Tumorigenesis of nuclear transfer-derived embryonic stem cells is reduced through differentiation and enrichment following transplantation in the infarcted rat heart

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Abstract. The aim of the present study was to evaluate the tumorigenic potential of nuclear transfer-derived (nt) mouse embryonic stem cells (mESCs) transplanted into infarcted rat hearts. The nt-mESCs were cultured using a bioreactor system to develop embryoid bodies, which were induced with 1% ascorbic acid to differentiate into cardiomyocytes. The nt-mESC-derived cardiomyocytes (nt-mESCs-CMs) were enriched using Percoll density gradient separation to generate nt-mESCs-percoll-enriched (PE)-CMs. Ischemia was induced by ligating the left anterior descending coronary artery in female Sprague-Dawley rats. Immunosuppressed rats (daily intraperitoneal injections of cyclosporine A and methylprednisolone) were randomly assigned to receive an injection containing 5×10^6 mESCs, nt-mESCs, nt-mESC-CMs or nt-mESC-PE-CMs. Analysis performed 8 weeks following transplantation revealed teratoma formation in 80, 86.67 and 33.33% of the rats administered with the mESCs, nt-mESCs and nt-mESC-CMs, respectively, indicating no significant difference between the mESCs and nt-mESCs; but significance ($P < 0.05$) between the nt-mESC-CMs and nt-mESCs. The mean tumor volumes were 82.72 ± 6.52 , 83.17 ± 3.58 and 50.40 ± 5.98 mm³, respectively ($P > 0.05$ mESCs, vs. nt-mESCs; $P < 0.05$ nt-mESC-CMs, vs. nt-mESCs). By contrast, no teratoma formation was detected in the rats, which received nt-mESC-PE-CMs. Octamer-binding transcription factor-4, a specific marker of undifferentiated mESCs, was detected using polymerase chain reaction in the rats, which received nt-mESCs and nt-mESC-CMs, but not in rats administered with nt-mESC-PE-CMs. In conclusion, nt-mESCs exhibited the same pluripotency as mESCs, and teratoma formation

following nt-mESC transplantation was reduced by cell differentiation and enrichment.

Introduction

The processes of generation and maturity of cardiomyocytes (CMs) are completed *in utero*, therefore, CMs are considered a source of terminally differentiated cells and CMs are unable regenerate if the heart is injured. For example, myocardial tissue destroyed following myocardial infarction is replaced typically by noncontractile scar tissue (i.e., fibrosis), which leads to congestive heart failure (1,2). Heart transplantation is currently the gold standard therapy for patients with end-stage heart failure. However, its application is limited in clinical practice due to a shortage of donor hearts and the side effects of immunosuppressive drugs (3,4). Thus, cell-based cardiac repair has attracted interest as an alternative method to ameliorate cardiac injury (5,6). Specifically, significant attention has been paid to embryonic stem cell (ESC)-based cardiac repair, particularly for infarcted hearts (5).

Fetal and neonatal CMs can improve the biological function of damaged hearts (7-9); however, their use is restricted by the shortage of cell sources and ethical problems. By contrast, adult stem cells are abundant, however, whether these have the ability to differentiate into CMs remains to be elucidated. ESCs are multipotent cells derived from the inner cell mass of blastocysts, which are able to differentiate into all cell types of the body (10). They have an unlimited capacity for self-renewal (11) and unquestioned cardiomyogenic potential (12,13).

Previous studies using ESC transplantation to repair infarcted hearts have achieved much acclaim. Xie *et al* (14) reported that undifferentiated human ESCs (hESCs) can be induced towards a cardiac lineage under a locally injured environment in the heart, which may provide a potential method for regenerating *de novo* myocardium to treat myocardial infarction. Caspi *et al* (15) reported that the transplantation of hESC-derived CMs (hESC-CMs) in rats following extensive myocardial infarction results in the formation of stable CM grafts, attenuates the remodeling process, and provides functional benefit. Numerous studies have demonstrated that ESC-CMs exhibit a developmentally appropriate program

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of cardiac gene expression, as well as the expected electrophysiological and contractile phenotype (16,17). It was also demonstrated that, following transplantation into infarcted rodent hearts, ESC-CMs survive and formed stable cardiac implants with improved heart contractile function (18-20). However, immunological rejection and teratoma formation induced by ESC transplantation are the two most significant challenges limiting its clinical application (21-23).

