Electron microscopic study of mouse embryonic stem cell-derived cardiomyocytes

Masoumeh Fakhr Taha · Mojtaba Rezazadeh Valojerdi ·
Leili Hatami · Arash Javeri

Received: 21 September 2011 / Accepted: 11 November 2011 / Published online: 9 December 2011
© Springer Science+Business Media B.V. 2011

Abstract Differentiation of embryonic stem cell (ESC)-derived embryoid bodies (EBs) is a heterogeneous process. ESCs can differentiate in vitro into different cell types including beating cardiomyocytes. The main aim of the present study was to develop an improved preparation method for scanning electron microscopic study of ESC-derived cardiac bundles and to investigate the fine structural characteristics of mouse ESCs-derived cardiomyocytes using electron microscopy. The mouse ESCs differentiation was induced by EBs' development through hanging drop, suspension and plating stages. Cardiomyocytes appeared in the EBs' outgrowth as beating clusters

that grew in size and formed thick branching bundles gradually. Cardiac bundles showed cross striation even when they were observed under an inverted microscope. They showed a positive immunostaining for cardiac troponin I and α -actinin. Transmission and scanning electron microscopy (TEM & SEM) were used to study the structural characteristics of ESC-derived cardiomyocytes. Three weeks after plating, differentiated EBs showed a superficial layer of compact fibrous ECM that made detailed observation of cardiac bundles impossible. We tried several preparation methods to remove unwanted cells and fibers, and finally we revealed the branching bundles of cardiomyocytes. In TEM study, most cardiomyocytes showed parallel arrays of myofibrils with a mature sarcomeric organization marked by H-bands, M-lines and numerous T-tubules. Cardiomyocytes were connected to each other by intercalated discs composed of numerous gap junctions and fascia adherences.

Electronic supplementary material The online version of this article (doi:[10.1007/s10616-011-9411-4](https://doi.org/10.1007/s10616-011-9411-4)) contains supplementary material, which is available to authorized users.

M. F. Taha (✉) · A. Javeri
Department of Medical Biotechnology, National Institute
of Genetic Engineering and Biotechnology (NIGEB),
Tehran, Iran
e-mail: mftaha@nigeb.ac.ir

M. R. Valojerdi
Department of Anatomy, School of Medical Sciences,
Tarbiat Modares University, Tehran, Iran

M. R. Valojerdi
Department of Embryology, Royan Institute, Tehran, Iran

L. Hatami
Department of Anatomy, School of Medical Sciences,
Shahrood University of Medical Sciences, Shahrood, Iran

Keywords Mouse embryonic stem cell ·
Cardiomyocyte · Scanning electron microscopy ·
Ultrastructure

Introduction

Embryonic stem cells (ESCs) which are derived from the inner cell mass of blastocyst-stage embryos (Evans and Kaufman 1981; Martin 1981) have unique characteristics which distinguish them from adult stem cells

and also make them an important cell source for biology research. ESCs can be maintained in an undifferentiated state *in vitro*. However, when allowed to differentiate in suspension or in hanging drops, they aggregate to form embryoid bodies (EBs). Differentiation from EBs is a heterogeneous process, and different cell types can be derived from ESCs during EB culture. Cardiac differentiation of ESCs is now a feasible and interesting field of research. Some growth and differentiation factors have been shown to induce cardiomyocyte differentiation from ESCs (Baharvand et al. 2005; Behfar et al. 2002; Bremer et al. 1999; Dell’Era et al. 2003; Kawai et al. 2004; Klinz et al. 1999; Paquin et al. 2002; Sauer et al. 2000; Taha and Valojerdi 2008; Takahashi et al. 2003; Ventura and Maioli 2000). Several researchers have studied the patterns of gene expression during cardiac differentiation of ESCs (Czyz and Wobus 2001; Fijnvandraat et al. 2003; Kehat et al. 2001; Wobus and Guan 1998; Xu et al. 2002). Moreover, structural and functional characteristics of ESC-derived cardiomyocytes have been assessed in several studies (Baharvand et al. 2005; Hatami et al. 2007; Kehat et al. 2001; Metzger et al. 1995; Snir et al. 2003; Taha et al. 2007; Taha and Valojerdi 2008).

