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Full Length Research Paper

Genetic enrichment of cardiomyocytes derived from mouse embryonic stem cells

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Pluripotent embryonic stem cells (ESC) have the ability to differentiate into a variety of cell lineages *in vitro*, including cardiomyocytes. Successful applications of ESC-derived cardiomyocytes in cell therapy and tissue engineering were limited by difficulties in selecting the desired cells from the heterogeneous cell population. We describe a simple method to generate relatively pure cardiomyocytes from mouse ESCs. A construct comprising mouse cardiac α-myosin heavy chain (MHC) promoter driving the neomycin resistance gene and SV40 promoter driving the hygromycin resistant gene designated pMHC-neo/SV40-hygro, was stably transfected into mouse ESCs. The transgenic ESC line, designated MN6 retained the undifferentiated state and the potential of cardiogenic differentiation. After G418 selection, more than 99% of cells expressed α-sarcomeric actin. Immunocytological and ultrastructural analysis demonstrated that, the selected cardiomyocytes were highly differentiated. Our results represent a simple genetic manipulation used to product essentially pure cardiomyocytes from differentiating ESCs. It may facilitate the development of cell therapy in heart diseases.

Key words: Embryonic stem cells, α-myosin heavy chain promoter, cardiomyocytes, differentiation, genetic enrichment.

INTRODUCTION

Many cardiovascular diseases are associated with acute or chronic myocardial damage. Because cardiomyocytes cannot regenerate, loss of cardiomyocytes in the adult mammalian heart will lead to the formation of scar tissue and weaken the heart function, eventually causing heart failure (Delcayre and Swynghedauw, 2002). Recent studies have confirmed that, the isolated cells directly transplanted into damaged parts of the heart could improve cardiac function (Dimmeler et al., 2005; Hristov and Weber, 2006; Laflamme and Murry, 2005; Menasché, 2008; Ye et al., 2006). This is a promising method for treatment of ischemic heart disease, but it requires a large number of myocardial cells.

Embryonic stem cells (ESC) are pluripotent cells derived from the inner cell mass of blastocyst stage embryos. They not only could proliferate unlimitedly *in vitro*, but also have the ability to differentiate into a variety of cell lineages, including caridomyocytes (Doetschman et al., 1985). Based on these unique properties, ESCs should be currently one of the most promising sources of donor cardiomyocytes (Zimmermann and Eschenhagen, 2007). Although, the methods for induction of ESC differentiation into cardiomyocytes had already been reported, the percentage of cardiomyocytes in ESC-derived differentiated

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Abbreviations: ESC, Embryonic stem cells; **EB,** emybryoid bodies; **MHC,** myosin heavy chain; **MEF,** mouse embryonic fibroblasts; **FBS,** fetal bovin serum; **AKP,** alkaline phosphatase; **LIF,** leukemia inhibitor factor.

cells was very low. The heterogenous population still contained a large number of non-myocardial cells and some of the undifferentiated ESCs, which would be a serious impediment for clinical applications of ESC-derived cardiomyocytes. Manual dissection of beating areas (Segev et al., 2005) and percoll gradient centrifugation (Xu et al., 2002) had been used to enrich cardiomyocytes, but the degree of purity would probably be insufficient for clinical or research purposes.

In this study, we described the generation of transgenic mouse ESC lines transfected with a vector designated pMHC-neo/SV40-hygro containing two transcriptional units to allow selection of differentiating cardiomyocytes. This method was based on using a cardiac-specific α-myosin heavy chain (MHC) promoter to drive the expression of neomycin resistance gene. Expression of the transgene in ESC-derived cardiomyocytes facilitated their selection with G418 after *in vitro* differentiation. Immunocytological and ultrastructural analyses showed that, the selected cardiomyocytes exhibited the expected cardiac markers and were highly differentiated. Thus, we demonstrated that a relatively simple genetic selection could be used to generate pure cardiomyocytes from ESCs.

MATERIALS AND METHODS

Plasmid construction

The 5.5 kb mouse cardiac α-MHC promoter was amplified from mouse genomic DNA by polymerase chain reaction using LA Taq DNA polymerase (TaKaRa). Primers for cloning of α-MHC promoter fragment contained *Mlu* I (forward: 5'-GATC ACGCGT GGA TCC TGC AAG GTC ACA CAA GGG TCT CCA CCC AC-3') and *Nhe* I (backward: 5'-GATC GCTAGC GTC GAC TCA AAC TCT TAT GGG GGA GAT AGG AGG GAT C-3') restriction sites. The CMV promoter in pCMV/SV40-hygro vector (based on the pcDNA3.1 (+) from Invitrogen) was replaced by α-MHC promoter to generate pMHC/SV40-hygro after digestion with *Mlu* I and *Nhe* I. Then, the *Nhe* I-*Not* I restriction fragment containing the neomycin resistance gene was inserted into the pMHC/SV40-hygro, resulting in pMHC-neo/SV40-hygro vector.

