Engineered heart tissue graft derived from somatic cell nuclear transferred embryonic stem cells improve myocardial performance in infarcted rat heart

Shuanghong Lü a, b, #, Ying Li a, #, Shaorong Gao c, #, Sheng Liu c, #, Haibin Wang a, Wenjun He a, d, Jin Zhou a, Zhiqiang Liu a, Ye Zhang a, Qiuxia Lin a, Cumi Duan a, Xiangzhong (Jerry) Yang a, e, Changyong Wang a, *

a Department of Tissue Engineering, Institute of Basic Medical Sciences and Tissue Engineering Research Center, Academy of Military Medical Sciences, Beijing, China
b Laboratory of Oncology, Affiliated Hospital of Academy of Military Medical Sciences, Beijing, China
c National Institute of Biological Sciences (NIBS), Beijing, China
d Institute of Bioengineering, Academy of Military Medical Sciences, Beijing, China
e Center for Regenerative Biology, University of Connecticut, Storrs, CT, USA

Received: February 9, 2010; Accepted: June 7, 2010

Abstract

The concept of regenerating diseased myocardium by implanting engineered heart tissue (EHT) is intriguing. Yet it was limited by immune rejection and difficulties to be generated at a size with contractile properties. Somatic cell nuclear transfer is proposed as a practical strategy for generating autologous histocompatible stem (nuclear transferred embryonic stem [NT-ES]) cells to treat diseases. Nevertheless, it is controversial as NT-ES cells may pose risks in their therapeutic application. EHT from NT-ES cell-derived cardiomyocytes was generated through a series of improved techniques in a self-made mould to keep the EHTs from contraction and provide static stretch simultaneously. After 7 days of static and mechanical stretching, respectively, the EHTs were implanted to the infarcted rat heart. Four weeks after transplantation, the suitability of EHT in heart muscle repair after myocardial infarction was evaluated by histological examination, echocardiography and multielectrode array measurement. The results showed that large (thickness/diameter, 2–4 mm/10 mm) spontaneously contracting EHTs was generated successfully. The EHTs, which were derived from NT-ES cells, integrated and electrically coupled to host myocardium and exerted beneficial effects on the left ventricular function of infarcted rat heart. No teratoma formation was observed in the rat heart implanted with EHTs for 4 weeks. NT-ES cells can be used as a source of seeding cells for cardiac tissue engineering. Large contractile EHT grafts can be constructed in vitro with the ability to survive after implantation and improve myocardial performance of infarcted rat hearts.

Keywords: myocardial infarction • tissue engineering • somatic cell nuclear transfer • embryonic stem cells

Introduction

Myocardial infarction (MI) leads to cardiomyocyte loss, ventricular remodelling and consequent impairment of myocardial function. Current therapeutic approaches have limited effects in attenuating disease progression. The only successful treatment is heart transplantation. Yet it is used for late-stage patients only and constrained by the shortage of organ supply [1–3]. Recent advancements in stem cell biology, cell therapy and tissue engineering have paved the way for establishing a novel discipline in regenerative medicine. The concept of developing cell-replacement strategies has gained increasing attention [4–7]. Such strategies aim to repopulate scar tissue with new contractile cells in an attempt to restore function in the failing heart.

