest

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research value among all stem cell types. The experimental methods of isolating and culturing ESCs *in vitro* have been well established, and ESCs can be greatly expanded in culture and differentiated into definitive cardiomyocytes.<sup>4-6</sup> In addition, cardiomyocytes induced from ESCs have powerful proliferative capacity both *in vitro* and *in vivo* following implantation,<sup>7-9</sup> implying that delivery of an initially subtherapeutic cell dose may suffice to obtain a functionally meaningful cardiac implant over time. But most methods are complicated and have low differentiation efficiency; there are many difficulties in clinical application. In order to create a differentiation method which is more simple and effective than other methods, this study used a direct adherent-culture method to induce hESCs, which has great significance in clinical application, to differentiate into cardiomyocytes successfully *in vitro* and achieve a high differentiation efficiency.

## Materials and Methods

### *Propagation of human ESC (hESC) and mouse ESC (mESC) lines*

The X-01 hESC line was obtained from Sidansai Biotechnology Co., Ltd. (Shanghai, China) Continuous cultures of the X-01 cell line were grown on 0.2% gelatin-coated mitomycin C (10 µg/ml; Sigma) inactivated mouse embryonic fibroblast (MEF) feeders in standard hESC culture medium consisting of 80% KO- (Dolbecco's modified Eagle's medium DMEM; Gibco, Grand Island, NY, USA), 20% serum replacement (Gibco), 1% non-essential amino acid solution (Gibco), 1 mM L-glutamine (Gibco), 0.1% beta-mercaptoethanol (Sigma, St. Louis, MO, USA), and human basic fibroblast growth factor (bFGF, Gibco). Media were replenished every day with media pre-equilibrated in an incubator for 2 hours at 37°C and 5% CO<sub>2</sub>, and cells were passaged every fifth/sixth day by incubation in 200 units/ml collagenase IV for 5–10 minutes at 37°C and then dissociated. The cultures were maintained at 37°C and 5% CO<sub>2</sub> in air and used for characterization and differentiation studies. MEF feeders were cultured in DMEM High Glucose (Gibco) supplemented with 10% fetal bovine serum (Gibco), 1% non-essential amino acid solution, and 1 mM L-glutamine.

### *Differentiation of cardiomyocytes via adherent culture*

Undifferentiated hESCs were dissociated into clumps using 200 U/ml collagenase IV at 37°C for 5–10 minutes and seeded onto Matrigel-coated plates at a density of 100 000 cells/cm<sup>2</sup>. Medium were refreshed daily with MEF-CM (management with mitomycin) plus 8 ng/ml bFGF for 6–10 days. To induce cardiac differentiation, MEF-CM was

replaced with Roswell Park Memorial Institute (RPMI) B27 medium (Gibco) supplemented with the following cytokines: 150 ng/ml human recombinant activin A (Gibco) for 36 hours, followed by 15 ng/ml human recombinant bone morphogenetic protein 4 (BMP4; Gibco) for 6 days. The medium was then exchanged for RPMI-B27 without supplementary cytokines; culture medium was refreshed every 1–2 days for 2–3 additional weeks. Widespread spontaneous beating activity was typically observed by day 13 after addition of activin A.