ESC culture and transfection

Mouse ESCs (ES-D3) were maintained in an undifferentiated state by culturing on a feeder layer of mitomycin C-treated mouse embryonic fibroblasts (MEF) on 0.1% gelatin-coated tissue culture dishes in ESC culture medium containing 85% high glucose DMEM (Invitrogen), 15% FBS (Hyclone), 0.1 mM β-mercaptoethanol (Sigma), 1% nonessential amino acids (Invitrogen) and 10^3 U/ml leukemia inhibitory factor (LIF, Millipore). ESCs were passed by disassociating with 0.25% trypsin and 0.04% EDTA every second day. 5×10^6 undifferentiated ESCs suspended in 800 µl PBS were transfected via electroporation (240 V/500 µF) with 50 µg nonlinearized pMHC-neo/SV40-hygro vector.

Establishment of stably transgenic ESC lines

After two days of recovery, the transfected cells were selected by growth in the presence of hygromycin B (200 µg/ml) for at least 7 days. The transgenic ESCs were digested by trypsinization for 3 min and then counted and plated in 96 wells plates at a concentration of a single cell/well covered with a layer of MEF. The single-cell derived clones were chosen for expansion in hygromycin B-supplemented ESC culture medium for an additional 7 days. Subsequently, the resulting stable cell lines were identified via genomic PCR using primers corresponding to the transfected plasmid. Primer sequences were: 5'-GAA AGT CAG GAC TTC ACA TAG AAG CCT AGC CCA CAC C-3' (forward) and 5'-TCA GAA GAA CTC GTC AAG AAG GCG ATA GAA GGC G-3' (backward). To verify that the transgenic ESC lines retained the undifferentiated state, the transgenic lines were stained for specific mouse ESC markers (SSEA-1, Oct-4) and alkaline phosphatase (AKP).

ESC differentiation

To induce differentiation, the transgenic ESCs were dispersed into individual cells using 0.25% trypsin and 0.04% EDTA, and preplated on tissue culture dishes for 60 min in differentiation medium (ES culture medium without LIF and replacing 15 for 20% FBS) to reduce the MEF and then inoculated into 100 mm Petri dishes at a density of 2×10^5 cells/ml to form embryoid bodies (EB). After 5 days in the suspension culture, the resulting EBs were plated onto 0.1% gelatin-coated tissue culture dishes or 24 well plates in differentiation medium supplemented with 0.1 mg/ml ascorbic acid. The cultures were monitored daily under a phase contrast microscope for the presence of contracting cardiomyocytes.

Selection and dissociation of cardiomyocytes

For selection of cardiomyocytes, the differentiation medium was supplemented with G418 (400 µg/ml) 12 days after induction to eliminate non-cardiomyocytes not expressing the neomycin resistance gene. The differentiated cultures were subjected to G418 selection for an additional 5 days before harvesting cardiomyocyte. For cell isolation, the selected cultures were washed with PBS and incubated with 0.05% trypsin and 0.02% EDTA at 37°C for 10 min. Cells were aspirated intensively by pipette for more complete dissociation. Dispersed cells were resuspended with fresh differentiation medium and seeded on 0.1% gelatin-coated plates for immunocytochemistry and electron microscope analyses.

AKP staining

After washing, the cultured cells were fixed with cold 4% paraformaldehyde for 15 min at room temperature. Alkaline phosphatase staining solution containing 50 mM NaCl, 100 mM Tris-HCl at pH 9.5, 50 mM $MgCl_2$, 0.1% Tween-20, 1 mg/ml NBT (Sigma), 0.1 mg/ml BCIP (Sigma) was added after washing. The reaction was incubated for 5 to 30 min at 37°C, and was stopped by addition of 10 mM EDTA. The wells were washed with PBS. Coloured colonies were scored using an inverted microscope (Leica, Germany).

Immunocytochemistry

Cells were rinsed briefly with PBS and fixed with 4% paraformaldehyde at room temperature for 15 min. The fixed cells were washed three times in PBS and treated with 0.3% Triton X-100 for 30 min. Unspecific binding sites was blocked by 5% BSA for 30 min. The primary antibodies were applied without washing and incubated at 4°C overnight. After washing, cells were treated with the secondary antibodies for 2 h at room temperature in the dark. Actin labeling was performed with 5 µg/ml phalloidin-TRITC (Sigma)

Table 1. Primer sequences and reaction conditions for RT-PCR analysis.

Gene	Primer	Tm (℃)
GATA-4	5'-CTCGATATGTTTGATGACTTCT-3' 5'-CGTTTTCTGGTTTGAATCCC-3'	53
Nkx2.5	5'-AGCAACTTCGTGAACTTTG-3' 5'-CCGGTCCTAGTGTGGA-3'	52
α-MHC	5'-ACCGTGGACTACAACAT-3' 5'-CTTTCGCTCGTTGGGA-3'	52
ß-MHC	5'-ACCCCTACGATTATGCG-3' 5'-GTGACGTACTCGTTGCC-3'	52
MLC-2v	5'-GCCAAGAAGCGGATAGAAGG-3' 5'-CTGTGGTTCAGGGCTCAGTC-3'	53
Oct-4	5'-GGCGTTCTCTTTGGAAAGGTGTTC-3' 5'-CTCGAACCACATCCTTCTCT-3'	60
GAPDH	5'-AACGACCCCTTCATTGAC-3' 5'-TCCACGACATACTCAGCAC-3'	53