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doi:10.1111/j.1582-4934.2010.01112.x
Two principal strategies were proposed for myocardial repairs: the cell therapy and the tissue-engineering approach. The former focuses on the direct cell transplantation into the dysfunctional myocardial areas [4–7]. The latter attempts to generate a tissue-engineered muscle construct followed by in vivo engraftment of the engineered tissue [8, 9]. Despite progress in the former strategy, problems such as the low rate of retention and survival still exist [10, 11]. Notwithstanding initial donor cell retention and survival following in vivo engraftment can be improved by using scaffolds, it still falls short for the injected cells to regenerate cardiac tissues to fit the shape of the infarcted area (especially when the distribution of injected cells were uneven) [12–15, 16]. However, it may theoretically allow complete replacement of diseased myocardium or reconstitution of cardiac malformations to implant engineered heart tissue (EHT) [16]. In recent years, progress is made in generating EHT. Among the studies in heart tissue engineering, various sources of seeding cells were used to construct EHT, including terminal differentiated, mesenchymal stem (MSCs) and embryonic stem (ES) cells [7, 8, 16–18]. ES cells have many advantages in direct differentiation into cardiomyocytes [19], capable of integrating with the host heart and electrical conduction [20, 21]. Therefore in theory, ES cells could potentially provide an unlimited supply of cardiomyocytes for cell therapy to regenerate functional myocardium. However, the immunological rejection after the ES cells transplantation makes the application of cell-replacement strategies difficult.

The immunological rejection in cell-replacement strategies can be avoided by using patient-specific cells derived from the induced pluripotent stem (iPS) cells [22] or the nuclear transferred embryonic stem (NT-ES) cells [23]. Although iPS cells can shun ethical controversies, the virus integration and oncogenes application hamper further clinical application. Therapeutic cloning has been successfully performed in mice and non-human primates, whereby somatic cell nuclear transfer is used to generate customized ES cells from differentiated somatic cells of specific individuals [23, 24]. In theory, the NT-ES cells carried the same genome as the donor somatic cells. After directed induction, the differentiated cells could rescue the damaged tissues without immune rejection [25]. However, the persistence of abnormalities in cloned animals has doubted whether ES cells derived by somatic cell nuclear transfer may pose risks in their therapeutic application. [26]. Although it has been demonstrated that the transcriptional profiles and developmental potentials of ES cells derived from cloned blastocysts are identical to those of ES cells derived from fertilized blastocysts (F-ES) [27], there is no report focusing on their ability in engineered tissue construction and damaged tissue repairs up to date. The results from the studies shall provide more sufficient support to the notion that the ES cells derived from cloned blastocysts have a strong therapeutic potential.

In this study, we produced engineered spontaneously contracting heart tissue constructs in vitro from the mouse NT-ES cell-derived cardiomyocytes. After 7 days of static and mechanical stretching, respectively, the EHTs were implanted to the infarcted rat heart. Four weeks after transplantation, the suitability of EHT in heart muscle repairs after MI was evaluated by histological examination, echocardiography and multielectrode array (MEA) measurement.

Materials and methods

Mouse NT-ES cell culture

NT-1 ES cell line was used in the following experiments of this study. Derivation of NT-ES cell lines from cloned blastocysts and pluripotency analysis of NT-ES cells were described in our recent studies, which showed that the NT-ES cell line – NT-1 (proved to be pluripotent) could differentiate into cardiomyocytes in vitro [28]. In the present study, NT-1 ES cells (about 20 passages) were used. (Details of NT-ES cell culture medium and differentiation medium can be found in the Supporting Information.)

NT-EB production in slow-turning lateral vessel (STLV) rotating bioreactor

Nuclear transferred embryoid body (NT-EB) in STLV was prepared by a method described previously by our laboratory [28]. In brief, the NT-ES cells were dissociated with 0.025% trypsin (Sigma-Aldrich, St. Louis, MO, USA) + 0.04% ethylenediaminetetraacetic acid (Sigma-Aldrich) at 37°C for 1 to 2 min., and 0.4–1 × 10^5 cells/ml in culture medium without leukaemia inhibitory factor were transferred into a 250 ml STLV (Synthecon, Inc., Houston, TX, USA) for cell expansion and EB formation. The rotating speed was 10 rpm for the first 12 hrs and was then gradually adjusted to 20 rpm. A total of 50% of the medium was replaced by fresh medium daily. After 3 days in the STLV, 1% ascorbic acid was added into the medium. Two days later, NT-EBs were transferred onto gelatine-coated plates (one to three NT-EBs/cm²). After cultivation in differentiation medium for additional 14 days, the cultures were then examined for the presence of beating cells and subjected to analysis of cardiomyocytes by immunostaining of cardiac troponin T (cTnT; for details, see Supporting Information [Table S1, Fig. S1]).