To accompany the rapid development of stem cell and nuclear transfer technologies, nuclear-transferred ESCs (nt-ESCs) emerged towards the end of the 20th century (24,25). By combining therapeutic cloning with nuclear transfer technology and embryonic stem cell technology, and using donor somatic cells (such as skin cells) as nuclear donor cells and nuclear maturation in oocytes of other animals as receptor cells, researchers obtained nt-ESCs with an identical genetic background to that of the donor (26). An nt-ESC has the same genetic material as the donor, therefore it is not associated with immunological rejection following transplantation (26). However, teratoma formation cannot be avoided due to the multipotent ability of nt-ESCs.

Teratoma formation following ESC transplantation has achieved substantial attention in the research community. Leor *et al* (27) reported that the transplantation of undifferentiated hESCs and embryoid bodies (EBs) into infarcted hearts leads to teratoma formation. He *et al* (22) reported that, following the injection of mouse (m)ESCs into the infarcted hearts of immunosuppressed rats, the incidence of teratomas was ~50% after 1 week and 100% after 4 weeks. Additional studies have demonstrated that teratoma formation following ESC transplantation is associated with the immune response, the site of transplantation and the number of undifferentiated ESCs (23,28,29). However, to date, few studies have reported teratoma formation following nt-ESC transplantation.

The aim of the present study was to investigate teratoma formation from ESCs of different origins (mESCs and nt-mESCs) and at different stages of maturity [nt-mESCs, nt-mESC-CM and Percoll-enriched-nt-mESC-CMs (nt-mESC-PE-CMs)]. Infarcted rat hearts were selected as the experimental model. The tumor incidence and volume were assessed to compare the tumorigenesis of different transplanted seed cells. The aim of the present study was to compare the pluripotency of mESC and nt-mESC by comparing their tumorigenesis and to observe the influence of differentiation and enrichment on tumorigenesis of nt-ESC following transplantation in the infarcted rat heart.

Materials and methods

Reagents. All reagents and chemicals were, unless otherwise specified, purchased from Beijing Chemical Reagent Company (Beijing, China).

Undifferentiated nt-mESC and mESC culture. The mESC ES-D3 lines and nt-ESCs were cultured as described previously (30). Briefly, the cells were maintained in an undifferentiated state in Medium I, containing Dulbecco's modified Eagle's medium (DMEM; Gibco, Thermo Fisher Scientific, Waltham, MA, USA) with 4.5 g/l glucose, 15% fetal calf serum (FCS; GE Healthcare Life Sciences, Logan, UT, USA),

0.1 mM β -mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA), 2 mM glutamine and 0.1 mM non-essential amino acids (NEAAs; Gibco), containing 1,000 U/ml leukemia inhibitory factor (Chemicon International, Temecula, CA, USA).

Bioreactor expansion of EBs. The nt-mESCs were enzymatically dissociated using 0.25% trypsin (Gibco) and 0.04% ethylene-diamine tetraacetic acid (Sigma-Aldrich) at 37°C for 5 min. To amplify the nt-mESCs and obtain the EBs, 2.5×10^6 ES-D3 cells were transferred into a 250 ml slow-turning lateral vessel (STLV; Synthecon, Inc., Houston, TX, USA) filled with Medium II, containing DMEM with 4.5 g/l glucose, 20% FCS, 0.1 mM β -mercaptoethanol, 2 mM glutamine and 0.1 mM NEAA. The speed of rotation was 15 rpm for the first 12 h and was adjusted to 45 rpm for 5 days. Half of the culture medium was replaced every 2 days.