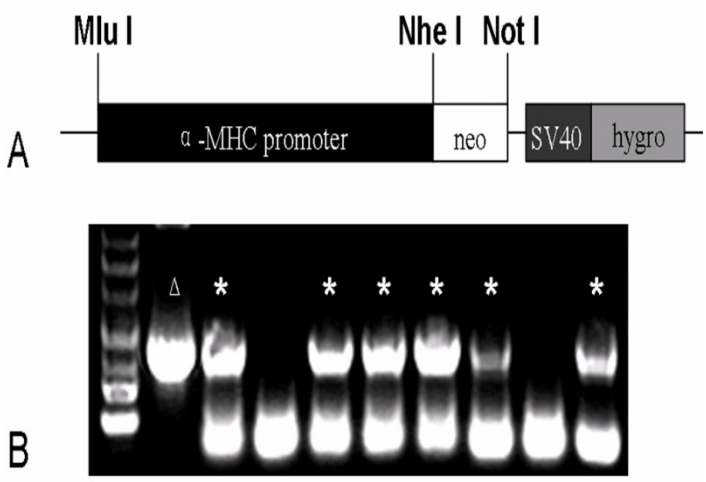

Figure 1. Establishment of stably transgenic ESC lines. (A) Structure of the vector pMHC-neo/SV40-hygro. The plasmid backbone was pcDNA3.1(+); (B) identification of stably transfected mouse ESC clones via genomic PCR using primers specific for the transfected plasmid. White asterisk presents positive clones revealing the expected band of 889 bp; white triangle presents positive control using the plasmid as template DNA.

at room temperature for 40 min. Nuclei were stained with 0.1 μg/ml of DAPI (Sigma) at room temperature for 15 min. The primary antibodies used were mouse monoclonal anti-SSEA-1 (Santa Cruz, 1:50), rabbit polyclonal anti-Oct-4 (Cell Signaling, 1:50), mouse monoclonal anti-Troponin T (Maixin, 1:100), mouse monoclonal anti-α-sarcomeric actin (Maixin, 1:100) and mouse monoclonal anti-α-sarcomeric actinin (Boster, 1:100). The second antibodies were FITC- or TRITC-labeled goat anti-mouse or anti-rabbit IgG (ZSGB-BIO, 1:50).

RT-PCR analysis

Total RNA was extracted from the undifferentiated MN6 cells, differentiated EBs and G418-selected cells using TRIzol (Invitrogen) according to the specification indicated by the manufacturer. RNA was quantified by UV spectrophotometer. RT-PCR was performed with 1 μg of RNA per sample using PrimeScript one step RT-PCR kit Ver.2 (TaKaRa). The primers and sequences are listed in Table 1 for GATA-4, Nkx2.5, α-MHC, β-MHC, MLC-2v, Oct-4 and GAPDH. 8 μl of RT-PCR products were taken for 1% agarose gel electrophoresis.

Ultrastructural analysis

Selected cell cultures were fixed in 2% glutaraldehyde, followed by postfixation in 2% osmium tetroxide. After subsequent overnight wash in PBS at 4℃, samples were dehydrated and embedded in epon. After trimming, the block was ultrathin sectioned and stained with uranyl acetate and lead citrate. Specimens were examined under a transmission electron microscope (HITACHI, H-7650).

RESULTS

Establishment of stably transgenic ES lines

The construct pMHC-neo/SV40-hygro containing two transcriptional units in a pcDNA3.1 (+) vector backbone was generated (Figure 1a). The first unit consisted of SV40 promoter and the hygromycin resistance gene used to select stably transfected ESCs before differentiation. The second unit contained α-MHC promoter driving the neomycin resistance gene used for selection of cardiomyocytes. Six stable transgenic mouse ESC lines were generated and identified via genomic PCR analysis with primers specific for the transfected plasmid (Figure 1b). One line, designated MN6 (Figure 2a) demonstrating robust and homogenous expression of the transgene, was chosen for propagation and subsequent analyses. MN6 cells exhibited a strong AKP activity (Figure 2b). They still expressed mouse ESC markers Oct-4 and SSEA-1 (Figure 2c, d). These results showed that, the transgenic ESC lines retained the undifferentiated state.

Induction of cardiogenic differentiation

We next sought to determine whether expression of the pMHC-neo/SV40-hygro transgene impacted negatively on cardiogenic differentiation of the transgenic mouse ESCs. Following selection and expansion of the stably transfected undifferentiated colonies, MN6 cells were allowed to differentiate by inducing EB formation. After 5 days in suspension culture, EBs were plated on gelatin-