Percoll enrichment of cardiomyocytes

Differentiated NT-ES cell clusters containing beating cells were dissociated, resuspended in differentiation medium and loaded onto a Percoll (Amersham, Uppsala, Sweden; Pharmacia, Piscataway, NJ, USA) gradient for enrichment of cardiomyocytes as previously described [18, 29]. In brief, Percoll was diluted in a buffer containing 20 mmol/l HEPES and 150 mmol/l NaCl. The gradient consisted of a 40.5% Percoll layer over a layer of 58.5% Percoll. After centrifugation at 1500 × g for 30 min., cell layers could be observed. Cells at different fractions were collected, washed, resuspended in the differentiation medium, plated into chamber slides and cultured for an additional 3 days for immunocytochemical staining (for details, see Supporting Information). A mixture of fractions IV and V was collected for the following experiments of this study (Fig. S2).

EHT construction

The casting moulds were prepared as described previously [30]. A total of 1.0 × 10^5 freshly isolated cardiomyocytes derived from mouse NT-ES cells
were mixed with 1 ml mixture of liquid collagen type I prepared from rat tails, a basement membrane protein mixture (Matrigel; Becton Dickinson Biosciences, San Jose, CA, USA), and concentrated serum-containing culture medium (2× Dulbecco’s modified essential medium [DMEM], 20% foetal bovine serum [FBS], 200 U/ml penicillin and 200 μg/ml streptomycin); pH was neutralized by titration with 0.1 M NaOH. The reconstitution mixture was pipetted into casting moulds and incubated for 45 to 60 min. at 37°C and 5% CO₂ to allow hardening of the reconstitution mixture [31]. Thereafter, 1.5 ml serum-containing culture medium (DMEM, 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin) was added to each dish. After 7 days in culture, EHTs were transferred into a modified stretch device and hooked on the working rods for unidirectional stretch (10%, 2 Hz) for an additional 7 days. Culture medium was changed 12 hrs after EHT casting and then every other day while the culture was maintained in casting moulds. Transferred into the stretch device, the culture medium was changed daily.

**In vitro evaluation of EHTs**

Seven days after culturing and static stretching in casting moulds, the EHTs were strong enough to allow for mechanical stretching. After additional 7 days, histological sections were prepared. Cardiomyocytes as well as non-cardiomyocytes in the EHTs were examined by immunohistochemical staining. Cell viability in EHTs was assessed by Live/Dead Viability/Cytotoxicity assay (Molecular Probes, Inc., Eugene, OR, USA) (for details, see Supporting Information).

**Myocardial infarction and EHT grafting**

We generated MIs through ligation of the left coronary artery (6–0 prolene suture) in sodium pentobarbital-anesthetized (1.5%) female Sprague-Dawley rats (250 ± 10 g) as described previously [15, 32] (for details, see the Supporting Information). Animals meeting the echocardiographic inclusion criterion (fractional shortening <40%) were used in the following experiments. Two weeks after MI, EHTs were sutured (6–0 prolene suture) onto a recipient heart (n = 10). We placed the sutures in healthy myocardium adjacent to the visible infarct scars to arrange the centre of the EHTs just above the latter. The non-infarcted group (n = 10) serving as sham-operation control underwent thoracotomy and cardiac exposure without coronary ligation (suturing without tying the left anterior descending coronary artery), and the untreated MI group (n = 10) serving as negative control was produced by ligation of coronary without implantation of EHT. While, non-contractile grafts (NCG) were produced by formaldehyde (4%) fixation of EHT [16]. Implantation of NCG served as tests for effects of NT-ES derived cardiomyocytes (n = 8). For immunosuppression all rats received immunosuppressants (mg/kg body weight/day: cyclosporin A, 5; methylprednisolone, 5) daily by subcutaneous injection.