### *Immunostaining*

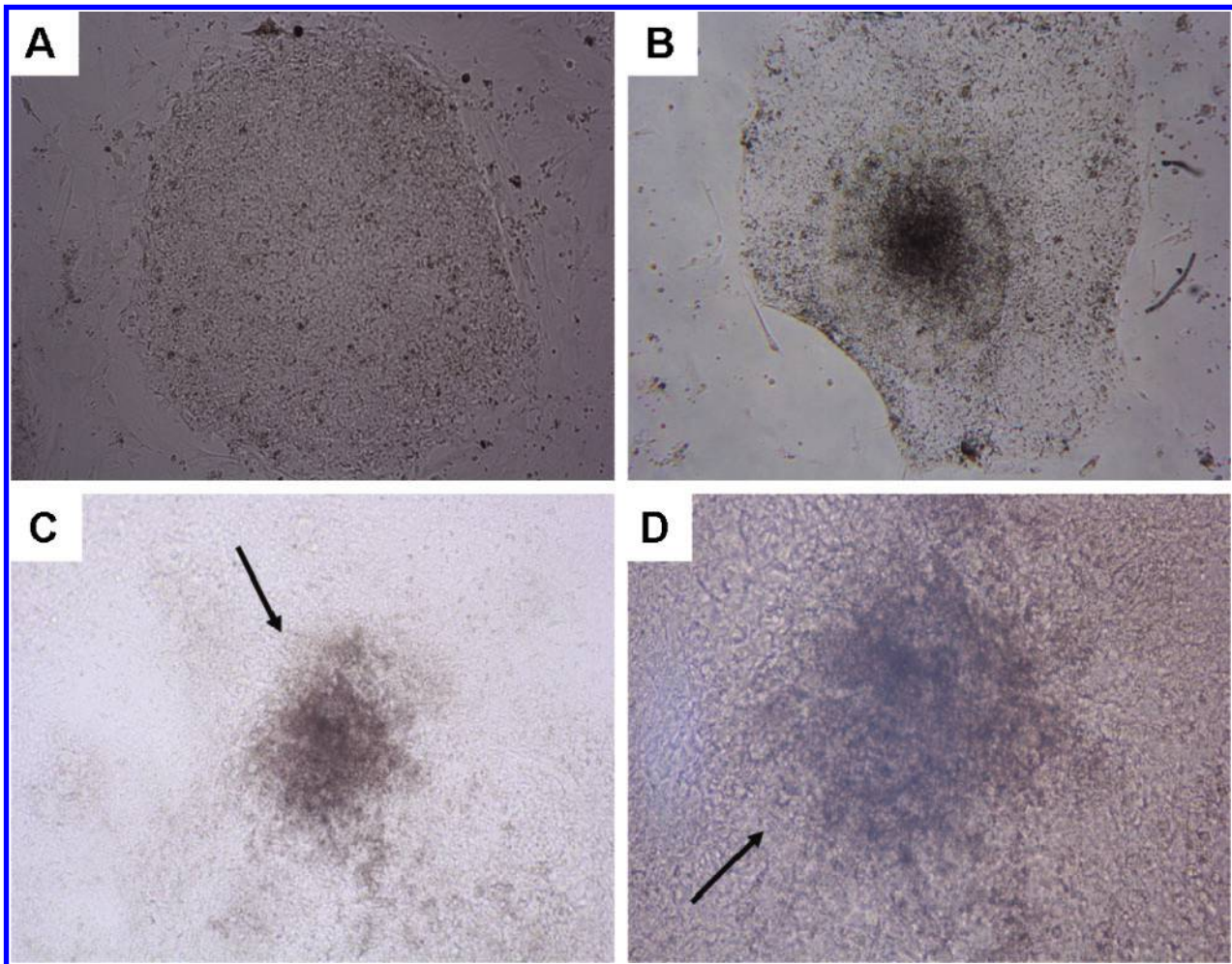
For immunostaining, hESC were induced to differentiate into beating colonies through the above protocol. Following this, beating colonies were fixed in 4% paraformaldehyde for 10 minutes, washed three times with phosphate buffered saline (PBS; Gibco), and permeabilized with 0.4% triton X-100 at room temperature for 10 minutes. Colonies were then washed three times with PBS, blocked with 5% bovine serum albumin (BSA) for 30 minutes, then incubated with primary antibody against cTnT at 4°C overnight. After washing, the colonies were exposed to the corresponding secondary antibody at room temperature for 45 minutes. The colonies were then washed again, and mounted with Vectashield medium for photomicroscopy.

### *Electrophysiological recordings*

Beating colonies were disassociated into isolated cardiomyocytes by collagenase or trypsin and, after culturing for 36 hours, beating cardiomyocytes were selected for patch-clamp experiments. Spontaneous action potentials of cardiomyocytes were recorded by current-clamp technique with an Axon-200 A amplifier. The glass microelectrodes were filled with a solution containing: 50 mM KCl, 80 mM KAsp, 1 mM MgCl<sub>2</sub>, 3 mM MgATP, 10 mM HEPES, and 10 mM ethylene glycol tetraacetic acid (pH 7.4 with KOH); the electrode resistance was between 2 and 4 MΩ. Cells were superfused with a bathing solution that contained: 140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl<sub>2</sub>, MgCl<sub>2</sub> 10 mM HEPES, and 10 mM D-glucose (pH 7.4 with NaOH). Experiments were conducted at 37°C.