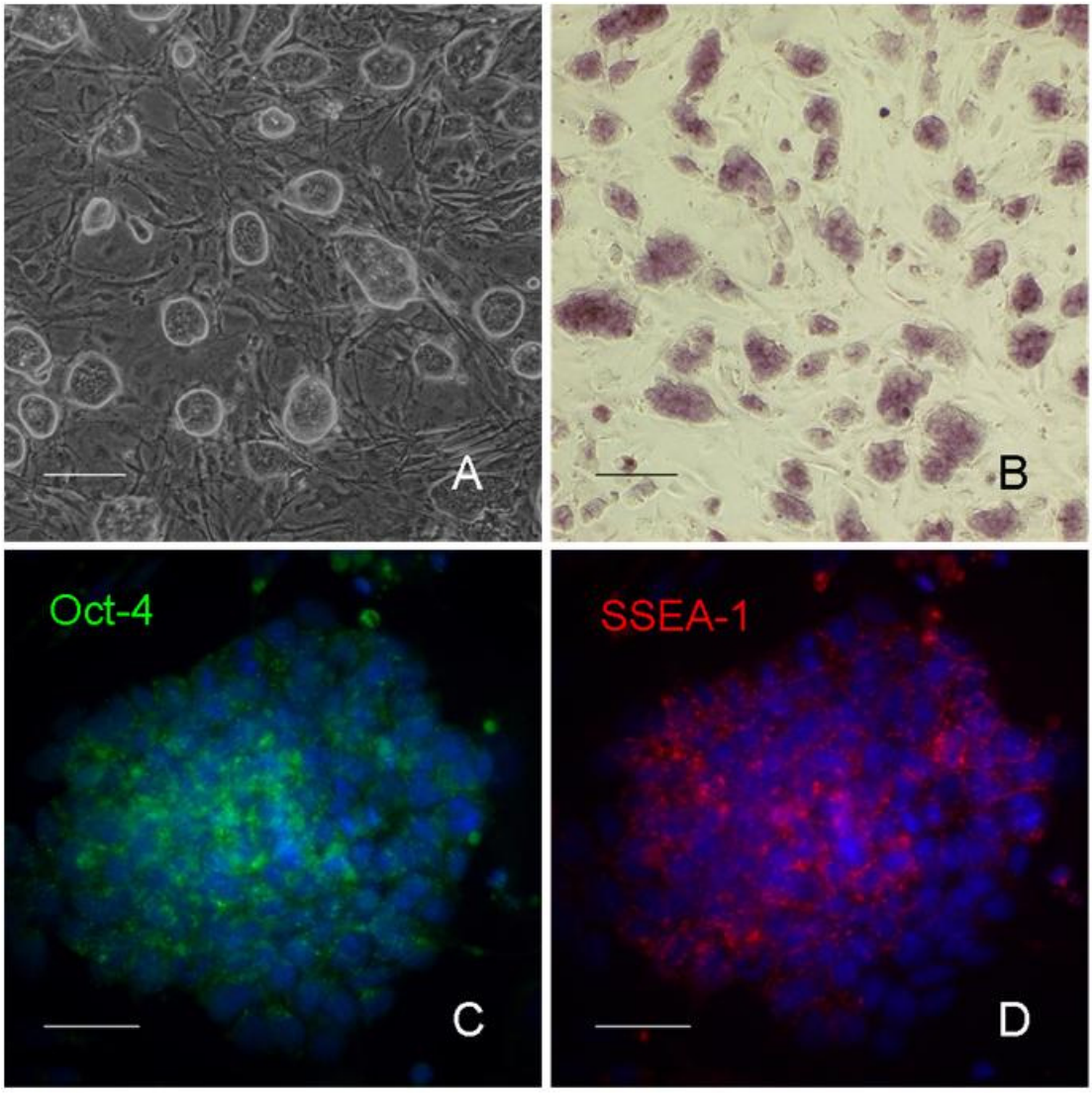

Figure 2. Characterization of the pluripotent properties of the transgenic mouse ESC line, MN6. (A) Phase contrast observation of colonies of undifferentiated MN6 cells; (B) alkaline phosphatase positive colonies showed purple; (C) Oct-4 immunostaining of MN6 cells showed green; (D) SSEA-1 showed red, nuclei staining with DAPI showed blue. Scale bars: 500 µm (A, B) and 100 µm (C, D).

coated tissue culture dishes and observed using light microscopy for the presence of beating cells. MN6 showed a similar developmental pattern with the wild-type ESC lines. EBs attached and continued to proliferate and differentiate into a heterogeneous population of cells, including beating cardiomyocytes. Spontaneously, contracting cells appeared as clusters after 2 days of adherence and induction. About 25% of EBs displayed contracting areas at differentiation day 10 and increased to as many as 70% of EBs by day 18. The percentage of beating EBs usually increased over time, reaching a maximum 18 days after differentiation (Figure 3a). After 30 days, the number of beating EBs decreased due to the overgrowth of other cells. Immunofluorescence analysis showed that, only a small percentage of differentiated cells expressed cTnT (Figure 3b), indicating that the majority of cells were not cardiomyocytes.

Selection of cardiomyocytes

After 12 days of induction, G418 selection was imposed and differentiated cells were cultured for additional 5 days. Selected cells were visibly spontaneous contracted with a rhythm of approximately 1 Hz, indicating a relatively pure population of cardiomyocytes. To determine the extent of cardiomyocytes enrichment obtained by genetic selection, the selected cultures were digested with trypsin and replated at a suitable density for immunocytologic analysis of individual cells.

