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Cardiac differentiation of mouse embryonic stem cells is influenced by a PPAR γ/PGC-1α—FNDC5 pathway during the stage of cardiac precursor cell formation

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ABSTRACT

Peroxisome proliferator-activated receptor (PPAR) γ co-activator 1α (PGC-1α) up-regulation induces *FNDC5* expression in muscle and consequently causes browning of white adipose tissue (WAT). In addition to skeletal muscle, *FNDC5* is mainly expressed in heart and brain tissues. Here, we demonstrate that *FNDC5* expression increased during the process of cardiac differentiation of mouse embryonic stem cells (mESCs) similar to *PGC-1α* and *PPARα*. To testify the correlation between PGC-1α and FNDC5 in cardiac cell differentiation of mESCs, we utilized specific PPARγ agonist and antagonist in two stages of cardiac differentiation, during and post-cardiac precursor cells (CPCs) formation. Our results indicated that a reduction in *PGC-1α* expression, via treatment with GW9662 during CPCs formation stage, down-regulated *FNDC5* transcript levels as well as mitochondrial markers which negatively influenced on the whole process of cardiac differentiation efficiency. On the other hand, increase *PGC-1α* expression during CPCs formation stage via rosiglitazone treatment increase *FNDC5* and mitochondrial markers transcript levels which enhanced cardiac differentiation efficiency. Importantly, such alteration in *PGC-1α* expression at post-CPCs formation stage did not affect overall cardiac differentiation rate as expression of *FNDC5* and mitochondrial markers were not significantly changed. We concluded that PPARγ agonist and antagonist induced up and down-regulation of *PGC-1α* and subsequently modulated the process of CPCs formation through an alteration in *FNDC5* and mitochondrial markers expression.

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Introduction

Postnatal mammalian heart formation depends on high turnover of ATP made by mitochondria through oxidative phosphorylation. Consistent with necessity of the heart to high energy, the cardiac myocyte differentiation needs a quite high energy which is produced by mitochondria. The importance of energy production in cardiac muscle is shown in the severe phenotype of inherited and acquired cardiomyopathy (Lehman and Kelly, 2002; Lopaschuk and Jaswal, 2010; Pohjoismäki et al., 2013).

Peroxisome proliferator-activated receptor (PPAR) γ co-activator 1α (PGC-1α) is a powerful transcriptional regulator of energetic pathways. PGC-1α attaches to PPARγ and accelerates the interaction of this protein with multiple transcription factors.

Abbreviations: ANOVA, one-way analysis of variance; BAT, brown adipose tissues; CPC, cardiac precursor cell; DMSO, dimethyl-sulfoxide; EBs, embryoid bodies; ES–FCS, ESC-qualified fetal calf serum; FCS, fetal calf serum; FNDC5, fibronectin type III domain containing 5; Gapdh, glyceraldehyde 3-phosphate dehydrogenase gene; GW, GW9662; HF, heart failure; KDMEM, knock out Dulbecco's modified Eagle medium; mESCs, mouse embryonic stem cells; OXPHOS, oxidative phosphorylation; PGC-1α, PPARγ coactivator 1α; PPARs, peroxisome proliferator-activated receptors; PPARγ, peroxisome proliferator-activated receptor; PVDF, poly-vinylidene-difluoride; Rosi, rosiglitazone; RT-qPCR, real-time quantitative PCR; α-MHC, alpha-myosin heavy chain.

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PPARγ belongs to the nuclear hormone receptor superfamily of the ligand activated transcription factors (Liang and Ward, 2006; Wu et al., 1999). PPAR γ/PGC-1α complex is involved in different metabolic activities through regulating of various genes expression (Lagouge et al., 2006).

Endurance exercise has been shown to up-regulate the *PGC-1α* gene in human skeletal muscle which is involved in the control of uncoupled mitochondrial respiration. Moreover, this process triggers thermogenesis in the fat tissues by secreting of a myokine, termed Irisin from the muscle cells (Boström et al., 2012). It has also been shown that *PGC-1α* is highly expressed in high energy demanded tissues, including heart which plays a central role in the control of mitochondrial function in cardiac tissue. During cardiac developmental process, PGC-1α controls the expression of proteins in mitochondrial fatty acid oxidation pathway through activating of PPARα (D'Errico et al., 2011). Furthermore, β-oxidation of fatty acids produces energy for continuous contractile activity of the cardiomyocyte (Lopaschuk et al., 2010). Previous studies have shown that knock down of cardiac *troponin T* led to down regulation of cardiac sarcomeric proteins and several special genes expression as *PGC-1α* which ultimately reduced cardiac differentiation (Ahmad et al., 2008). In fact, the increased expression of *PGC-1α* is parallel to increased mitochondrial DNA and gene expression of *OXPHOS* (*oxidative phosphorylation*) system in brown adipose tissues (BATs) (Garesse and Vallejo, 2001; Lehman et al., 2000). Moreover, loss of *PGC-1α* expression was identified in numerous models of cardiac failure (Geng et al., 2011). Boström et al., found that PGC-1α induces thermogenesis by stimulating the expression of *fibronectin type III domain containing 5* (*FNDC5*) gene. FNDC5 is a glycosylated type I membrane protein that cleaves proteolytically and forms Irisin. Irisin is responsible to induce the expression of *UCP-1* and other thermogenic genes in white adipose tissues. Therefore it has been suggested that Irisin may mediate some beneficial effects of exercise in human such as generating weight loss and blocking diabetes (Boström et al., 2012). Numerous studies have confirmed the positive correlation between increased *FNDC5* expression and resistance to obesity-linked insulin resistance (Huh et al., 2012; Moreno-Navarrete et al., 2013; Arias-Loste et al., 2014). In addition, contractile dysfunction are reported in obesity-linked insulin resistance mice with accumulate of intra-myocardial lipid (Ussher et al., 2012). Coding sequences of Irisin are conserved among mammals. For instance there is 100% identity between murine and human species, suggesting an important function for the respective protein (Boström et al., 2012). Our previous studies have demonstrated that *FNDC5* expression is induced by retinoic acid and responsible for appropriate neural differentiation of mouse embryonic stem cells (Ostadsharif et al., 2011; Seifi et al., 2014; Hashemi et al., 2012). Interestingly, *FNDC5* expression is also found in the heart and cardiac precursor cell (CPC) and in C2C12 muscle cells differentiated into myotube (Ferrer-Martines et al., 2002; Rabiee et al., 2014). Furthermore, *FNDC5* expression is well correlated with the heart contractile activity and aerobic performance as already was shown that heart failure (HF) patients with low *FNDC5* expression level have limited aerobic exercise performance (Lecker et al., 2012). Consistent with the high energy requirement of cardiomyocyte differentiation and connection of *PGC-1α* and *FNDC5* genes to regulate energy metabolism, led us to investigate whether such correlation between these genes is existed in cardiac differentiation. Here we show that alteration of *PGC-1α* expression during CPCs formation by PPARγ agonist and antagonist significantly changed *FNDC5* transcript levels and cardiac differentiation rate. Of note that variation in *PGC-1α* expression post CPCs formation affected neither on *FNDC5* transcript levels nor on cardiac differentiation rate.