In our previous studies (Taha et al. 2007; Taha and Valojerdi 2008), we evaluated the effect of bone morphogenetic protein-4 (BMP-4) on cardiomyocyte differentiation from ESCs. In the current study, we developed an improved preparation method for SEM study of ESC-derived cardiac bundles, and we assessed the fine structural characteristics of mouse ESC-derived cardiomyocytes by SEM and TEM. Since detailed results concerning TEM evaluation of cardiomyocytes have been reported (Taha et al. 2007), now we describe the result of SEM study, and the results of TEM analysis of cardiomyocytes are briefly reviewed.

Materials and methods

Mouse ESCs culture and differentiation

The mouse ESC line Royan B1, obtained from the inner cell mass of a C57BL/6 strain mouse blastocyst, was used in the present study (Baharvand and Matthaie 2003; Baharvand et al. 2004). Royan B1 ESCs were cultured on top of a feeder layer of mitomycin-C-treated mouse embryonic fibroblasts

(MEF) in the presence of leukemia inhibitory factor (LIF, Chemicon, ES-GRO, Boronia, Victoria, Australia), as described previously (Baharvand et al. 2004).

The ESCs differentiation was initiated by dissociation from the MEF feeder layer, and EBs’ development was induced through hanging drop, suspension and plating stages, as previously described (Taha et al. 2007). Growth and morphological changes of EBs were monitored daily using an inverted microscope.

Differentiation medium consisted of 0.1 mM β -mercaptoethanol (Sigma), 1 mM L-glutamine, 1% nonessential amino acid stock and 1% penicillin–streptomycin (all from Gibco) in knockout Dulbecco’s modified Eagle’s medium (KnockoutTM DMEM, high-glucose, with sodium pyruvate formulation; Gibco) supplemented with 15% fetal bovine serum (FBS, ESC qualified, Gibco). The medium was changed every 2 days.

Fluorescent immunostaining

For cardiac troponin I immunostaining, the contracting areas were mechanically dissected and dispersed using trypsin–EDTA (Gibco, 25300-054). Then, the cells were plated on gelatin-coated 4-well tissue culture plates at a low density and incubated for 60 h. For α -actinin immunostaining, the whole EB’s outgrowth, containing contracting areas, was used. Both groups of the cells were fixed using 4% paraformaldehyde, permeabilized using 0.5% Triton X-100 (Sigma, T-8787), blocked with 10% goat serum (Gibco, 16210-064) and incubated with primary and secondary antibodies for 45 min at 37 °C. Antibodies used in this study included mAb for cardiac muscle troponin I (CTnI; Chemicon, MAB169) at a dilution of 1:100, mAb for α -actinin (Sigma; A-7811) at a dilution of 1:800, mAb for nebulin (Sigma; N-9891) at a dilution of 1:75 and anti-mouse, FITC-conjugated IgG antibody (Sigma, F9006) at a dilution of 1:100. Preparations were examined using a fluorescent microscope (Nikon, Japan). Dispersed cells isolated from non-contracting EBs served as negative controls and cells isolated from mouse skeletal muscle served as positive control for nebulin staining.

Transmission electron microscopy

For TEM study, contracting areas were dissected and fixed in 4% glutaraldehyde in 0.1 M phosphate buffer

(pH 7.4) for 2 h, postfixed in 1% OsO₄ in the same buffer for 1.5 h, dehydrated in graded ethanol, and embedded in Araldite 6005 (Sigma). Ultrathin (50–70 nm) sections were double-stained with uranyl acetate and lead citrate and used for ultrastructural evaluation using a transmission electron microscope (EM900, Zeiss, Germany).