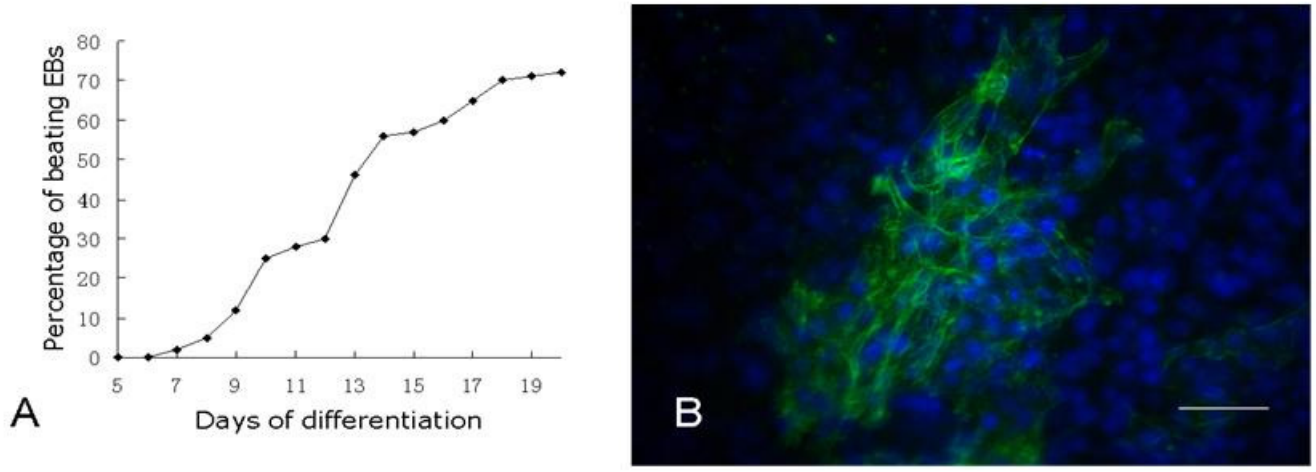

Figure 3. Differentiation of cardiomyocytes from the transgenic mouse ESCs. (A) Percentage of beating EBs derived from MN6 cells during differentiation; (B) EBs were transferred to gelatin-coated plates after 5 days in suspension culture to allow further differentiation into a heterogeneous cells, including cardiomyocytes that were positive for cTnT (green). Nuclei staining with DAPI showed blue. Scale bars: 100 μm.

Table 2. Cardiomyocytes content in the unselected and G418-selected differentiated cells.

Preparation	α-Sarcomeric actin positive cell	α-Sarcomeric actin negative cell	Percentage of cardiomyocyte (%)
No selection	13	1968	0.66
G418 selection	816	3	99.63

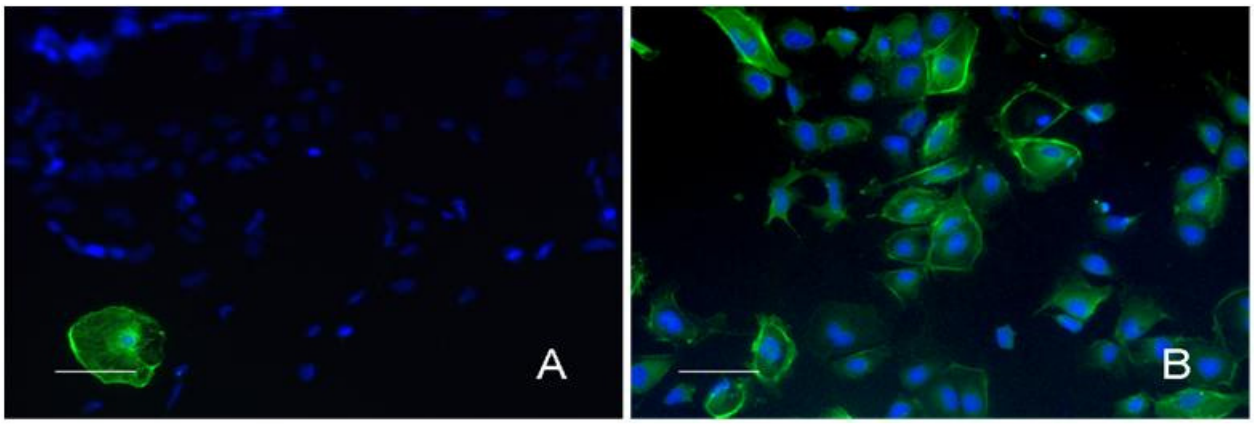

Figure 4. G418 selection of cardiomyocytes derived from transgenic mouse ES lines. (A) Only a very few cells in differentiated cultures were cadiomyocytes positive for α-sarcomeric actin without G418 selection; (B) although a difference in fluorescence intensity was observed among cells, more than 99% of G418-selected cells expressed α-sarcomeric actin indicating an efficient enrichment of cardiomyocytes. Scale bars: 100 μm.

Immunocytochemistry was used to demonstrate purity of the enriched cell population. Although, a difference in fluorescence intensity was observed among cells, more than 99% of G418-selected cells expressed cardiac markers α-sarcomeric actin indicating an efficient enrichment of cardiomyocytes in agreement with previous findings. In contrast, only a very few cells in differentiated cultures were cardiomyocytes without G418 selection (Table 2 and Figure 4). Confocal microscopy was used to characterize G418-selected cells and confirm the extent of differentiation. The selected cells exhibited a typical cardiac morphology and formed the characteristic myofilaments. Well-developed sarcomeric structures were detected by cTnT and α-actinin immunocytology (Figure 5).

