Biochemical and Biophysical Research Communications 461 (2015) 281–286

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Exogenous treatment with eicosapentaenoic acid supports maturation of cardiomyocytes derived from embryonic stem cells

Parisa Shabani [a, b], Zaniar Ghazizadeh [b], Sara Pahlavan [b], Shiva Hashemizadeh [b], Hossein Baharvand [b], Nasser Aghdami [b, *, 1], Mahmood Doosti [a, **, 1]

[a] Department of Clinical Biochemistry, Faculty of Medicine, Tehran University of Medical Sciences (TUMS), Tehran, Iran
[b] Department of Stem Cells and Developmental Biology at Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran

ARTICLE INFO

Article history:
Received 26 March 2015
Available online 12 April 2015

Keywords:
Embryonic stem cells
Cardiomyocytes
Eicosapentaenoic acid

ABSTRACT

Embryonic stem cells offer multiple advantages over adult stem cells in terms of achieving acceptable number of functional cardiomyocytes to be exploited in cell therapy. However, differentiation efficacy is still a major issue to be solved before moving to regenerative medicine. Although a vast number of chemical compounds have been tested on efficiency of cardiac differentiation, the effect of fish oil components, such as eicosapentaenoic acid (EPA) on developmental bioenergetics, and hence cardiac differentiation, remained unstudied. EPA has been reported to have several cardioprotective effects, but there is no study addressing its role in cardiac differentiation. After mesoderm induction of embryoid bodies (EBs) derived from mouse embryonic stem cells (mESCs) in hanging drops initiated by ascorbic acid, they were treated with various concentrations of EPA. Gene and protein expression and functional properties of cardiomyocytes derived from ESCs were evaluated following treatment with various concentrations of EPA. Exposure to low concentrations of EPA (10 μM) increased percentage of beating colonies and beating area. This treatment also resulted in up to 3 fold increase in expression of *NKX2-5*, *MEF2C*, *MYH6*, *TNNT2* and *CX43*. FACS analysis confirmed gene expression analysis with increased percentage of MYH6 positive cells in EPA-treated group compared to the control group. In contrast, the expression of genes coding for cardiac differentiation, remained constant or even declined with higher concentrations of EPA. In conclusion, we have demonstrated that treatment of mESCs undergoing cardiac differentiation with low concentration, but not high concentration of EPA up-regulate transcription of genes associated with cardiac development.

1. Introduction

Ischemic heart disease is the major cause of death worldwide [1]. Embryonic stem cells (ESCs) are promising therapeutic agents that can potentially generate an unlimited source for cell therapy. Several lines of evidence have shown that cardiomyocytes derived from ESCs can replenish infarcted heart and rescue heart function [2,3]. However, preclinical studies in animal models of myocardial infarction (MI), showed low cell survival and inefficient electrical coupling following transplantation [4]. Therefore, providing conditions that lead to better survival, higher differentiation and better expression of electrical coupling-related channels in cardiomyocytes developed from pluripotent stem cells, could help improve methods for producing efficient cells in terms of regenerative medicine.

Recently, a vast number of growth factors and compounds such as transforming growth factor-β (TGF-β) [5], 5-azacytidine [6], hepatocyte growth factor [7] and angiotensin II [8] have been reported to induce cardiac differentiation. Although ample number of studies has been conducted on cardiogenic effect of growth factors and chemical compounds, effect of providing metabolic and energetic requirements of beating cardiomyocytes during transition from stem cells requiring little energy to high-demand cardiomyocytes are only partly understood. Moreover, in addition to

* Corresponding author. Department of Stem Cells and Developmental Biology at Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, P.O. Box 19395-4644, Tehran, Iran. Fax: +98 21 22310406.
** Corresponding author. Department of Clinical Biochemistry, School of Medicine, Tehran University of Medical Sciences (TUMS), Tehran, Iran.
E-mail addresses: aghdami@royaninstitute.org (N. Aghdami), doostimd@sina.tums.ac.ir (M. Doosti).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.bbrc.2015.04.018
0006-291X/

being an important energy source in cardiomyocytes [9], fatty acids can modulate signaling pathways during heart development [10] and are also the major components of membranes and active ligands for receptors and transcription factors [11]. More recently a metabolomics study revealed the robust significance of metabolites such as fatty acids in directing fate of embryonic stem cell toward cardiomyocyte. They reported that treatment of ESCs with metabolites involving in oxidative metabolism such as saturated fatty acids enhanced cardiac differentiation [12]. However, effect of treatment with unsaturated fatty acids during cardiac differentiation has not been well defined yet.

n-3 polyunsaturated fatty acids (n-3 PUFA) such as eicosapentaenoic acid (EPA) have been reported to decrease risk of cardiovascular diseases and arrhythmia through various mechanisms [13–15]. Some studies showed that n-3 PUFA consumption leads to its incorporation into cellular membrane and also mitochondrial membranes, which could modify the activity of receptors and ion channels [16–20]. Moreover, increasing evidence indicated potential role of n-3 PUFAs in modulating bioenergetics of cardiac cells through increasing mitochondria number and improved mitochondrial function efficacy [21,22]. Besides, fatty acid treatment can modulate gene expression profile through various pathways, peroxisome proliferator-activated receptors (PPARs) being the most widely studied target [23]. Taking into account importance of coordination of genetic and bioenergetic transformations during differentiation into cardiomyocytes [24], it is of interest to study the possible effect of n-3 PUFA on cardiac differentiation and characteristics of cardiomyocytes derived from ESCs.