**In vivo evaluation of EHTs**

Four weeks after transplantation, the suitability of EHT in heart muscle repairs after MI was evaluated by echocardiography, MEA measurement and histological examination, (for details, see the Supporting Information).

**Statistical analysis**

Results are reported as mean ± standard deviation. ANOVA and Tukey’s multiple comparison tests were used to determine possible significant differences in the cardiac function between groups. ANOVA with Mann-Whitney U-test was used to determine possible significant differences in the total activation time between groups. Significance was accepted at P < 0.05.

**Results**

**Construction of the EHT**

A total of ≈8.7 \times 10^7 cells containing more than 49% cTnT⁺ cardiomyocytes were procured (see the Supporting Information). To improve the size and quality of EHTs, we used a self-made mould during the reconstruction. We have reported previously that the four glass columns standing in the moulds could provide static stretch and prevent the collagen/Matrigel from gradual contraction during the cultivation. Then helped to avoid thickening the construction and facilitate the survival of the cells in the centre of the reconstructed tissues [30]. In this study, the EHTs condensed after 1 day of culture and contracted gradually (Fig. 1A). The EHTs without the static stretching will contract into contact lenses-like structures in 7 days. They were unable to be transferred into the stretch device for mechanical stretching (Fig. 1D). Spontaneously contracting cell clusters could be observed in 4–9 days. The beating clusters increased in size with the time and contracted independently from each other. The coherent contraction of the complete EHT was achieved 7 days after mechanical stretching. Over time, contractions became more regular, and the beating rates could reach 1.1 Hz and even 1.2 Hz at day 10. After 7 days of mechanical stretching, the EHT beat regularly and unidirectionally along the direction of stretching (Fig. 1B). Upon removal from the stretching device, the EHT (diameter, ~10 mm; thickness, 2–4 mm) beat continuously at ~1.1 Hz for >10 days (Fig. 1C).

**In vitro evaluation of EHTs**

Sections of paraffin-embedded EHTs revealed that the complex of multicellular aggregates in the EHT patch was formed. The cells in the EHTs had the tendency of spread along the long axis of stretching, although many cells were haphazardly arranged. The cells had oval or round nuclei. The pyknotic nuclei could be found frequently, too. In addition, the cells exhibited less clear cross-striation, scant cytoplasm, high ratio of nuclei and plasma, and less organized distribution and compact overall structure (Fig. 2A). In comparison with mature adult rat myocardium (Fig. 2C), histological features of cells in EHTs resembled those of myocytes within immature neonatal rat more, especially in the high ratio of nuclei and plasma (Fig. 2B) [33]. In order to investigate the cell viability in EHTs, a Live/Dead Viability/Cytotoxicity assay was performed.
The results showed that the cell viability of EHTs in vitro was 92 ± 4.2% (see Supporting Information [Fig. S3]). These results illustrate the survival and rearrangement of the NT-ES-derived cells in the EHTs, a prerequisite for cardiac tissue engineering.