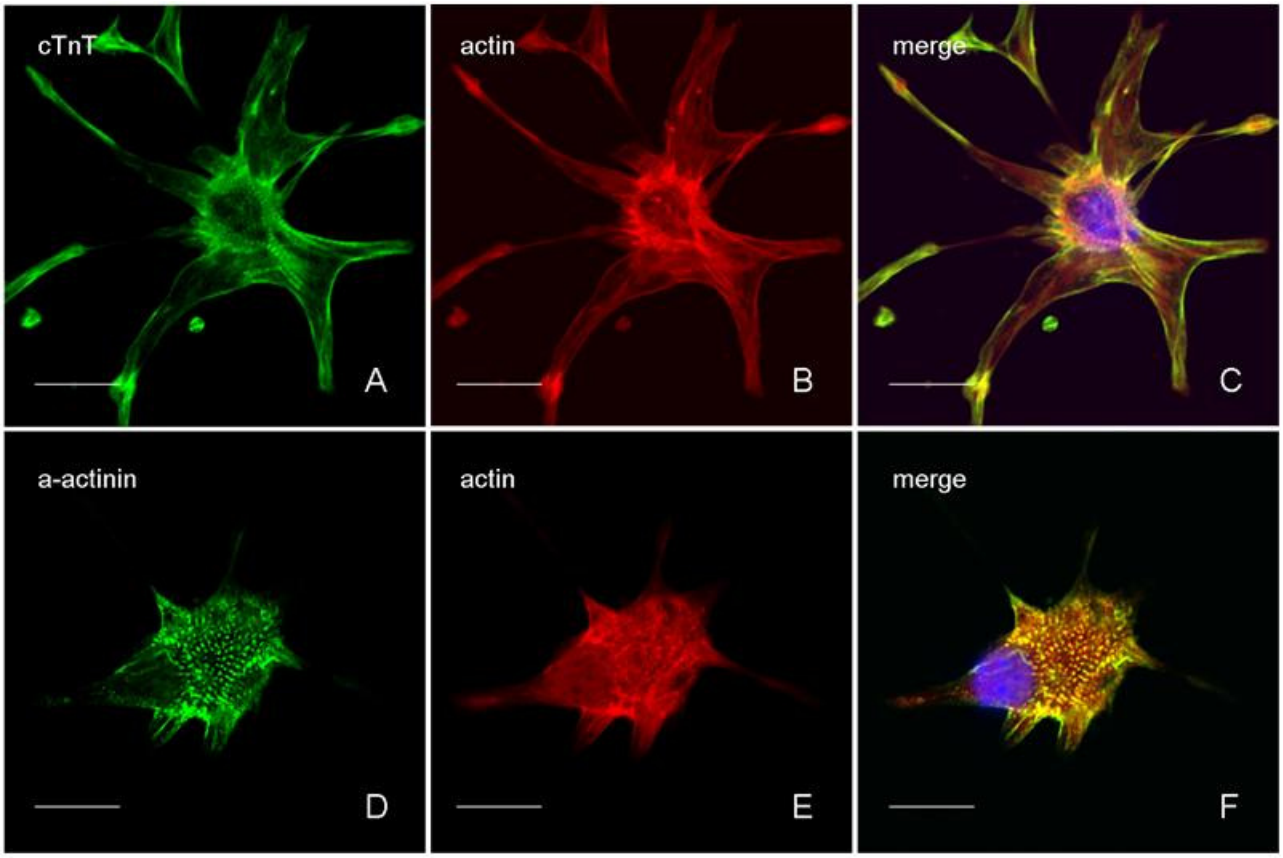

Figure 5. Confocal microscopy observation of immunofluorescence staining of G418-selected cardiomyocytes. Well-developed myofibrillar structures were detected using cTnT (A) and α-actinin (D) immunocytology and actin filaments (B, E) were labeled with phalloidin-TRITC. DAPI was used for staining nuclei showing blue in merged pictures (C, F). Scale bars: 20 μm.

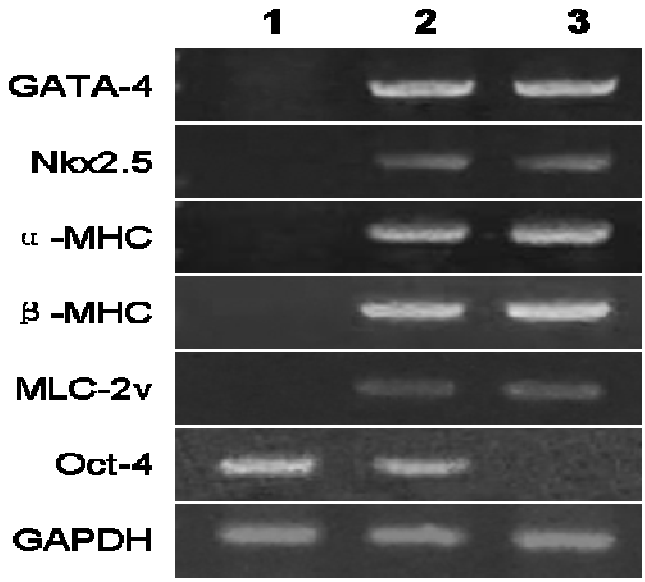

Figure 6. RT-PCR analysis of undifferentiated ESCs (1), differentiated EBs (2) and G418-selected cells (3). G418-selected cells expressed genes GATA-4, Nkx2.5, α-MHC, β-MHC and MLC-2v, whereas Oct-4 was not detected in the G418-selected cardiomyocytes.

RT-PCR analysis

RT-PCR results showed that G418-selected cells expressed genes GATA-4, Nkx2.5, α-MHC, β-MHC and MLC-2v. Undifferentiated ESCs expressed the pluripotent marker Oct-4 and did not express these cardiac-specific genes. Expression of Oct-4 in differentiated cultures of EBs decreased, whereas Oct-4 was not detected in G418-selected cardiomyocytes (Figure 6). The results indicated that, G418-selected cells may not contain undifferentiated ESCs.