This study aims to explore the possible effect of a range of EPA concentrations on cardiomyocytes differentiation from mouse embryonic stem cells (mESCs). Moreover, we addressed their potential effect on electrophysiological characteristics of cardiomyocytes derived from mESCs by multi-electrode array study of beating cardiomyocytes.

2. Methods

2.1. Cell culture and differentiation

mESCs were cultured and maintained on gelatin in knock out Dulbecco's minimum essential medium (Knockout™ DMEM, Gibco, 10829–018) growth media supplemented with 1000 U/mL leukemia inhibitory factor (LIF, Royan Institute), glutamine (Gibco, 25030–024), penicillin/streptomycin (Gibco, 15070–063), nonessential amino acids (Gibco, 11140–035), β-mercaptoethanol (Sigma Aldrich, M7522), and 15% fetal bovine serum (Gibco, 12483–020).

mESCs were differentiated using hanging drop method, a previously described protocol [25]. Briefly, mESCs were trypsinized and hanging drops of 800 cells in 20 μL of cultivation medium without LIF were generated. On day 3 of differentiation, the generated EBs were transferred to petri dishes and re-suspended for more 2 days. On day 5, the EBs were plated on gelatin-coated plates and observed for the appearance of beating EBs. From day 3 onwards, cells were treated with ascorbic acid (100 μM) and different concentrations of EPA (10, 50, 100 μM).

2.2. Quantitative real-time PCR

Total RNA was isolated using TRIzol reagent (Invitrogen, 15596-018). Subsequently RNA was reverse transcribed into cDNA using Revert Aid First Strand cDNA Synthesis Kit (Fermentas, K1632). In this reaction, the samples were incubated in a thermocycler at 37 °C for 15 min and then at 85 °C for 5 s. Quantitive real-time PCR was performed using SYBR Premix Ex TaqTM II (Takara Bio, Inc., SYBR® Premix Ex Taq™ II RR081Q) in a final volume of 20 μL on a Rotor Gene real-time thermocycler (Qiagen, Hilden, Germany). The thermal profile for SYBR Green PCR was 95 °C for 15 s, followed by 40 cycles of denaturation at 95 °C for 5 s and annealing/extension at 60 °C for 30 s. The sequence of primers for amplification was as shown in Supplementary Table 1. The specificity of amplification was monitored by DNA melting curve during gradual temperature increments. β-tubulin was used as the endogenous control. The comparative Ct method (ΔΔCt) was utilized to calculate the fold change in genes expression in EPA-treated groups compared to non-treated group.

2.3. Immunofluorescence staining

Contracting EBs were dissociated using collagenase type II for 40 min in a 37 °C incubator. After dissociation, single cells were plated on gelatin-coated 4-well plates and allowed to grow for two days. Cells were fixed with phosphate buffered saline (PBS, Gibco, 21600–051) containing 4% paraformaldehyde (Sigma–Aldrich, P6148) at 4 °C for 20 min, washed with PBS containing 0.05% (v/v) Tween, permeabilized and blocked in PBS containing 0.2% (v/v) Triton X-100 and 10% serum for 40 min at room temperature and then incubated with first antibodies against MYH6, TNNT2, NKX2-5, CX43 or respective isotype controls in PBS containing 1% BSA overnight at 4 °C. Cells were washed with PBS containing 0.05% (v/v) Tween for three times and stained with secondary antibodies in PBS containing 1% BSA for 2 h at room temperature. Cells were washed again and Nuclei were stained with 4,6-diamidino-2-phenylin-dole (DAPI, Sigma-Alrich, D8417). Immunocytochemistry-stained images were obtained using fluorescent microscope (Olympus, IX71). All primary and secondary antibodies, dilutions and suppliers are listed in Supplementary Table 2.

2.4. Flow cytometry

Dissociated cells were transferred to flow cytometry tubes and fixed with PBS containing 4% paraformaldehyde at 4 °C for 20 min, fixed cells were washed with PBS containing 0.05% (v/v) Tween, permeabilized and blocked in PBS containing 0.2% (v/v) Triton X-100 and 10% serum for 40 min at room temperature and then stained with first antibody against MYH6 overnight at 4 °C. Cells were washed as above and stained with secondary antibodythen washed again and Fluorescence was detected with a BD-FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA). Data were analyzed using Flowing software version 2.5.1.