Materials and methods

Culture and differentiation of mESCs

Mouse embryonic stem cells (mESCs), line Royan B20 derived from C57BL/6 strain were kept in an undifferentiated state as described before (Baharvand and Hassani, 2013; Rabiee et al., 2014). Meanwhile mESCs line Royan B1 was obtained from Royan Institute for Stem Cell Biology and Technology (Tehran, Iran). These cells were manipulated as previously described (Ghoochani et al., 2012). MESCs were differentiated into beating cardiomyocytes (Farokhpour et al., 2009). Briefly, the main steps of differentiation included cultivation of a definite number of mESCs (8×10^2) in 20 μl hanging drops with 10^{-4} M ascorbic acid/vitamin C (Sigma, USA) to produce embryoid bodies (EBs) for 2 days followed by cultivation in bacterial dishes (Grainer, Germany) for 5 days. On day 7, EBs were plated separately into 1% gelatin-coated wells of a 24 micro well tissue culture plate to allow adherence and outgrowth of the EBs and development of spontaneously beating cardiac muscle cells for an additional 5 days. The percentage of beating EBs was calculated as: (the number of beating EBs/the number of plated EBs) ×100.

EBs treatment by PPARγ antagonist and agonist

GW9662 (GW) (Sigma) (Collino et al., 2005; Bugge et al., 2009) as a selective specific antagonist and a potent specific PPARγ agonist, Rosiglitazone (Rosi) (Cayman Chemical, USA), were used (Burgess et al., 2005; Schuler et al., 2006). Both agonist and antagonist were dissolved in dimethyl-sulfoxide (DMSO). The equal amount of the solvent was used in all treatments including the control samples. EBs treated with effective concentrations of GW (10 μM) and Rosi (5 μM) as described (Ghoochani et al., 2012). This treatment was carried out during five days of the suspension culture and seven days of post plating in KDMEM supplemented with 15%ES–FCS medium, respectively.

Real-time quantitative polymerase chain reaction (RT-qPCR) analysis

Total RNA of the cell was extracted with RNeasy Mini Kit (Qiagen, Germany) and treated with *DNase*I (Thermo Scientific, USA). cDNA synthesis was performed using 1 μg of total RNA, MMLV reverse transcriptase (Thermo Scientific) and random hexamer (Thermo Scientific). RT-q PCR was carried out utilizing SYBR green (TaKaRa, Japan) in a Thermal Cycler Rotor gene 6000 (Corbett, Australia) according to the protocol (TaKaRa) as follows: 10 μl Rotor-Gene SYBR Green PCR Master Mix (Qiagen), 0.3 μM of each primer, and 25 ng cDNA in 20 μl final volume. Expression of target genes was normalized to transcript amount of two reference genes, *Glyceraldehyde 3-phosphate dehydrogenase* gene (*Gapdh*) and *beta-tubulin V*. The outcomes of two reference genes are similar. Supplementary Fig. S1 depicts the some diagrams of target genes were normalized to beta-tubulin V. All measurements were done in triple experiments and data were analyzed by ΔΔCt method. Primer pairs for target genes were designed by the Beacon designer (Version 7.2, USA) and ordered through Metabion Company (Germany; Supplementary Table 1).

Western blot

Protein extraction was carried out with TRI reagent (Sigma) according to the manufacturer's protocol. A solubilized protein fraction of each sample (30 μg) was separated by SDS–PAGE electrophoresis and transferred to polyvinylidenedifluoride (PVDF)