Ultrastructural analysis

The G418-selected cardiomyocytes exhibited spontaneous and rhythmic contractile activity. Ultrastructural analyses further demonstrated the differentiated status of cardiomyocyte cultures. Parallel arrays of myofibrillar bundles were apparent and the formation of early and more developed Z bands could be observed in many of the selected cardiomyocytes (Figure 7a). Desmosomes and gap junctions were observed to connect adjacent cells (Figure 7b, c). Myosin synthesis was observed to

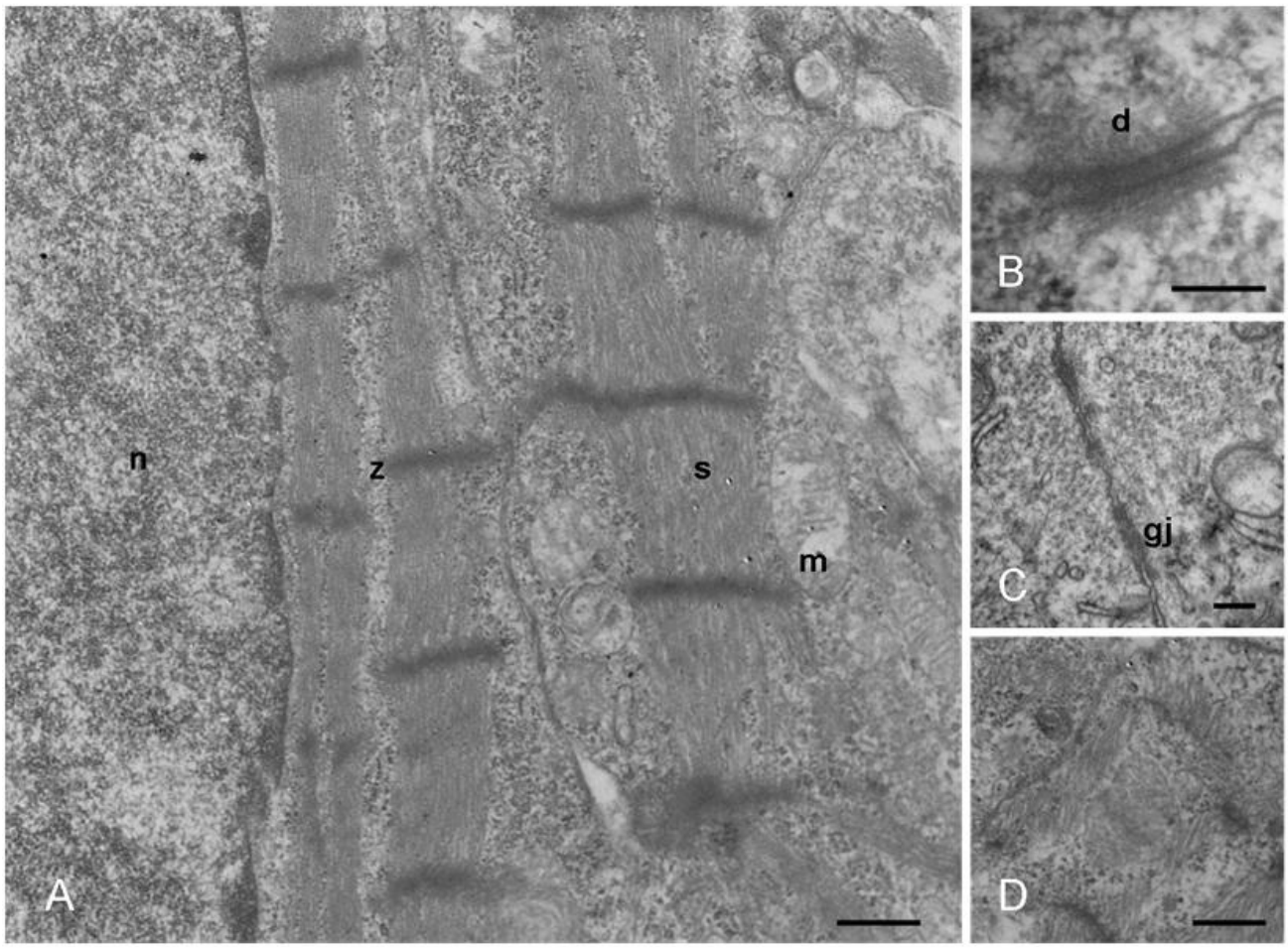

Figure 7. Ultrastructural analysis of selected cardiomyocytes. (A) Transmission electron micrograph of G418-selected cardiomyocytes. n, nucleus; m, mitochondria; s, sarcomere; z, Z band; (B) high-power electron micrograph showing the presence of a desmosome (d); (C) high-power electron micrograph demonstrating the laminar structure of the gap junction (gj); (D) high-power electron micrograph demonstrating the presence of myosin synthesis along free ribosomes. Scale bars: 500 nm.

occur along free ribosomes in many of the selected cells (Figure 7d).