### *Hoechst 33258 staining*

A Hoechst 33258 cell apoptosis staining kit (Beyotime; Nanjing, China) was used to confirm morphological changes in the nuclei. Cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature, then washed three times with cold PBS, and exposed to 10 mg/l Hoechst 33258 in the dark for 5 minutes. Following this, cells were washed again with PBS, and the stained cells were examined and immediately photographed under a fluorescence



**Figure 1** (A) hESC clone; (B) the second day of differentiating of hESC clone; (C and D) beating colony induced from hESC.

microscope at an excitation wavelength of 330–380 nm. Apoptotic cells were identified on the basis of morphological changes in their nuclear assembly by observing chromatin condensation and fragmentation by Hoechst 33258 staining. In each group, 10 microscopic fields were randomly selected and counted.

#### *Statistical analysis*

All experiments were performed at least three times, and data were expressed as mean  $\pm$  standard deviation and analyzed by Student's *t*-test or one-way ANOVA with post-hoc analysis. A value of  $P < 0.05$  was considered statistically significant.

## **Results**

### *Generation of beating cardiomyocytes*

Under the microscope, hESC clones had clear boundaries, flat nest-like shapes, and high nucleus to cytoplasm ratios (Fig. 1A). The hESCs grew relatively slowly, and the passage time was about

5–6 days. After culture in matrigel-coated dishes for 6–10 days, cells grew to clones of normal size. Then activin A was added and BMP4 was put in after 36 hours. After the beginning of differentiation, the clones extended outward, with the middle part raised slightly (Fig. 1B). After about 7-day differentiation, beating colonies began to appear, and most clones were widely spread. The thick inner sections of the clones were unequal and, sometimes, there were multiple beating areas with different sizes, different frequencies, and different amplitudes (Fig. 1C and D). Videos were also recorded.

### *Immunostaining and electrophysiological recordings*

After hESCs were induced to differentiate into beating colonies, they were immunofluorescently stained for the cardiomyocyte-specific marker cTnT. Almost the entire beating colony was positive for cTnT (Fig. 2A and B). The beating colonies were then disassociated