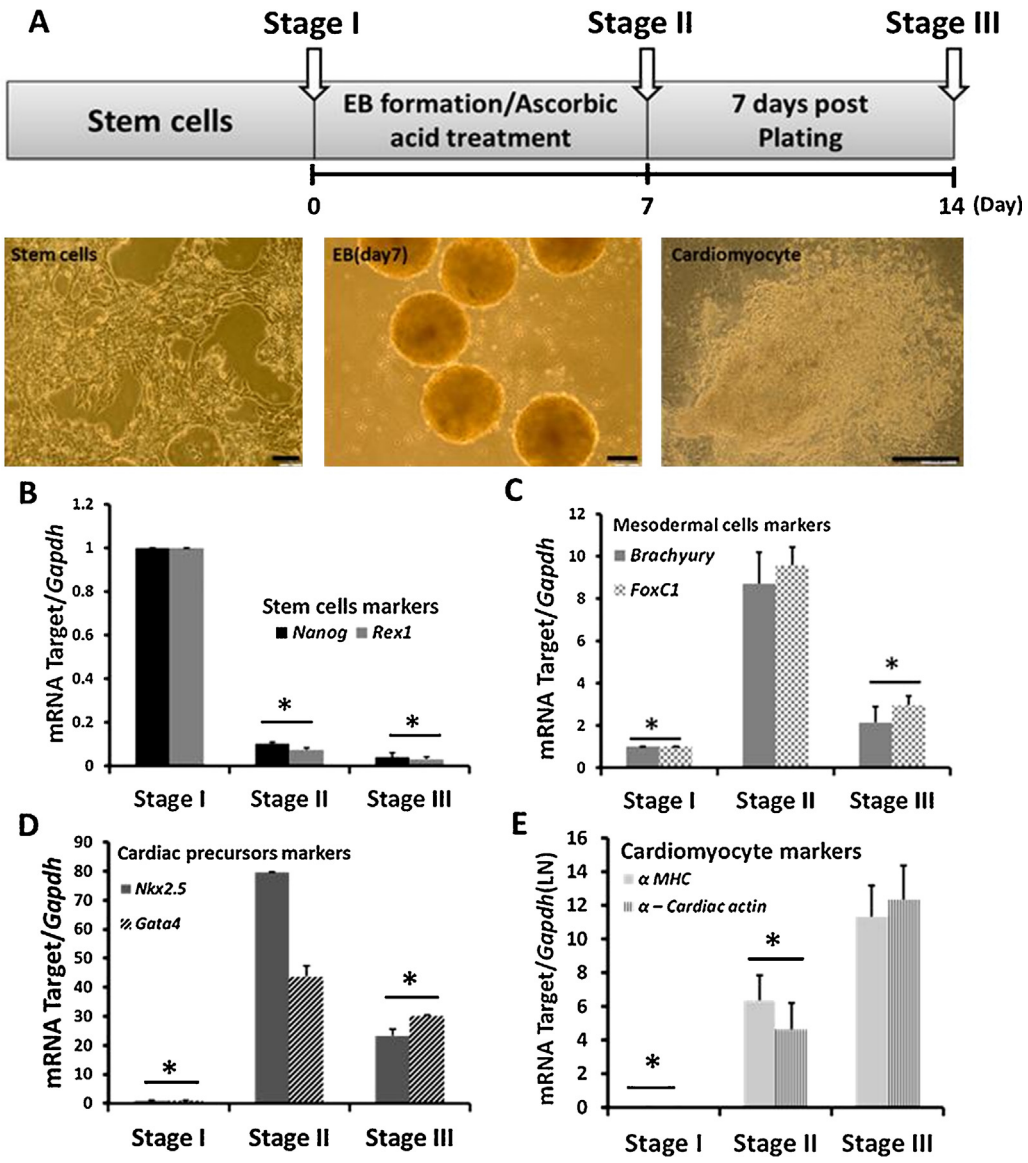

Fig. 1. (A) Illustrated protocol and morphological characterizations of mESCs during cardiac differentiation in three stages: undifferentiated cells (Stage I), cell aggregates or embryoid bodies (EBs) post treatment with 10^{-4} M ascorbic acid/vitamin C (Stage II), post plating of CPCs for seven days (Stage III). Phase contrast pictures of the cells in three stages are shown. Bar is 100 μm. (B) RT-qPCR analysis of *Nanog* and *Rex1* (Stem cells markers), (C) *Brachyury* and *FoxC1* (Mesodermal cells markers), (D) *Nkx2.5* and *Gata4* (Cardiac precursors markers) and (E) *αMHC* and *α-Cardiac actin* (Cardiomyocyte markers) during three stages of cell differentiation as described in the Materials and methods section. Represented values bars are the mean of triplicate independent experiments ± SEM. * Is $p < 0.05$ by ANOVA-test.

membranes. The primary antibody used was mouse anti CYP3A4 (HL3) (1:800 dilution, Santa Cruz) and mouse anti-GAPDH antibody (1:800 dilution, Sigma-Aldrich). HRP-conjugated secondary antibody (Dako) was used at concentration of 1:5000. HRP-conjugated IgG bound to each protein band was visualized by an Amersham ECL Advance Western Blotting Detection Kit (GE Healthcare).

Immunofluorescent staining of the cell

To evaluate the presence of α-MHC in beating cardiomyocytes, EBs on day 7 were plated in tissue culture dishes. After 3 days of incubation, beating cells were used for immunostaining. Cells were rinsed with PBS, fixed with 4% paraformaldehyde at room temperature and incubated with primary antibody against alpha-myosin heavy chain (α-MHC) in (1:300, Abcam) for 60 min at 37 °C in a humid chamber. At the end of the incubation time, cells were rinsed with PBS and incubated with Fluorescein Isothiocyanate (FITC)-conjugated goat anti mouse IgG (1:50,Chemicon, USA) for 60 min at 37 °C. After rinsing with PBS, nuclear staining was performed with DAPI and cells were analyzed under a fluorescent microscope (Olympus, Japan) and images were acquired with an Olympus DP70 camera (Olympus).

Statistical analysis

Microsoft Excel (2007) and SPSS (version 16) were used to express data as means ± SEM obtained from three independent observations. The difference between groups was analyzed with one-way ANOVA. Also independent *t*-test analysis was carried out to identify statistical differences between the two observations. The difference between data were considered to be significant at $p < 0.05$.

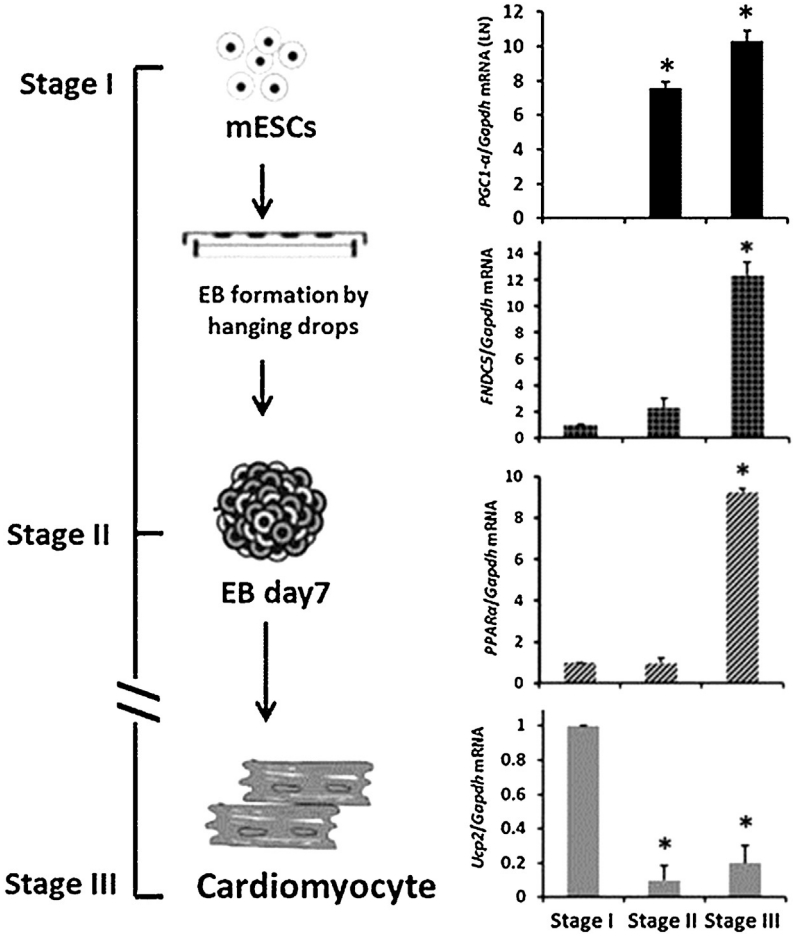

Fig. 2. Increased transcript levels of *PGC-1α*, *FNDC5* and *PPARα* accompanied with a decrease in *UCP2* expression level during three stages of cardiac differentiation of mESCs. Data were obtained by three independent experiments for spontaneous cardiac differentiation. * Indicates significant difference of gene expression at $p < 0.05$.