Adherence culture and ascorbic acid induction. The cells were cultured for 5 days in the STLV. Subsequently, the formed EBs were transferred onto gelatin-coated plates (1-3 EBs/cm²) and cultured in Medium II. EB differentiation into CMs (nt-mESC-CMs) was induced by adding 5 mg/l ascorbic acid followed by incubation for 8 days, with medium containing 5 mg/l ascorbic acid refreshed at days 3, 5 and 7.

Dissociation and Percoll enrichment of the nt-mESC-CMs. The differentiated cell cultures containing CMs were washed once with phosphate-buffered saline (PBS), followed by incubation in PBS containing 1 mg/ml dispase (Roche Diagnostics, Basel, Switzerland) at 1×10^6 cells/ml at 37°C for 30 min. The cells were then resuspended in a solution containing 85 mM KCl, 30 mM K₂HPO₄, 5 mM MgSO₄, 1 mM ethylene-glycol tetraacetic acid, 2 mM Na₂ATP, 5 mM sodium pyruvate, 5 mM creatine, 20 mM taurine and 20 mM glucose, and incubated at 37°C for 15 min for complete dissociation. Following dissociation, the cells were centrifuged at $1,500 \times g$ for 30 min at room temperature, resuspended in 3 ml Medium II and loaded onto a discontinuous Percoll gradient for enrichment of the CMs. Percoll (GE Healthcare Life Sciences) was diluted in buffer containing 20 mM HEPES and 150 mM NaCl. The gradient consisted of a 40.5% Percoll layer over a layer of 58.5% Percoll. Following centrifugation at $1,500 \times g$ for 30 min, the cell layers were apparent. Fraction V contained the enriched CMs (mESC-PE-CMs), which were collected for cell transplantation and semi-quantitative reverse transcription (RT)-polymerase chain reaction (RT-PCR) analysis. Octamer-binding transcription factor-4 (OCT-4) was used as a specific marker of undifferentiated mESCs. Mouse fibroblasts obtained from the embryos (13-14 days old) of Kunming white mice served as negative controls (Institute of Basic Medical Sciences, Academy of Military Medical Sciences, Beijing, China). The second-generation mouse fibroblasts were used as ESC feeder cells. An RT-PCR kit (Promega Corp., Madison, WI, USA) was used. First, RNA was extracted with TRIzol (Tiangen Biotech Co., Ltd., Beijing, China) according to the manufacturer's instructions. The RNA content was determined by measuring its optical density at 260/280 nm (BD-600 Electrophoresis Densitometer Measurement System analysis; QHBODA Technology Co., Ltd, Beijing, China). The RT-PCR reaction was performed in a total volume of 50 μ l [template

100 ng, 10X primer mix (2 μ M each) 5 μ l, 10X Mutli HotStart buffer 5 μ l, dNTPs 200 μ M, Multi HotStart (5 U/ μ l), ddH₂O up to 50 μ l] with 1 μ g RNA using a Multiplex PCR Amplification kit [KT109; Tiangen Biotech (Beijing) Co., Ltd., Beijing China]. RT was performed at 45°C for 45 min, followed by PCR amplification under the following conditions: Initial denaturation at 95°C for 5 min, followed 35 cycles of amplification with annealing at 53°C, and a final extension at 72°C for 10 min. The PCR products were separated by 1% agarose gel electrophoresis and images were captured with a gel imager (haCmpGel-3200, Beijing Sage Creation, Beijing, China) for qualitative detection of Oct-4. The following specific primer sequences were used: Oct-4 forward, 5'-GGAGGAAGCCGA CAACAATGAG-3' and reverse, 5'-TGGGGGCAGAGGAAA GGATACAG-3' (331 bp); and GAPDH forward, 5'-AACGAC CCCTTCATTGAC-3' and reverse, 5'-TCCACGACATACTCA GCAC-3' (191 bp).