Scanning electron microscopic study

For scanning electron microscopic study, all samples were mechanically dissected from EBs' outgrowths, washed twice with 0.1 M phosphate buffer (pH 7.4), fixed with 4% glutaraldehyde solution for 2 h, washed with 0.1 M/L phosphate buffer (pH 7.4) solution, postfixed with 0.1% osmium tetroxide for 1 h and washed with the same buffer again. At this stage cardiac samples were transferred to 8 N HCl and incubated at 60 °C with shaking for 15 min. Samples were immersed in 0.1 M phosphate buffer overnight, then transferred to 1% Triton X-100 with shaking at room temperature for 3 h and immersed in 15 mg/10 mL collagenase II with shaking at 37 °C for 6–8 h.

After dehydration with graded series of alcohol, all samples were freeze dried, coated in a sputter-coater with a layer of gold and observed under a scanning electron microscope (SEM360, Cambridge, UK).

Results and discussion

Differentiation of mouse ESCs was started by formation of EBs through hanging drop method (Fig. 1A). Cardiomyocyte development began before plating, and contraction in some EBs started from the suspension stage. After plating, mouse ESC-derived EBs attached to the gelatinized wells of tissue culture plates and continued their differentiation to heterogeneous cell types including spontaneously contracting cardiac cells. One day after plating, about 95% of EBs showed contraction. At the beginning, cardiomyocytes appeared as one or two round clusters with slow beating, and then they grew in size and formed thick and branching bundles with stronger and faster pulse rates (supplement video). Two–three weeks after plating, cardiomyocytes showed mature sarcomeric organization, and cross striation of cardiac bundles was even observable under inverted microscope (Fig. 1B, C).

Three weeks after plating, dissociated cells from contracting areas were immunostained using anti-CTnI and anti-nebulin monoclonal antibodies. As shown in Fig. 1D, the cells showed positive immunostaining for CTnI protein, but they were negative for nebulin. Since nebulin is an abundantly expressed protein in skeletal muscle, this confirms the cardiac rather than skeletal nature of the cells. Immunostaining of the EBs' outgrowth using anti- α -actinin monoclonal antibody demonstrated a network of cardiac bundles with a fine myofibrillar organization and a distinct cross striation (Fig. 1E, F).

TEM and SEM were used to study the structural characteristics of ESC-derived cardiomyocytes. Our SEM analysis of the ESC-derived cardiomyocytes is the first report in this regard. At first, we used a standard protocol for sample preparation and studied the surface topography of the plated EBs by SEM. Three weeks after plating, differentiated EBs showed a superficial layer of compact fibrous ECM. This observation was in agreement with the previous reports. As described by Sachlos and Auguste (2008), during EBs' development, a fibrous ECM mainly consisting of collagen type I is secreted on the surface of EB and continues to form until the voids between cells are filled. By day 14, ECM deposition results in a smoother surface topography and the cell boundaries become indistinguishable. This makes detailed observation of cardiac bundles impossible. So, we tried several preparation methods to remove unsuitable cells and fibers to achieve sharp bundles of cardiomyocytes. When we processed the samples with a general protocol for sample preparation, we could reveal only a small part of cardiac bundles (Fig. 2B), but it was not satisfactory. Then the samples were exposed to collagenase II, Triton X-100 and 8 N HCl as mentioned in Materials and Methods section, and finally thick branching bundles of cardiomyocytes were revealed (Fig. 2C, D). In the current study we could not detect structural features of cardiac bundles in more detail, and better tissue preparation would be required in order to improve visualization of the structures such as intercalated disc and T-tubular system. In fact, ESC-derived cardiomyocytes are important tools for functional studies concerning the effects of drugs on beating cardiomyocytes *in vitro*. SEM analysis can be used for study of the branching pattern, diameter and other structural features of the cardiac bundles, especially when cardiomyocytes are treated with cardioactive

Fig. 1 Differentiation of mouse ESCs to cardiomyocytes; **A** Embryoid bodies (EBs) at the end of suspension stage, **B**, **C** Photomicrographs of cardiac bundles with two different magnifications. Cross striation of the bundles is observable with higher magnification. *Arrowheads* point to cardiac bundles,

D Immunostaining of ESCs-derived cardiomyocytes for cardiac troponin I. **E**, **F** Immunostaining of ESCs-derived cardiomyocytes for α -actinin (**E**) and staining of the nuclei with Propidium iodide (**F**)

drugs and hypertrophy inducing agents. SEM analysis may also be useful to study the formation of neuromuscular junctions in future.