2.5. Lactate dehydrogenase assay

Lactate dehydrogenase (LDH) release assay (LDH Cytotoxicity Detection Kit; Biovision; USA) was performed to evaluate the possible cytotoxic effect of EPA according to manufacturer's protocol. Briefly, 50 μL of cell medium and 50 μL of cell medium collected from the control group and the groups treated with different concentrations of EPA and 50 μL of reaction mixture were added to each well of a 96-well plate and incubated at room temperature for 30 min. The release of LDH was assayed at absorbance of 490 nm.

2.6. Microelectrode array

Extracellular recordings were performed with a microelectrode array (MEA) data acquisition system (Multi Channel Systems, Reutlingen, Germany). The MEA plates contained a matrix of 60 titanium nitride electrodes (30 μm) with an inter-electrode distance of 200 μm. The MEA plates were sterilized and coated with fibronectin for 2 h. The beating colonies were micro-dissected and

plated in the middle of coated MEA plates in a medium containing 50% FBS. After 2 h, the medium was exchanged to conventional medium and the colonies were allowed to adjust for 2 days before recording. In order to perform recording, MEA plates were connected to a head stage amplifier. Signals were acquired at 50 KHz and all recordings were performed at 37 °C. Recordings were performed for 100 s at baseline and at 5 min after drug application. The parameters including filed potential duration (FPD), corrected filed potential duration (cFPD) interspike intervals (ISI) and beating rate were analyzed using AxoScope software (Molecular Devices) and also CardioMDA [26].

2.7. Statistical analysis

All data were presented as mean ± SEM. Statistical analysis was performed using GraphPad Prism 6. Comparisons among groups were performed using the one-way analyses of variance (ANOVA) followed by Tukey post hoc tests. A value of $p < 0.05$ was regarded statistically significant.

3. Results

First, we investigated whether gene expression of cardiomyocytes derived from mESC were affected by EPA treatment and if these changes are dose dependent. After mesoderm induction of EBs in hanging drops initiated by ascorbic acid, they were treated with various concentrations of EPA. When differentiation was induced through, spontaneously contracting cell clusters developed at 7 days and reached to its peak level at day 10 (Fig. 1). Cardiomyocytes resulted from this protocol were highly positive for cardiac specific transcription factor, NKX2-5, and cardiac structural proteins, MYH6, TNNT2 and CX43 (Supplementary Fig. 1). After cells were treated with various concentrations of EPA from day 3–10, quantitative real-time PCR showed that all cardiac markers tested, such as *NKX2-5*, *MEF2C*, *MYH6*, *TNNT2* and *CX43*, were up-regulated by 10 μM EPA treatment, whereas the expression of these genes in cells treated with higher concentrations of EPA, was not altered significantly (Fig. 2A).

Second, FACS analyses revealed increased percentage of *α-MHC* positive cells treated with 10 μM EPA, confirming data obtained at gene expression level. Although the difference in percentage of MYH6 positive cells were not significant among different groups, but mean fluorescence intensity (MFI) of MYH6 in the group treated with 10 μM EPA was significantly higher relative to the control, and the groups treated with 50 μM and 100 μM EPA. Other concentrations of EPA, neither results in significant increase in the number of MYH6 nor MFI of MYH6 positive cells (Fig. 2B).

Then we assessed the possible effect of EPA treatment on the development of the structural organization in cardiomyocytes derived from ESCs. So, the contracting EBs from treated and non-treated groups at day 10 were dissociated into single cells for immunofluorescence staining analysis of TNNT2 and CX43 (Fig. 3A). TNNT2 positive cells were markedly higher in the group treated with 10 μM EPA compared to the control cells and cells treated with higher concentrations of EPA (Fig. 3B). Although we have not observed a significant difference in percentage of CX43 cells between treated and non-treated groups, but the percentage of cells co-expressing TNNT2 and CX43 were higher in the group treated with 10 μM EPA compared to the control group (Fig. 3B).

In order to rule out cytotoxicity related decrease in differentiation efficacy of higher EPA concentrations treatment, the concentration of LDH in the medium of EBs treated with different concentrations of EPA and control group have been measured. On day 4 of differentiation, similar amount of LDH was detected in all groups. Subsequent measurements on days 6, 8 and 10 of differentiation, showed decreased cell death in higher concentrations (Fig. 3C).

The effect of EPA on beating properties of mESC-derived cardiomyocytes was examined by beating number and beating area indices, as well as multi-electrode array (MEA). At day 10, the group treated with 10 μM EPA showed increase in the percentage of beating cell clusters and beating area compared to control (non-treated) group (Fig. 4A).