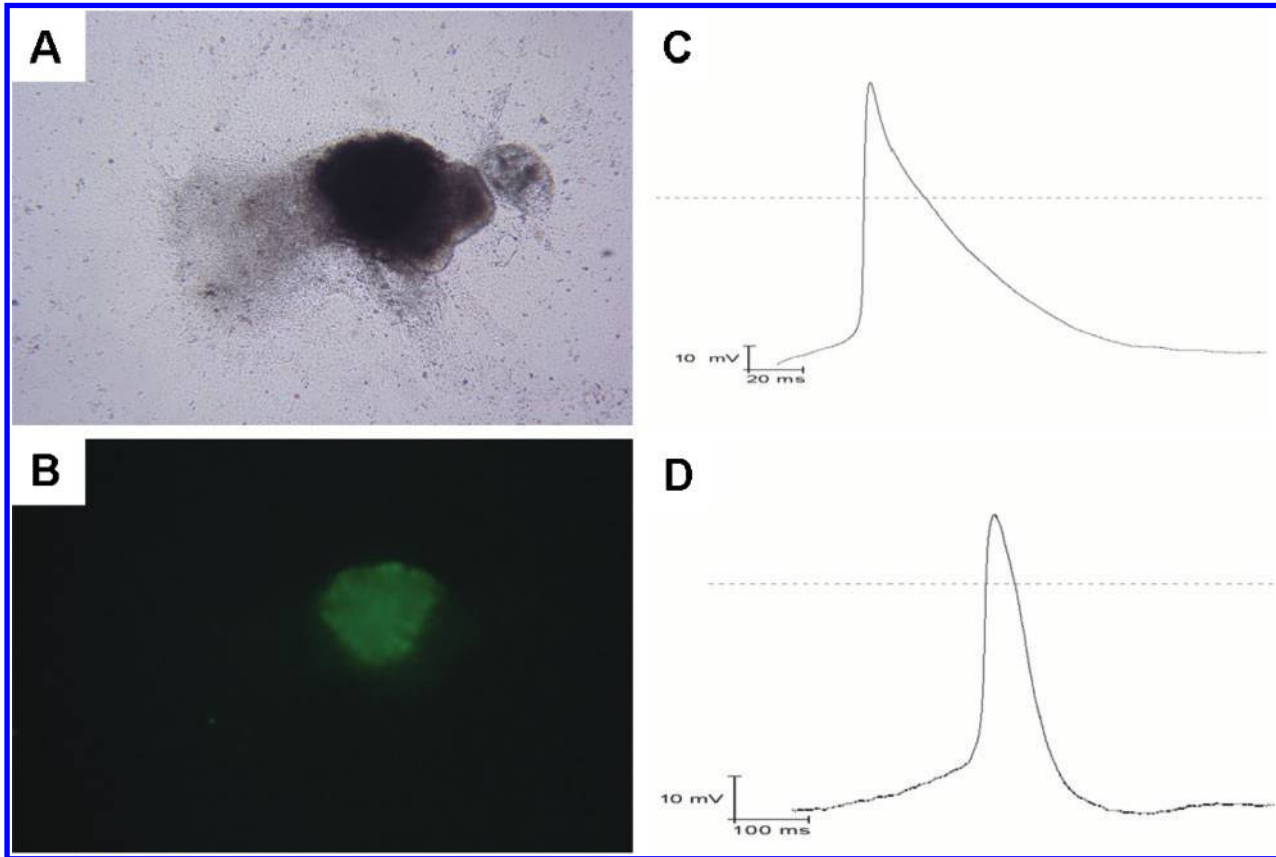


Figure 2 (A and B) Light field of beating colony induced from hESC and immunofluorescence staining of cTnT; (C) spontaneous action potential of atria-like cardiomyocytes induced from hESC; (D) spontaneous action potential of atria-like cardiomyocytes induced from hESC.

into isolated cardiomyocytes for patch-clamp experiments, and spontaneous action potentials were recorded from atria-like cardiomyocytes (Fig. 2C and D).

*Differentiation efficiency and frequency distribution*

From day 5 following adherent differentiation, the percentage of beating colonies and the frequency of

beating was observed and recorded (Fig. 3A and B). As can be seen from the figure, the percentage of beating colonies reached the highest point of 66.7% approximately on the fifteenth day of differentiation, and the beating colonies appeared between the eleventh and fifteenth days of differentiation. The mean time to appearance of beating cardiomyocytes was  $13.0 \pm 1.1$  days. The beating frequency of most

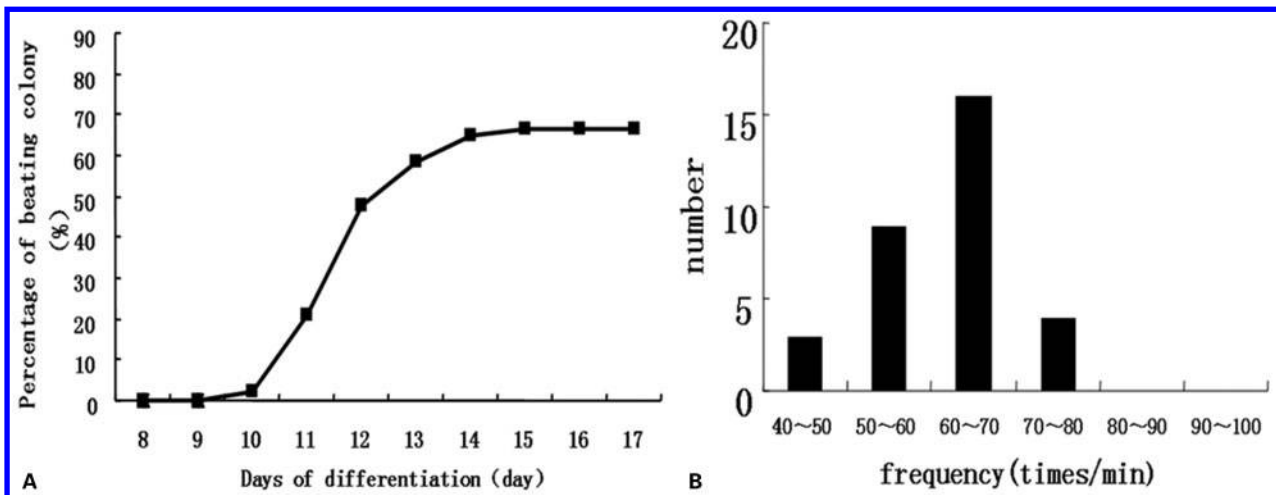
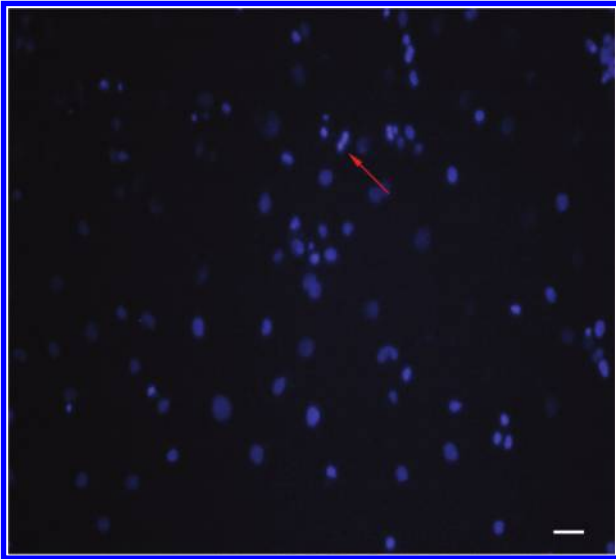