**Cell type identification in EHT**

The EHTs showed immunoreactivity for a variety of cardiac markers, including the cardiac transcription factors GATA-4 and Nkx2.5, cTnT and α-sarcomeric actinin. The transcription factors GATA-4 and Nkx2.5 are expressed in pre-cardiac mesoderm and persist in the heart during development. GATA-4 and Nkx2.5 immunoreactivity were found in nuclei of a large number of cells in EHTs, indicating the presence of NTES-derived cardiomyocytes (Fig. 2D, E). The percentages of GATA-4 nuclei and NKX2.5 nuclei were determined by dividing the number of GATA-4 nuclei and NKX2.5 nuclei by the total number of nuclei counted in each section, respectively. The results showed that the EHTs contained 51.6 ± 6.5% GATA-4 ‑ nuclei, and 53.2 ± 8.7% Nkx2.5 ‑ nuclei. The cTnT ‑ cells formed cell strands and interconnected cell bundles in most parts within the EHT patch (Fig. 2F). The α-sarcomeric actinin also expressed in the EHTs. However, the sarcomeric organization was obscure, indicating a low degree of development (Fig. 2G). Connexin43 (protein that expected to align following mechanical conditioning) immunostaining demonstrated the development of gap junction between the cells in EHTs (Fig. 2H). Besides myocytes, other types of cells – e.g. endothelial cells (positive for vWAg), fibroblasts (positive for smooth muscle α-actin) and neurons (positive for β-III tubulin) – were also detectable in the EHT patch (Fig. 2I–K). The vWAg ‑ cells indicated the spontaneous formation of vessel-like structures within the EHT patch (Fig. 3I). Fibroblasts were well distributed in the EHTs (Fig. 2J). Neurons scattered throughout EHTs (Fig. 2K).

**Graft effects on ventricular function**

To determine the physiological consequences of implanting EHT into infarcted hearts, we compared the functional outcome with EHTs to those observed with sham-operated non-infarcted and untreated infarcted rats. A third control of NCG implantation was performed to specifically test effects of the NT-ES-derived cells in improving the heart function.

Four weeks after EHT transplantation, all three infarction groups showed ventricular dilation by echocardiography. All infarction groups showed increased LVEDD and LVESD compared with the non-infarcted group (P < 0.01). Yet there was a marked attenuation of an increase in LVEDD and LVESD of the hearts receiving EHT versus NCG and MI controls (P < 0.01) (Fig. 3A, B). LVEDD in EHT-engrafted hearts was 8.2 ± 0.3 mm versus 9.2 ± 0.5 mm in NCG, 9.2 ± 0.4 mm in MI rats. LVESD in EHT-engrafted hearts was 5.9 ± 0.3 mm versus 7.8 ± 0.4 mm in NCG, 8.0 ± 0.2 mm in MI rats. There was no difference in LVEDD and LVESD among NCG and MI control groups at 4 weeks (P > 0.05).

Left-ventricular systolic function (measured by fractional shortening and ejection fraction) declined significantly in infarction groups compared with that in the non-infarcted group (P < 0.01). There was a marked attenuation of the decrease in FS and EF in hearts receiving EHT versus NCG and MI controls (P < 0.01) (Fig. 3C, D). FS in EHT-engrafted hearts was 25.9 ± 1.3% versus 15.1 ± 1.9% in NCG, 13.1 ± 0.9% in MI rats. EF in EHT-engrafted...
hearts was 61.7 ± 2.6% versus 38.1 ± 3.2% in NCG, 34.6 ± 2.0% in MI rats. There was no difference in FS and EF among NCG and MI control groups at 4 weeks (P > 0.05).

Structural integration and integrity of EHT in vivo

Four weeks after grafting, the EHTs could be identified by their appearance and location. Haematoxylin and eosin stained paraffin sections showed that the putative EHT-derived tissue (diameter, 510 ± 26 μm) covered the infarcted myocardium above (Fig. 4A). Immunohistochemistry staining showed the formation of cardiac muscle-like structure of the putative EHTs above the infarcted area (Fig. 4B). The formation of blood vessel like structure in the putative EHT was observed by vWAg immunohistochemistry staining (Fig. 4C). Whether the vessels were of donor origin or had a role in de novo vascularization remains to be investigated. The connexin43 area proved structural intercellular connections inside the putative EHTs and with the putative host native myocardium (Fig. 4D). However, further studies are needed to evaluate whether true electrical coupling occurred.