Next, we tested whether cardiomyocytes derived from EPA-treated ESCs display functional electrophysiological properties and functional syncytium. Beating colonies were mechanically dissected and plated on top of MEA plates, directing attachment on microelectrodes (Fig. 4B). The extracellular field potentials recorded from MEA electrodes were monitored for all EPA-treatments. Control as well as different EPA treatments showed comparable electrograms that is a representation of developing electrophysiological machinery toward excitability (Fig. 4C). Subsequent application of isoproterenol (100 nM) resulted in a positive chronotropic effect in control as well as EPA-treated groups with a similar pattern. Altogether, these data demonstrated the electrophysiological functionality of cardiomyocytes derived from all concentrations of EPA treatments.

4. Discussion

Despite many approaches developed for cardiac differentiation, many challenges lie ahead in yielding acceptable number of pure and mature cardiomyocytes to be exploited in cell therapy. Thus far, various chemical molecules have been tested on cardiac differentiation, but only a few studies have investigated the possible impact of endogenous metabolites on cardiomyocyte differentiation [12]. During cardiac differentiation, expression of cardiac specific genes changes concurrent with metabolic transcriptome rearrangement. A recent study has provided evidence that metabolites including saturated fatty acids play key role in differentiation of ESCs to cardiomyocyes [12]. Fatty acids comprise the major metabolites providing energy in cardiac cells [9]. Moreover, they can also alter expression of cardiac specific genes and activity of ion channels that are responsible in heart beating [18–20,23].

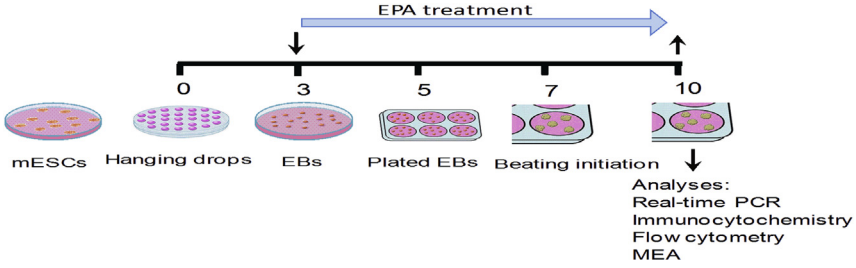

Fig. 1. Timeline of mESC differentiation into cardiomyocyte, showing the time window for EPA treatment and the days of real-time PCR, FACS and immunocytochemistry analyses.

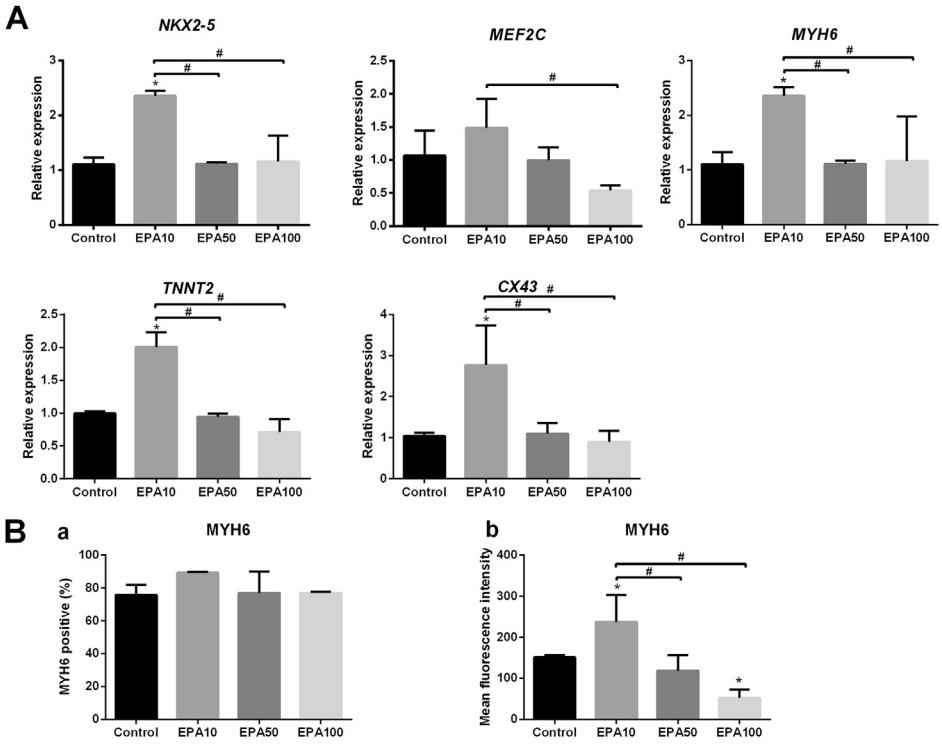

Fig. 2. Effect of EPA on the expression of cardiac markers in EBs derived from mESCs. (A) Quantitative Real Time PCR analysis of cardiac markers in EBs derived from mESCs in non-treated and EPA-treated groups. The relative gene expression was analyzed from three independent experiments. (B) FACS analysis for the percentage of cells exhibiting positive for MYH6 (a) and MFI of MYH6 positive population (b) in the total population derived from mESCs in non-treated and EPA-treated groups. The data are showed as mean ± SEM. Significant differences are indicated by * $p < 0.05$ with respect to control and #$p < 0.05$ between EPA-treated groups.