Figure 3 (A) Percentages of beating colonies at different differentiation times; (B) distribution of beating frequency.



**Figure 4** Detection of apoptosis by Hoechst 33285. Apoptotic cells ( $\times 100$ ) were identified as those displaying chromatin condensation and nuclear fragmentation. Scale bar = 50  $\mu\text{m}$ .

colonies distributed between 50 and 70 beats/min, and the average beating frequency was  $63.0 \pm 7.0$  beats/min.

#### Detection of apoptosis by Hoechst 33285

A Hoechst 33258 cell apoptosis staining kit was used to detect the apoptotic ratio of beating cardiomyocytes, which had received the 24-hour hypoxia treatment, and apoptotic cells were identified on the basis of morphological changes in their nuclear assembly by observing chromatin condensation and fragmentation (Fig. 4A). In each group, ten microscopic fields were randomly selected and counted. The apoptotic ratio of the hESC without inducers group was  $8.1 \pm 0.4\%$ , the hESC with inducers group was  $8.0 \pm 0.5\%$ , the mESC without inducers group was  $10.3 \pm 1.3\%$ , and the mESC with inducers was  $10.2 \pm 0.7\%$  (Fig. 4B). The data showed that there was a statistically significant difference between cardiomyocytes induced from the two different cell lines, implying that without other protective factors, the anti-hypoxic ability of cardiomyocytes induced from hESCs was stronger than that of mESCs.

#### Discussion

Cardiovascular diseases are the number one killer in human populations, of which myocardial infarction has the highest incidence. The heart has very limited capacity for regeneration.<sup>10,11</sup> When heart disease develops to the end stage, structural damage to cardiomyocytes leads to irreversible changes in cardiac function, while the compensatory capacity of surviving cardiomyocytes is unable to meet the needs of the

body. In addition, conservative treatment is unable to effectively treat the problem fundamentally, and heart transplantation is limited by the number of donors and immunological rejection.<sup>12</sup> This brings big challenges to the clinical treatment of cardiovascular disease, and there is an urgent need to find a more effective treatment option. With developments in the study of stem cells and tissue engineering technology, stem cell transplantation has become a hotspot in research for the treatment of cardiovascular disease.<sup>13–15</sup> In recent years, with the rapid developments in cell biology, especially stem cell biology, cell therapy has made great progress in the basic research field. At present, stem cells have a wide range of applications in the treatment and research of myocardial infarction, with inspiring achievement and a wide prospect for clinical application. However, as a therapeutic method for a variety of human degenerative diseases, the exact mechanism has not been fully elucidated and, at present, the effectiveness and safety of clinical application is still the focus of debate.<sup>16</sup>

Researchers have been trying to find a differentiation procedure to induce ESCs to differentiate into cardiomyocytes that is highly effective, economically viable, with high yield to meet the demand of clinical application. Studies show that ESCs have a high tendency to differentiate into cardiomyocytes.<sup>17</sup> This study used direct adherent-culture method to induce hESCs, which has great significance in clinical application, to differentiate into cardiomyocytes successfully *in vitro* and achieve a high differentiation efficiency.