Electrical coupling of EHT grafts to host myocardium

We assessed electrical coupling of EHTs to the host myocardium 4 weeks after engraftment by MEA. The total activation time on the region of MI, right, left and posterior segments of the investigated hearts were assessed. Sham-operated MI rats (n = 6) showed the expected delay of total activation time in MI segments. In contrast, the total activation time was normal in EHT grafted hearts (n = 6), indicating undelayed anterograde coupling of EHTs to the host myocardium (Fig. 5A). Acidification of the perfusion buffer to pH 6.7 led to uncoupling of EHTs from the native myocardium, proving direct but subnormal graft-host coupling (Fig. 5B).

Discussion

Cardiac tissue engineering approaches are designed to repair damaged heart tissues through the assembly of cells onto biomaterial scaffolds and their implantation into the areas of lesion [34].
Through this technology, functional EHTs can be manufactured to replace native ones. Despite the progress in the cardiac tissue engineering, challenges still remain for future clinical applications, such as immunological rejection after implantation and to construct EHTs at a size with contractile features supporting failing hearts [16]. In this study, we attempted to tackle both issues. By using the NT-ES cells as the source of seeding cells, the EHTs have the potential to solve immune rejection after transplantation. However, risk concerns of using NT-ES cells must be addressed first. By using the self-made mould, we were able to construct EHTs at a size with contractile features. This study is the first to report in vitro EHT constructs from NT-ES cell-derived cardiomyocytes. The data showed that EHTs derived from NT-ES cells integrated, electrically coupled to host myocardium, and exerted beneficial effects on left ventricular function of infarcted rat heart upon transplanting EHTs. They demonstrated that NT-ES cells could be used as a source of seeding cells for cardiac tissue engineering. Large contractile EHT grafts can be constructed in vitro with the ability to survive after implantation and improve myocardial performance of infarcted rat hearts. Our approaches to construct EHTs had the potential for the therapy of heart regeneration.

A qualified EHT construction requires careful calculation and optimization at each step of cardiac tissue engineering, especially in the preparation of seeding cells. We demonstrated the derivation of the EHTs through improved techniques to produce NT-EBs, cardiogenic differentiation and enrichment of cardiomyocytes from differentiated cell mixtures in large amounts. In our previous study, we demonstrated that the NT-EBs formed in STLV bioreactor were more uniform in size [28]. In addition, the STLV-produced NT-EBs differentiated into cardiomyocytes more efficiently (Online Supporting Information). These results suggested that STLV bioreactor provided a more ideal culture condition by facilitating the formation of better quality NT-EBs.

Purification of NT-ES-derived cardiomyocytes is vital in using these cells in tissue engineering or transplantation. This is because there is a mixture of non-cardiac cell types in the cells dissociated from NT-EBs. In our previous works, Percoll enrichment method proved successful in enriching cardiomyocytes from a mouse ES cell line to compare with neonatal rat cardiomyocytes.
tation damage EHTs and host myocardium and thereby destroy structural integration suggested that the procedures of transplanting multiloop EHTs (diameter, 15 mm; thickness, 1–4 mm) beat continuously. The static stretch may also exert positive effect on the EHTs before the mechanical stretch. Under these conditions, the EHT (diameter, ~10 mm; thickness, 2–4 mm) beat continuously at ~1.1 Hz for >10 days upon the removal of the stretching device (Fig. 1C). This study made bold moves to report the addition of static stretch in the construction of EHTs.

Engraftment and functional integration of EHTs in the infarcted heart is critical for successful regenerative therapy. In this study, we took care to study functional consequences of EHTs transplantation by morphological examination, echocardiography and MEA measurement. The results demonstrated that EHTs integrated and electrically coupled to host myocardium and exerted beneficial effects on the left ventricular function of infarcted rat heart. Four weeks after grafting, the connexin43 area demonstrated structural intercellular connections inside the EHTs and with the host native myocardium (Fig. 4D). A possible explanation for the structural integration suggested that the procedures of transplantation damage EHTs and host myocardium and thereby destroy physiological barriers [16]. NCG seemed to exert some beneficial effects on ventricular ejection fraction, possibly resulting from ventricle-stabilizing effects. A marked attenuation of the increase in LVEDD/LVESD and decrease in FS/EF in hearts receiving EHT versus NCG and MI controls (P < 0.01) (Fig. 3) suggested that the EHT engraftment improves the heart function in lieu of scar stabilization. The results form MEA measurement indicated the coupling of EHTs to the host myocardium (Fig. 5). The results demonstrated that NT-ES cells could be used as a source of seeding cells for cardiac tissue engineering. Our approach to construct EHTs has the potential for the therapy of heart regeneration.