Myocardial infarction model and cell transplantation. Female Sprague-Dawley rats (8-week-old, weighing 230 $\pm$ 20 g) were purchased from the Laboratory Animal Center of the Chinese Academy of Military Medical Science (Beijing, China). All animal experiments were performed under the authority of the Institutional Animal Care and Use Committee of the Chinese Academy of Military Medical Science (Beijing, China). The study was approved by the ethics committee of the First Affiliated Hospital of Dalian Medical University (Dalian, China). The animals were individually housed in cages at a constant room temperature of 24 $\pm$ 2°C under a 12-h light/darkcycle with access to water and rat chow *ad libitum*. The rats were then prepared for the induction of ischemia, as described in detail by Miyahara *et al* (31). Briefly, the rats were anesthetized via an intraperitoneal (i.p.) injection of 30 mg/kg sodium pentobarbital (China National Medicine Group, Beijing Chemical Reagent Company). Subsequently, limb-lead electrocardiography was performed using an RM6240BD type multichannel physiological instrument (Chengdu Instrument Factory, Chengdu, China), and rats were ventilated with a volume-regulated respirator for the entire duration of the procedure. The surgical approach to induce ischemia involved a left lateral thoracotomy, pericardiectomy and identification of the left anterior descending (LAD) coronary artery. The LAD was ligated with a 6-0 Prolene suture (Ningbo Medical Needle Co. Ltd., Ningbo, China) 2-3 mm from its origin between the pulmonary artery conus and the left atrium. Following ligation, the infarcted area of the left ventricle became immediately pale and contraction was limited. Typical myocardial infarction waves were simultaneously visible on the electrocardiogram. The rats were randomly assigned to receive either mESCs, nt-mESCs, nt-mESC-CMs or nt-mESC-PE-CMs (n=15 per group). For administration $\sim$ 5 $\times$ 10⁶ of each cell type, resuspended in 100 μ l PBS, was injected through a 28 gauge needle into the center of the infarcted area 5 min after the induction of myocardial infarction. The injections were verified by marginal lightening in color of the myocardium as the solution entered the infarcted wall. Subsequently, the chest was closed. To avoid graft rejections, all rats received daily i.p. injections of cyclosporine A (15 mg/kg) and methylprednisolone (2 mg/kg).

Evaluation of teratoma incidence and volume. The rats were sacrificed at 8 weeks after cell transplantation by anesthesia with sodium pentobarbital (30 mg/kg, i.p.), left lateral thoracotomy to expose the heart and injection of 5 ml 10% KCl into the heart. The hearts were rapidly excised, and the cardiac cavities rinsed in PBS to remove blood and thrombi. The incidence and volume of teratomas in the transplant area were calculated in a blinded-manner. Owing to fibrosis, the ventricular wall was sufficiently thin to permit observation of a teratoma, and the volume of the teratoma was measured as that, which formed subcutaneously. Tumor incidence was expressed as a percentage of the number of samples in each group. Tumor volume was calculated using the following formula: Tumor long diameter $\times$ (tumor short diameter)² / 2.