DISCUSSION

ESC-derived cardiomyocytes have several potential applications including cell transplantation for myocardial repair, but the heterogeneity of differentiated cells derived from ESCs represents a considerable hurdle for widespread use of these cells in scientific and clinical applications. This highlights the requirement for effective strategies to enrich cardiomyocytes. In this report, we described the establishment of transgenic mouse ESC lines that allowed selection of differentiated cardiomyocytes. This method is based on using a cardiac-specific α-MHC promoter to drive the expression of neomycin resistance gene. ESC-derived cardiomyocytes expressing the fusion gene could survive after G418 selection. In contrast, non-cardiomyocytes derived from transgenic ESCs did not express neomycin resistance gene and consequently were eliminated by G418 selection. The selected cells were stained positively for cardiac-specific proteins, expressed cardiac-specific genes and were highly differentiated.

Myosin is the main component of thick filaments of cardiac sarcomere and consists of the heavy chain and light chain proteins. In cardiac muscle, two MHC isoforms were present, namely α-MHC and β-MHC. β-MHC is the predominant isoform during embryogenesis and fetal development, but in the post-birth period, α-MHC becomes the major form in adult myocardium (Liew and Jandreski, 1986). Gulick et al. (1991) described the isolation of mouse cardiac MHC locus and the characterization of the promoter region of the α-cardiac MHC gene with respect to its ability to direct expression of the CAT gene in myocardium in a tissue-specific manner. The use of cardiac-specific promoters for successful selection of cardiomyocytes was previously reported (David et al., 2008; Fijnvandraat et al., 2003; Huber et al., 2007; Klug

et al., 1996; Kolossov et al., 1998). We isolated mouse α-MHC promoter and constructed a vector α-MHC-EGFP to enable cardiac-specific EGFP expression. Neonatal mouse cardiomyocytes transfected α-MHC-EGFP could appear green fluorescence, but cardiac fibroblasts transfected positively did not express EGFP (He et al., 2009). We also demonstrated that α-MHC promoter had the ability to drive the specific expression in myocardial cells and would be suitable for selection of ESC-derived cardiomyocytes.

Interestingly, the transgenic mouse ESCs retained the ability to propagate in undifferentiated state, because the ESC colonies were stained positively for mouse ESC markers SSEA-1 and Oct-4, as well as a strong AKP activity. Expression of the pMHC-neo/SV40-hygro transgene also did not impact negatively on cardiogenic differentiation, as evidenced by their ability to differentiate into spontaneously beating cardiomyocytes when compared with wild-type lines. However, α-MHC promoter is active in the entire adult heart including a trial and pacemaker cells and do not possess specificity distinguishing between different types of cardiomyocytes (Müller et al., 2000). All the different cardiac phenotypes would be positively selected by α-MHC promoter-based enrichment method. Refinement of the selection procedure should be possible by choosing an appropriately cell type-restricted promoter. Combinatorial approaches using multiple promoters and selectable markers also can be envisioned (Anderson et al., 2007; Kolossov et al., 2005).

Cell transplantation as a potential therapy for heart disease has been extensively demonstrated (Laflamme and Murry, 2005). Although, several types of cells have been transplanted into the hearts of experimental animals, cardiomyocytes are theoretically the best candidate donor cell to restore lost cardiac function because of their intrinsic electrophysiological, structural and contractile properties, which collectively allows them to functionally integrate with the host myocardium. To achieve cellular transplantation in larger animals and humans, a higher number of cardiomyocytes will be required. We recently showed that treatment of EBs with ascorbic acid is advantageous (data not shown). Another possibility to increase the quantity of differentiating cardiomyocytes might be employing a sequential cytokine induction protocol (Yang et al., 2008). However, the inherent variability of cardiogenic induction in differentiating ESCs can have a significant impact on the ultimate yield. The yield of G418-selected cardiomyocytes currently is somewhat variable, but it is likely that optimization and standardization of the induction and selection protocols will result in improved and reproducible preparations.

Most importantly, the ability to select and generate pure cardiomyocytes may be very important for the future utilization of human ESCs in cell replacement therapy. The presence of any remaining pluripotent stem cells in the cell grafts may result in ESC-related tumors such as teratomas (Gepstein, 2002). Our results showed that more than 99% of the selected cells were positive for α-sarcomeric actin and the pluripotent marker Oct-4 was not detected in G418-selected cells. Genetic enrichment was effective for purification of cardiomyocytes derived from ESCs. However, it still needs extensive studies in future to ensure the security of cell therapy. Regardless of stem cell source, the approach described here provides a useful tool to generate relatively pure cardiomyocytes for cell transplantation. The next challenge will be to determine the most effective route to scaling up production of ESC-derived cardiomyocytes and to test functional integration in animal models with cardiac dysfunction. Furthermore, it will be of great interest to transfer the promoter-based approach to the recently described ESCs derived from somatic cell nuclear transfer (Byrne et al., 2007), as well as to induced pluripotent stem cells (Yu et al., 2007). This may be the basis for future autologous cellular sources and future clinical use of cardiovascular progenitor cells.

In summary, this report describes the generation of unique transgenic mouse ESC lines allowing selection of pure cardiomyocytes. This ability may have important implications for several cardiovascular research fields, including basic developmental studies, pharmacological and physiological studies, cell therapy and tissue engineering. The selection procedure should be applicable to all ESC-derived cell lineages, provided that suitable lineage-specific promoters are available. It is likely that, ESC-derived cellular transplantation strategies can be extended to other organ systems.

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