The vascularization of the construct remains a major challenge for future studies of myocardial tissue engineering. In our study, liquid collagen was used as a scaffold. In the EHTs, the gelled collagen still allows for mass transfer and medium diffusion through the construct. This was further improved during the mechanical stretch. Additionally, the EHTs not only contained cardiomyocytes but represented organoids because of the presence of non-cardiac cell types – e.g. endothelial cells, fibroblasts, neurons (Fig. 2). The presence of vascular endothelial cells and fibroblasts in the construct may engender vasculature formation [35]. These factors may constitute the major (if not the sole) reason that we could generate EHTs with a thickness up to 4 mm. It is intriguing that formation of blood vessel-like structure in the EHT was observed 4 weeks after transplantation (Fig. 4C). Whether the vessels were of donor origin or had a role in de novo vascularization remains to be investigated.

The study has limitations. First, mouse EHTs were xenotransplanted into rat myocardium because rat MI model was more appropriate compared with mouse model because (1) the relatively large heart would facilitate the transplantation and observation of EHTs and (2) the surgery was relatively easier to increase the survival chances of animals. No obvious immune rejection was observed 4 weeks after EHTs transplantation. Nonetheless, allo-transplantation or autologous transplantation will be more useful practically for therapeutic cloning purpose. Second, the tumour formation was not assessed systematically after EHT transplantation. Because NT-ES cells are pluripotent stem cells, the possibility of inducing tumour formation has to be evaluated when the cells are transplanted in vivo. In this study, no tumour formation was observed in the histopathological sections of infarcted myocardium 4 weeks after EHTs transplantation. Exhaustive long-term investigations remain to be studied to ensure the safety of potential therapy for EHT transplantation. Third, in vivo visualization and quantification of the engrafted tissue were not performed in animal study to investigate the integration and cell migration or fusion. In this study, in order to determine the therapeutic effect of EHTs derived from NT-ES cells, no NT-ES cells were genetically labelled for tracking. EHTs could only be identified by their appearance and location in this study. Without the markers to identify graft cells, it is difficult to identify the graft from residual host muscle. So these structures could only be identified as the putative graft or host. More appropriate studies need to be undertaken for infect NT-ESC with luciferase or GFP labelled slow virus or to label NT-ESC with ferric oxide nanoparticle.
Nevertheless, this study is the first to describe the EHT construction from NT-ES cells and transplanted EHTs in infarcted rat heart for therapeutic application. Our results strongly suggest that NT-ES cells can be used as a source of seeding cells for tissue engineering, especially for those tissues or organs based on cell types difficult to obtain elsewhere, e.g. cardiac tissue. In addition, the EHTs increased the size and facilitated transplantation, with a strong potential for the therapy of heart regeneration.

### Acknowledgements

This work was supported by National High Technology Research and Development Program of China (No. 2006AA02A105 to C.W. and No. 2005AA21093 to S.G.), Nature Science Foundation of China (NO.30530220), Beijing Nature Science Foundation of China (NO.7062053). The authors thank Yuemei Hou (Laboratory of Cardiac Electrophysiology, Cardiac Centre, First Affiliated Hospital, Xinjiang Medical University) for technical assistance in MEA measurement.

### Conflict of interest

The authors confirm that there are no conflicts of interest.

### Supporting Information

Additional Supporting Information may be found in the online version of this article:

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