Histology, histochemistry and immunohistochemistry. The hearts were fixed with 10% formalin for 16 h, embedded in paraffin and cut into 4- μ m thick sections. The tissue sections were then stained with hematoxylin and eosin for histological examination. Chondrogenesis differentiation was identified by immunohistochemical staining of type II collagen (1:200; Chemicon Incorporated). Prior to immunohistochemistry, the sections were digested with pepsin at 1 mg/ml in TrisHCl (pH 2.0) for 10 min at 37°C. For immunohistochemistry, the intrinsic peroxidase activity was blocked by incubation with 5% H₂O₂ in PBS for 30 min following deparaffinization. Nonspecific antibody binding was blocked with 5% bovine serum albumin (Medgenics Inc., Philadelphia, PA, USA) in PBS for 1 h at 37°C. Immunohistochemical staining was performed using an avidin-biotin technique, followed by visualization with 3,3'-diaminobenzidine tetrahydrochloride dehydrate (0.006%) and H₂O₂ (0.003%). The following primary antibodies were used: Mouse monoclonal anti-type II collagen (cat. no. MAB8887; Chemicon), mouse monoclonal anti-Nestin (1:200; cat. no. ab93666; Wuhan Boster Biological Technology, Ltd., Wuhan, China), as a neural precursor cell marker, and rabbit polyclonal anti- α -smooth muscle actin (α -SMA; 1:200; cat. no. A2547; Wuhan Boster Biological Technology, Ltd.), as a smooth muscle cell marker. Samples were then incubated with secondary antibody, Cy3 goat anti-mouse or rabbit immunoglobulin G (1:50 dilution; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China), at 37°C for 30 min. Blank controls were performed by omitting the primary antibodies. Following washing with distilled water, counterstaining was performed with hematoxylin for 1 min. Samples were observed and images were captured using a microscope (BX51; Olympus, Tokyo, Japan).

Statistical analysis. The tumor volume data are expressed as the mean $\pm$ standard deviation. Fisher's exact test and two-tailed Student's t-test were used to evaluate the incidence and volume of teratomas, respectively. Statistical analysis was performed using SPSS 16.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Differentiation and enrichment of mESC-CMs. The mESC ES-D3 lines (Fig. 1A) and nt-mESCs (Fig. 1B) were successfully

Figure 1. Differentiation and enrichment of the seed cells. Cultures of the (A) undifferentiated mESCs and (B) nt-mESCs. The nt-mESCs were dissociated and cultured in (C) a 250 ml slow-turning lateral vessel to form (D) embryoid bodies, which were then transferred to gelatin-coated plates following 5 days in suspension culture, to allow further differentiation into (E) spontaneously contracting cardiomyocytes (nt-mESCs-CMs). (Fa1) nt-mESC-CMs were enriched through a Percoll gradient consisting of (Fa2) a 40.5% Percoll layer over a (Fa3) a layer of 58.5% Percoll. Following Percoll separation, six fractions were observed, with the enriched nt-mESC-PE-CMs predominantly localized in fraction 5 (Fb5). Scale bar=100 μ m. nt-mESCs, nuclear transferred mouse embryonic stem cells; nt-mESC-PE-CMs; nt-mESC-derived-percoll-enriched cardiomyocytes.

Figure 2. Reverse transcription-polymerase chain reaction analysis demonstrated that the expression of octamer-binding transcription factor-4 was detected in the nt-mESCs and nt-mESCs-CMs, but not in the nt-mESC-PE-CMs or in the negative control. nt-mESCs, nuclear transferred mouse embryonic stem cells; nt-mESC-PE-CMs; nt-mESC-derived-percoll-enriched cardiomyocytes.

cultured and the nt-mESCs were expanded to EBs using an STLV bioreactor system (Fig. 1C). At 5 days post-expansion, $\sim 4 \times 10^6$ EBs of 100 ± 30 μ m diameter were obtained. The EBs were spherical in shape with an intact structure and no signs of apoptosis (Fig. 1D).

The EBs were inoculated onto culture plates at low density to induce differentiation into CMs. The EBs grew in an attachment pattern and differentiated into beating CMs in the presence of ascorbic acid. After 5 days, the CMs began to spontaneously contract (Fig. 1E). At 14 days post-differentiation, the nt-mESC-CMs became dissociated and subsequently underwent discontinuous Percoll gradient separation (Fig. 1Fa). Following centrifugation, six layers of

cells were observed (Fig. 1Fb). As demonstrated previously, mESC-CMs are predominantly concentrated in fraction 5 (Fig. 1Fb5) (32), therefore, these cells were collected for subsequent cell transplantation. The RT-PCR assays demonstrated that OCT-4, a marker of early-stage ESCs, was expressed in the nt-mESCs and nt-mESC-CMs, but was absent in the nt-mESC-PE-CMs (Fig. 2).