We have previously reported the ultrastructural characteristics of mouse ESC-derived cardiomyocytes (Taha et al. 2007; Taha and Valojerdi 2008), and here we briefly explain it with two new EM micrographs (Fig. 2E–F). Fourteen days after plating, cardiomyocytes were revealed as mononuclear cells at different developmental stages. Most cardiomyocytes showed tightly packed parallel arrays of myofibrils. They

showed a mature sarcomeric organization marked by H-bands, M-lines and numerous T-tubules. These cardiomyocytes were connected to each other by intercalated discs composed of numerous gap junctions and fascia adherences. It should be mentioned here that T-tubular system and M-band formation are considered the endpoint of myofibrillar maturation that only develops postnatally (Viragh and Challice 1969).

In conclusion, mouse ESCs were differentiated to cardiomyocytes through hanging drop method.

Fig. 2 SEM and TEM micrographs of ESCs-derived cardiomyocytes; **A** SEM micrograph of differentiated EBs 3 weeks after plating. A superficial layer of compact fibrous ECM is observable. **B** Small part of a cardiac bundle (*Asterisk*) that was revealed after sample preparation with the general protocol. **C**,

D Branching bundles of cardiomyocytes were revealed when the samples were exposed to collagenase II, Triton X-100 and 8 N HCl. **E**, **F** Transmission electron micrographs of cardiomyocytes at day-14 after plating; *Fa* fascia adherence, *Gj* gap junction, *Mf* myofibrils, *Ml* M line, *Z* Z line

Contracting bundles showed positive immunostaining for cardiac markers as well as clear striation that was due to sarcomeric organization of the cardiomyocytes. An improved preparation method was also developed for SEM study of the ESC-derived cardiac bundles. SEM and TEM analyses demonstrated the structural characteristics of cardiac bundles in more detail and provided evidence that ESCs are suitable for production of nearly matured cardiomyocytes.

Acknowledgments This study was supported by research grants from Jihad Daneshgahi (137-1-5). We thank Dr. Hossein Baharvand for providing ESCs, Mrs. Ilnaz Sadeghi Sadr for

assistance in the SEM imaging and Abbas Piriaii and Shahram Pour Beiranvand for technical assistance in the TEM study.

References

- Baharvand H, Matthaehi KI (2003) The ultrastructure of mouse embryonic stem cells. *Reprod Biomed Online* 7:330–335
- Baharvand H, Ashtiani SK, Valojerdi MR, Shahverdi A, Taei A, Sabour D (2004) Establishment and in vitro differentiation of a new embryonic stem cell line from human blastocyst. *Differentiation* 72:224–229
- Baharvand H, Azarnia M, Parivar K, Ashtiani SK (2005) The effect of extracellular matrix on embryonic stem cell-derived cardiomyocytes. *J Mol Cell Cardiol* 38:495–503