In the present study, EPA was chosen as fatty acid substrate for differentiating cardiomyocytes, as their multifunctional properties have been proven [9,16–23]. In addition, previous studies indicated that these fatty acids are readily taken up by cardiomyocytes [16,17]. However, their possible contribution in cardiac differentiation from pluripotent stem cells has not been studied.

Along with the marked increase in the mRNA levels of genes involved in cardiac differentiation, such as *NKX2-5*, *MEF2C*, *MYH6*, *TNNT2* and *CX43*, cells treated with 10 μM EPA resulted in higher percentage of MYH6 positive cells at the end of the differentiation. However, the changes observed in high concentrations of EPA appeared to be less significant or unchanged. Even though we noted a significant increase in mean fluorescent intensity of MYH6 in differentiated cardiomyocytes treated with 10 μM EPA, it did not provoke the same increase in percentage of these cells in differentiating population. One potential explanation is that EPA not able to promote cardiogenesis from embryonic stem cells, but it provides necessary energy source for efficient cytoplasmic protein organization. Therefore, each cardiomyocyte would harbor more MYH6 proteins, consistent with quantitative real-time PCR data. In order to rule out the possibility of toxicity having a role in results obtained from higher concentrations of EPA treatment on cardiac differentiation, we performed LDH assay on cells treated with varying concentrations of EPA. Our findings have revealed that EPA did not only have any cytotoxic effect, but also decreased cell death.

Although Yanes et al. [12] proposed that unsaturated fatty acids, such as EPA, are essential in embryonic state, and not differentiated state, our data showed that EPA treatment at 10 μM concentration, would help in having more mature cardiomyocytes. This hypothesis has been further validated by observing increased expression of *CX43* responsible in cardiac conduction, and higher percentage of beating colonies in cells treated with 10 μM EPA.

Several studies have unraveled strong effect of ascorbic acid in differentiation of pluripotent stem cells into cardiomyocyes [27–29]. Our findings demonstrated that EPA could even augment ascorbic acid effect in cardiomyocyte differentiation. Similarly, after screening a series of natural products on cardiomyocyte differentiation, Chan and colleagues were able to show that salvianolic acid could enhance induction of cardiac differentiation by ascorbic acid in a synergistic manner [30].

EPA can influence cardiac differentiation through multiple pathways, the most possible pathway being through affecting gene expression by binding to peroxisome proliferator-activated receptors (PPARs) [23]. Previous studies implicated increased expression of PPARα during cardiomyocyte differentiation and showed that its agonists induced cardiac differentiation [29]. A recent study showed a dose–response relationship between different fatty acids and activity of PPARα, with low EPA doses (1, 5, 10 μM) associated with activation of PPARα, while high dose of EPA (100 μM) repressed PPARα activity [31]. We concluded that cardiogenic effect of EPA at low dose might be attributed to its ability to activate PPARα. Based on these evidences, measuring PPARα activity might help achieve a mechanistic view of EPA role in cardiac differentiation. However, the potential mechanism of EPA in cardiac differentiation needs to be further investigated.

Several lines of evidence show that n-3 PUFAs modulate the electrophysiological properties of cardiomyocytes. It has to be noted that acute administration resulted in different profiles of electrophysiological effects compared to long-term treatment [32]. Acute application of n-3 PUFAs altered gating properties of I_{Na} and $I_{Ca,L}$ [18–20]. However long term fish oil diet did not affect the excitability of ventricular myocytes isolated from porcine heart [33]. Another study show that acute EPA application altered the electrical excitability of both guinea-pig and rat cells in a dose-