Tumor formation is induced by seed cell transplantation in the infarcted heart. Tumor formation was observed in the infarcted area of the hearts administered with the mESC, nt-mESC and mESC-CM grafts (Fig. 3A-C), but not in the hearts administered with the nt-mESC-PE-CM grafts (Fig. 3D). Hematoxylin

Figure 3. Teratoma formation following seed cell transplantation in infarcted rat hearts. At 8 weeks post-cell transplantation, tumors were readily identified in the (A) mESC, (B) nt-mESC and (C) nt-mESC-CM groups, but were absent in the (D) nt-mESC-PE-CM group. nt-mESCs, nuclear transferred mouse embryonic stem cells; nt-mESC-PE-CMs; nt-mESC-derived-percoll-enriched cardiomyocytes.

Figure 4. Teratoma development and differentiation following mESC, nt-mESC and nt-mESCs-CM transplantation in infarcted rat hearts. (A) Hematoxylin and eosin staining revealed the structure of the teratoma, which was different from the myocardial tissue. Immunohistochemical analysis demonstrated that the tumor tissue was positive for (B) type II collagen, (C) α -smooth muscle actin and (D) nestin. Scale bar (A and B)=50 μ m; (C and D)=12.5 mm. nt-mESCs, nuclear transferred mouse embryonic stem cells; nt-mESC-PE-CMs; nt-mESC-derived-percoll-enriched cardiomyocytes.

Figure 5. Comparison of tumorigenesis in different seed cells. No differences in the incidence of tumorigenesis or the average tumor volume were identified between the mESC group and nt-mESC group. (A) Incidence of tumorigenesis was significantly lower in the nt-mESC-CM group, compared with that in the nt-mESC group. (B) Average tumor volume was significantly reduced in the nt-mESC-CM group, compared with that in the nt mESC group. No teratomas were found in the nt-mESC-PE-CM group. The incidence of tumorigenesis and the average tumor volume of the nt-mESC-PE-CM group were markedly lower, compared with those in the nt-mESC-CM group. * $P > 0.05$, * $P < 0.05$, # $P < 0.05$. nt-mESCs, nuclear transferred mouse embryonic stem cells; nt-mESC-PE-CMs, nt-mESC-derived percoll-enriched cardiomyocytes. The tumor volumes are presented as the mean $\pm$ standard deviation.

and eosin staining revealed that the formed tumor was entirely different from the myocardial tissue, and predominantly consisted of cartilage (Fig. 4A). Immunohistochemistry indicated that the tumor tissue was positive for type II collagen (Fig. 4B), α -SMA (Fig. 4C) and nestin (Fig. 4D).

Comparison of tumorigenesis in different seed cells. Tumors were observed in 86.67% of the rats that received nt-mESCs and in 80% of the rats that received mESCs. No significant difference was observed in tumor incidence between these two groups (Fig. 5A). However, tumors were observed in 33.33% of the rats administered mESC-CMs, which differed significantly from that of the mESC-treatment group ($P < 0.05$; Fig. 5A). The tumor volumes were 83.17 ± 3.58 and 82.72 ± 6.52 mm³ in the rats administered with nt-mESCs and mESCs, respectively (Fig. 3A and B and Fig. 5B). No difference in mean tumor volume was observed between the two groups. By contrast, a mean tumor volume of 50.40 ± 5.98 mm³ was observed in the nt-mESC-CM group, which was significantly reduced, compared with that in the nt-mESC group ($P < 0.05$; Fig. 3B and C and Fig. 5A). As expected, the infarcted rat hearts transplanted with nt-mESC-PE-CMs did not exhibit signs of tumor formation (Figs. 3D and 5A). The incidence of tumorigenesis and mean tumor volume in the nt-mESC-PE-CM group were significantly lower, compared with those in the nt-mESC-CM group ($P < 0.05$; Fig. 3C and D and Fig. 5).