- Behfar A, Zingman LV, Hodgson DM, Rauzier J, Kane GC, Terzic A, Puceat M (2002) Stem cell differentiation requires a paracrine pathway in the heart. *FASEB J* 16:1558–1566
- Bremer S, Van Dooren M, Paparella M, Kossolov E, Fleischmann BK, Hescheler J (1999) Effects of embryotoxic chemicals on the in vitro differentiation of genetically engineered embryonic stem cells into cardiac cells. *Toxicol In Vitro* 13:645–650
- Czyz J, Wobus AM (2001) Embryonic stem cell differentiation: the role of extracellular factors. *Differentiation* 68:167–174
- Dell'Era P, Ronca R, Coco L, Nicoli S, Metra M, Presta M (2003) Fibroblast growth factor receptor-1 is essential for in vitro cardiomyocyte development. *Circ Res* 93:414–420
- Evans M, Kaufman M (1981) Establishment in culture of pluripotent cells from mouse embryos. *Nature* 292:154–156
- Fijnvandraat AC, Van Ginneken ACG, de Boer PAJ, Ruijter JM, Christoffels VM, Moorman AFM, Lekanne Deprez RH (2003) Cardiomyocytes derived from embryonic stem cells resemble cardiomyocytes of the embryonic heart tube. *Cardiovas Res* 58:399–409
- Hatami L, Valojerdi MR, Mowla SJ (2007) Effects of oxytocin on cardiomyocyte differentiation from mouse embryonic stem cells. *Int J Cardiol* 117:80–89
- Kawai T, Takahashi T, Esaki M, Ushikoshi H, Nagano S, Fujiwara H, Kosai K (2004) Efficient cardiomyogenic differentiation of embryonic stem cell by fibroblast growth factor 2 and bone morphogenetic protein 2. *Circ J* 68:691–702
- Kehat I, Kenyagin-Karsenti D, Snir M, Segev H, Amit M, Gepstein A, Livne E, Binah O, Itskovitz-Eldor J, Gepstein L (2001) Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. *J Clin Invest* 108:407–414
- Klinz F, Bloch W, Addicks K, Hescheler J (1999) Inhibition of phosphatidylinositol-3-kinase blocks development of functional embryonic cardiomyocytes. *Exp Cell Res* 247:79–83
- Martin G (1981) Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci USA* 78:7634–7638
- Metzger JM, Lin W, Johnston RA, Westfall MV, Samuelson LC (1995) Myosin heavy chain expression in contracting myocytes isolated during embryonic stem cell cardiogenesis. *Circ Res* 76:710–719
- Paquin J, Danalache BA, Jankowski M, McCann SM, Gutkowska J (2002) Oxytocin induces differentiation of P19 embryonic stem cells to cardiomyocytes. *Proc Natl Acad Sci USA* 99:9550–9555
- Sachlos E, Auguste DT (2008) Embryoid body morphology influences diffusive transport of inductive biochemicals: a strategy for stem cell differentiation. *Biomaterials* 29:4471–4480
- Sauer H, Rahimi G, Hescheler J, Wartenberg M (2000) Role of reactive oxygen species and phosphatidylinositol 3-kinase in cardiomyocyte differentiation of embryonic stem cells. *FEBS Lett* 476:218–223
- Snir M, Kehat I, Gepstein A, Coleman R, Itskovitz-Eldor J, Livne E, Gepstein L (2003) Assessment of the ultrastructural and proliferative properties of human embryonic stem cell-derived cardiomyocytes. *Am J Physiol Heart Circ Physiol* 285:H2355–H2363
- Taha MF, Valojerdi MR (2008) Effect of bone morphogenetic protein-4 on cardiac differentiation from mouse embryonic stem cells in serum-free and low-serum media. *Int J Cardiol* 127:78–87
- Taha MF, Valojerdi MR, Mowla SJ (2007) Effect of bone morphogenetic protein-4 (BMP-4) on cardiomyocyte differentiation from mouse embryonic stem cell. *Int J Cardiol* 120:92–101
- Takahashi T, Lord B, Schulze PC, Fryer RM, Sarang SS, Gullans SR, Lee RT (2003) Ascorbic acid enhances differentiation of embryonic stem cells into cardiac myocytes. *Circulation* 107:1912–1916
- Ventura C, Maioli M (2000) Opioid peptide gene expression primes cardiogenesis in embryonal pluripotent stem cells. *Circ Res* 87:189–194
- Viragh S, Challice CE (1969) Variations in filamentous and fibrillar organization, and associated sarcolemmal structures in cells of the normal mammalian heart. *J Ultrastruct Res* 28:321–334
- Wobus AM, Guan K (1998) Embryonic stem cell-derived cardiac differentiation: modulation of differentiation and 'loss of function' analysis in vitro. *Trends Cardiovas Med* 8:64–74
- Xu C, Police S, Rao N, Carpenter MK (2002) Characterization and enrichment of cardiomyocytes derived from human embryonic stem cells. *Circ Res* 91:501–508