Results

Cardiomyocyte differentiation of mESCs induced by ascorbic acid/vitamin C

Fig. 1A illustrates the schematic representation of the cardiac differentiation procedure of mESCs upon ascorbic acid/vitamin C treatment and the morphology of cells at different stages. Previous data have indicated that ascorbic acid/vitamin C enhanced cardiomyocyte differentiation of mESCs. Thus, hereafter, ascorbic acid/vitamin C was implemented for efficient cardiomyocyte differentiation. Moreover, RT-qPCR was carried out to evaluate the expression level of marker genes in each step which was in good agreement with the results obtained by morphological analysis. Expression of the stem cells markers *Rex1* and *Nanog* was down-regulated during cardiac induction (Fig. 1B) whereas transcript level of mesodermal cells markers (*Brachyury*, *Foxc1*) (Fig. 1C) and CPCs markers (*Nkx2.5*, *Gata4*) (Fig. 1D) increased in stage II (CPCs formation). However mRNA levels of CPCs markers decreased markedly when CPCs differentiated into cardiomyocytes. Differentiated cells were characterized in terms the expression levels of matured cardiomyocyte markers including *αMHC* and *α-Cardiac actin*. Results showed that the expression levels of *αMHC* and *α-Cardiac actin* (Matured cardiomyocyte markers) (Fig. 1E) in cardiomyocytes were increased compare to mESCs and CPCs. These data were reconfirmed by implementing a different mESCs linage cells (Royan B1, Supplementary Fig. S2). Therefore, these results confirmed the accuracy of this protocol for cardiac differentiation.

Expression analysis of PGC-1α, FNDC5, and down-stream genes during cardiac differentiation of mESCs

Boström et al. (2012) have indicated that elevation in *PGC-1α* gene expression in exercised muscle enhanced RNA levels of *UCP1* through an increase in muscle *FNDC5* and fat *PPARα* expression levels. Thus, RT-qPCR analysis was applied to assess expression level of *PGC-1α, FNDC5, PPARα* and *UCP2*, a dominant expressed gene than *UCP1* in muscle and heart (Ricquier, 1999), during cardiac differentiation of mESCs. The results revealed increased transcript levels of *PGC-1α*, *FNDC5* and *PPARα* in cardiomyocytes while *UCP2* expression decreased significantly (Fig. 2). In order to confirm whether such phenomenon is universal and does not depend on the cell type, same experiment was performed in another mESCs linage cell (Royan B1) and similar results were obtained (Supplementary Fig. S3). Interestingly increased expression of *PGC-1α* was also obvious at stage II which CPCs were emerged. Such trend in enhancement for *FNDC5* and *PPARα* transcripts were not discernible at stage II. This was interpreted to mean that significant increased expression levels of *PGC-1α* was occurred preceding to increased transcript levels of *FNDC5* and *PPARα*.

Effects of PGC-1α expression alteration during CPCs formation on expression of downstream genes

To study the effect of PGC-1α on transcription rate of *FNDC5*, *PPARα* and *UCP2* genes during cardiac differentiation, mESCs were treated by PPARγ antagonist and agonist during CPCs formation

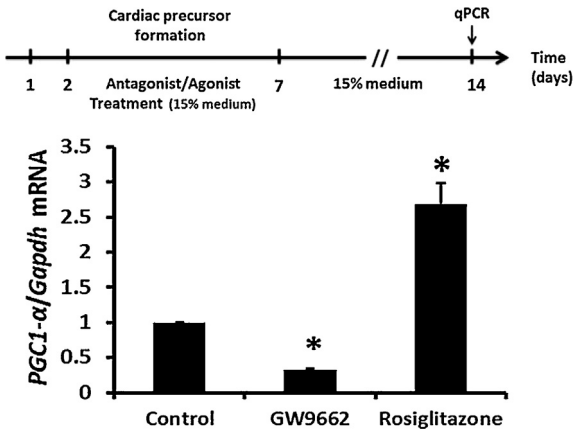

Fig. 3. Schematic illustrated protocol of mESCs treatment with PPARγ antagonist (GW) and agonist (Rosi) during CPCs formation (for six days). RT-qPCR analysis of *PGC-1α* expression level of cardiomyocyte on day 14 was assessed. Relative expression of *PGC-1α* gene was quantified and normalized with *Gapdh*. * Indicates significant difference between treated samples and control at $p < 0.05$.

and cells were allowed to differentiate into cardiomyocytes. According to RT-q PCR data, 10 μM of GW9662 (GW) treatment, decreased transcript contents of *PGC-1α*, compared to control significantly (Fig. 3). Moreover, 5 μM concentrations of Rosiglitazone (Rosi), PPARγ agonist, increased *PGC-1α* expression (Fig. 3). Data indicated a reduction in *PGC-1α* expression by GW treatment during CPCs formation resulted in a significant decrease in *FNDC5* expression (Fig. 4A), whereas there was no change in *PPARα* and *UCP2* expression levels (Fig. 4B). Interestingly, expression of mitochondrial markers (*Ndufb5* and *ATP5b*) was similarly reduced under GW treatment (Fig. 4C). In addition, Western blot using antibodies against CYP3A4 (mitochondrial marker) and GAPDH confirmed the data obtained by real time PCR (Fig. 4D). In the induced *PGC-1α* by Rosi treatment during CPCs formation, *FNDC5* expression increased while *PPARα* and *UCP2* expression levels were not influenced significantly (Fig. 4A and B). Also Expression level of mitochondrial markers (*Ndufb5* and *ATP5b*) increased upon the induction of *PGC-1α* expression (Fig. 4C). Similar to real time PCR results increased CYP3A4 (mitochondrial marker) level was observed in Western blot (Fig. 4D).