The directed differentiation system employed in this study, involving serial application of activin A and BMP4, was developed in response to the need for a clinically scalable system for human cardiomyocyte development. The selection of these two factors and the timing of their application was based on previous research. Activin was proved to be an effective inducer of mesoderm and endoderm in *Xenopus* embryonic explants,<sup>18</sup> and analysis of factors secreted by the chick hypoblast and anterior endoderm showed that the transforming growth factor (TGF)-beta family of secreted factors is expressed at the appropriate stages of development (activin A and TGF-beta in the hypoblast; BMPs in the anterior endoderm) to play a role in cardiac induction.<sup>19–23</sup> Therefore, it is hypothesized that activin would induce generic mesendoderm of undifferentiated hES cells and that subsequent BMP4 would further determine its fate of differentiating into myocardial cells. Indeed, this activin/BMP4-directed differentiation system could largely promote the hESCs to

differentiate into cardiomyocytes and give more consistent yields and purities. However, the human hearts need a much higher proportion of cell preparations. The present work shows that the activin/BMP system scales to larger formats with only minor modifications. This means that large-animal experiments are a realistic possibility and suggests scalability to humans could be possible. Of course, success in the scaled production of these cells will require an improved understanding of the molecular events in hESC self-renewal and early cardiac differentiation, as well as the identification of novel approaches to optimize the maintenance, enrichment, proliferation, and maturation of hESC-derived cardiomyocytes *in vitro*. It is essential to find a method for high differentiation efficiency. This result confirms the acceleration of inducers in cardiomyocyte differentiation but, in reality, inducers are expensive, especially the inducing factors of hESCs, which increase the cost of differentiation. In addition, it was also found that the beating frequency of cardiomyocytes differentiated from hESC was closer to the human heart rate, but more research is needed to confirm whether the cardiomyocyte beating coincides with the beating of the heart after transplantation to the ischemia site. Vitamin C could obviously improve the differentiation of mouse ESCs into cardiomyocytes,<sup>24</sup> and this method is simple and cost-effective. This is really an innovation and this method has great significance. However, vitamin C had no distinct effect on the differentiation into cardiomyocytes from hESC. Only with the dual effects of the inducing factors activin A and BMP4 did the differentiation efficiency of hESC increase. This implied that the induction of mESC into cardiomyocytes was more simple and efficient, while it is more demanding and complex for hESCs to differentiate into cardiomyocytes.

Although the adult cardiac tissue is composed of post-mitotic and terminally differentiated cells, there exists a small group of cardiomyocytes that display similar characteristics to stem cells.<sup>25,26</sup> Nevertheless, their limited availability, difficulty in isolation and restricted growth potential prevent them from being widely used in clinical applications. The method of acquiring ESC-derived cardiomyocytes *in vitro* potentially meets the demand for clinical applications, but there are still many problems that have not been resolved. These are as follows: the efficiency of differentiation into cardiomyocytes is not high enough;<sup>27</sup> the purity of ESC-derived cardiomyocytes is low and there are many other cells types present;<sup>24</sup> the cost of cardiogenic growth factors, time required,

and the scalability is significant; the survival rate of cardiomyocytes is low after transplantation;<sup>28</sup> the risk of arrhythmias following ESC–cardiomyocyte transplantation in injured hearts has not been determined;<sup>29</sup> and, finally, the electromechanical integration of ESC–cardiomyocytes in injured hearts has not been demonstrated, so it is unclear whether these cells improve contractile function directly through the addition of new force-generating units.<sup>30,31</sup> Investigations into the dynamic process of heart development *in situ* have provided critical insights for the improvement of *in vitro* cardiomyocyte differentiation protocols.

## Conclusion

The adherent method of inducing hESCs with activin A + BMP4 is more simple and effective than other methods. This is really an innovation and this method has great significance. In future research, the time-liness and spatiality of cardiomyocyte differentiation should be explored, because their differentiation is controlled by many complicated factors, many of which have unknown roles. Although there is a long way before stem cell research is applied in clinical applications, we believe that in the near future, the herein mentioned problems will be resolved and the widespread clinical application of stem cells will become a reality.

## Disclaimer Statements

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**Funding** None.

**Conflicts of interest** There are no conflicts of interest.

**Ethics approval** We complied with ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration.

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