Discussion

ESCs offer significant promise in regenerative medicine, however, several critical obstacles must be overcome prior to the use of ESCs in clinical medicine. Possibly the most important obstacle from a safety standpoint is the immunogenicity of ESCs and their tumorigenic ability (22-24). nt-ESCs are distinctly superior in that these cells do not induce immunological rejection following transplantation, however, due to their multipotency, nt-ESCs have been associated with tumorigenesis. It was reported in our previous study that tumorigenesis in the infarcted rat heart is eliminated through the differentiation and enrichment of transplanted mESCs (32). In the same study, it was demonstrated that mESCs also prevent teratoma formation through differentiation and enrichment.

In the present study, an established infarcted rat heart model was used, and it was demonstrated that the transplantation of mESCs and nt-mESCs resulted in tumor formation. Considering that no discernible differences were identified in tumor incidence and volume between these two groups, it was concluded that nt-mESCs exhibit the same level of tumorigenicity as mESCs. Teratoma formation is generally considered to represent the multipotency of ESCs (33,34). Therefore, it is appropriate to conclude that nt-mESCs have the same degree of multipotency as mESCs.

In the present study, pre-differentiated mESC-CMs induced by ascorbic acid also resulted in tumor formation in the infarcted hearts. However, the incidence of tumorigenesis and mean tumor volume in the nt-mESC-CM group were markedly lower than those observed in the nt-mESC group. The detection of OCT-4, a marker of undifferentiated mESCs, in the two groups suggested that undifferentiated nt-mESCs remained in the ascorbic acid-induced differentiated CMs.

This finding is consistent with that of a previous report concerning mESCs (32) and indicates the importance of transplantation using differentiated CMs to decrease the incidence of tumorigenesis. The pre-differentiation of nt-mESCs into CMs reduces the possibility of differentiation into other cell lineages and reduces the number of undifferentiated nt-mESCs. By contrast, the proliferative and self-renewal ability of the remaining undifferentiated nt-mESCs may be subject to the effects of the inducer, ascorbic acid, and the microenvironment formed by the pre-differentiated CMs. This may explain why the incidence of tumorigenesis resulting from the undifferentiated nt-ESCs decreased significantly in the implanted areas. This finding was also suggestive of teratoma formation. Histochemical analysis revealed that the teratomas that developed in the rat ischemia model in the present study were formed predominantly of bone and cartilage tissue.

As demonstrated in a previous study, to obtain maximally differentiated CMs, the CMs were enriched with ascorbic acid-induced EBs using Percoll gradient centrifugation (32). No teratomas developed in the rats injected with nt-mESC-PE-CMs, as expected. Teratoma were derived from undifferentiated ESCs. RT-PCR analysis demonstrated that OCT-4 was not expressed in the nt-mESC-PE-CMs group; therefore, it was speculated that the content of undifferentiated nt-mESC in nt-mESC-PE-CMs was further reduced, even without treatment, through differentiation and enrichment. Furthermore, no OCT-4 was detected in this treatment group. Laflamme *et al* (35) reported that when hESCs are inductively differentiated into CMs and subsequently enriched with Percoll, they fail to form teratomas following transplantation into normal rat hearts, but rather form stable grafts of human myocardium with a period of 4 weeks. This finding is consistent with our previous study, which revealed that mESC-PE-CMs were not associated with teratoma formation following transplantation (32). It was further demonstrated in the present study that the nt-mESC-PE-CMs were not associated with tumorigenesis following transplantation in the ischemic rat heart model.

In conclusion, the present study demonstrated that nt-mESCs exhibited the same multipotency as mESCs. In addition, transplantation of nt-mESC-PE-CMs prevented the formation of teratomas in an infarcted rat heart model. These findings suggested that nt-ESCs are an ideal cell resource for myocardial cell regeneration.

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