Attenuated PGC-1α–FNDC5 pathway during CPCs formation affected the process of cardiomyocyte differentiation

Alteration levels of PGC-1α during CPCs formation, as depicted in Fig. 3, was testified on the expression levels of *Brachuruy* and *FoxC1* as specific mesodermal markers on day 4 from the beginning of the experiment. RT-qPCR data indicated that *Brachuruy* and *FoxC1*expression levels were not influenced by GW and Rosi induced PGC-1α reduction and induction compare to the control significantly (Fig. 5A). Similar outcomes were obtained on cardiovascular progenitor markers, *Mesp1* and *FLK1*, transcription levels on day 7 from the beginning of the experiment (Fig. 5B). In the same conditions, the relative expressions of CPCs markers, *Gata4* and *Nkx2.5*, was assessed. Data indicated that reduction of *PGC-1α* expression resulted in a significant decrease in *Gata4* expression whereas RNA level of *Nkx2.5* did not change (Fig. 5C). Moreover, 5 μM of Rosi, PPARγ agonist, did not change transcript contents of CPC markers significantly, compared to control (Fig. 5C). However, this treatment attenuated the overall process of cardiac differentiation. Morphological observations of expanded beating bodies showed smaller size of beating bodies and reduced content of

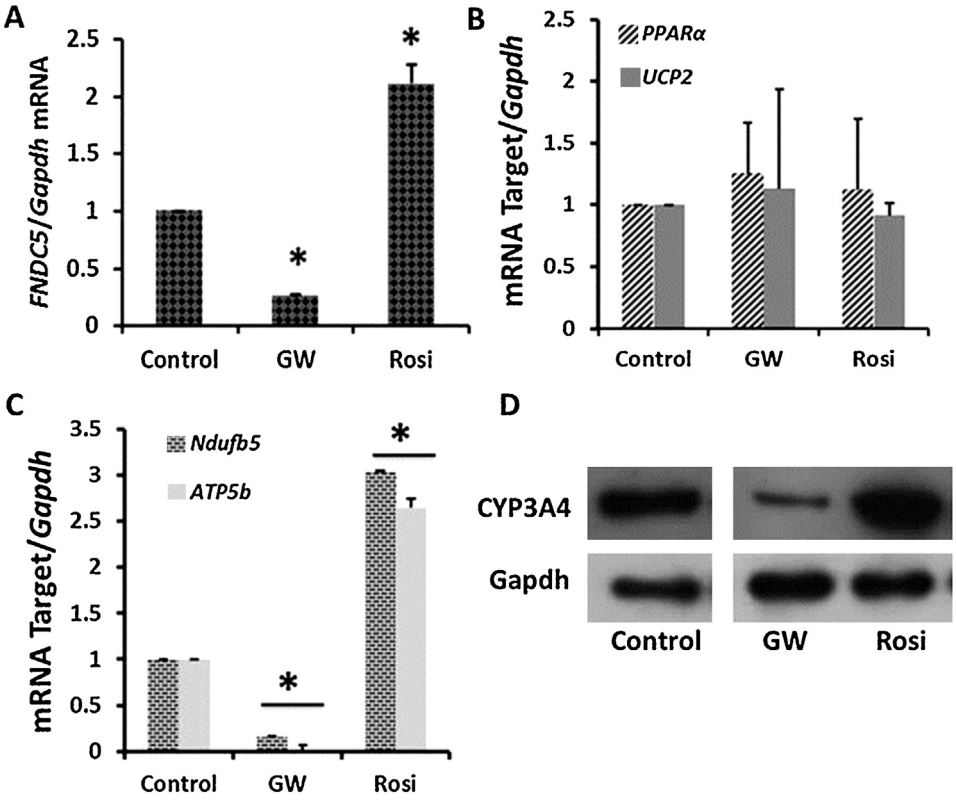

Fig. 4. Innovated level of *PGC-1α* transcript altered the expression level of *Fndc5* and mitochondrial markers during CPCs formation. (A) RT-qPCR analysis for *Fndc5*, (B) *PPARα* and *UCP2* and (C) mitochondrial markers (*Ndufb5* and *ATP5b*). Relative expression of target genes was quantified and normalized with *Gapdh*. Star indicates significant difference between same sample and control at $p < 0.05$. (D) Western blot analysis for mitochondrial marker (CYP3A4). Intracellular protein amounts were calculated relative to the Gapdh content.