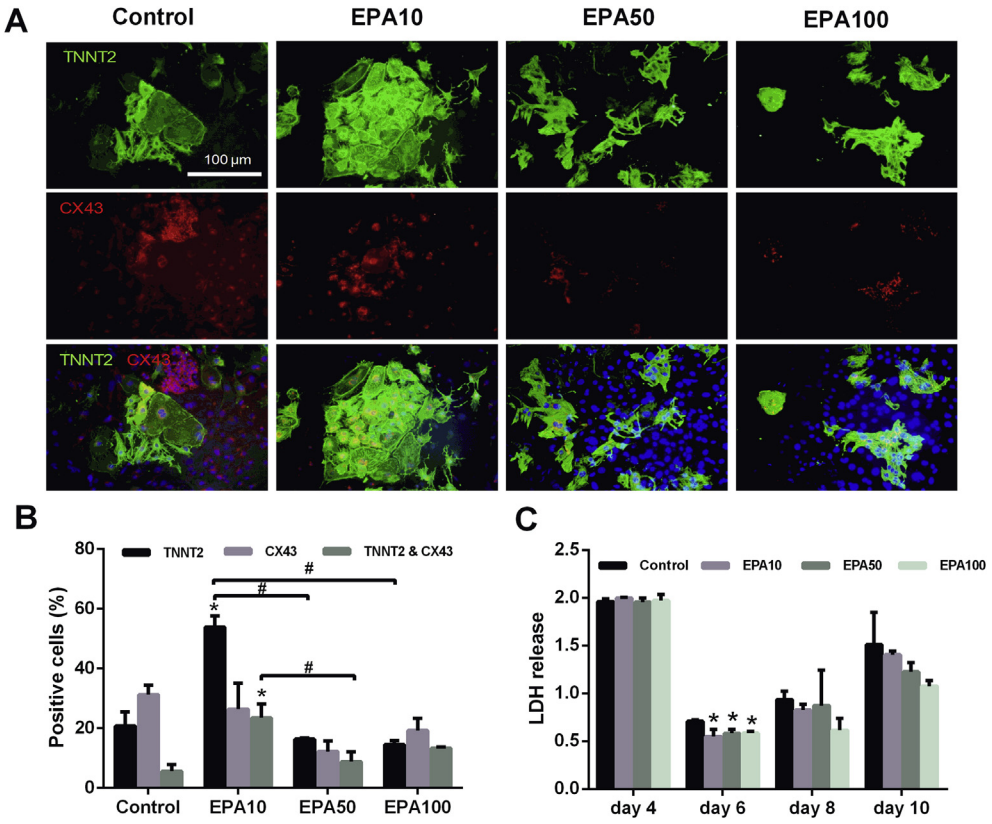

Fig. 3. Effect of EPA on the content of maturity indicative cardiac markers in cardiomyocytes derived from mESCs. (A) Representative immunofluorescence staining for TNNT2 (green) and CX43 (red) in single cells dissected from beating EBs in non-treated and EPA-treated groups (B) Quantification of TNNT2, CX43 and TNNT2 & CX43 positive cells. (C) The time-course of LDH release into the medium of EBs in the control group and the groups treated with different concentrations of EPA. . The data are showed as mean ± SEM, Significant differences are indicated by * $p < 0.05$ with respect to control and #$p < 0.05$ between EPA-treated groups. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

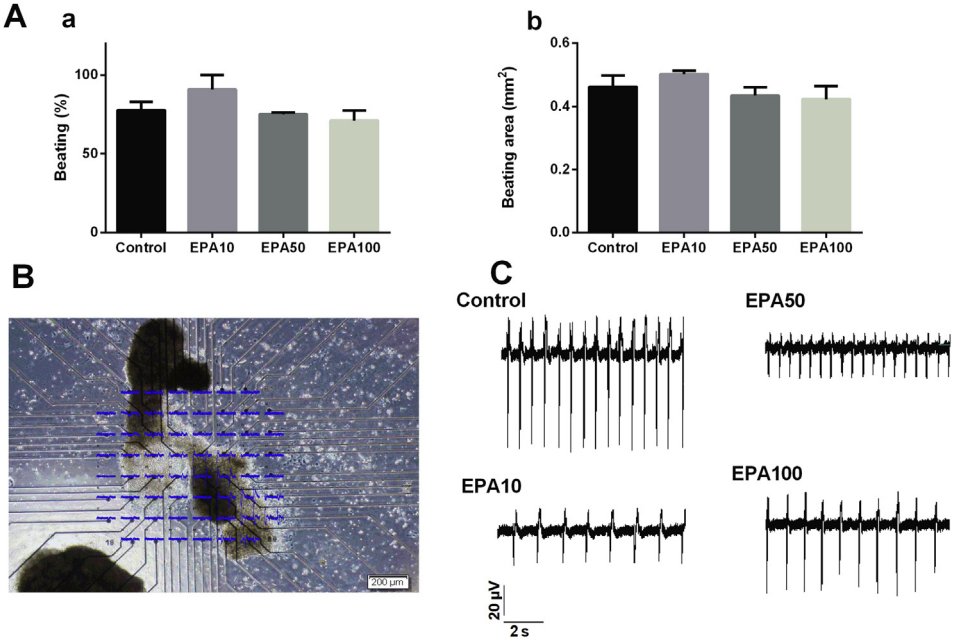

Fig. 4. Effect of EPA on beating properties of EBs derived from mESCs. (A) The percentage of beating EBs and beating area determined visually on day 10 of differentiation in non-treated groups and EPA-treated. (B) Spontaneously beating EBs plated on MEA plate (C) Representative baseline MEA recording trace from contacting EBs in EPA-treated and non-treated groups.

dependent manner. EPA treatment results in a dose-dependent negative inotropic response in guinea-pig cells. But EPA treatment in rat cells initially resulted in a dose-dependent positive inotropic effect at low concentrations (<10 mM) followed by a negative inotropic effect at higher concentrations (>10 mM) [34]. In the present study we recorded similar field potentials in ESC-derived cardiomyocytes treated with various EPA concentrations. Moreover, EPA treatment did not alter the β-adrenergic response triggered by 100 nM of isoproterenol application in ESC-derived cardiomyocytes. This is the first study showing the electrophysiological properties of cardiomyocytes derived from Eicosapentaenoic acid-treated ESCs. However, complementary current-clamp and voltage-clamp studies are required to better understand the effects of micromolar concentrations of EPA on the electrophysiology of cardiomyocytes derived from ESCs.

In the current study we have demonstrated that EPA could enhance differentiation of ESCs into cardiomyocytes. In particular, it promotes structural maturation of ESCs derived cardiomyocytes. **Error! Hyperlink reference not valid.**http//dx.doi.org/10.1016/j.bbrc.2015.02.166.

Conflict of interest

None.

Acknowledgments

This work was supported by Tehran university of medical sciences and Royan institute.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.04.018.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.04.018.

References

[1] A.S. Go, D. Mozaffarian, V.L. Roger, E.J. Benjamin, J.D. Berry, et al., Heart disease and stroke statistics–2013 update: a report from the American Heart Association, Circulation 127 (2013) e6–e245.
[2] S.L. Hendry 2nd, K.E. van der Bogt, A.Y. Sheikh, T. Arai, S.J. Dylla, et al., Multimodal evaluation of in vivo magnetic resonance imaging of myocardial restoration by mouse embryonic stem cells, J. Thorac. Cardiovasc Surg. 136 (2008) 1028–1037 e1021.
[3] W.Z. Zhu, K.D. Hauch, C. Xu, M.A. Laflamme, Human embryonic stem cells and cardiac repair, Transpl. Rev. Orl. 23 (2009) 53–68.
[4] S.Y. Liao, Y. Liu, C.W. Siu, Y. Zhang, W.H. Lai, et al., Proarrhythmic risk of embryonic stem cell-derived cardiomyocyte transplantation in infarcted myocardium, Heart Rhythm. 7 (2010) 1852–1859.
[5] M. Schuldiner, O. Yanuka, J. Itskovitz-Eldor, D.A. Melton, N. Benvenisty, Effects of eight growth factors on the differentiation of cells derived from human embryonic stem cells, Proc. Natl. Acad. Sci. U S A 97 (2000) 11307–11312.
[6] B.S. Yoon, S.J. Yoo, J.E. Lee, S. You, H.T. Lee, et al., Enhanced differentiation of human embryonic stem cells into cardiomyocytes by combining hanging drop culture and 5-azacytidine treatment, Differentiation 74 (2006) 149–159.
[7] C. Roggia, C. Ukena, M. Bohm, H. Kilter, Hepatocyte growth factor (HGF) enhances cardiac commitment of differentiating embryonic stem cells by activating PI3 kinase, Exp. Cell. Res. 313 (2007) 921–930.
[8] L. Wu, Z. Jia, L. Yan, W. Wang, J. Wang, et al., Angiotensin II promotes cardiac differentiation of embryonic stem cells via angiotensin type 1 receptor, Differentiation 86 (2013) 23–29.
[9] G.D. Lopaschuk, J.R. Ussher, C.D. Folmes, J.S. Jaswal, W.C. Stanley, Myocardial fatty acid metabolism in health and disease, Physiol. Rev. 90 (2010) 207–258.
[10] M. Van Bilsen, G.J. Van der Vusse, Phospholipase-A2-dependent signalling in the heart, Cardiovasc Res. 30 (1995) 518–529.
[11] V. Mollace, M. Gliozzi, C. Carresi, V. Musolino, F. Oppedisano, Re-assessing the mechanism of action of n-3 PUFAs, Int. J. Cardiol. 170 (2013) S8–S11.
[12] O. Yanes, J. Clark, D.M. Wong, G.J. Patti, A. Sanchez-Ruiz, et al., Metabolic oxidation regulates embryonic stem cell differentiation, Nat. Chem. Biol. 6 (2010) 411–417.
[13] M.K. Duda, K.M. O'Shea, W.C. Stanley, omega-3 polyunsaturated fatty acid supplementation for the treatment of heart failure: mechanisms and clinical potential, Cardiovasc Res. 84 (2009) 33–41.
[14] D. Mozaffarian, J.H. Wu, Omega-3 fatty acids and cardiovascular disease: effects on risk factors, molecular pathways, and clinical events, J. Am. Coll. Cardiol. 58 (2011) 2047–2067.
[15] R. Marchioli, G. Levantesi, n-3 PUFAs and heart failure, Int. J. Cardiol. 170 (2013) S28–S32.
[16] R.J. Khairallah, J. Kim, K.M. O'Shea, K.A. O'Connell, B.H. Brown, et al., Improved mitochondrial function with diet-induced increase in either docosahexaenoic acid or arachidonic acid in membrane phospholipids, PLoS One 7 (2012) e34402.
[17] G.E. Peoples, P.L. McLennan, Long-chain n-3 DHA reduces the extent of skeletal muscle fatigue in the rat in vivo hindlimb model, Br. J. Nutr. 111 (2014) 996–1003.