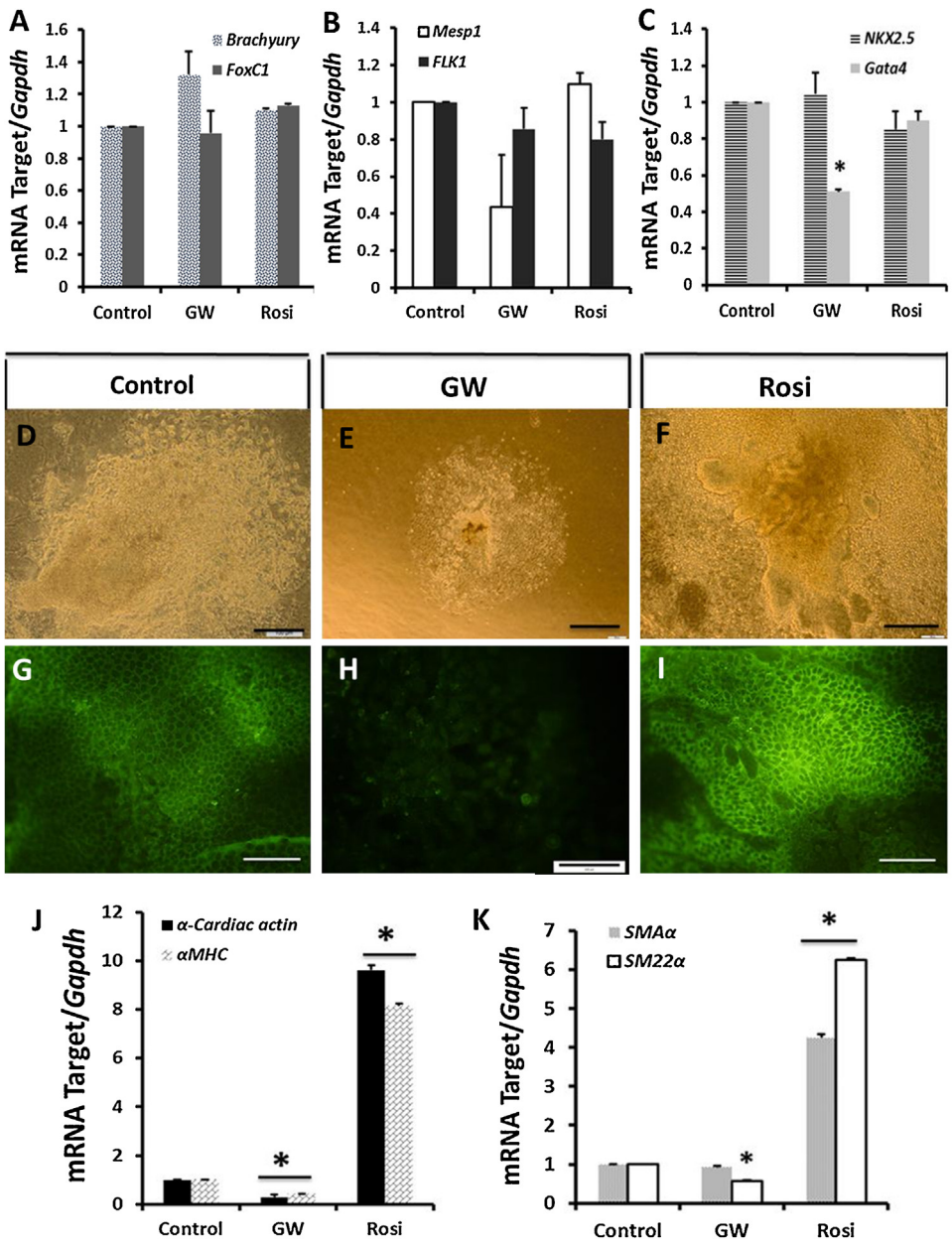

Fig. 5. The effect of *PGC-1α* alteration on cardiac differentiation efficiency during CPCs formation treated with GW9662 and Rosi. (A) RT-qPCR of mesodermal markers (*Brachyury* and *FoxC1*), (B) cardiovascular progenitor markers (*Mesp1* and *FLK1*) and (C) cardiac progenitor markers (*NKX2.5* and *Gata4*). (D–F) Morphological illustration of beating bodies derived from CPCs in treated samples and control. Scale bar is 20 μm. (G–I) Immunostaining of beating bodies against α-MHC (mature cardiomyocyte marker). Scale bar is 100 μm. RT-qPCR of (J) mature cardiomyocyte markers (*α-Cardiac actin* and *αMHC*) and (K) smooth muscle cells markers (*SMAα* and *SM22α*). Relative expression levels of target genes were normalized to *Gapdh* amount. Star shows significant difference between sample and control at $p < 0.05$.

α-MHC (Fig. 5E and H) with weakened beating percentage (Supplementary Fig. S4) in antagonist treated samples compare with the control sample (Fig. 5D and G). Furthermore, at this stage, the expression levels of cardiac markers (*α-Cardiac actin* and *α-MHC*) (Fig. 5J) and smooth muscle cell marker (*SM22α*) (Fig. 5K) were significantly reduced in the GW treated sample when compare with the control cell. Such observation was not confirmed for transcript level of *SMAα* (Fig. 5K). Moreover, the expansion size of the beating cardiac cells in the Rioglitazone treated sample was higher than those of GW-treated cells and control (Fig. 5F). Immunostaining of the beating cardiac cells with antibody against α-MHC, a cardiomyocyte marker, showed an enhanced level of alpha-myosin heavy chain concentration in the Rosiglitazone treated cell. Interestingly, this treatment caused a sharp increase in the expression of cardiac markers (*α-Cardiac actin* and *α-MHC*) (Fig. 5J) and smooth muscle cell markers (*SMAα and SM22α*) (Fig. 5K).

Effects of PGC-1α alteration in post-CPCs formation on specific gene expression

Similar to previous result (Fig. 3), GW treating cell at post-CPCs formation stage, caused a significant decrease in *PGC-1α* expression. Inversely, Rosi application increased expression of *PGC-1α* in similar situation (Fig. 6). Suppressing effects of reduced *PGC-1α* expression on down-stream target genes were also testified post-CPCs formation. As depicted, no significant changes was made on expression levels of *FNDC5* (Fig. 7A), *PPARα* and *UCP2* (Fig. 7B) and mitochondrial markers (*Ndufb5*, *ATP5b*) (Fig. 7C). Also,

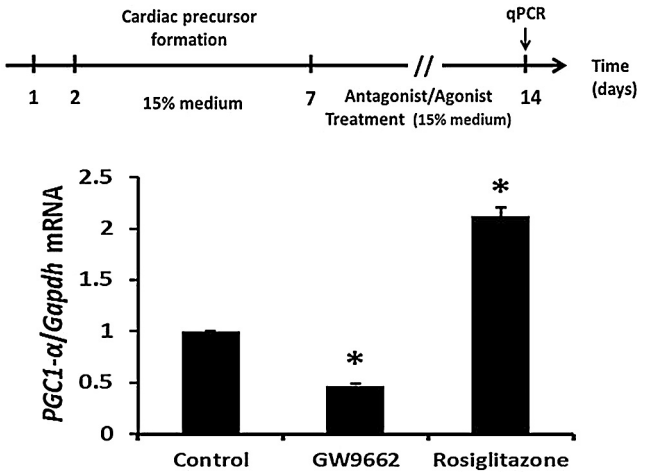

Fig. 6. Schematic illustrated protocol of mESCs treatment with PPARγ antagonist (GW) and agonist (Rosi) in post-CPCs formation on EBs for 7 days. RT-qPCR analysis of *PGC-1α* expression level of cardiomyocyte on day 14 was assessed. Relative expression of *PGC-1α* gene was quantified and normalized with *Gapdh*. * Indicates significant difference between treated samples and control at $p < 0.05$.

PGC-1α induction was not affective on *FNDC5* (Fig. 7A), *PPARα* and *UCP2* (Fig. 7B) and mitochondrial markers (*Ndufb5*, *ATP5b*) expression level (Fig. 7C). These data was also confirmed with Western blotting of the mitochondrial marker with CYP3A4 antibody (Fig. 7D).