[18] W.R. Leifert, E.J. McMurchie, D.A. Saint, Inhibition of cardiac sodium currents in adult rat myocytes by n-3 polyunsaturated fatty acids, J. Physiol. 520 (Pt 3) (1999) 671–679.
[19] Y.F. Xiao, A.M. Gomez, J.P. Morgan, W.J. Lederer, A. Leaf, Suppression of voltage-gated L-type Ca2+ currents by polyunsaturated fatty acids in adult and neonatal rat ventricular myocytes, Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 4182–4187.
[20] Y.F. Xiao, Q. Ke, S.Y. Wang, K. Auktor, Y. Yang, et al., Single point mutations affect fatty acid block of human myocardial sodium channel alpha subunit Na+ channels, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 3606–3611.
[21] G.E. Peoples, P.L. McLennan, Dietary fish oil reduces skeletal muscle oxygen consumption, provides fatigue resistance and improves contractile recovery in the rat in vivo hindlimb, Br. J. Nutr. 104 (2010) 1771–1779.
[22] S. Pepe, P.L. McLennan, (n-3) Long chain PUFA dose-dependently increase oxygen utilization efficiency and inhibit arrhythmias after saturated fat feeding in rats, J. Nutr. 137 (2007) 2377–2383.
[23] A. Georgiadi, M.V. Boekschoten, M. Muller, S. Kersten, Detailed transcriptomics analysis of the effect of dietary fatty acids on gene expression in the heart, Physiol. Genomics 44 (2012) 352–361.
[24] S. Chung, P.P. Dzeja, R.S. Faustino, C. Perez-Terzic, A. Behfar, et al., Mitochondrial oxidative metabolism is required for the cardiac differentiation of stem cells, Nat. Clin. Pract. Cardiovasc Med. 4 (Suppl. 1) (2007) S60–S67.
[25] F. Rabiee, M. Forouzanfar, F. Ghazvini Zadegan, S. Tanhaei, K. Ghaedi, et al., Induced expression of Fndc5 significantly increased cardiomyocyte differentiation rate of mouse embryonic stem cells, Gene 551 (2014) 127–137.
[26] P. Pradhapan, J. Kuusela, J. Viik, K. Aalto-Setala, J. Hyttinen, Cardiomyocyte MEA data analysis (CardioMDA)–a novel field potential data analysis software for pluripotent stem cell derived cardiomyocytes, PLoS One 8 (2013) e73637.
[27] H. Sato, M. Takahashi, H. Ise, A. Yamada, S. Hirose, et al., Collagen synthesis is required for ascorbic acid-enhanced differentiation of mouse embryonic stem cells into cardiomyocytes, Biochem. Biophys. Res. Commun. 342 (2006) 107–112.
[28] T. Takahashi, B. Lord, P.C. Schulze, R.M. Fryer, S.S. Sarang, et al., Ascorbic acid enhances differentiation of embryonic stem cells into cardiac myocytes, Circulation 107 (2003) 1912–1916.
[29] L. Ding, X.G. Liang, Y.J. Lou, Time-dependence of cardiomyocyte differentiation disturbed by peroxisome proliferator-activated receptor alpha inhibitor GW6471 in murine embryonic stem cells in vitro, Acta Pharmacol. Sin. 28 (2007) 634–642.
[30] S.S. Chan, J.H. Chen, S.M. Hwang, I.J. Wang, H.J. Li, et al., Salvianolic acid B-vitamin C synergy in cardiac differentiation from embryonic stem cells, Biochem. Biophys. Res. Commun. 387 (2009) 723–728.
[31] H.E. Popeijus, S.D. van Otterdijk, S.E. van der Krieken, M. Konings, K. Serbonij, et al., Fatty acid chain length and saturation influences PPARalpha transcriptional activation and repression in HepG2 cells, Mol. Nutr. Food Res. 58 (2014) 2342–2349.
[32] H.M. Den Ruijter, G. Berecki, T. Opthof, A.O. Verkerk, P.L. Zock, et al., Pro- and antiarrhythmic properties of a diet rich in fish oil, Cardiovasc Res. 73 (2007) 316–325.
[33] A.O. Verkerk, R. Wilders, W. de Geringel, H.L. Tan, Cellular basis of sex disparities in human cardiac electrophysiology, Acta Physiol. (Oxf) 187 (2006) 459–477.
[34] J.C. Macleod, A.D. Macknight, G.C. Rodrigo, The electrical and mechanical response of adult guinea pig and rat ventricular myocytes to omega3 polyunsaturated fatty acids, Eur. J. Pharmacol. 356 (1998) 261–270.