PGC-1α transcript alteration in post-CPCs formation did not effect on the expression of FNDC5 and down-stream genes

Importance of PGC-1α on transcription rate of *FNDC5* and down-stream genes was testified post-CPCs formation as shown in Fig. 7. Different morphology and size of generated beating bodies was not noticeable between the treated and untreated samples (Fig. 8A–C). The same observation was also obtained for the percentage of beating bodies (Supplementary Fig. S4). Immunostained α-MHC indicated no difference between treated groups and control (Fig. 8D–F).The relative expression of cardiac markers (*α-Cardiac actin* and *α-MHC*) and smooth muscle cell markers (*SM22α* and *SMAα*) was also was assessed. Data indicated that GW induced reduction of *PGC-1α* expression and Rosi induced *PGC-1α* expression resulted in a non-significant change in *α-Cardiac actin, α-MHC, SM22α* and *SMAα* expression (Fig. 8G and H). Thus, PGC-1α alteration at post-CPCs formation did not affect cardiomyocyte differentiation as monitored expression analysis and morphological observations. These data revealed that amount of PGC-1α at post-CPCs formation is not a critical determining factor for cardiac differentiation.

Discussion

Recently, Boström et al. (2012), have shown that the secretory type of FNDC5, Irisin, secreted from muscle into blood under the control of PGC-1α, causes an increase in energy expenditure. In the present study, we have delineated that expression of both, PGC-1α and *FNDC5* was increased in terminal stage of cardiomyocyte differentiation of mESCs. To investigate further involvement of increased PGC-1α and *FNDC5* transcripts upon cardiac differentiation, this

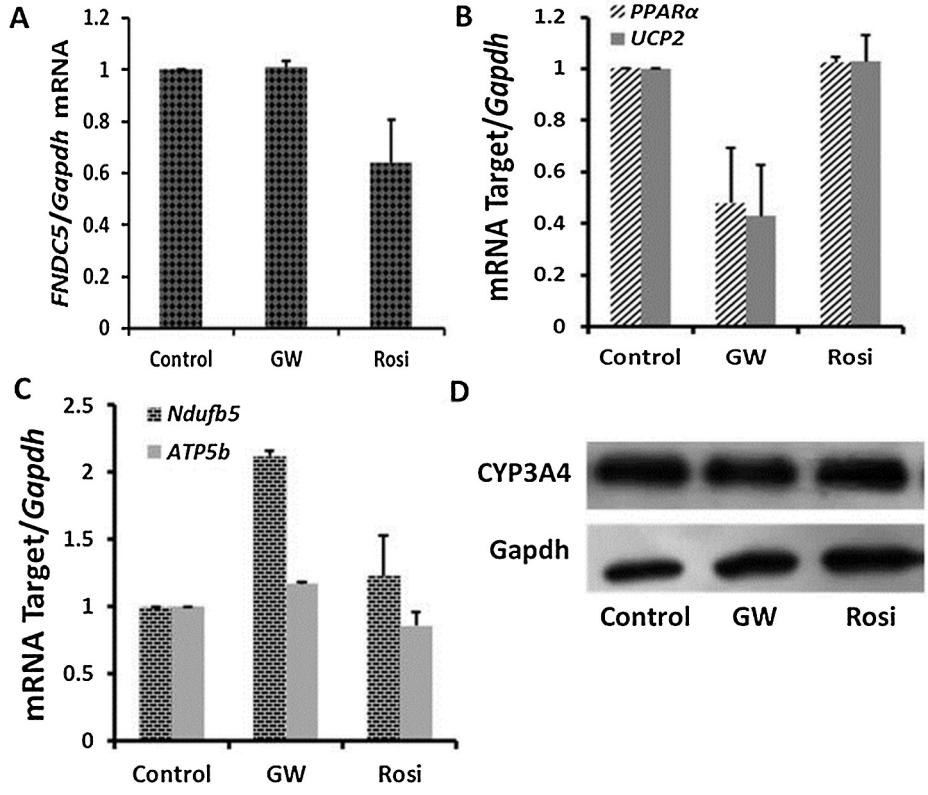

Fig. 7. The evaluation of PGC-1α alteration on transcript levels of downstream genes and mitochondrial markers post-CPCs formation. (A) RT-qPCR assessment was carried out on (A) *Fndc5*, (B) *PPARα* and *UCP2*, (C) Mitochondrial markers (*Ndufb5* and *ATP5b*). Relative expression of target genes were quantified and normalized with *Gapdh*. The number of independent repeats was 3 for each experiment ($n = 3$). Value bars are mean ± SEM. As depicted in the figure there is no significant difference between treated and control samples. (D) Western blot analysis for mitochondrial marker (CYP3A4). Intracellular protein amounts were calculated relative to the Gapdh content.

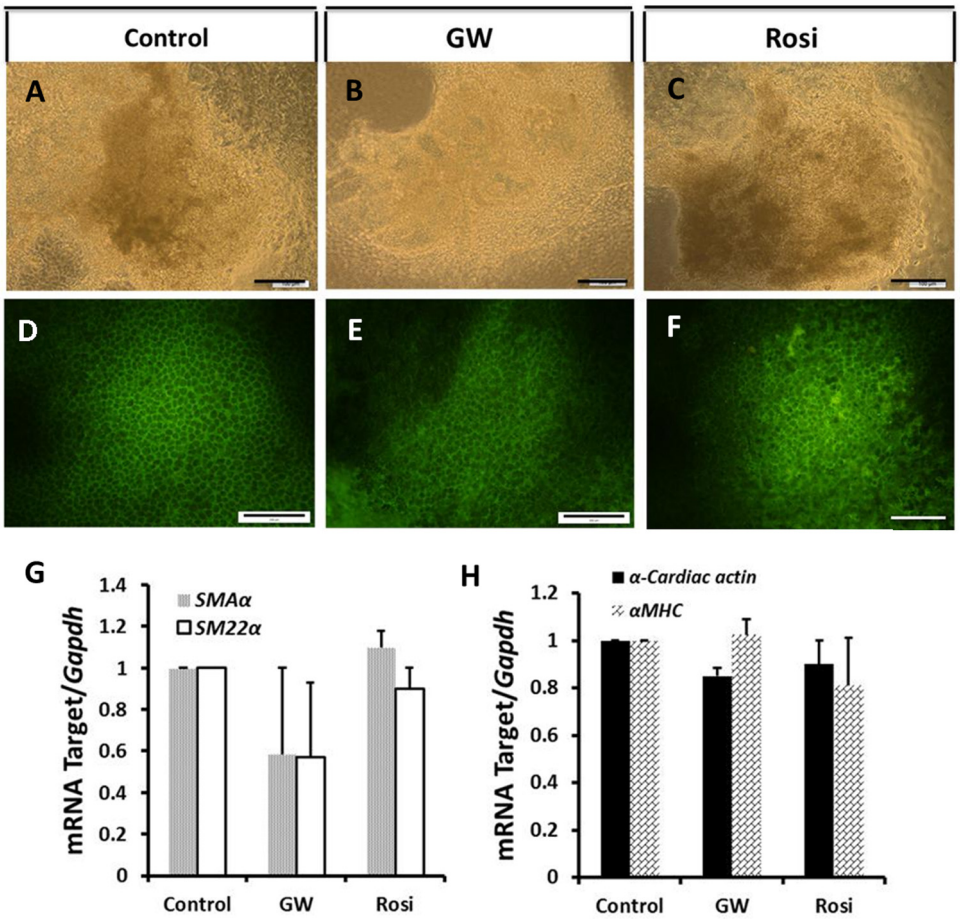

Fig. 8. Altered amount of PGC-1α, post-CPCs formation, did not effect on cardiac differentiation efficiency. (A–C) Morphological illustration of beating bodies derived from CPCs in treated samples and control. Scale bar is 20 μm. (D–F) Immunostaining of beating bodies with anti α-MHC (mature cardiomyocyte marker) antibody. Scale bar is 100 μm. RT-qPCR for (G) mature cardiomyocyte markers (*α-Cardiac actin* and *αMHC*) and (H) smooth muscle cells markers (*SMAα* and *SM22α*). The relative expression level of target genes were quantified and normalized with *Gapdh*. The number of independent repeats was 3 for each experiment ($n = 3$). Value bars are mean ± SEM. As depicted in the figure there is no significant difference between treated and control samples.

study was carried out. Several published evidences have suggested that PGC-1α plays critical roles in regulating cardiac energy metabolism genes which knockout of PGC-1α correlates with defects in ATP generation and susceptibility to induced heart failure (Arany et al., 2005, 2006; Huss et al., 2007; Leone et al., 2005). For instance, Garnier et al. (2003) observed consequence of PGC-1α decline, a defect in mitochondrial oxidative capacity would occur. Inversely, expression of *PGC-1α* can cause loss of pressure overload on vascular changes after myocardial infarction (Sun et al., 2007). *FNDC5*, as a transmembrane protein, is expressed during myoblast differentiation and embryo development similar to heart, skeletal muscle, and brain tissues with high rate of energy demands (Ferrer-Martines et al., 2002). In our previous study, down-regulation of *FNDC5* during neurogenesis resulted in decreasing of mature neuronal and astrocyte markers expression levels and neuronal differentiation efficiency (Hashemi et al., 2012). Also, we have shown that *FNDC5* overexpression in early stage of cardiac differentiation enhanced cardiac differentiation efficiency via increased expression levels of mesodermal markers, cardiac precursor and cardiomyocyte markers (Rabiee et al., 2014). Aydin et al. (2013) declared that cardiomyocytes are the main generating cells for *FNDC5*. Previous study by Lecker et al. (2012) has also indicated that *FNDC5* expression might affect aerobic performance in human heart failure (HF) patients. They have shown that plasma Irisin level was higher in aerobic exercise performed HF patients compare to those HF patients who could not perform aerobic exercise (Lecker et al., 2012). By administration of GW, a potent PPARγ antagonist to down-regulate PGC-1α level in cells, we addressed the importance of PGC-1α on cardiac differentiations of mESCs. In our experiments, stage dependence of PGC-1α reduction was considered. Therefore PGC1-α reduction assessed in both stages during and post stages of CPCs formation. Insufficient amount of PGC-1α during CPCs formation significantly decreased both expressions of smooth muscle cell marker (*SM22α*) and mature cardiac markers (*α-Cardiac actin* and *α-MHC*) in differentiated cardiac cells. In addition, the size of plated EB and percentage of beating EBs were reduced markedly. However, expression levels of mesodermal and cardiac precursor markers were not changed in CPCs. Noticeably, *FNDC5* and mitochondrial genes expression levels were efficiently suppressed under this condition. Notably, using PPARγ agonist, moderately improved the rate of cardiac differentiation as an enhancement in expression of cardiac markers was observed. In this condition the expression levels of *FNDC5* and mitochondrial markers were raised moderately. Therefore, we postulated that a threshold level of FNDC5 is required for appropriate cardiac differentiation during CPC formation. In post-CPCs formation stage, altered level of PGC-1α did not effect on transcript level of smooth muscle cell and mature cardiac markers genes. Furthermore, the size of plated beating bodies and their percentage were not affected in this condition. This condition was also did not change the expression of *FNDC5* and other down-stream genes as well as mitochondrial genes. These results clearly demonstrated stage dependent role

of PGC-1α–FNDC5 pathway on cardiac differentiation of mESCs. Consistent reduction in *PGC1α* and *FNDC5* expression levels with mitochondrial marker genes notified the importance of the role of PGC-1α–FNDC5 pathway on energy homeostasis. Numerous preceding reports have documented positive health beneficial effects of physical exercise especially physiological cardiac hypertrophy (Boström et al., 2012). Interestingly, exercise increases *FNDC5* transcript level suggesting that *FNDC5* may have a functional role in physiological cardiac hypertrophy by producing energy for efficient cardiac differentiation. No reduction/modulation of *PPARα* and *UCP2* suggests that PGC-1α–FNDC5 pathway may act through different mechanisms to regulate the expression level of downstream targets during the process of cardiac differentiation. These mechanisms are needed to be elucidated in further studies. In addition, another complication which should be clarified yet is due to intracellular/intercellular function of FNDC5. To answer this concern, high quality antibody and different strategies are needed to elucidate whether FNDC5 is secreted as Irisin or acts at cellular levels. Unfortunately, we were not able to perform suitable detection of endogenous FNDC5 to overcome this obstacle.

Author contributions

Faezeh Ghazvini zadegan: Experimental design, collection and/or assembly of data, data analysis, interpretation, and manuscript writing. Kamran Ghaedi: Conception and design, financial support, data analysis, interpretation, manuscript writing, and final approval of manuscript. Seyed Mehdi Kalantar: Conception, design, data analysis, interpretation, and final approval of manuscript. Maryam Peymani: Data analysis, interpretation. Motahare-Sadat Hashemi: Data analysis, interpretation. Hossein Baharvand: Conception, design, data analysis, interpretation, manuscript writing, and final approval of manuscript. Mohammad Hossein Nasr-Esfahani: Conception, design, data analysis, interpretation, and final approval of manuscript.

Conflict of interest statement

None of the authors has any conflicts of interest to disclose and all authors support submission to this journal.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejcb.2015.04.002

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